

**NEW METHODS FOR THE REPAIR AND
ASSESSMENT OF PERIPHERAL NERVE INJURY**

BY

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ABSTRACT

The present study was designed to investigate factors which could influence the regeneration of peripheral nerves which had been cut and repaired using a variety of grafting techniques.

These methods were: repair with a microwaved muscle autograft, a freeze-thawed muscle autograft, and several groups where repair involved a controlled release biodegradable glass tube containing a variety of factors which have been shown to influence nerve regeneration.

Assessment of all of these experimental groups involved the use of established electrophysiological and morphometric techniques but also the development of new techniques for measuring the conduction velocity of the slowest fibres and the variability of reinnervation at the neuromuscular junction (stimulated jitter).

The experiments revealed that the microwave muscle graft provided the structural support needed for regeneration, however difficulties in preparing the graft made its use in the clinical setting doubtful. The controlled release glass tube did not interfere with regeneration and supported similar levels of regeneration when compared with an established surgical technique. Jitter proved to be an excellent and highly discriminatory test for assessment of the progression and quality of reinnervation of skeletal muscle.

The potential for using these techniques in the experimental and clinical settings is discussed.

DECLARATION

I, David Lenihan, declare that this thesis has been composed totally by myself and that the work reported here is my own work. Any technical assistance obtained has been acknowledged.

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year with my father and son is probably the main reason that I finished this work. Although we often disagreed on my work and his criticisms were often hard, I have come to consider him a good friend. I truly wish I could do more than say thank you.

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TABLE OF ABBREVIATIONS

| <u>Abbreviations</u> | <u>Definition</u> |
|----------------------|--|
| °C | degree Celsius |
| μm | micrometer |
| μs | microseconds |
| Ω | ohm |
| ARP | absolute refractory period |
| ATP | adenosinetriphosphate |
| cm | centimeter |
| CRG | controlled release glass |
| CRG-GAP | an empty CRG tube |
| CRG-M | a CRG tube filled with freeze thawed muscle |
| CRG-MN | a CRG tube filled with a muscle nerve sandwich graft |
| CRG-N | a nerve filled CRG tube |
| CV_{\max} | maximum conduction velocity |
| CV_{\min} | minimum conduction velocity |
| EDL | extensor digitorum longus |
| EMG | electromyographic |
| FTMG | freeze thawed muscle graft |
| Hz | hertz |
| kg | kilogram |
| kHz | kilohertz |
| l | litre |
| $m s^{-1}$ | meters per second |
| m | meter |
| M | molar |
| mA | milliamps |
| MCD | mean consecutive difference |
| mg | milligram |
| MHz | megahertz |
| min | minute |
| ml | millilitre |
| mm | millimeter |
| mM | millimolar |
| MMG | microwave muscle graft |
| MUAP | motor unit action potential |
| mV | millivolts |
| mVs | millivolt-seconds (a measure of area) |
| M-wave | muscle wave |
| N | newtons |
| p | probability |
| s | second |

| | |
|--------|-------------------------------|
| SF-EMG | single fibre electromyography |
| TA | tibialis anterior |
| TTI | time tension index |
| UK | United Kingdom |
| USA | United States of America |
| V | volts |

THE INTRODUCTION

CHAPTER 1.0

INTRODUCTION

| <u>SECTION</u> | <u>TITLE</u> |
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1.1 OVERVIEW

Division of a peripheral nerve will result in the nerve fibres distal to the site of injury undergoing Wallerian degeneration. If conditions are favourable, the proximal fibres may regenerate across the injury to make functional connections in the periphery and possibly allow regeneration to occur.

Attempts to restore nerve function after nerve injury dates back to the thirteenth century (Seckel *et al.*, 1984). Despite the recent advances in medicine, such as the utilization of microsurgical techniques, the best possible outcome for the repaired peripheral nerves is limited co-ordination of motor control and diminished sensory localization. Unfortunately, these results are the norm rather than the exception. Some authors have hypothesized that the reason for the poor quality of regeneration is a function of altered central organization while others have said that it is a result of inaccurate connection being made in the periphery.

Nerve reconstruction has been attempted using many different methods. Direct end to end suture of a transected peripheral nerve is the simplest approach to promote repair. However, direct end to end suture must not have tension at the suture line, as this will reduce the recovery of function after repair (Terzis *et al.*, 1975). Currently, gaps that are too long to allow for direct end to end suture without tension are repaired using nerve autografts. Concerns regarding the harvesting of normal nerve to be used as a donor have given rise to research into alternative

grafting techniques. The Introduction will cover some of the alternative grafting techniques employed in the experimental setting today.

1.2 DEGENERATION AND REGENERATION OF A PERIPHERAL NERVE

Division of a peripheral nerve can occur owing to a variety of injuries. After division of the nerve the distal segment undergoes Wallerian degeneration. Axons migrate from the proximal to the distal stump if there is a supportive pathway for them to travel through. If there is no pathway available, a neuroma forms on the proximal stump. This is why providing a pathway supportive of regeneration is paramount for the recovering patient.

1.2.1 Degeneration

When a peripheral nerve is divided, the proximal end-bulb swells owing to non-specific inflammatory reaction. There is some retrograde degeneration of the axons, usually one internode, although this depends on the type of injury. The result is that collagen is deposited on the distal portion of the proximal segment.

The distal segment of a divided peripheral nerve will undergo Wallerian degeneration. The mitochondria swell and the microtubular structure in the axoplasm degenerates. Within 4 days after injury the myelin begins to separate and break-up. These fragments are engulfed by macrophages and Schwann cells. Over the next 3-7 days there is increased proliferation of Schwann cells and fibroblasts (Abercrombie & Johnson, 1942). The Schwann cells basement membrane shrinks

with increased collagen deposition. The basement membrane forms into folds that result in a tubular structure containing Schwann cells.

The Schwann cells and fibrocytes undergo multiplication at the distal end-bulb. The increase in the amount of cells causes considerable swelling at the end-bulb. Thomas (1966) showed that the Schwann cells grew out in columns that are surrounded by basement membrane. While these columns were surrounded by endoneurial fibrocytes and blood vessels they are preparing for regeneration. Thomas further showed that the perineurium grew to surround the Schwann cell columns (Thomas & Jones, 1967)

1.2.2 Regeneration

If the proximal and distal nerve ends are connected with a supportive graft the advancing neurites from the proximal stump join with the Schwann cell columns from the distal stump. These advancing neurites then enter the empty Schwann tubes and grow distally. It has been postulated that the rate of regeneration is 1-2 mm per day. However, it seems unlikely that all neurites will grow at the same speed and that the value of 1-2 mm per day is an average of the fastest fibres. This hypothesis will be considered in Chapter 13.

If these neurites reach the periphery and form function connections, the neuron may begin to mature. Maturation will only occur if the neurite re-establishes itself in the periphery with a muscle or a sensory organ (Sanders & Young, 1946). Even in ideal conditions, there will still be a reduction in the number of functioning

connections when compared with normal. The function of the doctor is to provide a means to allow the greatest number of connection to be made.

1.3 DIFFERENT TYPES OF CONDUIT

There have been numerous experiments conducted in the past three decades using a variety of different graft materials for the repair of peripheral nerves. Conduits have one specific advantage when compared to nerve grafts: they are abundant and do not cause loss of function owing to harvesting of nerve.

When a nerve is divided cleanly, as with a knife, it is often possible to repair it using direct end to end sutures without tension. Microsurgical techniques over the last decade have made apposition of the nerve ends more accurate. However, the ends of the nerve often require trimming to make the stumps more accessible for the advancing neurite. This often makes repair without tension difficult.

Highet and Holmes (Highet & Holmes, 1943) tried to resolve the problem of tension at the suture line by fixing the joint so that no tension would exist at the suture site initially. Then over time, the joint was gradually extended using a series of splints. The results of this technique were not favourable and the technique was subsequently discarded.

Because repair of a peripheral nerve without tension is seldom achieved there exists a need for graft material to be used to repair these injuries.

In the present section some of the different materials used to repair nerve peripheral nerves where direct end to end suture without tension is impossible will be discussed.

1.3.1 Veins

Wrede was the first to suggest that veins could be used as conduits for nerve repair. (Doolabh *et al.*, 1996). However, vein grafts were not used in the surgical field because of the belief during World War II that they would collapse and the result would be fibrotic tissue formation around the regenerating fibres (Chiu *et al.*, 1988). Chiu *et al* renewed interest in the possibility of using the vein as a graft for nerve repair. He compared a vein graft with no graft for the repair of a 10 mm gap in the sciatic nerve of rats (Chiu *et al.*, 1982). Not surprisingly he found improved recovery of function in the rats that were grafted. This study was confirmed by Rice *et al* (Rice & Bernstein, 1984) who demonstrated that the vein graft supported nerve regeneration across a 25 mm gap in the rat sciatic nerve.

The vein graft provides the scaffolding that is required for successful regeneration and allows for a ‘more orderly’ regeneration with less indiscriminate axonal migration than the nerve graft group (Ristano *et al.*, 1989). Brunneli *et al* used the vein graft filled with muscle to prevent collapse and assessed the recovery 3 months after repair (Doolabh *et al.*, 1996). They found that these conduits were similar to nerve grafts and superior to either vein grafts or muscle grafts alone.

Wang *et al* (1993) showed that inside-out vein grafts expose the advancing neurite to collagen rich adventitia which resulted in increased vascularity and increased speed of regeneration and myelination when compared to standard vein grafts. However Bento-Rutz *et al* (1994) found no difference in histology or electrophysiology between the inside out and normal vein graft 6 months after repair.

