COMPARATIVE STRUCTURAL ANALYSIS OF REINNERVATED MUSCLE FOLLOWING NERVE INJURY AND REPAIR

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Declaration

I declare that the work described in this thesis and its composition are entirely my own.

8/1/02.

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Abstract

The repair of peripheral nerve injuries is a major reconstructive problem, particularly when direct suturing of a transected nerve is not a feasible option. This arises because mechanisms of nerve repair are not fully understood. The purpose of the first group of experiments was to compare methods of nerve repair, including use of novel, biodegradable glass tubes. The possible use of these tubes as a means of confining humoral or cellular substances at the site of repair was also assessed.

In a further study, experiments were designed to establish first, the optimal timing of nerve repair (immediate or delayed); second, whether there was a difference in the level of recovery between neonates and adults after nerve repair; third, to assess the freeze-thawed muscle graft (FTMG) method as a surgical technique and to determine whether the FTMG is at least as good as a conventional nerve graft. This investigation was carried out in an animal model (lambs and sheep) of obstetrical brachial plexus palsy (OBPP).

Assessment of experimental outcome in both studies was by measurement of the structural and cytochemical changes which occurred in the target muscle after peripheral nerve injury and repair. Morphometric, histochemical and immunocytochemical measurements showed alterations in muscle fibre size and architecture, as well as connective tissue content and the proportions and the distributions of the different fibre types. These changes indicated that after repair with controlled-release glass tubes there was reinnervation of the target muscle, although the results were superior after repair by FTMG. As to whether the potential of nerve to regenerate after repair decreases with age, or whether immediate or

delayed repair is best in the treatment of OBPP, the experiments have contributed to solving but have not entirely resolved the dilemma associated with these issues. In the context of the design of the experiments it was established that neither age nor a delay in repair adversely affected neuromuscular recovery. However, the present study has established that in the repair of the brachial plexus root injury, the FTMG compares favourably, and may even be superior, to the nerve autograft as a choice of treatment.

The thesis additionally compares different morphometric methods for analysis of muscle fibre type distributions in the context of nerve repair studies.

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Abbreviations

^{0}C degree Celsius

µm micrometer

AChR acetylcholine receptors

ATPase adenosine triphosphatase

cm centimetre

C contralateral

CDI codispersion index

CRG controlled release glass

CRG-GAP an empty CRG tube

CRG-M a CRG tube filled with freeze-thawed muscle graft

CRG-MN a CRG tube filled with freeze-thawed muscle and nerve

CRG-N a CRG tube filled with nerve graft

EDL extensor digitorum longus

Exp experimental

g gram

FTMG a coaxial freeze-thawed autologous muscle graft

H & E haematoxylin and eosin

HC heavy chains

J1 tenascin

kg kilogram

l litre

M-HSPG matrix-associated heparan sulfate proteoglycan

MHC myosin heavy chain

MRC medical research council

NADH-Tr nicotinamide adenine dinucleotide tetrazolium reductase

N-CAM neural cell adhesion molecule

NMJ neuromuscular junction

NO nitric oxide

NOS nitric oxide synthase

OBPP obstetrical brachial plexus palsy

RSE relative standard error

s second

SEM standard error of the mean

SS supraspinatus

TS toe spreading

W watts

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CHAPTER 1

Introduction

1. INTRODUCTION

The restoration of normal function following nerve injury continues to be a clinical challenge despite significant advances in the understanding of anatomy and pathology, and the use of antibiotics. The introduction of microsurgical techniques has improved the results of surgical nerve repair, but diminished motor control and defective sensory localization are often persistent after such procedures. An understanding of the effects of nerve injury and repair on target muscles may go some way to increasing the knowledge of the processes at work, and thereby improving the clinical management of such conditions. This thesis focuses on the recovery of muscle following nerve injury and various strategies designed to encourage nerve repair. The following is a review of the organization of muscle and its motor innervation, that provides an essential backdrop to the experiments that I describe subsequently.

1.1 SKELETAL MUSCLE

It has long been recognised that skeletal muscles are not a single homogeneous population. As early as 1678 observation of animal muscles exposed a varying degree of red colouration which was later found to be due to myoglobin content (Kuhne, 1865). Based on this difference in colour, muscle was subsequently referred to as either red or white. Ranvier (1873) established that red and white muscles differed in their physiological properties. He demonstrated that red muscle both contracted and relaxed more slowly than white muscle. He also confirmed the

findings of earlier studies that the red muscle had a more extensive blood supply than the white. Later studies of the anatomical distribution of both red and white muscles in a wide range of species led to a consensus view of the functions of red and white muscles. It was concluded that red muscles, although more slowly contracting, are required for the sustained activity required to maintain posture. White muscles, on the other hand, are capable of rapid, powerful action but lack staying power and are used therefore for more vigorous intermittent activity (Landon, 1992). Corresponding with these functions, Paul and Sperling (1952) demonstrated that white muscle had a low content of mitochondria and low respiratory activity, while red muscle was rich in mitochondria and had high respiratory enzyme activity.

1.1.1 The structure of skeletal muscle

Striated skeletal muscle is the main tissue component of the mammalian body, comprising 40 to 50% of its total wet weight (Landon, 1992). It consists of parallel bundles of long, multinucleate muscle fibres within a connective tissue framework. The individual muscle fibres are closely packed to each other and in transverse section, are polygonal in shape.

The collagenous connective tissue framework is organised into three discrete but interconnected sheaths (Borg and Caulfield, 1980). The epimysium surrounds each whole muscle and is composed of orientated collagen fibrils merging at either end with a tendon, aponeurosis or periosteum. Perimysium is a well-defined layer which subdivides the muscle into fascicles or bundles of fibres and is formed by deep extensions of collagen from the epimysium. Nerve branches, blood vessels and

neuromuscular spindles lie within the perimysium. The endomysium separates the individual muscle fibres from one another by a delicate network of fine collagen fibrils. Although barely visible in normal muscle viewed with the light microscope, proliferation of the endomysium is a striking feature of certain pathological conditions of skeletal muscle (Landon, 1992). The endomysium contains a variety of structures and cells which include capillaries, fine motor and sympathetic nerve branches, fibroblasts, mast cells, and macrophages as well as a network of extracellular fibrils (Sanes, 1994).

Muscle fibres are often referred to as "muscle cells" as they consist of a single cytoplasmic mass within one cell membrane, the sarcolemma. Electron microscopy of the cell membrane known as the sarcolemma has revealed that it is composed of several concentric layers. The inner most layer, the plasma membrane, is coated by a thin carbohydrate-rich glycocalyx (Rambourg and Leblond, 1967). A narrow electron-lucent gap separates the glycocalyx from the basal lamina and just beyond the basal lamina lies a reticular lamina containing collagen fibrils. Further ultrstructural studies have revealed subdivisions within the basal lamina itself which include a 10 to 15 nm thick lamina densa that is separated from the plasma membrane by a relatively electrolucent layer, the lamina rara 2 to 5 nm thick (Inoue, 1989). The basal lamina and reticular lamina together comprise the basement membrane (Sanes *et al.* 1978). The term basement membrane may often be used to refer to the basal lamina alone. Likewise the term sarcolemma has been adopted by many writers as a synonym for the plasmalemma of muscle cells.

Visible by light microscopy and located at the periphery of the fibres and immediately beneath the sarcolemma are the myonuclei. When examining a

transverse section of muscle if more than 3% of the fibres contain a nucleus in the substance of the muscle fibre and not at its periphery the specimen is said to demonstrate internal nuclei (Greenfield *et al.* 1957). The migration of nuclei to an internal position in the muscle fibre represents an abnormal but nonspecific response and is a consistent feature of a number of myopathies (Landon, 1992). It is important when assessing for the presence of internal nuclei that biopsies are from the belly of the muscle as internal nuclei are common in the normal muscle fibre at the myotendinous junction (Loughlin, 1993).

Electron microscopy has revealed that a proportion of the apparent population of myonuclei visible by light microscopy are extrinsic to the fibres and belong to small flattened satellite cells. As originally described by Mauro (1961), the satellite cells of mature muscle is a mononucleated cell lying between the basement membrane and the plasma membrane of the muscle fibre. Satellite cells consist of a nucleus, enclosed by sparse granular sarcoplasm, containing abundant free ribosomes, Golgi apparatus, endoplasmic reticulum and mitochondria (Bischoff, 1994). They are found in all known mammalian muscles, including muscle spindles. In adult extrafusal muscle fibres, the average percentage of satellite cell nuclei is less than 5% of muscle nuclei (Allbrook, 1981). Satellite cells have been shown to be a potential source of myogenic cells during muscle growth (Moss and Leblond, 1970, 1971), regeneration after injury (Bischoff, 1975, Konigsberg *et al.* 1975, Snow, 1977) and after denervation (Aloisi *et al.* 1973, McGeachie and Allbrook, 1978).

The major subcellular constituents of the muscle fibre are the myofibrils. Myofibrils occupy between 85% and 90% of the volume of each muscle fibre and are separated from each other by the intermyofibrillar space. Each myofibril is composed

of a bundle of myofilaments of two types, myosin (thick) and actin (thin) filaments. Actin and myosin together account for more than 70% of myofibrillar protein with actin contributing approximately 20%, and myosin, 54% (Perry, 1955, Huxley and Hanson, 1957, Hanson and Huxley, 1957). In addition to myosin, the thick filaments contain small quantities of nonmyosin proteins including C protein, H protein, X protein, AMP deminase, end filaments, myomesin, M protein and creatine kinase. The thick filaments are also associated along their length with the giant protein titin. The components of the thin filaments other than actin include troponin and tropomyosin, and a giant myofibrillar protein, nebulin (Craig, 1994).

The myofilaments are regularly aligned to form serially repeating identical structures known as sarcomeres. The precise natural alignment of the contractile filaments, actin and myosin within each sarcomere gives rise to the characteristic striated pattern of skeletal muscle seen in longitudinal sections. An individual sarcomere consists of a dark central band, (A band), bordered by two paler bands (I band) which are divided at their midpoints by a narrow dense line (Z line or disc). The A band consists of a regular parallel of thick myofilaments whilst the principal component of the I band are thin myofilaments. α-Actinin is a major component of the Z line (Craig, 1994). Electron microscopy reveals that the lengths of the thick and thin filaments do not change during muscle contraction. Instead and according to the sliding filament theory of contraction, the sarcomere shortens by the sliding of thick and thin muscle filaments past one another producing greater overlap of the filaments without a change in length of the filaments themselves (Huxley and Hanson, 1954, Huxley and Niedergerke, 1954). Huxley (1957) further observed that the thick filaments bore projections on their surfaces that formed links with the thin filaments

in the region of filament overlap. These are called "cross-bridges" and refer to the myosin "heads". The myosin heads are part of the bulbous end of the myosin filament which are attached to a long thin "tail" (Lowey *et al.* 1969, Elliott and Offer, 1978).

The components of each thin myofilament include two strands of fibrous actin (F-actin), and 300 to 400 globular subunits (G-actin) which are arranged in a double row and twisted to form a helix. Tropomyosin lies within the groove between the F-actin strands, and attached to tropomyosin, rather than directly to the actin, is troponin (Craig, 1994). In combination with tropomyosin, troponin is a regulator of the contractile activity of muscles. In a relaxed muscle, the position of the tropomyosin in the thin filaments is such that it physically blocks the cross-bridges from bonding to the actin. When muscle is stimulated to contract, Ca^{2+} is released from the sarcoplasmic reticulum (an elaborate endoplasmic reticulum), and the concentration of Ca^{2+} in the cytoplasm of the muscle cell rises. Troponin then binds Ca^{2+}, causing a conformational change that moves the troponin and its attached tropomyosin out of the way so that the cross-bridges can attach to actin. Each cycle is powered by the hydrolysis of ATP at the ATPase site on the myosin head (Adelstein *et al.* 1982, Cooke, 1986). Other components of the myofilaments, titin and nebulin, may function as protein rulers that define the precise lengths of the actin and myosin filaments respectively (Trinick, 1991).

The cytoplasm of the myofibre, the sarcoplasm, intervenes as a thin layer between the outermost myofibrils and the sarcolemma as well as between individual myofibrils in the core of the fibre. The sarcoplasm is not a structureless fluid but includes a collection of organelles which provides structural and energetic support

for the contractile apparatus, as well as serving the general metabolic requirements of the cell. It contains an elaborate endoplasmic reticulum (the sarcoplasmic reticulum), the membrane systems of the T tubules, a supportive framework of intermediate and finer filaments, microtubules, a Golgi complex, mitochondria, free ribosomes, glycogen and droplets of neutral lipid (Landon, 1992).

Another important component of the muscle cell is the cytoskeleton, which functions in the establishment and maintenance of cell shape, cell movement, cytoplasmic flow, and morphogenesis (Franzini-Armstrong and Fischman, 1994). The sarcoplasm located between the outermost myofibrils and the deep surface of the sarcolemma contains a number of cytoskeletal proteins. These are thought to play a structural role in strengthening the sarcolemma and adjacent layer of sarcoplasm, as well as linking both to the underlying myofibrils. These proteins include desmin, which forms a network around individual myofibrils and links the edge of contiguous Z-discs to maintain their lateral registration, and dystrophin (Hoffman *et al.* 1987, Koenig *et al.* 1988). Dystrophin is of particular interest since it has been shown to be the protein product of the defective gene in Duchenne and Becker muscular dystrophy (Menke and Jockusch, 1991).

1.1.2 Muscle fibre types and histochemistry studies

The development of histochemical techniques and their application to frozen, fresh or fixed muscle resulted in the ability to localize enzyme systems and other

chemical components at a cellular level. This unfolded the way for direct correlation of the functional activity of individual muscle fibres with their morphology.

The enzymes that have aroused attention have been those associated with glycogen synthesis and breakdown (such as phosphorylase), oxidoreductases (such as various dehydrogenases) and hydrolases (such as adenosine triphosphatase and various esterases).

The first sequence of such investigations involved the use of various animal muscles. Semenoff (1935) noted a variation in succinate dehydrogenase activity in amphibian muscle fibres, whilst Padykula (1952) demonstrated a similar modification in succinate dehydrogenase activity in the muscles of the rat. Nachimas and Padykula (1958) observed that red muscle such as rat soleus, had a large proportion of fibres which displayed a strong enzyme reaction for succinate dehydrogenase. They noted that in mixed fibres such as the biceps femoris and tibialis anterior, there was marked variation in succinate dehydrogenase activity between fibres. George and Scaria (1958) performed similar investigations on the breast and leg muscles of the pigeon and fowl and were able to associate strong oxidative enzyme activity and high mitochondrial content with red muscle.

Dubowitz and Pearse (1960a,b) later compared the results of oxidative enzyme techniques with phosphorylase activity and demonstrated a reciprocal relationship between the two. They suggested that both the human and the animal muscle could be divided into two-fibre types: type I have a high oxidative and low glycolytic activity, and type II having a low oxidative and high glycolytic activity. In his study of human biopsy material, Engel (1962) also approved of a two-fibre type system and based this on the myofibrillar actomyosin adenosine triphosphatase

(ATPase) activity. This distinction of two myofibrillar actomyosin ATPase (mATPase)-based fibre types, type I and type II, was made possible by the use of a histochemical assay for ATPase activity (Padykula and Herman 1955). According to Engel (1962), type I fibres were defined as small, red, rich in oxidative enzymes and poor in phosphorylase and ATPase activity after preincubation at pH 9.4, while the larger type II, white, fibres showed the direct inverse of these properties.

More refined methods for mATPase-based fibre type delineation were developed after the observation that fast and slow myosins have different alkaline and acid stabilities (Sreter *et al.* 1966, Seidel, 1967). One of the most commonly employed techniques for fibre typing utilises this varying sensitivity of mATPase to acid or alkaline pH. In this "Brooke and Kaiser method" the type II fibres may be subdivided on the basis of their susceptibility to acid preincubation prior to the routine myofibrillar ATPase staining reaction. This results in the delineation of fast fibre subtypes IIA and IIB and the rarely occurring fibre type called IIC (Brooke and Kaiser, 1969, 1970).

After acid preincubation at pH 4.3, type I fibres stain intensely and type II fibres remain unstained (often referred to as type II fibres being acid-labile). The reverse of this is seen after alkali preincubation at pH 9.4 (often referred to as type I fibres being alkali-labile). Preincubation at 4.6 produces a third ATPase staining pattern in which type I and type IIB fibres stain, while type IIA fibres remain unstained. The third subtype of type II fibres, type IIC, stain at pH 9.4 and pH 4.6 and have residual positive reaction at pH 4.3, but become negative at still lower pH. Although this method was originally primarily developed for use in human muscle specimens, Brooke and Kaiser (1970) have shown that after suitable adjustment of

the pH of the preincubation medium, the same system of classification was also applicable to muscles of some common experimental animals.

Brooke *et al.* (1971) suggested that the rarely occurring type IIC fibres were a precursor of the type I, IIA and IIB fibres. Histochemical studies of developing human muscle by Farkas-Bargeton *et al.* (1977) and Colling-Saltin (1978) have shown that undifferentiated fibres during the first phase of development were type IIC fibres. After about the 20th week of gestation type I fibres began to appear, and after about 30 weeks of fetal life type IIA and IIB. The process of differentiation was not yet complete at birth as there was still between 15 to 20% of undifferentiated type IIC fibres. During the first year of life a gradual increase in type I, IIA and IIB fibres was observed at the expense of the undifferentiated type IIC fibres. At one year of age the type IIC was only 3 to 5%, whereas the type I fibres amounted to 60 to 65% and the type II fibres 30 to 35%. The type IIA fibres were the predominant type II fibres.

As distinct from type IIC fibres in developing muscle, Kugelberg (1976) considered type IIC fibres in adult muscle to be transitional fibres. As transitional or intermediate fibres Kugelberg (1976) suggested that they are the temporary result of transition from type IIA to type I fibres. In the rat soleus muscle between birth and 34 weeks Kugelberg (1976) noted a large scale conversion of IIA to type I fibres.

Gates *et al.* (1991) investigated whether the number of type IIC fibres in the fourth deep lumbrical muscle of the rat which remains constant from near birth to 60 days, remains constant in older rats. At 3 months the number of type IIC fibres was not significantly different from that in neonates but between 3 and 4 months there is a marked reduction in the number of fibres. From 4 months onwards there is no

progressive reduction and at 2 years of age there are still type IIC fibres present. Another finding was of an unusual, minority type of motor unit that contained muscle fibres of type IIC and type IIA. Gates *et al.* (1991) suggested that it was possible that these IIC/IIA motor units in the fourth deep lumbrical muscle are also transitional however, they would represent transition from slow to fast and not fast to slow as in the rat soleus muscle.

In addition to the Brooke and Kaiser method, another frequently used technique base their fibre typing on alkaline preincubation of formaldehyde fixed muscle sections (Guth and Samaha, 1970), and recognise three fibre types: the slow β fibres, and the two fast fibre types, α and $\alpha\beta$ (Samaha *et al.* 1970).

The two different mATPase-based classification schemes of Brooke and Kaiser (1970) and Guth and Samaha (1969, 1970) have commonly been considered to produce equivalent staining patterns i.e., I = β, IIA = α and IIB = $\alpha\beta$. These two classification systems however, do not appear to be entirely compatible. Green *et al.* (1982) revealed complete correspondence between type I and β fibres in each of the species they examined. The correlation between IIA and α as well as between IIB and $\alpha\beta$ fibres was species dependent and was of variable degree. They concluded that the two classification schemes were not interchangeable for all species and that the two terminologies should be used only with the methods from which they were derived. However, such a lack of correspondence between the two mATPase techniques may indicate the presence of systematic differences in mATPase properties that are not detected by one technique alone. This possibility is strongly suggested by the immunohistochemical demonstration of a new variety of myosin, referred to as type IIX, and whose mATPase has been found to stain like type IIA fibres after

formaldehyde-alkali preincubation (Guth and Samaha method) but like type IIB fibres after acidic preincubation (Brooke and Kaiser method) (Schiaffino *et al.* 1989, Gorza, 1990). Indeed studies by Lind and Kernell (1991) have verified this possibility. Their investigation is, for rat limb muscles, the first quantitative comparison between the mATPase reactions of muscle fibres when stained according to the Brooke and Kaiser (1969, 1970) and the Guth and Samaha (1969, 1970) methods. This was achieved by an objective assessment of staining intensity by measuring the light absorption with microphotometric techniques. Their results demonstrated that as with earlier studies, either technique isolated three types of muscle fibres (I, IIA, IIB). However, the combined use of both mATPase staining techniques resulted in the classification of nearly all muscle fibres into one of four groups: type I, type IIA, and two subdivisions of type IIB which they referred to as type IIBd and type IIBm. Furthermore, Lind and Kernell (1991) found that the histochemical properties of the type IIBd corresponded to those described for type IIX fibres (Schiaffino *et al.* 1989).

1.1.3 Muscle fibre types and immunohistochemical studies

Recent immunohistochemical analysis of muscle fibres using antibodies raised against myosin of fast or slow twitch muscle have complemented the histochemical classification of fibre types. Each of the main fibre types type I, IIA and IIB have different myosin heavy chain (MHC) composition.

Using electrophoretic procedures that permit the separation of MHCs and immunoblotting analysis with several monoclonal antibodies, a fourth fibre type, type IIX (as discussed above), has been delineated in muscles of small animals. These fibres are characterised by a specific MHC isoform distinct from IIa-MHC and IIb-MHC (Schiaffino *et al.* 1985, 1986, 1989, Gorza, 1990). Muscles composed of mainly type IIX fibres exhibit a velocity of shortening intermediate between that of slow muscles and that of fast muscles containing predominantly type IIB fibres (Schiaffino *et al.* 1989). Type IIX fibres are numerous in most leg muscles and are notably abundant in the diaphragm (Schiaffino *et al.* 1989), are rich in oxidative enzymes (Schiaffino *et al.* 1986), and are resistant to fatigue (Larsson *et al.* 1991).

It is important to note and was previously discussed that with mATPase staining, the type IIX fibres stain like type IIA fibres after formaldehyde-alkali preincubation and like type IIB fibres after acidic preincubation (Schiaffino *et al.* 1989). As mATPase staining after preincubation at pH 4.6 (Brooke and Kaiser, 1969, 1970) is frequently used for fibre typing, it appears that IIX fibres have been erroneously included into the type IIB class in many histochemical studies. In addition, as type IIX fibres display moderate succinate dehydogenase activity (Schiaffino *et al.* 1989, Gorza, 1990), it is likely that they would be identified as type IIA fibres in studies using histochemical methods for oxidative enzymes.

Bär and Pette (1988) have identified another fast myosin heavy chain isoform in various skeletal muscles of the rat by an improved gradient gel electrophoretic technique. This isoform was abundant in muscles specialized for sustained activity in particular, the diaphragm, and so was tentatively named MHC IId (Bär and Pette, 1988, Termin *et al.* 1989a,b, 1990). Despite the fact that Schiaffino *et al.* (1989) were

not able to electrophoretically separate MHCIIx from the MHCIIa band, examination of their published immunoblast analyses suggests that MHCIIx and MHCIId are identical. Indeed, later studies by La Framboise *et al.* (1990) propose that MHCIIx and MHCIId correspond to the same isoform.

There are unique myosin heavy chains for fibres of specific muscles in particular, extraocular muscles (Wieczorek *et al.* 1985, Sartore *et al.* 1987), and muscles derived from the first branchial arch (the masseter, temporalis, pterygoideus, tensor veli palatini, tensor tympani, digastricus anterior, and mylohyoideus muscles; Mascarello *et al.* 1982, 1983, Rowerlson *et al.* 1981, 1983, Shelton *et al.* 1988). It is now recognised that there are nine distinct MHC isoforms in adult mammalian skeletal muscles including five fast, two slow and two developmental heavy chains (Pette and Staron, 1990).

Most muscle fibres contain only one MHC isoform and are called pure fibre types. They are fast fibre types IIA (MHCIIa), IIB (MHCIIb), and IID/X (MHCIId/x), and the slow type I (MHCI). Muscle fibres may also coexpress two MHCs in the same fibre and these are referred to as hybrid muscle fibres. The coexistence of two MHC isoforms in a given fibre may occur at different ratios. Based on the MHCs present and their ratio, hybrid fibre types are designated as: IIBD (MHCIIb > MHCIId), IIDB (MHCIId > MHCIIb), IIDA (MHCIId >MHCIIa), IIAD (MHCIIa > MHCIId). Hybrid fibres containing fast MHCIIa and slow MHCI are called C fibres. Hence, type IIC fibres have a higher proportion of MHCIIa than MHCI, and type IC fibres have a lower proportion of MHCIIa than MHCI (Staron and Pette 1986, 1987a,b, Hamalainen and Pette, 1995). In the rabbit, MHCIIa also coexists with MHCIID in type IIDA and IIAD fibres (Aigner *et al.* 1993). The number of hybrid

fibres vary in different species and increase in muscles undergoing fast-to-slow transition under the influence of chronic low frequency stimulation (Staron and Pette, 1987c, Maier *et al.* 1988, Termin *et al.* 1989a). The presence of hybrid fibres, although at lower amounts in untreated muscles, suggests the possibility that normal mature muscle may undergo a continuous adjustment of its fibre type composition (Aigner *et al.* 1993). However, a recent quantitative analysis of myosin heavy chain mRNA and protein isoforms in single fibres of normal rabbit muscle has revealed a pronounced heterogeneity of the fast fibre population. This was demonstrated by the numerous hybrid fibres in normal muscles coexpressing MHCIIb and MHCIId/x. The detection of high amounts of IIBD and IIDB fibres suggests that hybrid fibres may represent functional elements within a continuum of finely tuned, functionally different fibres (Peuker and Pette, 1997).

1.1.4 Muscle fibre types of rabbit extensor digitorum longus muscle

Although rabbits have been commonly used laboratory animals it is only until recently that studies have provided a database on normal fibre type composition of some of their more experimentally important muscles. One such muscle is the extensor digitorum longus (EDL) muscle which is studied in this research project on muscle reinnervation.

The EDL muscle is located in the anterior compartment of the lower hind limb of the rabbit, and contains a predominance of fast-twitch fibres. It is pennate in

shape and acts across several joints including the ankle, and phalanges. The functions of the EDL muscle include ankle dorsiflexion, and spreading of the toes.

The fibre type composition of the EDL muscle of the rabbit includes the slow type I, as well as two fast fibre types, IIA and IID (Hämäläinen and Pette, 1993). Electrophoretic separation of myosin heavy chains has demonstrated HCIId as the predominant fast isoform in the rabbit EDL, as well as almost devoid of the HCIIb isoform (Aigner et al, 1993, Hämäläinen and Pette, 1993, Janmot and d'Albis, 1994). Furthermore HCIId is the predominating fast MHC isoform in rabbit skeletal muscle. Unlike mouse and rat, type IIB fibres expressing HCIIb are less frequent in homologous muscles of the rabbit. In these muscles type IID represent the major fast fibre type (Aigner *et al.* 1993, Hämäläinen and Pette, 1993).

Assessment of the spatial distribution of fibre types I and II within the EDL muscle of the rabbit reveals a systematic arrangement of type II fibres, and a significantly higher proportion of type I fibres in the medial and deep parts than in the lateral, superficial part of the muscle. Studies also show a small but significant increases in the proportion of type I fibres in the proximal-to-distal direction. Further comparison of the fibre type proportions and fibre densities between EDL muscles from right and left limbs, and between male and female rabbits, found no significant difference (Lexell *et al.* 1994).

The organization of innervation in the EDL muscle has been studied in the rat but not in the rabbit. In the rat it has been established that each extramuscular branch formed from the bifurcation of its muscle nerve innervates a separate region or "compartment" in the muscle (Balice-Gordon and Thompson, 1988). The term compartment refers to a further subdivision of skeletal muscle recognised by the

nervous system that is larger than a motor unit. Anatomically, it has been defined as a contiguous group of muscle fibres and muscle receptors that are innervated by a branch of a muscle nerve (English and Letbetter, 1982a,b).

In the rat EDL muscle, Balice-Gordon and Thompson (1988) observed that the branch entering the muscle nearer the knee innervates fibres in the anteromedial half of the muscle, whilst the branch entering closer to the foot innervates fibres located posterolaterally. They found a sharp delineation between the compartments. There was no obvious anatomical feature within the rat EDL muscle which would explain its division into two distinct areas such as a tendinous or connective tissue "barrier" that prevents axons from branching between the two compartments. Investigation of a segmentotopic projection from the spinal cord to the muscle established that it was too weak to explain the presence of neuromuscular compartments. Balice-Gordon and Thompson (1988) concluded that compartments in the rat EDL muscle are primarily the result of the separation of the EDL motoneurones into two nerve branches which are directed into different regions of the muscle.

Investigation of the fibre type composition of muscle compartments have demonstrated marked differences (Galvas and Gonyea, 1980, English and Letbetter, 1982a,b, Bodine *et al.* 1982). In the rat EDL although there was no significant difference in the fast fibre composition of the two compartments, there was a marked disproportion in the slow fibre composition (Balice-Gordon and Thompson, 1988). Other studies suggest that neuromuscular compartments may function independently under different physiological conditions (Herring *et al.* 1979, English and Letbetter, 1982a,b, Bodine *et al.* 1982, English, 1984, Richmond *et al.* 1985, Richmond and

Armstrong, 1988, Mu and Sanders, 1998). Future studies to determine the neuromuscular compartments of the rabbit EDL muscle would certainly be of interest.

Each of the studies cited above in relation to the rabbit, were based on experiments conducted on a strain called the "New Zealand White" which is the experimental animal in the present study. To reduce any systematic error it is essential to quote from a database which is derived from studies on animals from the same species and strain. As Pette and Staron (1990) point out, the unique feature of skeletal muscle is its diversity with "even homologous muscles exhibiting differences in fibre composition between species and strains".

1.2 PERIPHERAL NERVES

1.2.1 The structure of peripheral nerves

Peripheral nerves are formed by aggregations of a variable number of axons, each lying side by side. Their size is determined by the number of axons: the greater the number of axons, the larger the peripheral nerve.

Axons are the extended processes of nerve cell bodies located in the anterior horn of the spinal cord (motoneurones), the dorsal root ganglia (sensory neurones), or sympathetic ganglia (sympathetic neurones). An axon may extend over a distance corresponding to many thousands of times the cell body diameter and as a consequence, may contain more than 90 % of the total cytoplasmic volume of the nerve cell. The cytoplasm of the nerve cell or axoplasm is a viscous fluid enclosed in

a plasma membrane: the axolemma. Contained in the cytoplasm are neutotubules, neurofilaments, mitochondria and smooth-surfaced endoplasmic reticulum (Cormack, 1987).

Axons are either myelinated or unmyelinated. Myelin is a sheath of lipid which functions as a protective and insulating coat for the axon whilst enhancing the speed of contraction of electrical impulses along it. Although both myelinated and unmyelinated axons are surrounded by a chain of Schwann cells arranged end to end, the particular relationship between the Schwann cell and the axon differs. The Schwann cells are the glial cells of the peripheral nervous system. In myelinated axons they form the myelin sheaths or in unmyelinated axons, the Schwann cells enclose the axons (Weinberg and Spencer, 1975, Aguayo, 1976).

In myelinated axons, each Schwann cell is related only to a single axon. The Schwann cell membrane or the myelin sheath wraps spirally around the axon and creates a multilayered sleeve of lipids and proteins. The point where the myelin sheath of one Schwann cell ends and the sheath of the next Schwann cell begins are known as "nodes of Ranvier". At these junctions there is an exchange of ions between the axon and the surrounding extracellular fluid which allows for saltatory propagation of impulses from node to node to occur. This results in the rapid propagation of impulses in myelinated nerve fibres (Bogduk, 1998).

Unmyelinated axons, instead of being enveloped by a tightly spiralling sheath of myelin, are embedded in invaginations of Schwann cell membranes so that a single Schwann cell may encapsulate several axons. The lack of myelin means that unmyelinated axons are afforded less physical protection than myelinated axons however, the principal difference between the two types of axons is that the

unmyelinated axons are of smaller diameter and have slower nerve conduction velocities.

Peripheral to the myelin sheath is the cell membrane and cytoplasm of the Schwann cell. The outermost layer of the Schwann cell is referred to as the neurolemma. Besides myelin proteins, Schwann cells synthesize components of the extracellular matrix (Bunge *et al.* 1980, Cornbrooks *et al.* 1983, Mehta *et al.* 1985), cell adhesion molecules (Nieke and Schachner, 1985, Noble *et al.* 1985, Daniloff *et al.* 1986), and also neurotrophic factors such as nerve growth factor (Richardson and Ebendal, 1982, Rush, 1984, Heumann *et al.* 1987).

The axons are held together by connective tissue sheaths which provide additional coverings to protect them from external physical and chemical insults. Individual myelinated axons are surrounded by a tubular sheath of connective tissue called endoneurium, whilst bundles of axons are enclosed by a larger connective tissue sheath called perineurium. In the case of unmyelinated axons, these are not surrounded by endoneurium but run in separate bundles parallel to myelinated axons and are enclosed with them in the perineural sheath. The endoneurium includes a thin inner endoneurial sheath that immediately surrounds the Schwann cells and is inflected at the nodes of Ranvier (Plenk, 1927, Laidlaw, 1929). This is surrounded by a thicker outer endoneurial sheath or sheath of Key and Retzius (Young, 1942), with a longitudinal orientation that crosses the nodes. The main function of the endoneurium is to prevent elongation under tension (Bradley *et al.* 1998).

The bundle of axons surrounded by a single perineural sheath is called a nerve fascicle. The perineurium maintains an optimum intrafascicular environment by providing a diffusion barrier to a number of extrinsic macromolecules. It also acts

as a mechanical barrier to external trauma. The number and size of fascicles may vary along the length of nerve (Sunderland, 1990). The fascicles are bound together by an external sheath of well-vascularized areolar connective tissue called the epineurium. The deep layers of epineurium separate fascicles and keep them loosely together. Superficially, it is condensed to a sheath which delimits nerve from surrounding structures to which it is very loosely attached. This arrangement plays an important role in the provision of a certain amount of gliding of nerve with movement of the extremity (Lundborg and Dahlin, 1992).

Peripheral nerves are well vascularized structures comprising segmental extrinsic vessels entering along the course of the nerve, as well as an intrinsic blood supply within the endoneurium, perineurium and epineurium. There are extensive connections between the two blood systems (Berry *et al.* 1995).

Axons may be classified into three groups based on the relationship between the shape of the compound axon potential and the fibre size (Erlanger and Gasser, 1937). Group A fibres are the largest and have the fastest conduction velocities in the range of 12 to 120 ms^{-1}. These fibres are myelinated somatic afferents and efferents. Group A fibres are further subdivided into A-α, A-β, A-γ and A-∂. The A-α and A-γ fibres represent motor fibres to voluntary muscles, and include specific sensory fibres that transmit position sensation from skeletal muscles. A-β are associated with touch, and A-∂ mediate pressure, pain and temperature sensations from skin, pain and pressure from muscle, and pain, pressure and position sense from ligaments and joints. Group B fibres are slowly conducting myelinated, preganglionic sympathetic efferent fibres. Group C fibres are slowly conducting axons with conduction

velocities in the range of 0.5 to 2 ms^{-1}. These fibres are thin, unmyelinated visceral afferent and somatic afferent fibres (Bogduk, 1998).

Neuronal function is dependent on the transport of materials from the cell body to the synapse and an important part of this is axonal transport. Axonal transport is the movement of proteins and other materials from their site of synthesis in the cell body, through the axon to synapses and growth cones. It is divided into separate fast and slow axonal transport (Yarden and Ullrich, 1988, Schlessinger and Ullrich, 1992). Fast axonal transport is the movement of all membranous organelles and membrane proteins along microtubules (Schlessinger and Ullrich, 1992). It includes the motor protein kinesin which moves membranous organelles in the anterograde direction, whilst cytoplasmic dynein moves membranous organelles in the retrograde direction (Letwin *et al.* 1988).

Slow axonal transport is composed of two subcomponents. Slow component a, which travels at 0.2 to 1 mm/day, consists of microtubules and neurofilaments. Slow component b, which travels at 2 to 8 mm/day, consists of microfilaments and the remaining proteins of the axon, including metabolic enzymes, which are collectively referred to as the cytomatrix (Schlessinger and Ullrich, 1992). Little is known about the mechanisms of slow axonal transport however, it has been proposed that in slow axonal transport the motor protein dynein generates movement of the microtubules by sliding them toward the synapse (Dillman *et al.* 1996).

1.3 THE MOTOR UNIT

1.3.1 The structure of the motor unit

Skeletal muscle fibres are richly innervated by motoneurones whose cell bodies lie within the spinal cord or brainstem and whose axons extend peripherally to the muscles. The motor innervation of somatic muscles is thus organized in motor units. A motor unit consists of a single motoneurone plus all the striated muscle fibres to which it is connected through its axonal branches.

The actual size of a motor unit is defined in terms of the number of muscle fibres it contains, with a small motor unit being one with relatively few muscle fibres and a large motor unit being one with many muscle fibres. In a small number of muscles all the constituent motor units are usually small with less than ten muscle fibres per unit. Examples include the extrinsic ocular muscles which are responsible for very delicate eye movements. In these muscles each constituent motor unit contains between three and five muscle fibres. However, in most other striated muscles each motor axon branches in such a way so that it innervates many muscle fibres. Examples include the trunk muscles that are responsible for maintaining posture in which each motor unit may contain several hundred muscle fibres.

The ratio of total number of muscle fibres to total number of motoneurones is called the innervation ratio of the muscle. Its value expresses the average size of the motor units that make up the muscle.

Within a muscle the fibres belonging to one motor unit are distributed over a wide area, without regard to fascicular boundaries, and intermingle with the fibres of other motor units (Edstrom and Kugelberg, 1968, Burke *et al.* 1973). In an adult, a

single muscle fibre receives only one axonal branch from a motoneurone and any one branch innervates only one muscle fibre.

The motor unit is the unit of all voluntary and reflex muscle contraction. The total tension in a muscle can be varied by adjusting the number of motor units that are activated. Motor units are activated according to a size principle and first are primarily the neurones having the smallest axon diameter which have the lowest threshold for synaptic activation (Henneman and Mendell, 1981). As the strength of the afferent input increases, progressively larger motoneurones are recruited, increasing muscle tension. Summation of muscle force is produced not only by recruitment of previously inactive motor units but also by more rapid firing of already active units. Both these mechanisms operate simultaneously (Petajan, 1991).

In normal motor units muscle fibres are not homogeneous in size (Bodine *et al.* 1987, Tötösy de Zepetnek *et al.* 1992). This fibre heterogeneity within motor units indicates that muscle fibre size is not entirely neurally regulated. Some of the size variation is due to regional differences in cross sectional area along the transverse axis (Pullen, 1977, Rafuse and Gordon, 1996b), as well as the length of each muscle (Ounjian *et al.* 1991). Muscle fibres located closer to the bone tend to be smaller than more superficial fibres. This gradation has been attributed to the derivation of muscle fibres from primary and secondary myotubes which is nerve-independent at early stages of myogenesis (Butler *et al.* 1982, Dhoot, 1985, Miller and Stockdale, 1987). Another contributing factor which is not under neural control is differential loading conditions of muscle fibres in different regions of muscle (Gordon and Pattullo, 1993).

1.3.2 Muscle fibre types and physiological properties

Close (1967) distinguished motor units in rat muscle which have fast, slow and intermediate contraction times. Bárány (1967) demonstrated that the intrinsic speed of contraction of muscle is proportional to the specific activity of myosin ATPase. From these studies it became clear that fast and slow skeletal muscles contain distinct myosin isoforms and that these correlate with differences in the speed of shortening.

An attempt to correlate directly the histochemical type of fibre with its physiological function was made by Barnard *et al.* (1971) and this resulted in a different system of nomenclature. According to this method, fibres were classified according to their histochemical reaction for nicotinamide adenine dinuleotide tetrazolium reductase (NADH-Tr), a reference enzyme for aerobic oxidative metabolism. The fibres were classed as red, white or intermediate based on strong, weak and intermediate NADH-Tr activity, respectively. They also observed that contraction speed correlated more exactly with the histochemical reaction for ATPase than with NADH-TR. Fibres with higher ATPase activities were fast, whereas fibres with lower ATPase activity were slow. Hence the name "fast-twitch white" was recommended for those fibres which were histochemically strongly reactive for ATPase and therefore presumably "fast", and weakly reactive with NADH-TR and therefore presumably "white". The other fibres included in the classification were "fast-twitch red" (strongly reactive for ATPase and strongly reactive for NADH-TR) and "slow-twitch intermediate" (weakly reactive for ATPase

and intermediate for NADH-TR). Subsequent to this Peter *et al.* (1972) recommended that as an alternative to slow-twitch intermediate, fast-twitch white, and fast-twitch red, the fibres should more appropriately be named slow-twitch oxidative (SO), fast-twitch glycolytic (FG), and fast-twitch oxidative glycolytic (FOG), respectively. This classification was based on studies on guinea pig and rabbit limb muscles using three criteria: the contraction time of the muscle fibre relative to others within the same animal, the glycolytic capacity, and the oxidative capacity.

In a study of single motoneurones in the gastrocnemius of the cat and using as criteria both contraction speed and resistance to fatigue, Burke *et al.* (1971, 1973) were also able to separate motor units into three classes. The individual motor units were designated as either slow fatigue-resistant (S), fast fatigue-resistant (FR), or fast rapidly fatiguable (FF). This resistance to fatigue of motor units seems to be correlated with the metabolic properties of their muscle fibres. The fatigue-sensitive motor units have muscle fibres which are rich in glycolytic enzymes and low in enzymes of aerobic oxidative metabolism, whilst muscle fibres of fatigue-resistant motor units are rich in enzymes of aerobic oxidative metabolism (Olson and Swett, 1966, Edström and Kugelberg, 1968, Kugelberg and Lindegren, 1979, Nemeth *et al.* 1981). Thus, the fast glycolytic (FG) fibres belong to the fast fatigable (FF) motor units, the fast oxidative-glycolytic (FOG) to the fast fatigue-resistant (FR), and the slow oxidative (SO) to the slow fatigue-resistant units.

This classification based on fatigability and metabolic properties of the muscle fibres does not necessarily coincide with the mATPase-based grouping system. Several histochemical studies have compared fibres typed using the

mATPase-based (Brooke and Kaiser, 1970) and the metabolic-based (Barnard *et al.* 1971) methods and have indicated their incompatibility (Sjögaard *et al.* 1978, Nemeth *et al.* 1979, Nemeth and Pette, 1980, 1981). This may be explained by a large variation in metabolic profiles within each of the mATPase-based fibre types I, IIA and IIB (Pette and Vrbová, 1985), as well as the existence of type IIX fibres (Schiaffino *et al.* 1989).

1.3.3 Neural determination of muscle properties

The influence of nerve on muscle characteristics was clearly demonstrated by the cross-innervation experiments of Buller *et al.* (1960). They performed a cross-union of fast and slow motor nerves in the cat which resulted in an increase in the speed of contraction of slow muscle and a decrease in the speed of contraction of fast muscle. As a result it was established that the contractile properties characteristic of a muscle are not inherent but are influenced by a motor nerve. Application of histochemical techniques to cross-innervation experiments showed a corresponding change in fibre type pattern (Dubowitz 1967, Karpati and Engel, 1967, Romanul and Van der Meulen, 1967, Gordon *et al.* 1988).

This influence of nerve on muscle properties was initially considered to be mediated by a "trophic" substance released from the nerve terminals (Buller *et al.* 1960). Another explanation was proposed by A. F. Huxley, who suggested that the motor nerve maintains the slow course of contraction of the soleus muscle fibres by imposing on it a slow frequency activity (see Buller *et al.* 1960). However, Buller

and his colleagues did not favour this interpretation and it was not until later that the role of activity patterns in determining muscle fibre properties was established.

In these experiments the effects of different imposed activity patterns were initially tested without interfering with the muscle's own innervation. This was accomplished by inhibiting the muscle's afferent inputs by cutting the tendons (tenotomy) of the muscle. After tenotomy of the soleus muscle its continuous low frequency activity was reduced or abolished and it became a faster contracting muscle (Vrbová, 1963a,b). Other studies included long-term stimulation of intact motor nerves to tibialis anterior and extensor digitorum longus muscles of rabbits at a low frequency. As a result it had a dramatic slowing effect on the normal phasic activity of these fast muscles (Salmons and Vrbová, 1969, Pette *et al.* 1973, 1975, 1976).

Another approach to determine whether the neural influence on muscle properties is mediated by the pattern of neural activity is to physically cut the nerve supply and replace the neural activity with direct electrical stimulation. In the case of denervated rat soleus muscles stimulated with a continuous low frequency pattern, the muscle remained as slow as normal (Lømo *et al.* 1974). However, when the denervated muscle was stimulated with high frequency intermittent bursts, the muscles developed force as rapidly as a fast twitch muscle (Gorza *et al.* 1988). This increased contraction speed was attributed to the pattern of stimulation rather than any effects of denervation (Al-Amood and Lewis, 1987, Gorza *et al.* 1988). Thus, these results indicated that the nerve exerts its influence by imposing a specific pattern of activity. Recent studies have confirmed this conclusion (Hämäläinen and Pette, 1996, Windisch *et al.* 1998).

Hämäläinen and Pette (1996) observed that denervation alone of a slow twitch muscle induced appreciable changes in myosin heavy chain composition. However, the effects of phasic high frequency stimulation of the denervated muscle exceeded by far those of denervation alone. Windisch *et al.* (1998) observed a similar response after denervation of a fast twitch muscle. They also stimulated innervated and denervated fast twitch muscles with a "slow" pattern of impulse activity and demonstrated that the effects on myosin heavy chain composition was identical for both groups. Their results indicated that the slow pattern of evoked muscle activity, rather than nerve derived trophic influences or denervation, was primarily responsible for the changes in muscle fibre type. Indeed, it is now generally accepted that neurally transmitted impulse patterns have a specifying effect on the phenotypes of muscle fibres. This impact on muscle phenotypes has been demonstrated by sequential transitions in myosin heavy chain isoforms after different patterns of neural activity. Chronic low frequency stimulation of fast twitch muscle involves sequential transitions in its myosin heavy chain isoforms in the order of MHCIIb → MHCIId/x → MHCIIa → MHCI (Termin *et al.* 1989a, Leeuw and Pette, 1993, Windisch *et al.* 1998). Conversely, phasic low amount, high frequency stimulation of slow twitch muscles results in sequential transitions in myosin heavy chain expression in the order of MHCI → MHCIIa → MHCIId/x →MHCIIb (Hämäläinen and Pette, 1996).

In the response of denervated muscle to electrical stimulation there may be some species differences. Lewis *et al.* (1997) observed that after denervation and chronic stimulation of the soleus muscle in both guinea-pig and rat, there was a slow to fast conversion of the soleus muscle in the rat but not in the guinea-pig.

Furthermore, the conversion of the rat soleus muscle was due to the formation of "fast" regenerated fibres and not the transformation of slow muscle fibres. Indeed some difficulty in the conversion of slow muscle to fast muscle by denervation-stimulation has been observed, particularly when compared to the transformation of fast muscles after denervation-stimulation of these muscles (Vrbová *et al.* 1995).

1.4 THE NEUROMUSCULAR JUNCTION

1.4.1 The structure of the neuromuscular junction

The neuromuscular junction (NMJ) is a chemical synapse that is anatomically and functionally specialized for the transmission of a signal from a motoneurone to a muscle fibre. The junction involves distinctive structural specializations of both the neurone and the muscle cell and of certain surrounding tissues.

As a motor axon approaches its target muscle cell, it loses its myelin sheath and forms a flattened expansion that lies adjacent to the sarcolemma of muscle in an indentation in the surface of the muscle fibre. A shallow gutter is formed beneath the nerve terminal and is known as the primary synaptic cleft. At the site of the NMJ, focal accumulation of sarcoplasm and organelles within the muscle cell results in its elevation above the general contour of the muscle fibre to produce the "Doyere eminence" or soleplate (Bogduk, 1998).

The terminal expansion of the motoneurone is specialized for neurotransmitter release. It contains synaptic vesicles filled with the neurotransmitter, acetylcholine, as well as a number of mitochondria which provide the source of

energy for the synthesis and release of transmitter. Many of the vesicles are focused at dense patches on the presynaptic membrane known as active zones. Here vesicles fuse with the presynaptic membrane and release their contents into the synaptic cleft (Sanes and Lichtman, 1999). The active zones are associated with voltage-sensitive potassium and calcium channels. Their localization and transmitter release sites play a major role in neuromuscular transmission by allowing calcium ion influx into motoneurone terminals and thereby effecting acetylcholine release (Rabitaille *et al.* 1993, Sugiura *et al.* 1995, Day *et al.* 1997).

The postsynaptic membrane is specialized to react swiftly and consistently to neurotransmitter released from the overlying nerve terminal. Acetylcholine receptors are highly concentrated which ensures that the postsynaptic response to acetylcholine is sufficient to initiate an action potential. Indeed, the concentration of acetylcholine receptors is 1000-fold higher under the nerve terminal as compared to the extrasynaptic membrane (Shyng and Salpeter, 1989). Two signalling molecules, agrin and neuregulin, are concentrated in the post synaptic membrane. There is good evidence that both mediate the clustering of acetylcholine receptors at the neuromuscular synapses (Bowe and Fallon, 1995, Chu *et al.* 1995, Gautam *et al.* 1996, Meier *et al.* 1997).

The postsynaptic membrane of the muscle fibre is further invaginated into about 1 μm deep junctional folds known as secondary synaptic clefts (Sanes and Lichtman, 1999). In the crests and part way down the sides of these folds acetylcholine receptors are concentrated, whereas voltage-gated sodium channels and the neural cell adhesion molecule (N-CAM) are concentrated in the depths of the folds (Covault and Sanes, 1986, Flucher and Daniels, 1989). Although the function

of the postsynaptic folds remain to be defined, they are thought to amplify the effects of postsynaptic currents. Wood and Slater (1997) studied the contribution of this effect to the "safety factor" for neuromuscular transmission. They defined the safety factor as the ratio of the normal quantal content of transmitter released per nerve impulse to the number of quanta required to reach threshold. Normally at the NMJ, the excitatory effect of nerve stimulation on the muscle fibre is greater than that required to generate an action potential. Hence, a margin of safety is established for times when neuromuscular transmission is under stress for example, during high frequency activity.

Not only the postsynaptic folds but also the voltage-gated sodium channels contained within them make an important contribution to the high safety factor. There is a high density of voltage-gated sodium channels at the NMJ and this facilitates action potential generation by reducing the amount of depolarization required to trigger an action potential (Wood and Slater, 1995). A high density also exists in the perijunctional region, and here the channels presumably ensure the initial spread of depolarization (Le Treut *et al.* 1990, Boudier *et al.* 1992).

The postsynaptic folds also include an elaborate cytoskeletal apparatus. The cytoskeletal proteins, rapsyn, utrophin and α-dystrobrevin-1 are colocalized with acetylcholine receptors at the top of the folds, while ankyrin, β-spectrin, α -dystrobrevin-2, and dystrophin are most concentrated in the depths of the postjunctional folds along with voltage-gated sodium channels (Sealock *et al.* 1984, Flucher and Daniels, 1989, Bewick *et al.* 1996, Wood and Slater, 1998, Peters *et al.* 1998). The functions of the cytoskeletal elements are likely to be generation of the postsynaptic folds as well as maintenance of the different areas within them (Sanes

and Lichtman, 1999). Utrophin is implicated in stabilising acetylcholine receptors at the tops of the postsynaptic folds (Apel and Merlie, 1995, Sanes *et al.* 1998), rapsyn is involved in the clustering of acetylcholine receptors at the synapse (Gautam *et al.* 1995, Sanes *et al.* 1998), whilst β-spectrin and ankyrin help to maintain the concentration of voltage-gated sodium channels at the NMJ (Wood and Slater, 1998).

Dystrophin, which has been detected with increased expression at neuromuscular and myotendinous junctions, is also found in the sarcolemma of skeletal muscle fibres (Zubrzycka-Gaarn *et al.* 1988, Arahata *et al.* 1988, Bonilla *et al.* 1988, Carpenter *et al.* 1990, Zubrzycka *et al.* 1991, Byers *et al.* 1991). It binds cytoskeletal protein actin, and a complex of four transmembrane glycoproteins at its N- and C- terminal domains, respectively (Koenig *et al.* 1988). The glycoprotein complex associates with an extracellular component named α-dystroglycan, a peripheral membrane protein known to bind laminin (Ibraghimov-Beskrovnaya *et al.* 1992, Ervasti and Campbell, 1993, Sunada *et al.* 1994, Pall *et al.* 1996). The role of the dystrophin-glycoprotein complex is believed to be one of linking the actin-based, cortical cytoskeleton with the extracellular matrix (Ervasti and Campbell, 1993).

Schwann cell processes cap the nerve terminal and insulate the neuromuscular junction from the external environment. The basal lamina which ensheaths each muscle fibre, passes through the synaptic cleft and extends into the junctional folds. Apart from the major components of muscle basal lamina, synaptic basal lamina also contains a collagen-tailed form of acetylcholinesterase (Krejci *et al.* 1997), a set of glycoconjugates (Scott *et al.* 1988), and as previously mentioned, the two signalling molecules agrin and neuregulin (Sanes and Lichtman, 1999). Although highly concentrated in junctional basal lamina, acetylcholinesterase is

present in a lower concentration throughout the length of muscle fibres (Sketelj, 1997). It is an essential component of the NMJ as it is responsible for the rapid hydrolysis of acetylcholine released from presynaptic nerve terminals (Brank *et al.* 1998).

1.4.2 Muscle fibre types and the NMJ

Studies of the NMJ in fast- and slow-twitch muscles have demonstrated a number of differences. The safety factor for neuromuscular transmission is higher in fast than in slow-twitch muscles (Gertler and Robbins, 1978, Wood and Slater, 1997). In comparison with NMJs in slow-twitch muscles, those in fast muscles are reported to have a higher quantal content of transmitter released per nerve impulse (Tonge, 1974, Gertler and Robbins, 1978, Wood and Slater, 1997), more functional acetylcholine receptors (Sterz *et al.* 1983), a higher density of voltage-gated sodium channels in both junctional and extrajunctional regions, (Milton *et al.* 1992, Ruff, 1992), and a different organization of postsynaptic folds such that they are shorter, broader and sparser in slow- than in fast-twitch muscles (Padykula and Gauthier, 1970, Ellisman *et al.* 1976, Ogata, 1988). The total acetylcholinesterase activity per unit of muscle weight is also higher in fast than in slow muscles (Groswald and Dettbarn, 1983, Sketelj *et al.* 1992). Each of these factors contribute to enhanced efficiency of neuromuscular transmission which is greater in fast-twitch than slow-twitch muscles (Wood and Slater, 1997).

1.5 DENERVATION

Denervation of skeletal muscle results in a number of changes in the structure and properties of muscle fibres. Animal models of denervation have provided a significant amount of data concerning these changes and as a result have contributed to the understanding of nerve muscle interrelationships. The prevalent features of skeletal muscle following denervation are discussed.

1.5.1 Muscle weight

The most prominent morphological change in denervated muscle is atrophy which reduces the gross bulk of the muscle and the dimension of its constituent fibres (Tower, 1939). The loss of muscle mass tends to be rapid initially, the process then slows and a relatively stable state is reached. Gutmann (1948) noted a marked reduction in muscle mass in the first two months following denervation of the tibialis anterior muscle in the rabbit and thereafter there was little change. There is general agreement that the loss of muscle mass is most marked within the first two months of denervation (Sunderland and Ray, 1950).

1.5.2 Muscle fibre size and shape

The loss in muscle bulk has been attributed to a reduction in muscle fibre cross-sectional area rather than to fibre degeneration (Vrbová, 1995). Fibre degeneration is defined as a disintegration of individual muscle fibres with phagocytosis (Vrbová, 1995), as apposed to atrophy, where there is a reduction in the size of the muscle fibre without disturbing its form (Tower, 1939). However in long

term denervated muscle fibre degeneration may occur (Tower, 1935, Gutmann, 1945, Anzil and Wernig, 1989, Schmalbruch *et al.* 1991, Lu *et al.* 1997).

The decrease in muscle fibre size and associated loss of sarcoplasm is described by Sunderland and Ray (1950) as the "earliest and most characteristic change in the morphology of denervated fibre". Sunderland and Ray (1950) suggest that the fibre "calibre" is a more accurate measure of the effects of denervation rather than the weight of the muscle. In their studies on the Australian opossum it was apparent that after comparing the loss in weight of the entire muscle and the rate and extent of the fibre atrophy (assessed in this study by measuring fibre cross-sectional area), the muscle fibres had suffered more severely than the loss in weight of the muscle indicated. This difference was accounted for by an increase in the connective tissue content of the muscle which compensates to some degree for the greater atrophy of the fibre.

In transverse sections the denervated fibres lose their polygonal shape and as atrophy proceeds they become more round or oval in shape. Angular fibres may develop particularly if the atrophic fibres become compressed in the narrow spaces between other fibres.

Denervation-induced atrophy appears to be largely due to an elevation in protein degradation with an accompanying reduction in muscle protein mass (Goldspink, 1976, Goldspink *et al.* 1983). Indeed, an increase in activity of many proteases has been noted following denervation (Mantle *et al.* 1992, Haycock *et al.* 1996). It is the lysosomes which seem to be recruited as part of this protein destruction (Tischler *et al.* 1990). Weinstein *et al.* (1997) studied the mechanism of accelerated proteolysis in denervated rat soleus muscles. The soleus was denervated

by severing either the tibial nerve (proximal, short stump) or sciatic nerve (distal, long stump). After 48 and 72 hours, denervation resulted in a decline in protein content, an increase in *in vitro* protein degradation, and a decrease in lysosomal latency, all of which were greater in proximally denervated muscles. It was hypothesized that a neurotrophic factor released by the nerve may attenuate protein breakdown by lowering lysosomal proteolysis. Hence, a longer nerve stump will delay atrophy by decelerating protein degradation and increasing lysosomal latency when compared with a short nerve stump (Weinstein *et al.* 1997).

Boudriau *et al.* (1996) investigated the effect of denervation-induced atrophy on the cytoskeletal lattice in rat gastrocnemius (fast-twitch) and soleus (slow-twitch) muscles. The fast-twitch gastrocnemius was found to be less affected by denervation. Furthermore, the relative cellular content of the two structural proteins, dystrophin and desmin, were reduced in the soleus muscle, while significant increases were shown in the gastronemius muscle. This increase is to possibly maintain the cellular integrity during rapid and progressive atrophy.

The significant difference in membrane skeleton between the soleus and gastrocnemius muscles may explain the differences in the susceptibility of fibre types to atrophy. However, using the denervation-induced atrophy model, several investigators have observed a greater degree of atrophy in type I fibres rather than type II fibres (Bajusz, 1964, Engel *et al.* 1965, Karpati and Engel, 1968a,b, Guth *et al.* 1971, Gauthier and Dunn, 1973, Tomanek and Lund, 1973, Neiderle and Mayr, 1978, Lu *et al.* 1997, Viguie *et al.* 1997). However, other studies suggest that the response of type I fibres to denervation is not the same in different skeletal muscles. Atrophy of type I fibre has been reported to be less pronounced in fast muscles than

in slow muscles (Tomanek and Lund, 1973, Jaweed *et al.* 1975). By contrast, Romanul and Hogan (1965) found little difference when comparing denervation atrophy of type I fibres in the slow-twitch soleus and the fast-twitch gastrocnemius.

In different portions of one and the same muscles, type I fibres can exhibit different behaviour after denervation. Guth *et al.* (1971) observed an equal response to denervation of all muscle fibre types in the superficial portion of cat gastrocnemius muscle, whereas in the deep portion of the same muscle, the atrophy of type II fibres exceeded that of type I fibres.

1.5.3 Muscle fibre types

Denervation may bring about a conversion in fibre type. Windisch *et al.* (1998) observed that after denervation of the EDL muscle in the rat, the percentage of type IIB fibres slowly declined to less than 10%, while type IIX fibres disappeared. Corresponding to these changes, the percentage of type IIA fibres gradually increased from 25 to 90% and remained at the higher value, while the percentage of type I fibres remained low at less than 5%.

The alteration in fibre type could be due to a change in muscle activity brought about by denervation. Following denervation, the EDL is no longer subject to the high frequency, low amount activity which is typical of many EDL motor units (Hennig and Lømo, 1985), but instead receives low frequency fibrillatory activity that can be significant (Purves and Sakmann, 1974). Furthermore, when the denervated EDL muscle was stimulated electrically with a "slow" pattern resembling the activity in soleus motor units, the type IIB and the type IIX fibres disappeared,

while the percentages of type IIA and type I fibres increased to around 75 and 20%, respectively. The stimulation of innervated EDL muscles had a similar effect on transformation of fibre types. Although these effects of electrical muscle stimulation are specifically different from those of denervation alone, both bring about a change in activity of the denervated muscle which results in a transformation of fibre types (Windisch *et al.* 1998).

Studies of innervated muscle have shown that the alteration in muscle activity and subsequent change in fibre type is associated with transitions in MHC expression at the mRNA level (Jaschinski *et al.* 1998). However, the denervation effects on myosin heavy chain (MHC) expression may also vary according to muscle type and species. In rat slow muscles, denervation causes a shift to faster isoforms (MHC I to MHC IIA/X), while in fast muscles, denervation causes a shift to slower isoforms (MHC IIB/X to MHC I/IIA) (Jakubiec-Puka *et al.* 1990, Haddad *et al.* 1997, Midrio *et al.* 1998). However, in the slow twitch semimembranous proprius muscle of the rabbit, the predominant MHC I isoform pattern was maintained after denervation (Bacou *et al.* 1996). After denervation of the fast twitch semimembranous accessorius, minor forms of MHC I and IIA disappeared but the proportions of MHC IIB and MHC IIX gradually shifted from a predominance of MHC IIB to MHC IIX (Bacou *et al.* 1996). Most authors however, agree that in fast twitch muscle of both rat and rabbit, MHC IIB isoforms are the most susceptible to lack of innervation (Jakubiec-Puka *et al.* 1990, Bacou *et al.* 1996, Haddad *et al.* 1997, Shiotani and Flint, 1998). Reexpression of embryonic and neonatal MHC has also been detected in denervated muscle fibres (Cerny and Bandman, 1987, Schiaffino *et al.* 1988, Bacou *et al.* 1996, Haddad *et al.* 1997, Jakubiec-Puka *et al.* 1990, Midrio *et al.* 1998).

1.5.4 Nuclei

In the initial stages of denervation, nuclei usually remain in a peripheral position and some may become enlarged and vesicular in appearance (Jennekens, 1992). As atrophy proceeds and the cross-sectional areas of the muscle fibres decrease, central nucleation becomes increasingly prominent (Tower, 1935, Bowden and Gutmann, 1944, Pelegrino and Franzini, 1963, Stonnington and Engel, 1973, Tomanek and Lund, 1973, Snow, 1983, Anzil and Wernig, 1989, Schmalbruch *et al.* 1991, Lu *et al.* 1997.

Viguie *et al.* (1997) completed a detailed study of nuclear number and cytoplasmic volume on isolated muscle fibres in the rat extensor digitorum longus muscle after long term denervation. Results of their study clearly showed that after denervation the loss of myonuclei is preceded by a significant loss of cytoplasmic volume. The mean cytoplasmic volume/millimetre of muscle fibre declined dramatically over the denervation period when compared with normal controls. Cytoplasmic volume declined to 4% and 2% of control values by 4 and 7 months, respectively, following denervation. The number of nuclei also declined as a function of the time of denervation. Mean nuclear numbers per millimetre of fibre decreased from 34 ± 5 in control muscle to 22 ± 2 at 2 months and 11 ± 2 at 7 months of denervation. The number of nuclei per muscle fibre in normal EDL muscle was approximately 410 nuclei however, this declined by 36% at 2 months and 68% at 7 months of denervation. The reduction in nuclei number was correlated with an increased incidence of nucleus-free regions of cytoplasm alternating with clusters of nuclei. No explanation for this clumping could be offered by Viguie *et al.* (1997).

1.5.5 Ultrastructural change - satellite cells

Accompanying the pronounced decline in the number of myonuclei per muscle fibre Viguie *et al.* (1997) noted that during the first 2 months following denervation there was a threefold increase in satellite cells over control levels. A number of other investigators have observed an increase in the percentage of satellite cells after denervation (Tomanek and Lund, 1973, Ontell, 1974, Hanzlikova *et al.* 1975, McGeachie and Allbrook, 1978, Murray and Robbins, 1982, Snow, 1983, Anzil and Wernig, 1989, Rodrigues and Schmalbruch, 1995).

Earlier reports (Lee, 1965, Hess and Rosner, 1970, Ontell, 1974) suggested that the increase in satellite cells may be the result of sequestrated myonuclei from atrophying muscle fibres. Other studies however, disprove this process and show that denervation leads to activation and proliferation of preexisting satellite cells (McGeachie and Allbrook, 1978, Schultz, 1978, Larocque *et al.* 1980, Snow, 1983, Lu *et al.* 1997). Lu *et al.* (1997) reported morphological evidence of activation of individual satellite cells by 2 months of denervation. They observed elongated satellite cell processes extending between the muscle fibre and the surrounding basal lamina. Furthermore, some muscle fibres appeared to have the morphology of early regenerating fibres.

The newly formed satellite cells may fuse with their affiliated muscle fibres (McGeachie, 1989, Lu *et al.* 1997), move away from the muscle fibres and fuse to form new myotubes in the interstitium, (Konigsberg *et al.* 1975, Schultz, 1978, Snow, 1983, Lu *et al.* 1997), or form new myotubes in association with atrophic muscle fibres (Anzil and Wernig, 1989, Schmalbruch *et al.* 1991). The reaction of

satellite cells to denervation may prepare the muscle for ensuing reinnervation but it cannot amend the severe atrophy that results from the absence of innervation.

During long term denervation the satellite cell numbers steadily decline (Rodrigues and Schmalbruch, 1995, Viguie *et al.* 1997) or may eventually be depleted (Anzil and Wernig, 1989). The satellite cells however, are still capable of substantial recovery upon reinnervation (Irintchev *et al.* 1990) or stimulation (Al-Amood *et al.* 1991, Schmalbruch *et al.* 1991). The basis for the decline in satellite cell numbers after prolonged denervation remains to be defined. A number of options exist including the possibility that particular satellite cells die without being replaced in long term denervated muscle. Another possibility is that the rate at which satellite cells are incorporated into atrophying or newly forming muscles exceeds the rate at which they are formed.

1.5.6 Further ultrastructural changes

A number of other ultrastructural changes in denervated muscles have been observed. During the first 2 weeks after nerve section there is loss of fibrils at the periphery of muscle fibres (Pellegrino and Franzini, 1963) and by 1 month of denervation, myofibrils in the fibre interior have atrophied (Engel and Stonnington, 1974). Other changes during the first two weeks of denervation include "streaming" of the Z discs, disarrangement of the contractile material although this does not occur in more than two or three sarcomeres at a time, and the appearance of small autophagic vacuoles and lipofuscin granules (Pellegrino and Franzini, 1963, Engel and Stonnington, 1974, Schmalbruch *et al.* 1991).

Accompanying these changes is proliferation or vesiculation of the sarcoplasmic reticulum with a somewhat disordered spatial arrangement (Pellegrino and Franzini, 1963, Muscatello *et al.* 1965, Tomanek and Lund, 1973, Engel and Stonnington, 1974, Anzil and Wernig, 1989). Transverse tubules become disoriented and displaced and the associated triads become pentadic in structure (Pellegrino and Franzini, 1963, Tomanek and Lund, 1973, Lu *et al.* 1997).

With increasing denervation times highly disorganized arrays of filaments are common (Pellegrino and Franzini, 1963, Gauthier and Dunn, 1973, Schmalbruch *et al.* 1991, Lu *et al.* 1997). However, there can still be dramatic differences in sarcomere structure and cytoplasm appearance between adjacent muscle fibres. Even at 18 months of denervation Lu *et al.* (1997) could still find some well-ordered sarcomeres.

Folding of the sarcolemma occurs throughout the denervation process and in severely affected fibres, the sarcolemma may become indistinct or disappear and the fibre may appear fragmented (Tomanek and Lund, 1973). Clefts or indentations of the sarcolemma eventually pinch off sarcoplasmic portions and give rise to appendages from the parent fibre (Pellegrino and Franzini, 1963, Tomanek and Lund, 1973). These appendages may contain products of degeneration such as lysosomes, degenerative mitochondria, fibril fragments, dense granules, myelin figures or membrane fragments (Tomanek and Lund, 1973).

During the course of denervation mitochondria decrease in size (Tomanek and Lund, 1973, Engel and Stonnington, 1974, Lu *et al.* 1997) and become more globular in shape (Tomanek and Lund, 1973, Lu *et al.* 1997). The mitochondrial matrix loses its normal density (Tomanek and Lund, 1973, Lu *et al.* 1997), and the

cristae either disappear (Tomanek and Lund, 1973), or are sparsely arranged (Pellegrino and Franzini, 1963, Lu *et al.* 1997). There is also a reorientation of mitochondria as normally they are elongated in the transverse plane whilst after denervation their long axis is parallel to the long axis of the myofibril. A possible explanation for this change is that the three-dimensional arrangement of mitochondria is affected by the contractile activity of the myofibrils (Engel and Stonnington, 1974).

Macrophages in extracellular areas but in close continuity with the sarcolemma have also been observed in denervated muscle (Pellegrino and Franzini, 1963, Tomanek and Lund, 1973). Anzil and Wernig (1989) however, observed an occasional macrophage engulfing a muscle fibre after entering its basal lamina sheath. The macrophages involved in the response are mainly resident histiocytes, as prior labelling of white cells in the bone marrow and elsewhere show that leukocytes are not found in denervated muscles in any substantial numbers (Murray and Robbins, 1982).

Denervation results in a pronounced increase in the number of fibroblasts in the connective tissue between muscle fibres (McGeachie and Allbrook, 1978, Murray and Robbins, 1982a,b). Following denervation these connective tissue cells make up the largest class of dividing cells and are found throughout the muscle (Murray and Robbins, 1982b). Their association with fibrosis in long-term denervated muscles has been acknowledged for some time (Gutmann and Young, 1944, Zak *et al.* 1969, Murray and Robbins, 1982a,b).

1.5.7 Connective tissue

An increase in the amount of interstitial connective tissue or fibrosis in long-term denervated muscles has been noted by a number of authors (Tower, 1935, Bowden and Gutmann, 1944, Sunderland and Ray, 1950, Pellegrino and Franzini, 1963, Hogenhuis and Engel, 1965, Tomanek and Lund, 1973, Savolainen *et al.* 1988, Al-Amood *et al.* 1991, Lu *et al.* 1997). It has been observed in the epimysium, the perimysium, and the endomysium regions of the muscle (Hogenhuis and Engel, 1965, Lu *et al.* 1997). With the increase in interstitial collagen this may surround the smaller blood vessels with substantial layers of thick collagen and consequently isolate them from the muscle fibres. In long term denervated muscles residues of degenerated capillaries have been observed (Borisov *et al.* 1996, Lu *et al.* 1997). The thick deposition of collagen fibres around both muscle fibres and fascicles following prolonged denervation may also act as a physical barrier to nerve ingrowth if reinnervation does occur (Lu *et al.* 1997). Indeed muscle fibre degeneration has also been reported by a number of observers (Tower, 1935, Chor *et al.* 1937, Anzil and Wernig, 1989, Schmalbruch *et al.* 1991, Lu *et al.* 1997). After prolonged denervation the ultrastructural changes may worsen to a point where the fibre degenerates and eventually all trace of the original muscle fibre structure is lost. At the end stage of degeneration the muscle fibres are replaced to a greater or lesser degree by connective tissue and fatty tissue depending on animal species and muscles. Dulor *et al.* (1998) noted that in the rabbit, and after two months of denervation, muscles were transformed into a white type adipose tissue characterized by the expression of a specific gene. Furthermore the extent of this transformation was dependent on the

fibre type as the constitution of the fast-twitch muscles were mostly adipose tissue, whilst for the slow-twitch muscles, the transformation was incomplete and slower.

1.5.8 Adhesive macromolecules

Shortly following denervation and preceding atrophy, four adhesive macromolecules: neural cell adhesion molecule (N-CAM), tenascin (J1) glycoprotein, fibronectin and a matrix-associated heparan sulfate proteoglycan (M-HSPG), accumulate in the interstitial spaces near synaptic sites (Covault and Sanes, 1985, Sanes *et al.* 1986, Gatchalian *et al.* 1989). N-CAM is normally present on the surface of embryonic myotubes but as development proceeds its levels decrease and in adult muscle, concentrate near neuromuscular junctions (Covault and Sanes, 1985, Gatchalian *et al.* 1989). Fibronectin and M-HSPG are normally abundant in the basal lamina of muscle fibres, whilst tenascin (J1) is undetectable (Sanes *et al.* 1986). After denervation the four adhesive macromolecules (tenascin (J1) glycoprotein, N-CAM, fibronectin and M-HSPG) accumulate in perisynaptic interstitial spaces. This coordinated accumulation is thought to provide an attractive substrate for regenerating axons as each of these molecules are known to be capable of interacting with neurons (Covault *et al.* 1987, Gatachalian *et al.* 1989). Fibroblasts selectively accumulating in perisynaptic interstitial spaces from two days after denervation are likely to be the cellular source of these four adhesive molecules (Gatchalian *et al.* 1989).

1.5.9 Muscle membrane

Various properties of the muscle membrane are altered as a result of denervation. First, there is an almost immediate fall in resting membrane potential, although the degree of change is small as it is just a few millivolts (Albuquerque *et al.* 1971, Card, 1977a, Kirsch and Anderson, 1986). This continues over the first few days before recovering a little after approximately three weeks (Kirsch and Anderson, 1986). The fall in resting membrane potential is first seen at the endplate and spreads gradually to the rest of the muscle fibre (Albuquerque *et al.* 1971). However, by three days after nerve injury, there is complete failure of both neuromuscular transmission and nerve conduction (Miledi and Slater, 1970).

The length of nerve stump left attached to the muscle influences the time at which changes in membrane properties occur. The longer a nerve stump, the later such changes develop (Slater, 1966, Harris and Thesleff, 1971, Guth *et al.* 1981). Also dependent on nerve stump length are nerve terminal degeneration (Ribchester *et al.* 1995) and decline of acetylcholine release (Card, 1977b).

The acetylcholine receptors (AChR), which are normally confined to the endplate region of adult muscle fibres, are found over the entire muscle surface shortly after denervation (Axelsson and Thesleff, 1957, Miledi, 1960a, Albuquerque and McIsaac, 1969, Albuquerque *et al.* 1971, Dreyer and Peper, 1974, Guth *et al.* 1981). However, the endplates lose their AChRs after denervation. A period of denervation of up to two months reduced the number of endplate AChRs by 65% (Andreose *et al.* 1995). Indeed, Andreose *et al.* (1995) hypothesized that nerve-derived trophic factors and the evoked muscle activity act together to control

the number of junctional AChRs. They suggested that trophic factor is released by impulse activity in the nerve, and following denervation, is degraded. This process of degradation is also accelerated by the acute effects of nerve degeneration.

Motor terminals do degenerate rapidly after nerve section, and it has been hypothesized that this is due to the axotomized neuronal cell bodies being no longer able to provide their terminals with the cellular components required for synaptic transmission and function (Albuquerque *et al.* 1972, Perisic and Cuneod, 1972, Hudson *et al.* 1984). However, Ribchester *et al.* (1995) suggest that the trigger for motor nerve terminal degeneration after nerve section is more complex, and it cannot be accounted for simply by this suggestion that motoneurone cell bodies fail to provide essential maintenance factors for their terminals. Their data support the view that nerve section activates cellular processes already present, but latent, in motor nerve terminals.

The increase in extrasynaptic AChRs following denervation results from a rise in the transcription of AChR subunit genes by extrasynaptic nuclei (Merlie *et al.* 1984, Tsay and Schmidt, 1989). Both innervation and electrical stimulation dramatically down-regulate the expression of AChR subunit genes (Eftimie *et al.* 1991, Witzemann and Sackmann, 1991, Huang *et al.* 1994). This control of AChR gene expression is linked to protein kinase C (Klarsfeld *et al.* 1989, Huang *et al.* 1992). It participates in the signalling pathway, connecting electrical membrane activity to the inhibition of AChR subunit genes. Influx of extracellular calcium is required to activate protein kinase C and is an essential component of the membrane depolarization-receptor gene inactivation process (Huang *et al.* 1992). The targets of protein kinase C are the myogenic factors. These are transcriptional activators

required for initiation of the myogenic program which includes the initial induction of AChR subunit genes (Rudnicki and Jaenisch, 1995). The myogenic factors (MyoD, My-5, MRF4 and myogenin) all bind to a number of critical sites in AChR subunit genes (Bessereau *et al.* 1998).