One study in 1988 by Chiu demonstrated that recovery through a vein graft had a similar conduction velocity when compared with a nerve graft although the action potentials were decreased. (Chiu *et al.*, 1988). Although not stated in the paper, this result implies that the number of muscle fibres innervated was less in the vein graft group compared with the nerve graft.

Because of the argument of collapsing vein grafts and the result of Chiu in 1988, the use of vein grafts alone will probably not enter the clinical setting. Modification to the vein graft to provide improved structural support may hold promise, however, the technical difficulties of performing the modification may make the technique unrealistic.

1.3.2 Arteries

One of the first conduits used was artery. As early as 1891, Bungner demonstrated that regeneration of a peripheral nerve could occur through arterial grafts. However, the discrepancies between Weiss's and Taylor's experiments ended research at the time for using this form of graft (Doolabh *et al.*, 1996). Politis *et al* renewed interest in this form of graft in the 1980's (Politis *et al.*, 1982). Anderson *et al* demonstrated that the peroneal nerve axons of a rat would cross 5 mm gaps within arterial grafts (Anderson & Turmaine, 1986). It is interesting to note that both Anderson and Politis demonstrated preferential growth towards a distal lure. Although arterial grafts may support nerve growth and regeneration, the difficulties of harvesting donor artery segments is a major problem.

1.3.3 Muscle

The use of muscle grafts had been attempted in the late 1800's (Glasby & Hems, 1993) however this research seems to have been lost until the 1970's. Modern research on the use of muscle tissue as a graft began when Ide demonstrated that a regeneration of a peripheral nerve does not require the presence of Schwann cells, only their basement lamina (Ide *et al.*, 1983). This led to experiments to determine the similarities between the skeletal muscle basement membrane and degenerated nerves endoneurial tubes and the possible uses of muscle grafts in nerve repair.

Initially there was extensive research into the use of muscle grafts for the repair of peripheral nerves. A muscle graft would hold several advantages when compared to nerve grafts in that they can be tailored to size, material is plentiful and a second harvesting procedure would not be required. Early work by Keynes *et al* (Keynes *et al.*, 1984) showed that denatured muscle could support nerve regeneration in the mouse. This was further supported when Glasby *et al* demonstrated that the denatured muscle graft and nerve graft allowed for similar levels of regeneration when compared by histological and electrophysiological analysis. It was also shown that the speed of regeneration was accelerated when the muscle was arranged in a co-axial arrangement (Glasby *et al.*, 1986a).

Muscle autografts have been found to be comparable to nerve grafts with respect to recovery of function after nerve repair. Fawcett and Keynes (1986) demonstrated that maximum nerve conduction velocity and motor refractory period were similar when the two methods of repair were compared. Glasby *et al* (1986b) further demonstrated similar return of axon numbers and maturation in marmosets with ulnar nerve transection. Gattuso *et al* confirmed these finding in the marmosets median nerve (Gattuso *et al.*, 1988).

One difficulty with denatured muscle grafts is the limit on length of repair. Hems *et al* (Hems & Glasby, 1992) found inferior regeneration of rabbit peroneal nerve across gaps of 5 cm or longer.

From these experiments it is possible to conclude that muscle grafts can serve as effective conduits for nerve regeneration. They have several advantages when compared to nerve grafts in that they are easy to harvest, can be tailored to size and the basal lamina matrix provides little resistant to regeneration axons.

1.3.4 Microwave muscle grafts

One problem associated with the freeze-thawed muscle grafts is that the freezing process can produce considerable shrinkage (Glasby *et al.*, 1986a). Several researchers have attempted to overcome this difficulty by altering the methods by which the muscle is prepared. One of the different methods of producing a denatured muscle graft the use of chemicals such as 10mM CaCl₂ in 2 mM imidazole (Feneley *et al.*, 1991).

Whitworth *et al* published a paper where grafts were prepared by heating muscle in a microwave oven (Whitworth *et al.*, 1995). He showed that muscle grafts may be prepared in this manner to reduce the amount of shrinkage of the graft when compared to muscle grafts prepared by freezing (Whitworth *et al.*, 1995). Whitworth's paper stated that the MMG provided similar levels of axonal regeneration when compared with FTMG

1.3.5 Tubes

Even with the studies demonstrating the effectiveness of denatured muscle grafts for the repair of small nerve deficits the technique has not developed in the

surgical field. This is probably a result of the techniques required to denature the muscle as being too complicated or time consuming in the operating theatre. The lack of utilization of the denatured muscle graft and the desire to provide more effective methods of nerve repair have led many researchers to explore other biological and non-biological materials as conduits for nerve repair.

Lundborg (1982) was one of the first to use synthetic conduits for the repair of peripheral nerves. He used silicon tubes to repair small gaps in the rat sciatic nerve. Although this was fashionable at the time and attempted clinically (Lundborg *et al* 1991) this was impractical because a second procedure was required to remove the tubes.

Some of the different materials used to make conduits are mesothelial chambers, collagen chambers, pseudosynovial membrane, vascular human amnion, polyglolic acid, hyaluronic acid and polyesters. These have all been used as a possible material for providing growth across a nerve gap. Gibby *et al* (Gibby *et al.*, 1983) found that reinnervation in the cat was superior in the nerve repaired with collagen chambers when compared with direct end to end suture. The collagen chamber was one of the first bioabsorbable repair conduits and has been used in the clinic setting.

Biodegradable materials such as polyglolic acid, hyaluronic acid and some polyesters have all been used as a conduit for nerve repair. Seckel *et al* (Seckel *et al.*, 1984) demonstrated successful regeneration with 'negligible' inflammatory

reaction in the repair of a rat sciatic nerve. However, Henry *et al* (Henry *et al.*, 1985) found that the more biodegradable a conduit is, the narrower and more distortable the conduit became as time increased after repair and this deterred regeneration. From this they concluded that conduits must be flexible, lack antigenicity, can be easily made, and are of easy to use. They further stated that a primary criticism of biodegradable conduits is the dimensional instability.

The flexibility of the conduit is not as clean cut as has been suggested and may be one of the causes of dimensional instability. Given that these injuries are often severe, stabilization of the joint is to be expected. Thus, a rigid tube is less likely to lose its structural support.

1.4 THE SURGICAL ADVANTAGE OF BIODEGRADABLE TUBES

A tube composed of synthetic materials for peripheral nerve repair must meet the following requirements to be successful clinically. 1) the tube must be biodegradable and be completely reabsorbed by normal metabolic pathways. 2) the tube must not provoke an inflammatory reaction. 3) the tube must be nontoxic, nonantigenic and non-carcinogenic. 4) the internal lumen of the tube must be large enough to accommodate the initial swelling of the nerve while allowing the axons to migrate without obstruction and not be distorted during re-absorption. 5) the tube must be completely reabsorbed once the migrating neurites have established appropriate connection in the periphery and not before. 6) the tube must maintain its

structural integrity for enough time to allow the slowest neurites to migrate into the distal stump. 7) the nerve should be secured to the tube away from the transected ends to avoid further trauma. 8) the tube should be made of substances which are amenable to custom fabrication so that the diameter of the lumen, rate of degradation, and permeability can be controlled. The purpose of the experiments presented in this thesis was to test the CRG tube with respect to these 8 criteria above and then to compare the CRG tube with other more conventional methods of repair. The hypothesis of this thesis was that the CRG tube would support a similar level in the quality of recovery of function when compared with nerve grafts.

1.5 JITTER

Hakelius *et al* (1975) showed that when regenerating motor axonal sprouts reached their target muscles, new end plates began to form and establish new neuromuscular junctions. In immature neuromuscular junctions there was an increased variability in the latency of neuromuscular transmission (Stålberg and Trontelj, 1994). Jitter is the measurement of the variability in neuromuscular transmission.

Jitter was first measured by Sålberg in 1964 to assess patients with diseases that affected the neuromuscular junctions such as motor neurone disease and multiple sclerosis. Initially, this technique was difficult to perform because it required patient co-operation and needed two motor unit action potentials (MUAP's) to occur at the

same time within the capabilities of the oscilloscope sweep speed. Trontelj *et al* (1986) subsequently developed a method of measuring jitter without the need of patient co-operation or two simultaneous MUAP's. This technique was termed stimulated jitter and involves stimulating the terminal motor axons directly and recording the associated MUAP. This adaptation to the jitter technique proved extremely popular with electrophysiologists and propelled it into the clinical EMG laboratories across the world. The technique was simple to perform and quick to use and is extremely adaptable to the animal model. Because the processes of degeneration and reinnervation associated with motor neurone disease are similar to a regenerating nerve caused by transection, the measurement of jitter to assess nerve regeneration after injury seems logical. However, there has not been any controlled studies where jitter was used to assess the neuromuscular junction after injury.

1.6 HYPOTHESIS STATEMENT

That there was a potential clinical use for the microwave muscle graft (MMG), and to determine whether the CRG tube would support nerve regeneration and whether jitter could be used to assess recovery of function after nerve repair.

THE METHODS

CHAPTER 2.0

EXPERIMENTAL GROUPS

| <u>Section</u> | <u>Title</u> |
|-----------------------|---|
| 2.1 | <u>INTRODUCTION</u> |
| 2.2 | <u>PREPARATION OF EXPERIMENTAL ANIMALS</u> |
| 2.3 | <u>DESCRIPTION OF EXPERIMENTAL GROUPS</u> |

2.1 INTRODUCTION

The animal chosen for the experiments presented in this thesis was the rabbit. Over the last four decades, rabbits have been used in many experiments to assess the recovery of function after nerve repair. During the Second World War, Sanders and Young (Sanders & Young, 1944) (Sanders & Young, 1942; Sanders & Young, 1946) used rabbits for peripheral nerve research. Young *et. al.* (Young *et al.*, 1940) observed that peripheral nerves in the rabbit could regenerate across gaps of up to 2 cm. Cragg and Thomas (Cragg & Thomas, 1961; Cragg & Thomas, 1964) demonstrated that the maximum conduction velocity was reduced after peripheral nerve repair. The rabbit provides an excellent pool of data with which to compare the results of these experiments.

The left peroneal nerve of the rabbit was chosen as the model for these experiments based on the following criteria: a) it provides sufficient length of nerve to provide accurate electrophysiological measurements, b) the extensor digitorum muscle is completely supplied by the peroneal division of the sciatic nerve c) dividing the peroneal nerve results in an acceptable loss of function for the animal, and d) the nerve is easily accessible through a lateral incision in the thigh.

2.2 PREPARATION OF EXPERIMENTAL ANIMALS

Adult female New Zealand White rabbits were supplied by City Farms Ltd. (Willie Walker, City Farms Ltd., West Calder, West Lothian, UK). They were bought at weights between 2.75 and 3.50 kg which represents an approximate age of 2 months. The rabbits were housed in the Medical Faculty Animal Area and were allowed a minimum period of 1 week to adjust to their new environment before any procedure was carried out (University Federation for Animal Welfare Handbook, 1989). All the animals were housed together in a pen large enough to provide 2600 cm² per animal as required by the Home Office (Home Office (H.M.S.O.), 1986). The animals were provided with a special diet to help prevent obesity and were given unlimited access to water. Straw, toys and hiding locations were provided to help reduce stress and improve the welfare of the animals (Sandford, 1996). Daily water and food checks were performed by a member of the Medical Faculty Animal Area staff. The animals were weighed and inspected for infection or disease weekly.

2.3 DESCRIPTION OF EXPERIMENTAL GROUPS

A population of 35 rabbits was separated into seven equal sized groups. Previous experiments have shown that a group size of 5 animals is large enough to compare different groups with respect to histology and electrophysiology after nerve repair (Hems, 1993). The groups consisted of normal animals and animals whose left peroneal nerve had been divided and repaired using one of the following procedures:

- Group A A CRG tube filled with a 1 cm length of chopped freeze-thawed muscle in the centre of the tube (CRG-M).
- Group B A CRG tube with chopped nerve inserted in the centre of the tube (CRG-N).
- Group C A CRG tube filled with a 1 cm length of mixed freeze thawed muscle and chopped nerve (CRG-MN).
- Group D A 1 cm freeze thawed muscle graft orientated co-axially (FTMG).
- Group E An empty CRG tube with 1 cm gap (CRG-GAP).
- Group F A 1 cm microwave muscle graft (MMG).

CHAPTER 3.0

THE CONTROLLED RELEASE GLASS TUBE

| <u>Section</u> | <u>Title</u> |
|----------------|--|
| 3.1 | <u>INTRODUCTION</u> |
| 3.2 | <u>PREPARATION OF THE CONSTITUENTS AND GLASS</u> |
| 3.3 | <u>PREPARATION OF THE GRAPHITE MOULD</u> |
| 3.4 | <u>MAKING OF THE CRG TUBE</u> |

3.1 **INTRODUCTION**

The controlled release glass (CRG) was developed by Giltech Limited (Ayr, Scotland). The initial intent of developing the CRG was to use it as a method of introducing bactericidal silver salts into the urinary tract of patients to help improve the recovery time of urinary tract infections. The idea of using the CRG as a tube to repair peripheral nerves was first thought of by Dr. Lynn Myles FRCS (Sn) however, this idea was soon abandoned owing to an almost 100% infection rate after insertion of the tube into animals. The present author took up the project several years later and introduced a mechanism to reduce the rate of infection by changing the sterilization procedures to gamma-irradiation.

3.2 **PREPARATION OF THE CONSTITUENTS AND GLASS**

A BBC computer was used to calculate the correct amount of constituents to be used in the preparation of the CRG. The constituents were fused as described below to produce the CRG. The CRG used in the experiments presented in this thesis was a modified calcium phosphate where sodium was used to break up the cross-link chain in the CRG. The more sodium in the CRG, the weaker the cross-links in the crystalline structure of the CRG and, therefore, the quicker the CRG will dissolve. By changing the amount of sodium in the CRG, the solubility rate may be altered. The composition of the CRG was 32 mole% Na₂O, 21 mole% CaO and 47 mole% P₂O₅.

The initial components were weighed and mixed by hand in a plastic drum. The mixed components were then placed in a 1 meter silicon crucible and fused to produce the CRG. Placing the silicon crucible in a furnace at a temperature of 1050 °C completes the fusing process. The amount of time the components were placed in the furnace was determined when all the water resultants from the reaction were evaporated. The amount of time of the fusing process was approximately 1 hour.

The molten CRG was then poured on to a polished steel casting slab. The glass was cooled at room temperature. It was important to keep the CRG in a clean environment while it was cooling to avoid dust contaminating the glass. Once the CRG was cooled to room temperature it was broken into small pieces (approximately 10 to 100 cm in size) with a polished steel hammer. The CRG fragments were then stored in a clean dry place until needed.

A portion of the molten CRG was cast into rods using a graphite mould. The rods were annealed to remove stresses in the CRG by gradually cooling it under controlled conditions. The annealing process makes the CRG equal and homogeneous throughout. These rods were then placed in an ionized water solution to assess the solubility rates of the CRG.

During the handling of the molten CRG it was important to wear the appropriate safety devices. Goggles, a molten metal splash-proof visor and Mercury 400 heat resistant gloves were worn at all times when handling the molten CRG.

3.3

PREPARATION OF THE GRAPHITE MOULD

The mould used to make the CRG tube was composed of graphite to avoid adhesion of the molten CRG. To make the mould, a graphite rod was cut to approximately 200% of the desired length of the CRG tube. This graphite rod was machined with a circular lathe (Ford Motor Co., UK) to a diameter greater than 150% of the diameter of the desired CRG tube. A bore was made in the centre of the graphite rod with the circular lathe to the desired diameter of the CRG tube. The length of this bore was approximately 80% of the length of the desired CRG tube. A 2 mm diameter hole was then bored in the remaining 20% of the graphite mould to accommodate an ejection pin. The ejection pin made removing the CRG tube from the graphite mould easier.

A stainless steel holder was made to hold the graphite mould in the 'spinner'. The 'spinner' was a modified Black and Decker (London, UK) drill where the governor was removed. The drill had been modified to reach 30,000 rotations per minute in less than 1 second. The stainless steel holder was mounted on the 'spinner' and the mould was inserted in the holder.

3.4 MAKING OF THE CRG TUBE

Some of the smaller CRG fragments were placed in a 10 cm diameter silicon basin and then inserted into an furnace with a preheated temperature of 1100 °C. The basin was left in the furnace until the CRG melted and was glowing red. The reason the CRG was heated to a temperature greater than the fusing temperature was to make the CRG less viscous and therefore easier to pour into the graphite mould.

The graphite mould was pre-heated to 330 °C to prevent cracking and to decrease the chance of thermal shock stress when the molten CRG was poured into the mould. Once the graphite mould was heated to 330 °C it was then taken out of the oven and inserted into the stainless steel holder. The basin was then removed from the furnace with thermal resistant tongs and a quantity of molten CRG was quickly poured into the graphite mould to ½ the depth of the bore. The drill was immediately switched on and spun to maximum speed and then quickly switched off. This process took approximately 2 seconds. The centrifugal force of the spin created a hollow tube of CRG within the graphite mould. The excess CRG was thrown out the top of the mould.

When the machine came to a complete halt the graphite mould was removed from the stainless steel holder. The ejection pin was used to remove the tube from the graphite mould. The tube was ejected on to a ceramic fibre blanket that was in an oven pre heated to 330 °C.

The tube was then annealed by raising the temperature to 370 °C at a rate of 5 °C per minute. The temperature was held at 370 °C for 30 minutes and then cooled at 1 °C per minute until ambient temperature. Once the tube was at room temperature, it could be safely moved by hand. Any imperfections within the tube would cause it to break during the annealing process. Thus, if the CRG tube was strong enough to survive the annealing process it should succeed as the final product.

The tube was then ground to the desired length using a Draper wet grind stone. Two suture holes were drilled at 180 degrees apart on each end of the tube using a dental drill burr that was made of a diamond coated tip. To avoid cracking, the drill also had a water cooling apparatus to keep the CRG tube cool during drilling. Once the holes were bored, the tube was washed in de-ionized water and rinsed with isopropyl alcohol. The tube was allowed to dry in air and then packaged individually in a heat sealed foil pouch. Each pouch was then sent for sterilization by gamma-irradiation (Isotron plc, Bradford UK). The gamma-irradiation process changed the CRG tube from clear to a red colour as shown in figure 3.1. The sterile tube was then sent to us for use in these experiments.

The change in colour is of particular interest as it was unexpected and is probably a result of elevating the electron orbital position in the sodium ions. The colour change did not affect the solubility rate however, it did affect the ease of use associated with the CRG tube. It was slightly more difficult to see into the tube lumen without the visual assistance of a microscope.