Rapsyn, a 43 kDa peripheral membrane protein which is largely concentrated at neuromuscular synapses and closely associated with the AChRs, is increased after denervation (Baldwin *et al.* 1988, Froehner, 1989, Wang *et al.* 1999). Although the function of the rapsyn is not known, it is thought to play some role in anchoring or stabilizing AChR at synaptic sites (Walker *et al.* 1984, Bloch and Manow, 1989, Froehner, 1986, Wang *et al.* 1999). Denervation of adult muscle also leads to an increase in levels of Myogenin and MyoD. These are two proteins that bind to the regulatory proteins of a number of skeletal muscle genes and activate their transcription. Indeed, both myogenin and MyoD interact with the enhancer of the AChR α-subunit gene. Following denervation the increase in myogenin and MyoD mRNA levels precedes the rapid accumulation of AChR α-subunit transcripts (Eftimie *et al.* 1991, Witzemann and Sakmann, 1991).

There is a marked decrease in acetylcholinesterase enzyme activity after denervation (Bacou *et al.* 1982, Collins and Youkin, 1982, Lømo *et al.* 1985, Michel *et al.* 1994). These changes in enzyme activity are paralleled by similar decreases in acetylcholinesterase transcript levels, and a disappearance of the selective accumulation of acetylcholinesterase mRNAs within the postsynaptic sarcoplasm (Michel *et al.* 1994).

1.6 REINNERVATION

1.6.1 Time

The process of reinnervation will vary according to the time which elapses between nerve injury and the arrival of regenerating axons at the muscle. Reinnervation is most effective if it occurs shortly after the nerve has been injured (Gutmann and Young, 1944, Gutmann, 1948). However, in the case of delayed (as opposed to immediate) nerve repair or injuries, poor functional recovery of muscle is often observed.

The basis for this poor functional recovery after delayed nerve repair is not well understood. It has generally been attributed to the inability of denervated muscle to accept reinnervation and recover from denervation atrophy (Gutmann and Young, 1944, Gutmann, 1948, Irintchev *et al.* 1990). There may be other contributing factors such as a reduced ability of motoneurones to regenerate their axons after prolonged axotomy (Fu and Gordon, 1995a), and loss of trophic support for regenerating axons in long term denervated distal stumps (Li *et al.* 1997, You *et al.* 1997, Terenghi *et al.* 1998).

Fu and Gordon (1995b) studied the effects of prolonged denervation on nerve regeneration and muscle reinnervation. They established that the major contributing factor to poor recovery after long term denervation is a significant reduction in the number of axons that successfully regenerate through deteriorating intramuscular nerve sheaths, the normal pathways for axonal regeneration. This progressive deterioration in the ability of intramuscular nerve sheaths to support regenerating axons back to long term denervated muscle fibres may be explained by a number of

factors. A possibility is a reduction in the number of Schwann cells to a level at which adequate substrate and trophic support is no longer available (Weinberg and Spencer, 1978, Pellegrino and Spencer, 1985, Salonen *et al.* 1987, Li *et al.* 1997, You *et al.* 1997). The collagenization of the endoneurial tubes after prolonged denervation may also obstruct the axonal regeneration (Sunderland and Bradley, 1950a,b). Another contributing factor to poor functional recovery is the failure of reinnervated muscle fibres to resume their normal size (Gutmann, 1948, Irintchev *et al.* 1990, Fu and Gordon, 1995b). The endomysial fibrosis and connective tissue proliferation associated with long term denervation, may physically limit the size of muscle fibres (Gutman and Young, 1944, Savolainen *et al.* 1988, Fu and Gordon, 1995 b).

With prolonged denervation the regenerating axons escape from the deteriorating intramuscular nerve sheaths and grow directly on the surface of denervated muscle fibres (Gutmann and Young, 1944, Fu and Gordon, 1995b). The regenerating neurites are guided along denervated muscle by Schwann cells that migrate from the proximal nerve stump (Son and Thompson, 1995a), and the adhesive surfaces of the denervated muscle fibres (Covault *et al.* 1986). However, few of these regenerating axons succeed in reinnervating denervated muscle fibres as the distances over which they can grow is limited (Fu and Gordon, 1995b).

1.6.2 Type of nerve injury

The accuracy of muscle reinnervation is dependent on the type of nerve injury. After a crush injury, because the basal lamina surrounding each nerve fibre is

not cut (Haftek and Thomas, 1968, Richardson and Thomas, 1979, Swett *et al.* 1991), both accuracy and muscle recovery are excellent because regenerating axons elongate within their original endoneurial tubes to innervate their original muscle fibres. After a nerve is cut and repaired, the regenerating axons show little specificity for their original target muscles (Weiss and Hoag, 1946, Bernstein and Guth, 1961, Miledi and Stefani, 1969, Gordon and Stein 1982a,b, Thomas *et al.* 1987, Rafuse and Gordon, 1996a,b). This is probably due to loss of the "intrinsic guidelines" provided by intact basal lamina or endoneurial tubes. As a result, axons enter incorrect parent endoneurial tubes that guide them to inappropriate muscles.

The type of injury however, does not influence the capacity of regenerating motor nerves to form enlarged motor units. In reinnervation of partially denervated muscle, motor units enlarge by sprouts emerging from either the nodes close to the endplates or from the terminal regions of the axons (Brown *et al.* 1981). Provided 15 to 20% of the normal complement of motor units remains, the force of partially denervated muscle recovers completely (Thompson and Jansen, 1977, Brown and Ironton, 1978, Gorio *et al.* 1983, Fisher *et al.* 1989, Yang *et al.* 1990, Rafuse *et al.* 1992, Gordon *et al.* 1993). After severe nerve injury or delayed nerve repair, the number of regenerating axons is reduced (Fu and Gordon, 1995a). However, the regenerating motor axon can sprout and make functional connections with approximately three to five times the original number of muscle fibres it normally supplies (Rafuse and Gordon, 1996a).

1.6.3 Size of the motor axon

The size of the motor axon governs the number of muscle fibres it supplies, so a large axon will reinnervate more fibres than a small axon. However provided that during the process of reinnervation the axons regenerate within "the distal nerve sheaths", the normal relationship between axon size and contractile properties of motor units will be retained (Rafuse and Gordon, 1998). Hence, this means that the normal order of recruitment of motor units is maintained as in accordance with the size principle, motor units are recruited according to size and force (Cope *et al.* 1991).

1.6.4 Muscle fibre type

The cross-sectional areas of reinnervated muscle fibres recover to normal values and increase according to type in the normal manner so that type I < type IIa < type IIb, after crush injuries (Rafuse and Gordon, 1996a), whilst after nerve section, muscle fibres within reinnervated motor units may vary more widely in size than normal (Tötösy de Zepetnek *et al.* 1992a, Rafuse and Gordon, 1996a, Rafuse and Gordon, 1998). This apparent heterogeneity is indicative of limited conversion of the reinnervated muscle fibres and suggests that muscle fibre type is not entirely neurally regulated. Indeed, several other studies have indicated that neural conversion of muscle fibre properties is incomplete (Dum *et al.* 1985a,b, Gordon *et al.* 1986, Foehring *et al.* 1986a,b, Gillespie *et al.* 1987, Gordon *et al.* 1988, Cope *et al.* 1991, Tötösy de Zeptnek *et al.* 1992 b, Unguez *et al.* 1995, Rafuse and Gordon, 1998).

What limits the complete neural conversion of muscle fibre properties following reinnervation is not known. A possible explanation is that once denervated, the contractile and metabolic properties of muscle fibres are less conducive to neural control. There may also be intrinsic differences between different muscle fibre types that reduce the extent to which their properties can be modulated by the innervating motoneurone. Fast and slow myotubes arise from distinct and committed myoblast populations and express specific fibre type features that are independent of innervation (Miller and Stockdale, 1987, Stockdale, 1992, Donoghue and Sanes, 1994). Hence, fibre type depends on lineage as well as innervation (DiMario and Stockdale, 1997). The incomplete conversion of muscle fibre properties may also be due to a feature not of the muscle fibre themselves, but due to an inadequacy of the injured motoneurones to respecify contractile and metabolic properties of the muscle fibres (Rafuse and Gordon, 1998).

1.6.5 The NMJ

Following denervation the terminal Schwann cells which cover NMJs, sprout dramatically and produce elaborate processes (Reynolds and Woolf, 1992). During reinnervation in partially denervated muscle, the motor axons use these processes as guides as they return to synaptic sites. In doing so, the motor axons escape the confines of endplates and grow between them to reach denervated synaptic sites (Son and Thompson, 1995a,b, Son *et al.* 1996, Trachtenburg and Thompson 1996, 1997, Love and Thompson, 1998). Partial denervation induces division of terminal

Schwann cells at innervated, but not denervated endplates (Love and Thompson, 1998).

In partially denervated muscle, when regenerating axons return, they may reestablish connections with muscle fibres that are already innervated by sprouts and thus, this results in polyneuronal innervation (Guth, 1962, McArdle, 1975, Brown and Ironton,1978, Thompson, 1978, Taxt, 1983, Ribchester and Taxt, 1984, Ribchester, 1988, Barry and Ribchester, 1995). There is a subsequent competitive elimination of supernumerary terminals which closely resembles that seen in neonatal muscles during postnatal development (Redfern, 1970, Brown *et al.* 1976, Betz *et al.* 1979, Bixby, 1981, Fladby, 1987). This phenomenon is also observed in reinnervation of denervated muscle (Jansen and Van Essen, 1975, Rothshenker and McMahan, 1976, Gorio *et al.* 1983, Rich and Lichtman, 1989). How the surviving axon collaterals and terminals are selected is not known, but hypotheses include some function of endogenous proteases (Vrbová *et al.* 1988), competition for limited neurotrophic resources (Purves, 1988), and competition for space due to a limited distribution of AChRs and/or adhesion molecules (Ribchester and Barry, 1994, Ngyuyen and Lichtman, 1996). There is some involvement of neuromuscular activity as the rate of synapse elimination is reduced in paralysed neonatal muscle and accelerated in stimulated muscle (O'Brien *et al.* 1978, Thompson *et al.* 1979, Thompson, 1983, Barry and Ribchester, 1995, Costanzo *et al.* 1999, 2000). Selective chemical matching of motoneurone and muscle fibre types may also influence the outcome (Jones *et al.* 1987, Thompson *et al.* 1987, Fladby and Jansen, 1990, Gates and Ridge, 1992). There has been some debate as to whether the nerve terminals undergoing synapse elimination regress as a result of a process similar to

degeneration of terminals, as with that seen after nerve injury. However, Parson *et al.* (1997) found that Wallerian degeneration and synapse elimination occur by distinct and different mechanisms. They also observed that motor terminals have some autonomy from their cell bodies as they were able to support polyneuronal synaptic input after peripheral nerve section. Indeed, Parson *et al.* (1997) suggest that the rate and outcome of synapse elimination is determined more by local factors in the area of the competing terminals.

As described previously, following denervation the muscle membrane becomes more sensitive to acetylcholine outside the endplate region. However, the AChRs at the old motor endplate are remarkably stable and do not disappear, so this region remains the most sensitive area of the muscle fibre (Miledi, 1960a, McArdle and Albuquerque, 1973). The regenerating axons form their new synapses at these original motor endplates (Gutmann and Young, 1944, Letinsky *et al.* 1976, Rotshenker and McMahon, 1976, Sanes *et al.* 1978, Rich and Lichtman, 1989). The synaptic basal lamina contains cues that guide this selective reinnervation of synaptic sites (Sanes *et al.* 1978, Glicksman and Sanes, 1983, Kuffler, 1986) and in particular, components such as the synaptic laminins (Hunter *et al.* 1989, Sanes, 1995, Patton *et al.* 1997). The perisynaptic accumulation of adhesive macromolecules (N-CAM, tenascin (J1), fibronectin, M-HSPG) following denervation could also account for this preferential reinnervation of original motor endplates as they may play a role in guiding axons to synaptic sites (Covault *et al.* 1986, Gatachalian *et al.* 1989, Caroni and Schneider, 1994).

Neuromuscular transmission is restored almost as soon as the regenerating motor nerve terminals arrive at the endplate (Miledi, 1960b). Such rapid recovery of

transmission is only possible when the muscle is denervated for a short period by a nerve crush close to the entry of the nerve to the muscle. However, in the later stages of denervation atrophy, the nerve fibres often fail to find the original endplates and they tend to establish new "ectopic" endplates on these very atrophic muscle fibres (Gutmann and Young, 1944).

1.7 PERIPHERAL NERVE INJURY AND REPAIR

1.7.1 Classification of nerve injuries

Currently, there are two classifications of nerve injury used in clinical practice which describe the nature of the lesion. The first is based on the pioneering work of Sir Herbert Seddon (1943) who described 3 types of nerve injuries: neurapraxia, axonotmesis and neurotmesis. Neurapraxia refers to a local conduction block in which axonal continuity is preserved. Axonotmesis is charaterized by total interruption of the axons and their myelin sheaths at the site of the lesion, although the endoneurial tubes are still intact. Neurotmesis refers to loss of continuity of the nerve trunk. This was later amplified by Sunderland (1978) who classified nerve injuries into 5 types based upon the pathoanatomy of the various tissue components of the nerve trunk. Types 1 and 2 of the Sunderland injuries correspond to the Seddon groups neurapraxia and axonotmesis respectively, while the more severe nerve injuries Sunderland prefers to subdivide into three different groups, depending upon the continuity or discontinuity of the individual tissue components.

1.7.2 Nerve degeneration

Upon injury there are numerous cellular processes which are initiated in the neuronal cell body and the axon. These are reviewed according to the changes that take place after axotomy in the cell body, the proximal stump and the distal stump.

1.7.2.1 Changes in the neuronal cell body

Following axotomy, the corresponding cell bodies undergo a number of characteristic structural and functional changes (Lieberman 1971, Cragg 1970, Grafstein 1975, Guntinas-Lichius *et al.* 1996). These changes are a fast event, becoming apparent in the rat 4 to 8 hours after axotomy (Guntinas-Lichius *et al.* 1996)

The morphological changes, first observed by Nissl (1892), include swelling of the cell body, migration of the nucleus to an eccentric position in the cell, nucleolar hypertrophy and an apparent disappearance of basophilic material from the cytoplasm, referred to as "chromatolysis". Ultrastructurally, chromatolysis represents disorganization of the structure of rough endoplasmic reticulum and ribosome clusters (Vrbová, 1995). The reaction reflects a change in the arrangement and concentration of RNA-containing material in the cell, leading to changes in protein synthesis from that required for the production of neurotransmitters needed for synaptic activity, to the production of materials for axonal repair and growth.

Upon nerve injury, unidentified signals are relayed back to the cell body via retrograde transport to initiate the repair process (Ambron *et al.* 1992, 1995, Gunstream *et al.* 1995, Ambron and Walters, 1996, Ambron *et al.* 1996, Smith and

Skene, 1997). As soon as these signals reach the cell body, the cell body begins to synthesize and transport large amounts of actin and tubulin to lengthen the axon shaft and growth-associated glycoproteins to support membrane expansion at the growth cone (Routtenberg, 1985, McQuarrie, 1989, Baetge and Hammang, 1991, Jacob and McQuarrie, 1993, 1996, Lund and McQuarrie, 1996). Thus, the cell body up-regulates the synthesis of mRNAs and proteins necessary for the repair of the damaged axon, while down-regulating the synthesis of mRNAs required for the production of neurotransmitters which are not critical to the repair process (Tetzlaff *et al.* 1988, Bisby and Tetzlaff, 1992, Kinderman and Jones, 1993).

Other changes in the neuronal cell body after axotomy include a rise in the concentration of enzymes of the oxidative pentose phosphate shunt which are required for RNA synthesis (Sinicorpi and Kauffman, 1979). Putrescine, a polyamine associated with proliferating cells, is also expressed (Fischer and Schmatolla, 1972, Ingoglia *et al.* 1977). There are specific alterations in gene expression of cytoskeletal proteins including tubulin, actin and neurofilament protein, upregulation of calcitonin-gene related peptide (Streit *et al.* 1989) and induction of novel proteins including specific tubulin isoforms, T-alpha 1- and Class IIb tubulin (Miller *et al.* 1989), growth factor receptors (Hayes *et al.* 1992), and growth-associated protein GAP-43 (Benowitz and Routtenberg, 1987, Tetzlaff *et al.* 1989, Bisby and Tetzlaff, 1992). Each of these changes has been viewed as associated with and necessary for regeneration. The synthesis of GAP-43 in particular, is increased 20 to 100 times during successful regeneration of peripheral nerves, and is a major component of the growth cone membrane (Skene *et al.* 1986).

Most adult motoneurones survive axotomy even though they are deprived of putative target deprived trophic support. This support is primarily in the form of neurotrophic factors which play an important role in the development, maintenance and plasticity of the nervous system (Davies, 1994, Lindholm *et al.* 1994, Lindsay *et al.* 1994, Sendtner *et al.* 1994). Kobayashi *et al.* (1996) established that after axotomy, rat facial motoneurones increased the expression of mRNA for brain-derived neutrophic factor (BDNF) and its receptor trkB. Their findings suggest that BDNF may act locally on axotomised motoneurons and provide support for these injured motoneurons during the first weeks after axotomy. The BDNF increase as well as other possible sources of trophic support from the glial environment and the proximal stump, might contribute to the survival of motoneurones after axotomy (Curtis *et al.* 1993, Masu *et al.* 1993, Gehrmann *et al.* 1994). Indeed, Schwann cells in the proximal stump play a key role in regulating neuronal Nitric Oxide Synthase (nNOS) expression. This is an enzyme necessary in the synthesis of NO, a free radical compound believed to signal neuronal death (Thanos and Terzis, 1995). Hence, Schwann cells may help enhance the survival of axotomized neurones by suppressing NOS expression, and consequently reducing its neurodestructive effect (Yu, 1994, 1996).

1.7.2.2 Changes in the proximal stump

When a nerve is transected the ends of the nerve fibre retract under the influence of the elastic endoneurium. The proximal stump swells as a result of the oedema and the non-specific inflammatory reaction that occurs in any recently damaged tissue. There is retrograde degeneration which follows the same pattern as

that occurring in the distal stump. The axons degenerate as far as the first node of Ranvier, creating a small area of Wallerian degeneration (Terenghi, 1999).

1.7.2.3 Changes in the distal stump

Distal to the nerve injury the whole peripheral stump degenerates in a process known as Wallerian degeneration (Waller, 1850). The first signs of degeneration are evident 12 to 48 hours after the injury (Sunderland, 1978). Axons in the distal stump become amorphous granular masses. The mitochondria in the axoplasm swell and form clumps, and the neurotubules and neurofilaments disintegrate so that the axoplasm becomes filled with a granular material. The axon swells and develop an irregular "varicose" appearance. The myelin lamellae at the nodes of Ranvier exhibit fragmentation, and the myelin sheath retracts from the nodes and fuses to form ellipsoids. Within 24 hours the Schwann cells undergo nuclear and cytoplasmic hypertrophy and move to occupy the entire circumference of the internode. By 3 days the Schwann cells exhibit signs of active phagocytosis of myelin ellipsoids with thick vimentin-rich processes. Resident macrophages phagocytose myelin debris sequestrated into the endoneurium by Schwann cells after 3 days, but they do not enter the nerve tube until the fifth day post lesion (Reichert *et al.* 1994, Liu *et al.* 1995). Although Schwann cells initiate myelin phagocytosis, the completion of Wallerian degeneration relies on the phagocytic ability of macrophages to degrade myelin and axonal debris (Beuche and Freide, 1984, Hann Bonnekoh *et al.* 1989, Lunn *et al.* 1989, Griffin *et al.* 1992).

The proliferation and infiltration of macrophages is one of the most striking cellular responses during Wallerian degeneration in the peripheral nervous system

(Griffin *et al.* 1993, Bruck, 1997). In the rat the macrophage response begins within 24 hours of axonal injury and reaches its peak by 14 to 21 days (Monaco *et al.* 1992, Avellino *et al.* 1995). There is some debate as to whether resident macrophages contribute (Griffin *et al.* 1993, Vass *et al.* 1993, Liu *et al.* 1995), or whether the majority of macrophages are recruited predominantly from the circulating pool of hematogenous monocytes (Ramon y Cajal, 1928, Beuche and Freide, 1984). The precise mechanisms responsible for macrophage recruitment during Wallerian degeneration of peripheral nerves remain unknown. A recent study by Dailey *et al.* (1998) supports a role for serum complement in both the recruitment and activation of macrophages during peripheral nerve degeneration. Apart from the phagocytic ability of macrophages to degrade myelin and axonal debris (Beuche and Freide, 1984), macrophages can degrade molecules inhibitory to axonal regeneration (David *et al.* 1990). Macrophages can also release factors such as interleukin-1 (1L-1), which can promote axonal growth via the induction of neurotrophic factors such as nerve growth factor (Heumann *et al.* 1987, Lindholm *et al.* 1987).

From the fourth day on, many Schwann cells undergo active mitosis (Reichert *et al.* 1994, Liu *et al.* 1995). In vitro it has been demonstrated that axonal membrane and myelin remains stimulate Schwann cell mitosis (Salzer and Bunge, 1980, Salzer et al. 1980), and that macrophages that have phagocytosed myelin produce a medium that is mitogenic for Schwann cells (Baichwal et al, 1988, Liu *et al.* 1995). Each of these cells account largely for the increase in nuclear population that is observed after nerve injury. Abercrombie and Johnson (1946) found that after injury to the sciatic nerve in rabbits, the nuclear population increased eight-fold by the twenty fifth day and was still five-fold higher at 225 days.

As the myelin and axonal debris are removed, the basal lamina or endoneurial tubes that ensheath the myelinated nerve fibres persist. The proliferating Schwann cells align longitudinally within the confines of the basal lamina tube and form a continuous column of cells called "the bands of Büngner" (Nathaniel and Pease, 1963, Thomas, 1964, Giannini and Dyck, 1990). This proliferation continues for approximately 2 weeks, with the Schwann cells forming a conduit by which regenerating axons grow through to their target in the peripheral nervous system (Son and Thompson, 1995a,b). Some regenerating axons may fail to follow the band of Büngner and enter the connective tissue compartment resulting in no further growth (Thanos *et al.* 1998).

Shortly after axotomy the distal stump becomes compartmented by fibroblast processes, and a zone of fine collagen fibrils develops around the bands of Büngner (Röyttä *et al.* 1987, Bradley *et al.* 1998). After 26 months of denervation, Bradley *et al.* (1998) observed that the sites of previous nerve fibres were denoted by areas of approximately circular outline in transverse section, consisting of densely packed longitudinally oriented collagen fibrils. These collagen domains sometimes contained atrophic Büngner bands composed of isolated or small groups of Schwann cell processes surrounded by a basal lamina. Fibroblast processes or a perineurial cell ensheathment enclosed the collagen domains. The Schwann cells, deprived of axonal contact, atrophy and progressively disappear (Weinberg and Spencer, 1978, Bradley *et al.* 1998).

It is of interest to note that if a viable proximal stump is linked to a transected distal stump that has remained unreinnervated for 12 to 16 months, the regenerating axons grow into the areas of thin collagen fibrils in the centre of the collagen

domains (Vuorinen *et al.* 1995). However, other studies suggest that a chronically denervated distal nerve stump is unable to support axonal regeneration (Li *et al.* 1997, You *et al.* 1997, Terenghi *et al.* 1998). In chronically denervated adult rats, Schwann cells down-regulate expression of receptors for axonally-derived ligands, whilst after acute denervation, expression of these receptors is up-regulated (Li *et al.* 1997, You *et al.* 1997). Moreover, a close correlation has been established between the levels of receptor expression by denervated Schwann cells, and the extent to which distal stumps are reinnervated (Li *et al.* 1997).

What initiates Wallerian degeneration is still unknown; however, hypotheses include loss of trophic support from the cell body and activation of a neural protease called calpain by calcium influx (Glass *et al.* 1994). Recent studies on the C57BL/slow Wallerian degeneration mutant mouse suggest that Wallerian degeneration could be an active process similar to apoptosis (or programmed cell death) (Buckmaster *et al.* 1995, Ribchester *et al.* 1995, Gillingwater and Ribchester, 2001). In the case of the slow Wallerian degeneration mutation, a series of transplantation experiments have shown that the phenotype is inherent in the axon (Perry *et al.* 1990, Glass *et al.* 1993). It has been proposed that this mutation could affect gene function by a mechanism causing gene disruption, or by affecting the coding region of the gene or by upsetting gene dosage (Coleman *et al.* 1998).

1.7.3 Nerve regeneration

Within a few hours after damage axonal sprouts arise from the proximal stump at the point of retrograde degeneration. The number of sprouts is in excess of

the number of axons originally in the nerve fascicles, a phenomenon which possibly maximizes the chances for each neuronal cell reaching its target organ (Terenghi *et al.* 1999). For at least 2 days after axotomy the regenerating neurites consist of "naked axons" i.e., axons not ensheathed by Schwann cells, but only partially covered with a basal lamina-like configuration. Thereafter, Schwann cells from the proximal stump travel along a network of regenerating axons and after closely attaching to the axons, ensheath them (Torigoe *et al.* 1996). Associated with the axonal sprouting is a proliferation of not only Schwann cells but also fibroblasts (Hall, 1997). The terminal tip of the regrowing axons, or growth cones, uses integrin-class extracellular matrix receptors and at least two cell adhesion molecules including N-cadherin and L1/Ng-CAM, to mediate interactions between Schwann cell membranes and the regenerating neurites (Bixby *et al.* 1988, Rutishauser, 1993). The cell adhesion molecules are expressed by both the growth cone and Schwann cells (Rutishauser, 1993). The growth factors released by Schwann cells may also potentialize axonal regeneration by upregulating neuronal cell adhesion molecules expression (Hall, 1997).

The axonal outgrowths traverse the gap in a severed nerve, guided by columns of Schwann cells, and reinnervate the distal stump along the bands of Büngner (Allt, 1976). If, however, the growing nerve fibres do not find any "guiding" structures they form a neuroma which is a combination of immature nerve fibres and connective tissue. As the length of the gap increases, the probability that axons will grow successfully across a gap diminishes (Hall, 1997).

The terminal tip of the axon growth cone responds to contact guidance signals and actively seeks a suitable matrix and environment to support axonal growth

(Bixby *et al.* 1988, Rutishauser, 1993). The distal stump plays an important part in this process. Numerous studies have demonstrated that following peripheral nerve section, axons regenerate preferentially towards the distal stump (Cajal and Ramon, 1928, Politis *et al.* 1982, Anderson and Turmaine, 1986, Abernethy *et al.* 1992, Doubleday and Robinson, 1995). It is assumed that axonal growth can be directed by a chemical gradient of diffusible substances secreted by cells within the distal stump and operating over distances up to 10 mm (Politis *et al.* 1982). This is the concept of neurotropism (chemotaxis) which was apparently first proposed by Forssman in 1898 (cited by Lundborg, 1988). Recent work however, suggests that neurotropic factors are unlikely to include the protein nerve growth factor (Doubleday and Robinson, 1995), and that they may not be exclusively *neuro*tropic (Williams *et al.* 1993, Abernethy *et al.* 1994). Instead, these factors may well act upon the Schwann cells and endoneurial fibroblasts which escort the regenerating axons (Williams *et al.* 1993, Abernethy *et al.* 1994).

Schwann cells play a crucial role in regeneration of peripheral axons as in their absence, regeneration is deficient (Hall, 1986, Williams *et al.* 1993). Indeed, it has been suggested that Schwann cell processes lead and guide peripheral axonal regeneration. Son and Thompson (1995a) report that in the complete absence of axons, Schwann cells of the regenerating nerve establish processes that later serve as substrates for axonal growth. In their study they observed the growth of Schwann cells and axons from the cut end of the peroneal nerve over the surface of the soleus muscle. The Schwann cells and axons extended together from the cut end of the nerve and grew over the surface of the muscle. The tips of both the Schwann cells and axons furthermost from the cut end of the nerve were often coextensive however,

occasionally the Schwann cell tips were in advance of the axons. In these cases, axons appeared to be navigating along the Schwann cell processes. Furthermore, axons were always associated with Schwann cells although not all Schwann cells were occupied by axons. Torigoe *et al.* (1996) observed the behaviour of these "migratory" Schwann cells in peripheral nerve regeneration. Using a scanning and transmission electron microscope they observed that for at least 2 days after axotomy all the regenerating neurites consisted of "naked axons", i.e. axons not ensheathed by Schwann cells but only partially covered with a basal lamina-like configuration. Thereafter, Schwann cells from the proximal stump travelled along the regenerating axons and eventually ensheathed them. These migratory Schwann cells initially followed the axons, but later overtook them and provided a pathway for axonal regeneration.

It has been observed that the migratory Schwann cells "promote" axonal regeneration. Indeed, the Schwann cells from the distal stump also have this ability to stimulate axons to advance but at a higher speed. In partially denervated muscle, the terminal Schwann cells which cover the neuromuscular junctions extend elaborate processes upon denervation, and these may also guide regenerating axons (Son and Thompson, 1995a,b, Love and Thompson, 1998).

The regenerating axons also respond to trophic signals and grow back preferentially towards original target organs, although the mechanism controlling this specificity is not fully understood (Gu *et al.* 1995). Motor axons regenerating after transection of mixed nerve preferentially reinnervate muscle (Brushart, 1988) or muscle nerve (Brushart, 1993), a process called preferential motor reinnervation. Preferential motor reinnervation appears to be a constant feature of motor axon

regeneration (Madison *et al.* 1996). Its mechanism has been investigated in the rat femoral nerve (Brushart, 1990, 1993). The "pruning hypothesis" was established which suggests that preferential motor reinnervation is generated by pruning collaterals from cutaneous pathways while maintaining those in muscle pathways. Hence, a motoneurone that at first samples both pathways is converted to one projecting correctly and only to muscle (Brushart, 1993, Brushart *et al.* 1998).

The phenomenon of preferential motor reinnervation may be explained by a pathway marker, the carbohydrate epitope L2/HNK-1, that is associated with several neural cell adhesion molecules (Kunemund *et al.* 1988). The L2 epitope is selectively expressed on the Schwann cells and Schwann cell basement membrane of motor axons, but is rarely found on sensory axons (Martini *et al.* 1988). It persists in these positions during and after Wallerian degeneration (Martini *et al.* 1992). Furthermore, there is preferential expression of L2 during reinnervation by motor axon-associated Schwann cells (Martini *et al.* 1994). The L2 epitope thus selectively marks motor pathways, is present in the proper cellular location and at the proper time to influence regeneration.

Investigation into target specificity of sensory nerve regeneration has demonstrated a high degree of accuracy at the receptor (Burgess and Horch, 1973, Horch, 1979, Madison *et al.* 1996), and the cortical level (Dykes, 1984). However, it would seem that sensory nerves lack topographic specificity as afferent fibres have the capability of growing back to their particular class of receptor, but not necessarily to the same receptor field (Rath and Green, 1991). Their display of target organ specificity is derived from the presence of specific neurotrophic factors (Terenghi, 1999).

The two elements within the distal stump which are most likely to facilitate axonal growth are the Schwann cells and their basal laminae. After axotomy Schwann cells upregulate gene expression of the low affinity neurotrophin receptor p75 (Heumann *et al.* 1987, Taniuchi *et al.* 1986, Liu *et al.* 1995); the growth-associated proteins GAP-43 (Curtis *et al.* 1992, Scherer *et al.* 1994) and GFAP (Thomson *et al.* 1993); neurotrophins NGF (Matsuoka *et al.* 1991) and BDNF (Meyer *et al.* 1992, Korsching, 1993); cell-cell adhesion molecules N-CAM and L1 (Martini and Schachner, 1988); cell-extracellular matrix molecules laminin (Kuecherer-Ehret *et al.* 1990); J1/tenascin (Martini *et al.* 1990, Martini, 1994) and fibronectin (Lefcort *et al.* 1992, Matthews and FFRENCH-Constant, 1995).

As axons penetrate the bands of Büngner a second transient phase of proliferation of Schwann cells occurs (Pellegrino and Spencer, 1985). During the establishment of functional Schwann cell-axon contact, Schwann cells upregulate the expression of β integrin, an adhesion molecule. The Schwann cells of the distal stump also generate a basal lamina which is supportive of nerve growth. Molecules of the basal lamina which are known to promote axonal regeneration include laminin, fibronectin and heparan sulphate (Sandrock and Matthew, 1987, Giftochristos and David, 1988, Rogers *et al.* 1988, Tomaselli and Reichardt, 1988, Toyota *et al.* 1990, Wang *et al.* 1992a,b, Bryan *et al.* 1993, Kauppila *et al.* 1993). The inner surface of the basal lamina is particularly rich in laminin which affects the velocity and direction of growth cone neurites. Axons grow preferentially along the inner aspect of the Schwann cell derived lamina, rather than in association with its outer, extracellular surface (Hall, 1997).

The rate of regeneration of motor nerves varies and is thought to be determined by the speed at which the cell body can resynthesize and supply the necessary components for the reestablishment of the missing axon (Vrbová *et al.* 1995). Gutmann (1942) first obtained an accurate estimate of the rate of growth of motor axons. In this study, the common peroneal of the rabbit which supplies muscles that spread the toes when the animal is lifted off the ground was crushed. Upon crushing the nerve, the reflex disappeared and its reappearance was used as an indicator of reinnervation. Hence, to assess the rate of growth Gutmann (1942) crushed the nerve at different distances from the peroneal muscles and noted the times of appearance of the spreading reflex. From these studies it was calculated that the rate of motor nerve regeneration was 2.77 ± 0.09 mm/day (Gutmann *et al.* 1942).

1.7.4 Factors affecting quality of nerve repair

1.7.4.1 Age

The results of nerve repair are best in the very young (Larsen and Posch 1958, Stromberg *et al.* 1961, Önne, 1962, Almquist and Eeg-Olafsson, 1970, Seddon, 1975, Young *et al.* 1981). In man, teenagers and young adults have poorer functional nerve recovery than children, but their results after repair are better than the rest of the adult population (Önne, 1962, Sakellarides, 1962, Seddon, 1975). In Önne's (1962) detailed clinical study of digital nerve repair, he found that age was an important factor in the eventual recovery of sensation. Almost all the patients under 10 years recovered normal two-point discrimination. For patients over 30 years recovery was

poor, although functional two-point discrimination was recorded in some patients up to the age of 50 years.

As well as achieving better results, children also appear to recover more quickly. Children gain normal or near-normal motor and sensory function in distal nerve injuries by 2 years after nerve repair while adults may take up to 5 years to obtain their best results (Stromberg *et al.* 1961, Gaul, 1982).

In animals, the reduced capacity for nerve repair with increasing age is well documented (Black and Lasek, 1979, Pestronk *et al.* 1980, Jacob and Robbins, 1990a,b, Grieve *et al.* 1991, Tanaka and Webster, 1991, Vaughan, 1992, Verdú *et al.* 1995, Belin *et al.* 1996, Jacob and Croes, 1998). Experimental studies have observed that in aged animals nerve regeneration involves fewer axons than in young adults (Tanaka and Webster, 1991, Vaughan, 1992). In younger animals, neurones also have a greater ability to generate sprouts either regenerative (Gutmann *et al.* 1942), or collateral (Fagget *et al.* 1981, Navarro and Kennedy, 1988). Jacob and Croes (1998) observed the effect of age and time after nerve lesion on the rate of motor axon outgrowth. They determined that the age of the animal does not affect outgrowth rates at early periods after a nerve injury, but with longer post injury times, motor axon outgrowth is reduced in old rats.

Studies of the partially denervated neuromuscular junction have demonstrated that reestablishment of single axon innervation (Hopkins *et al.* 1986), and the maintenance of terminal size after expansion of the motor unit (Jacob and Robbins, 1990b), were not achieved in old animals.

These age-related changes in nerve regeneration may be due to a number of factors. Firstly, after Wallerian degeneration of distal nerves in aged rodents there are

fewer macrophages and myelin sheaths remain for a longer time than in young rodents (Tanaka and Webster, 1991, Tanaka *et al.* 1992, Vaughan, 1992). There is a progressive reduction of proliferating Schwann cells in aged nerves, and the levels of neurotrophic factors secreted by Schwann cells and target organs are lower in older animals (Komiyama and Suzuki, 1992). Although increased age has no effect on the rate of the fast component of axonal transport (Jacob, 1995), the slow component tends to decrease (Komiya, 1980, McQuarrie *et al.* 1989, Jacob and Croes, 1998). This finding in old animals may suggest that with age, the ability of the axon to transport the necessary factors for nerve repair is compromised (Tashiro and Komiya, 1992). Furthermore, the chromatolytic response of axotomised neurones tends to decrease and be delayed with increasing animal age (Vaughan, 1990a,b). However, experimental evidence suggests that axons from old animals can be stimulated to repair themselves at rates comparable to those seen in younger animals (Jacob and Croes, 1998). By using the conditioning lesion paradigm, Jacob and Croes (1998) observed an increase in outgrowth rates in axons in mature and old rats, and moreso in the old rats. Their data suggests this model can be used to accelerate outgrowth in animals of all ages, and that there is a maximal outgrowth rate which can be accomplished by axons that is independent of the age of the animal. The mechanism of the conditioning lesion paradigm is discussed in section 1.7.4.4.

There are several other possible explanations for superior clinical outcome after nerve injury in the young. Due to the length of the extremities in the young the distances that the regenerating axons must traverse are shorter. After nerve injury, neonates exhibit greater specificity of regenerating axons and a greater maintenance of somatotopy than that seen in adults (Aldskogius and Thomander, 1986, Laskowski

and Sanes, 1988). Young animals also display more central plasticity in their superior ability of cerebral adaptation to new peripheral innervation patterns than adults (Bowe *et al.* 1987).

It is of interest to note that although functional recovery from peripheral nerve injury is faster and better in the young, experimental studies have demonstrated greater nerve cell death after axotomy in neonates (Schmalbruch, 1984, Pollin *et al.* 1991). A possible explanation for this sensitivity of the neonate to axotomy-induced cell death is deficiency of ciliary neurotrophic factor which prevents the degeneration of neurones after nerve injury (Sendtner *et al.* 1990, Belin *et al.* 1996).

1.7.4.2 Level of injury

The level of injury has a significant effect on the quality of neural recovery following repair. Numerous clinical studies have shown that repair of distal nerve injuries are associated with a better prognosis than more proximal injuries (Schaffer and Cleveland, 1950, Nicholson and Seddon, 1957, Sakellarides, 1962, Omer, 1974).

The poorer results after repair of proximal nerve injuries are partly due to the distance over which the nerve fibres must regenerate to reach end organs. After proximal lesions the amount of total cell mass which may be lost can be 50% of the total intracellular substances (Ducker, 1972).

The chance of fascicular misalignment is increased after repair of more proximal lesions. Fascicles in proximal nerves contain greater mixtures of motor and sensory fibres whereas nerve fibres are often arranged in distal nerve into motor and sensory fascicles (Sunderland, 1990). Hence the further proximally the injury is located, the more widely dispersed are the fibres in the cross-section, and this favours

the prodigal regeneration of axons into functionally unrelated endoneurial tubes. Mismatched motoneurones can reinnervate other muscles but sensory nerves cannot innervate motor end-plates (Gutmann, 1945, Zalewski, 1970).

1.7.4.3 Type of injury

The type of injury is of considerable importance. It influences the capability of regeneration of the nerve and the chances of success in surgical attempts to restore nerve function.

Neural impulses may be interrupted due to be a local conduction block with preservation of axonal continuity. The conduction block can be caused by moderate nerve compression, temporary ischaemia, chemical insults, alterations of metabolism, or temperature extremes. Functional recovery usually returns within weeks or months (Brown, 1972, Lundborg, 1988). With more severe compression or traction injuries of peripheral nerves physical continuity of the nerve is still maintained. The proximal and distal stumps remain interconnected because the epineurial sheaths and the basal lamina surrounding each axon are not divided by this particular type of injury (Haftek and Thomas, 1968, Richardson and Thomas, 1979). The distal segment undergoes Wallerian degeneration whilst the proximal stump sends out new sprouts. The intact membranes maintain the exact alignment of all constituents of the nerve and ensure that axon sprouts will grow across the lesion site and into the bands of Büngner. Optimal functional recovery is observed after these injuries as the regenerating axons from the proximal stump are guided by the intrinsic guidelines provided by the endoneurial tubes, and regeneration of the appropriate target organ occurs (Lundborg, 1988, De Medinaceli and Merle, 1991).

In the case of some severe compression or traction type injuries to the nerve fibre, both the endoneurial tube and the connective tissue sheaths may be damaged. As a result, no tissue structures can guide the growing axons across the site of lesion and axonal misdirection becomes a problem. After nerve transection, epineurial, perineurial and endoneurial sheaths are disrupted and the stumps retract by inherent elastic forces, forming a gap. Thus, regenerating axons have to traverse an unknown domain before entering the bands of Büngner (Lundborg, 1988, Hall, 1997). With the disruption the formation of a scar or neuroma is common on the proximal stump. This interferes with the growth of regenerative axons and offers more opportunities for these regenerating axons to escape from the distal stump and not contribute to effective reinnervation (Sunderland, 1990).

In clinical practice the majority of severe injuries to nerves are due to blunt mechanisms such as stretch and contusion. These leave the nerve in continuity and as a result, are more difficult to deal with as the full extent of involvement may not be recognized. There can be a variable degree of intraneural damage. Swelling or neuroma formation at the injury site is not constant and if present, does not necessarily correlate with the severity of intraneural axonal injury (Kline, 1990). Associated with the more severe injuries are significant vascular and soft tissue damage which will affect nerve regeneration. Extensive muscle and soft tissue damage may produce a scarred hostile environment for the regenerating new fibres which increases tensile forces at the suture line, interferes with axoplasmic flow and slows neural recovery (De Medinacelli *et al.* 1983). The presence of either a complicating arterial injury (Glasby *et al.* 1998), a complicating long-bone injury (Fullarton *et al.* 1998), or a complex nerve injury comprising cavitation, fibrosis and

haematoma (Glasby *et al.* 1997), contribute to a poorer outcome in recovery of nerve function and maturation.

1.7.4.4 Timing of repair

The optimal timing for peripheral nerve repair has yet to be determined. There is some controversy as to whether immediate or early delayed (secondary) repair is the more conducive to effective nerve regeneration.

There are certain animal studies which would appear to support delayed nerve repair. Indeed, these investigations have shown the beneficial effects on axonal regeneration if a test nerve lesion has been preceded by a suitably timed earlier injury referred to as a "conditioning lesion" (McQuarrie and Graftstein, 1973, McQuarrie, 1978, Forman *et al.* 1980, Bisby, 1985, Sjöberg and Kanje, 1990a,b, Jacob and Croes, 1998). The optimal conditioning interval, or the period between an initial conditioning lesion followed by a test injury is reported to be 8 to 12 days (Forman *et al.* 1980, Sjöberg and Kanje, 1990b) however, shorter periods of 2 days (Sjöberg and Kanje, 1990a), or even 14 hours (Arntz *et al.* 1989), result in accelerated axonal growth. Sjöberg and Kanje (1990a) also observed that a conditioning lesion increases the regeneration velocity and its acceleration. As to the effect of repetitive conditioning lesions, Sjöberg and Kanje (1990b) demonstrated that the conditioning interval and not the number of conditioning lesions determine the outgrowth after a test lesion. The mechanism by which a priming lesion is able to influence axonal regeneration is not yet known. The main hypothesis refers to the changes in the cell body after the primary lesion whereby the metabolism of the cell body is altered from

that of homeostasis to one of cellular growth (Bisby and Tetzlaff, 1992, Jacob and McQuarrie, 1993).

In the 1954 Medical Research Council Report early secondary suture was the procedure of choice (Seddon, 1954). Early secondary suture allows better recognition of the damaged nerve for neurolysis as weeks after the injury it is often a palpable and, on section, visible zone of intraneural fibrosis. In addition, as the epineurium is thicker after delayed repair, it is better able to support sutures (Seddon, 1954). There are, however, a number of significant disadvantages associated with delayed nerve repair.

After nerve transection the two stumps retract due to nerve elasticity. In response to the trauma fibrosis develops in nerve tissue at the site of the lesion and a certain distance both proximal and distal to it. This results in loss of elasticity, and any formation of adhesions also reduces the gliding capacity of the nerve. In order to achieve end-to-end repair one must overcome not only the elastic retraction but also the fibrotic retraction by more extreme neurolysis. In addition, any normal nerve tissue which has preserved the capacity for elongation must be elongated further to compensate for the lack of extensibility of the fibrotic section. If adhesions have formed, the even distribution of forces along the length of the nerve may not be achieved. The consequences of this are that even after a "clean" transection at secondary repair there is higher tension at the site of coaptation than if a primary repair were performed. The deleterious effects of tension at the suture line are yet to be discussed however, the development of intraneural fibrosis and neuroma formation are directly related to the length of delay in nerve repair. These processes

act to impede nerve regeneration when axons are growing toward the distal Schwann cell tubes and target organs (Glasby *et al.* 1997).

In daily clinical practice immediate repair has become the treatment of choice wherever possible (Lawson and Glasby, 1995). Currently, the decision of either immediate or delayed repair is determined by the degree and nature of the associated injury.

Delayed nerve repair is indicated in complex cases of severe local soft tissue and/or bony injury associated with a significant region of nerve injury or indeed loss (Kilne, 1990). As to the practicality of this approach there is no question, particularly when the associated injury is life-threatening or when available services are not appropriate for careful reconstructive surgery. However, there have been a number of experimental studies evaluating the outcome of immediate versus delayed nerve repair in complex injuries involving either soft tissue cavitation (Glasby *et al.* 1997), arterial injury (Glasby *et al.* 1998), or long bone fracture (Fullarton *et al.* 1998). The results indicate that when each of these complex injuries is associated with a delay in nerve repair, this contributes to a poorer outcome in recovery of nerve function and maturation.

The preference of immediate nerve repair has been verified by a number of other experimental studies. Gutmann (1942) noted significant differences in the onset of motor recovery after a 6 month delay of nerve repair in rabbits. He cited irreversible end organ atrophy as the main reason for this, as well as degenerative changes in the peripheral stump. Bolesta *et al.* (1988) noted that after denervation of the EDL in the rabbit, delay of nerve repair from 3 weeks to 6 months adversely affected muscle morphology and function. Greater myelin content was also found in

rabbit sciatic nerve after primary nerve repair, as compared to those sutured at 3 weeks (Bora *et al.* 1976). However, in rhesus macque monkeys, after immediate suture results were superior than after a delay of 3 weeks (Grabb, 1968). Experimental work by Van Beek *et al.* (1975) in rats also suggested that primary suture produced the best results. A study by Bignotti *et al.* (1986) also in rats, found no significant morphological difference between immediate and delayed (14 days) nerve suture, but conduction velocities were better in the early repair group. Gattuso *et al.* (1989) investigated immediate and delayed nerve repair using freeze-thawed autologous skeletal muscle grafts in the rat. They observed that after shorter delays, myelinated nerve fibre numbers and diameters did not differ significantly from those seen after immediate repair. However, there was a significant reduction in the rate of remyelination of the fibres following a delay. In a large animal model such as the sheep, Lawson and Glasby (1995) noted that the peak nerve conduction velocities and mean fibre diameters were appreciably lower in the delayed repair group. Fu and Gordon (1995a) observed in the rat that prolonged axotomy significantly reduces the number of motor axons that regenerate and make functional connections with denervated muscle fibres. Nevertheless, prolonged axotomy did not compromise the capacity of regenerative axons to branch or the number of muscle fibres reinnervated by each motor axon.

A number of studies have investigated the effects of delay of nerve repair on reinnervation of *partially* denervated muscle (Guth, 1962, Thompson and Jansen, 1977, Thompson, 1978). Partially denervated refers to when the axons of a portion of the population of motoneurones are interrupted. In response there is collateral sprouting of the remaining undamaged axons and nerve terminals, followed by the

innervation of denervated muscle fibres (Thompson and Jansen, 1977). Thus, these motoneurones can increase the number of muscle fibres they innervate on average, about three to four times (Thompson and Jansen, 1977). Partial denervation can be induced by either resecting, cutting or crushing the nerve. Each provide for different nerve regeneration times (Frank *et al.* 1975). Nerve crush allows for more rapid reinnervation by the regenerating axons and a substantial number of muscle fibres become "hyperinnervated" as the regenerating axons begin forming synapses in the muscle before the sprouting nerve fibres have consolidated their new end-plates. However, if regeneration occurs later as after nerve section, the sprouting nerve fibres have time to complete expansion and so very few muscle fibres become hyper-innervated. Thus, after delayed nerve regeneration the sprouted motor units are able to attain their maximal size. After more rapid nerve regeneration as in nerve crush, the sprouted nerve fibres are the fibres preferentially eliminated from the hyperinnervated fibres and as a result, are never able to attain their maximal size. A possible explanation for this phenomenon may be that the expanded and large size of the sprouted motor units places their nerve terminals at a competitive disadvantage in comparison to the regenerating axons, which support much fewer terminals in the muscle (Thompson, 1978).

1.7.5 Types of peripheral nerve repair

One of the early attempts at repair of a divided nerve date back to 1608 when Ferrara of Italy gave a detailed account of an experimental neurorrhaphy. In spite of these early endeavours, most surgeons subscribed to either no surgery or amputation

of the involved limb until the nineteenth century. During the last 100 years advances in the surgery of peripheral nerves have coincided with world wars in which the number of nerve injuries greatly increased. The development of technology in the operating theatre in particular, the introduction of the operating microscope, synthetic monofilament sutures, and advances in instrument manufacture have contributed to improved results.

1.7.5.1 Nerve-to-nerve epineural suture

When a nerve has been cleanly sectioned and the proximal and distal stumps are in very close proximity, then the simplest form of repair is direct epineurial suture. This involves suturing the epineurium of the proximal and distal stumps together with individual interrupted sutures. It is important that there should be no tension between the distal and proximal nerve ends. The deleterious effects of tension at the suture line are well documented (Millesi *et al.* 1972, Terzis *et al.* 1975, Millesi *et al.* 1976, Miyamoto, 1979, Stevens *et al.* 1985). The complete absence of tension at the suture line is regarded as one of the most important factors for successful nerve repair (Millesi *et al.* 1972). Acute stretch may cause intraneural vessel damage with resultant scar formation and disseminated degeneration (Highet and Holmes, 1943, Highet and Sanders, 1943, Denny-Brown and Doherty, 1945, Miyamoto *et al.* 1979). The proliferation of connective tissue at the suture line presents an obstacle to regenerating axons (Millesi *et al.* 1972, Millesi, 1973). Mobilization of the nerve ends by dissecting them from anchoring tissues may gain some length however, this in itself may disturb the blood supply to the nerve and make the problem worse. It is now generally accepted that a nerve should not be

repaired under tension and if there is a significant gap which cannot be closed without tension, some form of graft should be used (Terzis *et al.* 1975, Millesi 1980, Rodkey *et al.* 1980).

1.7.5.2 Nerve graft

The first experiment on nerve grafting was reported in the literature in 1870 by Phillipeaux and Vulpian in which a segment of lingual nerve was used to bridge a gap in the hypoglossal nerve. Albert (1878) described the first clinical use of nerve grafting where defects in the median nerve were bridged by nerve grafts. Unfortunately the long term results of this were not reported but further experiments by Kilvington (1908) suggested that nerve regeneration was possible through nerve autografts.

During World War I efforts by surgeons to save nerves interrupted by penetrating trauma using nerve grafts were largely unsuccessful (Platt, 1919, Stopford, 1920). In many of these cases there was a considerable delay between injury and repair which would have jeopardized any nerve regeneration and subsequent recovery. Also, the nerve grafts were surrounded by a fascial sheath which subsequent experience has shown inhibits revascularization of the graft. As a result of the World War I experience many surgeons tended to avoid the procedure and instead used end-to-end repair. Included in the methods used, singly or in combination, were nerve mobilization, bone shortening, and flexion of adjacent joints.

There was a revival of interest in nerve grafting as a result of the work of Bunnell (1927) on digital nerve grafting; of Ballance and Duel (1932) on repair of the

facial nerve; and of Bunnell and Boyes (1939) on repair of major nerves in the limbs. Sanders and Young (1942) showed encouraging results in animals and incited further attempts at the use of nerve grafts in the treatment of patients. In the following years clinical evidence indicated that nerve grafts were worthwhile (Seddon, 1947, Seddon, 1963). Much of the clinical work performed by Seddon and others during and after the second world war resulted in the Medical Research Council (1954) report on peripheral nerve injuries. Many of the principles of peripheral nerve surgery were established by this report.

Nerve grafts can be divided into three types: autografts, xenografts and allografts.

1.7.5.3 Nerve autografts

These are grafts made from nerve removed from the same individual and are commonly used in the repair of peripheral nerve defects. In clinical practice the sural nerve is usually the first choice as a donor graft as it is a small cutaneous nerve which can be sacrificed with minimal deficit. It is also easily accessible, has a long course without branches, and due to knowledge of its fascicular arrangement which varies from a monofascicular to a polyfascicular nerve, the proper segment can be selected according to the fascicle groups to be united. (Millesi, 1984). Once removed from the donor nerve, an autologous nerve graft behaves like a distal stump and undergoes Wallerian degeneration. After the axons and myelin sheaths are removed, the remaining endoneurial tubes serve as guiding structures for regenerating axons entering the nerve graft. However, the nerve graft is more than just a framework as

the Schwann cells within the nerve graft ensheath and myelinate the regenerating axons (Aguayo *et al.* 1976a,b, 1977, Charron *et al.* 1976).

1.7.5.4 Nerve xenografts and allografts

An inevitable disadvantage of using nerve autografts for nerve grafting is the sacrifice of a healthy cutaneous nerve in order to provide a donor nerve. The patient may suffer sensory loss or may develop a painful neuroma at the donor site. To procure the donor nerve also requires a second operation which is time consuming for the surgeon, and results in another scar for the patient. Over the years researchers have tried to find an alternative source of material both neural and non-neural to bridge the gap. Many early experiments used xenografts (nerve taken from individuals of different species) and nerve allografts (nerve harvested from individuals of the same species).

After repair with nerve xenografts the results, both experimentally and clinically, have been consistently disappointing (Sunderland, 1978). Xenografts were found to induce a marked inflammatory reaction and to inhibit nerve regeneration (Sanders and Young, 1942). Although early experiments with nerve allografts reported some success, the superiority of nerve autografts in bridging a nerve gap was still acknowledged (Kilvington, 1908, Huber, 1920). Sanders and Young (1942) compared results after nerve repair with autografts and allografts in rabbits. They concluded that allografts can support nerve regeneration, but in some cases may set up an inflammatory reaction. They often noted a considerable lymphocytic reaction occurred around the grafts and although nerve fibres were seen entering the grafts, this was in smaller numbers than autografts. Attempts to improve the results of

allografting by a reduction in the immune reaction have included the experimental use of immunosuppressive drugs such as cyclosporin-A in rats (MacKinnon, 1985a, Jost *et al.* 2000). The application of this procedure in humans would require a dosage similar to that used for cardiac allografts and the logic of subjecting patients with peripheral nerve injuries to such an excessive form of treatment is questionable. However, reduction in peripheral nerve allograft antigenicity has been noted with preservation treatment of the allograft (Strasberg *et al.* 1996).

1.7.5.5 Fascicular repair

An alternative form of repair is fascicular repair whereby each individual fascicle is sutured to its counterpart in the distal fascicle. Fascicular suture was first advocated by Langley and Hashimoto (1917). The recommendation was largely disregarded until the introduction of the operating microscope when interest in fascicular repair was renewed.

Numerous studies indicate that fascicular repairs seem to fare better than epineurial repairs (Bora 1967, Grabb *et al.* 1970, Wise *et al.* 1976), whilst other authors report little or no difference in results after repair with either of these methods (Bora *et al.* 1976, Cabaud *et al.* 1976, 1980, Orgel and Terzis, 1977, Kline *et al.* 1981, Young *et al.* 1981). Although it would seem practical to unite proximal and distal stumps of sensory and motor fascicles so that regenerating axons have a greater probability of innervating applicable end organs, epineurial suture is still the technique of choice for most acute nerve transections. Compared to fascicular repair, epineurial suture technique is easier, faster, and requires less manipulation of the fragile inner neural structure. The disadvantages of fascicular repair include: the

increased time taken for the operation; the possibility of increasing scar tissue due to the greater mass of suture material placed in the nerve; and the division of blood vessels which might interfere with the blood supply of the isolated fascicle. With fascicular repair there is also a chance of fascicles being mismatched which has the potential of producing poorer results than after epineurial end-to-end suture.

1.7.5.6 Cable grafts

In the repair of large calibre nerves, multiple strands of cutaneous nerve are used in parallel to make a "cable graft" of equal diameter to the nerve being repaired. When there are a number of strands united to form a cable, there is a substantial infiltration of connective tissue which can obstruct the regenerating axons (Glasby, 1990). Indeed in the repair of sheep femoral nerve, Glasby *et al.* (1990) found that the cable graft appeared to acquire more connective tissue infiltration than the muscle graft although no formal quantification was attempted. They also found that more nerve fibres passed through the muscle graft to reach its distal stump than through the cable graft however, indices of fibre maturation were indistinguishable between the two groups. Further studies by Myles and Glasby (1991) demonstrated no significant differences in level of recovery after muscle or cable grafting. In considering the clinical application of either technique in the repair of a defect in a large nerve, the results show that the insertion of a muscle graft confers no disadvantage and is technically much quicker and easier.

1.7.5.7 Muscle grafts

The disappointing results from allografts and the consequences of sacrificing autogenous nerve have prompted attempts to find a non-neural tissue or material which would support axonal regeneration. These endeavours have included the trial of muscle as a graft material.

The concept of using muscle grafts instead of nerve grafts to repair defects in damaged peripheral nerves was stimulated by the work of Sanes *et al.* (1978). This study noted the ability of regenerating axons to reinnervate denervated skeletal muscle fibres at their original synaptic sites and to differentiate into nerve terminals on contact with the muscle fibres. They set out to determine the location of factors which might influence these processes and concluded that the muscle fibre basal lamina supported axonal regeneration.

Further studies by Ide *et al.* (1983) investigated the influence of the inner basement surface of nerve grafts on regeneration and found that a regenerating nerve does not require the presence of Schwann cells, only their basal lamina. The basal lamina scaffolds proved to be effective pathways for the maintenance of regenerating axons. Ide *et al.* (1984) went on to investigate whether the basal lamina of other cells could support axonal regeneration and confirmed the results of Sanes *et al.* (1978). In these studies muscle grafts killed by repeated freezing and thawing were placed onto the proximal stump of the transected sciatic nerve in mice. Analysis demonstated that over 2 to 5 days after grafting, dead muscle cells had disintegrated and were phagocytized, leaving behind the basal lamina as a series of parallel tubes into which regenerating axons and Schwann cells grew. There were few regenerating axons found outside the basal lamina scaffold.

Work by Keynes *et al.* (1984) reported similar findings. Skeletal muscle, degenerated by chemical means, was attached onto the proximal stump of the transected sciatic nerve in mice. By 4 days nerve fibres grew coaxially within the tubes of basement membrane of the muscle fibres in the graft, coinciding with the onset of degeneration of the sarcoplasm. Growth of axons did not occur into live-innervated muscle or glutaraldehyde fixed muscle in which muscle fibre architecture was preserved. Hence these grafts failed to provide empty basement tubes. Later studies by Glasby *et al.* (1986b) had similar findings.

Neither Ide *et al.* (1984) or Keynes *et al.* (1984) considered the possibility of the regenerating axons leaving the muscle graft if offered an attached distal nerve stump. Thus, on the basis of their work the concept was developed of a degenerated muscle graft supporting axonal regeneration between the proximal and distal stumps of a transected nerve. The studies by Glasby *et al.* (1986a,b,c,d) and Fawcett and Keynes (1986) supported this idea.

A method advocated by Glasby *et al.* (1986a) for the degeneration of the muscle graft was to first freeze a piece of muscle in liquid nitrogen, and then osmotically shock the tissue by immersing it in distilled water. This procedure produces acute necrosis of myocytes, endothelial cells, intramuscular nerves and interstitial cells (Hall, 1997). This cellular debris is rapidly cleared by recruited macrophages, thus providing an efficient and a quick method of clearance of basement membrane tubes (Glasby *et al.* 1986b).

One of the problems associated with the freeze-thawed skeletal muscle autograft (FTMG) is that the freezing process produces shrinkage of up to 50% (Glasby *et al.* 1995). Several researchers have attempted to overcome this problem by

modifying the methods by which the muscle graft is prepared. Modifications have included repeated episodes of freeze-thawing at -25^{0}C followed by immersion in distilled water (Enver and Hall, 1994, 1997), heating the muscle in distilled water to 60^{0}C (Hall and Enver, 1994) and heating the muscle in a microwave oven at 300 W for 120 s (Whitworth *et al.* 1995). A recent study by Lenihan *et al.* (1998b) has shown that heating muscle in a microwave oven does produce a graft which is an acceptable alternative to the FTMG. However, they found that the preparation of a satisfactory microwave muscle graft was much more difficult than that of a FTMG and as a result, suggested that this reduces the potential use of this technique in the clinical setting.

Glasby *et al.* (1986b) investigated how orientation of the muscle graft affected regeneration. In this study grafts were placed in the rat sciatic nerve. In one group the FTMG was aligned so that its fibre axis and hence, basement membrane tubes were coaxial with the axons and Schwann tubes in the proximal and distal stumps respectively. Whilst in another group, the FTMG was implanted with its fibre axis at 90^0 to the nerve axis. Subsequent analysis demonstrated that the coaxially aligned muscle grafts supported the fastest regeneration although by 100 days there was no significant difference between the two groups.

The rapid growth associated with the coaxially aligned FTMG concentrated attention upon this group with a view to clinical nerve repair. However, prior to the clinical application of the coaxially aligned FTMG a number of experiments have been performed on various models of nerve injury and repair. These have included studies on the repair of the rat sciatic nerve (Glasby *et al.* 1986a,b,c, Davies *et al.* 1987, Gattuso *et al.* 1988, Glasby *et al.* 1988a,b, Gschmeissner *et al.* 1988, Gattuso

et al. 1989, Glasby *et al.* 1990, Huang *et al.* 1990, Gschmeissner *et al.* 1990), the marmoset radial, ulnar and median nerves (Glasby *et al.* 1986d, Gattuso *et al.* 1988), the primate ulnar nerve (Glasby *et al.* 1991), the sheep femoral nerve (Glasby *et al.* 1990), the guinea pig sciatic nerve (Glasby *et al.* 1989), and the rabbit common peroneal nerve (Lenihan *et al.* 1998a,b). The FTMG has been shown to be successful as a graft in each of these situations however, in clinical trials the results have been mixed.

Norris *et al.* (1988) used coaxially aligned FTMG to repair injured digital nerves. The report dealt with a small series of sensory nerves but seven of the eight cases achieved Medical Research Council (MRC) grade S3+ which was assessed as an excellent level of recovery. Pereira *et al.* (1991) compared the FTMG with end-to-end suture for the repair of human digital nerves and demonstrated superior results for the muscle graft group. This was possibly due to the lack of tension at the suture line for the grafted group.

In another study Stirrat *et al.* (1991) reported the results of clinical trials of FTMG used in the repair of major nerve trunk deficits and painful neuromata. In the nerve trunk defect group there were 5 patients and the average length of the gap was 7 cm. After a mean period of 20 months following repair there was no motor recovery and one patient had sensory recovery. These results are discouraging but the lesions were of the most severe type that they were considered unfavourable for conventional nerve grafting. It was felt unethical to use a new technique such as the FTMG in the repair of injuries which are likely to have a good result from nerve grafting. In addition, the average length of repair was in excess of that shown to be

successful for muscle grafts in animal models, i.e. less than 5 cm (Hems and Glasby, 1992).

In the painful neuromata group, there were 6 patients with the condition in the upper limb, and 6 with the disorder in the lower limb. After removal of the neuroma a FTMG was used to restore continuity in the nerve. In the upper limb neuromata group, 5 patients gained partial or complete relief of pain and hypersensitivity, and 4 had partial return of sensation. For the lower limb group, only 2 patients had partial relief of pain. Based on these results Stirrat *et al.* (1991) concluded that muscle grafting was the treatment of choice for upper limb neuromata.

Calder and Norris (1993) investigated the effectiveness of muscle grafts in the delayed repair of mixed peripheral nerves in a group of 12 patients. A satisfactory MRC S3+ level of sensory recovery was achieved for 5 patients whilst motor recovery was universally poor. These disappointing clinical results may be explained by deficiencies in Calder and Norris' method of muscle denaturation. A hand held freeze spray was used as there was a problem with the graft fracturing on freezing in the liquid nitrogen. When a failed graft was removed and examined histologically it was established that the graft had not been completely denatured by this method and probably contributed to the poor result.

It is clear that further clinical trials are required before the efficacy of the FTMG in comparison with conventional nerve grafting can be determined. However, the advantages of using muscle grafts include that they are cheap and are easy to harvest as skeletal muscle is usually near the site of injury and, being autogenous, induce no immune response. There is also the minimalization of donor morbidity. The performance of a muscle is unlikely to be affected by the amount of muscle

removed to make a muscle graft as opposed to the scar formation, sensory loss and, occasionally, the formation of neuroma which occurs as a result of removal of a cutaneous nerve to make a graft. It also appears to excite less scarring than cable grafts (Glasby *et al.* 1990). The wide basement membrane tubes which the muscle graft provides are large enough to accommodate the largest nerve fibres (Glasby *et al.* 1991). Furthermore grafts made from cutaneous nerves provide small Schwann cell basement tubes which may have a restrictive influence on the development of regenerating axons of large motor fibres (Simpson and Young, 1945, Berry and Hinsey, 1946). Although whether this is correct is somewhat debatable as Cragg and Thomas (1964) and Glasby *et al.* (1988) have suggested that large diameter nerves never regenerate.

The FTMG is not without its disadvantages. Firstly, it is not a reliable method of repair for long defects. Hems and Glasby (1992) compared nerve and muscle grafts over 5 cm gaps in rabbit peroneal nerve and found significant regeneration through the nerve grafts but not through the muscle grafts. This may well be explained by the nerve grafts complement of Schwann cells which the muscle graft lacks, as well as the limited migratory powers of the Schwann cells (Nadim *et al.* 1990, Hems and Glasby, 1993). However, the freeze-thawing process is rapid as opposed to the time-consuming chemical denaturing techniques (Keynes *et al.* 1984). The use of the liquid nitrogen may also shatter the muscle graft due to the severe thermal shock, resulting in loss of orientation of the basement membrane tubes (Glasby *et al.* 1992).

1.7.5.8 Entubulation

The concept of bridging a gap between nerve ends with a nonneural conduit is not a new idea and is known historically as tubulation or entubulation repair (Suematsu, 1989). The method was reported experimentally over 100 years ago by Glück (1880) who used decalcified bone to repair a divided nerve, but without successful regeneration. Vanlair (1882a,b) used decalcified bone to bridge a 3 cm gap of the sciatic nerve of dogs. These experiments were more successful as histological examination revealed regenerated fibres in the tissue replacing the resorbed bone, and in the distal nerve stump. Another technique that assisted in the development of the tubulization procedure was the practice of wrapping the suture line with various membranous materials. This method developed early in nerve suture and was designed to maintain an effective barrier to the ingrowth of scar tissue (Fields *et al.* 1989). Over the years numerous materials including arteries, veins, fascia, stainless steel, tantalum metal and various synthetic substances have been employed in an attempt to find the ideal nonneural conduit (reviewed by Fields *et al.* 1989).

In recent years there has been a revival of interest in this concept engendered by the belief that the entubulation method may ultimately prove to be superior to conventional nerve repair and autologous nerve graft techniques. The most obvious advantage of entubulation over autologous nerve grafts or the FTMG, is the absence of a donor site. Other theoretical advantages of entubulation include avoidance of surgical trauma to the nerve endings by suture as the nerve stumps are usually placed into the guide with only one epineurial suture and this probably results in less traumatic degeneration. The tube may prevent fibroblasts from entering the site of repair and interfering with the distal migration of the nerve sprouts. Also, the tube

may confine the nerve endings and prevent the loss of neurites to tissues other than end organs. Each of these would contribute to the maintenance of an optimal microenvironment for nerve regeneration around the site or repair, whilst preventing disturbance by any extrinsic influences. In addition, and perhaps most importantly for the future, the entubulation technique permits the introduction of various growth-promoting factors into the lumen of the tube and, thus, enable one to modulate or enhance the regeneration process. This may help in addressing the problem of specificity of regeneration which to date has not been achieved by other methods of nerve repair.

A conduit made from silicone was first used clinically in the repair of peripheral nerve injuries by Lundborg *et al.* (1991). As these chambers did not degrade, some of the patients complained of "irritation" at the site of the repair and as a result the silicone tubes were subsequently removed. Furthermore, compression of nerves regenerating through silicone tubes with reduction in nerve function has been demonstrated (Johnston *et al.* 1993). Hence the potential need for a second operative procedure after silicone chamber reconstruction raised doubts as to the use of these particular conduits in the repair of peripheral nerve injuries (Lee Dellon, 1994). As a result a biodegradable material was sought for entubulation repair. Some of these materials which have been used as a conduit for nerve repair include polyester, polyglycolic acid and hyaluronic acid (Seckel *et al.* 1984). However, it was observed that the more biodegradable the tube was, the more likely it was to harm nerve regeneration. In particular, Henry *et al.* (1985) noted that the lumens of the more degradable conduits such as polyester tubes, became narrowed and misshapen. Based on these experiments it was concluded if a biodegradable tube was to be used

clinically it must first be capable of fulfilling certain requirements (Doolabh *et al.* 1996). Some of these include: can be easily made and are widely available, are flexible, lack antigencity, and do not cause any compression or irritation at the site of repair which would require a second procedure to remove the conduit.

1.7.5.9 Biodegradable controlled release glass tubes

One tube which meets the requirements of the ideal biodegradable conduit is manufactured from controlled release glass. Controlled release glass (CRG) is a new material developed over the last decade by Giltech Limited (Ayr, Scotland). It is an inorganic polymer with the composition including 32 mole% Na_2O, 21 mole% CaO and 47 mole% P_2O_5. The constituents are converted into glass by heating them at temperatures of about 1100^0C. By varying the composition of the CRG the rate of solution can be selected. The CRG will dissolve in aqueous media such as blood, saliva, urine, lymph or soft tissue. Upon completion of the dissolution there are no residues (Burnie *et al.* 1981, Burnie and Gilchrist, 1983).

The constituents of the CRG can be altered so that elements which may be biologically active can be delivered as the CRG dissolves. A number of investigations have been conducted into determining the biocompatibility and level of toxicity if any, of CRG based on a Na_2O-(C, Mg)-O-P_2O_5 composition. In a range of applications which varied from orthodontic devices (Savage, 1982) to the administration of copper, cobalt and zinc elements to cattle (Drake and Allen, 1985), there were no deleterious effects reported. In experiments where the composition of CRG was based on Na_2O-CaO-P_2O_5, there have been no signs of bio-incompatibility after implantation in the soft tissue of sheep (Burnie *et al.* 1981) or in specimens of

bone (Burnie *et al.* 1981, Burnie and Gilchrist, 1983). However, in experiments where CRG pellets were implanted subcutaneously, intramuscularly and intraperitoneally in rats, sheep and cattle, there was a reaction at the site of implantation. This was in the form of a fibrous capsule surrounding the pellet although the reaction was less than that seen with other biocompatible surgical substances (Allen *et al.* 1978).

It was decided to compare the effectiveness of CRG as a conduit with FTMG in repairing severed peripheral nerves in this project.

1.8 AIMS OF THIS STUDY

While experiments which have used the rat model have been of considerable neurobiological significance, there has still be some doubt as to whether these findings made on rats have practical significance for nerve repair strategies in man. Indeed the FTMG has attracted attention as an effective substrate for peripheral nerve regeneration after encouraging experimental results (Glasby *et al.* 1986a,b,c,d, Davies *et al.* 1987, Gattuso *et al.* 1988a,b, Glasby *et al.* 1988a,b, Gschmeissner *et al.* 1988, Gattuso *et al.* 1989, Glasby *et al.* 1989, Glasby *et al.* 1990, Huang *et al.* 1990, Gschmeissner *et al.* 1990, Glasby *et al.* 1998). However in a report of the clinical results of mixed peripheral nerve repair by FTMGs, some of the patients achieved a satisfactory level of sensory recovery but motor recovery was universally poor (Calder and Norris, 1993). These results were disappointing. Mindful of the rat's superior regenerative ability, in the current study it was thought important to pursue

the study of nerve injury and repair in larger animal models such as rabbit and sheep, where the results might have more relevance to nerve repair in humans.

Hence, the aims of the project were as follows:

1. To compare biodegradable controlled release glass tubes and freeze-thawed muscle grafts in the repair of a peripheral nerve injury in rabbits.

2. To assess fibre type proportions and distribution in the rabbit after nerve injury and repair.

3. To compare nerve autografts and freeze-thawed muscle grafts in the repair of obstetrical brachial plexus palsy in sheep.

The method of evaluation was by morphometric assessment of muscle.

The main reason for using the morphology of cross-sections of muscle as an assay for reinnervation efficacy and recovery following peripheral nerve repair is that examination of muscle in this manner provides data about the structure and function of an important component of the motor unit, the muscle fibre. Detailed information can be gathered about any change in muscle fibre size, shape and architecture, as well as connective tissue content and the proportions and distributions of the different fibre types. These alterations can dramatically affect the functional capabilities of the muscle fibre and of its respective motor unit and therefore affect the level of recovery of the target muscle. A weakness of a muscle may be a consequence of connective tissue proliferation and/or atrophy of muscle fibres, whilst a change in the relative abundance of the different fibres present may result in the inability of a muscle to contract quickly or to maintain prolonged activity. The primary goal of nerve repair is to achieve a degree of useful function of the target organ. Hence in repairing a motor

nerve it is important to establish whether the method of repair influences any morphological changes in the muscle fibres.

CHAPTER 2

Materials and Methods

2.1 Introduction

The New Zealand White rabbit was chosen as the experimental animal for this study on muscle grafts and biodegradable CRG tubes. The rabbit is a commonly used laboratory animal and has a long history of use in the field of peripheral nerve research (Gutmann, 1942, 1948, Gutmann *et al.* 1942, Gutmann and Sanders, 1942, Haftek, 1970, Lundborg and Rydevik, 1973, Wall *et al.* 1991, 1992, Hems and Glasby, 1992, Gulati and Cole, 1994, Hou and Zh, 1998, Kim *et al.* 1998).

Additional experiments were performed on sheep. The purpose of these experiments were to establish first, the optimal timing of nerve repair (immediate or delayed), second, whether there was a difference in the level of recovery between neonates and adults after nerve repair, third, to assess the FTMG as a surgical technique and to determine whether the FTMG is at least as good as a conventional nerve graft. The experiments were designed to establish whether these three variables influenced the degree of recovery in a clinical model, the obstetrical brachial plexus palsy (OBPP).

The peroneal division of the sciatic nerve was selected as it is readily accessible through a lateral incision in the thigh and studies can be performed using the extensor digitorum longus as a muscle specifically innervated by the nerve. The anatomy of the rabbit sciatic nerve is shown in Figure 2.1.

The two divisions of the sciatic nerve (peroneal and tibial) run side by side as far as the mid-thigh where they diverge with the peroneal nerve remaining more superficial. The peroneal nerve passes into the anterior compartment of the leg where it divides into the superficial peroneal nerve and the deep peroneal nerve. The

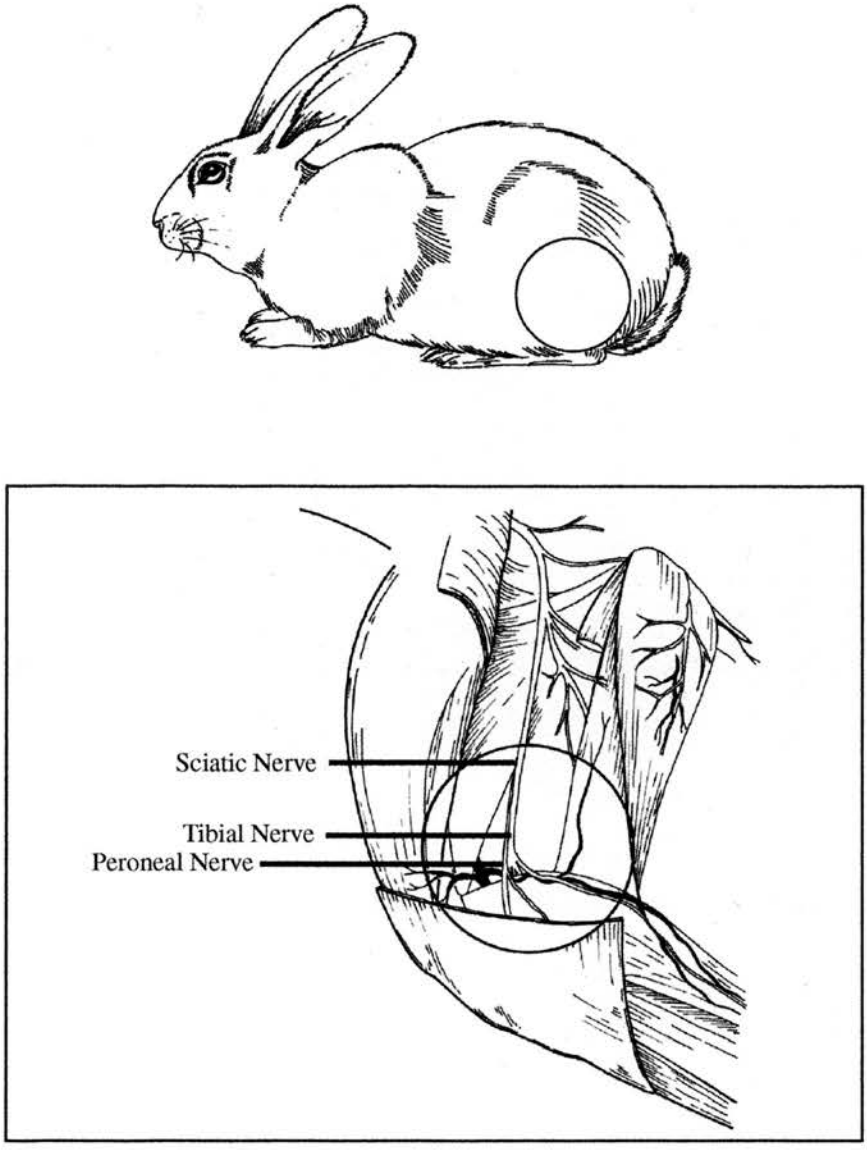


Figure 2.1 The anatomy of the rabbit sciatic nerve (left side).

muscles innervated by the peroneal nerve include tibialis anterior, extensor digitorum longus, peroneus longus, peroneus secundus, peroneus tertius and peroneus quartus. The actions of each of these muscles are: tibialis anterior, dorsiflexion and supination of the foot; extensor digitorum longus, dorsiflexion of the toes; peroneus longus, pronation and some plantar flexion of the foot; peroneus secundus, abduction of the fourth toe and some abduction of the other toes; peroneus tertius, dorsiflexion of the third toe and some abduction of the second and third toes; and peroneus quartus, plantar flexion and some abduction of the second and third toes. Hence, the combined functions of these muscles innervated by the peroneal nerve are dorsiflexion of the toes and the foot, pronation of the foot and abduction of the second, third, and fourth toes (Gutmann, 1942).