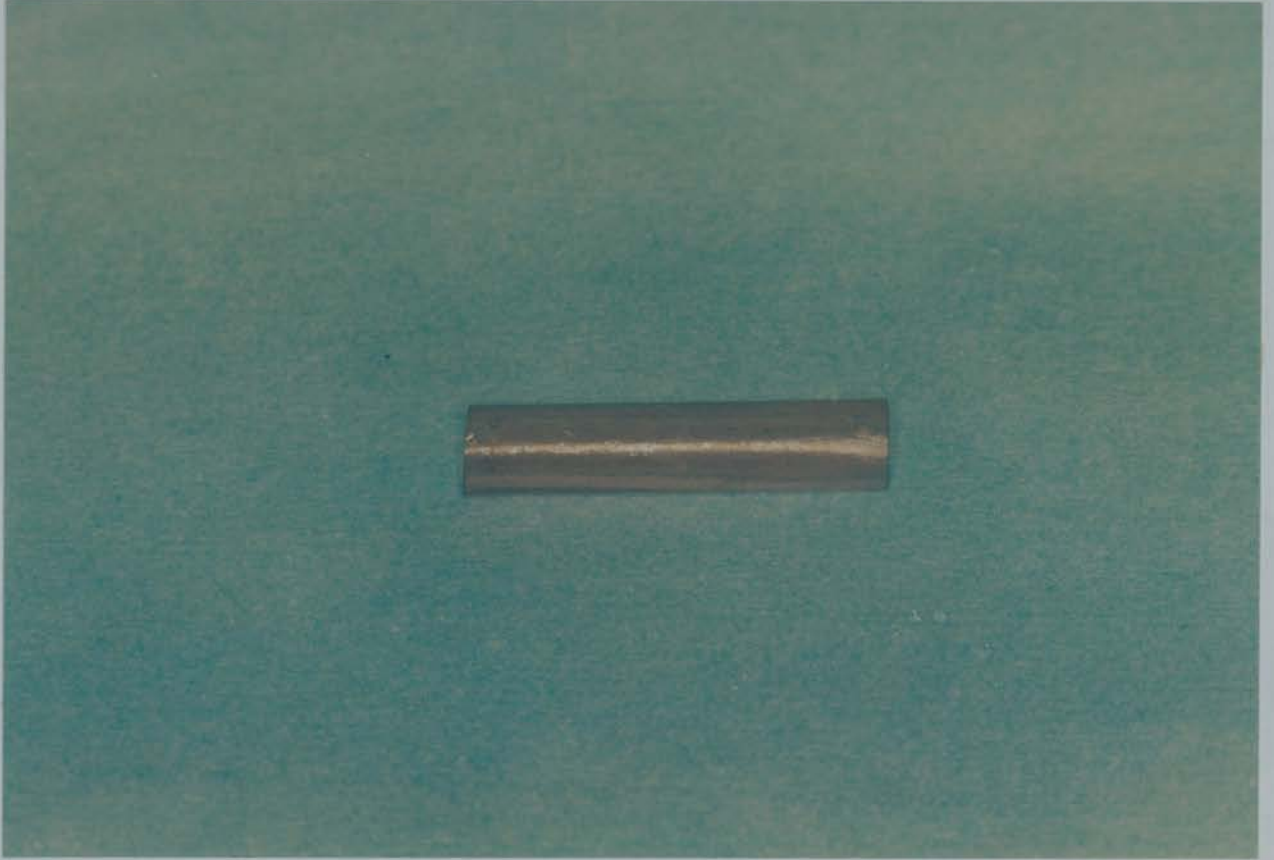


Figure 3.4.1

The CRG tube

CHAPTER 4.0

ANAESTHESIA

| <u>Section</u> | <u>Title</u> |
|-----------------------|--|
| 4.1 | <u>PRACTICAL ASPECTS OF ANAESTHETIC</u> |

4.1 ANAESTHETIC

General anaesthesia was induced with a combination of Hypnovel and Hypnorm. Each animal was given an intraperitoneal injection of Hypnovel (Midazolam: Roche Products Ltd., UK) 2 mg kg^{-1} , followed immediately by an intramuscular injection of Hypnorm (fentanyl 2.5 mg ml^{-1} and fluanisone 0.07 mg ml^{-1} , Janssen Pharmaceuticals Ltd., UK) 3 ml kg^{-1} in the right gluteus superficialis muscle.

In earlier experiments during procedures lasting more than 45-60 minutes, a subsequent dose ('top-up') of Hypnorm (i.m.) had been administered to maintain anaesthesia (Hems, 1993). However, it was found that maintenance of anaesthesia using the 'top-up' method was unreliable and resulted in varying degrees in the depth of anaesthesia. Owing to the length of the procedures in the present experiments and the strength of current applied to the peroneal nerve during assessment, it proved difficult to maintain a constant level of anaesthesia with the 'top-up' method. The animal often regained consciousness, therefore a more stable and controllable method of maintaining general anaesthesia was required.

Ten minutes after induction, by which time the rabbit was unconscious, general anaesthesia was maintained with a Bain circuit using a mixture of O_2 1 l min^{-1} , N_2O 0.5 l min^{-1} and 2% halothane (May & Barker, Rhone-Poulenc Group, UK). These gases were delivered from a Fluotec IV vaporizer and administered through a face mask. The expired gases were collected with a Veterinary Fluosorber

(International Market Supply, Cheshire, England) (Hunter *et al.*, 1984). Anaesthesia with gases provided improved control with respect to speed of application and depth of anaesthesia. This method also resulted in a quicker recovery time after surgery. A Pulse Oximeter (MicroSpan 3040, BCI International, UK) was used to continuously monitor blood oxygen saturation (SaO₂).

CHAPTER 5.0

SURGERY

| <u>Section</u> | <u>Title</u> |
|----------------|---|
| 5.1 | <u>PREPARATION FOR SURGERY</u> |
| 5.2 | <u>INITIAL OPERATION</u> |
| 5.2.1 | <u>CRG tube filled with freeze thawed muscle (CRG-M)</u> |
| 5.2.2 | <u>CRG tube filled with nerve (CRG-N)</u> |
| 5.2.3 | <u>CRG tube filled with freeze thawed muscle and nerve (CRG-MN)</u> |
| 5.2.4 | <u>Freeze thawed muscle graft (FTMG)</u> |
| 5.2.5 | <u>CRG tube only (CRG-GAP)</u> |
| 5.2.6 | <u>Microwave muscle graft (MMG)</u> |
| 5.3 | <u>CLOSURE OF THE INCISION AND OBSERVATION</u> |
| 5.4 | <u>ACUTE OPERATION</u> |

5.1 PREPARATION FOR SURGERY

Once anaesthesia was established, the fur from the lateral aspect of the thigh and the lateral and posterior leg was shaved using electric clippers (Oster Professional Products, Milwaukee, USA). Immac cream (Reckitt and Coleman Ltd., Hull, UK) was applied to the shaved area for 5 minutes to remove any extra hair. The skin was washed with warm tap water to remove the Immac cream and then washed with chlorhexidine (0.5% in 70% alcohol) to remove any oil and dirt. Correct preparation of the required surgical area cannot be overly emphasized. In the initial operation, extra hairs may penetrate the wound site causing infection. Excessive hair, dirt or oil on the skin during the acute operation may produce erroneous electrophysiological results.

After the skin was prepared, the animal was placed on a heating blanket and covered with an earthed aluminium foil blanket to help maintain body temperature. A digital readout thermistor probe (Technoterm 110, RS Products Ltd., West Germany) was used to continuously monitor the rectal temperature. Despite this, the temperature fell by more than 1.5°C in every procedure extending beyond 1.5 hours. During assessment, the average time was 3 hours and the decline in temperature was consistent for all animals. It was concluded that any change in the value of electrophysiological variables caused by the decline in temperature would be

consistent among the groups. A 5 ml bolus of isotonic saline at 37 °C was given subcutaneously in the scruff of the neck to help prevent dehydration.



Figure 5.1.1 Photograph showing the rabbit's leg completely shaved and cleaned.

5.2 **INITIAL OPERATION**

All surgery was performed under sterile conditions. Sterile drapes were used to isolate the area not within the operative field and sterile instruments were used during the operation. The exposed skin was washed with an iodine antiseptic solution (Betadine, Seton Health Care, England). An incision was made in the skin from a point over the sciatic notch to the proximal portion of the knee joint. The skin edges were mobilized and haemostasis was obtained using bipolar diathermy. The fascial plane between the gluteus superficialis and the coccygeofemoralis muscles was developed, extending from the sciatic notch to the knee, to expose the sciatic (ischadicus) nerve.

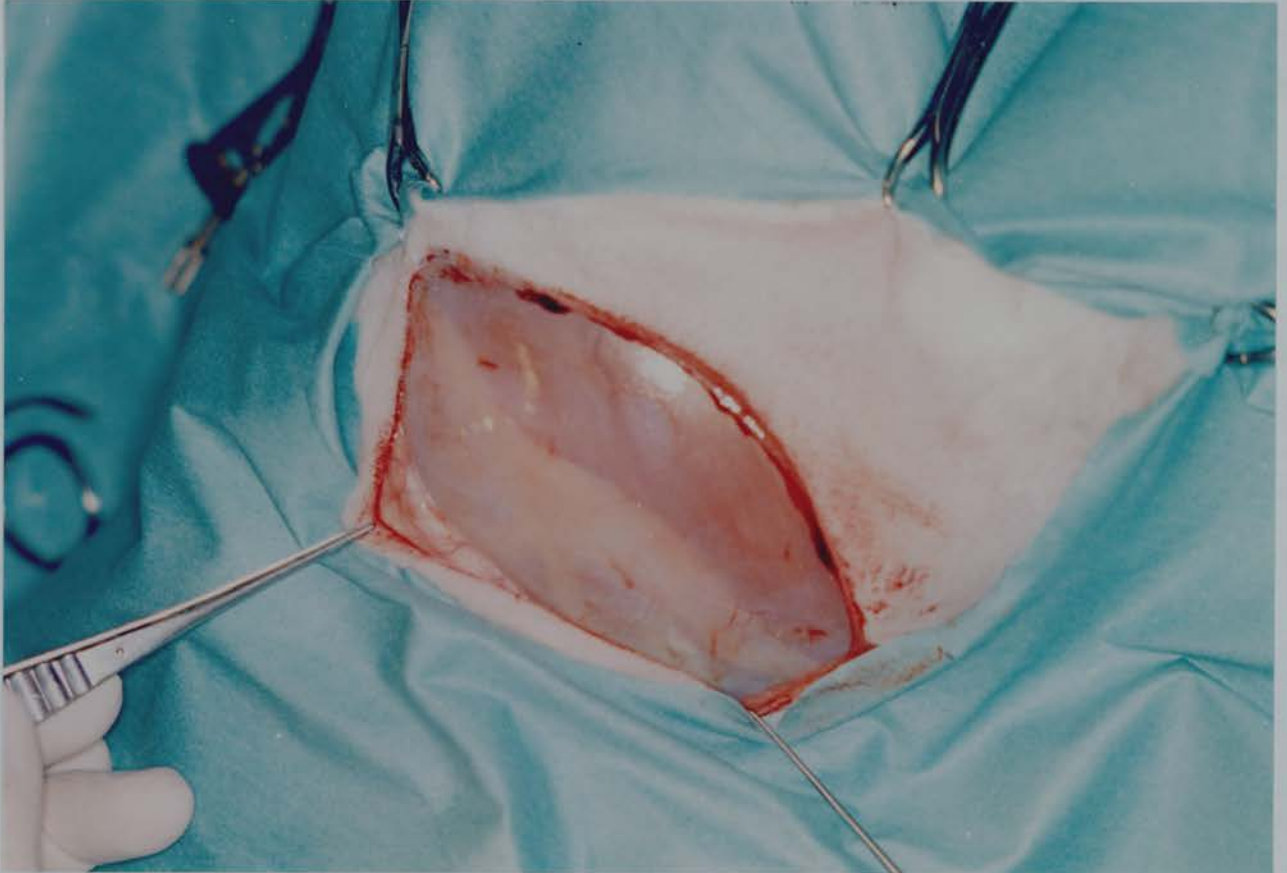


Figure 5.2.1

Photograph showing the fascial plane between the gluteal superficialis and the coccygeofemoralis muscles of the thigh.

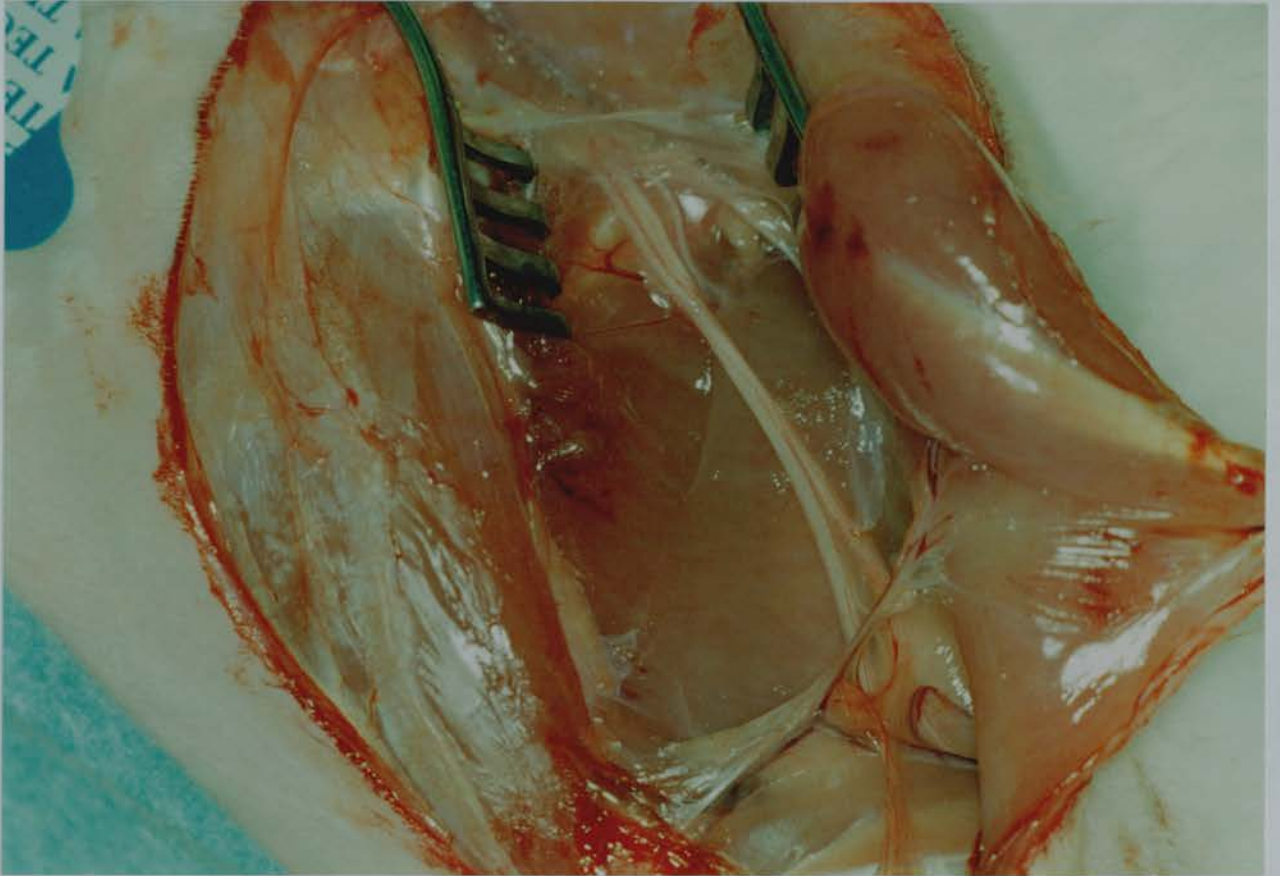


Figure 5.2.2

Photograph showing the fascial plane divided and exposing the sciatic nerve.

Beginning 1 cm above the popliteal artery, the peroneal nerve was separated from the tibial nerve using microsurgical forceps (note: the anatomy of the rabbit's leg is considerably different from the human — see figure 5.2.2). The separation between the peroneal and tibial nerves was extended for a distance of 3 cm in the proximal direction. Care was taken to ensure that trauma and tension to the peroneal and tibial nerves were kept to a minimum. The peroneal nerve was divided approximately 2 cm above the location where the popliteal artery crosses the nerve in the distal compartment of the thigh. A piece of nerve was removed from the distal segment of the peroneal nerve to create a 1 cm gap after retraction. This piece of nerve was set aside and used in preparing the CRG-N and CRG-MN grafts (see sections 5.2.2 and 5.2.3). During this procedure vision was assisted with an operating microscope (Wild M690, Heerbrugg, Switzerland) with stepless variable magnification of 7 to 36 times. Figure 5.2.3 shows the peroneal nerve divided.

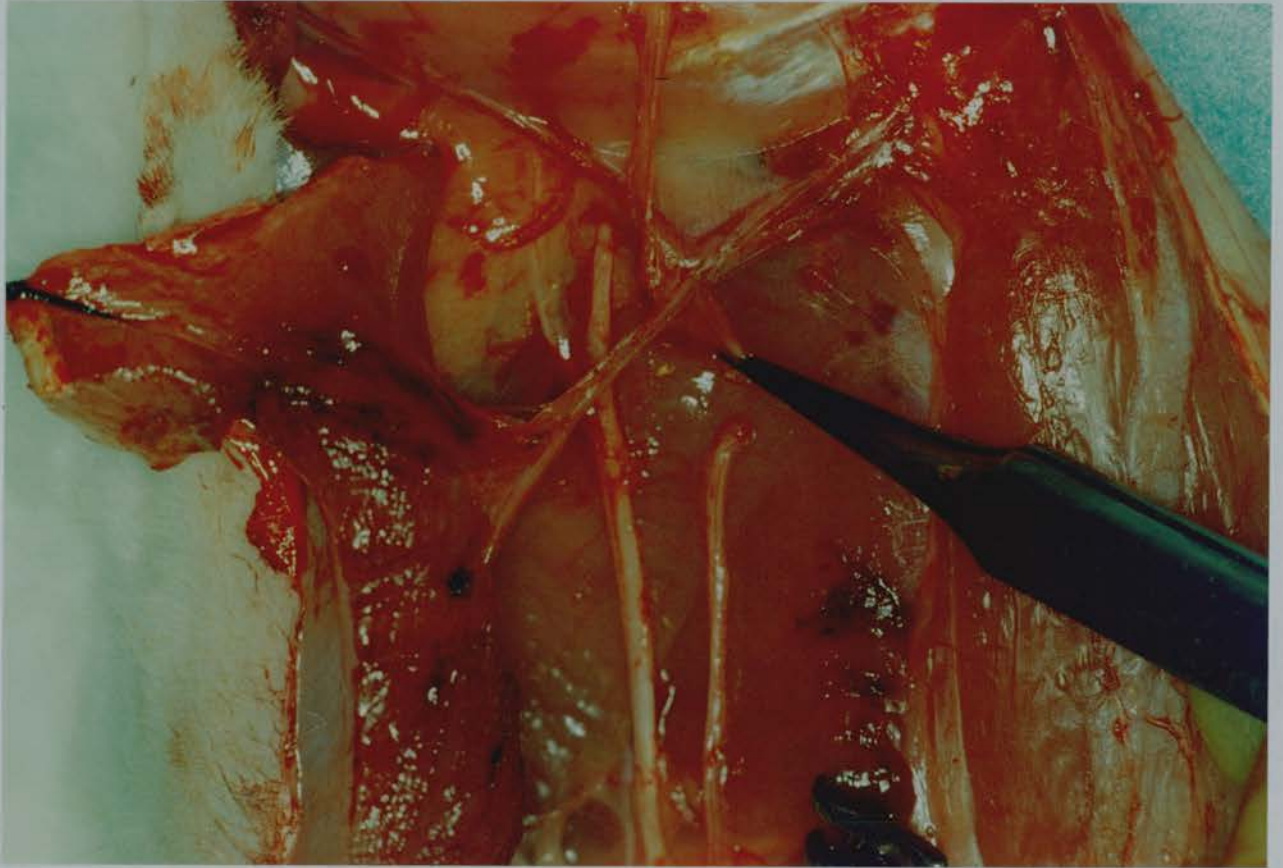


Figure 5.2.3 **Photograph of the divided peroneal nerve.**

The nerve gap was repaired with one of the following methods:

5.2.1 CRG tube filled with freeze thawed muscle (CRG-M)

A 2 cm × 2 cm × 1 cm block of parallel muscle fibres was removed from the coccygeofemoris muscle and wrapped in aluminium foil, then submerged in liquid nitrogen. When the liquid nitrogen stopped boiling the muscle block was at thermal equilibrium (-196°C). The frozen block of muscle was thawed in distilled water and then minced into tiny pieces with a razor blade. These tiny pieces were stuffed into the centre of the CRG tube to fill a distance of 1 cm. Originally, an attempt was made to keep the muscle fibres in a parallel arrangement. This proved impossible, owing to the small size of both the CRG tube and the chopped freeze-thawed muscle. The pieces of freeze-thawed muscle had to be inserted in a random arrangement. The proximal and distal ends of the transected peroneal nerve were placed in opposite ends of the CRG tube. Care was taken to ensure that the nerve ends were flush to the freeze thawed muscle inside the tube and that the nerve ends were in the correct geometric alignment. The nerve was secured to the tube by means of two interrupted 10/0 Ethilon sutures (Ethicon Ltd, Edinburgh, UK) at each end which were passed through a hole drilled through the tube at each end. The sutures were placed in the epineurium of the peroneal nerve 5 mm away from each cut nerve end. Placing the sutures away from the cut nerve ends helped to reduce the trauma and tension at the injury site .



Figure 5.2.1.1 **Photograph of the CRG tube filled with pieces of chopped freeze thawed muscle.**

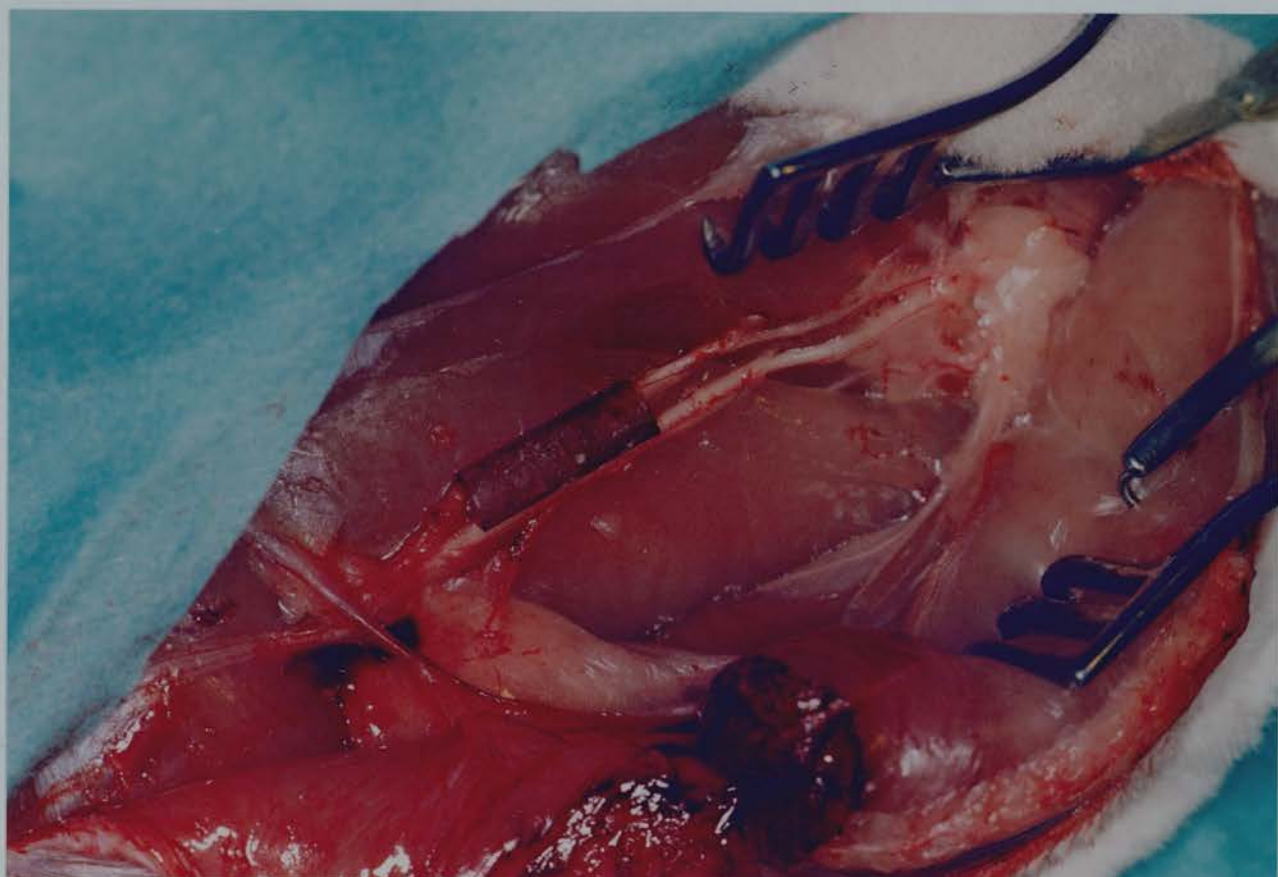


Figure 5.2.1.2

Photograph of the CRG-M graft inserted in the peroneal nerve gap.

5.2.2 CRG tube filled with nerve (CRG-N)

The segment of peroneal nerve that was removed (Section 5.2) was chopped into tiny pieces. These pieces were stuffed into the CRG tube. There was not enough peroneal nerve harvested to fill the tube with 1 cm of chopped nerve, so the pieces were placed in the centre of the CRG tube. The proximal and distal ends of the peroneal nerve were placed in opposite ends of the CRG tube and positioned to create a 1 cm gap with the chopped peroneal nerve between them. The nerve was secured to the tube as described in section 5.2.1.

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

In the CRG-MN group, the muscle was prepared as described in section 5.2.1 and the nerve was prepared as described in section 5.2.2. The tube was stuffed with the chopped freeze-thawed muscle and with chopped nerve to create a 1 cm graft. The order of the components of the graft was: a) 0.3 cm of chopped freeze thawed muscle, b) 0.05 cm of chopped nerve, c) 0.3 cm of chopped freeze thawed muscle, d) 0.05 cm of chopped nerve and e) 0.3 cm of chopped freeze thawed muscle. This is diagrammatically shown in figure 5.2.3.1. The proximal and distal segments of the peroneal nerve were inserted and secured to the CRG tube as described above in section 5.2.1.