2.2 Rationale for experimental design

The purpose of the present experiments was to examine the practicality of the CRG tube for bridging irreducible nerve gaps and to assess the possible use of the tube as a means of confining humoural or cellular substances at the site of repair. This latter rationale has important implications for the future as the isolation of the site of the repair in a tube may allow for manipulation of the regenerative process. Hence the present study was designed to compare CRG tubes filled with either freeze-thawed muscle, chopped nerve or combined freeze-thawed muscle and chopped nerve to enclose the ends of a nerve which had been cut and separated by a 1 cm gap. To determine whether any of these materials had any influence on the regenerative process a CRG tube was used not filled with any substances, but with

the 1 cm gap between the nerve ends. The FTMG was used as a standard as it is an established technique for the repair of a transected peripheral nerve (Glasby *et al.* 1986a,b,c,d, Davies *et al.* 1987, Gattuso *et al.* 1988, Glasby *et al.* 1988a,b, Gschmeissner *et al.* 1988, Gattuso *et al.* 1989, Glasby *et al.* 1989, Glasby *et al.* 1990, Huang *et al.* 1990, Gschmeissner *et al.* 1990, Glasby *et al.* 1998).

Three control groups were used. To provide a measure of normal values an age, sex and weight matched group which had undergone no procedure was used. For a measure of the "worst-case scenario" after nerve repair where reinnervation of the target muscle was reduced, a group where the peroneal nerve was cut, a gap created and then not repaired was used. A nerve crush injury was created in a further group to provide a comparative measure of reinnervation processes under optimal conditions ("best-case scenario"). As endoneurial tube continuity is maintained after a nerve crush this injury is less severe than after the transection of a nerve, and a greater number of regenerating axons which successfully reach and reinnervate their target muscle fibres would be expected.

2.3 Experimental groups

Forty adult female New Zealand White rabbits with initial weights in the range of 2.75 to 3.5 kg were used. The animals were divided into five experimental groups and three control groups. Each group consisted of five rabbits. The experimental groups were as follows:

I. A 1 cm coaxially aligned freeze-thawed autologous muscle graft (FTMG).

II. A CRG tube containing a 1 cm length of pieces of freeze-thawed muscle in the centre of the tube (CRG-M).

III. A CRG tube with chopped nerve placed in the centre of the tube (CRG-N).

IV. A CRG tube filled with a 1 cm length of combined freeze-thawed muscle and chopped nerve (CRG-MN).

V. An empty CRG tube with a 1 cm gap between the proximal and distal stumps of the divided peroneal nerve (CRG-GAP).

2.4 Preparation of animals

The rabbits were supplied by City Farms Ltd (Willie Walker, City Farms Ltd, West Calder, West Lothian, UK). They were obtained seven days prior to surgical procedure to allow an appropriate period for acclimatization to their new environment (University Federation for Animal Welfare Handbook, 1989). The animals were housed either in the Medical Faculty Animal Area, or in the Department of Tropical Veterinary Medicine in floor pens which were large enough to provide 2600 cm^2 area per animal (Figure 2.2). The weight of each animal was monitored on a weekly basis, as well as any sign of infection or disease. The housing and care of the animals were in accordance with the recommendations of the Home Office Animals (Scientific Procedures) Act 1986.

Figure 2.2 The rabbits in a floor pen in the Department of Tropical Veterinary Medicine, University of Edinburgh.

2.5 Anaesthesia

Each animal was weighed prior to anaesthesia. This allowed accurate calculation of drug dosages and assessment of any postoperative weight loss (Flecknell, 1996).

The anaesthesia was induced by a combination of Hypnorm and Hypnovel. Hypnorm (fluanisone 10 mg ml^{-1} and fentanyl citrate, 0.315 mg ml^{-1}; Janssen Pharmaceutical Ltd.) 0.3 ml kg^{-1} was administered first by intramuscular injection into the right thigh (non-experimental side), immediately followed by Hypnovel (midazolam hydrochloride 10 mg ml^{-1}; Roche Products Ltd.) 2 mg kg^{-1} by intraperitoneal injection.

Approximately ten minutes after induction the rabbit was unconscious. The depth of anaesthesia was assessed by the pedal withdrawal reflex. One limb of the rabbit was extended and the web of skin between the toes was pinched between the anaesthetist's fingernails. If the rabbit withdrew its limb, muscles twitched, or the animal vocalized, then the depth of anaesthesia was insufficient to allow surgical procedures to be carried out (Flecknell, 1996).

The anaesthesia was maintained with a Bain coaxial circuit using a mixture of oxygen 1 l min^{-1}, nitrous oxide 0.5 l min^{-1} and 2% halothane (May and Barker, Rhone-Poulenc Group, UK). A concentric face mask was used to connect the anaesthetic circuit to the animal. This mask design incorporates a gas scavenging system. The expired gases were collected through a Veterinary Fluosorber (International Market Supply, Cheshire, England). Anaesthesia with gases provided a more stable and controllable method of maintaining general anaesthesia in the rabbit.

2.6 Preparation for surgery

Just prior to surgery the fur over the lateral aspect of the left thigh was shaved with electric clippers (Oster Professional Products, Milwaukee, USA). To remove any extra hair immac cream (Reckitt and Coleman Ltd, Hull, UK) was applied to the shaved area. Once the immac cream and hair was removed, the skin was washed with warm tap water and then cleaned with an iodine antiseptic solution (Betadine, Seton Health Care Group plc, Oldham, UK) to remove any excess dirt and oil.

After the skin was prepared the animal was placed on a heating blanket and covered by an aluminium foil blanket to help maintain body temperature. In most cases body temperature was monitored during the operative procedure using a digital readout thermistor probe (Technoterm 110, RS Products Ltd West Germany) placed in the rectum. A pulse oximeter (Microspan 3040, BCI International, UK) was sometimes used to continuously monitor blood oxygen saturation (Sa O_2). None of these procedures proved essential for the animals to survive the operative procedure however and were not used for the latter groups of animals.

2.7 Surgical procedures

All surgery was performed using full sterile procedures. A drape was placed over the animal and a hole cut to reveal the operation site. An incision was made in the skin from the sciatic notch down the lateral aspect of the left thigh to a point just below the knee. A diagrammatic representation of the pelvic and lower limb muscles of the rabbit (lateral aspect) is shown in Figure 2.3. The skin edges were mobilized and haemostasis secured by means of a bipolar diathermy (Codman, Randolph USA).

Retraction of the skin exposed the gluteus superficialis and the coccygeofemoralis muscles. The plane dividing these muscles was then developed from the sciatic notch to the knee to expose the sciatic (ischiadicus) nerve. The exposure was maintained using a small self-retaining retractor.

Using an operating microscope (Wild M690, Heerbrugg, Switzerland) to assist vision, the peroneal nerve was separated from the tibial nerve from a location 1 cm above the popliteal artery (Figure 2.4). This was extended for a distance of approximately 3 cm proximally. Microsurgical forceps were used and care was taken to ensure that any tension or trauma to either the peroneal or tibial nerves was minimal. The peroneal nerve was cut approximately 2 cm above the point where the popliteal artery crosses the peroneal nerve. From the distal segment of the peroneal nerve a section of nerve was removed to create a 1 cm gap after retraction. This piece of nerve was later used in preparing the CRG-N and CRG-MN tube groups. The 1 cm deficit was filled by one of the five repair methods under investigation.

2.8 Repair groups

During each of the repair procedures vision was assisted with an operating microscope (Wild M690, Heerbrugg, Switzerland). With each repair group care was taken to avoid any tension at the suture line and any rotation of the nerve so as to maintain the correct geometric alignment of the two nerve ends. Details on each of the repair groups are as follows:

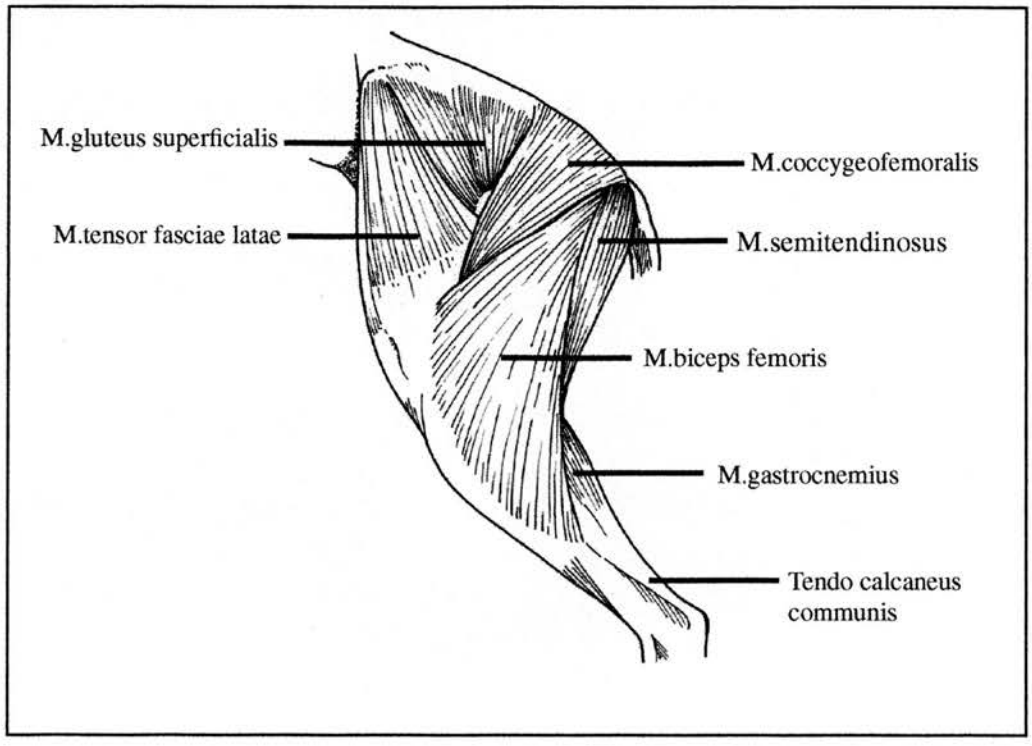
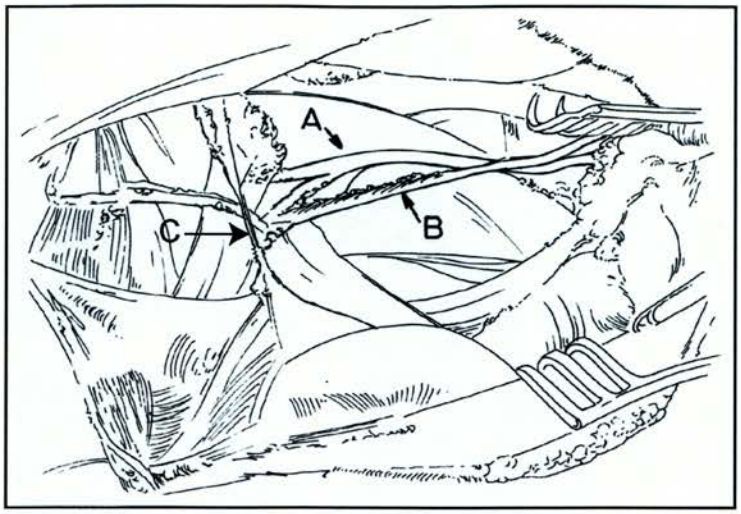
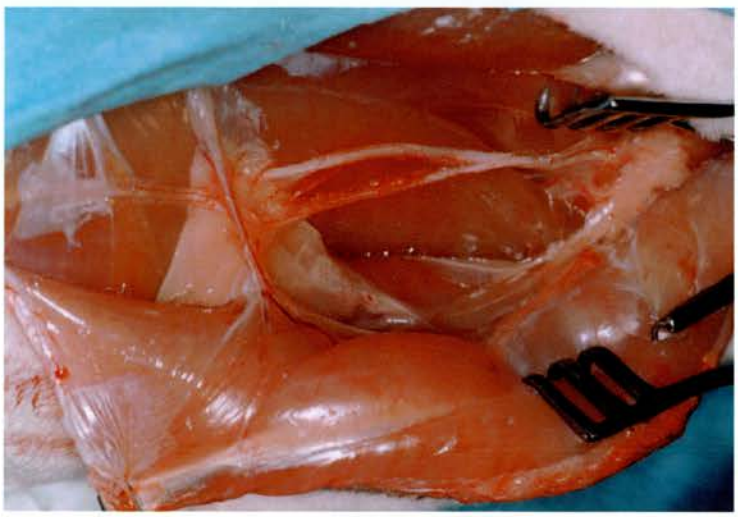


Figure 2.3 Muscles of the left pelvis and lower limb in the rabbit (lateral aspect).





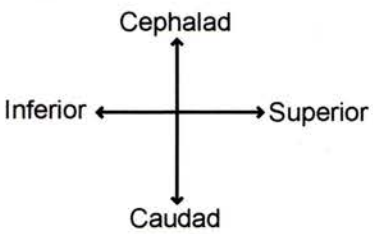


Figure 2.4 Exposure of the left sciatic nerve in the rabbit. (A) The Tibial Nerve. (B) The Peroneal Nerve. (C) The Popliteal Artery.

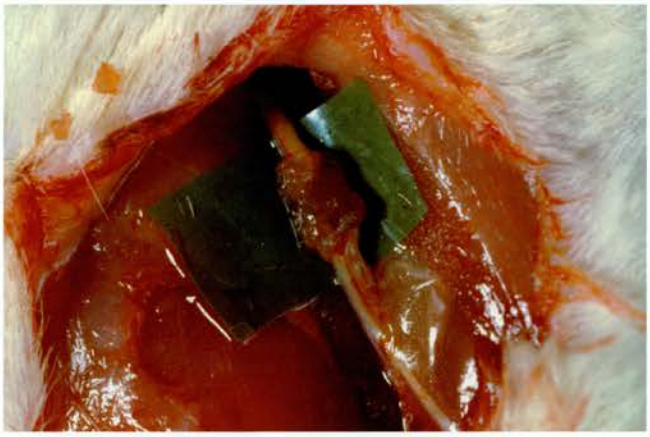
I. Coaxial freeze-thawed autologous muscle graft (FTMG)

A section of muscle 2 cm by 2 cm by 1 cm was excised from the coccygeofemoris muscle ensuring that the muscle fibres were parallel along its length. The piece of muscle was then wrapped in sterile aluminium foil and submerged in liquid nitrogen until thermal equilibrium was reached. At this point the muscle was transferred to sterile distilled water and allowed to thaw. This freezing-thawing procedure causes a shrinkage of the muscle of up to 50% of its original size (Glasby *et al.* 1992). Once thawed the muscle was placed on a sterile steel board and trimmed into a rectangular block using a scalpel. The muscle was cut to approximately 1 cm by 1 cm by 1 cm with the fibres running parallel to the long axis of the block. The FTMG was then interposed into the gap in the peroneal nerve and secured with 3 to 4 epineurial interrupted 10/0 sutures (Ethilon polyamide, Ethicon UK Ltd.) at the proximal and distal stumps. Figure 2.5 is a photograph of the FTMG *in situ*.

For each of the following groups biodegradable CRG tubes (Giltech, Ayr, UK) were used. Each tube was 2 cm in length with an internal diameter of 4 mm. At each end of the tube there were two small holes approximately 2 mm from the edge and 180^0 from each other. The controlled release glass tubes (CRG) tubes were individually packaged and sterilized by gamma-irradiation (Isotron plc, Bradford, UK). Figure 2.6 is a photograph of a controlled release glass (CRG) tube.

II. CRG tube filled with freeze-thawed skeletal muscle graft (CRG-M)

For the FTMG a 2 cm by 2 cm by 1 cm block of muscle was removed from



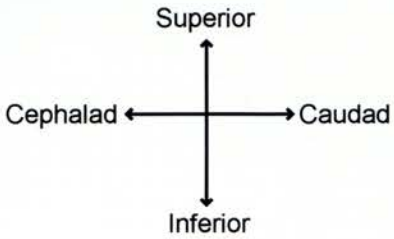


Figure 2.5 The FTMG *in situ*.

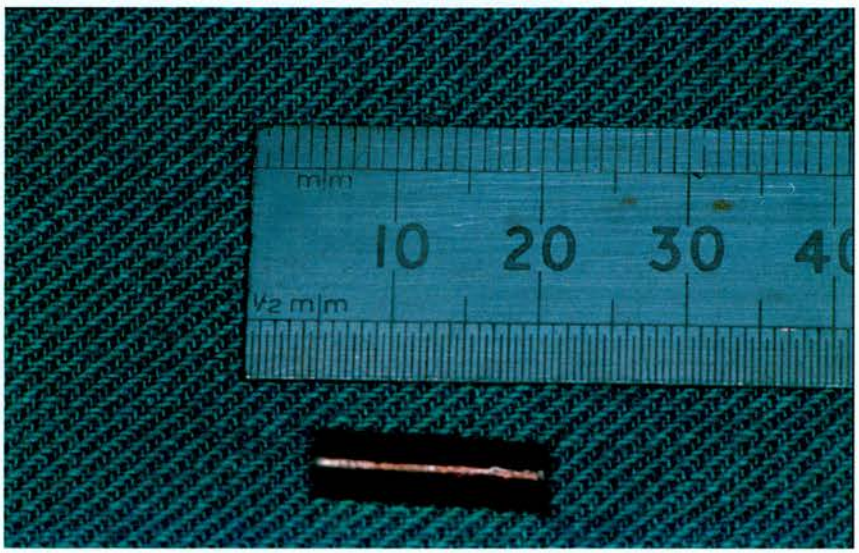


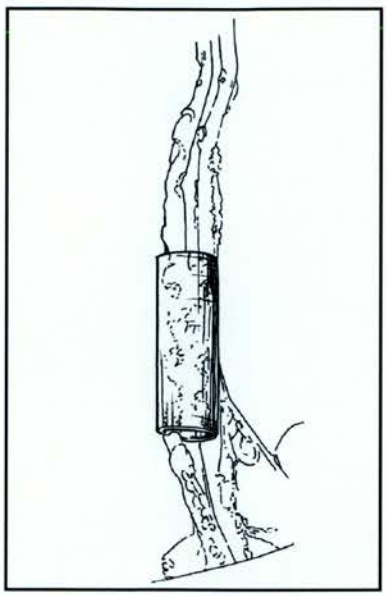
Figure 2.6 A controlled release glass (CRG) tube.

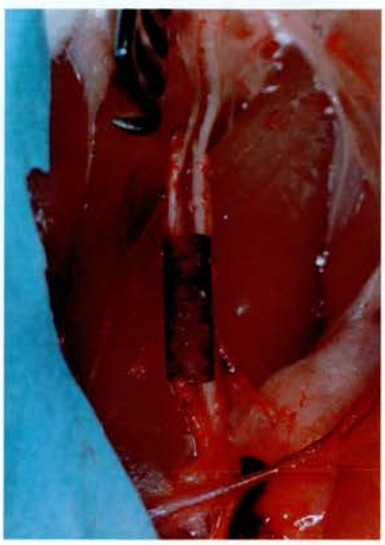
the coccygeofemoralis muscle. The graft was prepared as described above however, once the muscle had thawed it was cut into small pieces by a razor blade. The CRG tube was removed from its sterile packet and the FTMG pieces were randomly packed into the centre of the tube for a length of 1 cm.

The CRG tube was introduced into the gap in the peroneal nerve and the proximal and distal segments of the divided nerve were placed in opposite ends of the tube. The nerve segments were set in the tube so that they were flush with the FTMG pieces. Two interrupted epineurial 10/0 sutures (Ethilon polyamide, Ethicon UK Ltd.) were placed approximately 5 mm from each proximal and distal segment of the divided nerve, and were then passed through the holes drilled through the CRG tube at each end. This helped minimize damage or any tensile forces at the site of repair. Figure 2.7 is a photograph of a CRG tube containing FTMG sutured in place in the rabbit peroneal nerve. Figure 2.8b is an illustration of the CRG-M graft.

III. CRG tube filled with nerve graft (CRG-N)

For the CRG-N group, the section of nerve which was excised from the peroneal nerve was cut into tiny pieces and placed in the CRG tube. As there was not enough peroneal nerve gathered to fill the tube with 1 cm of chopped nerve, the pieces were placed in the centre of the CRG tube. This was then inserted into the gap in the peroneal nerve. The nerve was secured to the tube at each end using 2 interrupted 10/0 sutures (Ethilon polyamide, Ethicon UK Ltd.) as before. Figure 2.8a is an illustration of the CRG-N graft.





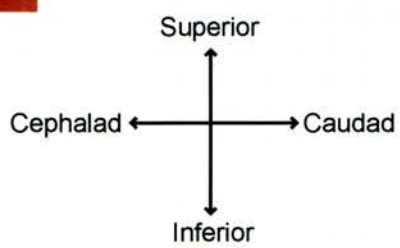


Figure 2.7 The CRG tube filled with pieces of chopped freeze thawed muscle (CRG-M) sutured in place in the peroneal nerve gap.

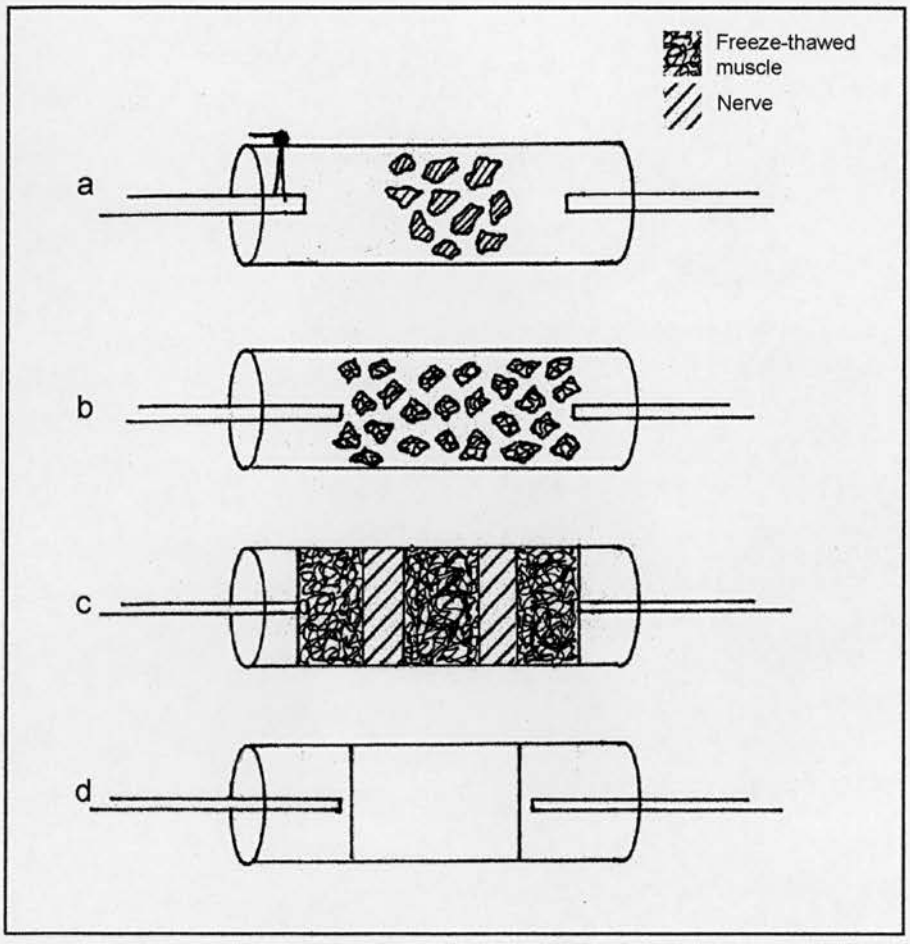


Figure 2.8 A diagrammatic representation of the contents of the CRG tubes.
(a) A CRG tube filled with nerve graft (CRG-N).
(b) A CRG tube filled with freeze-thawed skeletal muscle graft (CRG-M).
(c) A CRG tube filled with freeze-thawed muscle and nerve (CRG-MN).
(d) A CRG tube and 1cm gap between the nerve segments (CRG-GAP).

IV. CRG tube filled with freeze-thawed muscle and nerve (CRG-MN)

For the CRG-MN group, a section of muscle 2 cm by 2 cm by 1 cm was excised from the coccygeofemoris and a FTMG prepared as described in section 2.8.1. The preparation of the nerve component was as detailed in section 2.8.3. The chopped freeze-thawed muscle and nerve were placed into the centre of the CRG tube for a distance of 1 cm. The freeze-thawed muscle pieces were divided into 3 parts each 0.3 cm in length and separated by 2 sections of chopped nerve each approximately 0.05 cm long.

The distribution of the constituents of the graft were:

1. 0.3 cm of freeze-thawed muscle pieces
2. 0.05 cm of nerve pieces
3. 0.3 cm of freeze-thawed muscle pieces
4. 0.05 cm of nerve pieces
5. 0.3 cm of freeze-thawed muscle pieces.

The proximal and distal stumps of the divided nerve were set in the tube so that they were flush with the freeze-thawed muscle ends of the graft. The nerve was secured to the tube at each end using 2 interrupted 10/0 sutures (Ethilon polyamide, Ethicon UK Ltd.) as before. Figure 2.8c is an illustration of the CRG-MN graft.

V. CRG tube and gap (CRG-GAP)

For the CRG-GAP group, the proximal and distal stumps of the divided peroneal nerve were inserted in opposite ends of a CRG tube. Inside the tube there was approximately a 1 cm gap between the nerve segments. Care was taken to avoid

any rotation of the nerve ends so that the exact geometric alignment was maintained. The nerve was secured to the tube at each end using 2 interrupted 10/0 sutures (Ethilon polyamide, Ethicon UK Ltd.) as before. Figure 2.8d is an illustration of the CRG-GAP graft.

2.9 Control groups

As with the experimental groups adult female New Zealand White rabbits with initial weights in the range of 2.75 to 3.5 kg were used for the controls. There were three control groups with five rabbits per group. The control groups were as follows:

I. Left Peroneal Nerve crush.

II. Left Peroneal Nerve section with removal of a piece of nerve to create a 1 cm gap after retraction.

III. Unoperated.

The preparation of animals, anaesthesia, preparation for surgery and surgical procedures for the control groups were as described for the repair groups. For both the nerve crush and the nerve section groups, the section of peroneal nerve treated was as per that for the repair groups. The peroneal nerve was crushed with 3 mm-wide smooth bladed watchmakers forceps for a period of 10 seconds.

The majority of the animals were housed in the Medical Faculty Animal Area however, for those kept at the Department of Tropical Veterinary Medicine, one unoperated animal was used as a control to assess whether the different surroundings had any impact on the outcome of the experiments.

2.10 Closure of the wound

For all experimental and control groups the closure of deep tissues was accomplished with interrupted 6/0 polyglactin sutures (Vicryl, Ethicon Ltd, UK). The skin was closed with a continuous subcuticular stitch using 3/0 polyglactin sutures (Vicryl, Ethicon Ltd, UK). Antibiotic spray (Tribiotic, 3M Health Care Ltd) was applied to the deep tissues and permeable spray dressing (Smith and Nephew Medical Ltd.) was applied to the wound. Management of postoperative pain was by an intramuscular injection of Vetergesic (buprenorphine hydrochloride, 0.3 mg ml^{-1}; Reckitt and Colman Products Ltd) 0.03 ml kg^{-1}.

Once conscious the animal was placed in a plastic holding box and left to recover under observation. The bedding in the box was towelling and not straw as this will often adhere to the animal's eyes, nose and mouth (Flecknell, 1996). To prevent any loss of body heat the animal was wrapped in a foil blanket. Once recovered from anaesthesia the rabbits were then returned to the floor pens and kept in the animal area for a period of 6 months.

2.11 Toe spreading reflex and other criteria of muscle recovery

At 6 months after operation and prior to sedation and removal of the extensor digitorum longus muscle, each animal was tested for the toe spreading reflex. This is a postural reflex and is a useful indicator of recovery of motor function. In the rabbit this reflex is elicited by holding the animal by the loose skin of the back and then

suddenly lowering it. This causes a small group of muscles innervated by the peroneal nerve to contract and to spread the second, third, and fourth toes. The muscles involved in this reflex are the small peroneals (peroneus secundus, tertius and quartus). The reflex also includes abduction of the first toe by the abductor hallucis muscle which is innervated by the tibial nerve. Hence, reflex spreading of the second, third, and fourth toes establishes an index of peroneal nerve function, whilst reflex abduction of the first toe characterizes tibial nerve function (Gutmann, 1942).

For estimation of the degree of recovery of peroneal nerve function, an arbitrary scale called the "spreading index", was used. The degrees of spreading are as follows: degree 1 represents just visible spreading of the fourth toe alone; degree 2 slight spreading of the second, third, and fourth toes; degree 3 spreading of all three toes but to a degree that is less than that elicitable in the normal animal; degree 4 full spreading of all three toes with an amplitude equivalent to normal as determined before operation or by comparison with the opposite normal side (Gutmann, 1942). Elicitation of the toe spreading reflex in a normal rabbit and after cutting the peroneal nerve in a rabbit are illustrated in Figure 2.9.

At 6 months after operation other criteria of muscle recovery included general observation of stance and gait, palpation for muscle wasting of the limb in the region of the denervated muscles, and examination for degree of flexibility of affected joints and any joint contracture. However, although interruption of the peroneal nerve is characterized by drop foot and the absence of the spreading reflex, these features do not produce a marked influence on gait and stance. Apart from some abduction of the foot when sitting and some dragging of the foot when hopping, the gait and stance

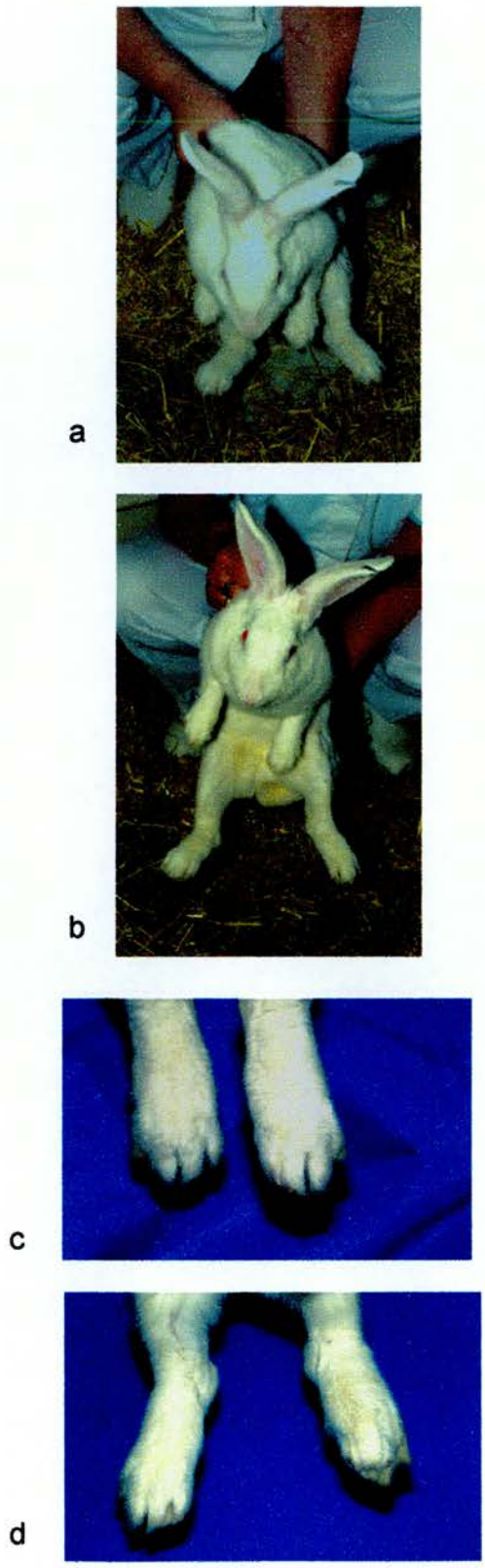


Figure 2.9 Elicitation of the toe spreading reflex (a and b) in a normal rabbit (c), and after cutting the peroneal nerve (d).

will appear normal. Indeed, although drop foot is most marked immediately after interruption of the peroneal nerve, it becomes less or disappears some time before reinnervation of the muscles. This is the result of the sitting posture of the rabbit which fixes the foot in a bent position. However, there are rare cases where the toes are kept in marked plantar flexion and the animal walks on the dorsum of the toes, where sores may develop (Gutmann, 1942). As a result, this was all taken into consideration when observing the stance of the animals and their general movement about the floor pens.

Visual examination of the extensor digitorum longus muscle *in situ* was performed 6 months after operation. The general form of the muscle was noted, as well as any differences in size between experimental and contralateral extensor digitorum longus muscles. The site of repair was observed for any evidence of the CRG tube or the FTMG and any fibrotic reaction. The general appearance and calibre of the peroneal nerve at the repair site was also noted.

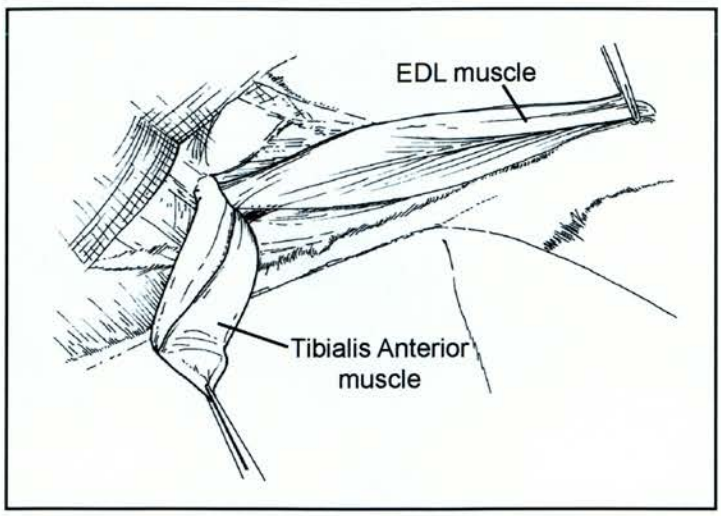
2.12 Removal of the extensor digitorum longus (EDL) muscle

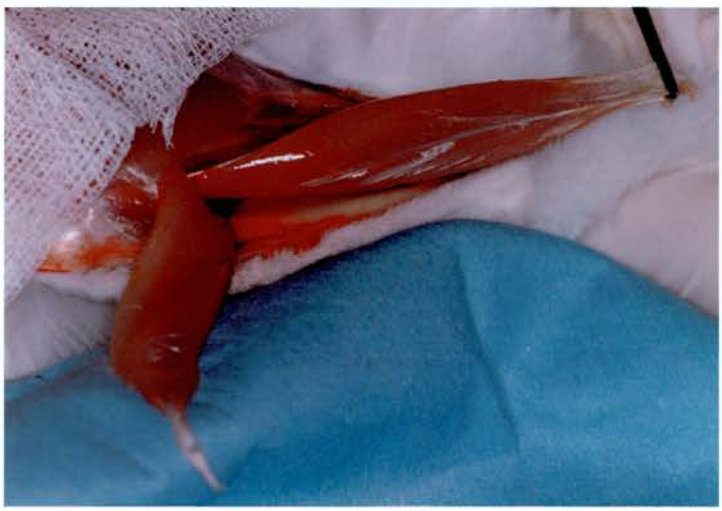
At 6 months after operation each animal was initially sedated with a dose of Hypnorm (fluanisone 10 mg ml^{-1} and fentanyl citrate, 0.315 mg ml^{-1}; Janssen Pharmaceutical Ltd.) 0.3 ml kg^{-1} by intramuscular injection into the right thigh (non-experimental side), before a lethal intravenous injection of phenobarbitone (Lethobarb, J.M. Loveridge plc, Duphar Veterinary Ltd, Southampton, UK body) 0.7 ml kg^{-1}.

Once the rabbit was dead the fur was shaved from the thigh and the front of leg. An incision was made down the lateral aspect of the thigh in order to expose the coccygeofemoralis and the gluteus superficialis muscles. The sciatic nerve in the thigh was exposed as before and the peroneal nerve examined to ensure that the surgical repair was intact. In a parallel study electrophysiological tests were performed using EMG recording to assess evoked responses (D.V. Lenihan, PhD Thesis, University of Edinburgh 2000). Stimulating electrodes were placed on the left peroneal nerve proximal and distal to the site of repair to check for nerve conduction. To prevent any electrical cross-interference the left tibial nerve was divided at the sciatic notch and at the knee. Presentation of the electrophysiological data obtained has not been duplicated in this thesis.

After the site of the repair was examined, the incision was extended distally along the front of the leg to the ankle to expose the anterior group of leg muscles and the extensor retinaculum. The extensor retinaculum was divided at the ankle together with the tendon of tibialis anterior. The lower part of both the tibialis anterior and the EDL muscles were mobilized and deflected proximally. Tibialis anterior was then detached from its tibial origin and removed to expose the EDL muscle. The tendons of the EDL at the ankle and the knee were divided as close to the myotendinous junction as possible and the complete muscle removed. Exposure of the left tibialis anterior and the left EDL muscles in a normal rabbit is shown in Figure 2.10.

The left (experimental) and the right (contralateral) EDL muscles were excised as discussed above.





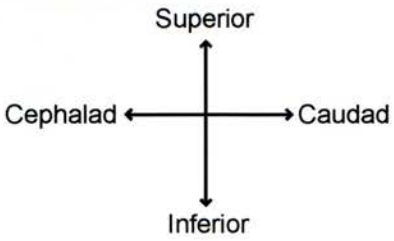


Figure 2.10 Exposure of the left extensor digitorium longus (EDL) and the tibialis anterior muscles in a normal rabbit.

2.13 Freezing of the EDL muscle

Immediately after excision any excess fat was dissected away from the muscles, the muscle blotted and their wet weight was recorded to the nearest 0.01 g. After weighing, the muscles were wrapped in foil to reduce drying out and stored in a fridge at 4^0C until preparations for the freezing process were complete which in most instances was no longer than one hour. Each muscle was transected through the mid-belly with a razor blade and a 1 cm section removed. This was mounted on an appropriately labelled cork disc with its cut face uppermost, supported by agar and tissue embedding medium (Tissue-tek O.C.T Compound, Miles Diagnostic). The block was then immediately frozen by plunging it quickly and completely into isopentane for about 30 seconds. The isopentane had been previously cooled to approximately -159^0C by suspending it over liquid nitrogen. The frozen blocks were then immediately transferred to a -70^0C freezer and stored until required for sectioning.

2.14 Tissue sectioning

Sectioning of the muscle was carried out in a Frigocut 2800E Cryostat (Reichert-Jung, Cambridge Instruments). When transferring the muscle specimens between the freezer and the cryostat utmost care was taken to avoid minor thawing or damage to the tissue block. If there is any thawing and refreezing of the tissue this can cause extensive damage due to the formation of ice crystal artefacts. These artefacts are in the form of ice crystal spaces which appear as regular rounded holes

within the muscle fibres and if they are large, they often disrupt the fibre outline (Cumming *et al.* 1994).

The tissue blocks were mounted on metal microtome chucks with embedding medium (Tissue-tek O.C.T Compound, Miles Diagnostic) and left for a minimum of 20 minutes to allow the tissue to adjust to the cryostat temperature of -22 ^{0}C before cutting. The initial sections were stained with toluidine blue to check for transverse orientation of the muscle fibres and tissue preservation. Once the orientation had been assessed, 20 consecutive sections were cut at 10 μm and then mounted on clean, pre-labelled, numbered glass microscope slides. The slides were then stored in a freezer at -70^{0}C until required for staining.

2.15 Histological and histochemical procedures

The following is a brief description of the histological and histochemical stains which were applied to the sections from all experimental and control groups. For more details of the staining techniques please refer to appendix.

I. Toluidine blue

This stain was used to check the orientation and preservation of the muscle block during sectioning. It stains the muscle fibres and the nuclei blue.

II. Haematoxylin and eosin (H & E)

This common staining method was used to provide information on fascicular architecture, muscle fibre shape and size, fibre splitting and position of nuclei. It stains the myofibres pink and the nuclei blue.

III. Masson's trichrome

This stain was used to assess connective tissue proliferation. It stains the muscle fibres red/brown, nuclei blue/black and the connective tissue green.

IV. Myofibrillar adenosine triphosphatase (ATPase) at pH 4.35, 4.6, 10.2

The two major types of muscle fibre (slow-twitch Type I and fast-twitch Type II) are distinguished by the myofibrillar ATPase stain. The reaction stains the muscle fibres pale, dark or intermediate depending on the pH of the preincubation medium. At an alkaline pH (10.2) type I fibres are pale and all type II fibres are dark, whilst at an acidic pH (4.35) type I fibres are dark and type IIa and b are pale. At pH 4.6 there is differentiation of the subclasses of type II fibres as type IIa fibres are pale and all other fibre types are dark.

2.16 Assessment of muscle

Sections from experimental and control groups were assessed for each of the following features:

I. Muscle wet weight

Immediately after excision any excess fat was dissected away from the muscles and their wet weight was recorded to the nearest 0.01 g.

II. Pathological features

Each section was stained with haematoxylin and eosin and examined for pathological features such as migrating nuclei, split fibres, angular or rounded shape, and granular or necrotic appearance. On average a total of 500 fibres were assessed for each section.

III. Connective tissue content

Each section was stained with Masson's Trichrome which stains the connective tissue a distinctive green colour. The connective tissue content of each muscle was measured by a point counting procedure (Aherne and Dunhill, 1982). An eye piece graticule was used to superimpose a 100 point lattice grid onto the muscle cross-section and a count was made of the number of grid intersections overlying connective tissue. These were recorded and expressed as a percentage of the total number of points counted. To determine the number of points that had to be counted to ensure a relative standard error of less than 5% a trial set of counts was completed and Hally's formula (Hally, 1964) applied where:

$$\text{Relative Standard Error} = \sqrt{\left(\frac{1-Vv}{n}\right)}$$

$$Vv = \frac{n}{N}$$

n = the number of points on connective tissue

N = the total number of points counted

2500 points per muscle were counted to ensure an error of less than 5%. All point counting was carried out at a magnification of x20 and sections from experimental and control groups were examined.

IV. Fibre type size and shape

Sections stained for ATPase at pH 4.35 and 10.2 were used to measure size and shape of type I and type II muscle fibres, and a total of 200 to 300 fibres were assessed for each muscle section. This was accomplished by the use of the VIDS III, (Analytical Measuring Systems Ltd, Pampisford, Cambridge, UK) which is a high resolution semiautomatic image analysis system. Muscle sections were viewed under a microscope (Zeiss) and utilizing a high performance television camera, images were displayed on a high resolution colour monitor. The monitor was connected to a digitizing tablet with a cursor. Features of interest were outlined or "marked" using the tablet and cursor, and the data handling was carried out by an IBM XT computer interfaced to the VIDS III system.

The narrow fibre diameter, which is defined as the maximum distance across the narrowest aspect of the muscle fibre was taken as a measure of fibre size (Brooke and Engel, 1969). This is recommended as it is the only measurement which is not altered by either obliquity of the section or kinking of the muscle fibre which are two common occurrences in muscle biopsies (Dubowitz, 1985). Furthermore, narrow fibre diameter is more frequently used in the clinical assessment of muscle pathology whilst cross sectional area is mostly used in human performance research. This is because the cross sectional area is a measure which seems best to correlate with the

functional capacity of muscle since it allows for calculation of the relative area of the muscle occupied by a given fibre type (Bar-Or *et al.* 1980, Mannion *et al.* 1995).

Fibre shape was assessed by calculating the form factor:

$$\text{Form factor} = \frac{4xaxπ}{p2}$$

a = the area

p = the perimeter

The area was calculated from the boundary and the perimeter was a total of the distances between the mid point of the boundary vectors. Form factor is unity for a perfect circle and less than unity for other shapes.

All measurements for fibre size and shape were carried out at an objective power of x20 and sections from experimental and control groups were examined.

V. Fibre type frequency

Sections stained for ATPase at pH 4.35 and 10.2 were used to assess frequency of type I and type II muscle fibres and a total of 500 fibres were assessed for each muscle section. In accordance with the recommendation of Cumming *et al.* (1994) the proportion of the different muscle fibre types was determined by counting contiguous fibres in a number of representative microscopic fields.

VI. Fibre type distribution

Sections stained for ATPase at pH 4.35 and 10.2 were used to assess the distribution of type I and type II muscle fibres in the EDL muscle. It was noted whether the fibre types were randomly scattered in the classic mosaic pattern or whether type grouping was evident. Some thought was given to the method of

assessment of fibre type grouping as historically, the identification of fibre type grouping has been dependent on somewhat arbitrary criteria. This is discussed at length in Chapter 4.

2.17 Preparation and sampling protocols of muscle

To avoid interanimal variations in fibre type population species, sex and weight were kept consistent among groups. In recording the muscle wet weight care was taken to ensure that the same amount of tendon was excised with each muscle and any excess fat removed. For optimum histochemical staining the muscles were frozen within an hour of removal from the animal (Cumming *et al.* 1994). Rapid freezing was also essential to prevent the problem of ice crystal artefacts.

All sections were removed from the mid-belly of the EDL muscle as there may be variations in muscle architecture along the length of normal muscle. Approximately 4% of fibres in normal muscle may possess internally located nuclei and this value can be much higher near myotendinous junctions (Cumming *et al.* 1994). There is also a greater proportion of connective tissue near the myotendinous junction than in the mid-belly of muscle (Kennedy, 1987), and fibre splitting is seen under normal circumstances at the myotendinous junction (Dubowitz, 1985). Thus, normal sections from the myotendinous junction could demonstrate pathological changes as seen with the denervation and reinnervation processes.

The sample size for the quantitative measurements of fibre size, shape and type was 200 to 300 fibres. The fields were selected along the midline of the greatest diameter of the muscle section by a systematic random scheme (Mayhew, 1991). In this method the first field is chosen randomly and by using a certain pattern determines the positions of all other fields in the sample. All selected fields are equidistant from one another. The systematic random scheme ensures that the muscle is sampled fairly (Mayhew, 1983, Wigmore *et al.* 1992).

In this study, an initial count was made of the number of consecutive fields which lay along the midline of the greatest diameter of the muscle section. It was then calculated whether every second, third or fourth field was counted to ensure that the whole length of muscle was evaluated. If for example every third field was to be counted, then numbers 1, 2 and 3 were placed in a box. The initial field was then chosen at random by lottery. Thus, the number drawn out of the box determined the area of the first field of vision.

To test the efficiency of the method of sampling a count was made of the total number of fibres per one section of muscle for three groups and the proportions of type I and type II muscle fibres were determined. The muscles assessed included experimental muscle from one animal from the muscle graft group, experimental muscle from one animal from the CRG-GAP tube group, and muscle from one animal from the unoperated control group. The results from these test counts established that there was reliability in the method of sampling utilized in the current study. These results are outlined in Table 2.1.

	Type of count	Fibre Type I (%)	Fibre Type II (%)
Muscle Graft	Experimental	7.68	92.32
	Test	7.39	92.61
CRG-GAP	Experimental	3.93	96.07
	Test	3.31	96.69
Unoperated Control	Experimental	3.39	96.61
	Test	3.87	96.13

Table 2.1 Comparisons of the total proportions of type I and type II muscle fibres from counting by the sampling method in the current study (experimental) and from a test count of all fibres per muscle section.

2.18 Statistical analysis

Prior to statistical analysis the distribution of the data was tested for normality. This was performed by plotting frequency histograms of sets of values and then evaluating the skewness of the curve. Parametric and non-parametric tests were then applied where appropriate. Apart from statistical parameters such as the arithmetic mean and the standard deviation, the variability coefficient was determined. The variability coefficient was used to indicate the spread of fibre sizes, and is the standard deviation multiplied by 1000, divided by the mean fibre diameter. A variability coefficient greater than 250 denotes abnormal variability in the size of muscle fibres (Dubowitz, 1985).

Within each experimental group paired Student's *t*-test were used to examine the differences between experimental and contralateral control muscles, and for the control groups, between left and right muscles. For comparisons of repair methods, and comparisons of repair methods and control groups, ANOVA was used to analyse the data. If ANOVA revealed a significant difference, unpaired Student's *t*-test were

applied to the results to determine which repair method was different from one another or which repair method was different from any of the control groups. Where a normal distribution could not be shown the Wilcoxon Signed Rank test was used for comparisons within experimental and control groups. For comparisons of repair methods and repair methods and control groups, the Kruskal-Wallis test was used. The Mann-Whitney test was used for comparing two independent samples. In all the statistical tests, a p value less than 0.05 was considered statisitically significant. The statistical analysis on all data was performed using STATISTICA 5.0 (Statsoft, USA).

2.19 Immunohistochemical procedures

The immunohistochemical procedures are described in Chapter 4.

2.20 Protocols and procedures for sheep experiments

The protocols and procedures for the sheep experiments are described in detail in Chapter 5. The muscle examined for these series of experiments in sheep was the supraspinatus muscle. The same methodology as described in this chapter for freezing, sectioning, histological and histochemical staining and assessment of muscle was applied in the evaluation of the supraspinatus muscle.

CHAPTER 3

A Morphological Comparison of Muscle Recovery following Repair of Peripheral Nerve Injuries using Biodegradable Controlled Release Glass or Freeze-Thawed Muscle Grafts in the Rabbit

3.1 INTRODUCTION

If entubulation is to become a viable method in nerve repair surgery, a number of criteria must be satisfied. First, the tube must be biodegradable and the reabsorption completed by normal processes of metabolism. Second, it must be inert and therefore nontoxic, nonantigenic and noncarcinogenic. Third, the tube should be made of materials that are agreeable to alteration so that size of the lumen, permeability and rate of solubility can be controlled. Fourth, the tube must not prompt an inflammatory reaction but be beneficial to processes of healing and regeneration. Fifth, it must prevent the surrounding non neural tissue, such as connective tissue, from invading the tube and resulting in scarring. Sixth, the size of the internal lumen of the tube must be big enough to facilitate the initial swelling of the nerve and the migration of any regenerating axons. Seventh, the nerve should maintain its correct original geometric alignment and be secured to the tube away from the transected ends to prevent any possible trauma to the nerve stumps at the suture site. Eighth, the tube must maintain its structural integrity beyond the period of time it takes the regenerating axons to cross the gap and penetrate the distal stump. Once the regenerating axons have established a connection in the periphery the tube must be completely reabsorbed.

The aim of the current study was to test whether the CRG tube fulfils the criteria as listed above, by assessing the level of regeneration after repair with the implant and then comparing it after repair with a more conventional method, such as the FTMG. It has already been demonstrated that repair of a peripheral nerve injury

using a CRG tube does not produce an immunological reaction in the host and is with time, completely reabsorbed (Gilchrist *et al.* 1998).

The chambers of other tubes used in entubulization repair have been filled with various substances to see if they could affect the quality and speed of regeneration. Rich *et al.* (1989) demonstrated that the administration of exogenous nerve growth factor within a tube enhanced regeneration. Tubes have been filled with laminin and other extracellular matrix molecules and these additions also appear to promote the regeneration of axons across gaps when compared to unfilled tubes (Madison *et al.* 1985, Yannas *et al.* 1985, Satou *et al.* 1986, Seckel *et al.* 1995). In the present study, the chambers of some of the CRG tubes were filled with various materials to see if these would enhance the quality and speed of nerve regeneration. The filling included chopped nerve and/or freeze-thawed muscle pieces.

Regenerating nerves can cross a 10 mm conduit as long as the distal stump is present in the nerve guide (Seckel *et al.* 1984). Numerous other studies have demonstrated the guiding influence of the distal stump (Lundborg *et al.* 1982, Politis *et al.* 1982, Politis and Spencer, 1983, Scaravilli, 1984, Williams *et al.* 1984, Kuffler, 1986a,b, Glasby *et al.* 1988b, Weis and Schröder, 1989, Abernethy *et al.* 1992). It is clear that the distal stump plays an important role in nerve repair and as discussed in chapter 1 two elements within a distal stump that are most likely to facilitate axonal regrowth are Schwann cells and their basal laminae.

The essential requirement of Schwann cells for significant axonal elongation has been indicated in a number of studies (Jenq and Coggeshall, 1986, Berry *et al.* 1989, 1992, Maeda *et al.* 1993, Reynolds and Woolf, 1993). There is little or no effective migration of axons when the proliferation of Schwann cells is inhibited

whilst Schwann cells will still migrate in the absence of axons (Ide *et al.* 1983, Hall, 1986, Madison and Archibald, 1994). There have been studies which have demonstrated that Schwann cells placed in the centre of a tube enhance nerve regeneration compared to similar groups without Schwann cells (Kosaka, 1990, Ikeda, 1991). Indeed the beneficial effect of Schwann cells has resulted in an attempt in the present study to incorporate them into the nerve gap in the CRG tubes. This was done by using the segment of nerve excised from the transected nerve (which would be a simple technique to apply in the clinical setting).

Another method of inserting Schwann cells in a non-neural graft is the use of a "nerve-muscle sandwich graft" (Calder and Green, 1995, Whitworth *et al.* 1995). This is composed of small pieces of nerve harvested from the distal stump sutured between freeze-thawed muscle grafts. The sandwich graft approach has led to encouraging results probably due to the neurotrophic factors provided by way of the Schwann cells, and structural support by way of sarcolemmal basal laminae. A disadvantage of the conventional sandwich graft is the increased amount of suture material introduced in the repair site. However this idea of the sandwich graft was incorporated into the current study and nerve-muscle sandwich grafts without sutures were inserted into the centre of CRG tubes. The entubulization of the sandwich graft thus removed the potential disadvantage of increased suture material at the repair site. The idea was that this approach would also help establish whether CRG tubes could enhance the effectiveness of the nerve-muscle sandwich by providing a supportive conduit.

It has been suggested that muscle grafts work by providing a solid matrix for Schwann cell migration (Feneley *et al.* 1991, Enver and Hall, 1994, Hall, 1997).

However the structure of the sarcolemmal basal lamina alone is insufficient to promote nerve regeneration as axons will not elongate into muscle grafts without accompanying Schwann cells from the proximal stump (Enver and Hall, 1994). It has been argued that unless the gap is short enough to permit the migration of Schwann cells across the graft from the nerve ends, then the sarcolemmal basal lamina will not support effective peripheral nerve regeneration (Enver and Hall, 1994, Hall, 1997). Furthermore it has been well established that axonal regeneration is poor when muscle grafts are used over distances greater than 2 cm (Hems and Glasby, 1992, 1993, Gu and Zhu, 1992). In the present study the gap length was short (1 cm) and so theroretically this should not hinder nerve regeneration through the FTMG or the CRG tube containing pieces of freeze-thawed muscle graft. Indeed comparison of nerve regeneration after these two forms of nerve repair should help provide further evidence of whether or not CRG tubes support peripheral nerve regeneration.

3.1.1 Experimental and control groups

Assessment of the quality of nerve repair was made in 5 experimental groups:

I. A 1 cm coaxially aligned freeze-thawed autologous muscle graft (FTMG).

II. A CRG tube containing a 1 cm length of pieces of freeze-thawed muscle in the centre of the tube (CRG-M).

III. A CRG tube with chopped nerve placed in the centre of the tube CRG-N).

IV. A CRG tube filled with a 1 cm length of combined freeze-thawed muscle and chopped nerve (CRG-MN).

V. An empty CRG tube with a 1 cm gap between the proximal and distal stumps of the divided peroneal nerve (CRG-GAP).

In the context of this study these groups will be referred to as the "experimental" groups.

In addition there were three control groups:

I. Left Peroneal Nerve crush

II. Left Peroneal Nerve section with removal of a piece of nerve to create a 1 cm gap after retraction

III. Unoperated

In the context of this study these groups will be referred to as the "operated control" (nerve crush and nerve cut) or "unoperated control" groups.

3.2 RESULTS

QUALITATIVE ASSESSMENT

3.2.1 Toe Spreading Reflex

In the current study the next day after the initial operation each animal was tested for any spreading of the toes and this was continued on a daily basis for the first signs of the reflex.

The initial signs of recovery of the toe spreading reflex which is usually abduction of the fourth toe, varied from 47 to 98 days after operation for the experimental groups. After repair with a CRG-GAP tube the first appearance of the reflex ranged from 82 to 98 days after operation, whilst for the CRG-N tube group it was 71 to 95 days, the CRG-M tube group 68 to 79 days and the CRG-MN tube group 75 to 88 days after operation. The FTMG group had one animal that exhibited first signs of appearance of the reflex at 47 days after operation however, this result was for one animal in the FTMG group which as assessed by other assays appeared to achieve full repair. For the other animals in the FTMG group it was 61 days before the first appearance of the toe spreading reflex, and by 70 days after operation all animals had shown the early signs of this reflex. For the nerve crush control group the first signs of recovery of the toe spreading reflex was 19 days after operation and by 26 days all animals had exhibited the first appearance of this reflex. The first appearance of toe spreading was at 42 days after operation for the nerve cut control group and by 66 days all animals in this group that did recover this reflex had displayed the first signs of it.

At 6 months after operation the toe spreading reflex could be elicited in 22 of the 25 experimental animals. Figure 3.1 includes photographs of the toe spreading reflex elicited for a normal animal, and after nerve crush and nerve cut (6 months after operation). Two animals whose peroneal nerves had been cut and then repaired by means of a CRG-MN tube, and a further animal whose repair included a CRG-N tube failed to regain their toe spreading reflex on the repaired side. In the case of the control groups, at 6 months after operation the toe spreading reflex could be elicited in 7 of the 10 operated control animals. Three animals whose peroneal nerves had been cut and not repaired failed to regain their toe spreading reflex on the operated side. The toe spreading reflex could be elicited for each of the animals of the nerve crush control group.

Table 3.1 shows the strength of the toe spreading reflex at 6 months after operation for each experimental and operated control group as assessed on a scale of 0 - 4 (see table). Of the animals which exhibited a toe spreading (TS) reflex, this varied in degree from just visible response of fourth toe alone (TS Index = 1), slight spreading of second, third, and fourth toes (TS Index = 2), spreading of all three toes but to a degree less than elicitable in normal animals (TS Index = 3), full spreading of all three toes equal to normal as determined by comparison with the contralateral control side (TS Index = 4). This scale was converted into percentages where 0% was equivalent to no reflex, 25% to TS Index of 1, 50% to TS Index of 2, 75% to TS of Index 3 and 100% to TS Index of 4 (full spreading of all three toes equal to normal as determined by comparison with the contralateral control side). Based on this scale the means of the toe spreading index for each experimental and nerve crush and nerve cut control group is shown in Table 3.2 and Figure 3.2.

No single experimental animal achieved full spreading of all 3 toes equivalent to normal, as determined by observation of the reflex on the contralateral unoperated side. This was also the case for each animal from the nerve cut control group however after crushing the peroneal nerve, four of the five animals for this group did achieve full spreading of all 3 toes (TS Index = 4).

The results for the experimental and operated control groups were compared to that seen with the contralateral unoperated side. The differences were statistically significant for all experimental groups as well as the nerve cut control group (Wilcoxon Signed Rank test, $p < 0.05$).

Overall there were significant differences when the results for the toe spreading reflex for all the different nerve repairs were compared with those after nerve crush and cut ($p < 0.01$, Kruskal-Wallis test). Individually each experimental group showed significantly weaker responses than the response after nerve crush. This difference was highly significant for the CRG-GAP and the CRG-MN tube groups ($p < 0.01$, Mann-Whitney *U* test), and significant for all other repair groups ($p < 0.05$). There was only one experimental group where the response to the toe spreading reflex was significantly stronger than that of the nerve cut control group, and this was the FTMG group ($p < 0.05$). Indeed the FTMG and the CRG-M tube groups were the only experimental groups which exhibited consistent "good" scores for the TS Index. In particular after repair with either a CRG-N or a CRG-MN tube, the score for the TS Index ranged from 0 (no reflex) to 3 (spreading of all three toes but to a degree less than elicitable in normal animals), whilst after repair with a CRG-GAP tube every animal in this group achieved only a TS Index of 2 (slight spreading of second, third, and fourth toes). However, a TS Index of 3 was

accomplished for the majority of the animals for the FTMG and the CRG-M tube groups.

For the FTMG group there was one animal which did appear to achieve full repair (based on the results from morphometric assessment of muscle). This result was removed from the data and the only difference that this had on the findings was that there was no significant difference in the response to the toe spreading reflex between the FTMG and the nerve cut control groups.

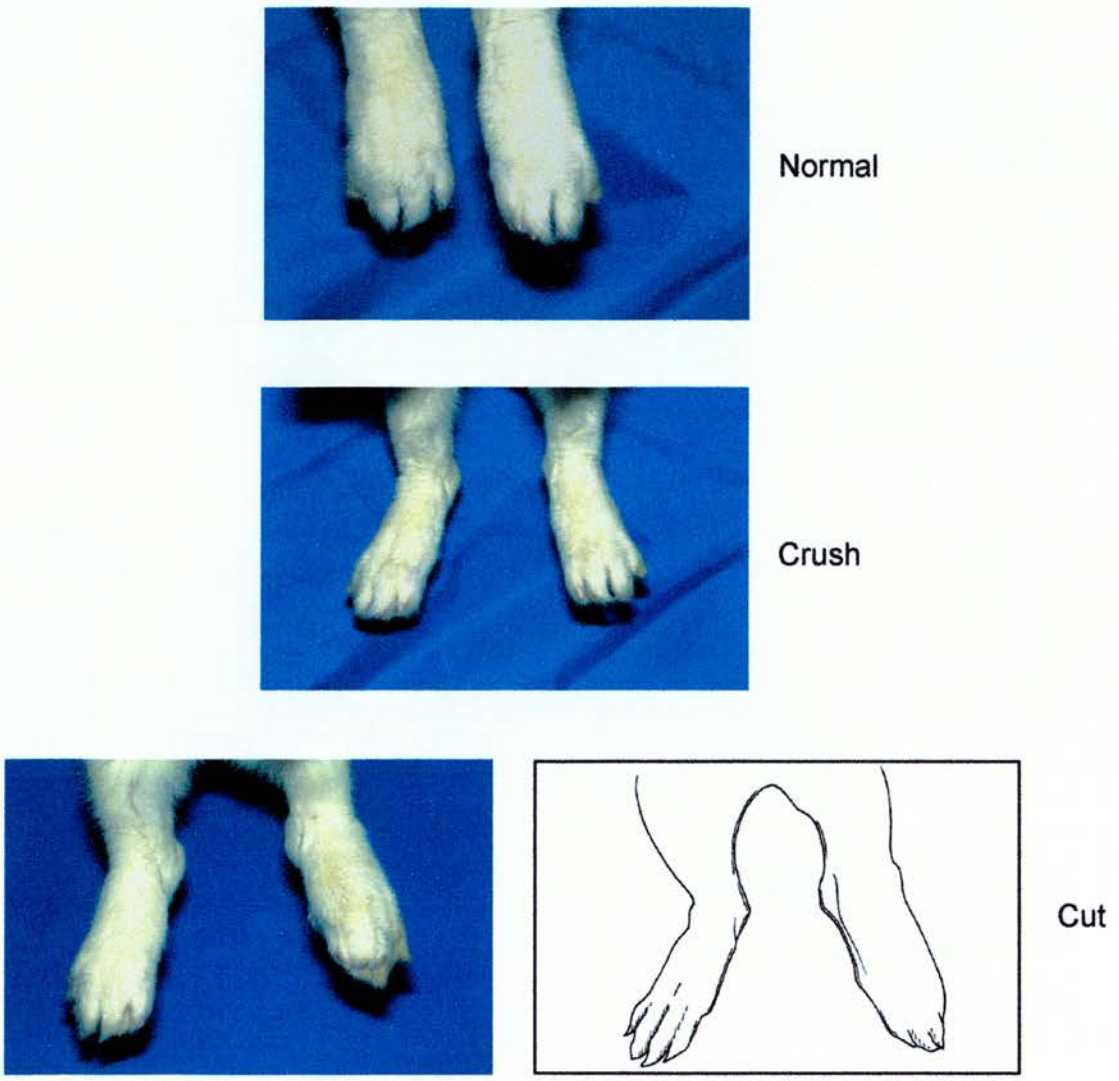


Figure 3.1 The toe spreading reflex as observed in a normal rabbit (TS=4) and at 6 months after operation, for peroneal nerve crush (TS=4) and cut (TS=0) animals.

Procedure	Animal Number	Toe Spreading Index
CRG-GAP	1	2 *
	2	2
	3	2
	4	2
	5	2
CRG-N	1	2 *
	2	3
	3	0
	4	2
	5	3
CRG-M	1	3 *
	2	2
	3	2
	4	3
	5	3
CRG-MN	1	0 *
	2	3
	3	0
	4	2
	5	2
FTMG	1	3 *
	2	3
	3	3
	4	3
	5	2
Nerve Crush	1	4
	2	3
	3	4
	4	4
	5	4
Nerve Cut	1	0 *
	2	0
	3	3
	4	2
	5	0

Table 3.1 - The toe spreading index at 6 months after operation for each experimental and nerve crush and nerve cut control group by comparison with the contralateral control side.

(0 no reflex, 1 just visible response of fourth toe alone, 2 slight spreading of second, third, and fourth toes, 3 spreading of all three toes but to a degree less than elicitable in normal animals, 4 full spreading of all three toes equal to normal as determined by comparison with the contralateral control side).

* A significant result for the experimental or operated control group when compared to those values for the contralateral/right side

Procedure	Mean (%)
CRG-GAP	50
CRG-N	50
CRG-M	65
CRG-MN	35
FTMG	70
Nerve Crush	95
Nerve Cut	25

Table 3.2 - The toe spreading index for each experimental and nerve crush and nerve cut control group expressed as a percentage of the contralateral EDL muscle.

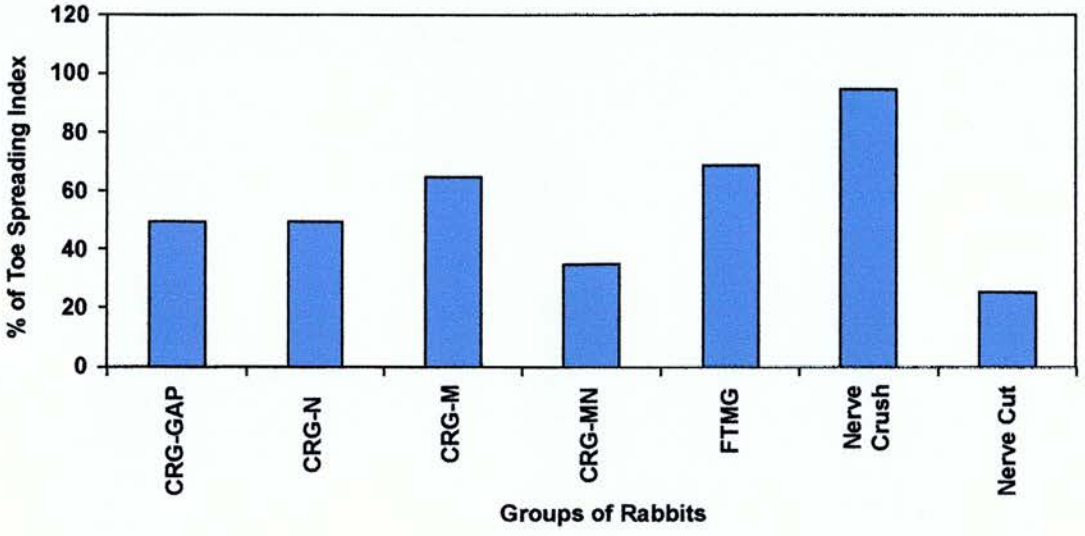


Figure 3.2 The toe spreading index expressed as a percentage of the contralateral EDL muscle for each experimental and nerve crush and nerve cut control group.

3.2.2 Other criteria of muscle recovery

At 6 months after operation passive movement of the ankle joint on the operated side exhibited stiffness and reduced range of passive movement for all experimental and nerve cut control animals when compared with the contralateral side. Athough passive movement was reduced on the repaired side none of the animals acquired a fixed deformity of the ankle joint. However, palpation of the limb in the region of the denervated muscles for each experimental and nerve cut control animal showed a marked reduction in muscle bulk when compared to the opposite or contralateral sides. For the nerve crush control animals, the majority exhibited no stiffness and a good range of passive movement of the ankle joint on the operated side. There was slight reduction of plantar flexion for two of the nerve crush control animals when compared to the range of movements of the corresponding contralateral ankle joints.

At 6 months after operation the stance and gait for each of the experimental or control animals did not display any abnormalities. As discussed previously, interruption of the peroneal nerve generally does not produce any marked influence on the stance and gait apart from rare cases where the toes are kept in marked plantar flexion and the animal walks on the dorsum of the toes where sores may develop. No such changes in gait nor development of sores were observed for any of the experimental or control animals.

3.2.3 Visual examination of the repair site

At 6 months after operation each animal was killed and the site of repair was examined. Figure 3.3 (a to d) includes photographs of a normal peroneal nerve and the site of repair after nerve crush, nerve cut and repair with a CRG-M tube (all 6 months after operation). Upon visual inspection there was no evidence of the CRG tube or the FTMG, although at the site of repair there was an extensive amount of connective tissue (fibrotic in appearance - please note the appearance of the repair sites for CRG-M, Figure 3.3d). Examination of the peroneal nerve distal to the graft revealed a full thickness nerve for all experimental groups apart for the CRG-GAP group where the peroneal nerve did not appear to have the same girth. In a parallel study utilising electrophysiological tests, it was determined that there was nerve conduction across the site of repair for each animal (Lenihan, 2000). Furthermore, the electrophysiological tests confirmed that there was evidence of some nerve regeneration after nerve repair for each animal.

In the case of the control groups, visual examination of the site of the nerve crush revealed adipose, and not fibrotic tissue surrounding the peroneal nerve. For all animals after nerve crush, the area of nerve distal to the site of injury was of normal thickness and colour (Figure 3.3b). However for the nerve cut control group, the area of nerve distal to the site of injury was thinner in width compared to the breadth of the normal peroneal nerve, as well as paler in colour. There was also a greater amount of adipose tissue than what was found after crushing the peroneal nerve (Figure 3.3c), although as with the nerve crush control group there was no evidence of fibrotic tissue (as seen after each form of nerve repair).

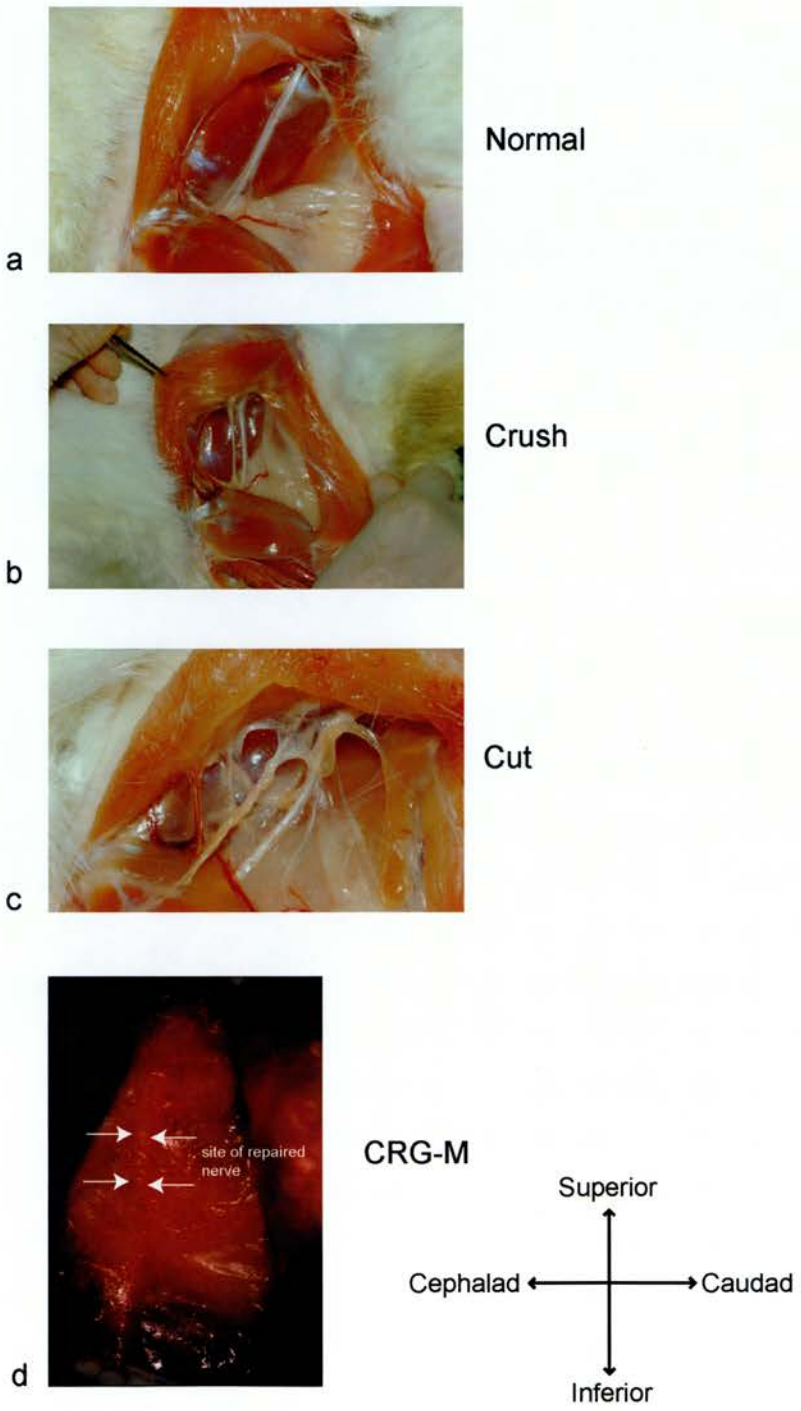


Figure 3.3 The normal peroneal nerve (a). The repair site 6 months after operation for the nerve crush (b), the nerve cut (c) and CRG-M tube group (d).

3.2.4 Visual examination of the EDL muscle

After the site of repair was examined, the incision was extended distally along the front of the leg and the anterior group of leg muscles, including the EDL, was exposed. Visual inspection of the EDL muscle *in situ* established that it could be easily identified and that the general form of the muscle was retained for all groups (both experimental and control). The muscle was easily removed from every animal, although for the majority of the experimental groups there was a most conspicuous reduction in the size of the EDL muscle when compared to the corresponding contralateral muscle. (Please refer to Figures 3.4 and 3.5 which include micrographs of the left and right EDL muscles for each of the experimental and control groups). The only exception to this was one animal in the FTMG group where the experimental and contralateral EDL muscles appeared to be of equivalent size and weight (as shown in Figure 3.5f). Some of the experimental muscle, in particular after repair with a CRG-GAP tube, was distinctly paler in colour when compared to the corresponding contralateral EDL muscle (see Figure 3.5a). However, in spite of this change in colour the muscles still retained the appearance that could identify them readily as muscle tissue.