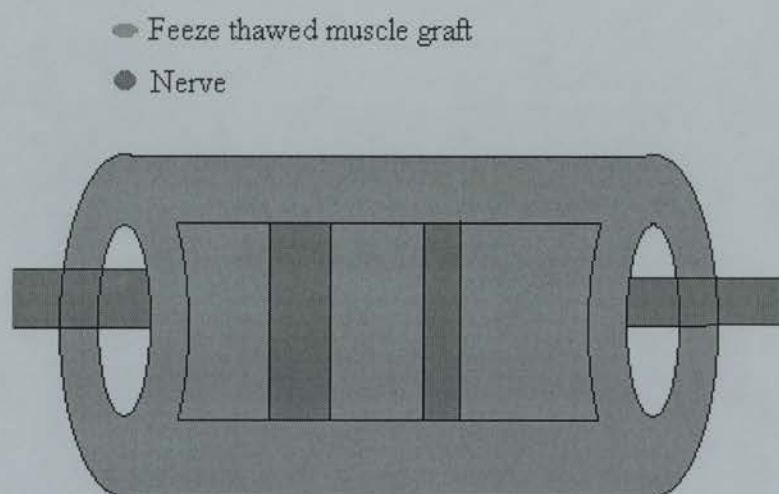


Figure 5.2.3.1 **Diagrammatic representation of the CRG-MN graft.**

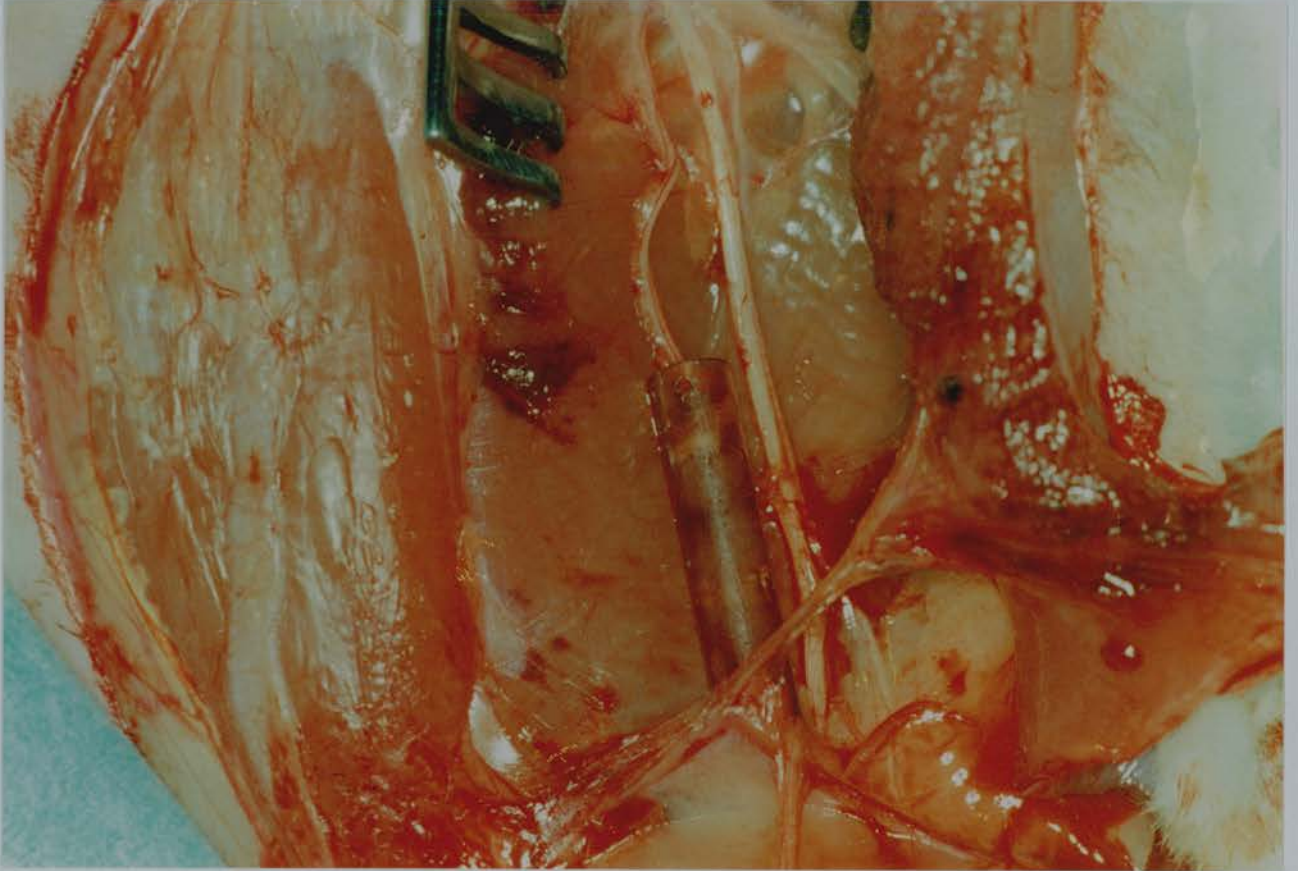


Figure 5.2.3.2 **Photograph of the CRG-MN graft inserted in the peroneal nerve gap.**

5.2.4 Freeze thawed muscle graft (FTMG)

In the FTMG group, a 2 cm × 2 cm × 1 cm sized block of muscle was removed and prepared as described in section 5.2.1. After the muscle block was thawed, it was cut to form a 1 cm × 1 cm × 1 cm graft and sutured in the peroneal nerve gap with 3 to 4 interrupted 10/0 Ethilon sutures.

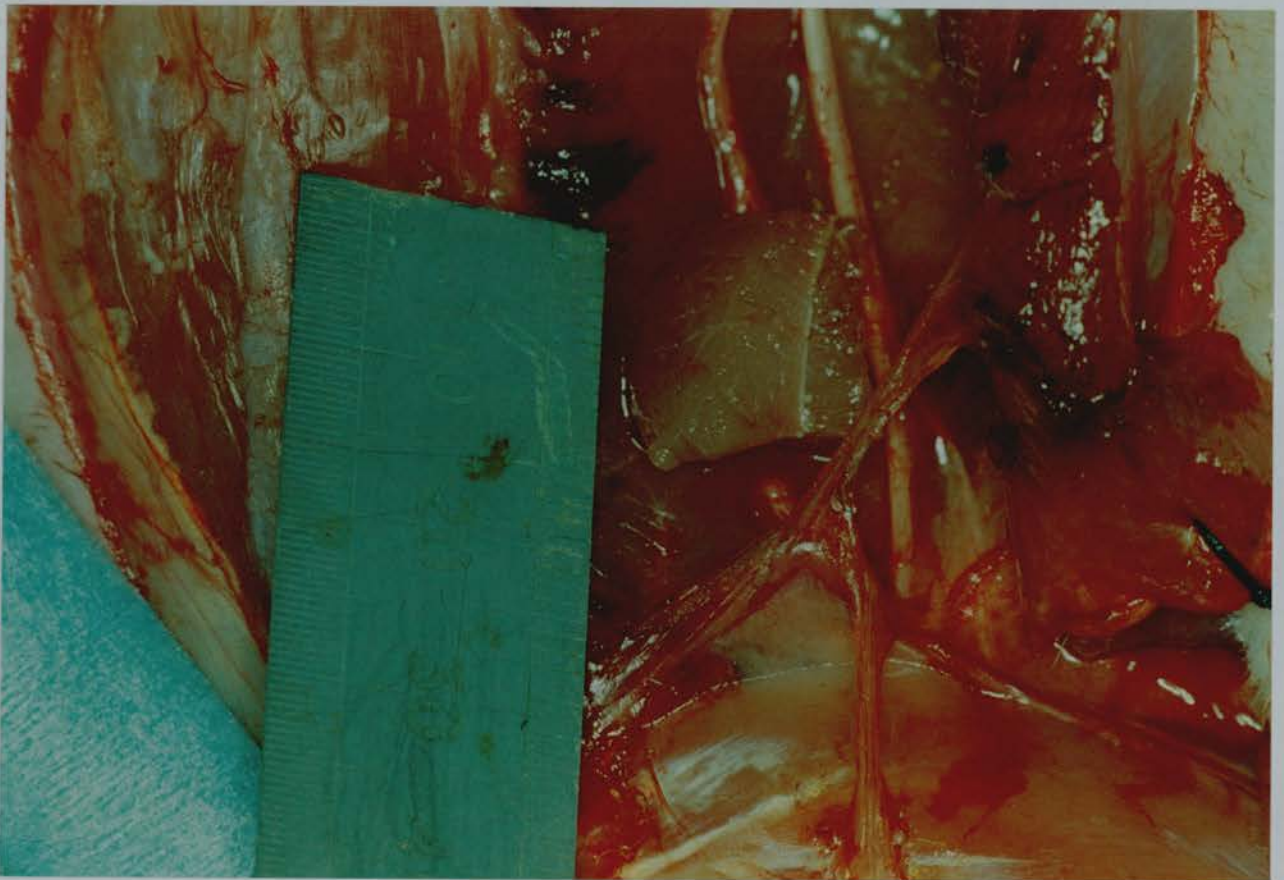


Figure 5.2.4.1 Photograph of the FTMG graft inserted in the peroneal nerve gap.

5.2.5 CRG tube only (CRG-GAP)

The proximal and distal ends of the peroneal nerve were placed in opposite ends of the CRG tube. Care was taken to ensure that trauma was kept to a minimum and that the nerve ends were placed in the tube without rotation and with the correct geometric alignment maintained. The nerve ends were secured to the tube as described in section 5.2.1.

5.2.6 Microwave muscle graft (MMG)

The MMG was prepared by removing a 2 cm × 2 cm × 1 cm block of parallel muscle fibres from the coccygeofemoralis muscle. This piece of muscle was placed in a sterile container and heated in a domestic microwave for 100 s on 'medium' (350 W). The MMG was cooled for 2 minutes after which the charred edges on the muscle block were removed. The block was then trimmed to form a 1 cm × 1 cm × 1 cm graft.

It was discovered that the size of the muscle block had to be consistent to ensure that each animal in the group received a graft that had been subjected to the same thermal shock. It has been suggested that to allow nerve regeneration to occur, it is essential that the muscle block is heated above 60°C (Hall & Enver, 1994) but not above 80°C (Goodman *et al.*, 1991). For the present experiment a large number

of trials were required to determine the correct “wattage” and time settings to heat the entire block of muscle within this narrow temperature range. This made the production of the MMG very difficult.