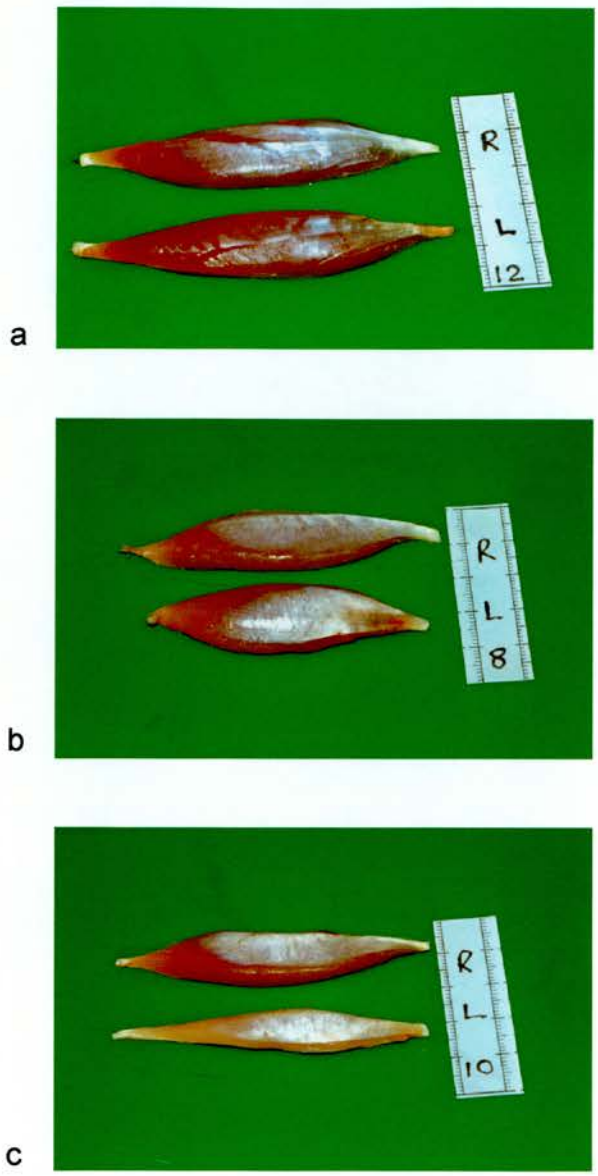


Figure 3.4 The left (L) and right (R) EDL muscles of the unoperated control. The operated (L) and contralateral (R) EDL muscles of the nerve crush and the nerve cut control groups, 6 months after operation.

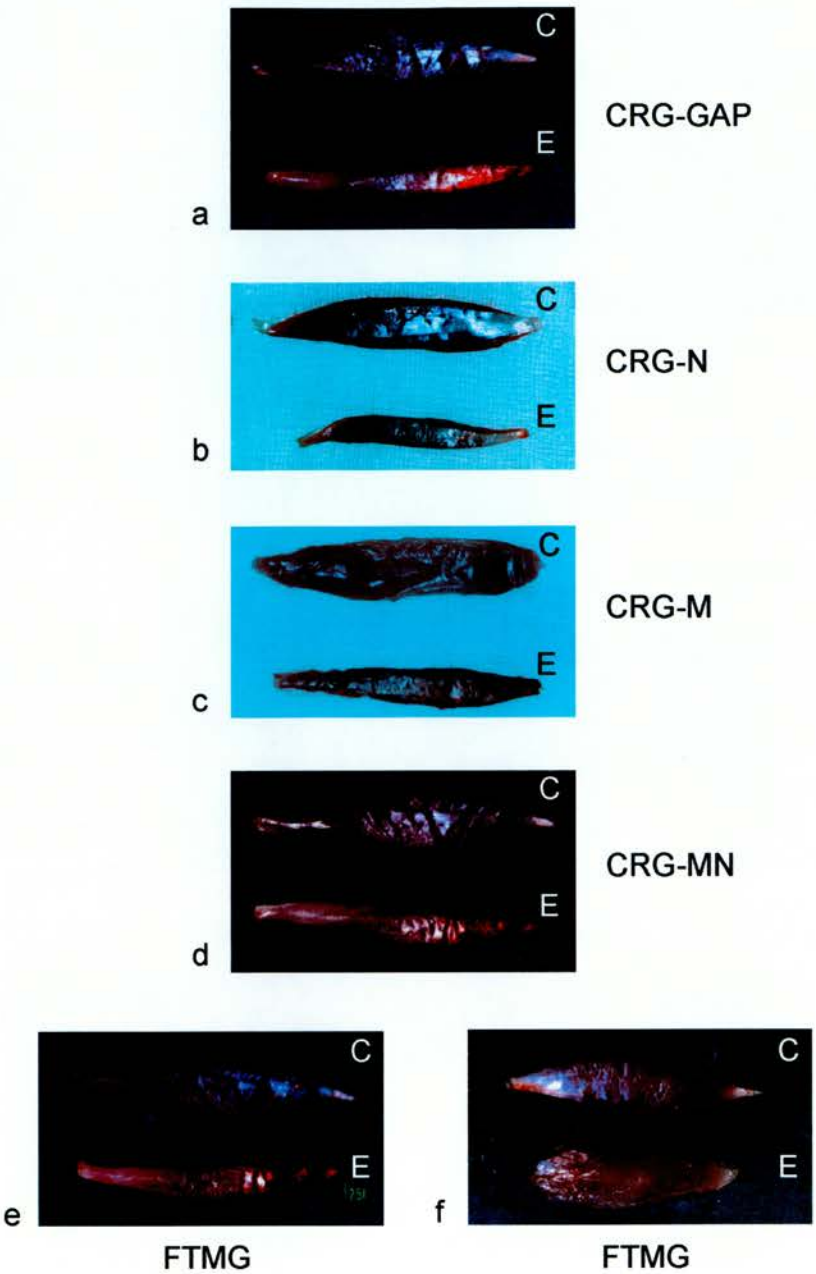


Figure 3.5 The experimental (E) and contralateral (C) EDL muscles of each of the experimental groups, 6 months after operation.

QUANTITATIVE ASSESSMENT

The focus of this study was muscle morphology and fibre type distribution as an assay of reinnervation after nerve repair. In this chapter the findings for muscle morphology are presented whilst those for fibre type distribution are discussed in Chapter 4.

3.2.5 Muscle wet weight

Experimental, contralateral, operated and unoperated control EDL muscles were weighed to within 0.01 g immediately following excision and blotting. The muscle wet weights were normalized to allow direct comparison, taking into account any weight loss of experimental animals. This was achieved by multiplying the muscle wet weight by 1000 and dividing by the weight of the animal.

Table 3.3 shows the values of the mean and the standard deviation of the standardized muscle wet weight of both the experimental and contralateral EDL muscles with respect to the method of repair, as well as data for the nerve crush, nerve cut and unoperated control groups. Figure 3.6 shows the mean and the standard error of the mean (SEM) for the standardized muscle wet weight of the EDL muscles for each of the experimental and control groups.

For all experimental and nerve crush and nerve cut control groups, the EDL experimental and operated muscle showed a decrease in mean standardized muscle wet weight when compared with values for the corresponding contralateral muscles. This difference in weight was significant for all experimental groups, as well the nerve cut control group ($p < 0.05$ for all groups, Wilcoxon Signed Rank test).

However, as assessed by muscle wet weight one animal in the FTMG group did appear to achieve full repair. For this particular animal the standardized experimental muscle wet weight of the EDL was 10.11 g, whilst its corresponding contralateral muscle weight was 10.21 g, compared with a mean of 9.32 g for unoperated controls.

Overall there were significant differences when the results for the standardized muscle wet weight for all the different nerve repairs were compared ($p < 0.05$, Kruskal-Wallis test). Individual comparisons of the repair groups revealed that after repair with a FTMG the EDL muscle wet weight was significantly greater than after repair with a CRG-GAP or a CRG-M tube ($p < 0.05$ for each case, Mann-Whitney *U* test).

There were significant differences when the muscle wet weights for all the five experimental groups were compared with those values for the left and right EDL muscles of the unoperated control, as well as the operated EDL muscles of the nerve crush and nerve cut control groups ($p < 0.05$, Kruskal-Wallis test). Individual comparisons of the repair methods and each of the different control groups revealed that after repair with either a CRG-GAP or a CRG-M tube the muscle wet weight was significantly less when compared to those wet weights for the unoperated, nerve crush and nerve cut EDL muscles ($p < 0.01$, Mann-Whitney *U* test). There were also highly significant differences when comparing muscle wet weights for CRG-N and CRG-MN tube groups to those values for the left and right EDL muscles of the unoperated control, as well as the operated muscles of the nerve crush group ($p < 0.01$). However, the difference in muscle wet weight was not as significant when the CRG-N and CRG-MN tube groups were compared to those values for the nerve cut group ($p < 0.05$). After repair with a FTMG, there was a significant difference in

muscle wet weight when compared with those values for the nerve crush control group ($p < 0.05$).

Overall there were significant differences in muscle wet weight between operated EDL muscles of the nerve crush and the nerve cut control groups, as well as the left and the right EDL muscles of the unoperated control ($p < 0.05$, Kruskal-Wallis test). Individual comparison of the groups revealed that the muscle wet weight of the nerve cut control group was significantly less than those values for the nerve crush control group, as well as the left and the right EDL muscle of the unoperated control ($p < 0.05$ for each case, Mann-Whitney *U* test).

There were significant differences when the muscle wet weights for all the contralateral muscles of the experimental groups, as well as the left and right EDLs of the unoperated control and all the contralateral muscles of the nerve crush and the nerve cut control groups were compared ($p < 0.001$, Kruskal-Wallis test). Individual comparison of the groups revealed that the muscle wet weights for contralateral muscles for each of the experimental groups were significantly less than those values for the contralateral muscles of the nerve crush group ($p < 0.01$ for CRG-GAP and CRG-M; $p < 0.05$ for CRG-N and FTMG, Mann-Whitney *U* test). For the contralateral muscles of the nerve cut group, the muscle wet weight was significantly greater when compared to those values for contralateral muscles of the CRG-GAP ($p < 0.01$), CRG-M and FTMG (both $p < 0.05$) experimental groups.

For a direct comparison of the level of recovery of the experimental and control muscle (in terms of muscle weight) to their corresponding contralateral muscles, the weights of the experimental and control muscles were expressed as a percentage of the weight of the contralateral muscles (Table 3.4 and Figure 3.7). Of

all the experimental groups the FTMG did appear to be the better performer in recovery of muscle weight as this group had the highest mean value. As discussed previously one animal in the FTMG group had achieved full repair as assessed by wet muscle weight. Indeed the recovery of muscle weight for this particular animal was 99.02%, whilst for the other animals in the muscle graft group the values ranged from 44.79% to 54.24%.

Overall there were significant differences when the level of recovery of muscle weight for all of the experimental and control groups (nerve cut, nerve crush and unoperated) were compared ($p < 0.001$, Kruskal-Wallis test). Individual comparison of the experimental and control groups revealed that for most experimental groups there was a significant decrease in the level of recovery of muscle wet weight when compared with those values for the nerve crush and the unoperated control groups ($p < 0.01$ for each group apart from after repair with a FTMG where $p < 0.05$, Mann-Whitney *U* test). For the nerve cut control group there was a significant increase in the level of recovery of muscle weight when compared to the recovery of wet muscle weight after most forms of nerve repair. This increase was highly significant when compared to those values for the CRG-GAP tube group ($p < 0.01$), and significant after repair with either a CRG-N, CRG-M or a CRG-MN tube ($p < 0.05$). However, when the level of recovery of muscle wet weight after nerve cut were compared to those after nerve crush and the values for the unoperated control, it was significantly less for both ($p < 0.05$ for each case).

The one control animal housed in the Department of Tropical Veterinary Medicine had a muscle wet weight of 8.62 g for the left EDL muscle, and 9.60 g for

the corresponding right. These figures were comparable to those muscle wet weights for both the left and the right EDL muscles of the unoperated control.

In summary, after each type of nerve repair and for the nerve cut control group, there was a decrease in muscle wet weight. For the experimental groups the best recovery in terms of muscle wet weight was the FTMG group. Of the remaining experimental groups there was not a great deal of difference in results for muscle wet weight amongst these types of nerve repair, although repair with either a CRG-GAP or a CRG-M tube produced consistently poor results. Indeed based on the findings for muscle wet weight all types of nerve repair other than the FTMG were worse than those results for the nerve cut control group. The values for muscle wet weight for the nerve crush control group were comparable to that of the normal or unoperated control group. The muscle wet weight of contralateral muscles of experimental groups was generally less when compared to those values for the contralateral muscles of operated control groups (nerve crush and nerve cut).

Removal of the data for the one animal in the FTMG group which did appear to achieve full repair resulted in a value for the mean standardized muscle wet weight of 4.27g and for the standard deviation, 0.66g. For the corresponding contralateral side after removal of this data the value for the mean standardized muscle wet weight was 8.50g and for the standard deviation, 0.81g. Statistical analysis of this data produced different results to those previously in that there was no significant difference in muscle wet weight between the FTMG group and other experimental groups. Furthermore, the difference in muscle wet weight was not only significant when compared with the muscle wet weights for the nerve crush control group, but also the nerve cut control group. as well as the left and the right EDL muscles of the

unoperated control ($p < 0.05$ for each case). For the level of recovery of muscle weight, removal of the data for the one animal in the FTMG group which did appear to achieve full repair resulted in a mean value of 50.02%. Statistical analysis of this data produced different results to those previously in that there was a significant difference in the level of recovery of muscle weight between the nerve cut control and the FTMG groups ($p < 0.05$).

Procedure	Muscle	Mean muscle wet weight (g)
CRG-GAP	Experimental EDL Contralateral EDL	3.62 ± 0.44 * 8.22 ± 0.69
CRG-N	Experimental EDL Contralateral EDL	4.37 ± 1.22 * 9.30 ± 0.84
CRM-M	Experimental EDL Contralateral EDL	3.65 ± 0.86 * 8.64 ± 0.99
CRG-MN	Experimental EDL Contralateral EDL	4.45 ± 1.10 * 9.55 ± 2.16
FTMG	Experimental EDL Contralateral EDL	5.44 ± 2.67 * 8.84 ± 1.04
Nerve Crush	Operated EDL Contralateral EDL	10.29 ± 1.07 10.52 ± 0.38
Nerve Cut	Operated EDL Contralateral EDL	6.89 ± 1.31 * 10.45 ± 0.77
Unoperated Control	Left EDL Right EDL	9.32 ± 1.07 9.52 ± 1.28

Table 3.3 - The mean and standard deviation of the standardized muscle wet weight (g) of the EDL muscle for each of the experimental and control groups.

* A significant result for the experimental, operated or unoperated control group when compared to those values for the contralateral/right side

Procedure	Mean (%)
CRG-GAP	44.04
CRG-N	46.99
CRG-M	42.25
CRG-MN	46.59
FTMG	61.53
Nerve Crush	97.58 *
Nerve Cut	66.94
Unoperated	99.84

Table 3.4 - The standardized muscle wet weight of the experimental and control EDL muscles expressed as a percentage of the contralateral EDL muscles.

* A significant result when the level of recovery of muscle wet weight was compared to those values for each experimental or operated control group

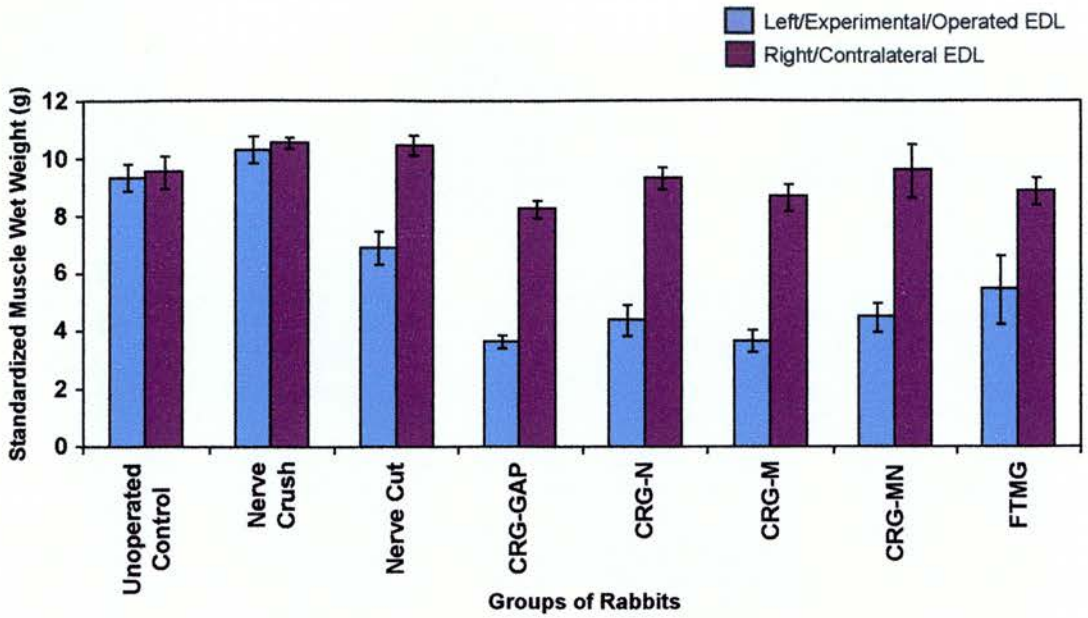


Figure 3.6 The mean and the SEM of the standardized muscle wet weight (g) of the EDL muscles for each of the experimental and control groups.

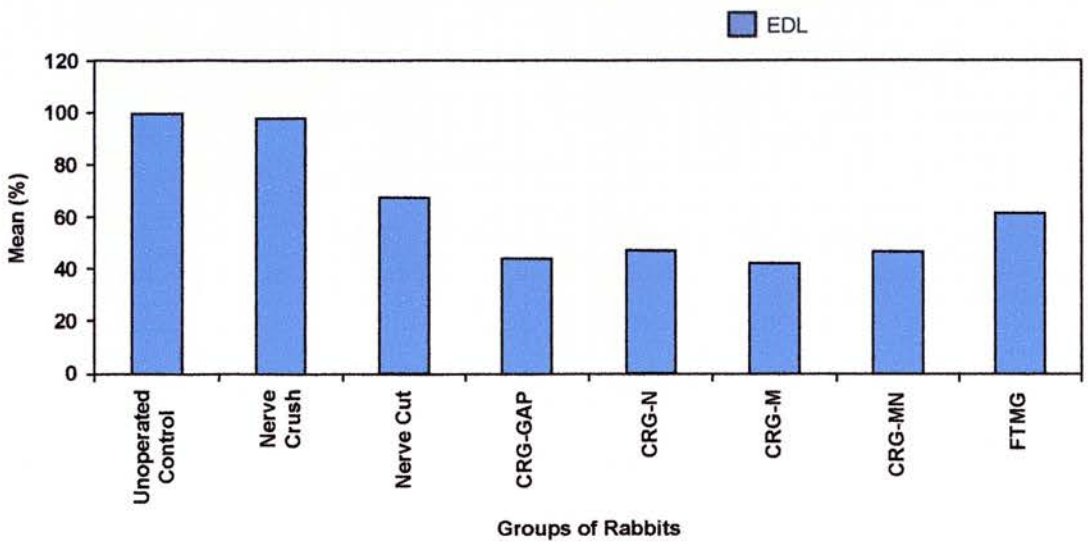


Figure 3.7 The mean standardized muscle wet weight of the experimental and control EDL muscle expressed as a percentage of the contralateral EDL muscles.

3.2.6 Incidence of muscle fibres with pathological features

The incidence of pathological features was assessed using sections stained with haematoxylin and eosin (H & E). The pathological features are structural alterations which may occur in muscle as part of the denervation process. The features examined after H & E staining included migrating or internal nuclei, split fibres, angular shaped fibres and any fibres granular or necrotic in appearance. On average a total of 500 muscle fibres were examined for each section.

Table 3.5 depicts the mean percentage of muscle fibres with pathological features after staining with H & E, for each type of nerve repair and for the contralateral muscles, the operated control groups (nerve crush and nerve cut) and the unoperated control (normal) group.

The most frequent pathological feature for all experimental, operated and unoperated control groups was internal nuclei (Figures 3.8 and 3.9). The average number of fibres with internal nuclei in experimental muscles was 22.18%, and in the operated muscles of the nerve crush and the nerve cut control groups it was 15.36%. This compares with an average of 0.24% for the number of muscle fibres that exhibited internal nuclei in the contralateral muscles of experimental and operated control groups, as well as the left and the right EDL muscles of the unoperated control. Indeed for each experimental and operated control group, the experimental EDL muscle showed a significant increase in the number of internal nuclei when compared to that seen in the contralateral muscle ($p < 0.05$ for all groups, Wilcoxon

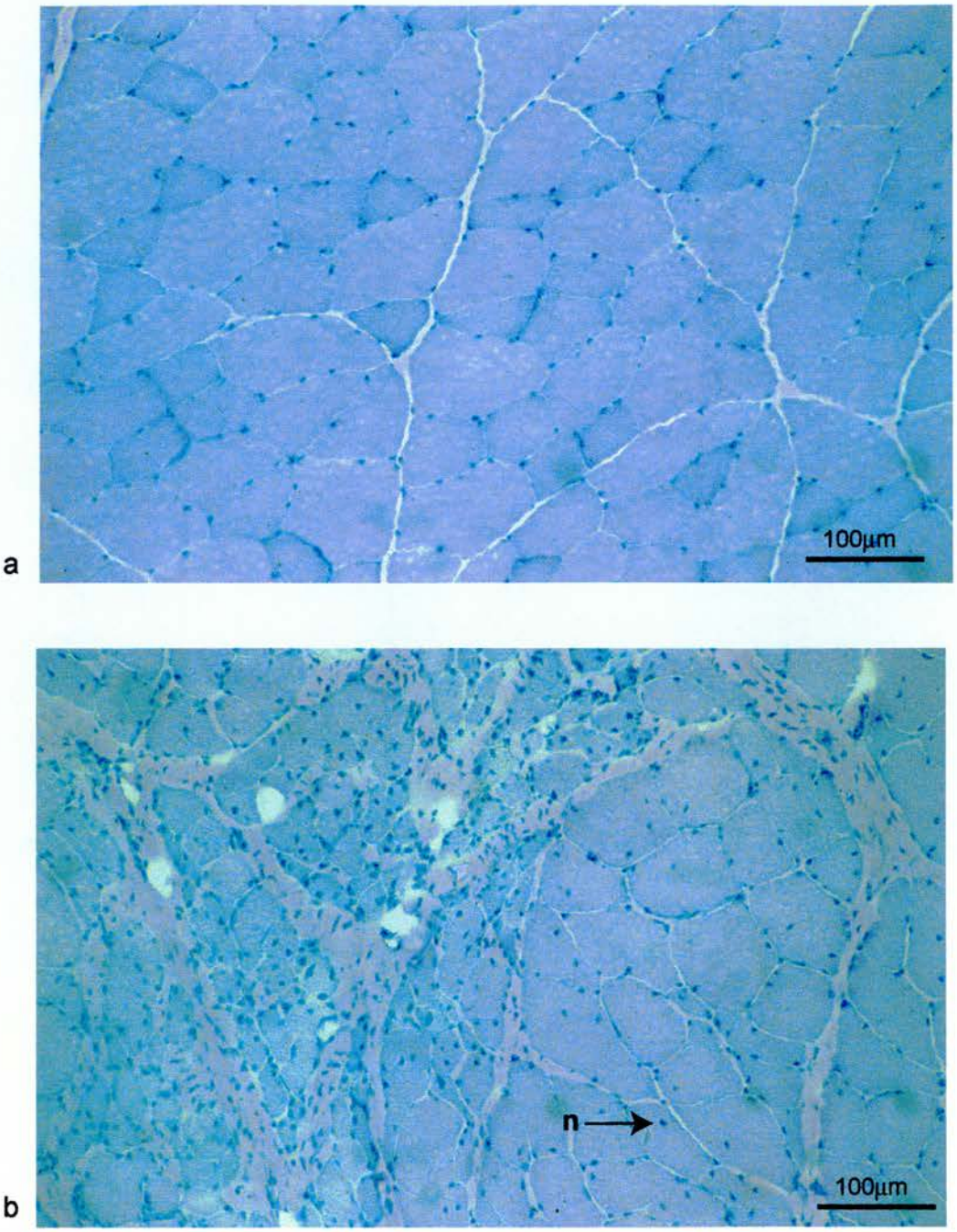
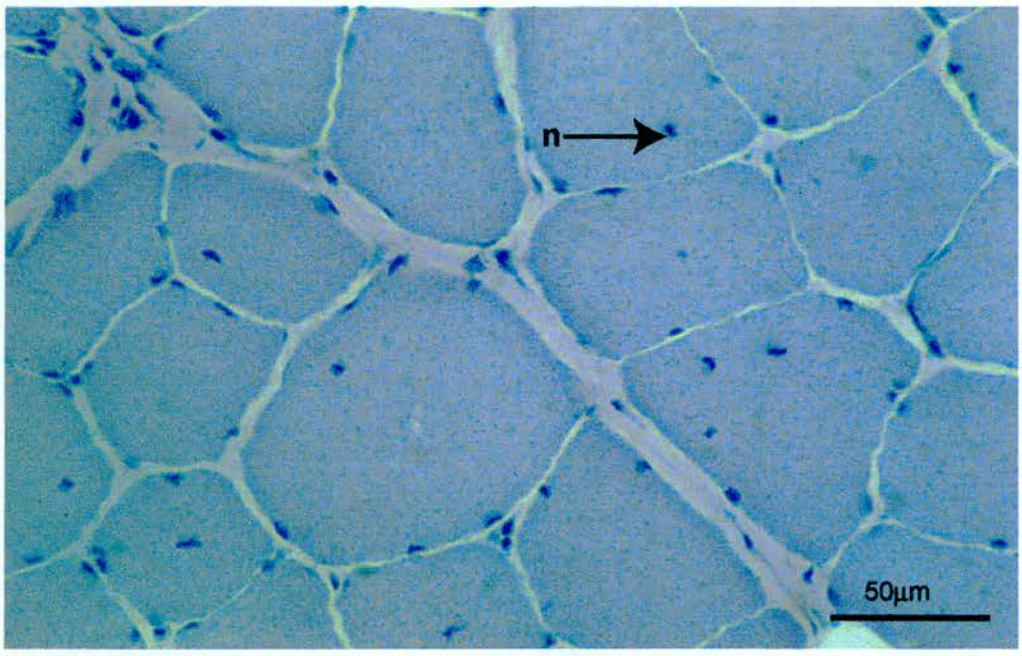


Figure 3.8 (a) The normal EDL muscle (H & E).
(b) The EDL muscle after repair with a FTMG. Note the internal nuclei (n) and the variation in fibre size.The fibre in the fasicle to the left of centre which are markedly atrophied compared to those in the fascicles to the right (H & E).



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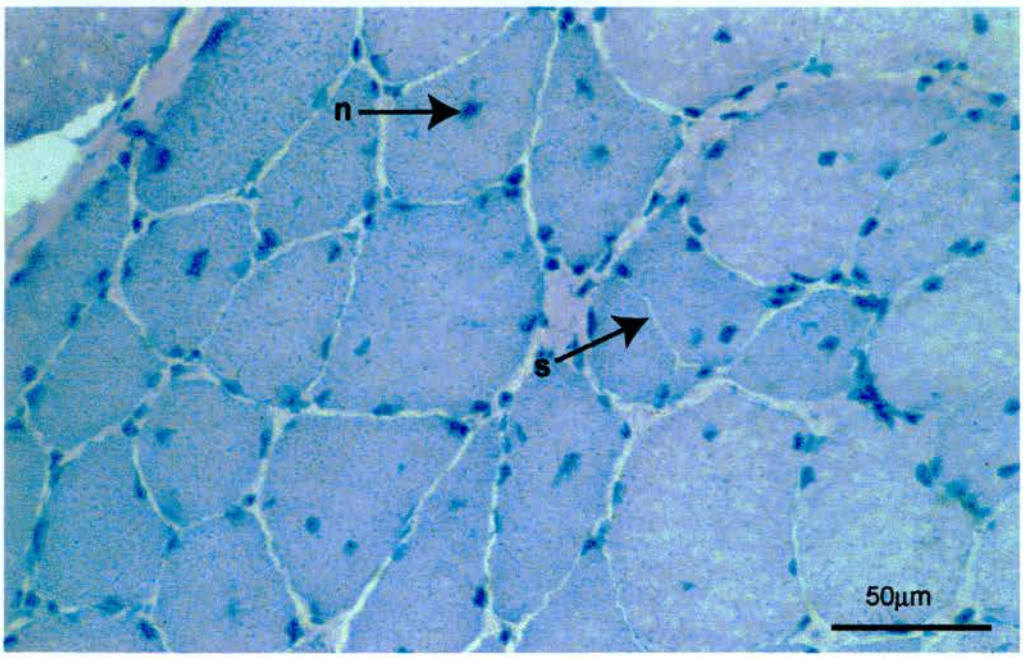


b

Figure 3.9 (a) The EDL muscle after peroneal nerve crush.Note the internal nuclei (H & E).
(b) The EDL muscle after peroneal nerve cut. Note the internal nuclei (n), the variation in fibre size and the split (s) fibre (H & E).

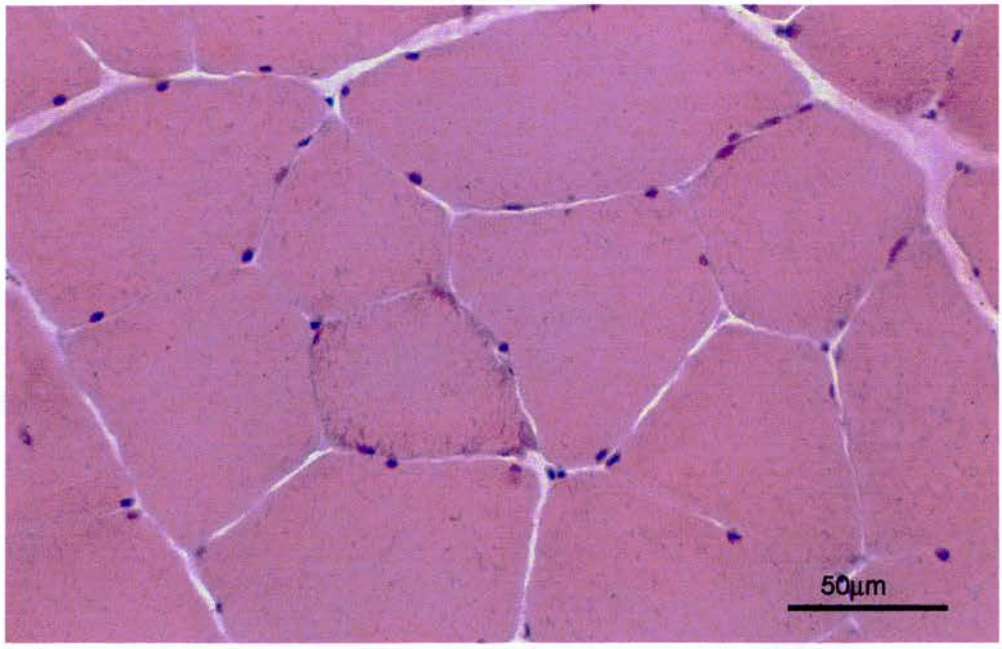
Signed Rank test).

Necrotic fibres were the next most common pathological feature followed by angular, split and granular fibres. Necrotic fibres (Figure 3.10) were found in experimental muscles after each type of nerve repair and in the operated muscles of the nerve cut control group. However, it was only after repair with a CRG-GAP tube that this increase was significant when compared to those values for the corresponding contralateral muscle ($p < 0.05$). Indeed the incidence of this pathological feature was much less than that of internal nuclei as the average number of necrotic fibres found in experimental muscles was 1.94%. There was a significant increase in angular fibres (Figures 3.11 and 3.12) after repair with a FTMG and after either a nerve crush or cut when compared to those values for the corresponding contralateral muscles ($p < 0.05$ for each group). After repair with either a CRG-GAP or CRG-N tube there was a significant increase in the number of split fibres (Figures 3.9b) in experimental muscle when compared to the numbers in contralateral muscle ($p < 0.05$ for each group).

Overall there were significant differences when the incidence of necrotic fibres for all the experimental groups were compared ($p < 0.01$, Kruskal-Wallis test). Individual comparisons of the experimental groups revealed that after repair with a CRG-GAP tube, there was a significant increase in necrotic fibres when compared to all other forms of repair (CRG-N tube $p < 0.01$; CRG-M, CRG-MN, and FTMG $p < 0.05$, Mann-Whitney *U* test).

There were significant differences when the incidence of internal nuclei for all the experimental groups, as well as all the operated EDL muscles of the nerve

a



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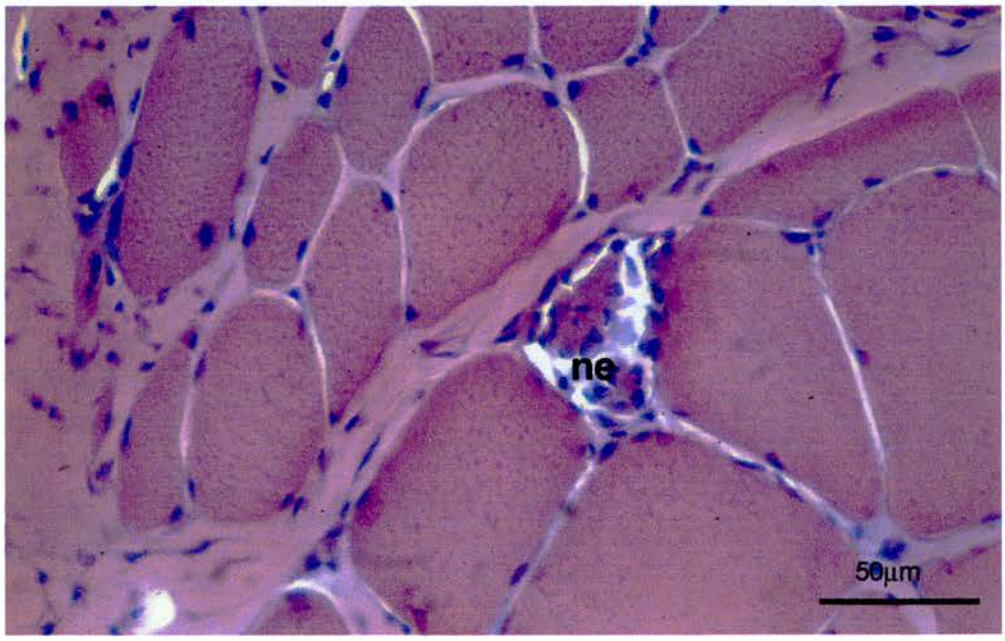


Figure 3.10 (a) The normal EDL muscle (H & E).
(b) Necrotic fibre (ne) after repair with a CRG-GAP. Note also the variation in fibre size and the proliferation of perimysial connective tissue (H & E).

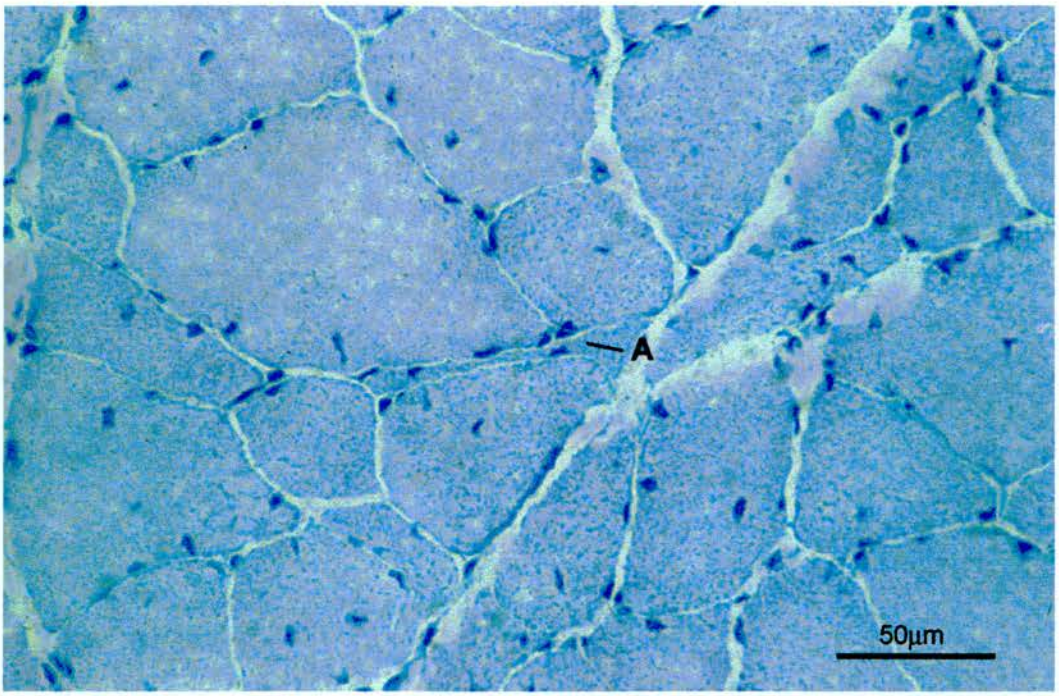


Figure 3.11 The EDL muscle after repair with a CRG-N. Note the angular (A) muscle fibres.

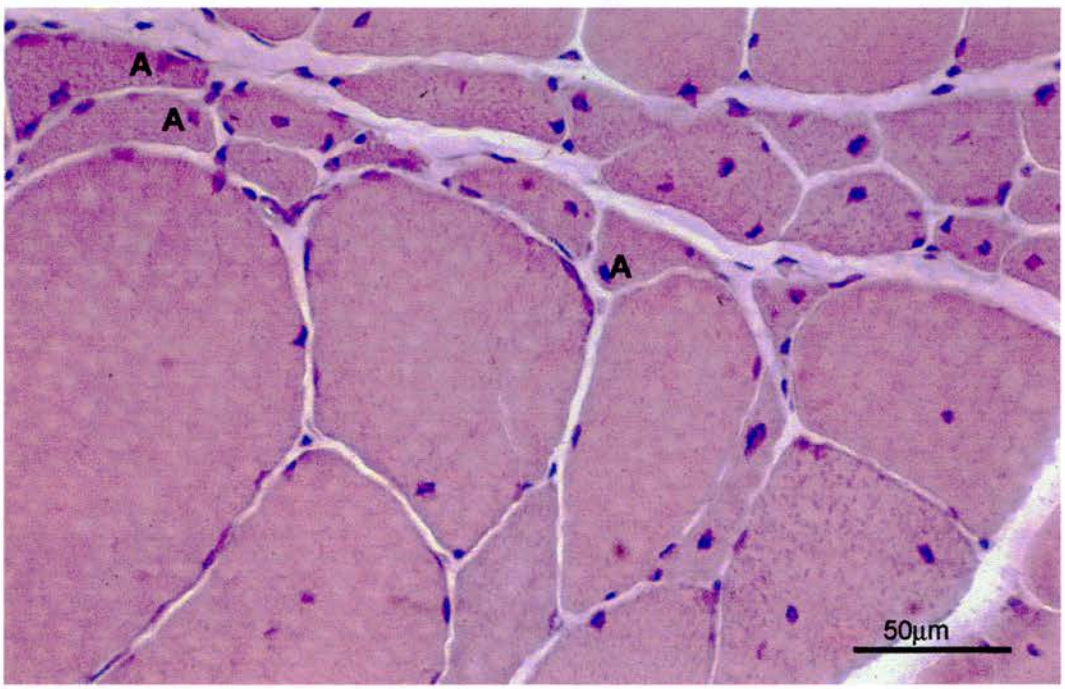


Figure 3.12 The EDL muscle after repair with a CRG-MN tube. Note some of the angular (A) muscle fibres are labelled.

crush and the nerve cut control groups, and the left and the right EDL muscles of the unoperated control groups were compared ($p < 0.01$, Kruskal-Wallis test). Individual comparisons of the experimental and control groups revealed that there was a highly significant increase in the number of internal nuclei for each experimental group when compared to the numbers in either the left or the right EDL muscle of the unoperated control ($p < 0.01$ for each case, Mann-Whitney *U* test).

Overall there were significant differences in the number of split fibres when the incidence for all experimental and operated and unoperated control groups were compared ($p < 0.01$, Kruskal-Wallis test). Individual comparisons of the experimental and control groups revealed that after repair with either a CRG-GAP, CRG-MN or a CRG-MN tube, or a FTMG, there was a significant increase in split fibres when compared to those values for the nerve crush control group ($p < 0.05$ for each case, Mann-Whitney *U* test). There was also a significant increase in split fibres in most experimental muscles when compared to the number in the left EDL muscle of the unoperated control (CRG-GAP, CRG-N and FTMG groups $p < 0.01$; CRG-N and CRG-MN groups $p < 0.05$).

There were significant differences in the number of angular fibres when the incidence for all experimental and operated and unoperated groups were compared ($p < 0.01$, Kruskal-Wallis test). Individual comparisons of the experimental and control groups revealed that the greatest number of angular fibres was for the nerve cut control group and this was significantly different to the numbers of this pathological feature after repair with either a CRG-M, CRG-MN or a CRG-N tube ($p < 0.05$ for each group, Mann-Whitney *U* test). There was also a significant difference in the incidence of angular fibres after comparison of the values for either the left or the

right EDL muscles of the unoperated control and those after repair with a CRG-M or a CRG-N tube ($p < 0.05$ for each case), or a FTMG ($p < 0.01$).

Overall there were significant differences in the number of granular fibres when the incidence of this pathological feature for all experimental, operated and unoperated control groups were compared ($p < 0.05$, Kruskal-Wallis test). However, individual comparisons of the experimental and control groups revealed that it was only after repair with a CRG-N tube that there was a significant increase in the incidence of this pathological feature when compared to the numbers for the operated or unoperated control groups ($p < 0.05$, Mann-Whitney *U* test).

There were significant differences in the number of necrotic fibres when the incidence for all experimental and operated and unoperated groups were compared ($p < 0.001$, Kruskal-Wallis test). Individual comparisons of the experimental and control groups revealed that after repair with either a CRG-GAP or a CRG-M tube there was a highly significant increase in the number of necrotic fibres in experimental muscle when compared to those values for control muscles ($p < 0.01$, Mann-Whitney *U* test). However after repair with either a FTMG or a CRG-N tube, there was a significant increase in the number of necrotic fibres in experimental muscle when compared to those values for the nerve crush and unoperated control muscles ($p < 0.05$).

The incidence of pathological features for all operated EDL muscles of the nerve crush and the nerve cut control groups were compared with the incidence of pathological features for the left and the right EDL muscles of the unoperated control. For the pathological feature of internal nuclei significant differences were found ($p < 0.01$, Kruskal-Wallis test). Individual comparisons of these operated and

unoperated control groups revealed that there was a significant increase in internal nuclei for both the nerve crush and the nerve cut control group when compared to those values of internal nuclei in either the left or the right EDL muscle of the unoperated control ($p < 0.01$ for each case, Mann-Whitney *U* test). The incidence of angular fibres was also significantly different between operated and unoperated control muscles ($p < 0.001$, Kruskal-Wallis test). As with internal nuclei, individual comparisons revealed that there was a significant increase in angular fibres for the operated control groups when compared to those values in either the left or the right EDL muscles of the unoperated control ($p < 0.01$ for each case, Mann-Whitney *U* test).

The incidence of pathological features for the one control animal housed in the Department of Tropical Veterinary Medicine included no features found for the left EDL muscle, and a mean value of 0.08% for the number of muscle fibres in the corresponding right EDL muscle with internal nuclei. No other pathological features were found and these figures for internal nuclei were comparable to the numbers found in the left and the right EDL muscles of the unoperated control.

In summary, there was an increase in the incidence of pathological features after all types of nerve repair, as well as for the nerve cut and the nerve crush control groups. Internal nuclei was the most common pathological feature for all experimental, operated and unoperated control groups. However, the incidence of internal nuclei was not significantly different amongst experimental groups nor between experimental groups and operated (nerve crush and nerve cut) control groups. Amongst experimental groups the only pathological feature to increase significantly was necrotic fibres and this was most marked after repair with a

CRG-GAP tube. Based on the incidence of all pathological features, the contralateral muscles of experimental and operated control groups were no different from those of the unoperated control (normal) group.

Removal of the data for the one animal in the FTMG group which did appear to achieve full repair resulted in no change in the findings for pathological features.

Figures 3.13 to 3.17 depict the mean and the SEM for the percentage of pathological features found in the EDL muscle after staining with H & E.

Procedure and Muscle	Internal Nuclei %	Split Fibres %	Angular Fibres %	Granular Fibres %	Necrotic Fibres %
CRG-GAP	*	*			*
Exp EDL	19.36 ± 7.40	1.32 ± 1.96	1.32 ± 2.00	0.12 ± 0.18	5.20 ± 3.02
C EDL	0.16 ± 0.17	0	0	0	0
CRG-N	*	*			
Exp EDL	18.92 ± 8.17	0.96 ±0.79	1.04 ±1.45	0.28 ± 0.23	0.32 ± 0.27
C EDL	0.28 ± 0.44	0.04 ± 0.09	0	0	0
CRG-M	*				
Exp EDL	23.12 ±19.02	0.40 ± 0.32	0.40 ± 0.57	0.24 ± 0.26	1.04 ± 1.56
C EDL	0.52 ± 0.59	0.56 ± 0.74	0	0	0
CRG-MN	*				
Exp EDL	20.40 ±10.47	1.44 ± 1.34	0.72 ± 1.10	0.04 ± 0.09	1.68 ± 1.98
C EDL	0.30 ± 0.13	0	0	0	0
FTMG	*		*		
Exp EDL	29.12 ±15.91	0.76 ± 0.36	2.52 ± 1.95	0.28 ± 0.33	1.44 ± 1.03
C EDL	0.28 ± 0.41	0.4 ± 0.42	0	0	0
Nerve Crush	*		*		
Op EDL	11.28 ±11.92	0.08 ± 0.18	1.04 ± 0.55	0	0
C EDL	0.08 ± 0.18	0	0	0	0
Nerve Cut	*		*		
Op EDL	19.98 ±15.35	0.28 ± 0.27	2.76 ± 2.21	0	0.12 ± 0.18
C EDL	0.12 ± 0.18	0	0	0	0
Unop Control					
Left EDL	0.08 ± 0.11	0	0	0	0
Right EDL	0.32 ± 0.41	0.04 ± 0.09	0	0	0

Table 3.5 - The mean percentage and standard deviation of each pathological feature present in the experimental (Exp), contralateral (C) and operated (Op) and unoperated (Unop) control EDL muscles after staining with H & E.

* A significant result for the experimental, operated or unoperated control group when compared to those values for the contralateral/right side

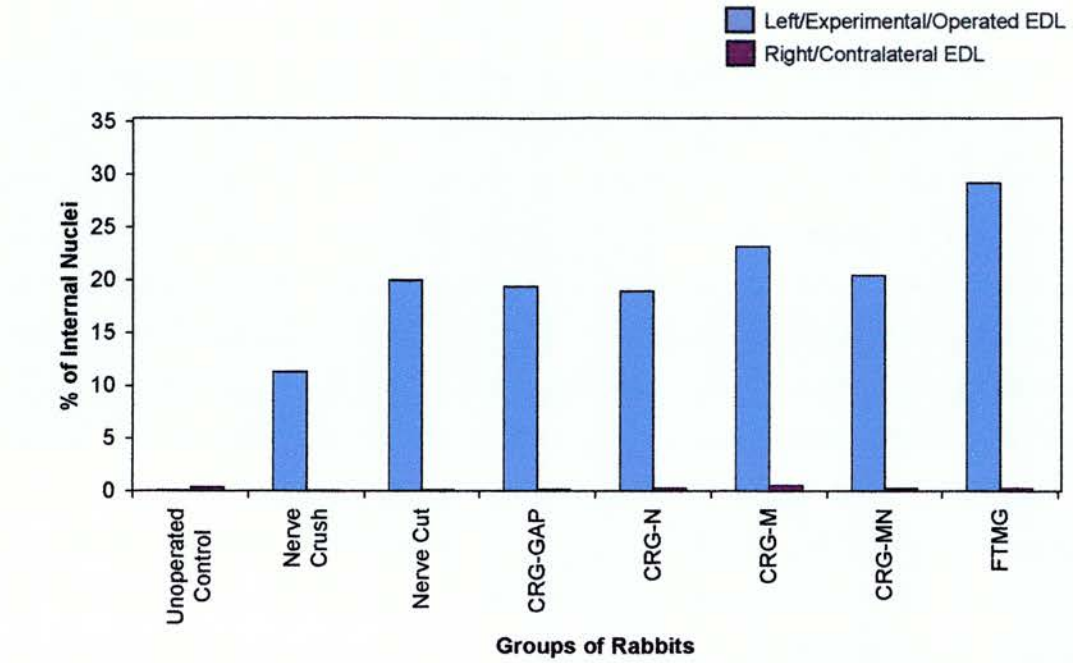


Figure 3.13 The mean percentage of internal nuclei present in the EDL muscles of the experimental and control groups.

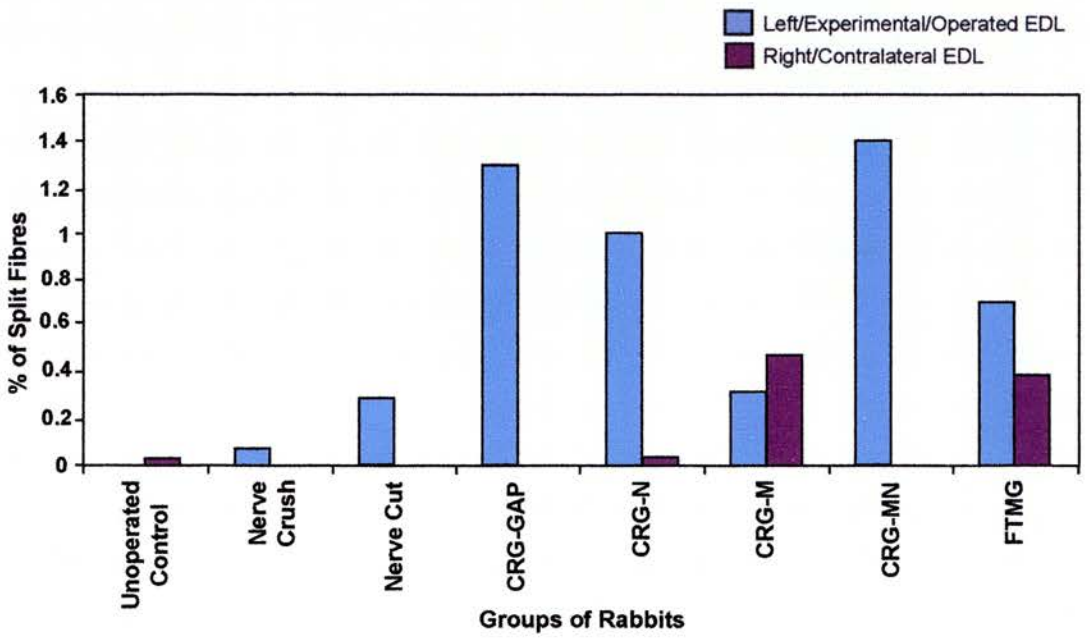


Figure 3.14 The mean percentage of split fibres present in the EDL muscles of the experimental and control groups.

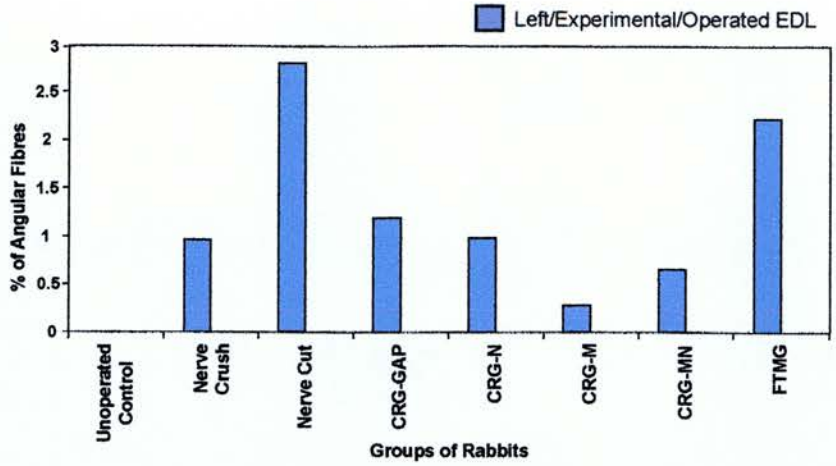


Figure 3.15 The mean percentage of angular fibres present in the EDL muscles of the experimental and control groups.

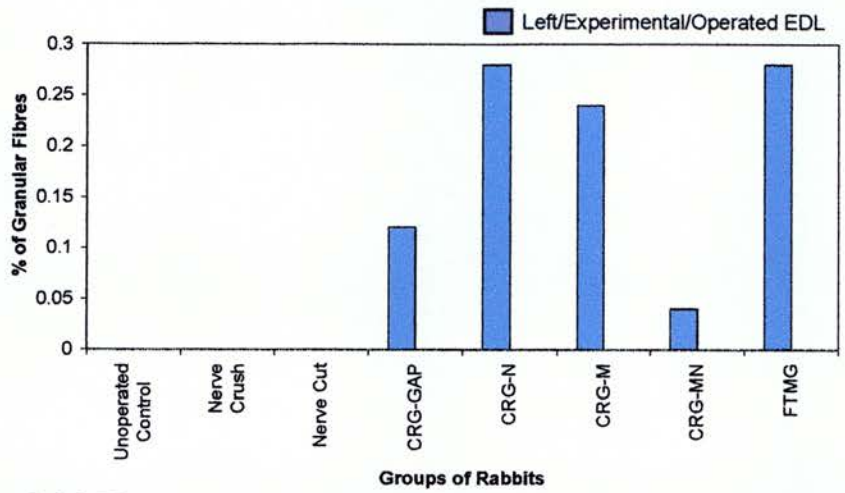


Figure 3.16 The mean percentage of granular fibres present in the EDL muscles of the experimental and control groups.

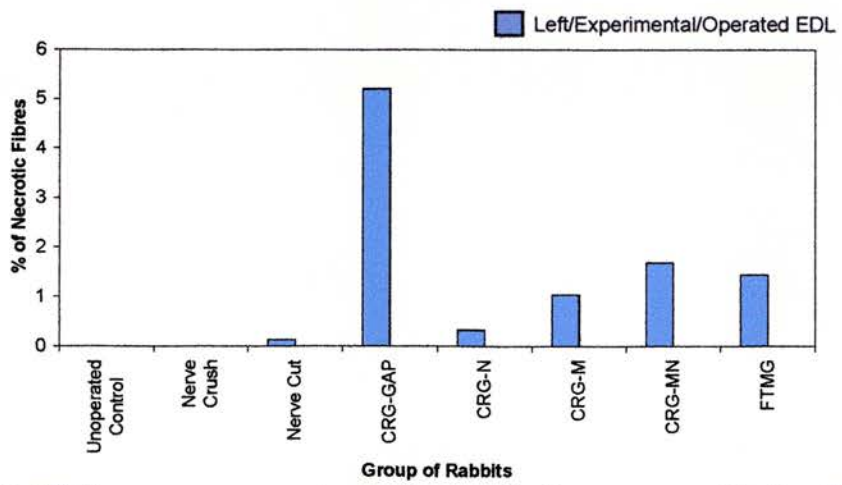


Figure 3.17 The mean percentage of necrotic fibres present in the EDL muscles of the experimental and control groups.

3.2.7 Muscle fibre diameter

In the study of changes observed in muscle specimens after denervation and reinnervation one of the most important parameters to be considered is the size of the muscle fibres. This is particularly important as the process of denervation is associated with muscle fibre atrophy, whilst reinnervation can produce compensatory hypertrophy of reinnervated fibres while neighbouring fibres remain denervated.

Sections stained for myofibrillar ATPase after pH 4.35 and 10.20 pre-incubation were also used to measure the diameter of type I and type II muscle fibres. Figure 3.18a is a photograph of a normal EDL muscle stained for myofibrillar ATPase after pH 10.20 pre-incubation, and Figure 3.18b after pH 4.35 pre-incubation. A total of 200 to 300 fibres were assessed for each muscle section and this was accomplished by the use of the VIDS III (Analytical Measuring Systems Ltd, Pampisford, Cambridge, UK), a high resolution semiautomatic image analysis system. The data handling was carried out by an IBM XT computer interfaced to the VIDS III system.

The measure of fibre size was determined by the minimum or narrow fibre diameter. This is defined as the maximum distance across the narrowest aspect of the muscle fibre (Brooke and Engel, 1969). The narrowest diameter measurement is least prone to the influences of sectioning obliquity or kinking of the muscle fibre which are two common occurrences in muscle biopsies (Dubowitz, 1985). Table 3.6 shows the mean and the standard deviation values for the minimum diameter of type I and type II fibres of the experimental and contralateral EDL muscles of the experimental groups, the operated and contralateral EDL muscles of the nerve crush and the nerve

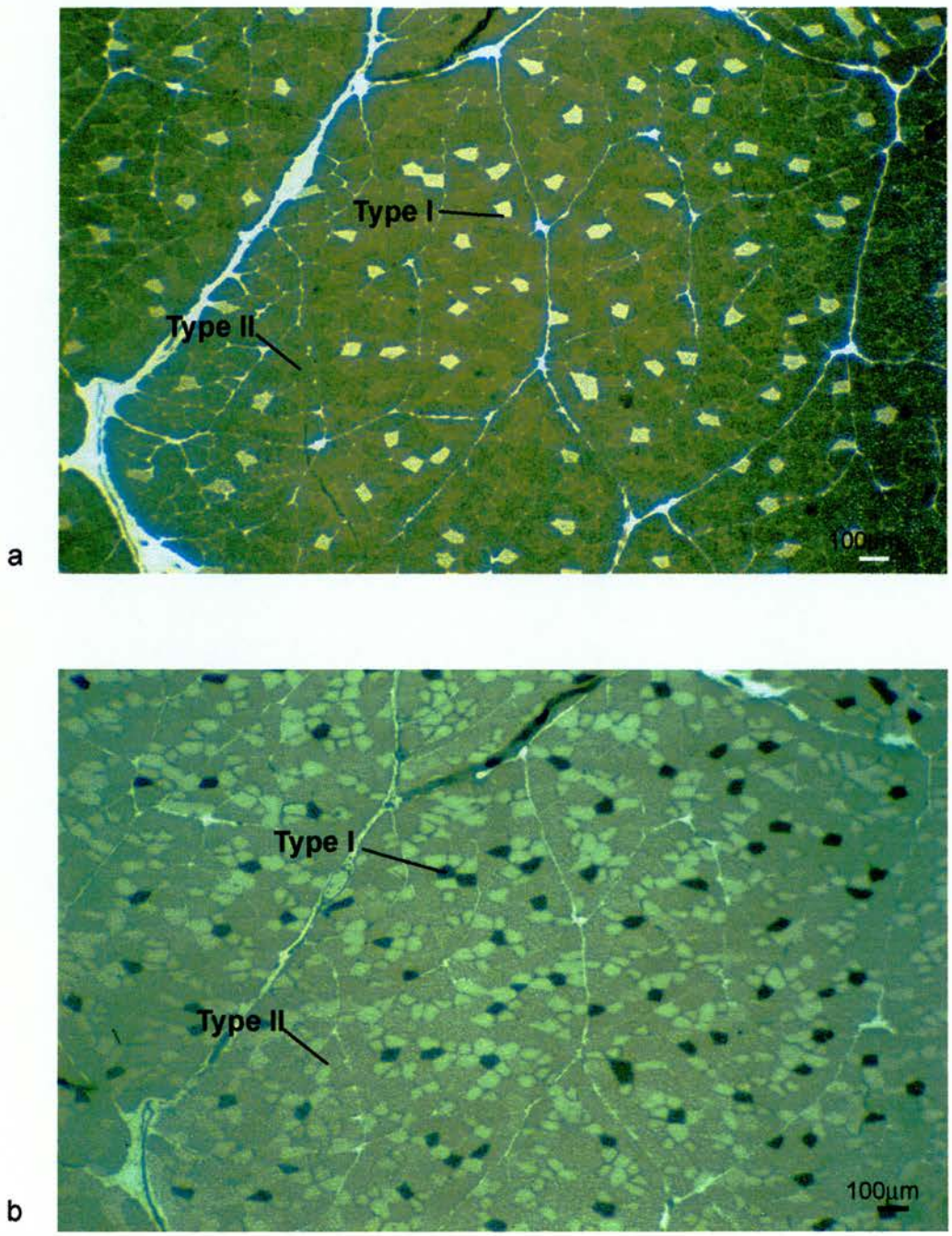


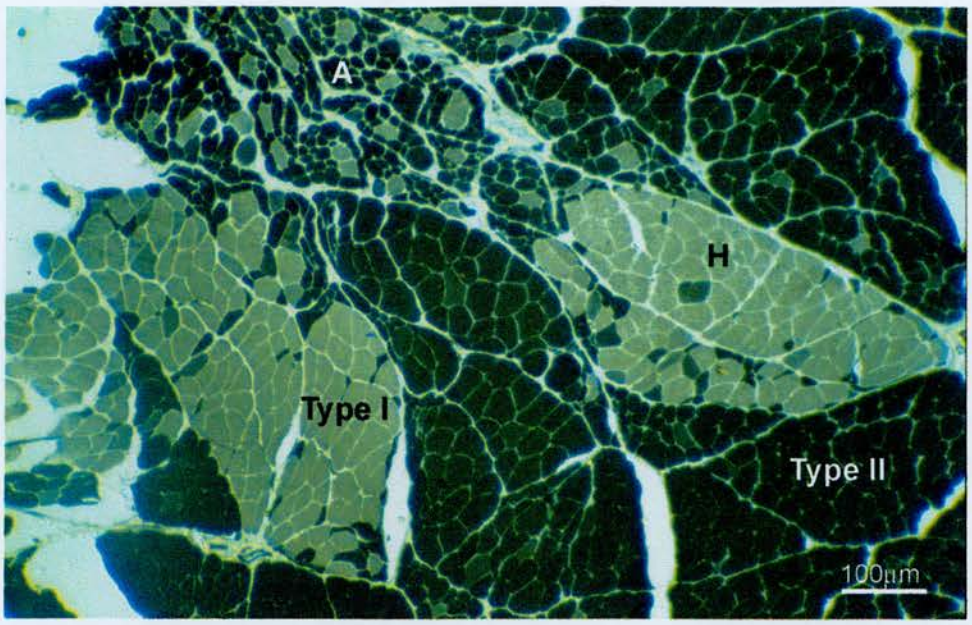
Figure 3.18 A normal EDL muscle.
(a) Myofibrillar ATPase after pH 10.2 pre-incubation.
(b) Myofibrillar ATPase after pH 4.35 pre-incubation.
Type I and type II fibres are labelled.

cut control groups, as well as the left and the right EDL muscles of the unoperated control.

For the majority of the experimental groups the mean minimum diameter of type I experimental EDL muscle fibres were lower than that of the corresponding contralateral muscles. It was after repair with CRG-N and CRG-MN tubes that the mean minimum diameter of type I fibres increased. On average this was a 1.51% or 0.69 μm increase on the mean minimum diameter of the contralateral type I fibres. For the experimental groups where the mean minimum diameter of type I muscle fibres was less after repair, the diameter decreased by an average 4.48% or 2.07 μm. The differences in diameter of type I fibres between experimental and contralateral EDL muscles were only significant after repair with either a FTMG or a CRG-M tube ($p < 0.05$ for both cases, paired Student's *t*-test). After both these methods of repair the mean minimum diameter of type I experimental muscle fibres was less than that of the corresponding contralateral muscles. Figure 3.19a is a photograph of the EDL muscle after repair with a FTMG stained for myofibrillar ATPase after pH 10.20 pre-incubation, and Figure 3.19b after pH 4.35 pre-incubation.

Of the operated control groups, it was only after a nerve cut that the mean minimum diameter of type I fibres was significantly greater in operated than corresponding contralateral EDL muscles ($p < 0.05$). There were also differences in the mean minimum diameter of type I muscle fibres between those values for the left and the right EDL muscles of the unoperated control, and in fact this was a highly significant difference ($p < 0.001$). The mean minimum diameter of the left EDL muscle was 16.73% or 7.22 μm greater than that of the right.

Overall there were significant differences when the results for the minimum



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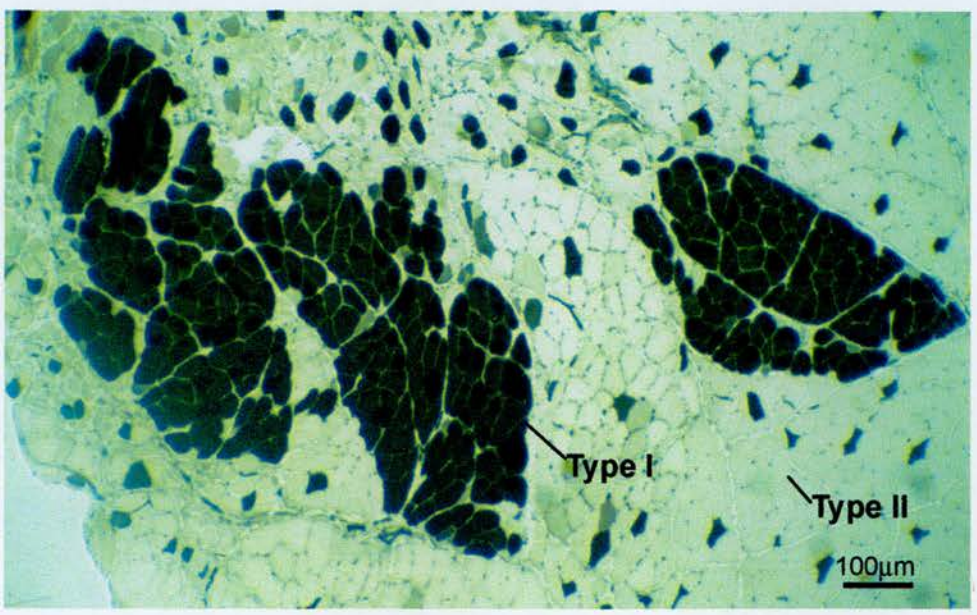


b

Figure 3.19 The EDL muscle after repair with a FTMG.
(a) Myofibrillar ATPase after pH 10.2 pre-incubation.
(b) Myofibrillar ATPase after pH 4.35 pre-incubation.
Type I and type II fibres are labelled.
Note the variation in fibre size and distribution of fibre types.Groups of smaller and atrophied (A) fibres and larger hypertrophied (H) fibres are labelled.

diameters of type I fibres for all the different nerve repairs were compared ($p < 0.001$, ANOVA). Individual comparisons of the repair groups revealed that after the insertion of a CRG-GAP tube the mean minimum diameter of type I fibres was significantly less than after repair by any of the other methods. Indeed, this decrease in diameter after repair with a CRG-GAP tube was highly significant when compared to the results after repair with either a CRG-N, a CRG-M or a CRG-MN tube (all $p < 0.001$, unpaired Student's *t*-test), and significant when compared to the diameters after repair with a FTMG ($p < 0.05$). After repair with a FTMG there was a highly significant decrease in the mean minimum diameter of type I fibres when compared to repair with either a CRG-N or a CRG-MN tube ($p < 0.01$ in each case).

Overall there were significant differences when the minimum diameters of type I fibres for all of the experimental and control groups (nerve cut, nerve crush and unoperated) were compared ($p < 0.001$, ANOVA). Individual comparison of the experimental and control groups revealed that the mean minimum diameter of type I fibres after nerve crush was greater when compared to those values for the majority of the experimental groups. This difference was highly significant for the CRG-GAP tube group ($p < 0.001$, unpaired Student's *t*-test), and significant after repair with a FTMG ($p < 0.05$). For the nerve cut control group, the values for mean minimum diameter of type I fibres were greater than those for each experimental group. This was highly significant after repair with either a CRG-GAP tube or a FTMG ($p < 0.001$ for each case), and significant after repair with a CRG-M tube ($p < 0.05$). There was a highly significant difference when the mean minimum fibre diameter for each experimental group was compared to the mean minimum fibre diameter for the left EDL muscle of the unoperated control ($p < 0.001$ for each case). However, the

mean minimum fibre diameter of type I fibres for the right EDL muscle of the unoperated control was less than those values for the majority of the experimental groups. This difference was significant after repair with a CRG-MN tube ($p < 0.001$). There was also a significant difference after repair with either a CRG-M ($p < 0.01$), a CRG-N ($p < 0.01$), or a CRG-GAP tube ($p < 0.05$).

There were significant differences in the minimum diameters of type I fibres between operated EDL muscles of the nerve crush and the nerve cut control groups, as well as the left and the right EDL muscles of the unoperated control ($p < 0.001$, ANOVA). Individual comparison of the groups revealed that these differences included a highly significant difference in mean minimum fibre diameter of type I fibres between the nerve crush control group and the left and the right EDL muscles of the unoperated control ($p < 0.001$, unpaired Student's *t*-test). For the nerve cut control group, the mean minimum fibre diameter of type I fibres was significantly less than those values for the left EDL muscle of the unoperated control ($p < 0.05$), and significantly greater than the mean minimum fibre diameter of type I fibres of the nerve crush control group ($p < 0.01$) and the right EDL muscle of the unoperated control ($p < 0.001$).

Overall there were significant differences when the minimum diameters of type I fibres for all of the contralateral EDL muscles of the experimental and operated control groups, as well as the left and the right EDL muscles of the unoperated control were compared ($p < 0.001$, ANOVA). Individual comparison of the contralateral EDL muscles from the experimental groups and those from the control groups revealed that the mean minimum fibre diameter of type I fibres for the contralateral muscles of the nerve crush control group were significantly greater than

those values for the CRG-GAP ($p < 0.001$, unpaired Student's *t*-test) and the CRG-N tube groups ($p < 0.01$). For the contralateral muscles of the nerve cut control group, the mean minimum diameter of type I fibres was significantly greater when compared to those values for contralateral muscles of the CRG-GAP and CRG-MN tube groups ($p < 0.05$ for each case), and significantly less than after repair with a CRG-M tube ($p < 0.01$). There was a highly significant difference in the mean minimum diameter of type I fibres of the left EDL muscle of the unoperated control when compared to similar values for each of the contralateral muscles of the experimental groups ($p < 0.001$ for each case). Indeed, the mean minimum diameter of type I fibres for the left EDL muscle of the unoperated control was greater in value than any of the diameters for the contralateral muscles of the experimental groups. For the right EDL muscle of the unoperated control there was also a highly significant difference in the mean minimum diameter of type I fibres in contralateral muscles when compared to values after repair with either a CRG-M or a CRG-MN tube or a FTMG ($p < 0.001$). As with the CRG-M, CRG-MN and FTMG experimental groups, the mean minimum diameter of type I fibres in contralateral muscles of the CRG-GAP and CRG-N tube groups were significantly greater although to a lesser extent, than those values for the right EDL muscle of the unoperated control ($p < 0.05$ for the CRG-GAP and $p < 0.01$ for the CRG-N tube groups).

There were significant differences in minimum diameter of type I fibres between contralateral muscles of the nerve crush and the nerve cut control groups, and the left and right EDL muscles of the unoperated control ($p < 0.001$, ANOVA). Individual comparisons of these groups revealed that these differences were highly significant when either the left or the right EDL muscle of the unoperated control

were compared to the contralateral muscles of the nerve crush and the nerve cut control groups ($p < 0.001$ for each case, unpaired Student's *t*-test).

For the type I fibres in experimental muscles of all repair groups as well as the operated muscles of the nerve crush and the nerve cut control groups, the variability coefficient was greater than a value of 250 (where the standard deviation of the mean minimum fibre diameter was greater than 0.25 of the value of the mean diameter in the experimental/operated muscles). This demonstrates that there was an abnormal variability in the size of type I muscle fibres in the experimental EDL muscles after nerve repair, and the operated muscles of the nerve crush and cut control groups (Dubowitz, 1985). For the contralateral muscles of the experimental groups, as well as the nerve crush and the nerve cut control groups and the left and the right muscles of the unoperated control, the standard deviation of the mean minimum diameter of type I fibres was less than 0.25 of the value of the mean diameter. These findings indicate that there was no abnormal variability in muscle fibre size in the contralateral muscles of experimental and control groups, or unoperated control muscles.

For each repair group the mean minimum diameter of type II muscle fibres of the experimental EDL was less than that of the corresponding contralateral muscle. On average, this was a 47.07% or 24.20 μm decrease on the mean minimum diameter of contralateral type II muscle fibres. This decrease in diameter of fibres of experimental muscle was highly significant after each form of nerve repair ($p < 0.001$ for each case, paired Student's *t*-test). For both the nerve crush and the nerve cut control groups, there was also a highly significant decrease in diameter of type II fibres of the operated muscle when compared to similar values for the corresponding

contralateral muscle ($p < 0.001$). However, the decrease was not as great as that observed for the experimental groups. On average, this reduction in the mean minimum diameter of type II muscle fibres for the nerve crush and cut control groups was a 17.18% or 9.09 μm decrease on the mean minimum diameter of contralateral type II muscle fibres.

Overall there were significant differences when the results for the minimum diameters of type II fibres for all the different nerve repairs were compared ($p < 0.001$, ANOVA). Individual comparisons of the different experimental groups revealed that after repair with a CRG-GAP tube the mean minimum diameter of type II muscle fibres was the smallest of all repair groups. This difference in diameter was highly significantly when compared to those values for any of the other methods of repair ($p < 0.001$ for each case, unpaired Student's *t*-test). The next smallest mean minimum diameter of type II muscle fibres was after repair with a CRG-MN tube group and this was significantly smaller than after repair with either a FTMG ($p <0.001$), a CRG-N ($p < 0.001$) or a CRG-M tube ($p < 0.01$). This was followed by repair with a CRG-M tube where the mean minimum diameter of type II muscle fibres was significantly lower than after repair with a FTMG ($p < 0.001$). Next was repair with a CRG-N tube where the mean minimum diameter of type II muscle fibres was significantly lower than after repair with a FTMG ($p < 0.001$). Hence, after repair by a FTMG the mean minimum diameter of type II muscle fibres was the greatest of all repair groups and there was a highly significantly difference in diameter when compared to values for any of the other methods of repair ($p < 0.001$ for each case).

There were significant differences when the minimum diameters of type II fibres for all of the experimental and control groups (nerve cut, nerve crush and unoperated) were compared ($p < 0.001$, ANOVA). Individual comparison of the experimental and control groups revealed that after each type of nerve repair the mean minimum diameter of type II fibres was less than the values for the nerve crush and cut control groups, as well the left and right EDL muscles of the unoperated control. These differences in type II fibre diameter were highly significant for every experimental group ($p < 0.001$ for each case, unpaired Student's *t*-test).

The differences in the mean minimum diameter of type II fibres between operated EDL muscles of the nerve crush and the nerve cut control groups, as well as the left and the right EDL muscles of the unoperated control were statistically significant ($p < 0.001$, ANOVA). Individual comparison of these groups revealed that of the three control groups, the nerve cut group was the poorest performer as measured by the minimum diameter of type II EDL muscle fibres. For the nerve cut control group, the mean minimum diameter of type II fibres was significantly less than those values for the nerve crush control group as well as the right and the left EDL muscles of the unoperated control ($p < 0.001$ for each case, unpaired Student's *t*-test). The mean minimum diameter of type II fibres of the left and the right EDL muscles of the unoperated control were also significantly larger than those of the nerve crush control group ($p < 0.001$).

Overall there were significant differences when the minimum diameters of type II fibres for all of the contralateral EDL muscles of the experimental and operated control groups, as well as the left and the right EDL muscles of the unoperated control were compared ($p < 0.001$, ANOVA). Individual comparison of

the contralateral EDL muscles from the experimental groups and those from the control groups revealed that for most contralateral muscles of experimental groups (apart for the CRG-MN tube group), the mean minimum diameter of type II fibres was less than that found for type II fibres in the contralateral muscles of the nerve crush and cut control groups, as well as the left and the right EDL muscles of the unoperated control. This reached statistical significance for the CRG-GAP ($p < 0.001$ for each case, unpaired Student's *t*-test), and the CRG-M tube groups ($p < 0.001$ when compared to the contralateral EDL muscles of the nerve crush control group and the right EDL muscle of the unoperated control, $p < 0.01$ when compared to the left EDL muscle of the unoperated control and $p < 0.05$ when compared to the nerve cut control group). There were also significant differences when the mean minimum diameter of type II fibres of contralateral muscles of the nerve crush control group were compared to those for the CRG-N tube group ($p < 0.001$) and the FTMG group ($p < 0.01$). The mean minimum diameter of type II fibres of the left EDL muscle of the unoperated control was significantly greater than those of the contralateral muscles of the CRG-N tube and the FTMG repair groups ($p < 0.001$ for each case). The contralateral muscles of the CRG-MN tube group were the only experimental group where the mean minimum diameter of type II fibres was greater than that found for type II fibres in the contralateral muscles of the nerve crush and cut control groups, as well as the left and the right EDL muscles of the unoperated control. This difference was statistically significant for the nerve cut control group ($p < 0.001$), and the right EDL muscle of the unoperated control ($p < 0.01$).

There were significant differences in minimum diameter of type II fibres between contralateral muscles of the nerve crush and the nerve cut control groups,

and the left and right EDL muscles of the unoperated control ($p < 0.01$, ANOVA). Individual comparisons of these groups revealed that the mean minimum diameter of type II fibres in the left EDL muscle of the unoperated control was significantly greater when compared to the value for the nerve cut control group ($p < 0.01$, unpaired Student's *t*-test). The mean minimum diameter of type II fibres in the nerve crush control group were significantly greater than those in the contralateral muscles of the nerve cut control group and the right EDL muscle of the unoperated control ($p < 0.05$ for each case).

For the type II fibres in experimental muscles of all repair groups as well as the operated muscles of the nerve crush and the nerve cut control groups, the variability coefficient was greater than a value of 250 which indicates that there was an abnormal variability in the size of type II muscle fibres (Dubowitz, 1985). Unlike the findings for type I fibres, the variability coefficient was greater than 250 for the contralateral muscles of every experimental group, as well as the contralateral muscles of the nerve crush and the nerve cut control groups and the left and the right muscles of the unoperated control. Again this indicates that there was abnormal variability in muscle fibre size in the contralateral and unoperated control muscles.

The mean minimum diameters of type I and type II fibres were compared in each experimental, contralateral, operated and unoperated control EDL muscle. For most experimental and operated control muscles the mean minimum diameter of type I fibres was consistently greater than for type II fibres. The only exception was from the nerve crush control group where the mean minimum diameter of type II fibres was greater. For all contralateral muscles of experimental and control groups, as well as the unoperated control muscles, the mean minimum diameter of type II fibres was

always the larger. These differences in diameter between type I and II fibres were significant for all groups ($p < 0.001$ for each case), apart from the experimental muscle of the nerve crush group where the difference in mean diameter between type I and II fibres was statistically insignificant.

The mean minimum diameter of type I fibres for the one control animal housed in the Department of Tropical Veterinary Medicine was 46.72 μm for the left EDL muscle, and 49.56 μm for the corresponding right. For the type II fibres the values were 50.41 μm for the left EDL muscle, and 53.22 μm for the right EDL muscle. These figures were comparable to the mean minimum diameter of type I and type II fibres for both the left and the right EDL muscles of the unoperated control.

In summary, there were several differences in diameters of muscle fibres of different fibre types in the experimental and the control groups. However in general it appears that there was a preferential atrophy of type II fibres for all experimental groups. For the experimental groups the best performer in terms of muscle fibre diameter was the FTMG group. Of the four CRG tube groups, there was not much difference in effectiveness as a form of nerve repair as measured by minimum fibre diameter, although repair with a CRG-GAP tube did produce consistently poor results. Indeed based on the findings for minimum fibre diameter all types of nerve repair were worse than those results for the nerve cut control group. The values for minimum fibre diameter for both the nerve crush and the nerve cut control groups were generally lower than that of the normal or unoperated control group. There were several differences in the diameters of muscle fibres of both type I and type II in the contralateral muscles of experimental and operated control groups, as well as the left and the right EDL muscles of the unoperated control. However no correlation

between method of repair and changes in muscle fibre diameter in contralateral muscles was found.

Removal of the data for the mean minimum diameter of type I fibres for the one animal in the FTMG group which did appear to achieve full repair resulted in a value of 39.34 μm and for the standard deviation, 16.16 μm. For the corresponding contralateral side after removal of these results the value for the mean minimum diameter of type I fibres was 46.76 μm and for the standard deviation, 11.58 μm. With this data excluded the value for the mean minimum diameter of type I fibres for the FTMG was less than **all** other experimental groups however, when included the mean minimum diameter of type I fibres after repair with a FTMG was greater than after repair with a CRG-GAP tube. Indeed statistical analysis of the findings after removal of the data for the one animal in the FTMG which appeared to achieve full repair produced different results to those previously. Firstly, the degree of significant difference in minimum fibre diameter between the FTMG group and its corresponding contralateral control was greater ($p < 0.001$). Secondly, the difference in minimum fibre diameter was significant for **all** experimental groups ($p < 0.05$ compared with repair with a CRG-GAP tube, $p < 0.001$ for all other groups). Thirdly, comparison with the control groups established that after removal of this data there was a significant difference in minimum fibre diameter between the FTMG group and the left and the right EDL muscles of the unoperated control ($p < 0.001$). The level of significant difference had also increased after comparing the values for the mean minimum fibre diameter of type I fibres after repair with a FTMG and those after a nerve crush ($p < 0.001$).

For the type II fibres, removal of the data for the mean minimum diameter of type II fibres for the one animal in the FTMG group which did appear to achieve full repair resulted in a value of 31.63 μm and for the standard deviation, 17.19 μm. The values for the corresponding contralateral side were 52.27 μm for the mean minimum diameter and 13.69 μm for the standard deviation. However, removal of this data resulted in no change in the findings for fibre diameter for type II fibres. This was also the case when the mean minimum diameters of type I and type II fibres were compared in each experimental and contralateral EDL muscle for the FTMG repair group.

Figure 3.20 depicts the mean and the standard error of the mean for the minimum diameter of type I fibres, and Figure 3.21 for type II fibres, in EDL muscles of each of the experimental and control groups.

Procedure	Muscle	Type I (μm)	Type II (μm)
CRG-GAP	Experimental EDL	42.68 ± 17.78	21.04 ± 10.23 *
	Contralateral EDL	44.18 ± 6.84	48.46 ± 13.42
CRG-N	Experimental EDL	45.52 ± 14.54	27.56 ± 13.38 *
	Contralateral EDL	44.76 ± 8.42	51.58 ± 12.81
CRG-M	Experimental EDL	45.38 ± 15.79 *	27.13 ± 12.45 *
	Contralateral EDL	47.45 ± 8.60	50.49 ± 13.35
CRG-MN	Experimental EDL	47.21 ± 15.56	25.64 ± 10.68 *
	Contralateral EDL	46.60 ± 6.51	54.90 ± 15.15
FTMG	Experimental EDL	43.72 ± 16.43 *	34.95 ± 18.65 *
	Contralateral EDL	46.36 ± 11.18	51.89 ± 13.44
Nerve Crush	Operated EDL	45.54 ± 11.99	46.20 ± 15.86 *
	Contralateral EDL	46.76 ± 7.46	54.16 ± 14.28
Nerve Cut	Operated EDL	48.05 ± 16.58 *	41.77 ± 15.46 *
	Contralateral EDL	45.55 ± 8.90	51.99 ± 13.82
Unoperated Control	Left EDL	50.38 ± 8.86 *	54.17 ± 14.59
	Right EDL	43.16 ± 7.31	52.41 ± 13.31

Table 3.6 - The mean and the standard deviation of the minimum fibre diameter (μm) of type I and type II muscle fibres for the experimental and contralateral EDL muscles of each of the experimental and control groups.

* A significant result for the experimental, operated or unoperated control group when compared to those values for the contralateral/right side

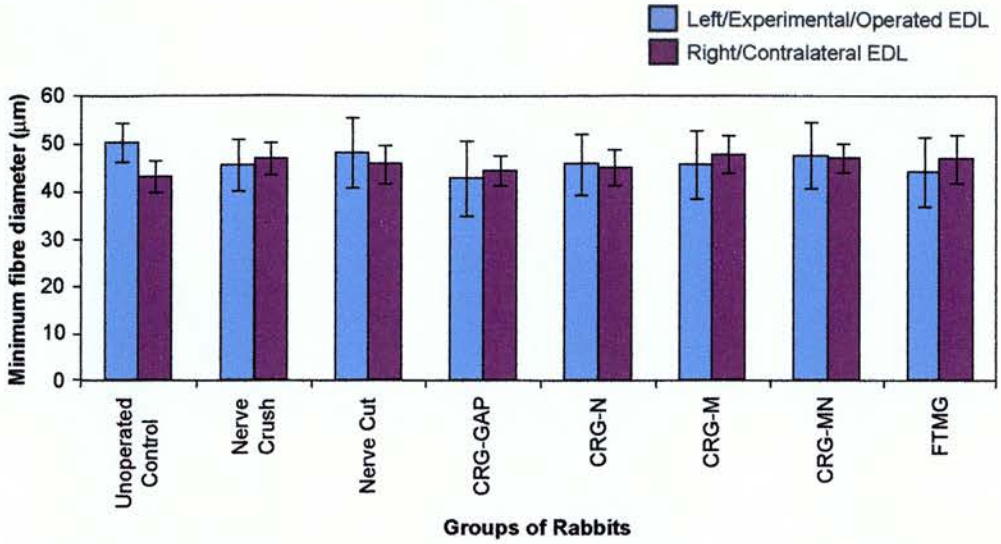


Figure 3.20 The mean and the SEM for the minimum fibre diameter (μm) of type I fibres in the EDL muscles of the experimental and control groups.

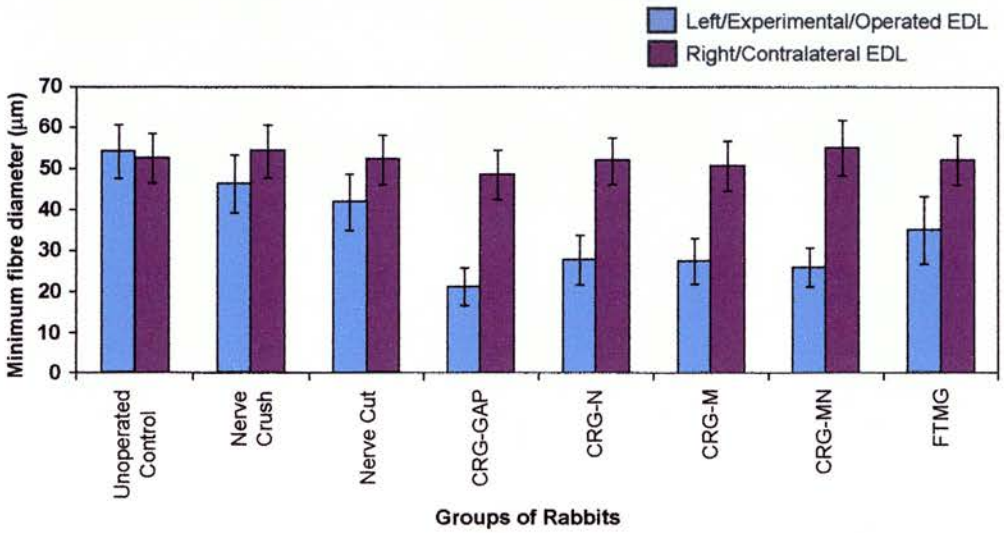


Figure 3.21 The mean and the SEM for the minimum fibre diameter (μm) of type II fibres in the EDL muscles of the experimental and control groups.

3.2.8 Connective tissue content

The proliferation of connective tissue is a consequence of the denervation of muscle. The connective tissue content (i.e. the proportion of tissue cross-sectional area occupied by non-muscle fibre components) was measured by a point counting procedure (Aherne and Dunhill, 1982). An eye piece graticule was used to superimpose a 100 point lattice grid onto the muscle cross-section and a count was made of the number of grid intersections overlying connective tissue. These were recorded and expressed as a percentage of the total number of points counted. To ensure that the histological sampling was adequate and that the sampling error was suitably small, e.g. 5% of the mean or less, the relative standard error was calculated. Calculation of this formula provides an indication of the number of points which must fall on the area whose volume fraction is being measured. In this study, 2500 points per muscle were counted to ensure an error of less than 5%.

Table 3.7 shows the mean and the standard deviation values for the volume fraction of connective tissue present in the experimental and contralateral EDL muscles of the experimental groups, the operated and contralateral EDL muscles of the nerve crush and the nerve cut control groups, as well as the left and the right EDL muscles of the unoperated control. The experimental EDL muscles for each experimental group as well as the operated muscles for the nerve crush and the nerve cut control groups showed an increase in connective tissue compared with contralateral levels (please refer to Figures 3.22, 3.23, 3.24 and 3.25). On average for the experimental groups this was a 291.33% increase in the volume fraction of connective tissue in contralateral muscle. For the nerve crush control group the mean

increase in connective tissue was 170.28%, whilst for the nerve cut group this value was 303.80%. This difference in connective tissue content was significant for most experimental groups, and the nerve crush and the nerve cut control groups ($p < 0.05$ for each group, Wilcoxon Signed Rank test, apart from $p > 0.05$ for the FTMG group). The one animal in the FTMG group which as assessed by wet muscle weight had achieved full repair also attained similar results as measured by the volume fraction of connective tissue. For this particular animal the volume fraction of connective tissue of the experimental EDL was 13.60%, whilst the volume fraction of connective tissue of its corresponding contralateral EDL muscle was 15.88%. The range in volume fraction of connective tissue in experimental EDL for the other animals in the FTMG group was from 28.08% to 56.56%.

Overall there were significant differences when the connective tissue content for all of the experimental and control groups (nerve cut, nerve crush and unoperated) were compared ($p < 0.001$, Kruskal-Wallis test). Individual comparison of the experimental and control groups revealed that after any form of repair the volume fraction of connective tissue was greater when compared to those values for the nerve crush and the left and right EDL muscles of the unoperated control groups. This difference was highly significant after repair with any of the tube groups and when comparing the connective tissue levels of the FTMG repair group to that of the left EDL muscle of the unoperated control ($p < 0.01$ for each case, Mann-Whitney U test). However when comparing the volume fraction of connective tissue between the FTMG and the nerve crush and the right EDL muscle from the unoperated control, the difference was significant ($p < 0.05$ for each case). All experimental groups had a greater volume of connective tissue in experimental muscles when compared to the

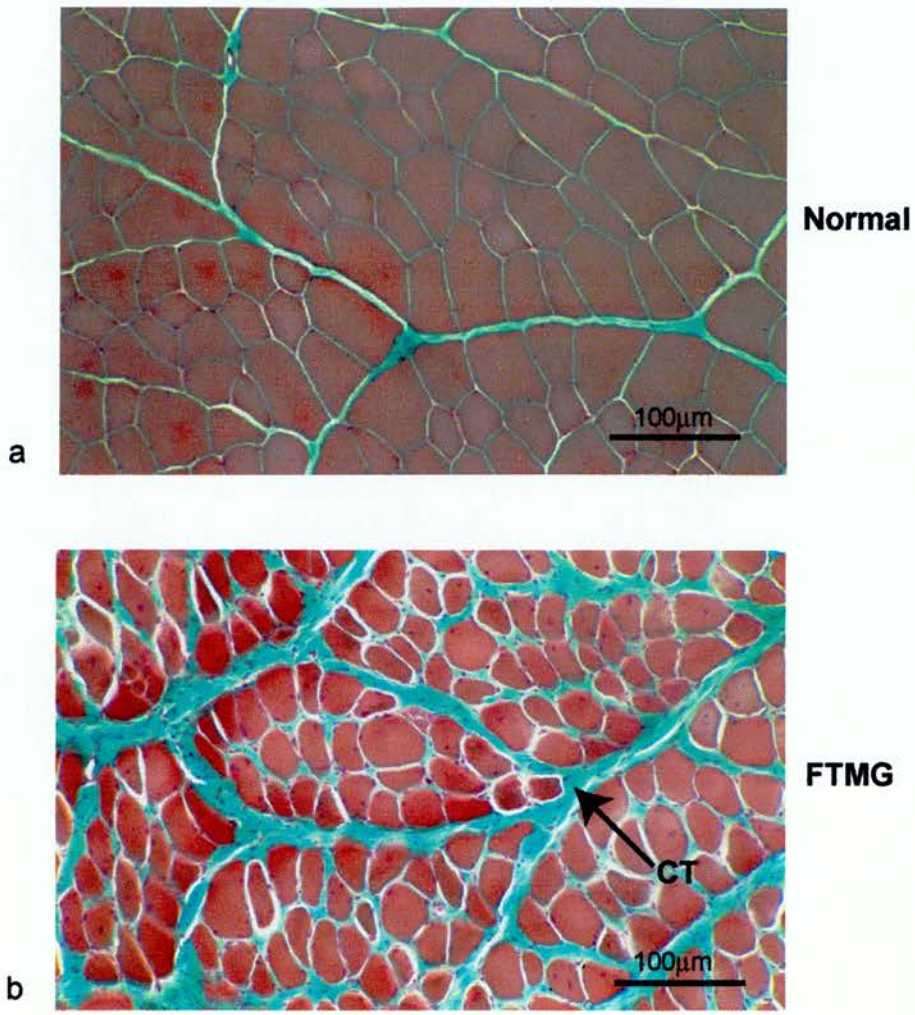


Figure 3.22 (a) The normal EDL muscle,note the connective tissue (CT) is stained blue/green (Masson's Trichrome).
(b) The EDL muscle after repair with a FTMG, note the proliferation of endomysial and perimysial connective tissue (Masson's Trichrome).

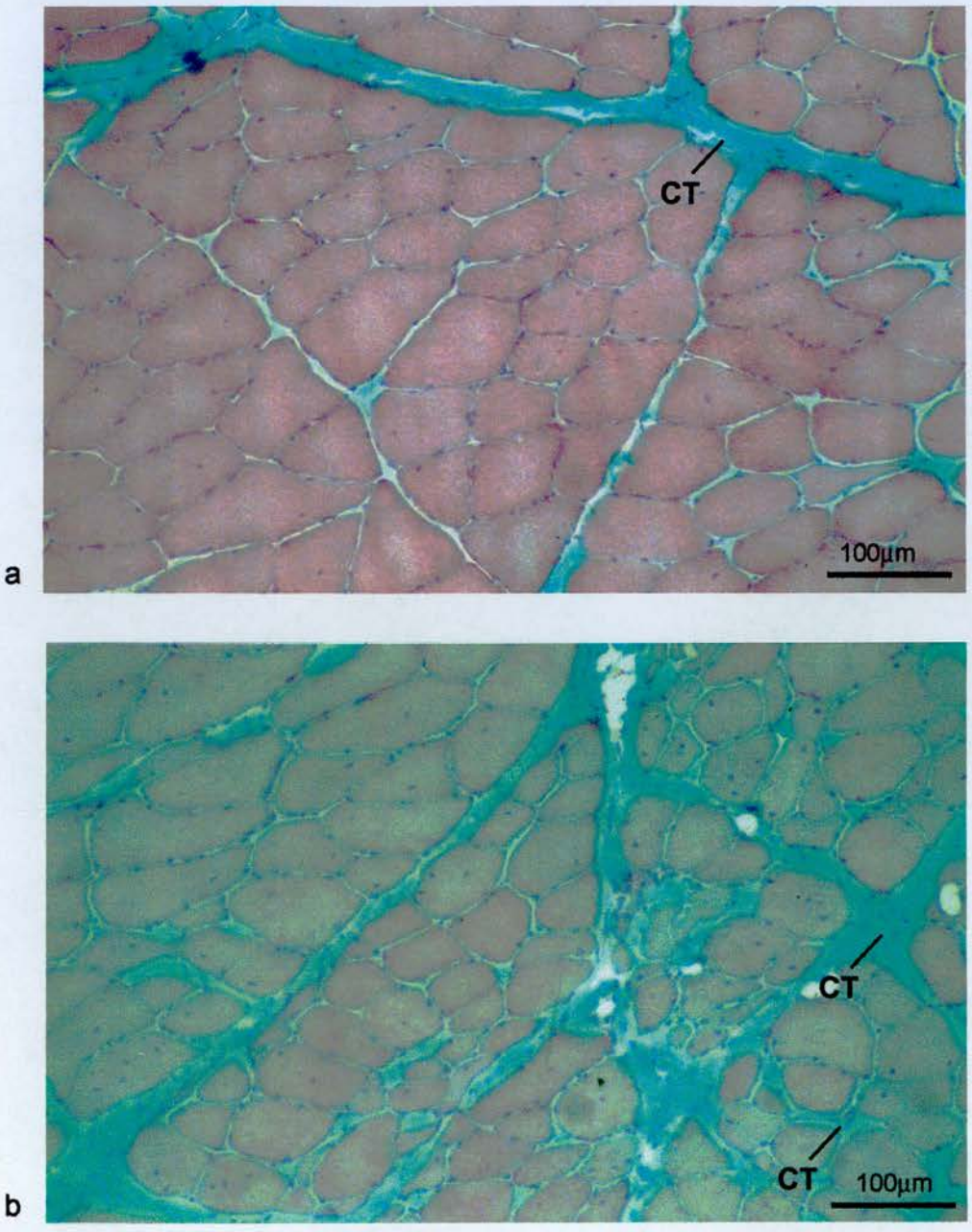
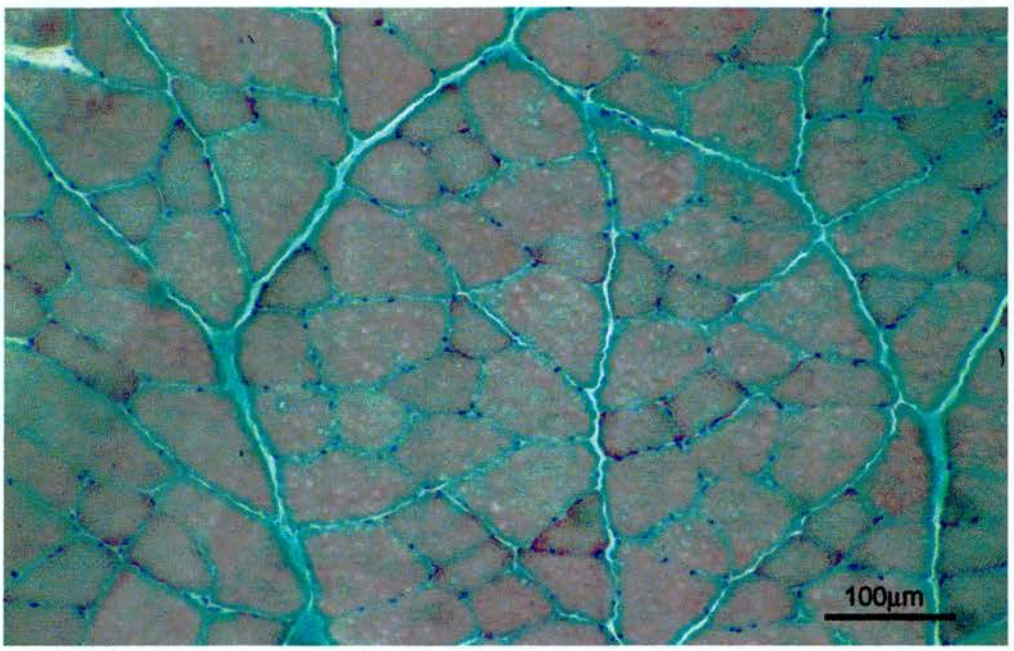


Figure 3.23 (a) The EDL muscle after peroneal crush (Masson's Trichrome). Note the increase in perimysial connective tissue (CT).
(b) The EDL muscle after peroneal nerve cut. There ia an increase in perimysial and endomysial connective tissue (Masson's Trichrome).



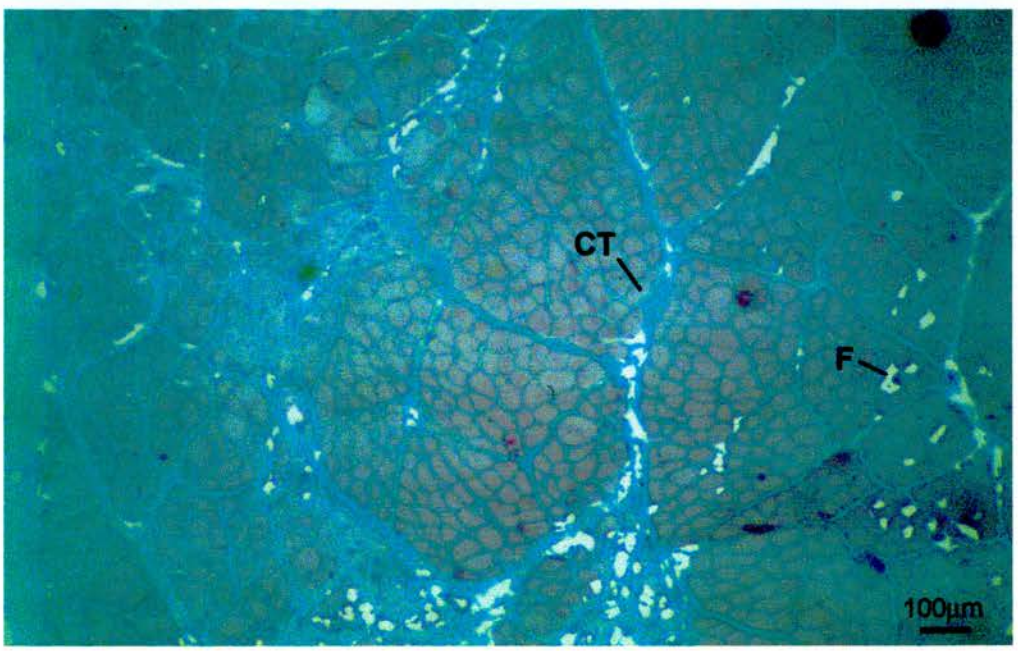


Figure 3.24 (a) The normal EDL muscle (Masson's Trichrome).
(b) The EDL muscle after repair with a CRG-M, note the increase in perimysial connective tissue and areas of fatty (F) replacement (Masson's Trichrome).

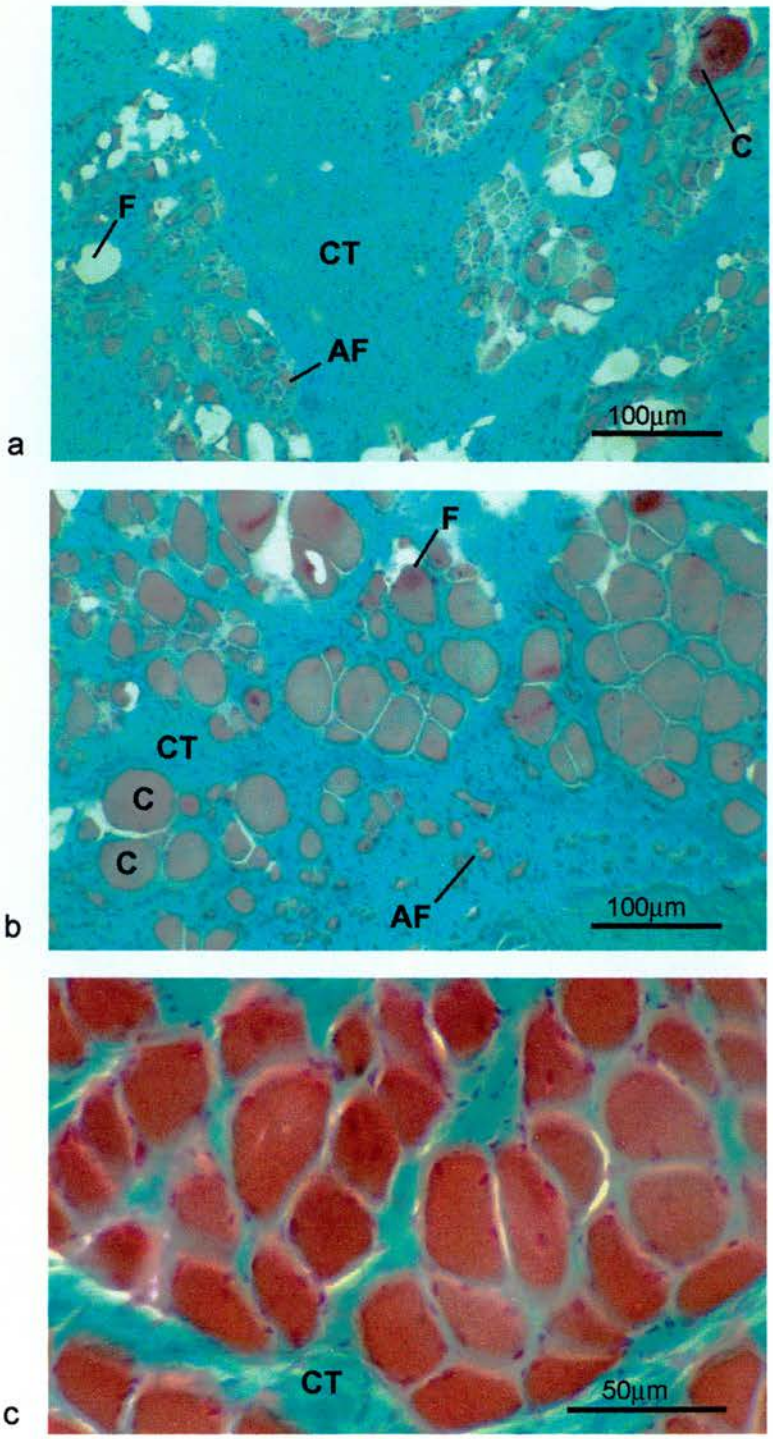


Figure 3.25 (a) The EDL muscle after repair with a CRG-GAP tube.Note the gross proliferation of connective tissue (CT) and the areas of fatty (F) replacement. One group of atrophied fibres (AF) and a large circular (C) fibre are labelled (Masson's Trichrome).
(b) The EDL muscle after repair with a CRG-N tube (Masson's Trichrome).
(c) After repair with a CRG-MN tube (Masson's Trichrome).

values for the nerve cut control groups, although this difference was only significant for the CRG-GAP, CRG-N and CRG-MN tube groups (all $p < 0.05$).

There were significant differences in the volume fraction of connective tissue between operated EDL muscles of the nerve crush and the nerve cut control groups, as well as the left and the right EDL muscles of the unoperated control ($p < 0.01$, Kruskal-Wallis test). Individual comparisons of these groups revealed that the volume fraction of connective tissue in the operated EDL muscles of the nerve cut control group was significantly greater when compared to that found in the left ($p < 0.01$, Mann-Whitney *U* test), or the right ($p < 0.05$) EDL muscles of the unoperated control or the operated muscles of the nerve crush control group ($p < 0.05$). The volume fraction of connective tissue for the left EDL muscle of the unoperated control was also significantly less than that for the experimental EDL muscle of the nerve crush control group ($p < 0.05$).

Overall there were significant differences when the connective tissue content for all of the contralateral EDL muscles of the experimental and operated control groups, as well as the left and the right EDL muscles of the unoperated control were compared ($p < 0.001$, Kruskal-Wallis test). Individual comparison of the contralateral EDL muscles from the experimental groups and those from the control groups revealed that there was a significantly greater volume fraction of connective tissue in the contralateral muscles of each of the experimental groups when compared to those levels in the contralateral muscles of the nerve crush and the nerve cut control groups ($p < 0.01$ for each case, Mann-Whitney *U* test). There was also significantly more connective tissue in the contralateral muscles of the experimental groups when comparing the right EDL muscle of the unoperated control to the contralateral

muscles of the CRG-N tube group, and the left EDL muscle of the unoperated control to all experimental groups ($p < 0.05$ for each case).

The volume fraction of connective tissue for the one control animal housed in the Department of Tropical Veterinary Medicine was 8.32% for the left EDL muscle, and 7.96% for the corresponding right. These figures were comparable to the connective tissue contents for both the left and the right EDL muscles of the unoperated control.

In summary, there was a marked increase in the connective tissue content of the EDL muscle after most forms of nerve repair. Of the four CRG tube groups, there was not much difference in effectiveness as a form of nerve repair as measured by the volume fraction of connective tissue. Indeed for most repair groups the findings were worst than those for the nerve cut control group. The volume fraction of connective tissue in contralateral muscles of experimental groups was greater when compared to those values for the contralateral muscles of operated and unoperated control groups. There was no relation between the method of repair and the level of connective tissue in contralateral muscles.

Removal of the data for the one animal in the FTMG group which did appear to achieve full repair resulted in a value for the mean volume fraction of connective tissue of 37.44% and for the standard deviation, 12.99%. For the corresponding contralateral side after removal of this data the value for the mean volume fraction of connective tissue was 12.97% and for the standard deviation, 3.02%. Statistical analysis of this data produced different results to those previously in that there was a significant difference in connective tissue content between experimental and contralateral muscles for the FTMG group ($p < 0.05$). Furthermore, the level of

statistical difference was greater when comparing the volume fraction of connective tissue for the FTMG repair group and the nerve crush group, and the FTMG group the right EDL muscle of the unoperated control ($p < 0.01$ for each case).

Figure 3.26 depicts the mean and the standard error of the mean for the volume fraction of connective tissue present in the EDL muscles of the experimental and control groups.

Procedure	Muscle	Vv (%)	RSE (%)
CRG-GAP	Experimental EDL	36.24 ± 9.44 *	2.73
	Contralateral EDL	11.14 ± 1.21	5.67
CRG-N	Experimental EDL	33.95 ± 5.08 *	2.82
	Contralateral EDL	14.30 ± 4.09	5.02
CRG-M	Experimental EDL	31.60 ± 12.13 *	3.12
	Contralateral EDL	11.10 ± 2.20	5.72
CRG-MN	Experimental EDL	40.24 ± 10.00 *	2.50
	Contralateral EDL	10.93 ± 1.41	5.74
FTMG	Experimental EDL	32.67 ± 15.50	3.15
	Contralateral EDL	13.55 ± 2.92	5.14
Nerve Crush	Operated EDL	12.13 ± 2.42 *	5.46
	Contralateral EDL	6.96 ± 1.22	6.38
Nerve Cut	Operated EDL	23.21 ± 6.91 *	3.77
	Contralateral EDL	7.64 ± 1.14	6.03
Unoperated	Left EDL	7.69 ± 2.38	6.15
	Right EDL	9.98 ± 3.13	6.16

Table 3.7 - The mean and the standard deviation for the volume fraction (Vv) of connective tissue present in the experimental and contralateral EDL muscles of each of the experimental and control groups. (RSE = Relative Standard Error).

* A significant result for the experimental, operated or unoperated control group when compared to those values for the contralateral/right side

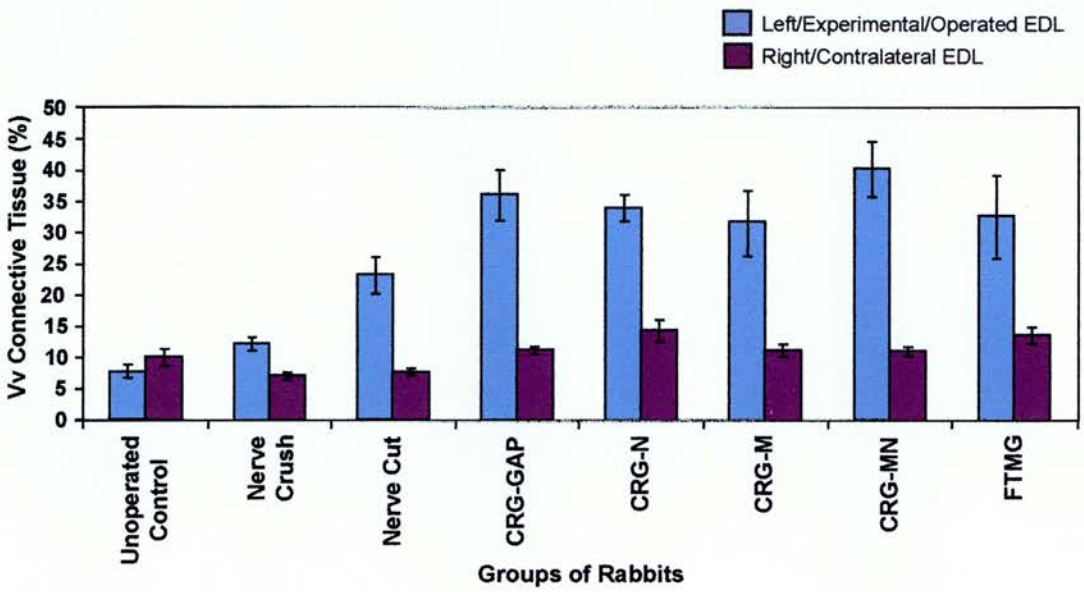


Figure 3.26 The mean and the SEM for the volume fraction (Vv) of connective tissue present in the EDL muscles of the experimental and control groups.

3.2.9 Muscle fibre shape

Fibre shape was assessed by calculating the "form factor". This is based on the muscle fibre cross-sectional area and perimeter. The area was calculated from the boundary and the perimeter was a total of the distances between the mid point of the boundary vectors. Form factor is a measure of "roundness" and equals unity for a perfect circle and less than unity for other shapes. Due to alterations in individual muscle fibre shape during denervation and reinnervation of muscle, form factor can potentially provide information on the extent of recovery. In particular, as atrophy proceeds the denervated muscle fibres lose their polygonal shape (as seen in transverse sections) and become more rounded in shape. Hence, the form factor of these fibres should increase.

As with assessment of muscle fibre diameter, sections stained for ATPase at pH 4.35 and 10.20 were used to measure the shape of type I and type II muscle fibres. A total of 200 to 300 fibres were assessed for each muscle section and, as with measurement of muscle fibre size, a high resolution semiautomatic image analysis system was used. Table 3.8 shows the mean and the standard deviation values for the form factor of type I and type II fibres of the experimental and contralateral EDL muscles of the experimental groups, the operated and contralateral EDL muscles of the operated control groups, as well as the left and the right EDL muscles of the unoperated control.

For most repair groups the mean form factor of type I fibres in experimental muscles were similar or no different to the form factor of type I fibres of corresponding contralateral muscles. However, it was only after nerve repair by a

CRG-MN tube that this difference was significant ($p < 0.05$, Wilcoxon Signed Rank test). For the nerve crush and the nerve cut control groups, the form factor of type I fibres in the operated muscles were significantly less than those values for the corresponding contralateral muscles ($p < 0.05$ for each group).

Overall there were significant differences when the form factor of type I fibres for all of the experimental and control groups (nerve cut, nerve crush and unoperated) were compared ($p < 0.01$, Kruskal-Wallis test). Individual comparison of the experimental and control groups revealed that after nerve repair the form factors of type I fibres were significantly greater than those after a nerve crush or a nerve cut ($p < 0.01$ after repair with either a CRG-GAP, a CRG-MN or a CRG-N tube; $p < 0.05$ after repair with a CRG-M tube or a FTMG, Mann-Whitney *U* test).

There were significant differences in the form factor of type I fibres between operated EDL muscles of the nerve crush and the nerve cut control groups, as well as the left and the right EDL muscles of the unoperated control ($p < 0.01$, Kruskal-Wallis test). Individual comparisons of these groups revealed that the form factor of type I fibres for both the left and the right EDL muscles of the unoperated control were significantly greater than those values for the nerve crush and cut control groups ($p < 0.01$ for each case, Mann-Whitney *U* test).

For type II muscle fibres there were significant differences when values for all of the experimental and control groups (nerve cut, nerve crush and unoperated) were compared ($p < 0.01$, Kruskal-Wallis test). For each experimental group the form factors of type II fibres was greater after nerve repair when compared to those values for the nerve crush and cut control groups. Individual comparisons of these groups revealed that this difference was statistically significant for every repair group when

compared with those values for form factor for the nerve cut ($p < 0.01$ after repair with either a CRG-GAP, a CRG-M, a CRG-MN tube or a FTMG, and $p < 0.05$ after repair with a CRG-N tube, Mann-Whitney *U* test), and the nerve crush control groups ($p < 0.05$ for each case).

There were significant differences when the form factors of type II fibres of operated EDL muscles of the nerve crush and the nerve cut control groups, and the left and the right EDL muscles of the unoperated control were compared ($p < 0.05$, Kruskal-Wallis test). Individual comparisons of these groups revealed that the form factor of type II fibres for both the left and the right EDL muscles of the unoperated control were significantly greater than those values for the nerve crush and cut control groups ($p < 0.01$ for comparisons of either the left or the right EDL muscles and the nerve cut group; $p < 0.05$ for comparisons of either the left or the right EDL muscles and the nerve crush group, Mann-Whitney *U* test).

The mean form factor of type I fibres for the one control animal housed in the Department of Tropical Veterinary Medicine was 0.78 for the left EDL muscle, and 0.77 for the corresponding right. For the type II fibres the values were 0.77 for the left EDL muscle, and 0.75 for the right EDL muscle. These figures were comparable to the mean form factor of type I and type II fibres for both the left and the right EDL muscles of the unoperated control.

In summary, there were several differences in form factor of type I and type II muscle fibres after nerve repair. There was no significant correlation between method of repair and changes in form factor of fibre types. However, the values for form factor of type I and type II muscle fibres for both the nerve crush and the nerve cut

control groups were generally lower than that of the experimental and normal or unoperated control groups.

Removal of the data for the one animal in the FTMG group which did appear to achieve full repair resulted in no change in the findings for form factor for either type I or type II fibres.

Frequency histograms were plotted of the values of form factor and minimum fibre diameter for both type I and type II fibres (these histograms are not included in this thesis). This was done to establish if there was any relationship between form factor and the diameter of muscle fibres however, no correlation was found.

Procedure	Muscle	Type I	Type II
CRG-GAP	Experimental EDL	0.82 ± 0.04	0.81 ± 0.02
	Contralateral EDL	0.85 ± 0.01	0.78 ± 0.04
CRG-N	Experimental EDL	0.84 ± 0.03	0.80 ± 0.06
	Contralateral EDL	0.84 ± 0.01	0.81 ± 0.03
CRG-M	Experimental EDL	0.84 ± 0.05	0.81 ± 0.03
	Contralateral EDL	0.84 ± 0.02	0.79 ± 0.03
CRG-MN	Experimental EDL	0.85 ± 0.03 *	0.79 ± 0.02
	Contralateral EDL	0.81 ± 0.02	0.78 ± 0.03
FTMG	Experimental EDL	0.79 ± 0.03	0.78 ± 0.03
	Contralateral EDL	0.85 ± 0.03	0.80 ± 0.01
Nerve Crush	Operated EDL	0.71 ± 0.04 *	0.72 ± 0.04
	Contralateral EDL	0.79 ± 0.01	0.76 ± 0.03
Nerve Cut	Operated EDL	0.72 ± 0.04 *	0.70 ± 0.02
	Contralateral EDL	0.78 ± 0.03	0.75 ± 0.04
Unoperated Control	Left EDL	0.81 ± 0.02	0.79 ± 0.01
	Right EDL	0.81 ± 0.03	0.80 ± 0.02

Table 3.8 - The mean and the standard deviation of the form factor of type I and type II muscle fibres for the experimental and contralateral EDL muscles of each of the experimental and control groups.

* A significant result for the experimental, operated or unoperated control group when compared to those values for the contralateral/right side

3.3 DISCUSSION

The main findings of the present chapter are (i) that as a form of nerve repair there appear to be no benefits of using the CRG tubes over doing nothing, and (ii) the advantages of the FTMG as a method of nerve repair are debatable.

The results for the CRG tubes were worse than those for the nerve cut control group. Furthermore this method of repair was not dependable in that only one of the CRG tube groups produced consistent results and the findings for this group, the CRG-GAP tube group, were poor. The FTMG proved to be more effective as a form of nerve repair than any of the CRG tubes although the results were often similar, or indeed no better to those for the nerve cut control group. However, there was one animal in the FTMG group which did appear to achieve full repair but removal of this data produced overall results for this experimental group which were often worst than the nerve cut control group. The toe spreading reflex was the first test to be conducted as part of this study and the findings confirmed some of these deductions. This reflex indicated the level of recovery of motor function of the peroneal nerve.

In the present study the first onset of the toe spreading response was at 19 days after nerve crush which was comparable to the findings of Gutmann (1942) after a similar study of crushing the peroneal nerve in rabbits. For the nerve cut control group it was at 42 days after operation. This was similar to the results of Gutmann (1942) however this was after severing (with no gap) and immediately suturing the peroneal nerve in rabbits, and in the present study it was after repair with a FTMG, where the first onset of the toe spreading reflex was at 47 days. However this result was for one animal in the FTMG group which as assessed by other assays appeared

to achieve full repair. It was 61 days after operation before any of the other animals in the FTMG group exhibited first signs of this reflex. Nevertheless the results for the CRG tube groups were poorer with the first onset of the toe spreading response varying from 68 to 98 days. Furthermore at 6 months all of the FTMG animals exhibited this reflex to some degree, whilst three of the CRG tube animals failed to exhibit the toe spreading response. Indeed at 6 months after operation there was some degree of difference in the level of return of this reflex between experimental groups but not one animal achieved full recovery of spreading. Gutmann (1942) had similar results in rabbits after severance and suture of the peroneal nerve and found that the toe spreading reflex was found to increase gradually in amount but never fully recover even up to one year after suture. However, all of these rabbits achieved spreading of all three toes but to a degree less than elicitable in normal animals (TS Index = 3). In the present study only ten out of the 25 experimental animals or 40% achieved this response, but six or 24% of these animals were from four CRG tube groups whilst four or 16% of these animals were from only one FTMG group. Furthermore none of the CRG-GAP tube animals achieved a TS Index = 3. Be that as it may four of the five nerve crush control animals did regain the full toe spreading reflex at six months after operation.

Of the three animals that failed to regain the reflex, two were repaired with a CRG-MN tube and one with a CRG-N tube. The other animals in these experimental groups exhibited a response which varied from slight (TS Index = 2) to spreading of all three toes but to a degree less than elicitable in normal animals (TS Index = 3). It is difficult to ascertain why these three animals failed to regain their toe spreading reflex when other members of their repair group did and to such a "reasonable" level.

It is unlikely to be due to any physiological or anatomical explanation as all other assays of reinnervation for these three animals (muscle wet weight, volume fraction of connective tissue, minimum fibre diameter, form factor) did not substantiate these results for the toe spreading reflex. It is more likely to be due to poor interpretation of the reflex as it is quite possible that there was a toe spreading response but it was not identified. The response and its degree was difficult to decipher at times often due to the long hair of the rabbits about their toes, and particularly when the response was poor. From this respect I found the toe spreading reflex test in rabbits somewhat of a subjective test and therefore it is likely that there would be some degree of inter-examiner variation, although the tests were conducted by the same person (myself) throughout this study. Nonetheless and as discussed, the results for the toe spreading reflex for the present study were comparable to those findings of other researchers.

At six months after operation other functional measures of muscle recovery such as stance and gait were similar for all experimental and control animals. However, palpation of the limb in the region of the denervated muscles confirmed that the results for the each of the experimental groups were similar to those for the nerve cut and not the nerve crush control group. Indeed upon removal and examination of the experimental and corresponding contralateral EDL muscle it was obvious that the muscle from the operated leg for all repair groups was wasted in appearance compared to the contralateral side. This observation was confirmed when the muscles were weighed as for all repair groups the experimental muscle weight was significantly less than the weight of the contralateral muscles. In fact this was also the case for the nerve cut but not the nerve crush control group. However the

nerve cut control did recover an average of 66.94% of the contralateral muscle weight whilst for each CRG tube group the results were much poorer. The mean level of recovery of wet muscle weight for the CRG tube groups was 44.97%. For the FTMG group the mean level of recovery of wet muscle weight was 61.53%, a figure that was not significantly different to that of the nerve cut control group. However, this value included the one animal in the FTMG group which as discussed previously, appeared to have achieved full repair. Indeed the recovery of muscle weight for this particular animal was 99.02%, whilst for the other animals in the muscle graft group the values ranged from 44.79% to 54.24%.

The results for the present study are similar to those of Gutmann (1948) who recorded a recovery of between 33.33% and 49.74% of the tibialis anterior muscle weight, a fast-twitch muscle similar to the EDL, 6 months after injury of the peroneal nerve in the rabbit. However, these values are after the peroneal nerve was cut, resected and not repaired and are similar to those after repair with CRG tubes rather than the nerve cut control group in the present study. This discrepancy may be due to a number of factors such as animal age, sex and strain of species as this information was not stated by Gutmann (1948) and may vary to that used in the present study. These are important elements, particularly the age of the animal, as the reduced capacity for nerve repair with increasing age has been well documented in animals (Black and Lasek, 1979, Pestronk *et al.* 1980, Jacob and Robbins, 1990a,b, Grieve *et al.* 1991, Tanaka and Webster, 1992, Vaughan, 1992, Verdú *et al.* 1995, Belin *et al.* 1996, Jacob and Croes, 1998). Furthermore once reinnervation starts in younger animals its rate is greater than in older animals (Campbell and Pomeranz, 1993, Choi *et al.* 1996). Nonetheless the results of Gutmann (1948) do substantiate the poor

findings after repair with CRG tubes, and confirm that as a form of nerve repair CRG tubes produce no improvement in nerve regeneration.

In the same study by Gutmann (1948) the peroneal nerve was crushed and the muscles regained initial weights approximately 5 months after crushing the nerve. The results for the present study confirm these findings as at six months the mean level of recovery of muscle wet weight was 97.58% for the nerve crush control group. This would be expected as after a nerve crush injury axonal continuity is maintained. Preservation of axonal continuity results in a more rapid and accurate restoration of reinnervation than injuries which involve transection of the nerve (Brown, 1972, Lundborg, 1988).

Comparing the different methods of repair there was not one group that was consistent in the number of pathological features present. All groups had features which indicate denervation such as internal nuclei and angulated fibres. For every method of repair there were also signs that denervation may have been prolonged for some muscle fibres and degeneration had taken place. In particular, the frequent incidence of necrotic fibres after repair with a CRG-GAP tube suggests that for these animals the period of denervation was longer than other repair groups. There was also signs of reinnervation for all groups such as split fibres. However, based on the measure of pathological features there was no one experimental group which seemed to be the more effective form of nerve repair.

The incidence of pathological features was certainly greater in experimental muscles than in any of the operated control muscles (nerve crush and nerve cut). The nerve cut control group did have the highest occurrence of angulated fibres and these are often a feature of reinnervation. Reinnervation produces compensatory

hypertrophy of innervated muscle fibres and while neighbouring fibres remain denervated, these hypertrophied fibres squeeze the denervated muscle fibres to produce "angular fibres". However many of the muscle fibres in the experimental groups, in particular the muscle fibres of the CRG tube groups, were small and rounded and atrophic in appearance. These smaller, atrophic fibres were not found in the nerve cut or the nerve crush groups. Hence although there was not one experimental group that was consistent in the number of pathological features present, when compared to the number of these features for the nerve cut control group it highlights the poor performance of CRG tubes and the FTMG as forms of nerve repair in rabbits.

For both experimental and operated control muscles there was a striking increase in the number of internal nuclei. If more than 3% of fibres in a transverse section of a human muscle contain internal nuclei, the muscle has undergone a pathological change (Dubowitz, 1985). Based on this measure there was pathological change in every experimental and operated control EDL muscle, except for two animals from the nerve crush control group. Indeed the presence of internal nuclei is a feature of denervation, particularly as atrophy proceeds and the cross-sectional area of muscle fibre decreases (Tower, 1935, Bowden and Gutmann, 1944, Pelegrino and Franzini, 1963, Stonnington and Engel, 1973, Tomanek and Lund, 1973, Snow, 1983, Anzil and Wernig, 1989, Schmalbruch *et al.* 1991, Lu *et al.* 1997). However, in the present study internal nuclei were common in atrophied fibres from all repair groups, but they were also present in other muscle fibres of different sizes and shapes in experimental and operated control groups. Some of these were hypertrophied fibres which are a feature of reinnervation (Swash and Schwartz, 1991). Furthermore

internal nuclei are frequent in the denervated muscle of small mammals such as rabbits (Schmalbruch, 1985). The incidence of internal nuclei can also increase after inserting needles into muscle (Engel, 1967). The experimental groups were also part of a parallel study (Lenihan, 2000) utilizing electrophysiological tests which included the use of a needle electrode. However care was taken to ensure that the electrode was not inserted into or near the area of the muscle biopsy. Furthermore no electrophysiological tests were done on any of the nerve cut or the nerve crush control groups and the incidence of internal nuclei in the operated muscles of these groups was not significantly different to that found in the experimental muscles.

Another striking histological appearance for the present study was the large volume of connective tissue, particularly in experimental muscles. There was a significant increase in connective tissue compared with contralateral levels for all experimental and operated control groups. As expected the size of this increase was not as great for the nerve crush control group. For experimental and nerve cut control groups the rise in the level of connective tissue was substantial. Moreover the results for the CRG tubes were worse than those for the nerve cut control group. The volume fraction of connective tissue was greater in experimental muscles of the CRG tube groups than that found in the operated muscles of the nerve cut control group. In fact three of the CRG tube groups (again including the CRG-GAP group) had a significantly greater volume of connective tissue than the nerve cut control group. After repair with a FTMG the connective tissue content was similar to those levels for the nerve cut control group.

The level of connective tissue in muscles of the experimental and the nerve cut control groups was probably related to the length of denervation and/or the slow

rate of reinnervation. For this amount of connective tissue to accumulate it is apparent that for each group the period of denervation was long. Increases in connective tissue after long-term denervation has been well documented in the literature (Tower, 1935, Bowden and Gutmann, 1944, Sunderland and Ray, 1950, Pellegrino and Franzini, 1963, Hogenhuis and Engel, 1965, Tomanek and Lund, 1973, Savolainen *et al.* 1988, Al-Amood *et al.* 1991, Lu *et al.* 1997). Furthermore, Savolainen *et al.* (1988) investigated the levels of enzymes involved in the biosynthesis of collagen and after denervation concluded that there was an increase in their activity which resulted in an increase in collagen synthesis with fibrotic changes in muscle. Virtanaen *et al.* (1992) noted that innervation had the opposite effect in suppressing the synthesis of collagen in muscle.

In the present study the fibrotic changes in some cases included an increase in connective tissue in both the perimysium and the endomysium regions of the muscle. After prolonged denervation these fields of collagen fibres around both muscle fibres and fascicles can increase and as Lu *et al.* (1997) suggests, may act as a physical barrier to nerve ingrowth when and if reinnervation does occur. With the extent of connective tissue proliferation observed and particularly in some of the experimental muscles, this suggestion may be feasible. Hence if the growth of regenerating axons is hindered this could contribute to a slow rate of reinnervation which could prolong denervation, and thereby promote collagen synthesis resulting in increased depositions of interstitial connective tissue. In fact for all repair groups there was evidence of fatty tissue in some of the experimental muscles although this was not quantified separately (it was measured as connective tissue). It does suggest a period of prolonged denervation in these areas of the experimental muscles where after the

fibre degenerates, the ultrastructural changes worsen to a point where eventually all trace of the original muscle fibre structure is lost. The muscle fibres are replaced to a greater or lesser degree by connective and fatty tissue. The appearance of fat has been well documented in the literature as a denervation event by Gutmann and Young (1944) in the rabbit, Chor *et al.* (1937) in the macaque, Bowden and Gutmann (1944) in the human, Karpati and Engel (1968a,b) in the guinea pig and Hogenhuis and Engel (1965), Schmalbruch *et al.* (1991) and Lu *et al.* (1997) in the rat.

After repair or either cutting or crushing the peroneal nerve, the changes in the mean minimum diameters of type I fibres compared to those values for the corresponding contralateral muscle fibres were not consistent and the differences were only significant for the FTMG and the CRG-M tube repair groups, as well as the nerve cut control group. Even so there was a significant difference in diameter of type I fibres between the left and the right EDL muscles of the unoperated control. Certainly based on these findings for the unoperated control it would appear that there is normally a degree of variation in the size of type I muscle fibres between the left and the right EDL muscle in any one "normal" rabbit. Indeed calculation of the variability coefficient indicated that this variability in diameters of type I muscle fibres in the muscles of the unoperated control group was normal. However for each experimental and operated control muscle there was an abnormal variation in the diameter of type I muscle fibres. These findings do suggest that there has been some effect on the size of type I muscle fibres through the processes of nerve injury and/or repair.

After repair with a CRG-GAP tube the mean minimum diameter of type I fibres was significantly less than other repair groups, which is in accordance with

other findings for the present study. However after repair with a FTMG the mean minimum diameter of type I fibres was significantly less than two other CRG tube groups, whilst for the nerve crush control group the diameters were only significantly greater than those for the CRG-GAP tube groups. Furthermore the nerve cut control group had the largest value for the mean minimum diameter of type I fibres for all experimental and operated control groups. These results are a contradiction to other findings for the current study and imply variability in the reaction of the type I muscle type in the rabbit EDL muscle during the processes of denervation and subsequent reinnervation. Nevertheless, after repair or either cutting or crushing the peroneal nerve, the type II fibres underwent a "preferential atrophy" which implies that type I fibres are also affected by atrophy but to a lesser degree than type II fibres.

For each experimental and operated control group there was a consistent decrease in the mean minimum diameter of type II fibres. After all types of nerve repair there was a significant decrease in the mean minimum diameter of type II fibres when compared to any of the control groups. Indeed the findings for the CRG tubes were worse than those for the FTMG group, with the CRG-GAP tube group having the poorest results. As was found with other variables in the present study, there was one animal in the FTMG which appeared to achieve full repair as measured by type II fibre diameter. For this particular animal the mean minimum diameters of type II fibres in the experimental muscle was 44.44 μm whilst the range of values for the other animals in the muscle graft were from 19.98 μm to 35.04 μm. This may explain why the minimum diameter of type II muscle fibres for the FTMG group were significantly greater when compared to values for any of the other methods,

although even when this animal is not included, the mean minimum diameter of type II fibres for the FTMG group are still the largest of the repair groups.

As predicted, the nerve crush control group showed the best recovery of all experimental and operated control groups as measured by type II fibre diameter. This would be expected as after a nerve crush injury axonal continuity is maintained which results in a more rapid and accurate restoration of reinnervation (Lundborg, 1988). Indeed the reactions of type II rather than type I fibres may provide a more accurate indication of the processes of denervation and reinnervation in the rabbit. The findings of the present study suggest that there may be more variability in the reaction of the type I muscle type in the rabbit EDL muscle, whilst it was apparant that upon denervation all type II fibres undergo denervation. Hence, based on this the results for the minimum diameters of type II fibres confirm that as a form of nerve repair there are no benefits of using the CRG tubes or the FTMG over no repair.

Why the variation in denervation behaviour of the different fibre types? Certainly the preferential atrophy of type II fibres seen in this study is in agreement with the results of Karpati and Engel (1968a,b) in the guinea pig, Jaweed *et al.* (1975), Niederle and Mayr (1978) and Lu *et al.* (1997) in the rat, and Vita *et al.* (1983) in the rabbit. However, data in the literature on the denervation conduct of different muscle fibre types does differ widely. Romanul and Hogan (1965) noted that all fibre types atrophied equally in the rat gastrocnemius and soleus muscles, whilst there are other authors who have found type I fibres to atrophy more than type II (Kennedy, 1987). It seems likely then that some of this disagreement is due to the different reactions of the two muscle fibre types depending on various factors. One factor could be the time after denervation the muscle is evaluated. Niederle and Mayr

(1978) noted that type I fibres of the rat EDL were affected by denervation atrophy only at rather late stages. Moreover, Vita *et al.* (1983) who studied the effect of nerve crush in the same muscle and species of rabbit as used in this study, noted at 50 days post operation a decrease in the mean diameter of type II fibres but an increase in the mean diameters of type I fibres. Other studies suggest that the response of type I fibres to denervation is not the same in different skeletal muscles. Type I fibre atrophy has been reported to be less pronounced in fast muscles than in slow muscles (Tomanek and Lund, 1973, Jaweed *et al.* 1975, Vita *et al.* 1983), which is applicable to the findings in the current study as the EDL muscle in the rabbit is a predominantly fast muscle. The more or less "resistance" of type I fibres to denervation atrophy may indicate an intrinsic difference in the motoneurones. This is supported by the observation that during development type I fibres, in contrast to type II fibres, are able to achieve a high level of differentiation following denervation (Engel and Karpati, 1968). The course and extent of denervation atrophy may also be explained by the influence of passive stretch.

The rat hemidiaphragm undergoes a transient hypertrophy after unilateral transection of the phrenic nerve (Sola and Martin, 1953, Gutmann *et al.* 1966, Hopkins *et al.* 1983). Similar changes have been observed in the mouse and rabbit (Stewart and Martin, 1956). The basis for this hypertrophy is the combination of the passive stretch exerted by the still innervated contralateral hemidiaphragm, and the exercise produced by the respiratory cycle (Sola and Martin, 1953). This modifies the expected changes in proteosynthesis and proteolysis following denervation resulting in muscle growth with increased synthesis of nucleic acids (Manchester and Harris, 1968, Zak *et al.* 1969), protein (Turner and Manchester, 1973), and polyamines

(Hopkins and Manchester, 1981). Hopkins *et al.* (1983) characterised more precisely the hypertrophy of the denervated rat hemidiaphragm through systematic evaluation of the main fibre types. The diameters of the white (type II) fibres decreased from the third day after denervation and by ten days after operation, were about 13% smaller than in the normal contralateral muscle fibres. Red (type I) fibres however, increased in size by the second day after denervation. This increase was most marked for the red fibres which after a week were about 35% larger than the contralateral fibres. Their findings indicated some influence of passive stretch on the rate of denervation atrophy of type I fibres. Indeed this process could be a possible explanation for the variability in the size of type I muscle fibres in the rabbit EDL muscle. Furthermore Goldspink (1978) demonstrated that passive stretch of the denervated EDL muscle of the rat either by the innervated antagonists, or by immobilization in extension, promoted protein synthesis and hence growth of the muscle even in the absence of an intact nerve supply.

Another assay of the process of denervation/reinnervation in muscle was the measurement of form factor. In transverse section muscle fibres are generally polygonal in shape. This characteristic polygonal shape is the result of the surrounding connective tissue, namely the perimysium, enforcing tight apposition of the muscle fibres as they grow. However, when the fibres atrophy upon denervation, this increases the free space within the muscle. With the increase in free space this reduces the compressive force the fibres put on one another and as a result allows the muscle fibres to assume a more rounded or circular shape. Hence, the more rounded a muscle fibre is in cross section the nearer the value for form factor is to unity. By this measure repair with a CRG-MN tube produced the poorest results for form factor

of type I fibres. However, when compared with other experimental values the form factor of type I fibres after repair with a CRG-MN were statistically insignificant. Indeed, the form factor results for both type I and type II fibres after any type of nerve repair were not consistent. The problem with the measurement of "form factor" is that it does not distinguish between large, round (i.e. innervated and hypertrophied) and small, round (i.e. denervated and atrophied) muscle fibres.

In sum the assays of muscle denervation/reinnervation as assessed in this project, promote concern as to the use of either CRG tubes or FTMGs as forms of nerve repair. There is no doubt that the period of denervation of the EDL muscle was prolonged after each of the types of repair. It has been well established that reinnervation is most effective if it occurs shortly after nerve injury and poor functional recovery is observed when it is delayed (Gutmann and Young, 1944, Gutmann, 1948). However why was there a period of prolonged denervation, and why was this so for every repair group in the present study? One factor could be that that there were two suture lines associated with every form of repair. This means that there is an increased probability that the regenerative axons do not locate appropriate endoneurial tubes and form effective and immediate reinnervation. Another factor could be that the material which forms the repair may impede the passage of the regenerating axons and contribute to a prolonged period of denervation and a greater degree of muscle atrophy. Associated with the atrophy of muscle fibres is the formation of spaces between the muscle fibres. It is thought that the development of these spaces stimulates the activity of fibroblasts and thereby increases the amount of connective tissue (Bowden and Gutmann, 1944). Indeed it has been observed that the accumulation of connective tissue has been preceded by a significant increase in

fibroblasts (Sunderland and Ray, 1950). After prolonged denervation fields of collagen fibres around both muscle fibres and fascicles can develop and increase and as Lu *et al.* (1997) suggests, may act as a physical barrier to nerve ingrowth when and if reinnervation does occur, thereby extending the denervation process and its effects. However as discussed in chapter 1, the basis for the poor recovery of muscle after prolonged denervation is poorly understood and the proliferation of connective tissue is one of many possible contributing factors. Nevertheless with the extent of connective tissue proliferation observed in the experimental muscles the suggestion that the material which forms the repair may impede the passage of the regenerating axons may be feasible. If this was the case then the effect was fairly uniform for the various forms of graft material used in the present study as the results were all poor, although the worst were after repair with a CRG-GAP tube. This would be expected with this form of repair as although the presence of the tube would stop migrating neurites being lost to tissues other than the target organ, with a gap between the proximal and distal nerve ends there was an increased probability of mismatch of regenerating axons and appropriate endoneurial tubes. However of the three remaining CRG tube groups the poor results for the CRG-MN tube were surprising. This form of repair theoretically should have provided two essential requirements for successful nerve regeneration which are structure by way of the CRG tube, and support by way of the nerve-muscle sandwich graft. In previous studies the use of an interposed nerve segment has been shown to enhance the effectiveness of FTMGs (Calder and Green, 1995, Whitworth *et al.* 1995). Indeed the whole concept of the FTMG is to provide a series of parallel basal lamina tubes into which regenerating axons and Schwann cells grew. It may be that by the time the dead muscle cells have

disintegrated and phagocytized in the FTMG and thereby provided a clear passage for the regenerating axons, the denervation of and reduction in the restorative capacity of muscle was well established.

One possible explanation for the uniform and deficient performance of the CRG tubes as forms of nerve repair may be a problem with the dimensional stability of the conduit. Indeed a primary criticism of biodegradable tubes has been structural instability (Doolabh *et al.* 1996). With instability this raises the possibility of a 'break' or a distortion in the tube which may result in the tube compressing, irritating or misdirecting the area of nerve regeneration, as well as allowing the ingrowth of any scar tissue. In another study biodegradable conduits made from a polyester compound, after a period of time became narrowed and distorted and resulted in deterring regeneration (Henry *et al.* 1985). If there was any "break" or disruption of the CRG tubes then this would not have been observed in the present study as assessment was at six months and by this time the tubes had degraded. Unfortunately this is one of the shortcomings of the present study. Evaluation would have been best accomplished by regular, short spaced intervals such as one, two, three and six months after operation, although this would have required more animals and hence an increase in experimental costs. This would have also assisted in providing information on the degradation rate of the CRG tubes. Studies have been conducted to ensure that the CRG tubes dissolve completely (Drake and Graham, 1976, Burnie *et al.* 1981, Burnie and Gilchrist, 1983). However none have been conducted in the same animal model as the present study.

Another criticism of biodegradable tubes is the degree to which fibrosis from the degeneration of the conduits affects nerve regeneration and thereby function

(Doolabh *et al.* 1996). Regrettably this does not appear to have been measured and if so, it has not been cited in the literature. However for the present study, the amount of fibrosis after repair with CRG tubes was striking. This appearance was highlighted when it was compared to the reaction after operation for the nerve crush and the nerve cut control groups as no such reaction was present. The CRG tubes are made from an inert material and so they should not provoke any inflammatory reaction. Studies have been conducted to test for the cytotoxicity of CRG tubes but the reactions have been reported to be minimal (Burnie *et al.* 1981, Burnie, 1982, Doherty, 1982, Burnie *et al.* 1983, Duff *et al.* 1984). Furthermore the amount of fibrosis after repair with the FTMG was equivalent (by observation) to that of each of the CRG tubes. This suggests that the length of time the repair site was exposed may have contributed to the reactions seen with the experimental groups. Indeed this time was significantly greater than that for either the nerve crush or cut control groups where the procedures were simpler. Nonetheless in view of the extent of the fibrotic reaction observed in the present study after repair with CRG tubes, the possibility of an inflammatory reaction to the tubes must still be considered and further work is required to elucidate this finding.

Other studies on the use of CRG tubes in the repair of peripheral nerve injuries have included experiments in sheep. Gilchrist *et al.* (1998) used CRG tubes of the same composition as used in the present study to repair facial nerves in the sheep. In this study it was reported that the CRG tube had dissolved completely without any adverse reaction and that electrophysiological assessment had shown results similar to that seen after repair with a FTMG. Indeed morphological assessment (axon diameter, fibre diameter and myelin sheath thickness) of the

repaired nerve showed a decrease in all measured variables but none of these changes were significantly different from normal. However in a study of CRG tubes in rabbits (the parallel study to the present), the same variables of nerve morphometry were measured and all were significantly different from normal (Lenihan, 2000). It is difficult to fully explain these discrepancies in results as essentially the same method of nerve repair has been used in both studies. An important consideration is that the nerve injuries were different as there was no gap at the site of repair for the sheep study. Furthermore the investigation of CRG tubes in sheep was only a small sample, four animals in total, so cautious interpretation of these results is warranted. It must also be mentioned that it is always difficult comparing results between different species as regeneration ability can differ.

The parallel study to the present on CRG tubes and the FTMG used not only variables of nerve morphometry but also electrophysiological tests in the evaluation of these forms of nerve repair (Lenihan, 2000). Unfortunately the only controls for the study were normal animals and statistical analysis of the data was such that the four CRG groups were not assessed together but in subgroups (CRG-GAP versus FTMG versus CRG-N; CRG-MN versus FTMG versus CRG-N). Nevertheless as discussed none of the repair groups achieved maturation of nerve fibres or myelination to the same levels as found in normal animals. Indeed the average percentage recovery of fibre diameter over control values was 44.84% and for axon diameter, 36.86%. For myelin sheath thickness the value was 56.85%. These poor results were supported by the electrophysiological findings. This outcome is in agreement with those of the present study although the results of Lenihan (2000) did differ slightly. Lenihan (2000) established that repair with either a CRG-M or a

CRG-MN tube was "at least as good" as a FTMG. I do not think this discrepancy is of great concern as the results for both studies show a clear trend in that all sets of data for both investigations (electrophysiological tests, muscle and nerve morphometry) were far worst for either CRG tubes or FTMGs when compared to normal values. It is unfortunate that Lenihan (2000) did not include further control groups such as the nerve crush and the nerve cut as used in the present study, and hence provide some measure of any differences from normal.

Previous investigations have verified nerve regeneration after repair with a FTMG (Glasby *et al.* 1986a,b,c,d, Davies *et al.* 1987, Gattuso *et al.* 1988a,b, Glasby *et al.* 1988a,b, Gschmeissner *et al.* 1988, 1990, Norris *et al.* 1988, Gattuso *et al.* 1989, Glasby *et al.* 1989, Findlater *et al.* 1990, Glasby *et al.* 1990, Huang *et al.* 1990, Mulroy *et al.* 1990, Grieve *et al.* 1991, Glasby *et al.* 1992, Myles and Glasby, 1992, Hems and Glasby, 1992, Glasby *et al.* 1993, Mountain *et al.* 1993, Hems *et al.* 1994, Drew *et al.* 1995, Glasby *et al.* 1995, Lawson and Glasby, 1995, Glasby *et al.* 1997, Carter *et al.* 1998, Lenihan *et al.* 1998a,b). However many of these studies have tried to establish that the FTMG was at least comparable to conventional methods of repair such as direct suture or autogenous nerve grafts. Hence although normal controls were often included, the results after repair with a FTMG were judged by how well they compared to the findings of established nerve repair techniques. There has been no other investigation of the FTMG other than the present which has attempted to quantify how bad or how good the results were from normal. The only other study of the FTMG to include comparisons with controls similar to that used in the present investigation was by Carter *et al.* (1998). In the rat they compared repair with either a FTMG, a direct nerve to nerve suture or an ideal-calibre (the same

nerve) autogenous nerve graft with nerve crush. As expected the nerve crush injury showed a significantly greater level of recovery of wet muscle weight than any of the methods of repair, however the difference in connective tissue between groups was insignificant (Carter *et al.* 1998). These results are surprising as the increase in connective tissue was one of the most striking and greatest changes in the morphology of the muscle for the present study. Furthermore for this investigation the mean recovery of the EDL muscle wet weight was 61.53% at 180 days after repair with a FTMG, whilst for Carter *et al.* (1998) it was 76% at 200 days. One possible explanation for these discrepancies in the results may be that the animal model used by Carter *et al.* (1998) was the rat and this particular species has a reputation for a prodigious regenerative ability (Mackinnon *et al.* 1985b).

In reference to the contralateral muscles of the experimental and operated control groups, and the unoperated control muscles, statistical analysis of the variables of muscle morphometry was undertaken to determine if there were any significant changes as a result of the procedures. Compensatory hypertrophy has been observed in contralateral muscles of denervation studies (Talesara *et al.* 1981, Freeman and Luff, 1982). In view of these findings it has been suggested that normal animals may serve as a better control than contralateral muscles (Talesara *et al.* 1981) and as a result, an effort was made to include an age, sex and weight matched group of rabbits in the present study. However the findings from comparison of results for contralateral muscles of the experimental and operated control groups, and the unoperated control muscles, indicated that none of the contralateral muscles for either the experimental or operated control groups had undergone compensatory hypertrophy. Pathological features such as internal nuclei and split fibres were found

in contralateral muscles as well as unoperated control muscles but the numbers were small and would appear to be a normal phenomenon. Freeman and Luff (1982) have reported that if there was any compensatory hypertrophy then this would most likely result in the addition of contractile material (by an increase in the number of sarcomeres) and not an increase in fibre diameter. Hence this would increase muscle wet weight. In the present study the muscle wet weights for the contralateral muscles of the nerve crush control group were significantly greater than those values for most contralateral muscles of the experimental groups, however the muscle wet weights for both groups were not significantly different from normal. Furthermore in contralateral muscles there was no consistency between method of repair or injury (nerve crush or cut) and changes in the volume fraction of connective tissue or muscle fibre diameter.

3.4 CONCLUSION

The experiments described in this chapter have suggested that are there are no benefits of using the CRG tubes as a form of nerve repair and raises doubts as to the use of FTMGs in this capacity. The results of tests of nerve regeneration (Lenihan, 2000) did correlate with the findings of this investigation but further work is required to elucidate the changes in muscle morphometry observed. In particular the establishment of functional neuromuscular junctions following repair was not examined by the present study. Additionally glycogen depletion studies would assist in determining whether or not there has been functional reinnervation of muscle by providing information on the motor units reinnervated.

CHAPTER 4

A Morphological Assessment of Fibre Type Proportions and Distribution in the Rabbit after Nerve Injury and Repair

4.1 INTRODUCTION

The histochemical delineation of muscle fibre types in reinnervated muscle after complete and partial denervation often reveals "type grouping" of muscle fibres. In a normal muscle, the muscle fibres of a single motor unit are scattered among the fibres of many different motor units within a defined area or territory (Edstrom & Kugelberg, 1968). This intermingling often results in a "mosaic pattern" of histochemical types seen in normal muscle (Kugelberg *et al.* 1970, Bodine-Fowler *et al.* 1993). After reinnervation the mosaic pattern is replaced by groups of fibres of the same histochemical type (Karpati and Engel, 1968c, Robbins *et al.* 1969, Kugelberg *et al.* 1970, Brooke *et al.* 1971, Jennekens *et al.* 1971, Jaweed *et al.* 1975, Warszawski *et al.* 1975, Lewis *et al.* 1982, Müntener and Srihari, 1984, Nemeth and Turk, 1984, Dum *et al.* 1985a,b, Foehring *et al.* 1986a,b, Gillespie *et al.* 1987, Gordon *et al.* 1988, Tötösy de Zepetnek *et al.* 1992a, Fu and Gordon, 1995a,b).

Type grouping is thought to be the result of reinnervation of adjacent muscle fibres by collateral sprouting of subterminal axons (Karpati and Engel, 1968c). Hence, muscle fibres of a single motor unit are grouped together instead of being intermingled with fibres of other motor units. Fibre type grouping is striking in reinnervated rat muscles such as tibialis anterior (Kugelberg *et al.* 1970, Parry and Wilkinson, 1990, Tötösy de Zepetnek *et al.* 1992a,b, Fu and Gordon, 1995a,b), EDL (Albani *et al.* 1988), lateral gastrocnemius and soleus, (Gillespie *et al.* 1987), but it is less obvious in the larger cat hindlimb muscles (Dum *et al.* 1985a,b, Foehring *et al.* 1986a,b, Gordon *et al.* 1988, Nemeth *et al.* 1993). This raises the question as to

whether fibre type grouping is a reliable diagnostic tool for muscle reinnervation in all muscles and in different species.

A study on the spatial distribution of muscle fibres demonstrated that reinnervated muscle fibres are more clumped than normal and that grouping of fibre types parallels the grouping of motor unit fibres (Rafuse and Gordon, 1996b). However, in the same study in reinnervation of a large skeletal muscle such as the cat medial gastrocnemius muscle, grouping was not visibly obvious unless the number of motor units was significantly less than normal. In a large skeletal muscle, axonal branching distributes motor unit fibres over a large portion of the muscle cross-sectional area. With more distal branching of regenerating axons this is not detected as fibre type grouping due to the large number of muscle fibres available for reinnervation within the motor unit territories. Rafuse and Gordon (1996b) did note that grouping of fibre types became increasingly more obvious in the cat medial gastrocnemius muscle as the number of regenerating axons declined. As this clumping resembled that seen in partially denervated muscle (Kugelberg *et al.* 1970, Gordon *et al.* 1991), it is likely that this reflects the increased number of axonal branches in the distal regions of the intramuscular nerve pathways (Rafuse and Gordon, 1996b). Thus, the proximal axonal branches determine the motor unit area and limit motor unit size to the number of muscle fibres that are available for reinnervation by distal axonal branches within the areas (Gordon *et al.* 1991, 1993, Rafuse and Gordon, 1996b). In smaller muscles such as reinnervated rat muscles where regenerating axons branch less in more proximal regions of the intramuscular nerve pathway, and more in distal sections closer to denervated pathways, fibre type grouping occurs more readily (Kugelberg *et al.* 1970, Gordon *et al.* 1991, Rafuse and

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Gordon, 1996b). Hence, in these cases fibre type grouping is a more "sensitive" method for detection of denervation and reinnervation (Rafuse and Gordon, 1996b).

In practice the identification of the fibre-type arrangement is often subjective and suffers from inter-examiner variation. Hence the need for a practical and an objective method for assessing the fibre type distribution. A number of methods for the detection of fibre-type grouping have been published, although most are not without limitations (Lexell, 1987). In this present chapter, four of these procedures for evaluating fibre-type arrangement have been applied and assessed.

An appraisal of the fibre type proportions was also made. The proportion of fibre types is of particular interest as the type of nerve supply to each individual muscle fibre (whether it is a fast or slow nerve) can influence the fibre type (Vrbová *et al.* 1995). Thus, as a result of the processes of denervation and reinnervation the innervation of muscle fibres can alter. Hence, after denervation and any subsequent repair knowledge of the proportion of fibre types may indicate which type of nerve has reinnervated the muscle first, and whether muscle function is likely to be altered as a consequence.

4.2 METHODS

4.2.1 Immunohistochemical procedure

In tissue sections proteins (antigens) may be accurately localized using antibodies specific to that protein. A marker that is visible via bright field or fluorescence microscopy, labels the antigen either directly or indirectly and assists in its identification. For skeletal muscle, antibodies are available to various myosin proteins (for example fast, slow). In the present study immunohistochemical methods were used to identify type I and type II fibres, and three subpopulations of type II skeletal muscle fibres, type IIA, IIB and IIX, as well as "neonatal"-type fibres.

The following is a brief description of the immunohistochemical technique used. For more details of the staining technique please refer to the appendix. The primary antibodies used were monoclonal antibodies, except for the neonatal where the antibody was polyclonal. The antbodies displayed differential reactivity with five myosin heavy chain (MHC) isoforms, as well as neonatal myosin in rabbit skeletal muscle. In brief, the primary antibiodies were BA-F8 which reacted with type I MHC; anti-skeletal myosin (fast) clone MY-32 with all type II MHC; SC-71 with type IIA MHC; Bff3 with type IIB MHC; and neonatal with neonatal MHC.

Serial cryostat sections of muscle were collected on glass slides coated with *poly*-L-lysine for adherence of the preparation to the slide. The sections were covered with the primary antibody and incubated for two days at room temperature in a humid chamber. Any unbound antibody was removed by repeated rinses in phosphate-buffered normal saline and tween 20 (PBS-Tween) solution. The sections were covered with a secondary antibody and incubated for several hours at room

temperature in a humid chamber. The secondary antibody was an anti-mouse immunoglobulin antibody conjugated with biotin (or anti-rabbit for those sections reacted with the neonatal polyclonal antibody). After repeated rinses with PBS-Tween, the sections were covered and incubated with a tertiary reagent for several hours. This was a perioxidase-linked and biotin binding protein. Further rinses with PBS-Tween were performed and then a colour reaction was completed with di-amino benzidine (DAB) to reveal the bound antibodies. Sections were then dehydrated, mounted and observed under a light microscope.

4.2.2 Methods for analysis of fibre type distribution

Figure 4.1 is a photograph of a small part of a whole cross-sectioned normal EDL muscle from a rabbit stained for ATPase at pH 4.35, and it shows how the type I fibre types form a regular pattern. Figures 4.2 and 4.3 illustrates "type grouping" of histochemically similar muscle fibres. However, as more than 90% of fibres in the EDL muscle of the rabbit are type II fibres, the randomness of the type II fibres distribution is more difficult to establish (Figure 4.1). Nonetheless, sub-typing of type II fibres on the basis of myosin isoforms can be used to evaluate whether type II grouping occurs, and this issue is considered later in this chapter.

Four methods of analysis of fibre type distribution were studied in the present chapter and one of the reasons for choosing these particular tests was that they could be easily applied using a light microscope. However, two other methods were also considered for assessment of fibre type arrangement. One was the technique as

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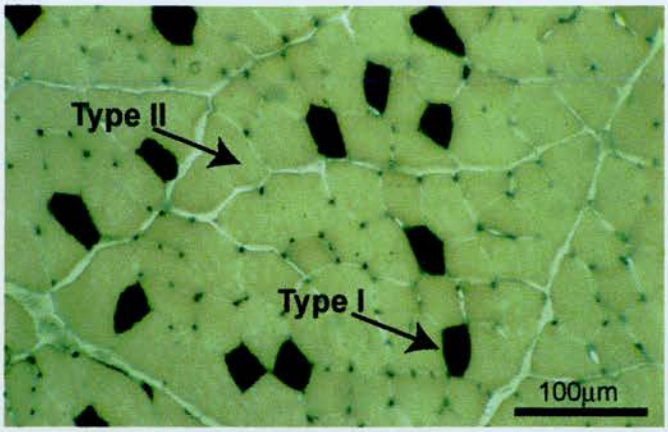

Figure 4.1 Small part of a whole cross-sectioned contralateral EDL muscle showing how the two fibre types, type I and type II form a regular pattern (Myofibrillar ATPase, pH 4.35 pre-incubation).

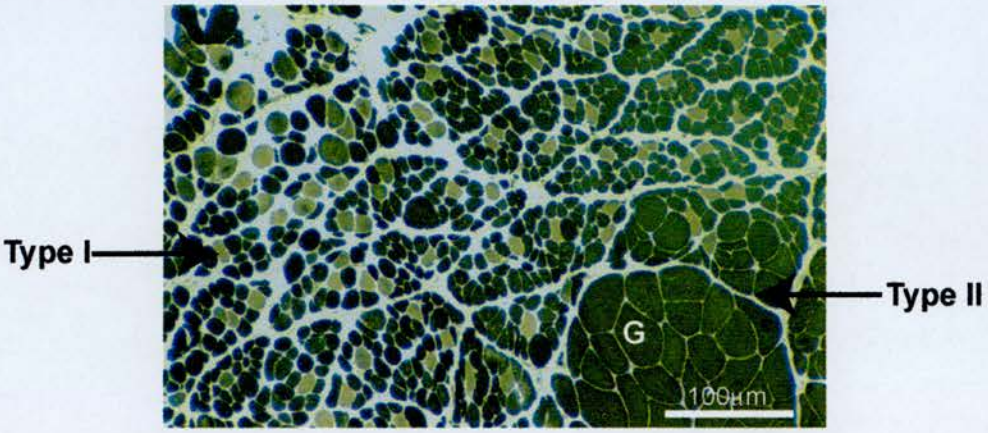

Figure 4.2 Small part of a whole cross-sectioned EDL muscle after repair with a FTMG, showing fibre type grouping (G) of type II fibres (Myofibrillar ATPase, pH 10.2 pre-incubation).

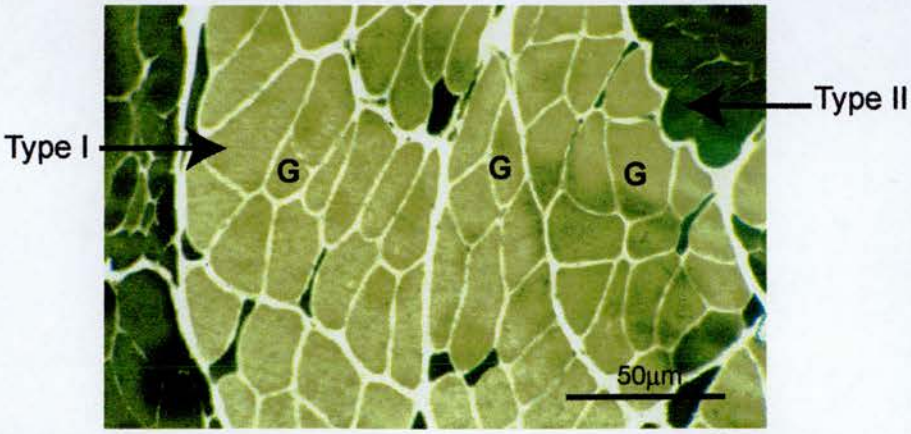

Figure 4.3 A section of EDL muscle after repair with a CRG-M, showing fibre type grouping (G) of type I fibres (Myofibrillar ATPase, pH 10.2 pre-incubation).

described by Gates and Betz (1993) but this required the use of a specific computer program if it was to be properly implemented. The other was a distance method as suggested by James (1971). This method involves acquisition of length data from a very large sample. It was not used in the present study either, because the aim here was to compare diagnostic methods that were potentially quick, accurate and effective.

In the present study the four Methods examined for the analysis of fibre type distribution of the EDL muscle in the rabbit included:

I. Run.

II. Enclosed fibre.

III. Point-sommet.

IV. Co-dispersion index.

The distribution of fibre types was also assessed qualitatively by scanning whole muscle cross-sections for each experimental, operated and unoperated control group. Descriptions of each of the four Methods examined for the analysis of fibre type arrangement follows.

4.2.3 Run method

If a line is drawn at random over a cross-section of muscle fibres stained to demonstrate muscle fibre types, a sequence of fibre types is formed. An example of a sequence of muscle fibres along a line is shown in Figure 4.4a. The run lengths (the number of fibres) of type I fibres is (1) and for type II fibres, (1, 10). If there is grouping of any fibre type there are likely to be longer runs of this fibre type than

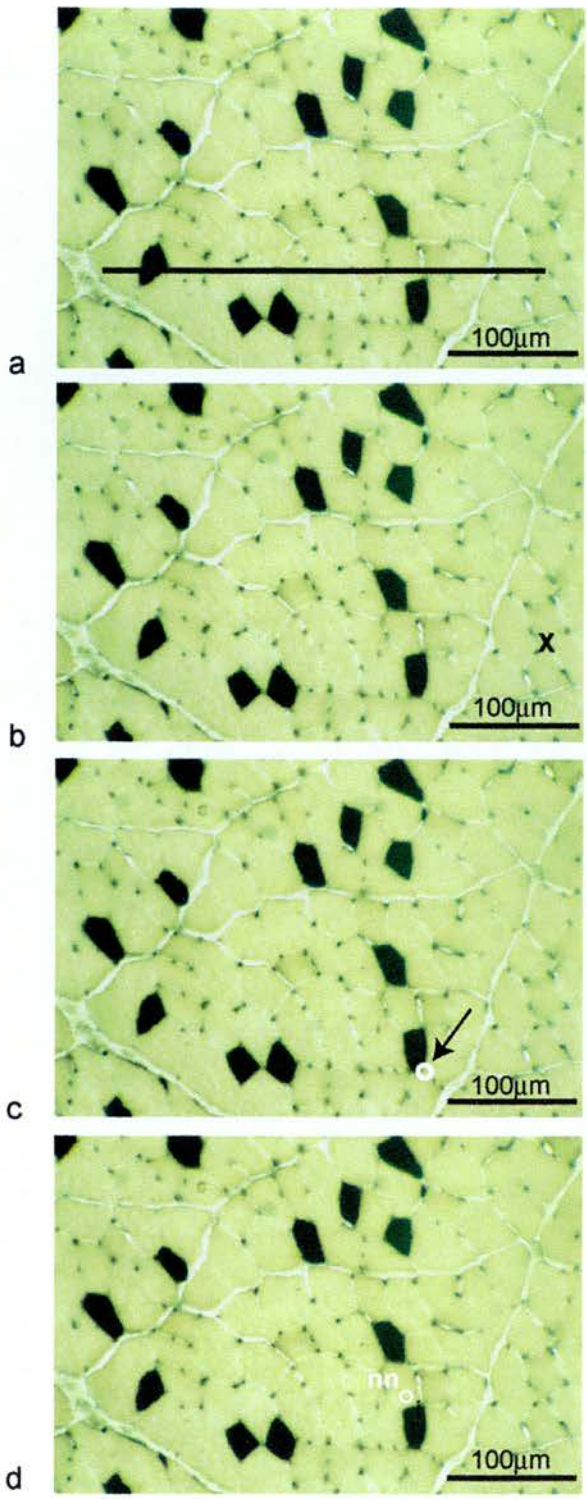

Figure 4.4 Four methods for the analysis of fibre type distribution: a. the run, b. the enclosed fibre, c. the point-sommet and d. the co-dispersion index methods.(Photographs of a normal EDL muscle, myofibrillar ATPase after pH 4.35 pre-incubation).

predicted and the sequence of type I and type II fibres along a line will not be random (James, 1972). In the present study the proportion of each fibre type in a number of fields of the cross-section was examined and the observed frequency of runs was compared with the expected numbers in a significance test.

The expected number of runs was calculated from the binomial distribution James (1972). The expected number of runs of type I fibres of length m per unit length of the transecting line was calculated by:

$$Np^{m-1}(1-p)^2$$

N = Total number of type I fibres measured

m = run length

p = Probability of occurrence of a type I fibre

A chi-square analysis was used to compare the observed and expected proportions of each fibre type:

$$\chi^2 = \Sigma \frac{(o-e)^2}{e}$$

o = Observed frequency of runs

e = Expected frequency of runs

A χ^2 value greater than 3.84 ($p < 0.05$) would suggest that there was a greater number of adjacencies (i.e. evidence of fibre type grouping) than would be expected if the fibre type was randomly distributed.

4.2.4 Enclosed fibre method

A muscle fibre is considered to be enclosed if, in cross-section, it is completely surrounded by fibres of the same histochemical type (Jennekens *et al.*

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1971). The number of enclosed type I and type II fibres is measured from cross-sections of muscle fibres stained to demonstrate muscle fibre types.

To test whether or not the observed number of type I and type II enclosed fibres were non-random, the simulation method of Lexell *et al.* (1983) was used. For this model, the expected number of type I enclosed points is calculated by:

$$MP^7$$

M = Number of internal type I + type II fibres

P = Probability of occurrence of a type I fibre

The expected number of type II enclosed points is given by:

$$M(1\text{-}P)^7$$

M = Number of internal type I + type II fibres

P = Probability of occurrence of a type I fibre

The probability of occurrence of a type I fibre was obtained by counting the proportion of type I fibres present in the region of the muscle analsyed (including enclosed and enclosing type I fibres), and dividing this figure by the total number of fibres (type I + type II) measured.

A chi-square analysis was used to compare the observed and expected proportions of each fibre type:

$$\chi^2 = \Sigma \frac{(o-e)^2}{e}$$

o = Observed frequency of runs

e = Expected frequency of runs

As in the runs method, a χ^2 value greater than 3.84 ($p < 0.05$) suggested that there was a greater number of adjacencies or evidence of fibre type grouping than would be expected if the fibre type was randomly distributed.

An example of the enclosed fibre method is shown in Figure 4.4b. In figure 4.4b, an enclosed type II fibre is identified by **X**.

4.2.5 Point-sommet method

A "point-sommet" or a "triad" is the point where three fibres meet (Rocques *et al.* 1973). From cross-sections of muscle fibres stained to demonstrate muscle fibre types the point-sommets are measured and each are classified into one of four categories according to the number of type I fibres. These categories are: I, I, I; I, I, II; I, II, II; and II, II, II. A fibre can belong to at most one point. If there is any fibre-type grouping there will be an excess of point-sommets with either I, I, I or II, II, II categories.

To determine the expected frequency of fibres in each category, a 2 X 2 contingency table was firstly set up (as adapted from Lester *et al.* 1983) and is shown in Figure 4.5. In this table the letters "A" through "D" are the observed frequencies for each of the four categories: I, I, I; I, I, II; I, II, II; and II, II, II. The column total represented by " A + C" indicates the number of point-sommets with I, I, I fibres + the number of point-sommets with I, I, II fibres. Likewise the row total represented by "A + B" equals the number of point-sommets with I, I, I fibres + the number of point-sommets with II, II, I fibres. The grand total, N, is the total number of point-sommets measured in the sample. The next step was to determine the expected

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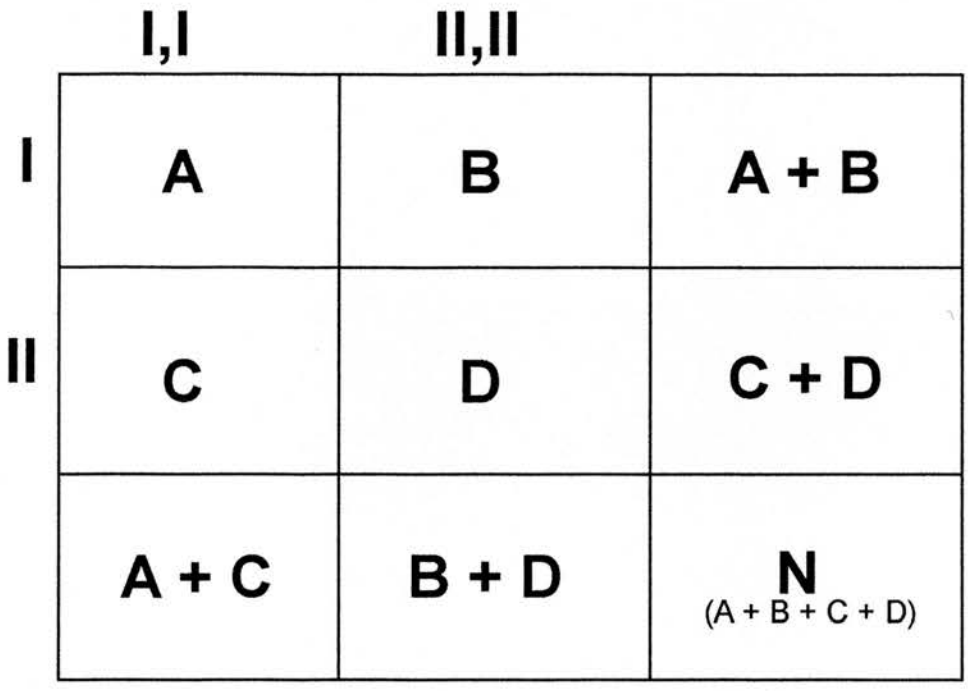

Figure 4.5 The contingency table as used for the point-sommet method (as adapted from Lester et al.1983). A = Observed frequency of fibre types I,I,I; B = II,II,I: C = I,I,II: and D = II,II,II. Row, column and grand totals are also shown (adapted from Lester *et al.* 1983).

values for each of the four cells. Hence these are the frequencies that would be expected if the fibre types were randomly distributed. The expected values for each cells were calculated as follows:

$$F_{xy} = \frac{(Rx)(Cy)}{N}$$

F_{xy} = Expected frequency of cell xy

Rx = Row total of row x

Cy = Column total of column y

N = Total number of point-sommets

Next, the chi-square was determined for each cell:

$$\chi^2 = \frac{(o-e)^2}{e}$$

o = Observed frequency of cell

e = Expected frequency of cell

The chi-square of the contingency table was the sum of the chi-squares of the individual cells. A χ^2 value greater than 3.84 ($p < 0.05$) would suggest fibre type grouping.

An example of the point-sommet method is shown in Figure 4.4c. In figure 4.4c, the point-sommet (the marked point) has one type I and two type II fibres.

4.2.6 Co-dispersion index method

The co-dispersion index is a measure of fibre type distribution introduced by Lester *et al.* (1983). In the calculation of this index each fibre in a sample is characterized by a point on its periphery (the upper left hand corner). A fibre is selected randomly and designated as the "index fibre", and the "nearest neighbour"

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of this fibre is identified. The type of both neighbours is then recorded and each are classified into one of four categories according to whether the fibre type is an index or a nearest neighbour fibre: I, I (I - index fibre, I - nearest neighbour fibre); I, II (I - index fibre, II - nearest neighbour fibre); II, II (II - index fibre, II - nearest neighbour fibre); and II, I (II - index fibre, I - nearest neighbour fibre). The criteria for the selection of index and nearest neighbour fibres as stated by Lester *et al.* (1983) and applied in the present study were as follows: (1) index fibres were chosen in random order; (2) a fibre was used either as an index or a nearest neighbour fibre, but not both; and (3) a fibre was used as a nearest neighbour more than once.

Once measured, a 2 X 2 contingency table as used by Lester *et al.* (1983) was formed to determine the expected frequency of fibres in each of the four combinations of the types of index fibres and nearest neighbours (Figure 4.6). In this table, the columns depict the two types of index fibres either I or II, and the rows show the two types of nearest neighbours, again either type I or type II fibres. The letters "A" through "D" are the observed frequencies of each of the four combinations of index/nearest neighbour. The column totals are represented by "A + C" and "B + D", and are the number of times each fibre was selected as an index fibre. The row totals are indicated by "A + B" and "C + D", and these represent the number of times each fibre type occurred as a nearest neighbour. The total number of index/nearest neighbour pairs observed in the sample is "N".

The next step in the calculation of the co-dispersion index was to determine the expected values for each of the four cells. Hence these are the

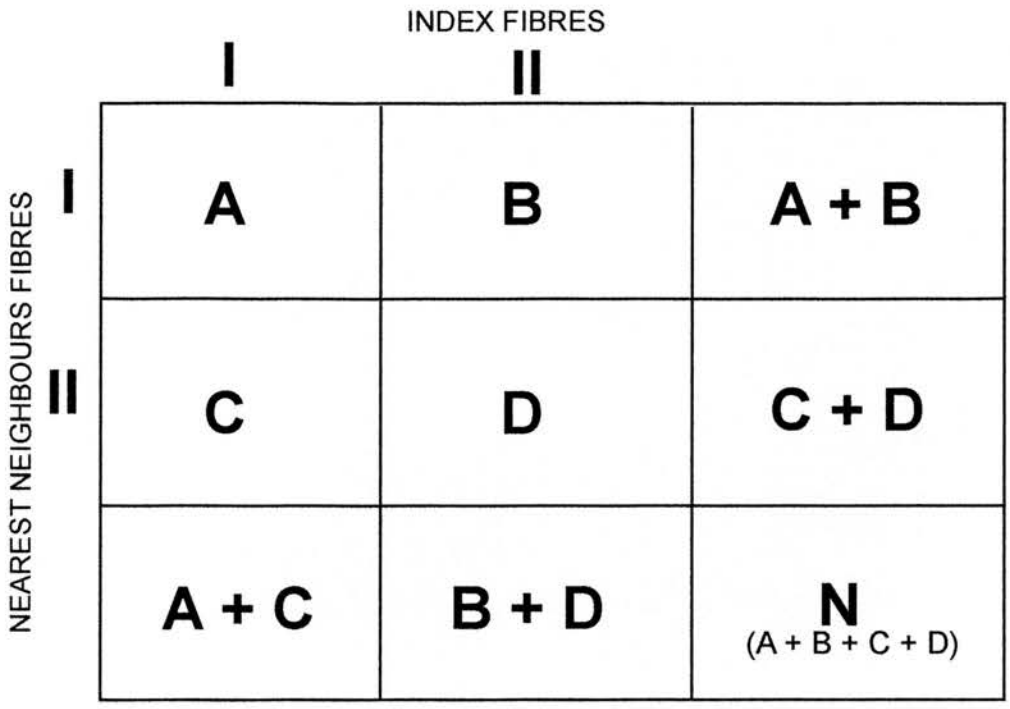

Figure 4.6 The contingency table as used for computing the co-dispersion index (CDI). "A" through "D" are the observed frequencies of each of the four combinations of index/nearest neighbour (as used by Lester *et al.* 1983).

frequencies that would be expected if the fibre types were randomly distributed. The expected values for each cells were calculated as follows:

$$F_{xy} = \frac{(Rx)(Cy)}{N}$$

F_{xy} = Expected frequency of cell xy

Rx = Row total of row x

Cy = Column total of column y

N = Total number of index/nearest neighbour pairs

Next, the chi-square was determined for each cell:

$$\chi^2 = \frac{(o-e)^2}{e}$$

o = Observed frequency of cell

e = Expected frequency of cell

The chi-square of the contingency table was the sum of the chi-squares of the individual cells.

Finally, the co-dispersion index (CDI) was calculated as a signed proportional chi-square:

$$CDI = + \sqrt{\frac{\chi^2}{N}} \text{ if } (B + C) < (E_B + E_C)$$

$$CDI = - \sqrt{\frac{\chi^2}{N}} \text{ if } (B + C) > (E_B + E_C)$$

χ^2 = Chi-square of the contingency table

B, C = Observed index/nearest neighbour frequencies of these cells

E_B, E_C = Expected index/nearest neighbour frequencies of these cells

N = Total number of index/nearest neighbour pairs

If the observed frequency of mixed pairs B + C (II, I + I, II) is greater than the expected frequency of mixed pairs, the value of the CDI will be negative. This indicates a random distribution pattern. If the observed frequency of mixed pairs B + C is less than expected, the CDI is positive and this denotes that the fibre type distribution pattern showed grouping of similar types. If the expected and observed mixed pairs are the same, the CDI is zero and the fibre type arrangement pattern is indistinguishable from a random distribution by the CDI test (Lester *et al.* 1983).

An example of the co-dispersion index method is shown in Figure 4.4d. In Figure 4.4d, a fibre has been selected randomly and is called the index fibre (i), whilst the nearest neighbour of this index fibre is also identified (nn).

4.2.7 Sampling procedures for analysis of fibre type distribution

All quantification was done on serial sections of EDL muscle stained by ATPase. This selection of fibre types I and II based on enzyme histochemistry was later confirmed by analysis of serial sections stained with antibodies directed against adult fast and slow myosins. A method was devised to measure the whole muscle cross-section but not measuring every fibre. A graticule with a square subdivided into 100 (10 x 10) small squares was placed in the eyepiece of the microscope. The central horizontal line of the graticule was nominated as the "point of measurement". That is, every fibre which crossed this line was selected for measurement. A x20 objective was chosen and once the fibres had been measured along the central horizontal line of the graticule, the next sample area was the adjoining field of vision.

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For each section, measurement of the fibre type distribution was along the longitudinal axis of the muscle. The number of sample areas for each section was between 38 and 102, depending on the size of the whole muscle cross-section. On average a total of 1330 fibres were examined for each muscle section.

4.3 RESULTS

4.3.1 Test of the methods of analysis of fibre type distribution

An initial test was conducted of each of the methods of fibre type distribution before using them in the analysis of the fibre type arrangement of **all** experimental and control groups. Two muscle sections were chosen where there was evidence of fibre type grouping by visual inspection. These were experimental EDL muscles from the CRG-GAP tube and the FTMG groups. Two further muscle sections were selected but these were "normal" muscles and therefore the distribution of fibre types would be expected to be random. These were an EDL muscle from the unoperated control group, and a contralateral muscle from the CRG-N group. It may be argued that since the primary myotubes are generally type I and all type II fibres are constant and established on a type I scaffold, the distribution of type I fibres is regular rather than random. However, although this may be the case the arrangement of type I fibres in normal muscle will still be referred to as "random" in the present study.

The sampling procedure of each of the cross-sections of muscle was as described in section 4.2.7 and the results are shown in Table 4.1. The enclosed fibre method failed to detect fibre type grouping of type I fibres in sections from both the CRG-GAP tube and the FTMG groups whilst the other tests of fibre type distribution detected a change in the type I fibre arrangement in at least one of the experimental muscles. As a result it was decided not to use the enclosed fibre method in the

evaluation of fibre type distribution for the remaining experimental, contralateral and control muscles of the present study.

Procedure	Runs	Point-sommet	Enclosed	CDI
CRG-GAP	R	R	R	NR
FTMG	NR	NR	R	NR
CRG-N Contralateral	R	R	R	R
Unoperated Control	R	R	R	R

Table 4.1 - The results for the arrangement of type I fibres after application of each of the methods of analysis of fibre type distribution.

(R = Random distribution of type I fibres, NR = Nonrandom distribution of type I fibres)

4.3.2 Fibre type proportions

In the evaluation of fibre type proportions the frequency of type I and type II fibre types was initially determined by classical enzyme histochemical tests and in addition, some analysis was performed on subgroups of type II as well as "neonatal"-type fibres, using immunohistochemical studies. The experimental and control groups were as described in Chapters 2 and 3.

4.3.3 Proportions of fibre types I and II identified with enzyme histochemistry

Serial sections stained for ATPase at pH 4.35, 4.6 and 10.2 were used to assess frequency of type I and type II muscle fibres in rabbit EDL muscles. A total of 400 to 500 fibres were examined for each muscle section. Table 4.2 and Figures 4.9 and 4.10, show the relative proportion of type I and type II fibres within all the experimental and control groups as described in Chapters 2 and 3.

Type II fibres were predominant in experimental, operated (Figure 4.8) and contralateral EDL muscles, as well as the left and the right EDL muscles of the unoperated control (Figure 4.7). On average, type II fibres made up 94.50% of the total number of fibres present in experimental EDL muscle after nerve repair and 95.19% in the corresponding contralateral muscle. After nerve crush and nerve cut the mean number of type II fibres in operated EDL muscle was 86.18%, and for the corresponding contralateral muscle, 92.90%. In general, for both experimental and control groups comparison of fibre type proportions for the experimental/operated and corresponding contralateral muscles indicated an increase in the number of type I fibres, and a corresponding decrease in type II muscle fibres. However, it was only after either nerve crush or cut that there was any significant change in proportions of fibre types when operated (or experimental or left) and contralateral (or right) muscles were compared ($p < 0.05$, Wilcoxon Signed Rank test).

Overall there were significant differences when the proportions of either type I or type II fibres for all of the experimental and control groups (nerve cut, nerve crush and unoperated) were compared ($p < 0.001$, ANOVA). Individual comparison

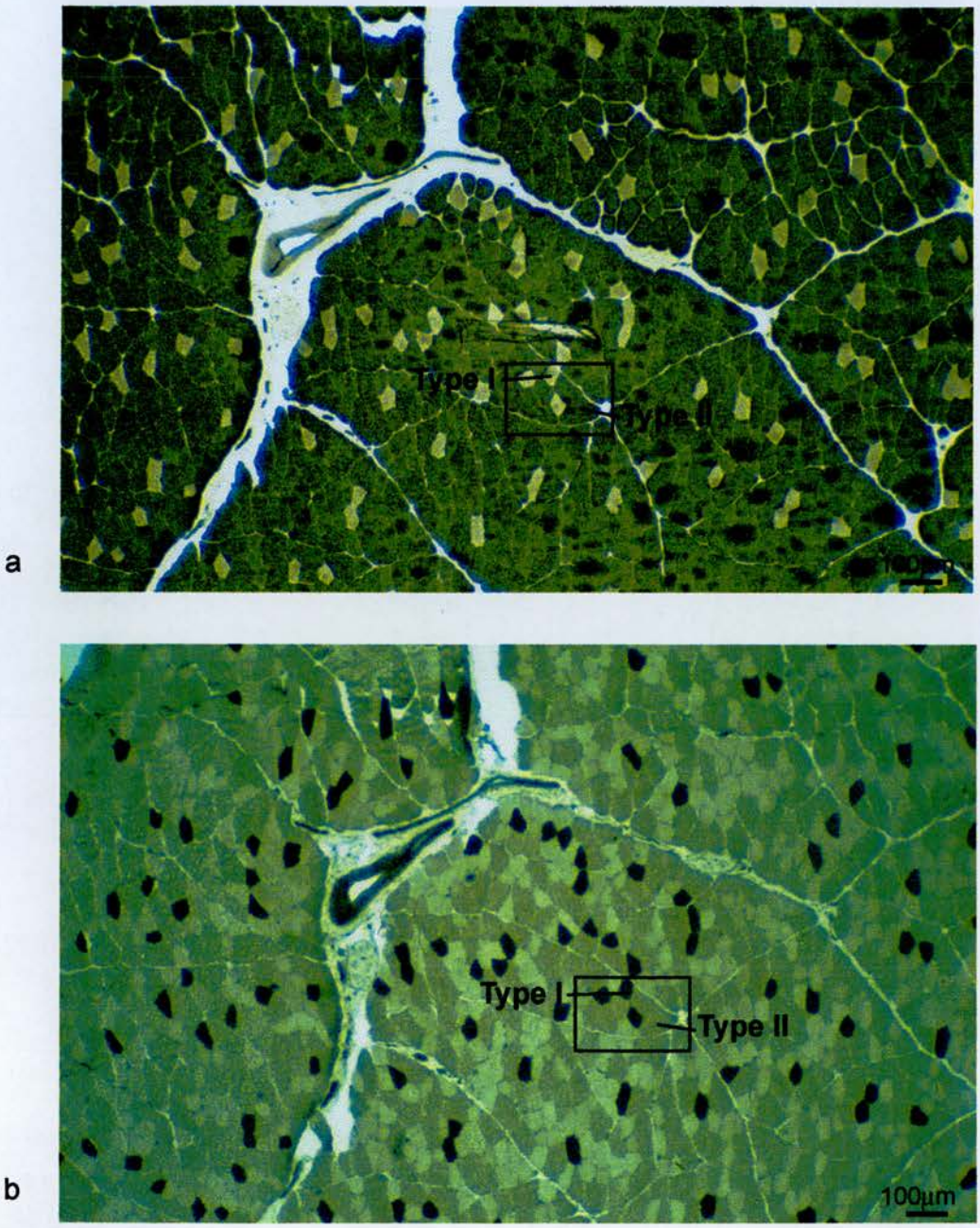

Figure 4.7 A normal EDL muscle.
(a) Myofibrillar ATPase after pH 10.2 pre-incubation.
(b) Myofibrillar ATPase after pH 4.35 pre-incubation.
Type I and type II fibres are labelled.

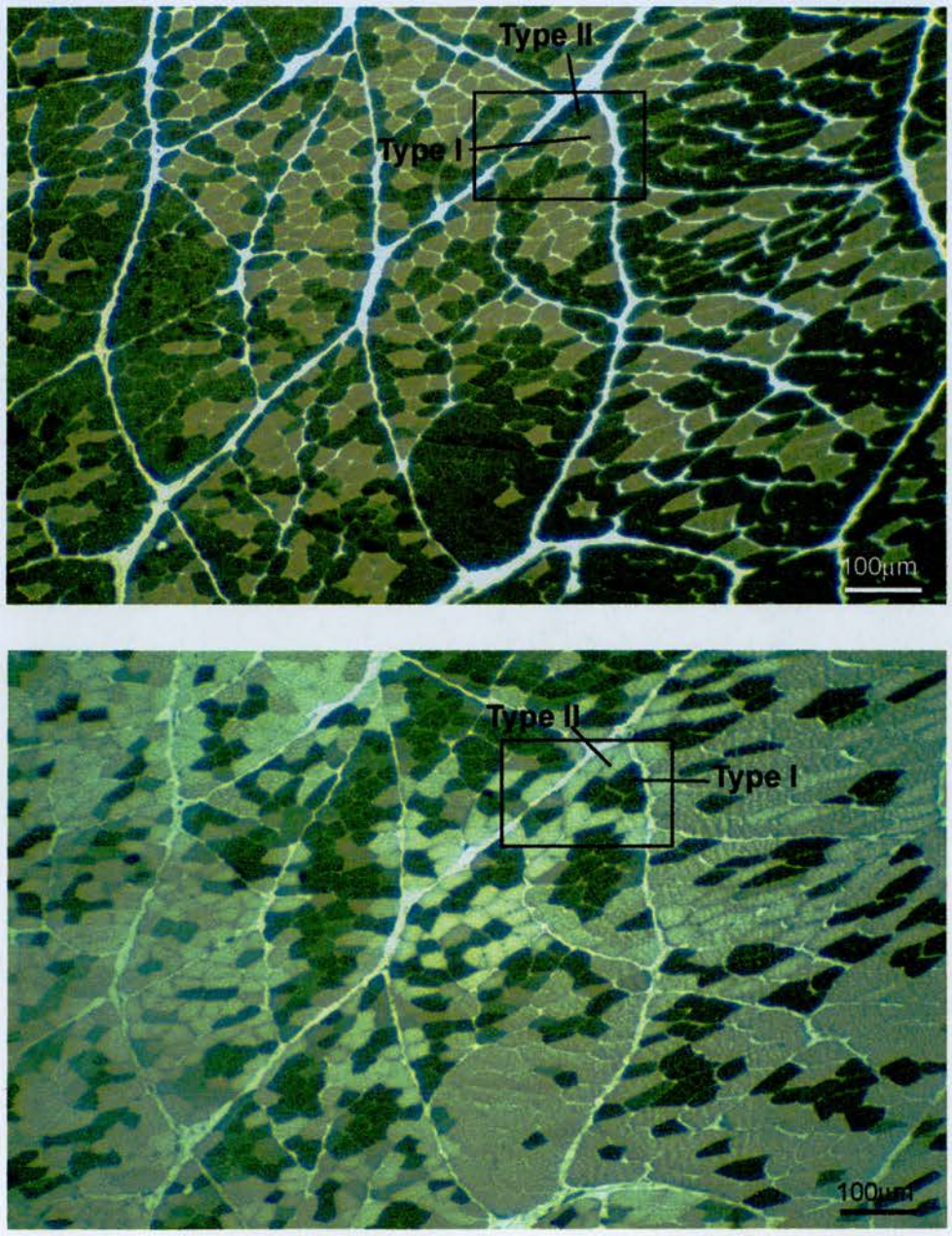

Figure 4.8 The EDL muscle after a peroneal nerve crush injury. Note the increase in proportion of type I fibres and the change in their distribution when compared with the normal EDL muscle.
(a) Myofibrillar ATPase after pH 10.2 pre-incubation.
(b) Myofibrillar ATPase after pH 4.35 pre-incubation.
Type I and type II fibres are labelled.

of the experimental and control groups revealed that there was significantly more type I fibres and significantly fewer type II fibres after nerve crush when compared with those values for each experimental group ($p < 0.05$ for each case, Mann-Whitney *U* test). After a nerve cut there was also an increase in type I fibres and a decrease in type II fibres compared to those findings for the experimental groups. This was significant when compared to those values after repair with a FTMG, and for the CRG-M and CRG-MN tube groups ($p < 0.05$ for each case, except $p < 0.01$ for the CRG-MN tube group).

There were significant differences in proportions of type I and type II fibres between operated EDL muscles of the nerve crush and the nerve cut control groups and the left and the right EDL muscles of the unoperated control ($p < 0.01$, Kruskal-Wallis test). Individual comparison of these groups revealed that for both the nerve crush and the nerve cut control groups, there were more type I fibres and fewer type II fibres when compared to the left or the right EDL muscles of the unoperated control. This difference was highly significant when compared to the findings for the right EDL muscle of the unoperated control ($p < 0.01$, Mann-Whitney *U* test), whilst for the left EDL muscle it was significant ($p < 0.05$).

Overall there were significant differences when the fibre type proportions for all of the contralateral EDL muscles of the experimental and operated control groups, as well as the left and the right EDL muscles of the unoperated control were compared ($p < 0.05$, Kruskal-Wallis test). Individual comparison of the contralateral EDL muscles from the experimental groups and those from the control groups revealed that the difference in proportion of type I and type II fibres between contralateral muscles of the nerve cut control group and those values of the CRG-M

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and CRG-MN tube groups was highly significant ($p < 0.01$ for each group, Mann-Whitney *U* test). However for the CRG-GAP tube and the FTMG groups, the difference in proportion of fibre types between contralateral muscles of the nerve cut control group and the contralateral muscles of these two repair groups was significant ($p < 0.05$ for each group). For the contralateral muscles of the nerve crush group there were significantly more type I and fewer type II fibres only when compared to the proportions of fibre types in the contralateral muscles of the CRG-M tube group ($p < 0.05$). The contralateral muscles of the CRG-N tube group had the greatest proportion of type I fibres (and smallest proportion of type II fibres) of all contralateral muscles of the experimental groups, but this difference was only significant when compared to those values for the right EDL muscle of the unoperated control ($p < 0.01$).

The proportions of type I fibres for the one control animal housed in the Department of Tropical Veterinary Medicine was 8.85% for the left EDL muscle, and 11.96% for the corresponding right. For the proportions of type II fibres these were 91.15% for the left EDL muscle, and 88.04% for the right EDL muscle. These figures are greater in number of type I fibres whilst smaller in proportions of type II fibres when compared to similar values for both the left and the right EDL muscles of the unoperated control.

In summary, type II fibres remained the predominant fibre type after all forms of nerve repair as well as for the operated control groups. There was a small increase in the proportions of type I fibres, although this was only significant for the nerve crush and the nerve cut control groups. For the nerve crush and the nerve cut control groups there was also a greater number of type I fibres in their contralateral EDL

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muscles when compared to the proportions of type I and type II fibres in the contralateral muscles of the experimental groups. However, the proportions of type I fibres in contralateral muscles of operated control groups was not significantly different from that found in normal or unoperated EDL muscles.

Removal of the data for the one animal in the FTMG group which did appear to achieve full repair (as discussed in Chapter 3) resulted in no change in the findings for the proportions of type I and type II fibres.

Procedure	**Muscle**	**Type I**	**Type II**
CRG-GAP	Experimental EDL	6.27 ± 5.29	93.73 ± 5.29
	Contralateral EDL	5.10 ± 1.08	94.90 ± 1.08
CRG-N	Experimental EDL	7.38 ± 3.43	92.62 ± 3.43
	Contralateral EDL	6.66 ± 1.38	93.34 ± 1.38
CRG-M	Experimental EDL	3.14 ± 3.15	96.86 ± 3.15
	Contralateral EDL	3.70 ± 1.61	96.30 ± 1.61
CRG-MN	Experimental EDL	5.13 ± 2.91	94.87 ± 2.91
	Contralateral EDL	3.87 ± 1.75	96.13 ± 1.75
FTMG	Experimental EDL	5.56 ± 2.28	94.44 ± 2.28
	Contralateral EDL	4.70 ± 2.18	95.30 ± 2.18
Nerve Crush	Operated EDL	16.19 ± 5.58 *	83.81 ± 5.58 *
	Contralateral EDL	6.62 ± 2.67	93.38 ± 2.67
Nerve Cut	Operated EDL	11.45 ± 2.83 *	88.55 ± 2.83 *
	Contralateral EDL	7.59 ± 1.70	92.41 ± 1.70
Unoperated Control	Left EDL	5.14 ± 3.23	94.86 ± 3.23
	Right EDL	4.35 ± 0.70	95.65 ±0.70

Table 4.2 - The mean and the standard deviation for the relative proportion (%) of type I and type II fibres for the experimental and contralateral EDL muscles of each of the experimental and control groups.

* A significant result for the experimental, operated or unoperated control group when compared to those values for the contralateral/right side

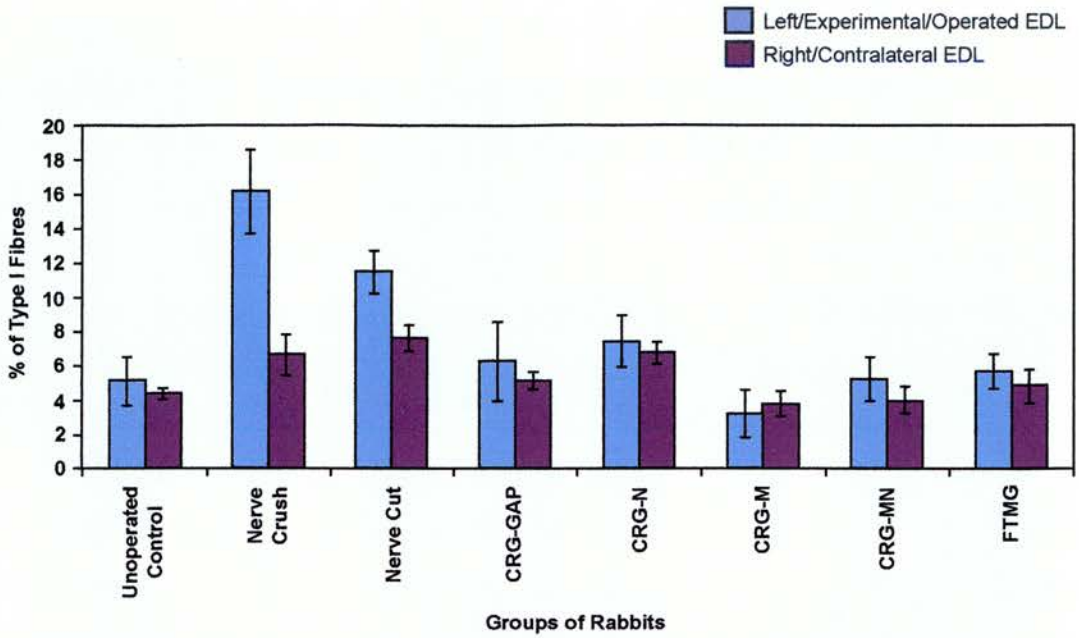

Figure 4.9 The mean and the SEM of the relative proportions of type I fibres in the EDL muscles of the experimental and control groups.

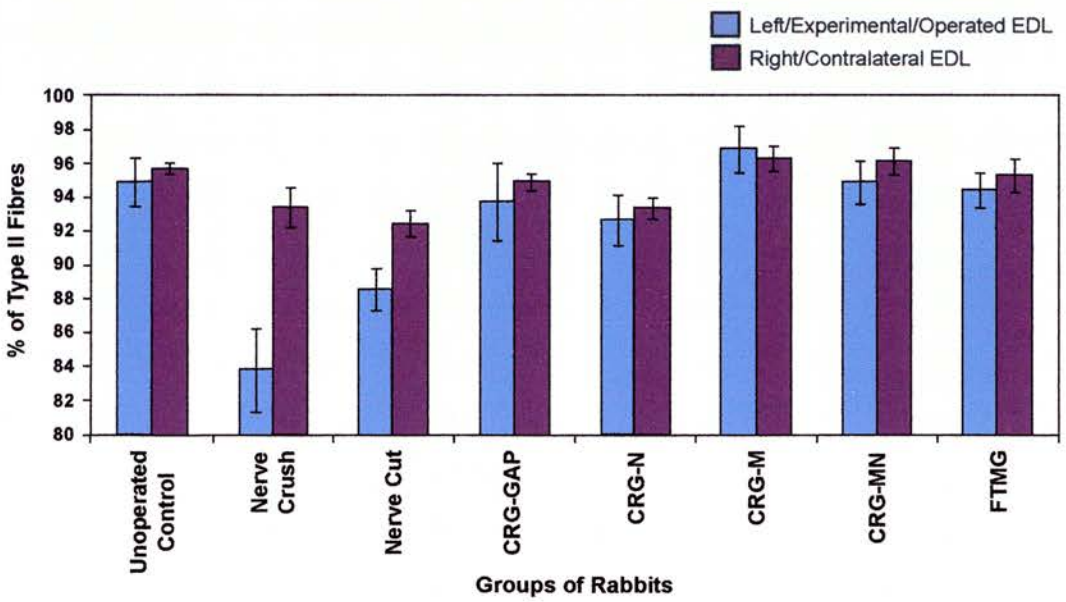

Figure 4.10 The mean and the SEM of the relative proportions of type II fibres in the EDL muscles of the experimental and control groups.(Please note that the ordinate has commenced at 80% in order to clearly display the SEMs).

4.3.4 Proportions of fibre types IIA, IIB and IIX, and neonatal muscle fibres identified with immunohistochemistry

Serial sections stained wth antibodies directed against adult fast and slow myosins as well as antibodies specific for type IIA, IIB and neonatal muscle fibres were used to assess frequency of type IIA, IIB, IIX and neonatal muscle fibres. A total of 400 to 500 fibres were examined for each muscle section. Figure 4.11 includes photographs of a normal EDL muscle after immunohistochemical labelling for fast, slow, IIA, IIB and neonatal myosin (please note that this is the same muscle and section of this muscle as in Figure 4.7).

In the present study type IIB fibres were not found in any EDL muscle for all experimental and control groups. However, neonatal fibres were present in experimental and operated control EDL muscles although the frequency was low. On average for all experimental and control groups there was one neonatal muscle fibre per whole muscle cross-section (a frequency of less than 0.0006%). Neonatal muscle fibres were found in all repair groups although the fibres were not present in every experimental EDL muscle. Furthermore there was no consistency between the method of nerve repair and the incidence of neonatal fibres.

Two fibre types which were present in all EDL muscles were type IIA and type IIX. Table 4.3 shows the mean and Figures 4.13 and 4.14, the mean and the SEM, for the relative proportions of type II fibres, type IIA and type IIX, within the experimental and contralateral EDL muscles of the experimental groups, the operated and the contralateral EDL muscles of the nerve crush and the nerve cut control

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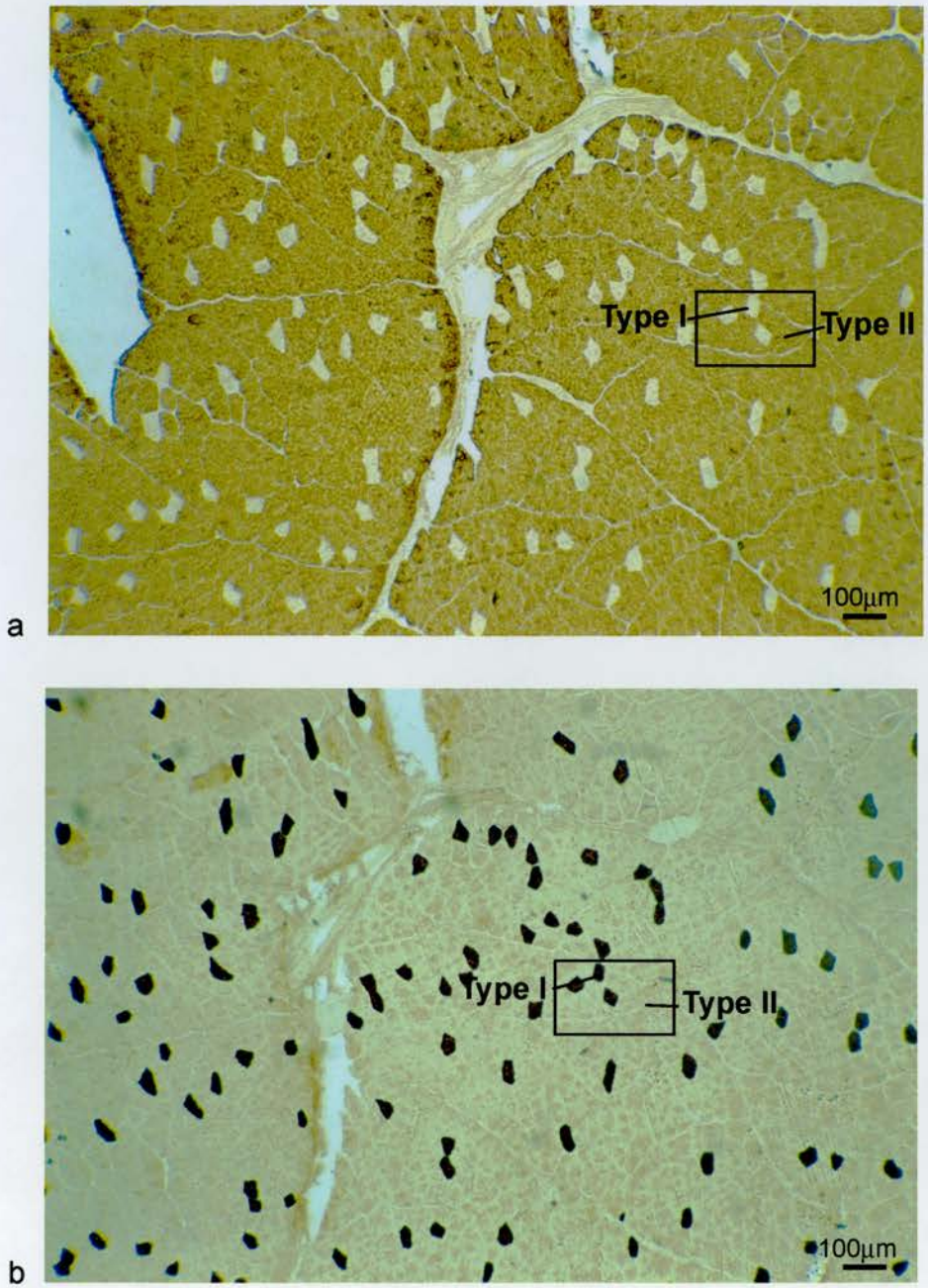
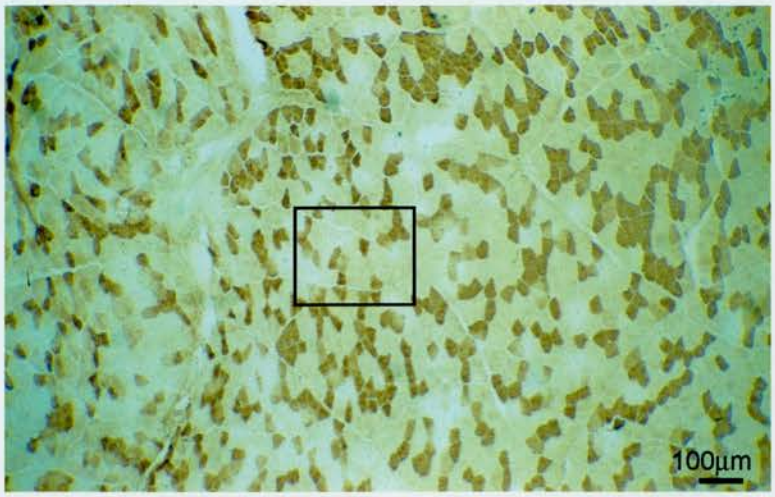
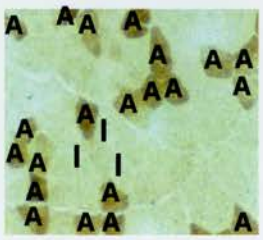


Figure 4.11 A normal EDL muscle stained with anti-MHC antibodies.
(a) MY-32, anti-type II MHC.
(b) BA-F8, anti-type I MHC.
Type I and type II fibres are labelled.
(Please note that this is the same muscle as shown in Figure 4.7).

c





I = Type I
A = Type IIA
Type IIX fibres are those not labelled

Figure 4.11 A normal EDL muscle stained with anti-MHC antibodies.
(c) SC-71 anti-type IIA MHC.
Type I and type IIA fibres are labelled.
(Please note that this is the same muscle as shown in Figure 4.7).

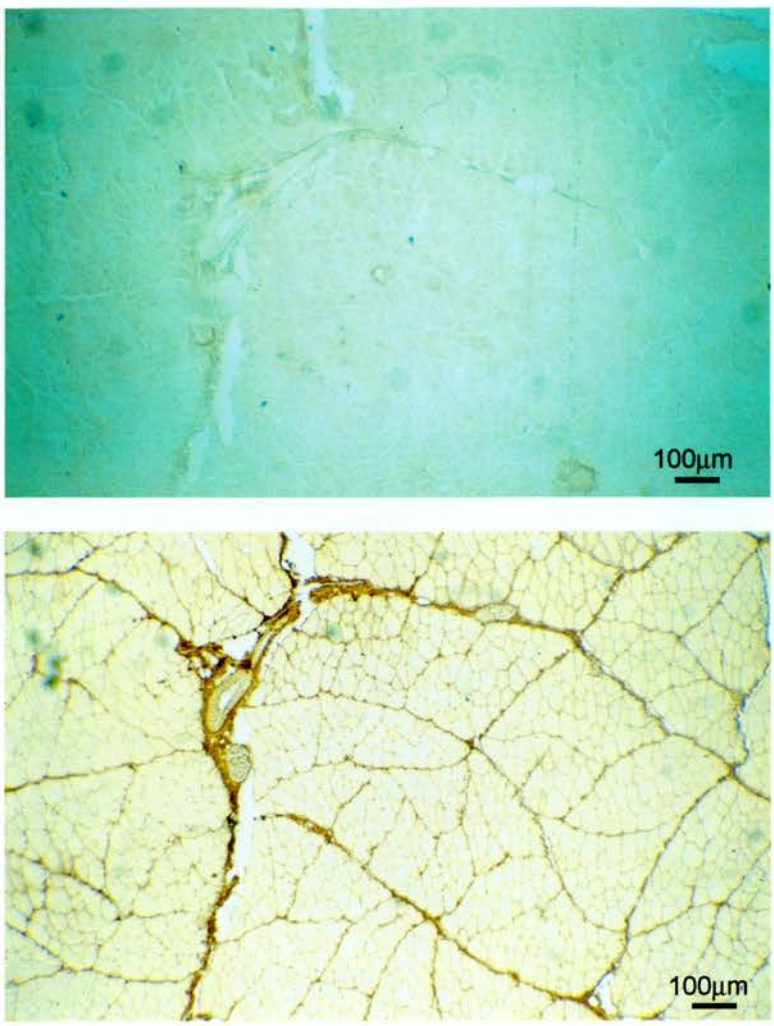


Figure 4.11 A normal EDL muscle stained with anti-MHC antibodies.
(d) BF-F3, anti-type IIB MHC.
Please note there were no positive fibres.
(e) Neonatal.
There were no positive fibres but note the staining of the connective tissue.
(Please note that this is the same muscle as shown in Figure 4.7).

groups, as well as the left and the right EDL muscles of the unoperated control. Figure 4.12 includes photographs of an EDL muscle after a peroneal nerve crush and after immunohistochemical labelling for fast, slow, IIA, IIB and neonatal myosin (please note that this is the same muscle and section of this muscle as in Figure 4.8).

On average type IIA fibres made up 44.42% and type IIX 55.58%, of the total number of type II fibres present in the contralateral muscles of the experimental groups. After nerve repair the incidence of type IIA fibres increased with a corresponding decrease in type IIX fibres, as the mean number of type IIA and IIX fibres in experimental muscle was 98.93% and 1.07% respectively. In fact after repair with either a CRG-N, a CRG-M or a CRG-MN tube no type IIX fibres were found.

For the nerve crush and cut control groups there was a similar increase in type IIA fibres in operated muscles. After nerve crush and nerve cut the mean number of type IIA fibres in operated EDL muscle was 87.74%, and for the corresponding contralateral muscle, 51.28%. For the type IIX fibres, the proportions of this fibre type decreased after either a nerve crush or a nerve cut as the mean number of type IIX fibres in operated EDL muscle was 12.27%, whilst for the corresponding contralateral muscle it was 48.72%. Indeed for all experimental and operated control groups (nerve crush and nerve cut) there was a significant difference between the percentage of type IIA or type IIX fibres present in the experimental and contralateral muscles ($p < 0.05$, Wilcoxon Signed Rank test).

Overall there were significant differences when the proportions of type IIA and type IIX fibres for all of the experimental and control groups (nerve cut, nerve crush and unoperated) were compared ($p < 0.01$, Kruskal-Wallis test). Individual

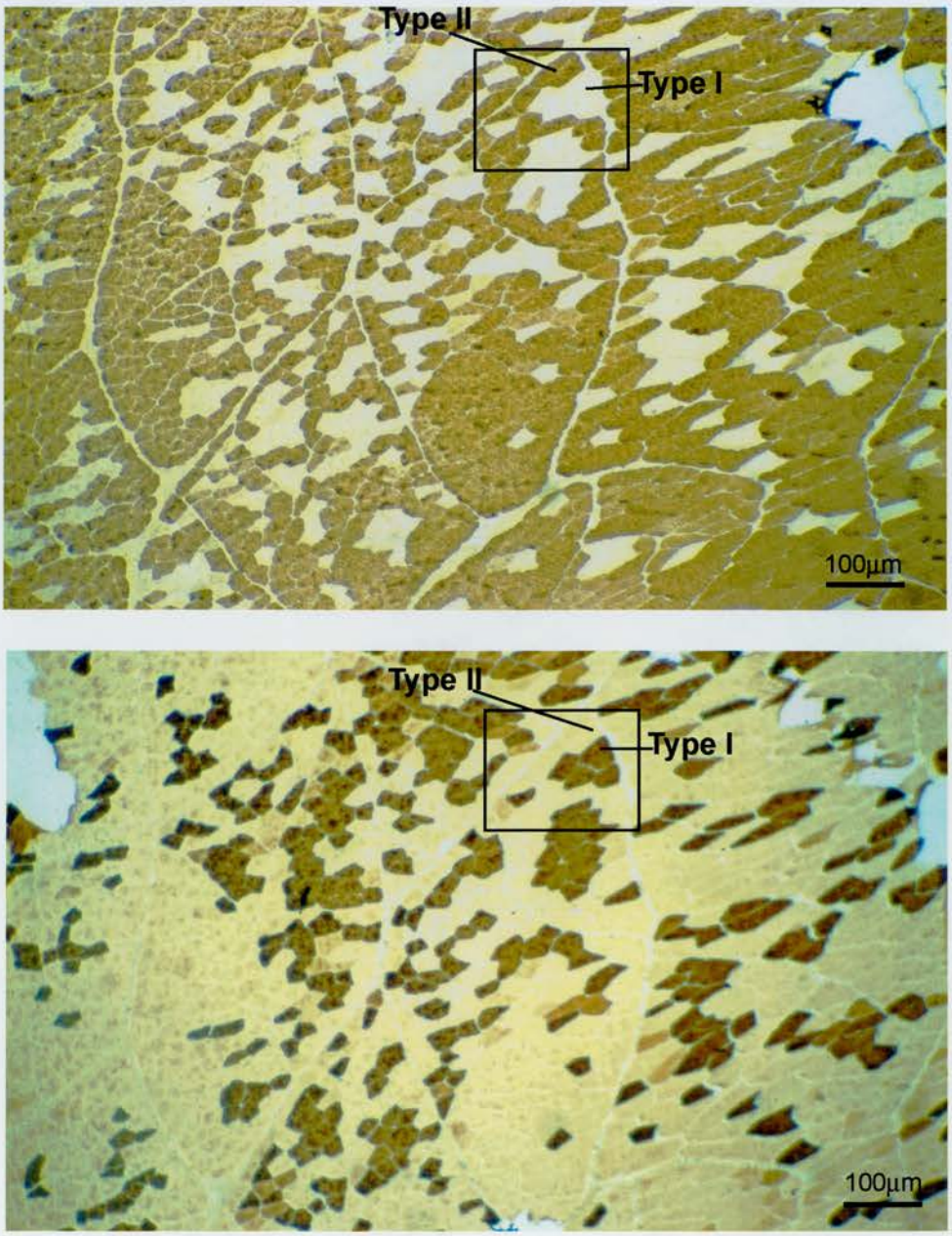
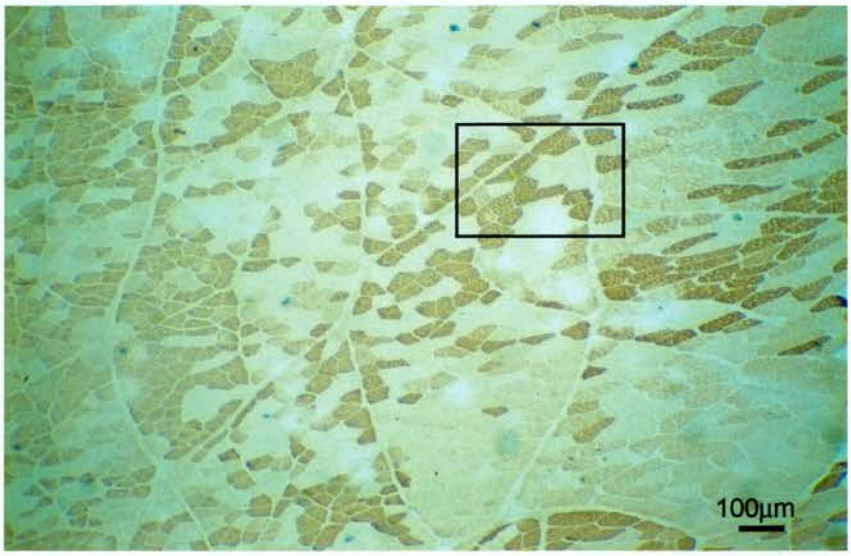
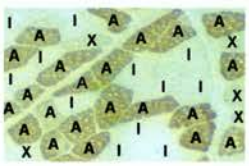


Figure 4.12 The EDL muscle after a peroneal nerve crush injury. Note the increase in proportion of type I fibres and the change in their distribution when compared with the normal EDL muscle.
(a) MY-32, anti-type II MHC.
(b) BA-F8, anti-type I MHC.
Type I and type II fibres are labelled.
(Please note that this is the same muscle as shown in Figure 4.8).





I = Type I
A = Type IIA
X = Type IIX

Figure 4.12 The EDL muscle after a peroneal nerve crush injury. Note the increase in proportion of type I fibres and the change in their distribution when compared with the normal EDL muscle.
(c) SC-71, anti-type IIA MHC.
Type I, type IIA and type IIX fibres are labelled.
(Please note that this is the same muscle as shown in Figure 4.8).

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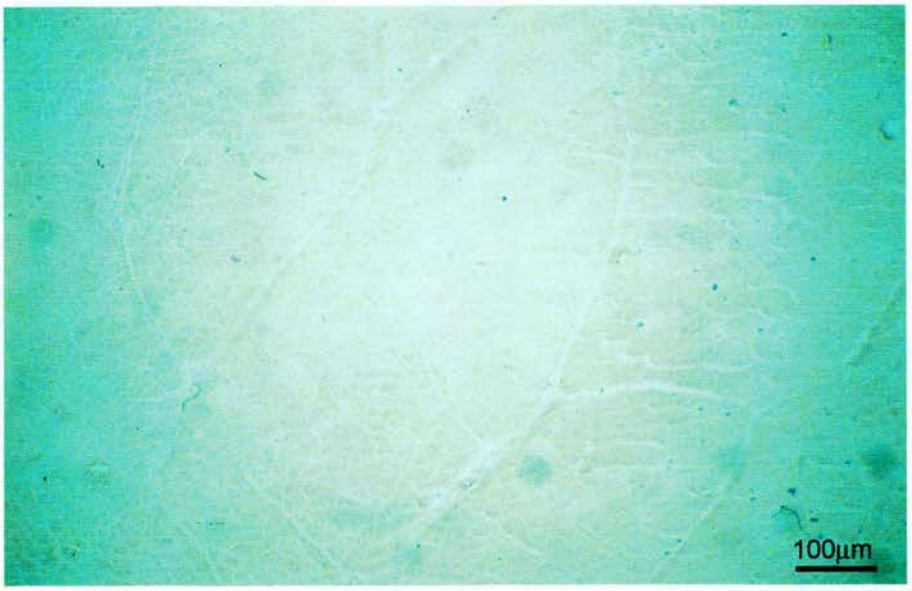

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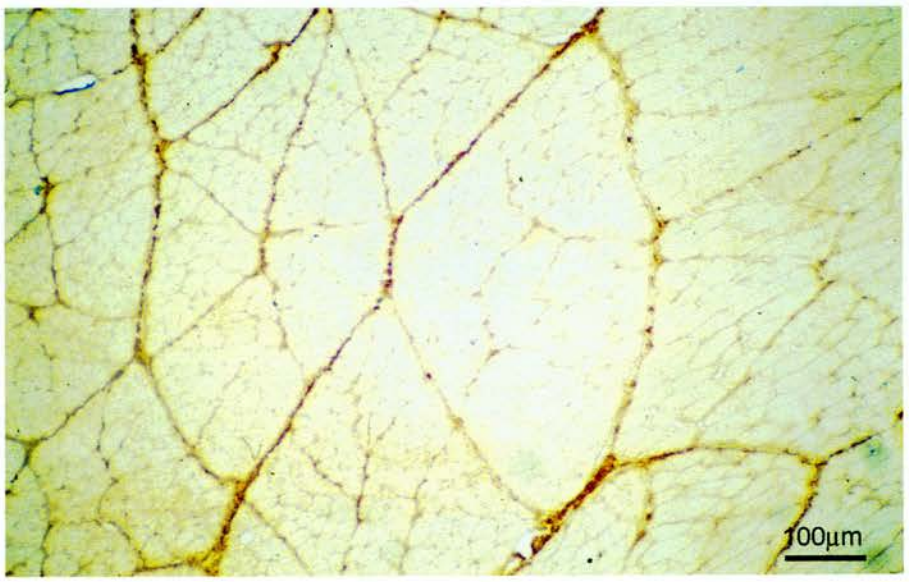

Figure 4.12 The EDL muscle after a peroneal nerve crush injury stained with anti-MHC antibodies.
(d) BF-F3, anti-type IIB MHC.
Please note there were no positive fibres.
(e) Neonatal.
There were no positive fibres but note the staining of the connective tissue.
(Please note that this is the same muscle as shown in Figure 4.8).

comparison of the experimental and control groups revealed that after nerve repair there were more type IIA and fewer type IIX fibres when compared to the proportions of these fibre types for the left and right EDL muscles of the unoperated control, or after nerve crush or cut. This difference was significant after a nerve crush ($p < 0.01$ for each case, Mann-Whitney *U* test), and for the the left and right EDL muscles of the unoperated control ($p < 0.01$ for the FTMG and CRG-GAP groups, $p < 0.05$ for the CRG-N, CRG-M, and CRG-MN groups).

There were significant differences in proportions of type IIA and type IIX fibres between operated EDL muscles of the nerve crush and the nerve cut control groups, as well as the left and the right EDL muscles of the unoperated control ($p < 0.01$, Kruskal-Wallis test). Individual comparisons of these groups revealed that after either a nerve crush or a nerve cut, there were significantly more type IIA and fewer type IIX fibres when compared to the left or the right EDL muscles of the unoperated control ($p < 0.01$ for each case, Mann-Whitney *U* test).

Overall there were significant differences when the proportions of type IIA and type IIX fibres for all of the contralateral EDL muscles of the experimental and operated control groups, as well as the left and the right EDL muscles of the unoperated control were compared ($p < 0.05$, Kruskal-Wallis test). Individual comparison of these groups revealed that the contralateral muscles of the CRG-MN tube group had the greatest percentage of type IIX fibres and this difference was significant when compared to those values for the contralateral muscles of the nerve crush control group ($p < 0.01$, Mann-Whitney *U* test) and the nerve cut control group, as well as the left EDL of the unoperated control ($p< 0.05$ for both cases). The contralateral muscles of the CRG-N tube group also had a significantly greater

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percentage of type IIX fibres when compared to the nerve crush ($p < 0.01$) and the nerve cut ($p < 0.05$) control groups.

The proportions of type IIA fibres for the one control animal housed in the Department of Tropical Veterinary Medicine was 47.12% for the left EDL muscle, and 61.38% for the corresponding right. For the proportions of type IIX fibres these were 52.88% for the left EDL muscle, and 38.62% for the right EDL muscle. These figures are greater in number of type IIA fibres whilst smaller in proportions of type IIX fibres when compared to similar values for both the left and the right EDL muscles of the unoperated control.

In summary, after all forms of nerve repair the predominant type II fibre was type IIA. There was a significant increase in the proportion of this fibre type, since for most controls, the percentage of type IIX fibres was greater than that of type IIA. Of the two operated control groups (nerve crush and nerve cut), it was only the nerve cut group which had similar changes in proportions of type IIA and type IIX fibres as was found for the experimental groups. In the nerve crush group, there were about twice as many type IIX fibres as in the nerve cut group and more than seven times as many as in the experimental muscles. However, the number of type IIX fibres in the nerve crush group was still only about one-third to one-quarter of the number of this fibre type observed in unoperated muscles.

Removal of the data for the one animal in the FTMG group which did appear to achieve full repair resulted in no change in the findings for the proportions of type IIA, IIB, IIX and neonatal type fibres.

Procedure	Muscle	Type IIA	Type IIX
CRG-GAP	Experimental EDL	97.23 ± 5.76 *	2.77 ± 5.76 *
	Contralateral EDL	45.60 ± 30.67	54.40 ± 30.67
CRG-N	Experimental EDL	100 ± 0 *	0 ± 0 *
	Contralateral EDL	39.65 ± 4.03	60.35 ± 4.03
CRG-M	Experimental EDL	100 ± 0 *	0 ± 0 *
	Contralateral EDL	36.64 ± 6.75	63.36 ± 6.75
CRG-MN	Experimental EDL	100 ± 0 *	0 ± 0 *
	Contralateral EDL	49.69 ± 7.60	50.31 ± 7.60
FTMG	Experimental EDL	97.44 ± 5.12 *	2.56 ± 5.12 *
	Contralateral EDL	50.83 ± 4.78	49.17 ± 4.78
Nerve Crush	Operated EDL	84.23 ± 12.25 *	15.77 ± 12.25 *
	Contralateral EDL	51.96 ± 2.16	48.04 ± 2.16
Nerve Cut	Operated EDL	91.24 ± 13.20 *	8.76 ± 13.20 *
	Contralateral EDL	50.60 ± 5.99	49.40 ± 5.99
Unoperated Control	Left EDL	44.23 ± 6.03	55.77 ± 6.03
	Right EDL	42.53 ± 8.19	57.47 ± 8.19

Table 4.3 - The mean and the standard deviation for the relative proportion (%) of type II fibres, type IIA and IIX, for the experimental and contralateral EDL muscles of each of the experimental and control groups.

* A significant result for the experimental, operated or unoperated control group when compared to those values for the contralateral/right side

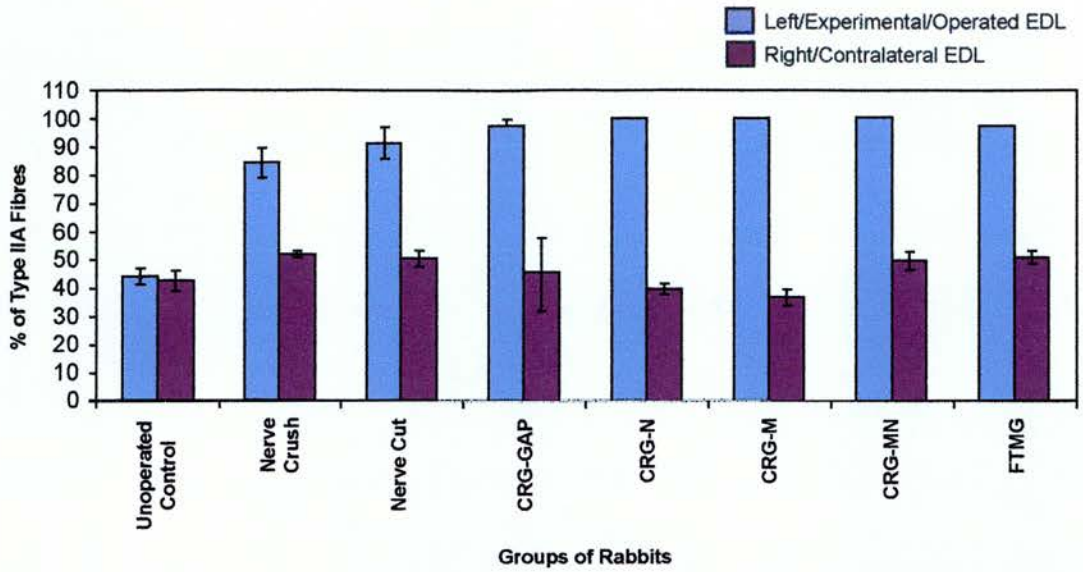

Figure 4.13 The mean and the SEM of the relative proportions of type IIA fibres in the EDL muscles of the experimental and control groups.

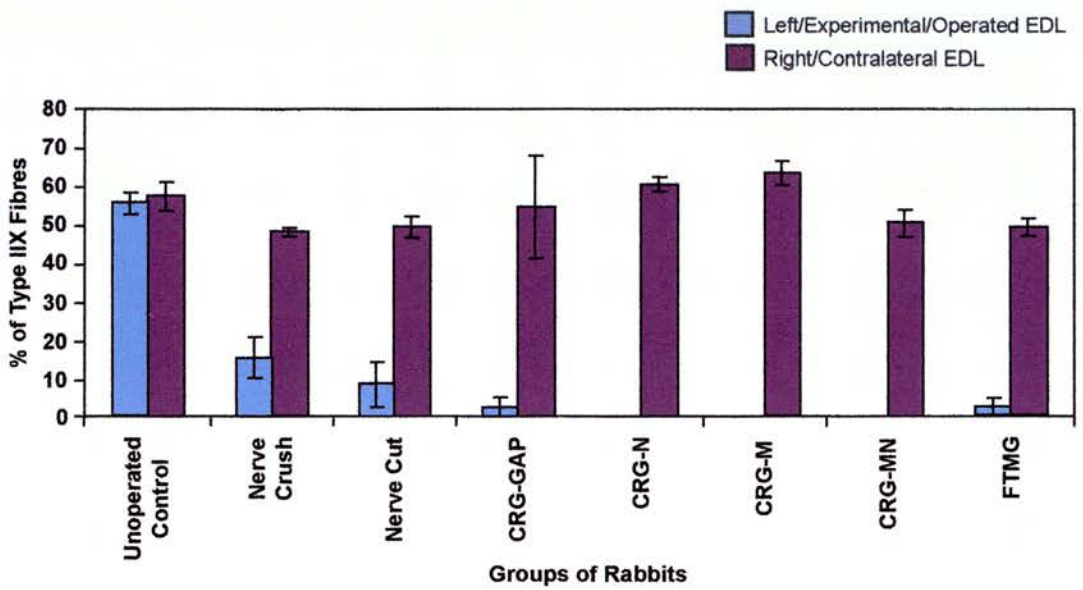

Figure 4.14 The mean and the SEM of the relative proportions of type IIX fibres in the EDL muscles of the experimental and control groups.

4.3.5 Distribution of fibre types I and II

The results for the arrangement of type I fibres in experimental and operated control muscles after analysis by each of the methods of assessment of fibre type distribution and qualitative assessment, is shown in table 4.4. The "verdict" column of this table refers to the majority ruling on the distribution of type I fibres for the methods used. Based on these results the distribution of type I fibres in the majority of experimental muscles was random. Indeed only 22.86% of the experimental muscles were shown to exhibit a nonrandom distribution or grouping of type I fibres. The greatest number of experimental muscles found to exhibit fibre type grouping after quantitative assessment was for the nerve cut control group, whilst the CRG-MN tube group had the least. In fact according to each of the methods of analysis of fibre type distribution, the CRG-MN tube group had none. Furthermore the majority ruling on the distribution of type I fibres indicated that there was a greater number of experimental muscles exhibiting a nonrandom arrangement of type I fibres after either a nerve crush or cut (a mean of 60%), then for the experimental muscles of experimental groups (a mean of 8%). Based on the majority ruling, it was only after repair with either a CRG-N tube or a FTMG that there was any evidence of grouping of type I fibres and the incidence was small (only 20% of experimental muscles for each group). In the evaluation of the different methods of analysis of fibre type distribution, it was the point-sommet technique which had the most consistent results. This conclusion was reached after comparing the findings on the distribution of type I fibres for each of the methods and the majority ruling. Indeed the findings on the distribution of type I fibres as gathered by the point-sommet

technique were in agreeance with 97% of the verdicts of the majority ruling. For the runs method of analysis of fibre type distribution this figure was 89%, whilst for the CDI it was 71%.

Qualitative assessment established that just over half (51%) of experimental muscles exhibited grouping which was a greater incidence of type I fibre grouping than what was found by any of the quantitative methods. Furthermore unlike the quantitative methods, evidence of type I grouping was found after every form of nerve repair when assessed qualitatively. The only repair group to exhibit similar results for both quantitative and qualitative assessment of fibre type distribution was the CRG-N tube group. However, both types of evaluation established that the greatest number of muscles exhibiting a nonrandom arrangement of type I fibres was for the nerve crush and the nerve cut control groups. Upon comparison with each of the methods of investigation of fibre type distribution, the results from qualitative assessment agreed with 77% of the findings of the CDI, 69% of the findings of the point-sommet and 60% of the findings of the runs systems of analysis. For the majority ruling on the distribution of type I fibres by the different methods of analysis of fibre type arrangement, the findings from qualitative assessment was in accord with 71% of these results.

A qualitative assessment was made of the distribution of type I fibres from the medial to the lateral portions of the EDL muscle for each experimental and control group. For the majority of these groups the type I fibres were distributed over most parts of the whole cross-section of all EDL muscles. There was a greater proportion of type I fibres to the lateral area of the EDL muscle for 28% of experimental muscles, whilst for the nerve cut control group a similar distribution of

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type I fibres was found in 20% of the operated muscles. Only 16% of experimental muscles were found to have a more medial arrangement of type I fibres, and for the operated muscles of the nerve crush and cut control groups, 30%. For the contralateral muscles of the operated control and experimental groups, as well as the left and the right EDL muscles of the unoperated control, 16% of these muscles exhibited a greater lateral distribution of type I fibres and none for a more medial arrangement.

Evaluation of the distribution of type I fibres of the contralateral muscles of both experimental and operated control groups, and unoperated control muscles was also made. However unlike the findings for the experimental muscles, all three methods (runs, point-sommets and CDI) found the type I fibre distribution in each contralateral and unoperated control muscle to be random.

The distribution of type I fibres for the one control animal housed in the Department of Tropical Veterinary Medicine was random for both the left and the right EDL muscles. This was comparable to the findings for the arrangement of type I fibres in the left and the right EDL muscles of the unoperated control.

In summary, as assessed by the methods I used to detect fibre type grouping and by qualitative evaluation, the incidence of a nonrandom arrangement or grouping of type I fibres after nerve repair was minimal. Furthermore the occurrence of any grouping of type I fibres was found to be greater after a nerve cut or crush. All contralateral muscles of both operated control and experimental groups, as well as the left and the right EDL muscles of the unoperated control exhibited a random distribution of the type I fibres. For the majority of experimental and control groups the type I fibres were distributed within most parts of the whole cross-section of

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muscle for each EDL muscle. In the evaluation of the different methods of analysis of fibre type distribution, it was the point-sommet technique which had the most consistent results of the four systems assessed based on the majority ruling. However, the CDI method detected the greatest number of nonrandom distributions.

The distribution of type I fibres in the EDL muscle of the one animal in the FTMG group which did appear to achieve full repair was nonrandom based on assessment by **every** quantitative method examined in this project, as well as by qualitative measures. Hence, removal of this data did not influence the evaluation of the different methods of analysis of fibre type distribution.

Procedure	Animal Number	Runs	Point Sommet	CDI	Verdict	Qualitative Assessment
CRG-GAP	1	R	R	R	R	R
	2	R	R	NR	R	R
	3	R	R	NR	R	NR
	4	R	R	NR	R	R
	5	R	R	NR	R	NR
CRG-N	1	R	R	R	R	R
	2	R	R	R	R	R
	3	R	R	R	R	R
	4	**NR**	**NR**	**NR**	**NR**	**NR**
	5	R	R	R	R	R
CRG-M	1	R	R	R	R	NR
	2	R	NR	R	R	R
	3	R	R	R	R	R
	4	R	R	NR	R	R
	5	R	R	NR	R	R
CRG-MN	1	R	R	R	R	R
	2	R	R	R	R	R
	3	R	R	R	R	NR
	4	R	R	R	R	R
	5	R	R	R	R	R
FTMG	1	R	R	R	R	R
	2	R	R	R	R	NR
	3	**NR**	**NR**	**NR**	**NR**	**NR**
	4	R	R	R	R	NR
	5	R	R	R	R	R
Nerve Crush	1	R	R	NR	R	NR
	2	R	R	NR	R	NR
	3	R	R	NR	R	NR
	4	R	**NR**	**NR**	**NR**	**NR**
	5	R	**NR**	**NR**	**NR**	**NR**
Nerve Cut	1	**NR**	**NR**	**NR**	**NR**	**NR**
	2	R	R	NR	R	NR
	3	**NR**	**NR**	**NR**	**NR**	**NR**
	4	R	**NR**	**NR**	**NR**	**NR**
	5	R	**NR**	**NR**	**NR**	**NR**

Table 4.4 - The distribution of type I fibres in experimental muscles of operated control and experimental groups, as assessed by methods of analysis of fibre type arrangement and qualitative assessment.

(R = Random distribution of type I fibres, NR = Nonrandom distribution of type I fibres, Verdict = Majority ruling on the distribution of type I fibres by the different methods of analysis of fibre type arrangement)

4.4 DISCUSSION

The main findings in this chapter are that (i) more type I muscle fibres were found after reinnervation following either a nerve crush or nerve cut compared with the experimental (graft) groups; (ii) there was a decrease in the proportion of type IIX fibres after nerve injury but this was significantly worst after repair with either a CRG tube or a FTMG; and (iii) the incidence of a nonrandom distribution of type I fibres for any of the repair groups was minimal but greater after either a nerve cut or crush. Based on these findings I would conclude that as a form of nerve repair, there are few - if any - benefits of using CRG tubes to guide nerve repair, over doing nothing. Furthermore, the data suggests that repair with either a CRG tube or a FTMG may, to some extent inhibit nerve regeneration and hence prolong the period of denervation of muscle. As to the methods for the detection of fibre type grouping that were reviewed, all had limitations. Indeed from observation of the results for the distribution of type I fibres after analysis by any of the methods, one may conclude that as the random arrangement of type I fibres was "restored" for most experimental muscles after repair, there were benefits from using the graft methods. However it is more likely that fibres were either not reinnervated or were, but did not transform. Fibre type grouping and thus, a nonrandom distribution, is thought to be the result of reinnervation of adjacent muscle fibres by collateral sprouting of the regenerated axons after denervation (Karpati and Engel, 1968c). However, after assessment of fibre type distribution by the various tests, the results for fibre type distribution for the nerve crush control group were not as good as those after nerve cut. This result would not be expected as the nerve crush is the "best scenario" after nerve injury, and

the nerve cut the worst. It is apparent that further work is required in the development of a system of analysis that will achieve an accurate and objective assessment of the changes in the spatial arrangement of fibre types after denervation and reinnervation of muscle.

4.4.1 Proportions of fibre types

Why was there a significant increase in the proportion of type I fibres after either nerve crush or nerve cut in the present study? It has been shown that following a nerve crush injury most myelinated axons in the proximal portion of the injured nerve survive, and indeed regenerate down their endoneurial tubes into the distal stump to reinnervate muscle (Toft *et al.* 1988). There has also been evidence of sprouting of intact axons after partial denervation by nerve crush (Edds, 1950, Karpati and Engel, 1968c, Warszawski *et al.* 1975, Bishop and Milton, 1997). Nemeth *et al.* (1993) established the potential for a similar process after self-reinnervation in the cat. Hence if there was an increase in the number of muscle fibres innervated by type I motoneurones after either nerve crush or nerve cut, this may go some way to explaining the concurrent increase in the proportion of type I fibres.

Increases in the proportion of type I muscle fibres have been reported in other studies, notably after denervation and self-reinnervation in the mouse (Desypris and Parry, 1990), and in the rat (Thomas and Ranatunga, 1993, Bobinac *et al.* 2000). One explanation for these observations is that the type I motoneurones exhibit a faster rate of axonal regeneration and thereby are able to reinnervate a majority of the

denervated muscle fibres before the arrival of the type II axons. However this is unlikely as in a study of a crush injury in the mouse, Bishop and Milton (1997) noted that it was the level of the lesion and not the rate of axonal growth which influenced the proportion of fibre types. In the investigation they observed that a denervation nearby to the nerve's point of entry resulted in significant increases in the percentage of type I fibres in the reinnervated muscle. After a denervation 4 mm from the muscle there was only a small and insignificant increase in type I fibres. Hence, if type I motoneurones exhibited faster axonal growth rates, then a significantly higher percentage of type I fibres should have been seen following the distant denervation. Furthermore the observations of Bishop and Milton (1997) do not help explain why there was a significant increase in the percentage of type I fibres after either nerve crush or cut in the present study since the level of denervation was at the same point for each experimental and control group.

Another possible explanation for these observations may be found in the differences in the degenerative and regenerative properties of type I and II motoneurones and the relationship of the stimulation patterns imposed on them. Artificial (electrical) stimulation has been shown to enhance the recovery of regenerating neurones (Nix and Hopf, 1983). The level of activity for type I motoneurones is such that although they transmit impulses at low frequencies, their period of activity is longer than the intermittent periods of high frequency impulses conducted by type II motoneurones (Eccles *et al.* 1958, Henning and Lømo, 1985). Thus, the greater total neural activity of type I motoneurones may accelerate regeneration following axotomy. As a result more type I motoneurones would reinnervate the muscle and increase their motor unit size.

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The increase in proportions of type I fibres seen in the present study could be a result of not only the properties of the reinnervating motoneurone, but due to some aspect of the muscle fibres themselves. Indeed following axonal regeneration there have been a number of studies which have indicated that motor unit properties are not entirely regulated by the reinnervating motoneurone (Dum *et al.* 1985a,b, Gordon *et al.* 1986, 1988, Foehring *et al.* 1986b, Gillespie *et al.* 1987, Cope *et al.* 1991, Unguez *et al.* 1995, Rafuse and Gordon, 1998). What limits the complete neural conversion of muscle fibre properties following nerve regeneration in the adult are not known. One possibility is that there may be intrinsic differences between different muscle fibre types that reduce the extent to which their properties can be modulated by the innervating motoneurone. Fast and slow myotubes arise from distinct and committed myoblast populations and express specific fibre type features that are independent of innervation (Rubinstein and Kelly, 1981, Miller and Stockdale, 1987, Condon *et al.* 1990a,b, Stockdale, 1992, Donoghue and Sanes, 1994). If this is the case it is likely when type II motoneurones sprout and reinnervate adjacent type I muscle fibres, features of type I fibres may be maintained. Unfortunately the extent to which specific motoneurones have regenerated and reinnervated the EDL muscle in these experiments is unknown, as the number or characteristics of the reinnervated motor units were not examined. Perhaps the completion of glycogen depletion studies as described by Rafuse and Gordon (1998) would have been helpful in providing this data.

The design of the present experiments may have not provided an explanation for the alteration in proportions of fibre types after nerve crush or cut, but these findings do suggest that the CRG tube as well as the FTMG may be inhibiting nerve

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regeneration. For the majority of the experimental groups there was an increase in the proportion of type I fibres after repair although the changes were insignificant. Nevertheless there was also an increase in type I fibre proportions after nerve crush or nerve cut but it was significant. This suggests that after nerve injury type I motoneurones are more adept at reinnervating muscle than type II motoneurones however, due to some factor and possibly some form of obstruction, the increase in the proportion of type I fibres was not significant after repair with either a CRG tube or a FTMG. To determine whether or not this hypothesis is correct, experiments would need to be conducted where after an initial crush injury it is repeated at several different time periods. The aim would be to provide some form of impedance to the passage of the regenerating axons without preventing the process of reinnervation. If there was effectively some form of blockage then the predicted results would be similar to those found for the present study.

In the present experiments the alteration in the proportions of type IIA and type IIX fibres was striking. In the normal EDL muscle it was found that type IIA fibres make up 44.20% and type IIX 55.58% of the type II fibre population. This observed predominance of the type II fibres IIA and IIX in the normal EDL muscle in the rabbit was in agreement with the findings of other immunohistochemical studies (Aigner *et al.* 1993, Janmot and d'Albis, 1994). However, for each of the experimental and control groups there was a dramatic decrease in the percentage of type IIX fibres with a concomitant increase in type IIA fibres. A similar change in fibre type proportions was observed by Bobinac *et al.* (2000) after denervation of the EDL muscle in the rat. In contrast, denervation of the fast-twitch semimembranosus accessorius muscle in the rabbit resulted in a shift of the type IIX fibre percentages

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from 22% to 98%, whilst the other type II fibre, type IIB, decreased from 70% to 2% (Bacou *et al.* 1996). It is difficult to explain this discrepancy with the results of the current study, although examination of the kinetics of the myosin heavy chain IIB and IIX expression may provide some reason. However, the study by Bacou *et al.* (1996) and that of d'Albis *et al.* (1994) did conclude that, in the rabbit, sensitivity to denervation is of the order of : IIB > IIX > IIA > I. The results of the present study concurs with these deductions.

The decrease in IIX fibres and concomitant increase in IIA observed for each experimental and operated control group was most marked for each of the experimental groups, as well as the nerve cut control group. Thus, based on these findings and as forms of nerve repair, there are no benefits of using either a CRG tube or a FTMG over doing nothing. The reduction in IIX and concomitant increase in IIA fibres was least after nerve crush. As previously discussed this would be expected since after nerve crush, the probability of axotomized motoneurones reinnervating their original muscle fibres is greatest. The preservation of the endoneurial tubes allows the regenerating axons to follow the original pathways back to their original muscle fibres (Kugelberg *et al.* 1970).

4.4.2 Distribution of fibre types

In terms of the quantitative and qualitative assessment of the type I fibre distribution, grouping was consistently greater for the nerve crush and nerve cut control groups. The type grouping has been a consistent feature of reinnervation of muscle in adult mammals (Dum *et al.* 1985, Foehring *et al.* 1986a,b, Gordon *et al.*

1988, Kugelberg *et al.* 1970, Nemeth *et al.* 1993, Rafuse and Gordon, 1996b). Based on this it would appear that reinnervation of the EDL muscle was greater after either nerve crush or cut, and this may add support to the hypothesis that the CRG tube as well as the FTMG inhibit nerve regeneration. It would clearly be of interest to apply similar methods to assess the grouping of type IIX fibres. The lower incidence of type IIX fibres would make these amenable to the same kinds of analysis as applied here to the type I fibres.

For the spatial distribution of type I fibres of the EDL muscle, qualitative assessment revealed that for most experimental and control groups the type I fibres were distributed within most parts of the cross-section of the muscle. This is in agreement with other studies where fibre type grouping was present and the spatial distribution of fibre types did not alter after the processes of denervation and reinnervation (Foehring *et al.* 1987a,b, Parry and Wilkinson, 1990, Rafuse and Gordon, 1996b). There are a number of possible explanations for this distinct regional distribution of fibre types in reinnervated muscles. Firstly, it is unlikely that motoneurones reinnervate their original muscle fibre types as several studies have shown that regenerating motor axons do not show any specificity for their original muscles (Weiss and Hoag, 1946, Bernstein and Guth, 1961, Miledi and Stefani, 1969, Gillespie *et al.* 1986, Thomas *et al.* 1987). However, it has been demonstrated that different subsets of axons preferentially innervate muscles from different levels. Hence, rostral muscles are innervated more frequently by rostral nerves, caudal muscles by caudal nerves and middle muscles by middle nerves (Wigston and Sanes, 1985). This selective innervation of muscle along its rostrocaudal axis by axons from corresponding levels of the spinal cord has been demonstrated in the serratus anterior

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muscle and the diaphragm (Laskowski and Sanes, 1987). Moreover, these topographic maps are reestablished during reinnervation following nerve damage (Laskowski and Sanes, 1988, De Santis *et al.* 1992, Reis and Laskowski, 1993, Laskowski *et al.* 1998). These studies suggest that muscles may differ in some quality that is graded along the rostrocaudal axis and that this may bias the synapses they receive. Or as Laskowski *et al.* (1998) suggests, the selective reinnervation may come about due to synaptic competition. The outcome of this is influenced by the "positional labels" associated with each regenerating axon from different levels in the spinal cord. Thus, the synaptic competition is biased due to differences in the rostrocaudal position of the motoneurones.

Another explanation for the reestablishment of regional differences in the distribution of fibre types after reinnervation is that muscle fibre types are resistant to histological modification by the reinnervated motoneurone (Chan *et al.* 1982, Gauthier *et al.* 1983, Dum *et al.* 1985a,b, Gillespie *et al.* 1986, Foehring *et al.* 1987a,b, Parry and Wilkinson, 1990). This resistance may be explained by intrinsic differences established during development, as studies in cell culture have shown that some myoblasts acquire a heritable commitment to form myotubes of a particular fibre type. Miller and Stockdale (1987) dissociated muscles from early chick embryos, plated mononucleated myoblasts, allowed them to divide and fuse, and then analysed the clones of myotubes that formed. They found that the myotubes exhibited different phenotypes, although all the myotubes within a single colony had the same phenotype. The phenotype for each myotube was also maintained through serial subculturing. It was concluded that the myoblasts were already segregated into distinct lineages at the time they were plated, and that they passed this commitment

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onto their progeny. Hence, the cloned embryonic chick myoblasts demonstrated an intrinsic control of MHC gene expression. Further studies which have reintroduced cloned myoblasts into embryonic chick limbs have shown that the myotubes that form *in vivo* and *in vitro* express the same MHC genes (Di Mario *et al.* 1993). Mammalian myoblasts injected into rat muscles have produced similar results (Pin and Merrifield, 1997).

Lexell *et al.* (1994) completed a detailed morphometric analysis of the normal EDL muscle in the rabbit and quantified the spatial distribution of type I fibres. Unfortunately the examination of the type I fibre distribution in the present study was qualitative so it is difficult comparing the findings to the investigation by Lexell *et al.* (1994). However, both studies did find that type I fibres were distributed systematically within the cross-section of the normal EDL muscle. Lexell *et al.* (1994) noted that there was a significantly higher proportion of type I fibres in the medial and deep areas than in the lateral, superficial part of the EDL muscle in the rabbit. It was hypothesized that "proximity to the bone, rather than depth per se, may be the determinant of these gradients", and that the spatial distribution of fibre types was influenced by processes during the embryological development of bone and muscle (Lexell *et al.* 1994).

The methods I used to detect fibre type grouping all showed limitations. The purpose of this review was to see if any of the standard methods could give reliable results quickly and efficiently. Of the four methods reviewed, the point-sommet technique was the most consistent in detecting grouping. However, it could be argued that the number of triads measured was influenced by the size of fibres (Venema, 1988). Furthermore there was some disagreement in findings of the point-sommet

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technique and those of the qualitative assessment. The qualitative assessment is a subjective measure but it did provide some form of another guide as to the distribution of the type I fibres.

For the other methods of analysis of fibre type grouping reviewed in the present study, the enclosed fibre technique was initially disregarded as it was determined that this count was unsuitable. This was due to the fact that this test is also influenced by differences in size. If one fibre type occurs infrequently as to some extent is the case with the proportion of type I fibres in the EDL muscle of the rabbit, few, if any, of these fibres would be expected to be found enclosed.

It has been argued that the CDI is sensitive to fibre type differences in size whereby if fibres are consistently smaller than fibres of the other type, they are more likely to be nearest neighbours and thus, less likely to be index fibres (Downham *et al.* 1984). The developers of the CDI have vigorously defended the CDI method and have suggested that the expected values for the parts of the contingency table used in the calculation of the CDI will show size differences (Lester *et al.* 1984). Thus, large fibres are less likely to be found as nearest neighbours than small fibres. In terms of the present study there were differences in the sizes of the fibre types (*cf.* type I and type II fibre diameter data for various experimental groups, Table 3.7, Chapter 3). If the CDI is sensitive to type-specific differences in fibre sizes then it would be expected that type I fibres would be grouped (in the present study type II fibres were consistently smaller than type I fibres and thus, are more likely to be nearest neighbours and less likely to be index fibres). This was not the case when the test was applied to every experimental EDL muscle after repair, but the CDI did have the highest incidence of detection of a nonrandom distribution of type I fibres. The runs

method of analysis of fibre distribution was the poorest performer of the tests reviewed although it has also been argued that it is also influenced by the size and indeed, the shape of muscle fibres (Lexell *et al.* 1987).

In sum, all of the methods of analysis of fibre type distribution were flawed. Some of the tests may have been influenced by the size of the muscle fibres. In normal muscle the variability in fibre size is not so great, but in pathologic muscle such as those examined in the present study, fibres exhibit a wide range of sizes (Chapter 3). An important assumption underpinning most of these methods is that there are no systematic differences in the sizes of the type I and type II fibres. As there was a great range in the size of fibre types in the present study it would appear that most of these methods were not sufficiently robust to overcome any departures from the underlying models. It is evident that the analysis of fibre type distribution requires a method that is insensitive to differences in fibre type proportion and type-specific differences in size and shape. Such a technique should also be objective and reproducible, as well providing a measure by which the fibre type distribution could be graded. This method has yet to be developed but it is possible that two, or more, may be required to correctly evaluate fibre type distribution.

4.5 CONCLUSION

The findings of this chapter have supported those in the previous chapter, that as a form of nerve repair there are few - if any - benefits of using the CRG tube or the FTMG. Furthermore it seems from the changes in the proportions of type I fibres that one of the reasons for the poor results after repair with either a CRG tube or a FTMG may be hindrance of the growth of regenerating axons by these grafts.

Evaluation of various methods of analysis of fibre type distribution also revealed their limitations and the continuing need for the development of new techniques which would objectively, efficiently and accurately summarise the distribution of fibre types in muscle cross-sections.

CHAPTER 5

A Morphological Comparison of Nerve Autograft and the Freeze-Thawed Muscle Autograft in the Repair of Obstetrical Brachial Plexus Palsy in Sheep

5.1 INTRODUCTION

The experiments described in this chapter were designed to establish whether the time of repair and the age of the recipient influenced the degree of recovery in a clinical model, the obstetrical brachial plexus palsy (OBPP). Obstetrical brachial plexus palsy is associated with birth trauma and results in a traction injury to the brachial plexus. It is classified into upper (involving C5, C6, and usually C7 roots), lower (predominantly C8 and T1), and total (C5, C6, C7, C8, ±T1) plexus palsies (Terzis *et al.* 1986, Terzis *et al.* 1987). An upper brachial plexus palsy was first described by Duchenne (1872) but bears the name of Erb's palsy (1874). A lower brachial plexus palsy is extremely rare in birth injuries and is known as Klumpke's palsy (Al-Qattan *et al.* 1995). Traction injuries to the brachial plexus are one of the most common and severe of all injuries of peripheral nerves (Birch, 1996). They usually occur after blunt trauma which results in traction on the whole or part of the plexus. In adults many of these closed traction injuries of the brachial plexus are after motorcycle accidents (Rosson, 1988).

At a cellular level a typical traction injury to a nerve generally consists of rupture of all neural elements whilst the fibrous epineurium is preserved (Sunderland, 1978). However a proportion of these injuries result in a lesser form of nerve injury and may resolve spontaneously. In these cases the nature of the injury is a temporary interruption of conduction or the axons are severed but the integrity of the endoneurial sheaths are maintained. At the time of injury it is impossible clinically or electrophysiologically to distinguish the type of injury so it has been

recommended for brachial plexus injuries to wait for a period of 3 months after injury before any surgical exploration is undertaken (Mackinnon and Dellon, 1988). By this time it might be expected that the injury would start to show a systematic advancement in recovery so that the degree of injury could be established. For OBPP injuries this approach has been largely utilized and unlike traumatic brachial plexus injury in adults, the majority of infants recover sufficiently so as not to require primary plexus surgery (Adler and Patterson, 1967, Eng, 1971, Greenwald *et al.* 1984, Tada *et al.* 1984, Jackson *et al.* 1988, Sjöberg *et al.* 1988, Michelow, 1994, Smith, 1996). However for those infants who do not show functional improvement primary brachial plexus exploration is usually undertaken at 3 to 6 months of age, some even up to 12 months depending on the centre (Francel *et al.* 1995, Clarke *et al.* 1996). There is a move towards earlier intervention in cases identified at birth since damage in the more serious forms of injury are often irreversible, particularly if it is 1 year before surgical intervention (Gilbert and Tassin, 1987, Birch, 1996). For these more severe forms of birth palsies it has been established in other fields of nerve repair that the earliest repair of these type of injuries is related to the best level of recovery (Lawson and Glasby 1995, Glasby *et al.* 1997, 1998, Fullarton *et al.* 1998). Furthermore it has been advocated that surgical exploration and repair is required as soon as possible in adult brachial plexus injuries (Narakas, 1993). Hence the question of when to treat a clearly identified "severe" (where there is rupture of all neural elements and connective tissue other than the epineurium) traction injury of the brachial plexus has been the subject of this chapter. The question of whether nerve regeneration after repair decreases with age is also addressed. This has

important implications in the management of OBPP particularly when a severe injury exists as a delay in treatment may be deleterious.

For traction injuries the current choice of treatment is the nerve autograft ("cable" graft). As has been discussed nerve grafting is associated with the sacrifice of a functioning nerve, with loss of sensation, scarring and in some cases the development of a painful neuroma at the donor site. Moreover, in neonates it is extremely difficult to find sufficient nerve graft (e.g. sural nerve), particularly if repair of more than 1 spinal nerve is required. Hence this often means taking cutaneous nerves from both legs and the injured limb which adds to the operating time, contributes to blood loss so there is often a need for blood transfusion, and results in considerable scarring of the donor limbs. The reasoning behind the present study was that if FTMG's can be shown to be equivalent to nerve grafting then their application in the repair of OBPP would reduce operating time, remove the need for blood transfusion and prevent scarring of normal limbs. Thus, the present study sought to compare FTMG and nerve grafts in newborn and adult animals in order to evaluate the clinical feasibility of using FTMGs.

5.2 METHODS

5.2.1 Rationale for experimental design

The present experiments were designed: to establish first, the optimal timing of nerve repair (immediate or delayed); second, whether there was a difference in the level of recovery between neonates and adults after nerve repair; third, to assess the FTMG as a surgical technique and to determine whether the FTMG is at least as good as a conventional nerve graft.

The experiments were intended to emulate OBPP. These birth palsies are the result of a traction injury and a "severe" injury (where there is rupture of all neural elements and connective tissue other than the epineurium) was to be studied. An obstetrical brachial plexus injury results after there is an increase in the angle between the neck and the shoulder, which is often at delivery. To produce an obstetrical brachial plexus injury experimentally by this kind of method is very difficult, particularly when a consistent lesion is required. Hence the method as described in this section was designed to produce a reproducible lesion which produced consistent visible effects on the injured nerve, in terms of both the length of the injury and the general morphology in the lesioned area.

The experimental model was the C6 root of the brachial plexus in sheep and lambs, which is equivalent to the human C5 (Hems and Glasby, 1992, Hems *et al.* 1994). This model was chosen as previous studies have shown it to be a good paradigm for human nerve repair (Hems and Glasby, 1992, Hems *et al.* 1994, Glasby *et al.* 1997, 1998, Fullarton *et al.* 1998, Lawson and Glasby, 1998).

The ovine brachial plexus is formed by the ventral rami of the sixth (C6), seventh (C7), eighth (C8) cervical nerves and the first (T1) thoracic nerve (Welch, 1994). The C6 root contributes to the innervation of the supraspinatus, infraspinatus and subscapularis muscles, and the formation of the musculocutaneous and median nerves. Since the loss of the C6 root would cause only slight proximal limb weakness (Dyce *et al.* 1987), and it is equivalent to a root most commonly injured in OBPP, the C6 root was chosen as the experimental model for this study.

5.2.2 Experimental and control groups

Twelve adult 1-year-old female Scottish Black-face sheep with initial weights of approximately 40 kg were used. The animals were divided into two experimental groups. A 1-year-old sheep corresponds approximately to a human age of 15 to 20 years and as there is no literature to confirm this, the estimate is arbitrary. A further twenty four newborn lambs of up to one week of age were used and were divided into four experimental groups. Each of these groups for both sheep and lambs consisted of six animals. The experimental groups were as follows:

I. In lambs, a traction injury of the C6 brachial plexus root and immediate repair by FTMG.

II. In lambs, a traction injury of the C6 brachial plexus root and repair at 10 weeks by FTMG.

III. In lambs, a traction injury of the C6 brachial plexus root and immediate repair by an interfascicular nerve autograft (cable graft).

IV. In lambs, a traction injury of the C6 brachial plexus root and repair at 10 weeks by an interfascicular nerve autograft (cable graft).

V. In sheep, a traction injury of the C6 brachial plexus root and immediate repair by FTMG.

VI. In sheep, a traction injury of the C6 brachial plexus root and immediate repair by an interfascicular nerve autograft (cable graft).

A further six age, sex and weight matched adult Scottish Black-face sheep were used as unoperated controls.

Obstetrical plexus injuries usually occur during birth in human clinical circumstance. However, for the present experiment it was difficult to acquire lambs immediately at birth so lambs of up to 1 week of age were used.

5.2.3 Preparation of animals

The sheep and lambs were supplied by the Moredun Foundation. They were obtained seven days prior to surgical procedure to allow an appropriate period for acclimatization to their new environment (University Federation for Animal Welfare Handbook, 1989). The animals were housed in the Medical Faculty Animal Area. The care of the animals were in accordance with the recommendations of the Home Office Animals (Scientific Procedures) Act 1986.

5.2.4 Anaesthesia

The night prior to surgery food but not water was withheld. Anaesthesia was induced with a combination of etomidate (Hypnomidate; Janssen-Cilag) 0.5 mg kg^{-1} and midazolam (Hypnovel; Roche) 0.5 mg kg^{-1} given by intravenous injection. Once unconscious and the jaw relaxed, laryngoscopy was performed and the trachea intubated with a cuffed endotracheal tube. The endotracheal tube was then attached to a mechanical ventilator (Blease Manley) and gas flow set at a 1 : 2 mixture of oxygen and nitrous oxide respectively. Anaesthesia was maintained with halothane administered from an out-of-circuit calibrated vaporizer (Fluotec Mark 3; Cyprane) set at 2-1.5% for the first ten minutes, and 1-1.5% thereafter.

During the operation, body temperature was maintained with a heating blanket. Saliva and rumenal fluid losses were replaced with lactated Ringer's solution at 10 ml kg^{-1} h^{-1} given by intravenuous injection. A bedside unit (Hellige Servomed SMV 104-S) was used to measure physiological variables and was equipped with modules for heart rate, respiratory rate and blood pressure monitoring. Arterial blood pressure was monitored using a strain-gauge transducer connected to a catheter in the auricular artery. A thermistor probe (Technoterm 110, RS Products Ltd West Germany) was placed in the oesophagus for measuring temperature. During the procedure the animal was also monitored using an electrocardiogram (ECG) and urinary catheter.

5.2.5 Preparation for surgery

For exposure of the left brachial plexus the surgical approach was the same for lambs and sheep. The animal was positioned in the lateral recumbent position with the left side up and the neck extended. Just prior to surgery the neck and the prescapular area were closely shaven with electric clippers (Oster Professional Products, Milwaukee, USA). To remove any extra hair immac cream (Reckitt and Coleman Ltd, Hull, UK) was applied to the shaved area for 5 minutes. The skin was washed with warm tap water to remove the immac cream and then cleaned with an iodine antiseptic solution (Betadine, Seton Health Care Group plc, Oldham, UK) to remove any excess dirt and oil.

5.2.6 Surgical procedures

All surgery was performed in an operating theatre under full sterile conditions. Sterile drapes were placed over the animal and around the operating site. From the spinous process of C6, an incision was made and extended down through the posterior triangle of the neck to a point just over the cranial border of the scapula. The skin edges were retracted with a self-retaining retractor and the underlying fascia divided with a cutting diathermy (Codman, Randolph USA). Haemostasis was obtained by means of a bipolar diathermy (Codman, Randolph USA) so that bleeding would not disturb the operative field. The platysma and splenius cervicis muscles were divided at their attachments to the scapula and the cranial border of the scapula was mobilised. A retractor was then placed deep to the scapula and its cranial border was removed away from the thoracic wall which allowed for excellent exposure of

the brachial plexus. The plexus was enveloped in a layer of adipose tissue although with careful dissection the suprasapular nerve or the upper trunk of the brachial plexus could be quickly and positively identified. The suprascapular nerve was traced centrally and the C6 root of the brachial plexus was identified.

To produce a traction injury that was consistent in morphology and extent, the C6 nerve was first clamped by two adjacent Dunhill clamps which had been closed to the third point of the ratchet. By applying a distraction force for 30 seconds between the two clamps an extension of the nerve of 0.5 cm was achieved. Including the section of nerve where the Dunhill clamps were attached, a crushed segment of nerve approximately 1.5 cm in length resulted. In preparatory studies this technique had preserved the epineurial sheath but produced total destruction of all nerve tissue and perineurium. In the current study and for each animal, histological examination of the specimen of C6 root removed at the time of injury and repair, presented such an image where all the neural elements had ruptured with preservation of the epineurium. The injury for each animal was therefore morphologically consistent with a Sunderland type IV lesion.

In four experimental groups, the sheep and lambs underwent immediate repair of the C6 nerve root and for two experimental groups in lambs, repair was delayed.

For those animals undergoing delayed repair, after the traction injury of the C6 root the operating microscope was removed and a pair of "Ligaclips" (Ethicon, Edinburgh, UK) were attached to muscle either side of the injury to act as markers. The wound was washed out with saline solution and an antibiotic spray (Tribiotic, 3M Health Care Ltd) applied to minimise infection. The closure of the deep tissues

was accomplished with interrupted 4/0 polyglactin sutures (Vicryl, Ethicon Ltd UK), and the skin with a continuous subcuticular stitch using 6/0 polyglactin sutures (Vicryl, Ethicon Ltd UK). Permeable spray dressing (Smith and Nephew Medical Ltd) was applied to the wound and the post-operative procedures were as described in the following section. However these animals returned 10 weeks later for nerve repair. General anaesthesia was induced and maintained as previously and the C6 root was re-exposed through the old wound site.

After delayed nerve repair, the C6 nerve root was intact but in some cases there was evidence of stenosis at the site of injury. In other animals after delayed repair there was a large neuroma-in-continuity. The crushed segment of nerve was removed and the nerve stumps were trimmed back to what looked like normal nerve. This resulted in a gap of between 1.5 and 2 cm long in every case.

The surgical repair of the C6 nerve root was as follows for both the immediate and repaired groups. For repair by FTMG, during the operating procedure a piece of muscle was obtained from the sternomastoid, wrapped in aluminium foil, frozen in liquid nitrogen, and then placed in distilled water to thaw (see Chapter 2). A piece of muscle was then cut to size with fibres longitudinally aligned. The FTMG was then inserted into the gap in the C6 root and secured with epineurial interrupted 10/0 or 11/0 sutures (Ethilon polyamide, Ethicon UK Ltd.) at the proximal and distal stumps.

For "cable" grafting, nerve grafts were obtained from superficial branches of cutaneous nerves in the neck. In all cases a three-strand cable graft was required to repair the C6 root. One large fascicle was identified in the majority of the cases and

this was repaired using one of the strands of cutaneous nerve whilst the rest of the C6 root was repaired using the two further strands of cutaneous nerve. The cable graft was secured to the C6 root with epineurial interrupted 10/0 or 11/0 sutures (Ethilon polyamide, Ethicon UK Ltd). Figure 5.1 is a diagrammatic representation of a cable graft.

For all cases, a minimum number of sutures was used at the site of repair. The suture lines at the site of repair were also reinforced with fibrin glue (Tisseel Immuno AG, Vienna, Austria). The operating microscope was then removed and the wound washed with a saline solution. An antibiotic spray (Tribiotic, 3M Health Care Ltd) was then applied to minimise infection. A pair of "Ligaclips" (Ethicon, Edinburgh, UK) were attached to muscle on either side at the level of the graft to assist in future identification. The closure of the deep tissues was accomplished with interrupted 4/0 polyglactin sutures (Vicryl, Ethicon Ltd UK), and the skin with a continuous subcuticular stitch using 6/0 polyglactin sutures (Vicryl, Ethicon Ltd UK). Permeable spray dressing (Smith and Nephew Medical Ltd) was applied to the wound.

5.2.7 Post-operative procedures

Once the operation was completed the halothane was gradually reduced and until the animal was breathing spontaneously, ventilation was assisted. Ewes were brought to the operating room to prompt lamb recovery. As soon as the endotracheal tube had been removed and the animal had gained consciousness, it was returned to a pen and positioned upright in hay. The animal was observed until it was weightbearing and moving freely on the operated limb. For post-operative analgesia

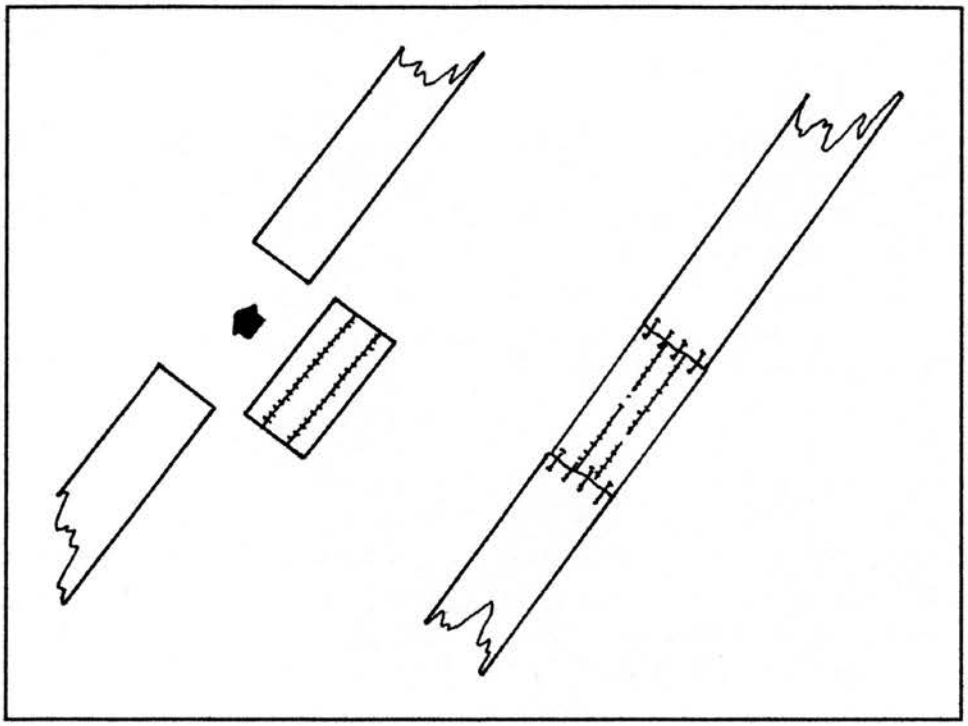


Figure 5.1 A diagrammatic representation of a cable graft.

an injection of flunixine (Finadyne: Schering-Plough Animal Health) 2.2 mg kg^{-1} was given with the first dose just prior to surgery. The postoperative medication consisted of three further doses of flunixine (Finadyne: Schering-Plough Animal Health) 2.2 mg kg^{-1} 12-hourly. An intravenous injection of a prophylactic antibiotic (Cefuroxime 750 mg 8-hourly) was given for 24 hours starting with the induction of the anaesthetic. After operation all animals recovered rapidly without obvious signs of sedation, discomfort or weakness. It was observed that all animals were able to walk within a very short time after the operation.

The animals were kept in the surgical unit for up to two weeks following operation and were then transferred to the field station without restriction of activity. Periodic checks were made to assess recovery and general well being of the animals.

5.2.8 Assessment

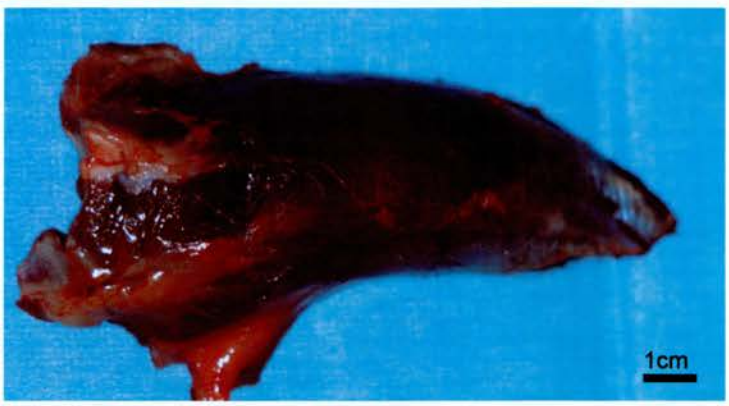
Assessment was at 1 year after the nerve-repair operation. At this time all animals were adult but in reference to the age of the animal at the time of operation, the terms "sheep" and "lamb" will still be used.

General anaesthesia was induced and maintained as previously, as was preparation for surgery and surgical exposure of the C6 root. In a parallel study electrophysiological tests were performed using an EMG to assess evoked responses and samples of nerve tissue distal to the nerve graft were taken (Fullarton *et al.* 2000). Presentation of the electrophysiological indices of nerve function has not been duplicated in this thesis. However, these data showed that when comparing immediate repair by nerve grafts in sheep and lambs, maximum conduction velocity

was significantly greater in lambs. Based on these findings, Fullarton *et al.* (2000) concluded that the potential for regeneration of nerve fibres was better in newborn animals.

After the electrophysiological measurements were complete, the animal was given a lethal intravenous injection of phenobarbitone (Lethobarb, J.M. Loveridge plc, Duphar Veterinary Ltd, Southampton, UK body) 0.7 ml kg^{-1}. Once the animal was dead, samples of nerve tissue were taken and a further incision was made from the T4 spinous process in the midline, to the inferior border of the scapula. The skin edges were retracted with a self-retaining retractor and the underlying fascia and trapezius muscle divided. This improved the exposure of the cranial border of the scapula and the supraspinatus muscle. A biopsy of muscle 1 cm by 1 cm by 1 cm was removed from the midbelly of the supraspinatus muscle but towards the cranial end (see section 5.12). Biopsies were taken from all experimental (left) and contralateral (right) supraspinatus muscles, as well as the unoperated control muscles (left and right supraspinatus muscles). Since it was found to be difficult to remove the supraspinatus muscle complete, biopsies were taken and the muscle wet weight not recorded. Figure 5.2 is a photograph of a supraspintus muscle after removal from a normal (unoperated) sheep.

The freezing of the supraspinatus muscle biopsy, as well as sectioning, histological and histochemical staining and assessment were as discussed in Chapter 2.



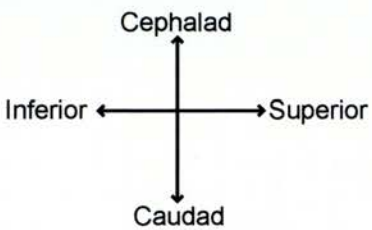


Figure 5.2 The supraspinatus muscle after removal from a normal (unoperated) sheep.

5.2.9 Removal and processing of nerve tissue

Once all electrophysiological measurements were concluded, specimens of nerve distal to the graft were taken. The nerve tissue was then processed by a standard method to produce resin embedded sections. The method is as described by Gschmeissner *et al.* (1990) with some modifications.

I. Fixation

Immediately upon removal from the animal the nerve specimen was laid on a piece of card and immersed in a solution of 4% glutaraldehyde in 0.1M sodium cacodylate buffer at 4^0C for 1 hour. This increased the firmness of the nerve section for cutting. The nerve was then removed from the solution and card and placed on dental wax. Using a dissecting microscope the damaged ends of the specimen were carefully trimmed with a new razor blade. The nerve was then cut into 1 mm thick transverse slices and replaced in the 4% glutaraldehyde in 0.1M sodium cacodylate buffer for a further hour before washing in 3 or 4 changes of 10% sucrose in 0.1M sodium cacodylate buffer. At this point the nerve sections were left overnight in the buffer solution.

The nerve tissue was placed in 1% cacodylate buffered osmium tetroxide at room temperature for 4 hours which is essential for myelin preservation. The excess osmium was removed by 3 X 20 minute washes in 10% sucrose solution, followed by 3 X 30 minute washes of 10% alcohol.

II. Embedding

At this point the nerve sections were transferred into plastic baskets and then placed in an automatic processor (Lynx, Microscopy Tissue Processor, Australian Biomedical Corporation Limited, Melbourne, Australia). The processor was preset with the following programme:

1.	70% alcohol	30 minutes	room temperature
2.	80% alcohol	15 minutes	room temperature
3.	90% alcohol	30 minutes	room temperature
4.	Absolute alcohol	30 minutes	room temperature
5.	Absolute alcohol	30 minutes	room temperature
6.	Absolute alcohol	30 minutes	room temperature
7.	Propylene oxide	30 minutes	room temperature
8.	Propylene oxide	30 minutes	room temperature
9.	Araldite / P.O. 1:1	30 minutes	room temperature
10.	Araldite / P.O. 2:1	30 minutes	room temperature
11.	Araldite	60 minutes	60^0C
12.	Araldite	12 hours	room temperature

As the Araldite had not hardened the sections were removed and placed into fresh Araldite in plastic moulds. A dissecting microscope was used to orientate the specimens parallel to the long axis of the mould and ensure a transverse section when cut. The moulds were then placed in an oven at 60^0C to polymerize for a minimum of 48 hours.

III. Cutting

The Araldite blocks were removed from the moulds and semi-thin (1μm) transverse nerve sections were cut using an ultramicrotome (Reichert OM U3) with glass knives. The glass knives were made using a LKB knifemaker (LKB Produkter, Stockholm, Sweden). Once cut the sections were laid on drops of water on clean glass microscope slides and dried flat on a hotplate. Each section was then checked under a light microscope to ensure that the whole nerve section was present and that no damage or folding had occurred.

IV. Staining

Two techniques were used for staining:

i. Toluidine Blue

Toluidine blue stains the tissue components blue and was used to check the correct orientation and preservation of the block during sectioning.

A 1% solution of toluidine blue in 1% borax (sodium tetraborate) and 1% pyronin B was used. A few drops of the stain was applied to the sections on the glass slides while still on the hot plate and left until a green line appeared around the edge of the specimen (approximately 60 seconds). The stain was then immediately washed off with cold water and the sections allowed to dry. The slides were appropriately labelled and stored unmounted. The mounting medium tended to lift the sections from the slide so cover slips were not used. Oil immersion was used when viewing the specimens.

ii. Paraphenylenediamine

This stain was useful when assessing sections for morphometric analysis as it is a more specific stain for osmiophilic material and clearly displays the myelin sheath.

Slides were placed in a Coplin jar containing a freshly filtered 1% solution of paraphenylenediamine for 20-30 minutes at room temperature. As this stain is light sensitive the Coplin jar was wrapped in aluminium foil. Sections were then washed with distilled water followed by 95% alcohol to remove any excess stain. Once dry the sections were stored unmounted.

5.2.10 Morphometric analysis of nerve tissue

The nerve sections were viewed using a compound microscope (Zeiss, Germany) at an objective power of x100 + oil immersion. A VIDS III image analysis system (Analytical Measuring Systems Ltd, Pampisford, Cambridge, UK) was used to measure the fibre and axon diameter.

Processing may cause some shrinkage of fibres due to fixation and dehydration, although it is unlikely to be more than 10% (Boyd and Davey, 1968). As all tissues were subjected to the same processing shrinkage would have been similar throughout the experimental groups.

Approximately 200 fibres were measured in each nerve section. A sample of 200 fibres is enough to represent the distribution of diameters for the entire nerve (Mayhew, 1990). An equivalent number of fibres was measured from each fascicle where the nerve was multi-fascicular. For each fascicle the nerve was divided into

equal sized sections. The number of fields required in order to obtain a measurement of approximately 200 fibres was calculated. The first field to be counted (starting from the top left of the specimen) was chosen at random by lottery and all the nerve fibres were measured for axon and fibre diameter. Following a pattern the section was then moved for example, nine fields from left to right and top to bottom and all nerve fibres in the second field were measured. The procedure was continued until 200 nerve fibres were measured. This systematic-random scheme of sampling ensured that the nerve was sampled fairly and eliminated random variation of different parts of the nerve specimen.

The axon and fibre diameter were measured at the maximum distance across the narrowest aspect of the fibre. This was recommended as it is the only measurement which is not altered by either obliquity or kinking of the section.

Once the 200 fibres were measured the myelin thickness and the G-ratio were calculated. The myelin thickness was calculated using the following formula:

$$\text{Myelin thickness} = \frac{FD-AD}{2}$$

FD = Fibre diameter (μm)

AD = Axon diameter (μm)

The G-ratio was calculated using the following formula:

$$\text{G-ratio} = \frac{AD}{FD}$$

FD = Fibre diameter (μm)

AD = Axon diameter (μm) in myelinated nerves

The G-ratio is a measure of nerve fibre maturation (Glasby *et al.* 1986b). The maturation of nerve fibres can be divided into two phases. The first is the attainment of normal axon diameter, and the second is the growth of the myelin sheath to

accomplish normal fibre diameters. If axon and fibre diameter vary "in concert", the G-ratio will remain constant whilst if myelination is reduced, the G-ratio will increase (Glasby *et al.* 1986b). For example, if axon diameter doubles and fibre diameter doubles, the myelin thickness also increases by the same factor as $\frac{AD}{FD}$ is constant.

5.2.11 Denervation of the supraspinatus muscle

The supraspinatus muscle of one age, sex and weight matched adult sheep was denervated. This was to determine the innervation of the supraspinatus muscle since it is supplied by the suprascapular nerve which takes origin from the sixth and seventh cervical nerves (Welch, 1994).

For this animal general anaesthesia was induced and maintained as previously, as was preparation for surgery and surgical exposure of the left C6 root. The left C6 root was cut and a 3 cm length of nerve was excised. No repair was made and the exposure closed as previously. The post-surgical procedure was as discussed and the animal was kept in the surgical unit for one week following operation. The animal was transferred to the field station without restriction of activity and periodic checks were made to assess recovery and general health of the animal. One month after leaving the surgical unit the animal was returned to the Medical Faculty Animal Area and was killed with an intravenous injection of phenobarbitone (Lethobarb, J.M. Loveridge plc, Duphar Veterinary Ltd, Southampton, UK body) 0.7 ml kg^{-1}.

The sheep was positioned in the lateral recumbent position initially lying on the right side. The scapular region was closely shaven and an incision was made down the cranial border of the scapula. The platysma and splenius cervicis muscles were divided at their attachments to the scapula and the cranial border of the scapula was mobilized. This exposed the supraspinatus muscle which was dissected from its attachments to the suprapinous fossa and the shoulder joint and the muscle was removed complete. After the wound was closed (to assist in the disposal of the carcass), the animal was turned onto its left side and the right supraspinatus muscle removed as per the procedure described. As mentioned already, it was difficult to remove the supraspinatus muscle completely and the muscle wet weight was not recorded. However, upon comparison of the gross appearance there was no visible sign of atrophy of the left supraspinatus muscle compared with the contralateral unoperated muscle. The muscles were wrapped in foil to reduce drying out and stored in a fridge at 4^{0}C until preparations for the freezing process were complete which was no longer than one hour.

In a parallel study, electrophysiological assessment (by stimulation of the C6 root), had determined that the motor point of the supraspinatus muscle was in the centre of the muscle belly (Fullarton *et al.* 2000). Each muscle was transected so that a centre section of the muscle measuring approximately 2 cm in length was removed. This was divided into 3 areas from cranial to caudal and these sections were further subdivided into superficial and deep. The area which would have been closest to the spine of the scapula or the most caudal section of the supraspinatus muscle was designated as area C with C1 as the superficial section and C2 the deep. The centre of the supraspinatus was called B whilst the cranial area was A. The superficial and

deep regions of areas A and B were named as per the nomenclature for area C. A 1 cm section was removed from each of these areas and with the use of a dissecting microscope the specimen was mounted on an appropriately labelled cork disc. Care was taken to ensure that the muscle fibres were appropriately orientated for freezing and transverse sectioning. The freezing, sectioning, histological and histochemical staining and assessment of these muscle specimens were as discussed in Chapter 2.

5.2.12 Statistical analysis

The distribution of fibre sizes in a normal nerve is bimodal (Sanders and Young, 1942). The data for fibre sizes from these experiments were tested and found to be bimodal. A uniform variance about the mean is required to perform a parametric test and as this does not occur in a bimodal distribution, a non-parametric test was used to analyse the data for nerve morphometry. The two tailed Kolmogorov-Smirnov test was used which is sensitive to differences in the general shapes of the distributions as well as differences in the location of the distributions (for example, differences in means).

For the data relating to muscle morphometry the protocol for statistical analysis was as discussed in Chapter 2. However, in this present study the three major variables are the age of the recipient, the method of nerve repair and the timing of repair. To measure the effects of the method of repair, immediate repair with a FTMG versus immediate repair with a nerve graft was tested between sheep or lambs. For the effects of age immediate repair with a FTMG or a nerve graft was tested for any differences between lambs and sheep. For the timing of nerve repair,

repair with a FTMG or a nerve graft was tested for any differences between immediate and delayed repair in lambs.

For comparison of data between experimental and unoperated contralateral supraspinatus muscles, matched or paired design tests (paired Student's *t*-test if parametric, Wilcoxon Signed rank test if nonparametric) were used. The paired Student's *t*-test is used to determine whether intervention makes a difference and often in the same subject. The first measurement is usually before treatment or intervention, and the second after treatment. In the current study, the right and the left supraspinatus muscles were matched and treated as though they were the same subject. Since these muscles are essentially the same except that one muscle is on the left and the other on the right side of the animal, it was considered that this was the appropriate test to use.

5.3 RESULTS

For all animals, there were no early or late complications of surgery. At 1 year after the nerve-repair operation, visual inspection of the C6 root distal to the graft revealed a full thickness nerve for all experimental groups. In the parallel study utilizing electrophysiological and morphometric indices of nerve function, it was determined that there was recovery of neuromuscular function after injury and repair of the C6 root in every experimental animal.

5.3.1 Effect of denervation on the supraspinatus muscle

The minimum fibre diameter and the form factor for type I and type II fibres were measured for each of the muscle specimens removed from the denervated supraspinatus muscle. Table 5.1 shows the values of the mean minimum diameter of type I and type II fibres of the denervated (left) and the innervated (right) supraspinatus muscles. Table 5.2 shows the values of the mean form factor of type I and type II fibres of the denervated (left) and the innervated (right) supraspinatus muscles.

For each area (A1 to C2) the left and the right muscles were compared for both minimum fibre diameter and form factor. Only the A1 and the A2 areas were significantly smaller in type I fibre diameter ($p < 0.001$, paired Student's *t*-test). For type II fibres only area A1 was significantly smaller in type II muscle fibre diameter ($p < 0.001$). Only Area A1 had a significantly greater value for form factor for both

type I and type II muscle fibres ($p < 0.001$). Based on these results the A1 area of the supraspinatus muscle was determined to be the section most affected by section of the C6 root. This area corresponded to the superficial region of the cranial end of the mid-belly of the supraspinatus muscle. Hence this was the section where all biopsies were taken for evaluation of experimental, contralateral and control supraspinatus muscle in the present study.

Area	Muscle	Type I (μm)	Type II (μm)
A1	**Denervated SS**	**37.22 ± 11.50**	**35.89 ± 10.60**
	Innervated SS	**47.52 ± 12.75**	**47.52 ± 12.75**
A2	Denervated SS	43.85 ± 12.06	41.22 ± 11.74
	Innervated SS	55.13 ± 9.85	40.36 ± 13.21
B1	Denervated SS	49.59 ± 17.20	37.22 ± 9.72
	Innervated SS	40.88 ± 13.02	34.49 ± 10.06
B2	Denervated SS	53.02 ± 14.66	39.30 ± 8.03
	Innervated SS	53.48 ± 8.41	34.42 ± 8.37
C1	Denervated SS	48.02 ± 16.01	36.42 ± 9.73
	Innervated SS	35.10 ± 11.23	30.13 ± 9.62
C2	Denervated SS	63.96 ± 13.06	49.07 ± 18.28
	Innervated SS	42.86 ± 9.67	34.72 ± 5.48

Table 5.1 - The mean and standard deviation of the minimum fibre diameter (μm) of type I and type II muscle fibres of the denervated (left) and innervated (right) supraspinatus muscles.
(The highlighted values are for the area of the supraspinatus muscle most affected by section of the C6 root that is, overall and based on the results for both minimum fibre diameter and form factor).

Area	Muscle	Type I (μm)	Type II (μm)
A1	**Denervated SS**	**0.89 ± 0.04**	**0.89 ± 0.05**
	Innervated SS	**0.85 ± 0.05**	**0.85 ± 0.06**
A2	Denervated SS	0.86 ± 0.05	0.86 ± 0.04
	Innervated SS	0.83 ± 0.06	0.81 ± 0.05
B1	Denervated SS	0.86 ± 0.08	0.84 ± 0.08
	Innervated SS	0.89 ± 0.05	0.81 ± 0.08
B2	Denervated SS	0.84 ± 0.07	0.81 ± 0.08
	Innervated SS	0.84 ± 0.06	0.75 ± 0.10
C1	Denervated SS	0.89 ± 0.05	0.85 ± 0.08
	Innervated SS	0.86 ± 0.07	0.81 ± 0.09
C2	Denervated SS	0.85 ± 0.07	0.83 ± 0.09
	Innervated SS	0.89 ± 0.04	0.83 ± 0.06

Table 5.2 - The mean and standard deviation of the form factor of type I and type II muscle fibres of the denervated (left) and innervated (right) supraspinatus muscles.
(The highlighted values are for the area of the supraspinatus muscle most affected by section of the C6 root that is, overall and based on the results for both minimum fibre diameter and form factor).

5.3.2 Incidence of muscle fibres with pathological features

The incidence of pathological features was assessed from sections stained with H & E, as discussed in Chapter 2. Muscles with pathological features (Figure 5.3a,b,c) were present in all experimental and control groups although the incidence was minimal. For any one feature (apart from granular or necrotic fibres which were not found in any experimental, contralateral or unoperated control muscle), less than 1% of fibres per experimental group exhibited each of these changes (internal nuclei, angular or split fibres) in muscle morphology. Indeed this is well below the measure by Dubowitz (1985), who argued if more than 3% of fibres in a transverse section of a muscle contain internal nuclei the muscle should be considered to have undergone a pathological change. Based on this measure it suggests that for all experimental groups, after nerve repair and injury there was minimal change and/or good recovery of muscle morphology.

5.3.3 Connective tissue content

The proportion of connective tissue was assessed by a point counting procedure as discussed in Chapter 2. However, 3000 points per muscle (as opposed to 2500 points for the rabbit study) were counted to ensure a relative standard error of less than 5%. Table 5.3 depicts the mean volume fraction of connective tissue present in the experimental, contralateral and unoperated control muscles.

All experimental muscles showed an increase in connective tissue compared with contralateral muscles (Figure 5.4), apart from after immediate repair with a

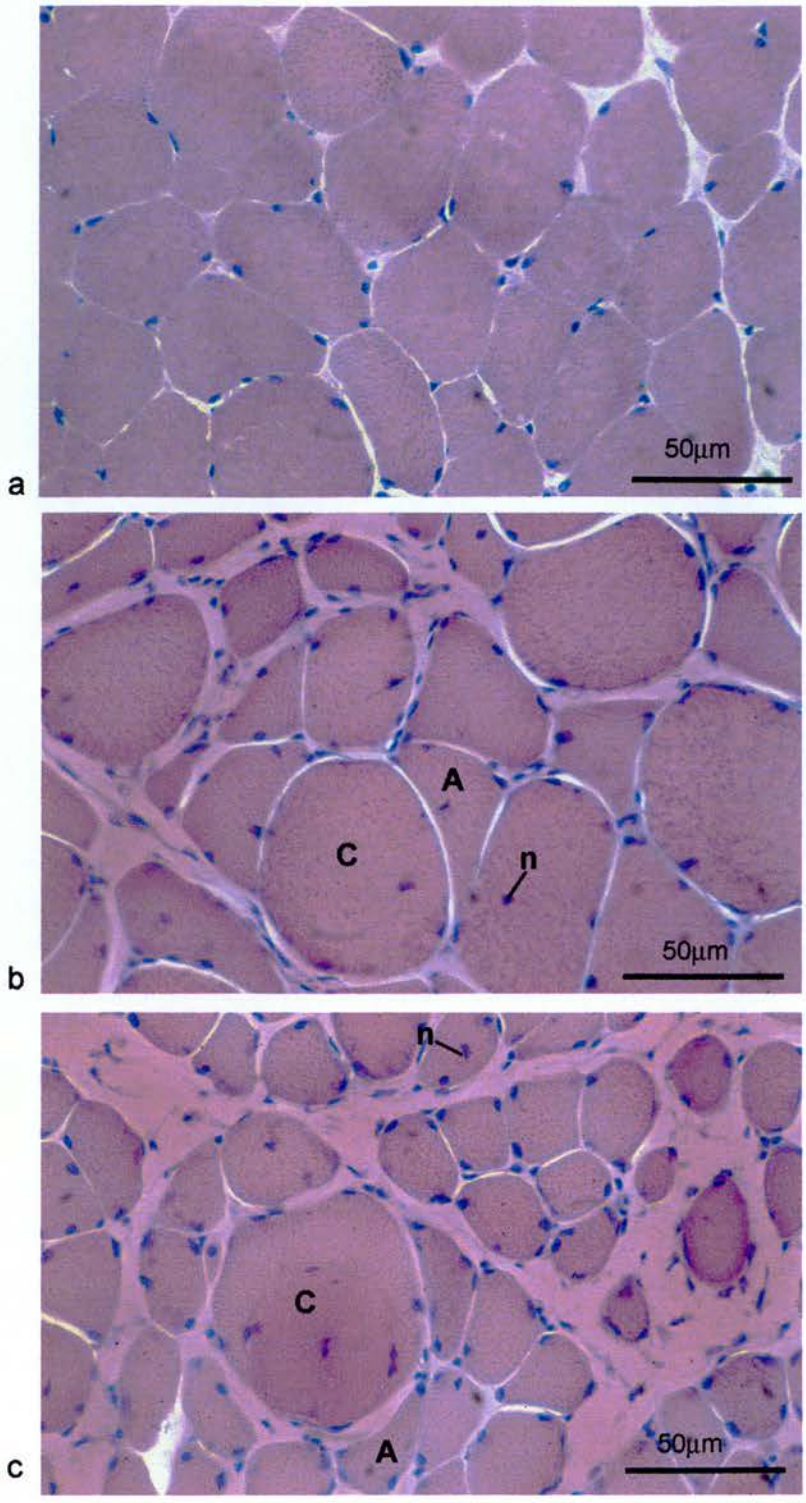


Figure 5.3 (a) The normal supraspinatus muscle (H & E).
(b) The supraspinatus after immediate repair with a FTMG in sheep (H & E).
(c) The supraspinatus after delayed repair with a nerve graft in lambs (H & E).
For both (b) and (c) note the variation in fibre size and shape. Angular fibres (A), large circular fibres(C) and internal nuclei (n) are labelled.

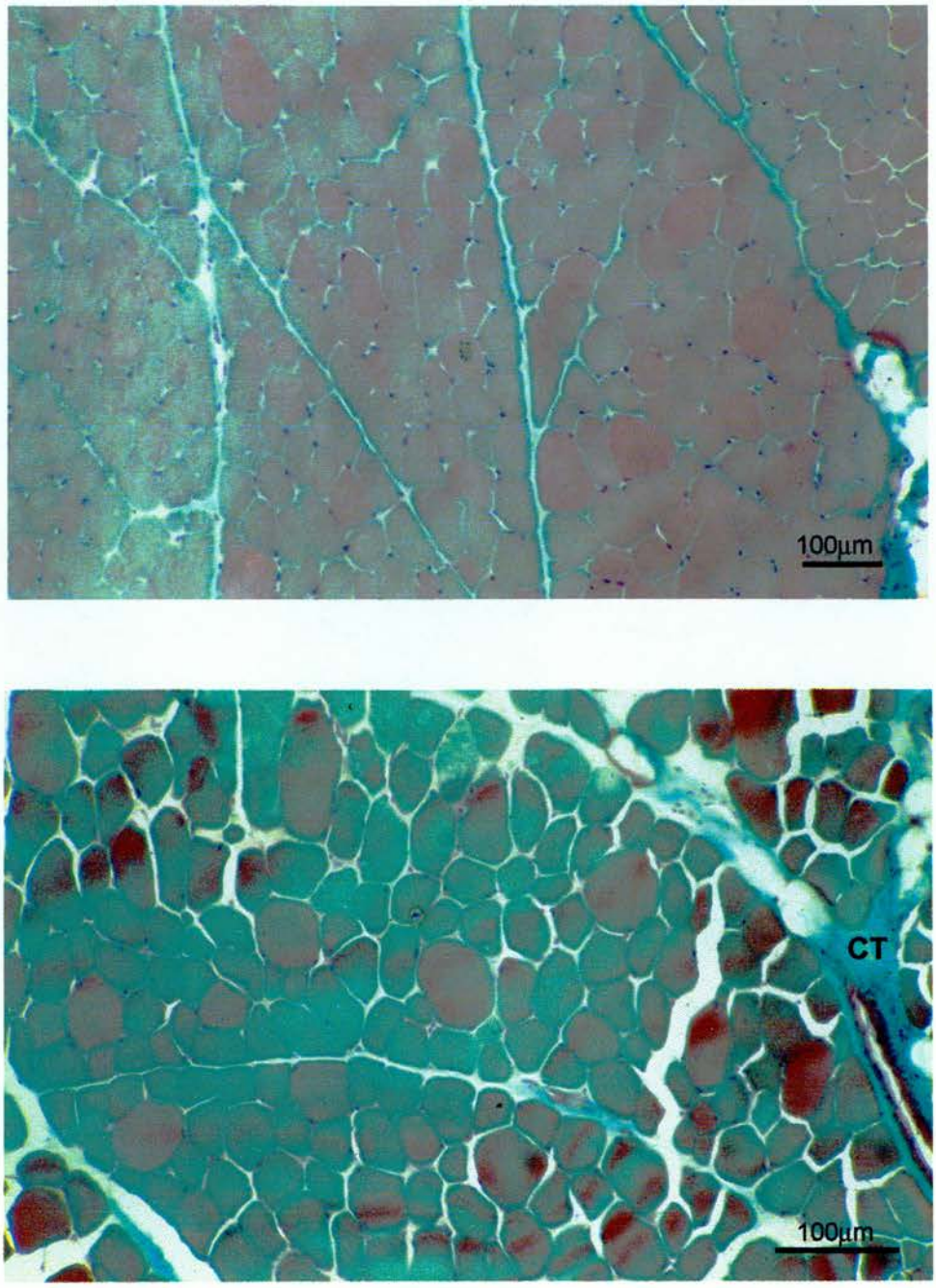


Figure 5.4 (a) The normal supraspinatus muscle (Masson's Trichrome). (b) The supraspinatus after immediate repair with a nerve graft in lambs (Masson's Trichrome). Note the increase in perimysial connective tissue (CT).

nerve graft in sheep. On average, this was a 34.10% increase on the mean volume fraction of connective tissue in contralateral muscle. For the group where connective tissue content decreased after nerve repair, this was a 3.08% decrease on the mean volume fraction of connective tissue in contralateral muscle. The difference in connective tissue content between experimental and contralateral muscles was significant after immediate repair with a nerve graft in lambs, and delayed repair with a FTMG and a nerve graft in lambs ($p < 0.05$ for each case, paired Student's *t*-test).

Comparison of the volume fraction of connective tissue with respect to repair revealed significant differences between immediate repair with a FTMG and a nerve graft in sheep ($p < 0.05$, unpaired Student's *t*-test). In terms of the age of the recipient, there was a significant increase in connective tissue after immediate repair with a nerve graft in lambs than with the same repair in sheep ($p < 0.05$).

Comparison of the volume fraction of connective tissue between experimental muscles and the unoperated control revealed significant differences ($p < 0.01$, ANOVA). Individual comparisons of the experimental groups and the unoperated control revealed that the differences were highly significant after delayed repair with a nerve graft in lambs ($p < 0.001$, unpaired Student's *t*-test). There was also a significant difference in the amount of connective tissue after immediate repair with a FTMG in sheep and a nerve graft in lambs ($p < 0.01$ for each case), and after delayed repair with a FTMG in lambs ($p < 0.05$).

In summary, there was an increase in the level of connective tissue in experimental muscle apart from after immediate repair with a nerve graft in sheep. There were three groups where the increase in the volume fraction of connective tissue was most significant, particularly in relation to levels in control muscles (both

contralateral and unoperated). These groups were immediate repair with a nerve graft in lambs, and delayed repair with a FTMG and a nerve graft in lambs. Figure 5.5 depicts the mean and the SEM for the volume fraction of connective tissue present in the experimental, contralateral and unoperated control muscles.

Procedure	Muscle	Vv (%)	RSE (%)
Sheep FTMG	Exp SS	18.33 ± 4.88	3.96
	C SS	15.40 ± 2.96	4.33
Sheep Nerve Graft	Exp SS	12.91 ± 1.53	4.77
	C SS	13.32 ± 3.41	4.77
Lambs FTMG	Exp SS	15.78 ± 4.92	4.33
	C SS	13.27 ± 3.84	4.83
Lambs Nerve * Graft	Exp SS	17.82 ± 3.85	3.98
	C SS	14.09 ± 2.16	4.54
Lambs Delayed * FTMG	Exp SS	16.71 ± 5.72	4.24
	C SS	10.23 ± 2.58	5.52
Lambs Delayed * Nerve Graft	Exp SS	22.28 ± 6.02	3.50
	C SS	15.61 ± 3.76	4.33
Unoperated Control	Left SS	12.64 ± 2.20	4.85
	Right SS	12.53 ± 2.38	4.89

Table 5.3 - The mean and standard deviation for the volume fraction (Vv) of connective tissue present in the experimental (Exp) and contralateral (C) supraspinatus muscles, and the left and the right supraspinatus (SS) muscles of the unoperated control group (RSE = Relative Standard Error).

* A significant result for the experimental or unoperated control group when compared to those values for the contralateral/right side

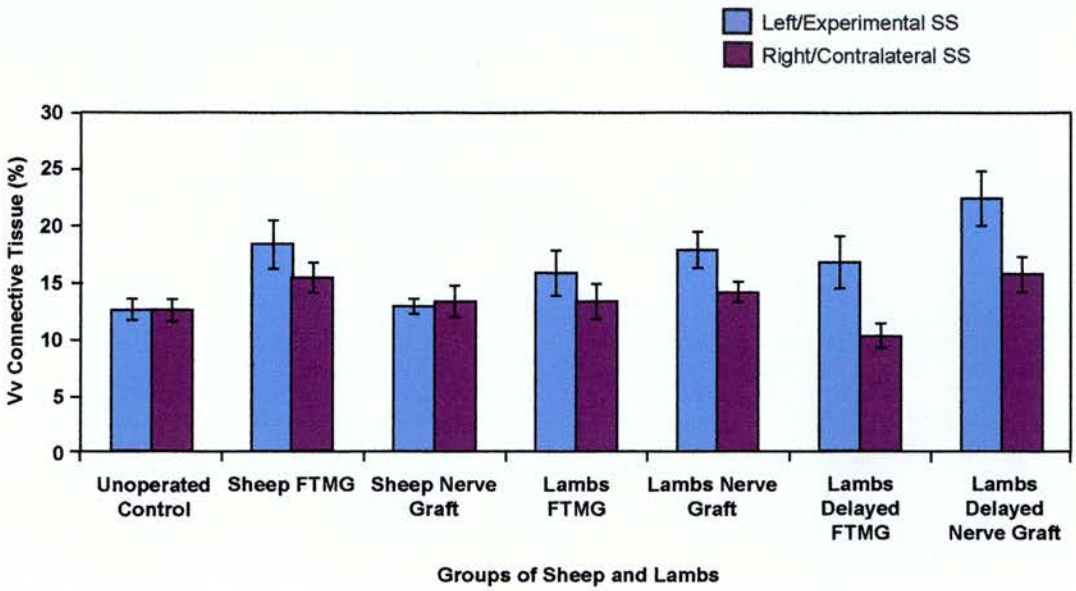


Figure 5.5 The mean and the SEM for the volume fraction (Vv) of connective tissue present in the supraspinatus muscles of the experimental and control groups.

5.3.4 Proportion of fibre types I and II

The frequency of type I and type II muscle fibres was assessed from sections stained for ATPase at pH 4.35 and 10.20, as discussed in Chapter 2. Table 5.4 shows the mean and the standard deviation values for the relative proportion of type I and type II fibres within the experimental, contralateral and unoperated control supraspinatus muscles.

In the supraspinatus muscle, type II fibres were predominant in the experimental and the contralateral muscles (Figure 5.6). However, comparison of fibre type proportions for the experimental and corresponding contralateral muscles show an increase in the number of type I fibres, and a decrease in type II fibres after nerve repair. The only exception to this was after delayed repair with a FTMG in lambs. In this case the number of type I fibres decreased whilst the numbers of type II fibres increased. On average, type II fibres make up 72.10% of the total number of fibres present in the experimental muscle and 77.94% in the contralateral muscles. There was only group where there was a significant difference in the percentage of type I or type II fibres present between experimental and contralateral muscles, and this was after delayed repair with a nerve graft in lambs. For this group, in experimental muscles there was a significant increase in the number of type I fibres with a significant decrease in the proportion of type II fibres ($p < 0.05$, Wilcoxon Signed Rank test).

On comparing the results with respect to the method of repair, there were significant differences in the proportions of type I and type II fibres. After immediate repair with a FTMG in lambs there was a significant increase in the number of type I

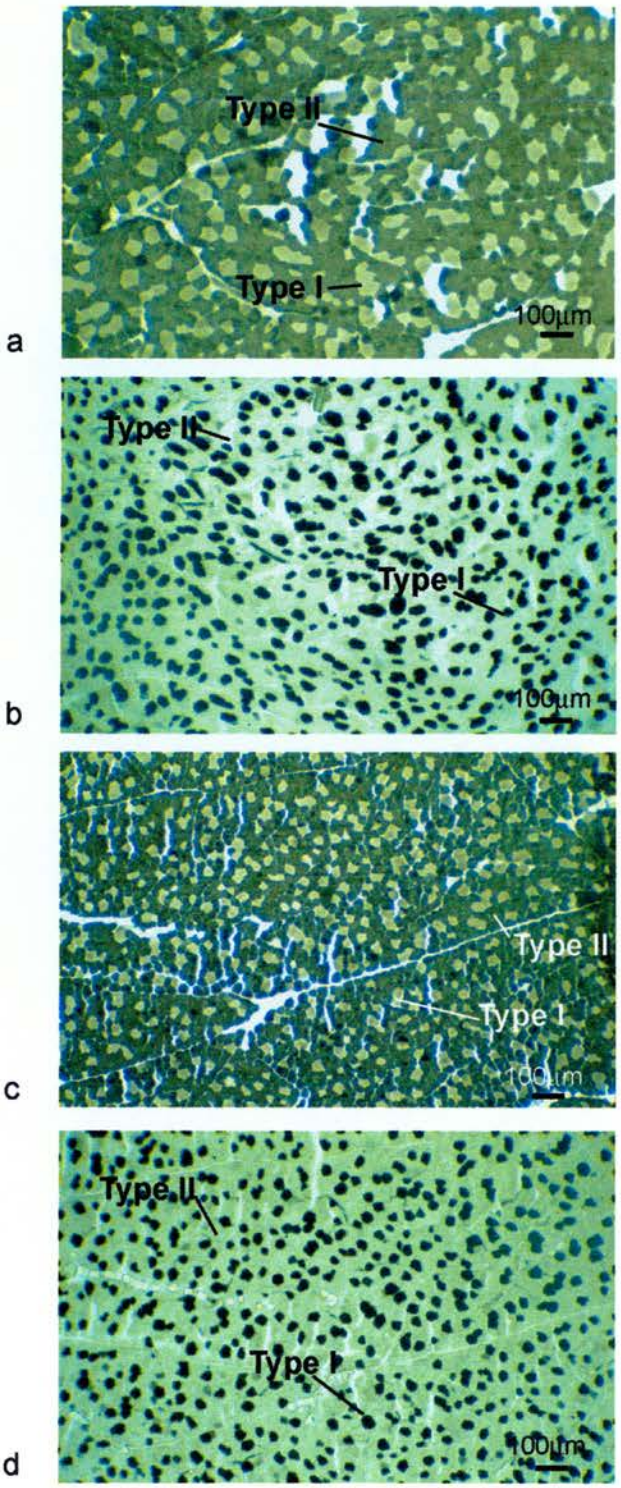


Figure 5.6 (a) A contralateral supraspinatus muscle from the sheep FTMG group (Myofibrillar ATPase after pH 10.2 pre-incubation).
(b) A contralateral supraspinatus muscle from the sheep FTMG group (Myofibrillar ATPase after pH 4.35 pre-incubation).
(c) A supraspinatus muscle after repair with a FTMG in sheep (Myofibrillar ATPase after pH 10.2 pre-incubation).
(d) A supraspinatus muscle after repair with a FTMG in sheep (Myofibrillar ATPase after pH 4.35 pre-incubation).
Type I and type II fibres are labelled.

fibres than after immediate repair with a nerve graft in lambs ($p < 0.05$, Mann-Whitney *U* test). As to the age of the recipient, there were significantly more type I and significantly fewer type II fibres after immediate repair with a nerve graft in sheep when compared with those values for immediate repair with a nerve graft in lambs ($p < 0.05$). With respect to the timing of repair, there were significantly more type I and significantly fewer type II fibres after delayed repair with a nerve graft in lambs than after immediate repair with a nerve graft ($p < 0.05$). After immediate repair with a FTMG in lambs there were significantly more type I and significantly less type II fibres than after delayed repair with a FTMG ($p < 0.01$).

Comparison of the proportions of type I and type II fibres between experimental muscles and the unoperated control revealed significant differences ($p < 0.001$, Kruskal-Wallis test). Individual comparisons of the experimental groups and the unoperated control revealed that after immediate repair with a FTMG in sheep there were significantly more type I and less type II fibres when compared with those values for the supraspinatus muscles of the unoperated control ($p < 0.01$ Mann-Whitney *U* test). Immediate repair with a nerve graft in sheep, and delayed repair with a nerve graft in lambs resulted in significantly more type I and less type II fibres when compared with those values for the unoperated control ($p < 0.05$ for each case). There were significantly fewer type I and more type II fibres in experimental muscles after immediate repair with a nerve graft in lambs, and delayed repair with a FTMG in lambs, when compared to the supraspinatus muscles of the unoperated control ($p < 0.05$ for each case).

Overall there were significant differences when the proportions of type I and type II fibres for all of the contralateral supraspinatus muscles of the experimental

groups were compared to that found in the supraspinatus muscles of the unoperated control ($p < 0.01$, Kruskal-Wallis test). Individual comparison of these groups revealed that after immediate repair with a FTMG or a nerve graft in sheep, the contralateral muscles had significantly more type I and less type II fibres when compared with those values for the supraspinatus muscles of the unoperated control ($p < 0.05$ for each case, Mann-Whitney *U* test) .

In summary, after nerve repair type II fibres remained the predominant fibre type in the supraspinatus muscle for all lamb and sheep groups. For some groups, there was an increase in the number of type I fibres and a decrease in the proportion of type II fibres after nerve repair. After delayed repair with a nerve graft in lambs this change in proportion of fibres types was most consistent.

Figure 5.7 depicts the mean and the SEM for the relative proportions of type I, and Figure 5.8 for type II fibres, in the supraspinatus muscles of the experimental and control groups.

Procedure	Muscle	Type I	Type II
Sheep FTMG	Experimental SS	37.97 ± 13.73	62.03 ± 13.73
	Contralateral SS	31.45 ± 10.54	68.55 ± 10.54
Sheep Nerve Graft	Experimental SS	31.64 ± 13.50	68.36 ± 13.50
	Contralateral SS	26.41 ± 7.24	73.59 ± 7.24
Lambs FTMG	Experimental SS	40.38 ± 29.93	59.62 ± 29.93
	Contralateral SS	27.20 ± 15.53	72.80 ± 15.53
Lambs Nerve Graft	Experimental SS	16.86 ± 7.69	83.14 ± 7.69
	Contralateral SS	16.17 ± 4.81	83.83 ± 4.81
Lambs Delayed FTMG	Experimental SS	12.56 ± 3.33	87.44 ± 3.33
	Contralateral SS	13.76 ± 2.91	86.25 ± 2.91
Lambs Delayed Nerve Graft	Experimental SS	27.98 ± 7.87 *	72.02 ± 7.87 *
	Contralateral SS	17.39 ± 2.86	82.61 ± 2.86
Unoperated Control	Left SS	19.01 ± 6.73	80.99 ± 6.73
	Right SS	18.66 ± 6.21	81.84 ± 6.21

Table 5.4 - The mean and standard deviation values for the relative proportions (%) of type I and type II fibres for the experimental (Exp) and contralateral (C) supraspinatus muscles, and the left and the right supraspinatus (SS) muscles of the unoperated control group.

* A significant result for the experimental or unoperated control group when compared to those values for the contralateral/right side

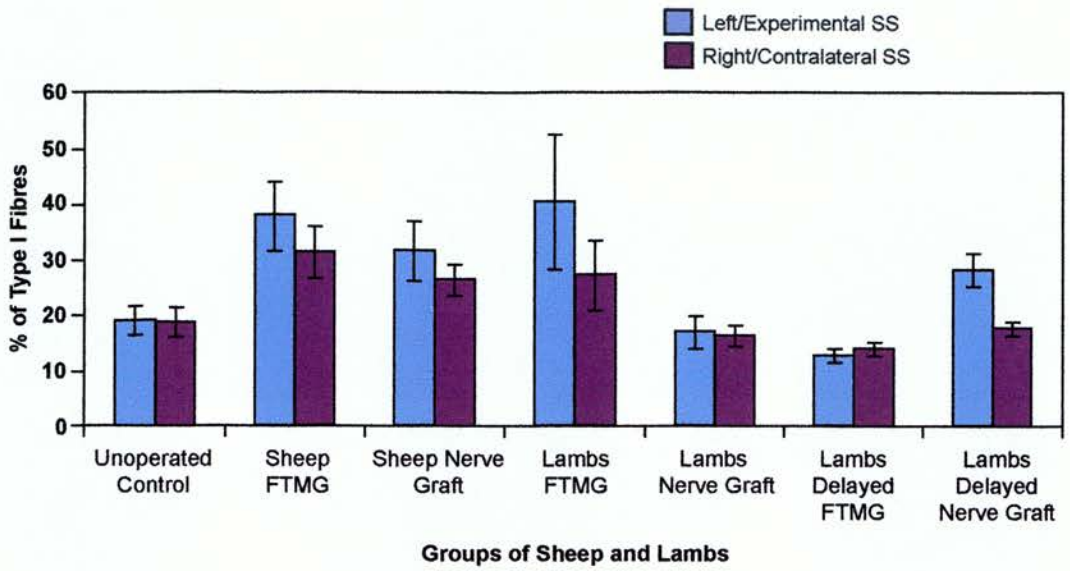


Figure 5.7 The mean and the SEM for the relative proportions of type I fibres in the supraspinatus muscles of experimental and control groups.

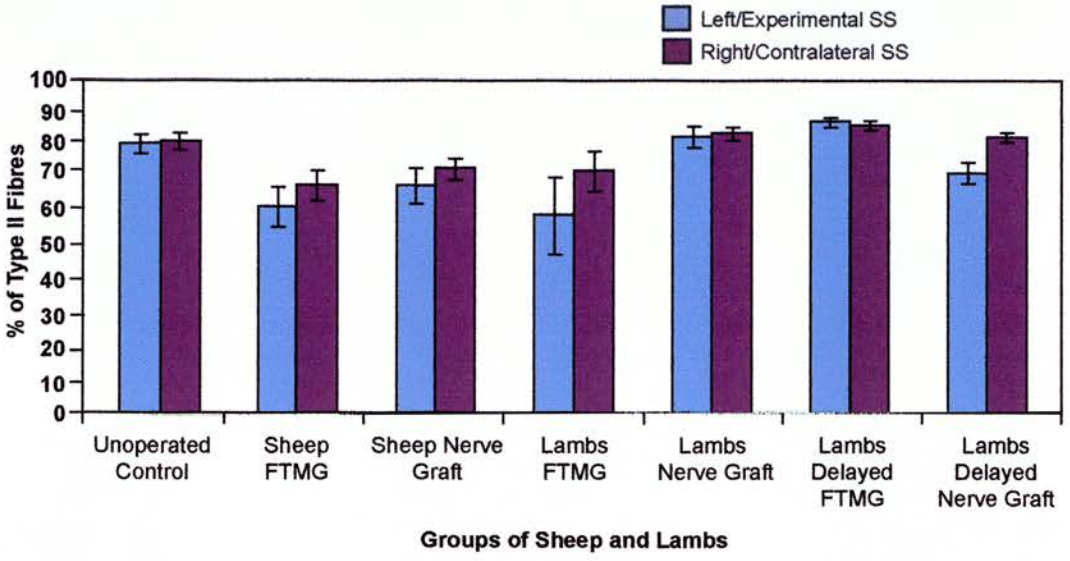


Figure 5.8 The mean and the SEM for the relative proportions of type II fibres in the supraspinatus muscles of experimental and control groups.

5.3.5 Muscle fibre diameter

The muscle fibre diameter was also assessed from sections stained for ATPase at pH 4.35 and 10.20, as discussed in Chapter 2. Table 5.5 shows the values of the mean minimum diameter of type I and type II fibres of the experimental, contralateral and unoperated control supraspinatus muscles. Figure 5.9 depicts the mean and the SEM for the minimum fibre diameter of type I, and Figure 5.10 for type II fibres, in the supraspinatus muscles of the experimental and control groups.

After repair the mean minimum diameter of some type I experimental muscle fibres increased when compared to the corresponding contralateral muscles. On average, this was a 9.74% or 4.52 μm increase on the mean minimum diameter of contralateral type I muscle fibres. The only exception to this was after delayed repair with either a FTMG or a nerve graft, or immediate repair with a nerve graft all in lambs where the mean minimum diameter of type I fibres decreased by an average 2.11% or 1.05 μm. The differences in diameter of type I fibres between experimental and contralateral muscles were only significant after immediate repair with a nerve graft in sheep and a FTMG in lambs ($p < 0.001$ for both cases, paired Student's *t*-test). Furthermore both of these groups had the largest increase in mean minimum diameter of type I fibres. After repair with a FTMG in lambs the mean diameter of type I fibres increased 15.30% or 7.15 μm, whilst after repair with a nerve graft in sheep, the rise was 10.90% or 4.85 μm. For the unoperated control, the mean diameter of type I fibres of the left supraspinatus muscle was significantly larger than the right supraspinatus muscle ($p < 0.01$). This was a 9.55% or 4.16 μm difference in

mean minimum diameter of type I fibres between the left and the right supraspinatus muscles.

With respect to the method of repair, the mean minimum diameter of type I fibres in both sheep and lambs was significantly greater after repair with a FTMG when compared to repair with a nerve graft ($p < 0.001$ for each case, unpaired Student's *t*-test). As to the age of the recipient, the mean minimum diameter of type I fibres was significantly greater after repair with a nerve graft in sheep than after the same repair in lambs ($p < 0.001$). For the timing of repair, the mean minimum diameter of type I fibres was significantly greater after immediate repair with a FTMG and after delayed repair with a nerve graft ($p < 0.001$ for each case).

Overall there were significant differences when the minimum diameters of type I fibres for all of the experimental and the unoperated control groups were compared ($p < 0.001$, ANOVA). Individual comparison of the experimental and control groups revealed that the mean minimum diameter of type I fibres was significantly greater in most experimental muscles when compared to that from the unoperated control ($p < 0.001$ all groups except delayed repair with a FTMG in lambs where $p < 0.01$ and immediate repair with a nerve graft in lambs where $p > 0.05$, unpaired Student's *t*-test).

There were significant differences when the mean minimum diameter of type I fibres for all of the contralateral supraspinatus muscles of the experimental groups were compared to that found in the supraspinatus muscles of the unoperated control ($p < 0.001$, ANOVA). Individual comparison of these groups revealed that in most contralateral muscles the mean minimum diameter of type I fibres was significantly greater when compared to that from the unoperated control ($p < 0.001$ all groups

except delayed repair with a FTMG in lambs where $p < 0.01$ and after immediate repair with a nerve graft in lambs and sheep where $p > 0.05$, unpaired Student's *t*-test).

For the type I fibres in all experimental, contralateral and unoperated supraspinatus muscles, the variability coefficient was greater than a value of 250 (where the standard deviation of the mean minimum fibre diameter was greater than 0.25 of the value of the mean diameter in the experimental muscles). This suggests that there was an abnormal variability in the size of type I muscle fibres in experimental and contralateral muscles, as well as the control or "normal" supraspinatus muscle (Dubowitz, 1985).

For most repair groups the mean minimum diameter of type II muscle fibres of the experimental supraspinatus was less than that of the corresponding contralateral muscle. On average, this was a 5.81% or 2.68 μm decrease on the mean minimum diameter of contralateral type II muscle fibres. The only exception to this was after delayed repair with a nerve graft in lambs where the diameter of type II fibres in experimental muscle was significantly greater ($p < 0.05$, paired Student's *t*-test). For this group it was a 3.55% or 1.34 μm increase on the mean minimum diameter of its corresponding contralateral type II muscle fibres. For the other groups, the decrease in diameter of experimental muscle was most significant after immediate repair with a nerve graft in lambs and a FTMG in sheep ($p < 0.001$ for each case). Next was immediate repair with a nerve graft in sheep ($p < 0.01$) and followed by immediate repair with a FTMG in lambs ($p < 0.05$). For the unoperated control, the diameters of the type II fibres of the left supraspinatus muscle were significantly larger than those of the right supraspinatus muscle ($p < 0.001$). This was

a 10.21% or 3.86 μm difference in mean minimum diameter of type II fibres between the left and the right supraspinatus muscles.

With respect to the method of repair, the mean minimum diameter of type II fibres in both sheep and lambs was significantly greater after immediate repair with a FTMG when compared to immediate repair with a nerve graft ($p < 0.001$, unpaired Student's *t*-test). In terms of the age of the recipient, the mean minimum diameter of type II fibres was significantly greater after immediate repair with a FTMG in sheep than after the same repair in lambs ($p < 0.001$). As to the timing of repair, the mean minimum diameter of type II fibres was significantly greater after immediate rather than delayed repair with a FTMG ($p < 0.01$).

Overall there were significant differences when the minimum diameters of type II fibres for all of the experimental and the unoperated control groups were compared ($p < 0.001$, ANOVA). Individual comparison of the experimental and control groups revealed that after immediate repair by a FTMG in sheep and lambs the mean minimum diameter of type II fibres of experimental muscle was significantly greater than that of the unoperated control ($p < 0.001$ for immediate repair with a FTMG in sheep, $p < 0.01$ for immediate repair with a FTMG in lambs, unpaired Student's *t*-test).

There were significant differences when the mean minimum diameter of type II fibres for all of the contralateral supraspinatus muscles of the experimental groups were compared to that found in the supraspinatus muscles of the unoperated control ($p < 0.001$, ANOVA). Individual comparison of these groups revealed that in most contralateral muscles the mean minimum diameter of type II fibres was significantly greater when compared to that from the unoperated control ($p < 0.001$ for all groups

except for delayed repair with a nerve graft in lambs where $p < 0.01$ and after delayed repair with a FTMG in lambs where $p > 0.05$, unpaired Student's *t*-test).

The variability coefficient was greater than 250 for type II fibres in all experimental, contralateral and unoperated muscles. This was similar to the findings for type I fibres and suggests that there was an abnormal variability in the size of type II muscle fibres in the experimental, contralateral and unoperated control supraspinatus muscles (Dubowitz, 1985).

The mean minimum diameter of type I and type II fibres was compared in each experimental, contralateral and unoperated control supraspinatus muscle. After nerve repair the mean minimum diameter of type I fibres was consistently greater than the mean minimum diameter of type II fibres. In the contralateral muscles, the mean minimum diameter of type I fibres was also always larger than the mean minimum diameter of type II fibres apart from after repair with a FTMG in sheep. For both left and right supraspinatus muscles of the unoperated control, the mean minimum diameter of type I fibres was also consistently greater than the mean minimum diameter of type II fibres. The difference in diameter between type I and II fibres for most experimental, contralateral and unoperated control muscles was highly significant ($p < 0.001$ for each case except for the contralateral muscles of the sheep FTMG repair group where $p > 0.05$, unpaired Student's *t*-test).

In summary, the diameter of type I muscle fibres was consistently greater in both experimental and contralateral muscles. Furthermore for most experimental groups it was apparent that there was a preferential atrophy of type II fibres after nerve injury and repair. Of all experimental groups, immediate repair with a FTMG

in lambs was the better performer based on diameters for type I and type II muscle fibres.

Procedure	Muscle	Type I (µm)	Type II (µm)
Sheep FTMG	Experimental SS	53.44 ± 17.22	46.67 ± 15.40 *
	Contralateral SS	51.87 ± 15.29	53.05 ± 13.37
Sheep Nerve Graft	Experimental SS	49.34 ± 18.27 *	38.41 ± 11.74 *
	Contralateral SS	44.49 ± 17.53	40.36 ± 13.21
Lambs FTMG	Experimental SS	53.88 ± 16.50 *	41.35 ± 12.23 *
	Contralateral SS	46.73 ± 15.75	42.87 ± 12.76
Lambs Nerve Graft	Experimental SS	43.32 ± 14.46	38.66 ± 12.05 *
	Contralateral SS	43.41 ± 13.33	41.21 ± 10.84
Lambs Delayed FTMG	Experimental SS	46.74 ± 17.48	39.02 ± 12.59
	Contralateral SS	48.28 ± 14.96	40.01 ± 10.47
Lambs Delayed Nerve Graft	Experimental SS	50.53 ± 18.30	39.10 ± 11.03 *
	Contralateral SS	52.06 ± 17.91	37.76 ± 10.19
Unoperated Control	Left SS	45.36 ± 14.11 *	41.68 ± 12.52 *
	Right SS	43.55 ± 14.21	37.82 ± 13.36

Table 5.5 - The mean and standard deviation of the minimum fibre diameter (µm) of type I and type II muscle fibres of the experimental (Exp) and contralateral (C) supraspinatus muscles, and the left and the right supraspinatus (SS) muscles of the unoperated control group.

* A significant result for the experimental or unoperated control group when compared to those values for the contralateral/right side

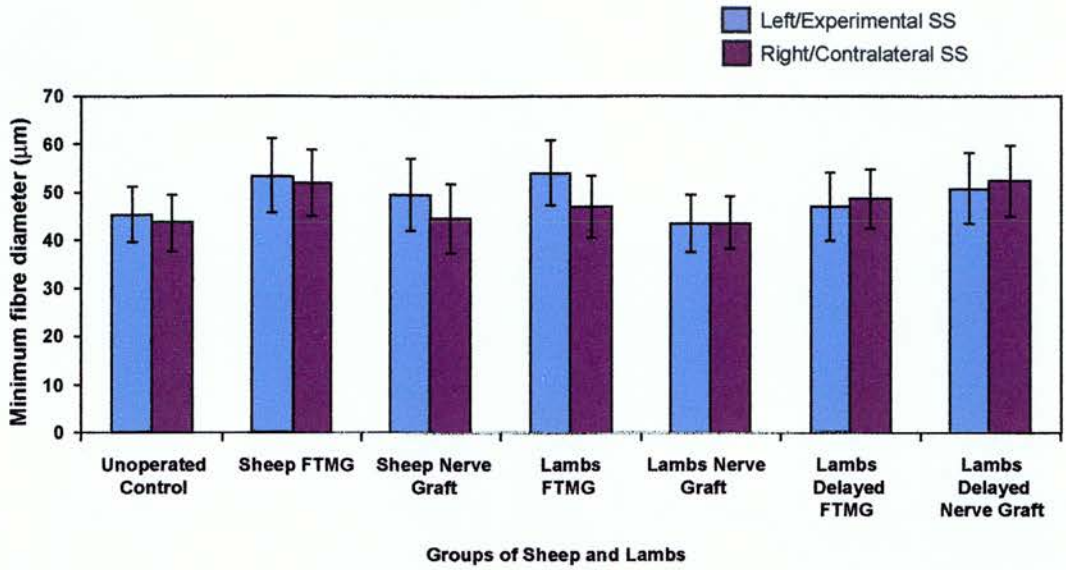


Figure 5.9 The mean and the SEM for the minimum fibre diameter (µm) of type I fibres in the supraspinatus muscles of the experimental and control groups.

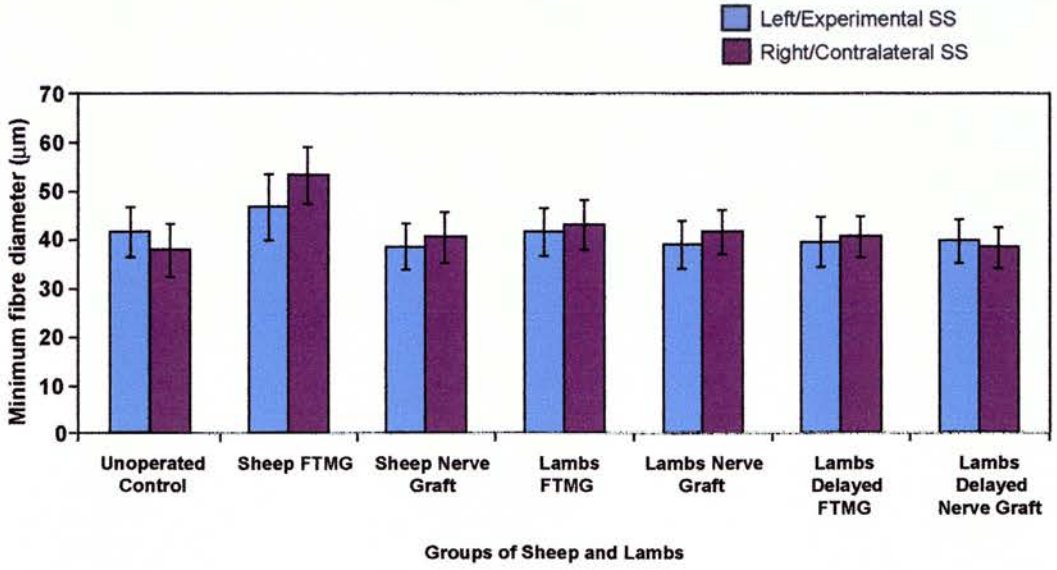


Figure 5.10 The mean and the SEM for the minimum fibre diameter (µm) of type II fibres in the supraspinatus muscles of the experimental and control groups.

5.3.6 Muscle fibre form factor

As with assessment of muscle fibre size, sections stained for ATPase at pH 4.35 and 10.20 were used to measure the shape of type I and II muscle fibres. Table 5.6 shows the values of the mean form factor of type I and type II fibres of the experimental, contralateral and unoperated control supraspinatus muscles.

For most experimental muscles the form factor of type I fibres were less than those of corresponding contralateral muscles. The only exceptions were after immediate repair with either a FTMG or a nerve graft in lambs where the mean form factor were similar in value. The difference in form factor of type I fibres between experimental and contralateral muscles was significant after delayed repair with a nerve graft in lambs, delayed repair with a FTMG in lambs and immediate repair with a nerve graft in sheep ($p < 0.05$ for each case, Wilcoxon Signed Rank test).

With respect to the method of repair, the form factor of type I fibres was significantly greater after repair with a nerve graft in sheep when compared to repair with a FTMG ($p < 0.05$, Mann-Whitney *U* test). As to the age of the recipient, the mean form factor of type I fibres was greater in lambs than sheep after repair with either a FTMG or a nerve graft ($p < 0.01$ for FTMG, and $p < 0.05$ for nerve graft).

Overall there were significant differences when the form factor of type I fibres for all of the experimental and the unoperated control groups were compared ($p < 0.01$, Kruskal-Wallis test). Individual comparison of the experimental and control groups revealed that for most repair groups the form factor of type I fibres in experimental muscle was significantly greater ($p < 0.05$, Mann-Whitney *U* test). An exception to this was after repair with a FTMG in sheep where the form factor of

type I fibres in experimental muscle was significantly less than that in the unoperated control muscles ($p < 0.05$).

For all repair groups there was a decrease in mean form factor of type II fibres when compared to the findings for the corresponding contralateral muscle. The difference was significant after immediate repair with either a FTMG or a nerve graft in sheep or lambs ($p < 0.05$, Wilcoxon Signed Rank test).

The findings for form factor of type II fibres in relation to method and time of repair as well age of the animal, were similar to those for type I fibres. With respect to the method of repair and as was found with form factor of type I fibres, the form factor of type II fibres was significantly greater after repair with a nerve graft in sheep when compared to repair with a FTMG in sheep ($p < 0.05$, Mann-Whitney *U* test). As to the age of the recipient and again as was found with form factor of type I fibres, the form factor of type II fibres was greater in lambs than sheep after repair with either a FTMG or a nerve graft ($p < 0.01$ for FTMG, and $p < 0.05$ for nerve graft). A delay in nerve repair with a FTMG resulted in a significant increase in form factor of type II fibres than after immediate repair ($p < 0.05$).

Overall there were significant differences when the form factor of type II fibres for all of the experimental and the unoperated control groups were compared ($p < 0.01$, Kruskal-Wallis test). Individual comparison of the experimental and control groups revealed that for both sheep groups (FTMG and nerve graft), the form factors of type II fibres in experimental muscle were significantly less than those values for the unoperated control muscle ($p < 0.01$, Mann-Whitney *U* test).

In summary, there was a decrease in form factor of both type I and type II muscle fibres after nerve injury and repair. For both type I and type II fibres the

smaller values for form factor were consistently found in older animals after immediate nerve repair, whilst the greater values were after delayed repair in the younger animals.

Frequency histograms were plotted of the values of form factor and minimum fibre diameter for both type I and type II fibres (these histograms are not included in this thesis). This was done to establish if there was any relationship between form factor and the diameter of muscle fibres however, no correlation was found.

Procedure	Muscle	Type I	Type II
Sheep FTMG	Experimental SS	0.79 ± 0.04	0.77 ± 0.02 *
	Contralateral SS	0.80 ± 0.03	0.82 ± 0.04
Sheep Nerve Graft	Experimental SS	0.82 ± 0.03 *	0.79 ± 0.04 *
	Contralateral SS	0.86 ± 0.02	0.83 ± 0.03
Lambs FTMG	Experimental SS	0.85 ± 0.03	0.82 ± 0.03 *
	Contralateral SS	0.85 ± 0.03	0.87 ± 0.02
Lambs Nerve Graft	Experimental SS	0.86 ± 0.06	0.83 ± 0.02 *
	Contralateral SS	0.86 ± 0.01	0.87 ± 0.01
Lambs Delayed FTMG	Experimental SS	0.86 ± 0.03 *	0.85 ± 0.03
	Contralateral SS	0.89 ± 0.02	0.86 ± 0.02
Lambs Delayed Nerve Graft	Experimental SS	0.86 ± 0.04 *	0.84 ± 0.03
	Contralateral SS	0.89 ± 0.01	0.85 ± 0.02
Unoperated Control	Left SS	0.82 ± 0.02	0.83 ± 0.03
	Right SS	0.83 ± 0.03	0.83 ± 0.03

Table 5.6 - The mean and standard deviation of the form factor of type I and type II muscle fibres of the experimental (Exp) and contralateral (C) supraspinatus muscles, and the left and the right supraspinatus (SS) muscles of the unoperated control group.

* A significant result for the experimental or unoperated control group when compared to those values for the contralateral/right side

5.3.7 Nerve morphometry

Table 5.7 shows the values of the mean and standard deviation for fibre and axon diameter, the G-ratio and myelin thickness for sheep, lambs and the unoperated control. Figure 5.11 depicts the mean and the SEM for axon and fibre diameter for each experimental and control group. Figure 5.12 depicts the mean and the SEM for myelin thickness for each experimental and control group.

In all cases after nerve repair the axon and fibre diameters, and the thickness of the myelin sheath were significantly smaller than those of the unoperated control, whilst the G-ratio was significantly greater ($p < 0.001$ for each case). This means that after repair, axon and fibre size and myelination did not attain normal levels and as the G-ratio was significantly different from controls, full maturation of nerve fibres had not been attained for any experimental group. However, the percentage recovery of the morphological indices over control values did indicate that the majority of the regenerated fibres were well matured. The average percentage recovery of fibre diameter over control values was 85.94% and for axon diameter, 91.23%. For myelin sheath thickness the value was 81.62%.

With respect to the method of repair, repair with a FTMG in sheep resulted in significantly larger fibre and axon diameters, as well as a thicker myelin sheath and greater maturation of nerve fibres when compared with a nerve graft repair in sheep ($p < 0.001$ for each variable apart for $p < 0.05$ for axon diameter, Kolmogorov-Smirnov test). This was also the case when immediate repair with a FTMG was compared with immediate repair with a nerve graft in lambs ($p < 0.001$ for each variable apart for $p < 0.01$ for axon diameter and $p < 0.05$ for G-ratio).

In terms of the age of the recipient, after repair with a FTMG in sheep there were significantly larger fibre diameters, as well as a thicker myelin sheath and greater maturation of nerve fibres ($p < 0.001$ for each variable apart for $p < 0.05$ for fibre diameter). For the nerve grafts it was repair in the younger animals which resulted in significantly larger fibre and axon diameters, as well as a thicker myelin sheath and greater maturation of nerve fibres ($p < 0.001$ for each variable).

A delay in nerve repair with a FTMG in lambs resulted in significantly larger fibre and axon diameters, as well as a thicker myelin sheath and greater maturation of nerve fibres ($p < 0.001$ for each variable apart for $p < 0.05$ for fibre diameter). This was also the case after a delay in repair with a nerve graft in lambs ($p < 0.001$ for each variable).

Procedure	Fibre diameter (µm)	Axon diameter (µm)	G-ratio	Myelin thickness (µm)
Sheep FTMG	9.40 ± 4.77 *	4.60 ± 2.85 *	0.48 ± 0.12 *	2.43 ± 1.22 *
Sheep Nerve Graft	7.99 ± 3.86 *	4.35 ± 2.76 *	0.51 ± 0.12 *	1.82 ± 0.69 *
Lambs FTMG	9.16 ± 4.25 *	4.90 ± 3.03 *	0.50 ± 0.12 *	2.13 ± 0.78 *
Lambs Nerve Graft	8.41 ± 4.43 *	4.52 ±2.88 *	0.51 ± 0.11 *	1.97 ± 0.92 *
Lambs Delayed FTMG	9.19 ± 4.71 *	4.47 ± 3.06 *	0.48 ± 0.12 *	2.39 ± 1.02 *
Lambs Delayed Nerve Graft	8.65 ± 4.80 *	4.53 ± 3.37 *	0.48 ± 0.14 *	2.09 ± 0.86 *
Unoperated Control	10.24 ± 3.67	5.00 ± 2.57	0.47 ± 0.13	2.62 ± 0.87

Table 5.7 - The mean and standard deviation for myelinated fibre and axon diameter, the G-ratio and myelin thickness for experimental and control groups.

* A significant result for the experimental group when compared to those values for the unoperated control

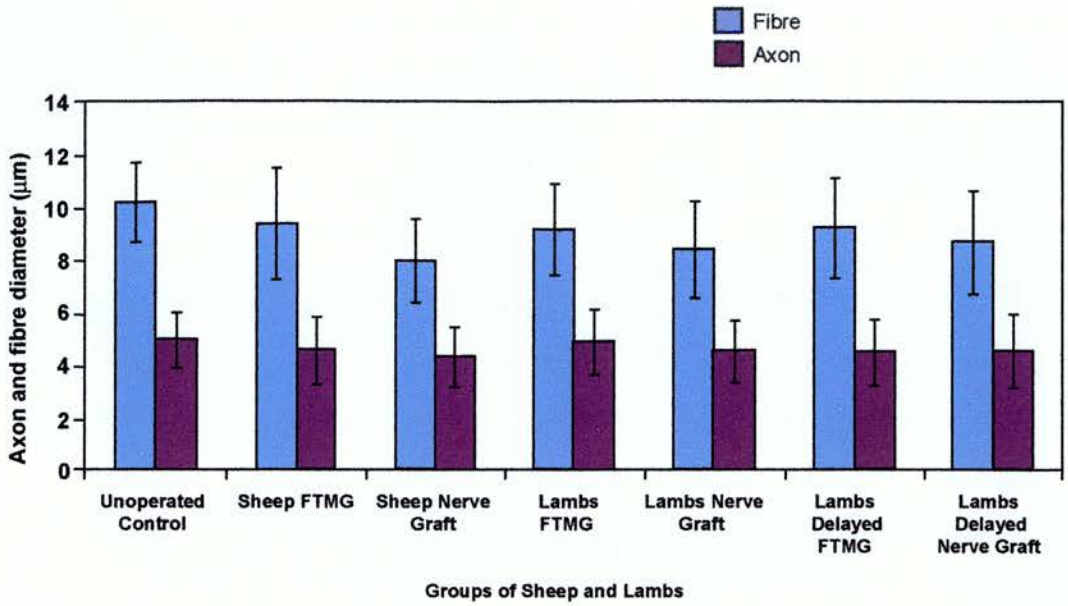


Figure 5.11 The mean and the SEM for the axon and the fibre diameter (µm) for each experimental and control group.

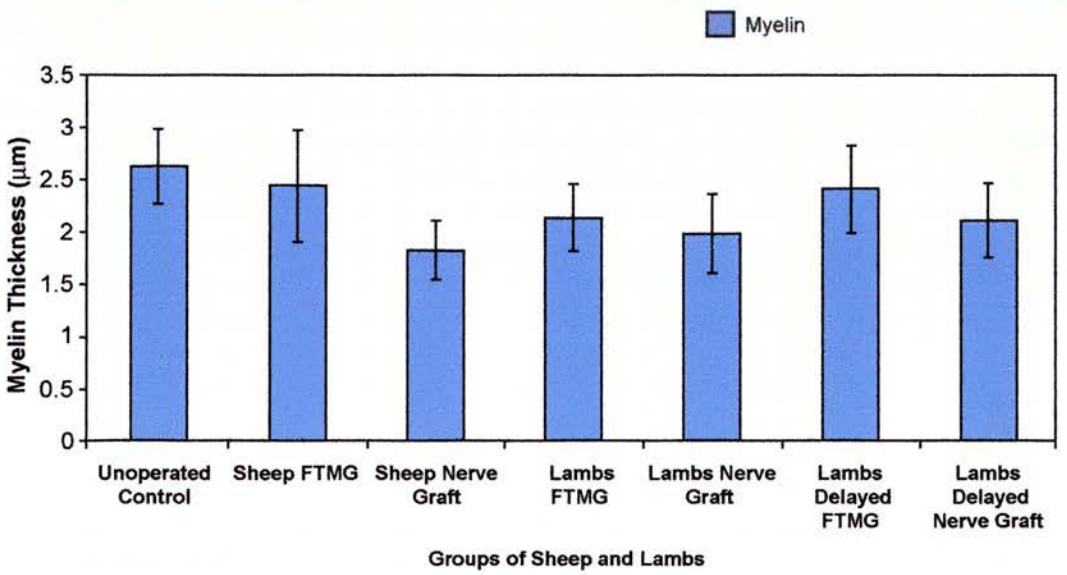


Figure 5.12 The mean and the SEM for myelin thickness (µm) for each experimental and control group.

5.4 DISCUSSION

In this chapter the three major variables were the method of repair, the age of the recipient, and the timing of repair. The conclusions are firstly, that the FTMG is on balance the best of the strategies attempted for immediate nerve repair. Secondly, for the two age groups examined (newborn - 1 week old, young adult 1-year-old), the age of the animal did not appear to adversely affect the recovery of neuromuscular function. Thirdly, after a 10 week delay in nerve repair recovery of muscle structure and function was not significantly different when compared with the findings after immediate repair. These conclusions are discussed further.

5.4.1 Method of repair

Table 5.8 shows a summary of morphological results for experimental supraspinatus muscles after comparing immediate repair with a FTMG and a nerve graft in sheep and lambs.

The data from the present study suggest that when autogenous FTMGs are used in repair of transected C6 nerve roots in sheep and lambs, the recovery in muscle fibre architecture is superior to that seen after repair by nerve autografts.

In sheep there was a significant increase in the volume fraction of connective tissue when comparing FTMG and nerve graft repair. This was also the case when comparing repair by a FTMG to the unoperated (normal) control but, the difference in connective tissue between experimental and contralateral muscle after repair by a FTMG did not reach statistical significance. It appears that the volume of connective

tissue in experimental muscle after repair by a FTMG in sheep was within normal limits for this repair group.

In lambs, when comparing FTMG and nerve graft repair, after repair with a FTMG there was a significant shift in the composition of the supraspinatus muscle from a fast to a slow muscle type although the majority of the fibres were still type II. This change in fibre type composition of the supraspinatus muscle would be due to type II fibres becoming innervated by type I motoneurones (Buller *et al.* 1960, Dubowitz, 1967, Karpati and Engel, 1967, Romanul and Van der Meulen, 1967, Gordon *et al.* 1988). Could it be that repair with a FTMG assists in the regeneration of neurones which innervate slow type muscles? This is unlikely to be the case as when comparing FTMG and nerve graft repair in sheep, the differences in fibre type proportions were insignificant. Furthermore, the differences in proportions of fibre types I and II between experimental and corresponding contralateral muscles after repair by a FTMG or a nerve graft in lambs or sheep, did not reach statistical significance. So the differences in fibre type proportions in supraspinatus muscles when comparing repair by a FTMG or a nerve graft was most likely due to individual variability and not the method of repair.

However, based on the findings for the present investigation, the method of repair does appear to influence the size of muscle fibres. In both sheep and lambs, repair with a FTMG resulted in significantly larger fibres than after repair with a nerve graft. This suggests that the number of regenerating axons which successfully reach and reinnervate either type I or type II muscle fibres was greater after immediate repair with a FTMG. This was confirmed by the findings for form factor. In sheep, after repair with a nerve graft there was a significant increase in form factor

for both type I and type II muscle fibres compared to that after repair by a FTMG. This suggests a larger number of rounded denervated muscle fibres and therefore, perhaps a longer period of denervation after repair by a nerve graft. These results are unexpected as it has been demonstrated that regeneration rates in muscle grafts and nerve grafts of equal diameter are comparable (Glasby *et al.* 1986c). However, in the repair of large diameter nerves and as was the case in the present study, "cable grafts" are used. A cable graft consists of a number of strands of nerve (forming a cable) to acquire a diameter equivalent to that of the host nerve. These grafts are not without problems as they may suffer from ischaemia, as well as the formation of fibrous connective tissue between strands (Glasby *et al.* 1990). Indeed, in a study comparing FTMG and cable grafts in the repair of large peripheral nerves, a greater number of axons had regenerated through the muscle graft (Glasby *et al.* 1990). The difficulty with the cable graft is that it appears to acquire a greater connective tissue infiltration than the FTMG, and this affects the spatial distribution within the perineurial cell sheath thereby influencing nerve regeneration (Glasby *et al.* 1990). In the present study the recovery of muscle fibre signs was better in the FTMG, suggesting (or at least consistent with) the number of regenerating axons which successfully reach and reinnervate either type I or type II muscle fibres being greater after immediate repair with a FTMG than with a cable nerve graft.

Myles and Glasby (1991) compared cable and muscle grafting and found no significant difference in the level of nerve function or nerve morphology. However, as they pointed out the cable grafts used in their study were large as each cable strand was from a mixed nerve with a large diameter endoneurial tube. In clinical practice and as was applied in the current study, small sensory nerves are used for the cable

strands, thus producing nerve grafts with smaller diameter tubes. However, the study by Myles and Glasby (1991) was in rats and mindful of this species superior regenerative ability, it is difficult comparing their investigation with the present one in sheep. This does raise the point that because this study was in a larger animal model, the results might have more relevance to nerve repair in humans. Furthermore, Myles and Glasby (1991) examined electrophysiological and morphometric indices of nerve function and not muscle morphometry. The results of the current investigation provided information on an important component of the motor unit, the muscle fibre. Hence any data on changes in muscle morphometry after nerve injury and repair, contributes to an understanding of the outcomes of such repair.

In conclusion, although there were a number of significant differences in the level of recovery of muscle morphology after muscle grafting, some of these changes appeared to be still within normal ranges. Particular assays of muscle reinnervation such as fibre diameter and shape suggest that after nerve repair with a FTMG, reinnervation is comparable if not slightly better, than after repair with a "cable" nerve graft. This was confirmed by the morphometric indices of nerve maturation (fibre diameter, axon diameter, myelin sheath thickness and G-ratio).

Assay	Sheep		Lambs	
	FTMG	Nerve Graft	FTMG	Nerve Graft
Connective Tissue Content	X	*	-	-
Proportions of Type I Fibres	-	-	*	X
Proportions of Type II Fibres	-	-	X	*
Minimum Diameter of Type I Fibres	*	X	*	X
Minimum Diameter of Type II Fibres	*	X	*	X
Form Factor of Type I Fibres	*	X	-	-
Form Factor of Type II Fibres	*	X	-	-

Table 5.8 - A comparison of the FTMG and the nerve graft in sheep and lambs, based on morphological assessment of the supraspinatus muscle.

(* = The best recovery for specified assay when FTMG and nerve graft are compared, **X** = The worst recovery for specified assay when FTMG and nerve graft are compared, - = No significant difference in specified assay when FTMG and nerve graft are compared).

5.4.2 Age of the recipient

Table 5.9 shows a summary of morphological results for experimental supraspinatus muscles after comparing immediate repair with a FTMG or a nerve graft in sheep with that in lambs.

The results of the current investigation show that after nerve repair, both young and adult age groups have a good prognosis for recovery of muscle function. Indeed morphometric indices of muscle structure such as muscle fibre diameter and

shape (after repair with either a FTMG or a nerve graft), would suggest that the degree of muscle recovery was similar for both age groups, and possibly slightly superior for the older animals. This result is surprising as the reduced capacity for nerve repair with increasing age has been well documented in animals (Black and Lasek, 1979, Pestronk *et al.* 1980, Jacob and Robbins, 1990a,b, Grieve *et al.* 1991, Tanaka and Webster, 1992, Vaughan, 1992, Verdú *et al.* 1995, Belin *et al.* 1996, Jacob and Croes, 1998). However, most of these studies have examined the influence of age on the rate and capability of nerve regeneration, and not the process of muscle recovery after denervation and nerve repair. Indeed, compared with mature muscles, immature skeletal muscles are more dependent on continued innervation for preservation of their structural integrity (Kumar and Talesara, 1977). Furthermore it has been demonstrated that muscle atrophy is greater following neonatal denervation than after adult denervation (Kumar and Talesara, 1977, Redenbach *et al.* 1988). This atrophy is primarily due to a decrease in individual fibre area as opposed to fibre loss (Redenbach *et al.* 1988). However, once reinnervation starts in younger animals its rate is greater than in older animals (Campbell and Pomeranz, 1993, Choi *et al.* 1996). These findings may help explain why in the present investigation after nerve repair the recovery of muscle structure was slightly superior in older animals. Hence, following nerve injury muscle atrophy was greater in the newborn lambs but due to a high capacity for nerve regeneration and recovery of muscle structure, reinnervation of the supraspinatus muscle in these younger animals was approaching or similar to that of the sheep by the time of assessment.

The findings for the present study are similar to those of Grieve *et al.* (1991) who investigated the effect of age on the recovery of reinnervated muscles after nerve

repair with a FTMG in juvenile, adult and elderly rats. They concluded that adult rats showed superior recovery (a similar finding to the present study), whilst the younger animals achieved "intermediate recovery". After nerve repair the elderly rats were left with "significant deficits in muscle function". It must be reiterated that the older animals used in the current investigation were not elderly but young adults. As discussed 1-year-old sheep were chosen which corresponds approximately to a human age of 15 to 20 years, although this estimate is somewhat arbitrary. It is important that this is specified since the potential for recovery of muscle structure and function after nerve repair is poor in elderly animals (Drahota and Gutmann, 1961, Grieve *et al.* 1991). Elderly animals were not part of this study as the design of the experiments was based on a clinical model, the obstetrical brachial plexus palsy which is a condition associated with newborns. As discussed, in clinical practice it is common practice to delay treatment of these injuries and so the purpose of the present investigation was to determine if age (in a finite time frame) would be detrimental to any future treatment.

In terms of the morphometric indices of nerve maturation the results were inconsistent as nerves in newborn lambs appeared to have a better potential for regeneration than that in older animals after nerve and not muscle grafting. This potential for superior recovery of neuromuscular function in lambs was confirmed in a parallel study by Fullarton *et al.* (2000). However, although Fullarton *et al.* (2000) studied electrophysiological and morphometric indices of nerve function, it was only the physiological data which established that recovery of neuromuscular function was superior in younger animals. Indeed, when the repaired groups of sheep and lambs were compared in respect of the morphological indices (fibre diameter, axon

diameter, myelin sheath thickness and G-ratio), there were no significant differences. The disparity in these results and those of the current study confirms the difficulty in evaluating the recovery of function of the motor unit. Indeed it has been reported that during regeneration, physiological indices of neuromuscular function do not agree with morphometric indices of muscle structure (Koenig and Pecot-Dechavassine, 1971). Perhaps an assessment of other components of the motor unit such as the neuromuscular junction would be helpful in determining the outcome after a delay in nerve repair.

In conclusion, for the age groups investigated (newborn - 1 week old, young adult 1-year-old), and based on the evaluation of muscle denervation/reinnervation, the age of the animal did not appear to adversely affect the recovery of neuromuscular function. However, the results for nerve maturation were inconsistent and indicate a possible shortcoming of the present study. Investigation of another element of the motor unit, the neuromuscular junction, may have assisted in the evaluation of the effect of age on recovery of neuromuscular function.

Assay	FTMG		Nerve Graft	
	Sheep	Lambs	Sheep	Lambs
Connective Tissue Content	-	-	*	X
Proportions of Type I Fibres	-	-	*	X
Proportions of Type II Fibres	-	-	X	*
Minimum Diameter of Type I Fibres	-	-	*	X
Minimum Diameter of Type II Fibres	*	X	-	-
Form Factor of Type I Fibres	*	X	*	X
Form Factor of Type II Fibres	*	X	*	X

Table 5.9 - A comparison of immediate repair with a FTMG or a nerve graft in sheep with that in lambs, based on morphological assessment of the supraspinatus muscle.

(* = The best recovery for specified assay when results for sheep and lambs are compared, **X** = The worst recovery for specified assay when results for sheep and lambs are compared, - = No significant difference in specified assay when results for sheep and lambs are compared).

5.4.3 Immediate versus delayed repair

Table 5.10 shows a summary of morphological results for experimental supraspinatus muscles after comparing immediate and delayed repair with a FTMG or a nerve graft in lambs.

The results from the current study suggest that a delay in nerve repair did not appear to assist or adversely affect the denervation/reinnervation process in muscle. There were minimal and inconsistent changes in morphometric indices of muscle structure and function when comparing immediate and delayed nerve repair with

either a FTMG or a nerve graft. Hence, the results suggest that both FTMGs and nerve grafts function after delayed repair (10 weeks before nerve repair) in a similar manner as after immediate repair in lambs.

The findings for the present study are surprising as Gattuso *et al.* (1989) found that after a delay of nerve repair with a FTMG of 8 weeks there was limited useful recovery. Motor endplates were poorly demonstrated and there was significant evidence of extensive muscle atrophy. Moreover, the study by Gattuso *et al.* (1989) was in rats which are presumed to have a considerable regenerative capability. However, even in the rat it has been established that if there is no coaptation of the cut nerve ends, there will be poor reinnervation of nerve due to the disorganized regeneration within the gap (Young, 1942, Jenq and Coggeshall, 1985, Mackinnon *et al.* 1985b, Jiming *et al.* 1986, Gattuso *et al.* 1988a, Glasby *et al.* 1988a,b). An explanation for the discrepancy in findings between the present study and that of Gattuso *et al.* (1989) may be the age of the animals. Gattuso *et al.* (1989) did not specify the age group of the rats used so if the animals were somewhat older this would account for the poor recovery of muscle function. With a greater capacity for nerve regeneration and reinnervation of muscle in younger animals, it would be expected that after a delay of nerve repair the impact on neuromuscular function would not be as great in newborn lambs. However, these findings do point out a shortcoming in the design of the present experiments as it would have been interesting to see the effect of immediate versus delayed repair in adult animals. The design of the current study was a clinical model, the obstetrical plexus palsy and this is a condition which affects neonates so the main objective was to examine the impact of timing of repair in the younger animals even though their prodigious

regenerative capability is well established. Nonetheless the data on the effect of immediate versus delayed repair in adult animals would have complemented the findings, particularly those relating to the age of the recipient.

Another explanation for the discrepancy in findings between the present study and that of Gattuso *et al.* (1989) may be interspecies variation. Gattuso *et al.* (1989) studied rats and observed a "maximal period of denervation" of between 4 and 8 weeks, beyond which the probability of successful repair was remote. Gutmann (1947) observed in the rabbit that the "maximal period of denervation" was 6 months, whilst in humans denervation of muscle of at least up to one year was compatible with good recovery (Sunderland, 1978, Carlson, 1981). These findings do suggest that the "maximal period of denervation" could be longer in sheep than that in rats, and certainly longer than 10 weeks as investigated in the present study.

Unfortunately the design of the experiments did not address the issue of a upper limit to the time period for denervation. As it was a large animal model this may have been of some relevance to nerve repair in humans. The design could have been altered so that nerve repair was delayed until a number of time periods after injury, for example 4, 8, 12, 16 and 20 weeks. However, with such a large animal model the research costs were high and to introduce multiple groups to an existing extensive study was difficult. This is a topic for future research as an understanding of the decline in the restorative capacity of a muscle following denervation has important clinical implications. Fu and Gordon (1995b) established that prolonged denervation after delayed nerve repair was very deleterious to functional recovery as there was a 90% reduction in the number of motor units. After prolonged axotomy the reduction was only 30%. This significantly reduced the number of motor axons

that regenerated and made functional connections with denervated muscle fibres, but it did not compromise the capacity of regenerative axons to branch or the number of muscle fibres reinnervated by each motor axon (Fu and Gordon, 1995a). Hence, a knowledge of the time frame and the effects of denervation atrophy could help in the establishment of a basis for surgical repair of nerve injuries.

The morphometric indices of nerve maturation support the findings that a delay in nerve repair did not appear to adversely affect the denervation/reinnervation process in muscle. Indeed after delayed repair with either a FTMG or a nerve graft, fibre and axon diameter, and the thickness of the myelin sheath were significantly greater. However, in a series of experiments in adult sheep it was established that delay of nerve repair contributed to a poorer outcome in recovery of nerve function and maturation (Lawson and Glasby, 1995, Fullarton *et al.* 1998, Glasby *et al.* 1997, Glasby *et al.* 1998). This disparity in results may be due to the fact that for these experiments the sheep were assessed at 6 months and not 12 months as in the present study. Sanders and Young (1944) noted that after delayed repair the fibre diameter of regenerating nerves were less than after immediate repair. However, it has been established that regenerated nerves do mature after delayed repair, but not as rapidly as after primary repair (Aitken *et al.* 1947). There is no reported findings on the time period for nerve fibre maturation in sheep but in rats it has been established that it continues for at least one year (Gutmann and Sanders, 1943, Simpson and Young, 1945, Cragg and Thomas, 1964, Schroder, 1972, Gattuso *et al.* 1988a, Glasby *et al.* 1988a). Given the rat's reputed prodigious regenerative capability the time period of maturation in sheep it is likely to be greater than one year. Hence, it is not surprising that at 6 months after delayed nerve repair fibre maturation was less than that found

at 12 months. As discussed further investigations where nerve repair was delayed until a number of time periods after injury would be required to confirm this hypothesis.

Assay	FTMG		Nerve Graft	
	Immediate	Delayed	Immediate	Delayed
Connective Tissue Content	-	-	-	-
Proportions of Type I Fibres	*	X	X	*
Proportions of Type II Fibres	X	*	*	X
Minimum Diameter of Type I Fibres	*	X	X	*
Minimum Diameter of Type II Fibres	*	X	-	-
Form Factor of Type I Fibres	-	-	-	-
Form Factor of Type II Fibres	X	*	-	-

Table 5.10 - Morphological results for experimental supraspinatus muscles after comparing immediate and delayed repair with a FTMG or a nerve graft in lambs.

(* = The best recovery for specified assay when results for immediate and delayed repair are compared, **X** = The worst recovery for specified assay when results for immediate and delayed repair are compared, - = No significant difference in specified assay when results for immediate and delayed repair are compared)

5.4.4 Overview of data

Based on the indices of denervation/reinnervation all repair groups achieved target muscle reinnervation. Pathological features associated with denervation were

minimal and within normal parameters (Dubowitz, 1985) for all groups. There were increases in interstitial connective tissue for the majority of the repair groups, although increases in interstitial connective tissue after nerve repair has been well documented (Bolesta *et al.* 1988, Grieve *et al.* 1991, Lenihan *et al.* 1998b, Carter *et al.* 1998). In the present study the average increase in connective tissue in experimental muscle when compared to contralateral muscles after immediate repair with a FTMG in sheep and lambs was 18.97%. This compares well with the results of Grieve *et al.* (1991) who had a mean increase of approximately 26% in connective tissue content 150 days after immediate repair with FTMGs in juvenile, adult and elderly rats. After nerve repair there was some variation in the mean minimum diameter of type I fibres between experimental and contralateral muscles. For those groups where there was a decrease in diameter, atrophy of type I fibres was less than 5%. This is comparable to another study of nerve repair where atrophy of type I fibres in the EDL muscle in the rat was 10% at 300 days after repair (Gilmour, 1994). Furthermore in the rabbit study (Chapter 3) at 6 months after repair atrophy of type I fibres in the EDL muscle was less than 7%. There was a preferential atrophy of type II fibres, but this has been well documented in the literature after denervation and reinnervation of muscle (Karpati and Engel, 1968a,b, Jaweed *et al.* 1975, Niederle and Mayr, 1978, Vita *et al.* 1983, Lu *et al.* 1997). In relation to the proportion of fibre types I and II, there was an increase in the number of type I fibres and a decrease in the number of type II. However, there was only one experimental group where this change in fibre type proportions was statistically significant. Nonetheless it must be mentioned that in the present experiments the denervation was done before the tertiary myotube formation was complete in sheep (Wilson *et al.* 1992,

Mascarello and Rowlerson, 1995). Furthermore the absence of innervation may have had some influence in decreasing the number of type II fibres whilst increasing the relative percentage of type I fibres present (Wilson and Harris, 1993).

In terms of indices of nerve maturation, in all cases after nerve repair the axon and fibre diameters, and the thickness of the myelin sheath were significantly smaller than those of the unoperated control. However, it has been well documented in the literature that control values are not accomplished even after repair by conventional direct suture or by autogenous nerve grafts of ideal calibre (Gutmann and Sanders, 1943, Schroder, 1972). Furthermore although full maturation of nerve fibres had not been attained for any repair group, the percentage recovery of the morphological indices (fibre diameter, axon diameter, myelin sheath thickness) indicated that the majority of the regenerated fibres were well matured. Since it is well established that end-organ contact is needed for nerve fibre maturation (Simpson and Young, 1945, Weiss *et al.* 1945, Sanders and Young, 1946, Aitken *et al.* 1947), these results indicate that reinnervation of target muscles had occurred.

In reference to the contralateral and unoperated control muscles, it has been observed that contralateral muscles can have significant changes in muscle morphology as a result of compensatory hypertrophy (Talesara *et al.* 1981, Freeman and Luff, 1982). In view of these findings it has been suggested that normal animals may serve as a better control than contralateral muscles (Talesara *et al.* 1981). Previous work has shown that division of the C6 root causes only slight proximal forelimb weakness as this root supplies the shoulder abductors and external rotators which are relatively unimportant in the sheep (Hems and Glasby, 1992, Hems *et al.* 1994, Fullarton *et al.* 2000). Nevertheless, in the present study an effort was made to

include an age, sex and weight matched group of sheep. The findings from comparison of results for experimental, contralateral and unoperated control muscles, indicate that none of the contralateral muscles had undergone compensatory hypertrophy. Split fibres which may be associated with functional overload (Bell and Conen, 1968, Salminen and Vihko, 1983, Kakulus and Adams 1985), were found in the contralateral muscles after muscle or nerve graft repair in sheep. However, the incidence was small and would appear to be a normal phenomenon and not due to a functional overload. Furthermore an analysis of the results showed that the incidence of internal nuclei was "normal" in some contralateral muscles. The results did indicate that there may have been some compensatory hypertrophy of type II fibres, although there were significant differences in muscle fibre size for both type I and II fibres between the right and the left supraspinatus muscles of the unoperated control. Indeed, the variability coefficient for fibre diameter indicated abnormal variability in the size of both type I and type II fibres for the experimental, contralateral and unoperated control muscles. However, other studies report that if there was any compensatory hypertrophy it would most likely result in the addition of contractile material (by an increase in the number of sarcomeres), and not an increase in fibre diameter (Freeman and Luff, 1982). This would increase muscle wet weight which was not measured in the present study. There was also no evidence of "conversion" of muscle fibres from one type to another in contralateral muscles, although this has not been found in muscles undergoing compensatory hypertrophy (Walsh *et al.* 1978, Freeman and Luff, 1982).

In conclusion, based on the indices of muscle morphometry and nerve maturation, after nerve repair there was reinnervation of target muscles for all groups.

There was no evidence of compensatory hypertrophy of contralateral muscles although there was some degree of "natural" variation in morphology in normal muscles. In view of these findings it was taken into consideration when interpreting results between experimental and contralateral muscles.

5.5 CONCLUSION

The present study has established that in the repair of the brachial plexus root injury, the FTMG compares favourably, and may even be superior, to the nerve autograft as a choice of treatment. As to whether the potential of nerve to regenerate after repair decreases with age, or whether immediate or delayed repair is best in the treatment of OBPP, these experiments have contributed to solving but have not resolved the dilemma associated with these questions. In the context of the design of the experiments it was established that neither age nor a delay in repair did not adversely affect neuromuscular recovery. However, there were faults in the design of the experiment (which were not evident at the design stage) which should be addressed in future research on this issue. In particular, the study did not address the issue of a "maximal period of denervation" of muscle. For such a large animal model which would be expected to achieve results more comparable to human situations, this has significant clinical implications. A knowledge of the time frame and the effects of denervation atrophy could assist the surgeon in the management of OBPP. Furthermore the experiments would have been enhanced by investigation of another important component of the motor unit, the neuromuscular junction. Recovery of motor function is essential to peripheral nerve reconstructive surgery and histochemical evidence of regeneration of neuromuscular junctions would help confirm this.

In conclusion, these experiments suggest a place for freeze-thawed skeletal muscle grafts in the management of the OBPP. However, further work is required to elucidate whether expectant treatment may be detrimental when a more severe form

of brachial plexus injury exists. It is hoped that this study will stimulate further investigations to resolve this dilemma.

CHAPTER 6

General Discussion

6. GENERAL DISCUSSION

An essential outcome of peripheral nerve reconstructive surgery is the recovery of motor function. However, surgical repair of peripheral nerve injuries often fails to achieve significant functional recovery (Sunderland, 1978, Kline and Hudson, 1995). The present study has contributed to some understanding of why this is the case as an important component of the motor unit, the muscle fibre, has been assessed after nerve repair. Indeed the findings have clearly demonstrated how significant the changes in muscle cytoarchitecture are when reinnervation is reduced after nerve repair.

A measure of the reinnervation process which was used in the present study was to examine the recovery of muscle after a nerve crush injury. Other researchers have compared different types of nerve injury and repair and established that, as with the present study, recovery was best after a crush injury (Gutmann *et al.* 1942, Cragg and Thomas, 1964, Hyde and Scott, 1983). It is generally accepted that recovery is better after injuries of this type due to the endoneurial tubes remaining intact (Thomas, 1964a) and providing guidance for regenerating axons to their original end organs. The findings from the work described here showed that after repair with a CRG tube or a FTMG in rabbits, the recovery of muscle was poorer than that observed after a nerve crush. It was established that the period of denervation was prolonged but the reasons why were not found.

Misdirection of regenerating axons from the proximal stump into inappropriate distal pathways has long been acknowledged as a factor contributing to poor functional recovery after nerve transection (Langley and Hashimoto, 1917,

Sunderland, 1978). Thus, if regenerating motor axons are directed to sensory end organs, they not only fail to establish appropriate functional contacts but prevent sensory axons from entering these pathways (Brushart, 1988). Indeed one of the concerns of using a non-neural graft in the repair of a mixed nerve is that it may result in less accuracy of regeneration, particularly when compared to interfascicular nerve grafting. However, the data from the present study suggested that the reasons for the poor recovery of muscle after repair with either a CRG tube or a FTMG in rabbits was not due to lack of specificity of regeneration, but that the regenerating axons may have been inhibited by the actual grafts. Further work is required to elucidate whether or not this is correct. The aim would be to simulate what was observed after repair with the CRG tubes and the FTMG. Thus the passage of the regenerating axons would need to be hindered without preventing any reinnervation. An experiment which may be of use would be to create a crush injury which was then repeated at several different time periods.

In a larger animal such as the sheep, the present study did establish that the FTMG compared favourably to the nerve autograft as a treatment of obstetrical brachial plexus palsy. In the clinical situation it is almost impossible to obtain grafts of comparable diameter to large injured nerves and as muscle grafts offer no significantly worse prospect of repair than the nerve autograft, it seems that there is an advantage in using these grafts for human nerve repair. In particular, the use of the FTMG rather than the nerve autograft in clinical practice would reduce the sacrifice of a functioning nerve and the associated loss of sensation, scarring and the possible development of a painful neuroma at the donor site. Indeed, the recovery of muscle appeared to be better after repair with a FTMG in sheep or lambs than in rabbits,

although it is difficult comparing results from the two studies because of inter-species variation. However, it does highlight the importance of pursuing the study of nerve injury and repair in a variety of animal models, rather than limiting it to one. In many investigations of peripheral nerve injury and repair the experimental animal has been the rat which has a reputation for a prodigious regenerative capacity (MacKinnon *et al.* 1985b).

As to whether the potential of nerve to regenerate after repair decreases with age, or whether immediate or delayed repair is best in the treatment of obstetrical brachial plexus palsy, this study has contributed to but has not resolved some dilemmas associated with the treatment of this condition. However, an understanding of the processes of denervation/reinnervation of muscle and the impact of this on the functional recovery of muscle after peripheral nerve repair is important in the management of conditions such as OBPP. Indeed Fu and Gordon (1995b) have shown that prolonged denervation results in a 90% reduction in the number of functional motor units which is very deleterious to functional recovery. No assessment of motor unit function was completed by the present investigation but in a parallel study (Lenihan, 2000), established that electrophysiological indices of nerve function were significantly worse after repair with either a CRG tube or a FTMG in the rabbit.

In terms of clinical implications efforts should be made to minimize the effects of denervation atrophy as a number of investigations have confirmed that reinnervated muscle fibres fail to recover fully, particularly if denervation of the fibres has been prolonged (Gutmann, 1948, Irintchev *et al.* 1990, Fu and Gordon, 1995b). Indeed the findings from the present study have demonstrated that after each

type of nerve repair the recovery of muscle from denervation was incomplete. The reasons for this were not found however, as discussed one possibility is that the implants delayed axon regeneration. If this was the case it may have been ascertained if the design of the experiments were modified. Indeed the strategy of the investigations for both the sheep and the rabbit studies was such that review was only at one time period (6 months for rabbits; 12 months for sheep). Evaluation would have been best accomplished by regular, short spaced intervals such as 1, 2, 3 and 6 months after operation in the case of the rabbit experiments, and 1, 2, 3, 6, 9 and 12 months for the sheep study.

A possible explanation for the poor performance of the CRG tubes may be dimensional instability so different periods of assessment of the site of repair would provide information on the structural integrity and degradation rate of the CRG tubes, as well as the FTMG, and the consequences of this at the site of repair. Various time periods of assessment would have also assisted in the appraisal of the rate of the denervation/reinnervation process in the supraspinatus and EDLs muscle following each type of nerve repair. This would have been helpful in establishing the time frame and the effects of denervation atrophy as a "maximal period of denervation" has been observed by a number of other studies beyond which the probability of successful repair was remote (Sunderland, 1978, Carlson, 1981, Gattuso *et al.* 1989). However, for the present project this would have required more animals and to introduce multiple groups to an existing extensive study was difficult. These issues should perhaps be addressed in future studies based on the present work. Despite the poor findings for the CRG tubes and indeed the FTMG, these methods of nerve repair would still seem to be worthy of further investigation. The purpose of both of

these methods of repair is to bridge a nerve defect and the deleterious effects of tension at the suture line have been well documented (Millesi *et al.* 1972, Terzis *et al.* 1975, Millesi *et al.* 1976, Miyamoto, 1979, Stevens *et al.* 1985). It is hoped that this study will stimulate further work in this field.

The results from the work presented here demonstrated and corroborated the findings of other researchers of the importance of motor innervation in the expression of rabbit muscle myosin isoforms (d'Albis *et al.* 1994, Bacou *et al.* 1996). These isoforms, as well as myosin light chain isoforms, are responsible for the speed of shortening of type II fibres (Schiaffino and Reggiani, 1994). Indeed another observation of the present study was the change in the proportions of type IIA and IIX fibres in the EDL muscle after repair with either a CRG tube or a FTMG. This has important implications in the recovery of function of the EDL muscle as these fibre types differ in their shortening velocity and degree of power output (Schiaffino and Reggiani, 1994). Furthermore there have been few studies such as the present which have investigated changes in the different myosin heavy chain isoforms of type II fibres after nerve injury and repair, particularly in a larger animal model such as the rabbit.

This study has established that there have been significant morphological and functional changes in muscle after nerve repair in both rabbits and sheep however, it has had limitations. Parallel studies of electrophysiological indices of nerve function were conducted for both the rabbit (Lenihan, 2000), and the sheep (Fullarton *et al.* 2000) investigations so recovery of neuromuscular function after repair was determined. However, the experiments would have been enhanced by examination of another important component of the motor unit, the neuromuscular junction.

Evidence of the regeneration of neuromuscular junctions would confirm recovery of motor function and show whether the shortfall in recovery of muscle form after the various treatments was due to incomplete reinnervation of motor end plates, or some other explanation. Glycogen depletion studies would have also been helpful in this respect as these studies can establish the size of reinnervated motor units (although only one motor unit can be assessed per muscle). It must also be stated that although the appearance of muscle does give an indication to the level of its reinnervation, its appearance may be influenced by other factors. In particular, the influence of activity on the contractile and biochemical properties of muscle fibres (Vrbová *et al.* 1995).

The surgical application of CRG tubes and FTMGs, as well as the management of obstetrical brachial plexus palsy has gone some way towards being defined by this project. Any future experiments should be directed at methods for investigating the process of degradation of the non-neural conduit and its impact on the regenerative capacity of the nerve. The evaluation of the various methods of analysis of fibre type distribution also revealed limitations and the need for future research in this area of diagnosis of denervation and reinnervation processes.

The future direction of peripheral nerve research needs to address an important outcome of this project. That is, the need for optimizing nerve regeneration and thereby counteracting any delay in reinnervation that may compromise functional outcome. Recent research has gone some way to achieving this. A short-period low-frequency electrical stimulation just proximal to the repair site has been shown to "dramatically" accelerate both axonal regeneration and preferential motor reinnervation (Al-Majed *et al.* 2000). As these researchers point out, a period of stimulation during the process of peripheral nerve surgery can easily be implemented.

In conclusion, unfortunately neither natural nor synthetic prostheses were shown to be sufficient to restore reinnervation to levels compared to that achieved following a simple crush injury. Indeed there appeared to be very little benefit when compared with nerve section without subsequent intervention, based on a variety of morphological criteria (Chapters 3 and 4). The present study demonstrated the importance of comparing tests of reinnervation with implanted prostheses against the important controls of nerve crush and nerve section, unfortunately neglected from a number of other studies. The importance of undertaking progressive studies of prosthetic enhancement of reinnervation in different animal species (rabbit, Chapters 3 and 4; sheep, Chapter 5) as well as the rat, before generalizing about the potential benefits to man are also emphasized by the present work.

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Appendix

Histological and Histochemical Procedures

1. Toluidine Blue

Method

1. Stain unfixed section for about 1 minute

2. Wash thoroughly in tap water the differentiate in 50% alcohol until any excess dye is removed.

3. View specimen.

4. If satisfactory and required, dehydrate, clear and mount in D.P.X.

Solution

1% Toluidine blue in 1% borax (disodium tetraborate) in water. Must be filtered before use, but keeps well.

Result

The tissue components are stained blue. Purpose of stain is to check orientation and preservation of block during sectioning.

2. Haematoxylin and Eosin (H & E)

Method

1. Stain unfixed sections in Harris's Haematoxylin for 1 to 3 minutes, depending on the freshness of the stain.

2. Section is blue by washing in warm tap water for 5 minutes.

3. Counterstain in eosin for 1 minute.

4. Wash briefly in cold tap water.

5. Dehydrate, clear and mount in D.P.X.

Solutions

I. Harris's haematoxylin

Harris's alum haematoxylin	5 g
Absolute ethanol	50ml
Ammonium or potassium alum	200 g
Sodium iodate	1 g
Glacial acetic acid	80 ml
Distilled water	make up to 2.5 litres

Dissolve haematoxylin in alcohol and add to alum solution. Bring to the boil for approximately 2 minutes and then remove from heat. Add sodium iodate. Allow to cool and then add glacial acetic acid.

II. Eosin

Eosin (alcohol soluble)	5 g
Absolute alcohol	350 ml
Distilled water	150 ml

Result

Stains nuclei and basophilic material dark blue and other cytoplasm pink. It is used to evaluate muscle fibre architecture, fibre splitting, position of nuclei or presence of vacuoles.

3. Masson's Trihcrome

Method

1. Stain unfixed sections in Harris's haematoxylin for 1 minute.
2. Blue by rinsing in running tap water for approximately 1 minute.
3. Differentiate by immersing in picric acid solution for 15 seconds.

4. Wash well in cold running tap water.

5. Stain cytoplasm red by immersion in ponceau solution for 2.5 minutes.

6. Wash well in cold running tap water.

7. Differentiate and mordant by immersion in PMA for 5 minutes.

8. Wash well in cold running tap water.

9. Counterstain connective tissue fibres in fast green for 15 seconds.

10. Wash in cold tap water. Dehydrate, clear and mount in D.P.X.

Solutions

I. Harris's haematoxylin - as for haematoxylin and eosin.

II. Picric acid - 1% picric acid in 70% alcohol (Caution).

III. Ponceau - 1% Ponceau de xylidine (ponceau 2R) in 0.5% acetic acid.

IV. PMA - 2% phosphomolybdic acid (PMA) in distilled water.

V. Fast green - 0.5% fast green in distilled water.

Result

Muscle fibres stain red/brown, nuclei blue/black and connective tissues green. It shows muscle fibre architecture and connective tissue proliferation.

4. Myofibrillar adenosine triphosphatase (ATPase)

1. Air dry unfixed sections for a minimum of 2 hours.

2. Pre-incubate at room temperature - 15 minutes for acids (pH 4.35 and 4.6), and 20 minutes for alkali (pH 10.2).

3. Wash 3 times in tap water, followed by 3 times in distilled water.

4. Incubate at 37^0C for 45 minutes for acids (pH 4.35 and 4.6), and 30 minutes for alkali (pH 10.2).

5. Wash in 2 changes of 1% calcium chloride.

6. Place in 2% cobalt nitrate for 3 minutes.

7. Quickly wash 3 times in tap water, followed by 3 times in distilled water.

8. Develop in dilute (1%) ammonium polysulphide in distilled water for approximately 5 minutes. Sections should immediately change to a black colour.

9. Wash well in tap water, followed by distilled water.

10. Mount in an aqueous mount, for example Uvinert.

Solutions

I. Pre-incubation solutions

pH 4.6

Sodium acetate 0.2M	30 ml
Acetic acid 0.2M	30 ml

Adjust to pH 4.6 with 1M sodium acetate

pH 4.35

Sodium acetate 0.2M	18 ml
Acetic acid 0.2M	42 ml

Adjust to pH 4.35 with 1M sodium acetate

pH 10.2

Tris-calcium buffer	60 ml

Adjust to pH 10.2 with either HCl or KOH

II. Incubation solution

ATP (disodium salt)	300 mg
Tris-calcium buffer	200 ml

Adjust to pH 9.5 with either HCl or KOH

III. Tris-calcium buffer

Tris(hydroxymethyl)methylamine 6.05 g

Calcium chloride (MW 147) 1.32 g

Distilled water make up to 500 ml

Should be made up fresh each time.

IV. 1% Ammonium polysulphide

Make up immediately before use and always use fume cupboard. Solution should a pale yellow colour but if it is bright yellow, the solution ha gone off.

V. 0.2M Sodium acetate

Sodium acetate 27.21 g

Distilled water 1 litre

VI. 0.2M Acetic acid

Acetic acid 11.6 ml

Distilled water 1 litre

VII. 1% Calcium chloride

VIII. 2% Cobalt nitrate

The solutions **V - VIII** should be made up in stock solutions and can last up to 1 month if stored at 4^0C.

Result

It is used to differentiate muscle fibre types. Serial sections stain differently depending on the pH of the pre-incubation solution.

	I	IIA	IIB	IIC
pH 4.35	Dark	Pale	Pale	Dark
pH 4.6	Dark	Pale	Int	Dark
pH 10.2	Pale	Int	Dark	Dark

(Int = Intermediate)

For more information on these histological and histochemical techniques please refer to Cumming *et al.* (1994).

5. Immunohistochemical procedures

1. Use 10 μm thick unfixed cryostat sections on slides pre-coated with *poly*-L-lysine.

2. Prepare primary antibody by diluting it to between 1/20 to 1/10000 (dependent on the antibody used). Dilute with PBS-albumin with azide solution. (For this project: anti-fast MY-32 1/3000; anti-slow BA-F8 1/200; IIA SC-71 1/200; IIB BF-F3 1/200; and neonatal 1/5000).

3. Incubate primary antibody on tissue section for 2 days at room temperature in a moist atmosphere (humidity chamber).

4. Remove antibody and wash in PBS + 0.025% Tween 20 for 5 minutes, 3 times. Briefly rinse in distilled water.

5. Incubate in biotinylated secondary antibody diluted to either 1/100 or 1/400 with PBS-albumin with azide for several hours or overnight. (For this project: secondary antibody dilution for anti-fast MY-32, anti-slow BA-F8, SC-71 and BF-F3 was 1/100; for neonatal 1/400).

6. Remove antibody and wash in PBS + 0.025% Tween 20 for 5 minutes, 3 times. Briefly rinse in distilled water.

7. Incubate in perioxidase-linked extravidin diluted to 1/100 in PBS-albumin with azide for several hours.

8. Remove antibody and wash in PBS + 0.025% Tween 20 for 5 minutes, 3 times. Briefly rinse in distilled water.

9. The colour reaction is produced by adding PBS + H_2O_2 + Di-amino benzidine (DAB). (Careful - wear gloves, perform reaction in the sink in a fume cupboard with the ventilation switched off).

10. The reaction should develop within 15 minutes or may appear in 3 to 5 minutes.

11. Empty the DAB solution but be careful to inactivate it with bleach immediately after use and before disposal.

12. Next gently wash with tap water and pour this first wash into beaker with DAB solution and bleach. Wash 3 times with water.

13. Dehydrate, clear and mount in D.P.X.

Solutions

I. PBS (Phosphate-buffered normal saline) 0.01M

Sodium chloride	8.7 g
Potassium dihydrogen phosphate	0.272 g
Disodium hydrogen phosphate	1.136 g

II. PBS-albumin with azide

As for PBS solution + 1% bovine serum albumin + a few grains of sodium azide

III. PBS-Tween 20

As for PBS solution + 0.025% tween-20.

IV. DAB colour reaction solution

As for PBS solution + H_2O_2 (1 drop per 20 ml) + Di-amino benzidine (DAB) at 0.5mg/ml.

Primary Antibodies

For this project suppliers are:

Anti-fast

Sigma, product no. M4276

Monoclonal MY-32.

Anti-slow

German collection of microorganisms and cell cultures. Department of human and animal cell cultures. DSM no. ACC 154.

Monoclonal BA-F8

Type IIA

German collection of microorganisms and cell cultures. Department of human and animal cell cultures. DSM no. ACC 217.

Monoclonal SC-71

Type IIB

German collection of microorganisms and cell cultures. Department of human and animal cell cultures. DSM no. ACC 217.

Monoclonal BF-F3

Neonatal

Kindly supplied by Dr Anthea Rowlerson.

Polyclonal neonatal.

Secondary Antibodies

For anti-fast MY-32, anti-slow BA-F8 and SC-71:

Sigma, product no. B7401

Anti-mouse IgG

Biotin conjugate

For BF-F3:

Chemicon, USA

Anti-mouse IgM

Biotin conjugate

For Neonatal:

Sigma, product no. B8895

Anti-rabbit IgG

Biotin conjugate

Peroxidase-linked Extravidin Solution

"Extravidin"

Sigma, product no.E2886