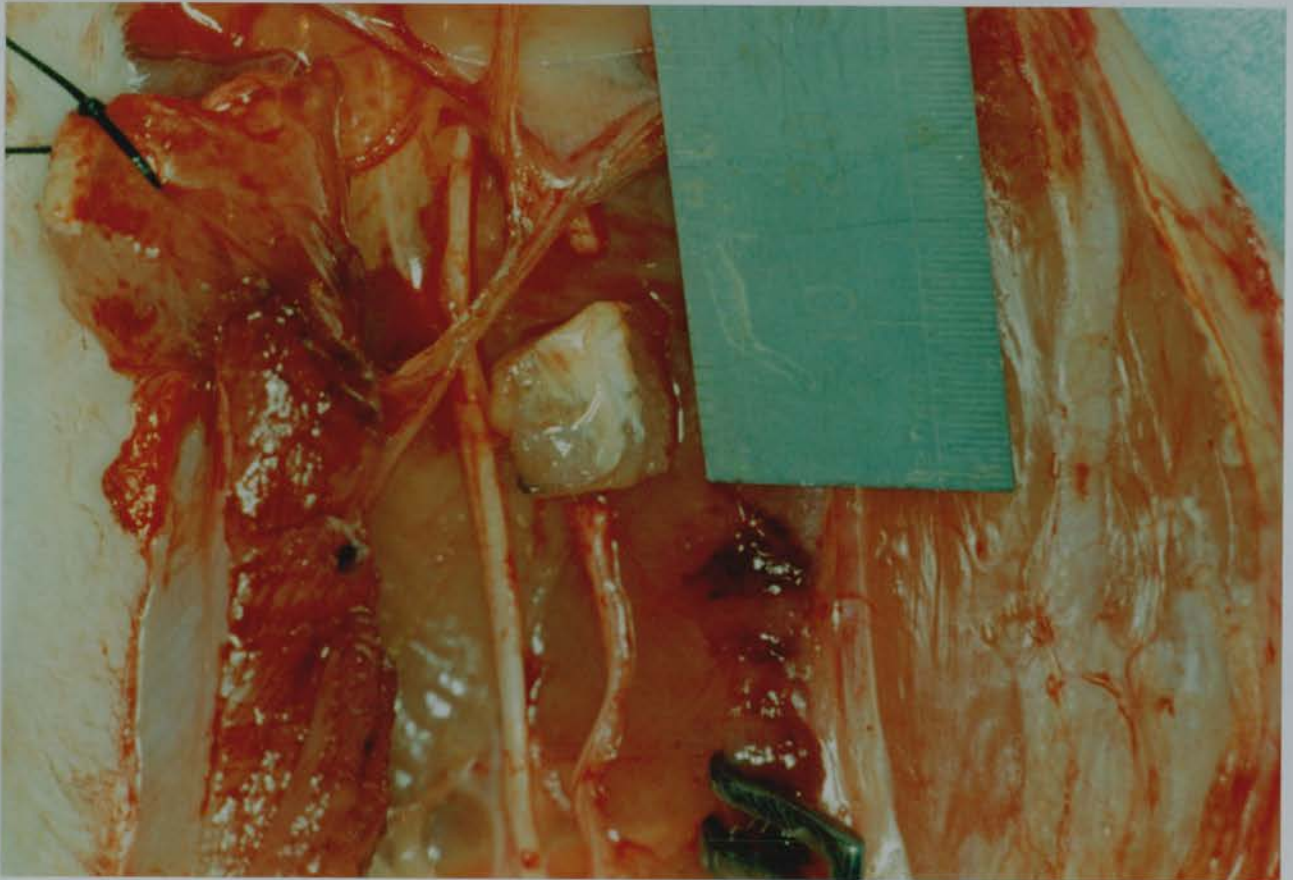


Figure 5.2.6.1 **Photograph of the MMG graft inserted in the peroneal nerve gap.**

5.3

CLOSURE OF THE INCISION AND OBSERVATION

After nerve repair the incision was closed by suturing the biceps femoris muscle to the gluteus superficialis and tensor fasciae latae muscles with 5-10 interrupted 6/0 polyglactin sutures (Vicryl, Ethicon Ltd. UK). The skin was closed with a subcuticular stitch using 3/0 polyglactin sutures (Vicryl, Ethicon Ltd. UK) and then the skin was cleaned with chlorhexidine. Antibiotic spray (Tribiotic, Riker laboratories, UK) was applied to the deep tissues and plastic film spray (Nobecutane, Astra Pharmaceuticals Ltd., UK) was applied to the skin. The rabbit was given buprenorphine (Vetagesic, Reckitt and Colman Products Ltd., Hull, UK) 0.3 mg ml⁻¹ for pain relief after the operation.

The animals were left to recover under observation for one hour and then returned to the pen with the other rabbits. Daily food and water checks were performed. The animals were also weighed and inspected for infection weekly.

All animals showed some degree of foot-drop on the operated side but they adapted quickly by using the extensors of the leg. There were three cases of wound infections. Two responded to antibiotic treatment. The third rabbit did not respond to antibiotic treatment and was removed from the experiment.

5.4 **ACUTE OPERATION**

Six months after the primary procedure the rabbits were assessed for recovery of function. The animal was prepared as described in section 5.1 with non sterile procedures. An incision was made in the lateral thigh from the sciatic notch to the knee joint as described in section 5.2. In the acute operation, a second incision was made from the knee joint to the mid-point of the leg and the skin was reflected to further expose the biceps femoris muscles. Haemostatis was achieved as before using bipolar diathermy.

The sciatic nerve was exposed as described in section 5.2. Once the sciatic nerve was located, the tendons of the coccygeofemoris and biceps femoris were divided and reflected to further expose the peroneal nerve in the anterior compartment of the leg.

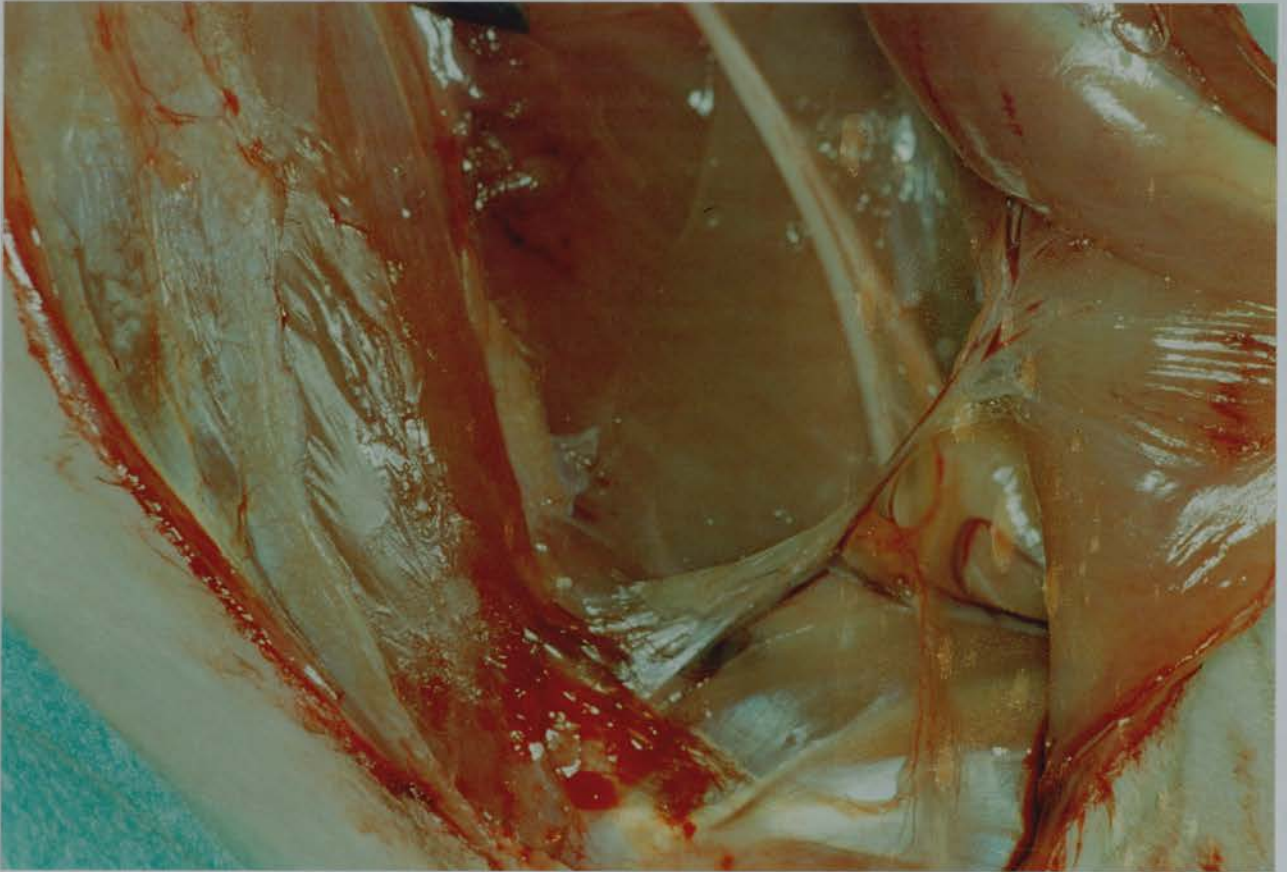


Figure 5.4.1

Photograph of the peroneal nerve extending into the anterior compartment of the leg.

Exposing the peroneal nerve in the anterior compartment of the leg increased the length of nerve that could be mobilized. This improved the accuracy of the electrophysiological recordings (see Appendix A). Using microsurgical forceps, the peroneal nerve was separated from the tibial nerve 1 cm proximal to the site of repair extending to the sciatic notch. Care was taken to ensure trauma to the peroneal nerve was kept to a minimum. The tibial nerve was divided in the gluteal region as close to the sciatic notch as possible. The tibial nerve was also divided distal to the popliteal artery in the leg. Dividing the tibial nerve at these locations prevented erroneous results during assessment owing to cross current from the peroneal nerve to the tibial nerve. During this procedure vision was aided with an operating microscope.

Once assessment of the animal was completed, the animal was killed by a Schedule One method (Home Office (H.M.S.O.), 1986) with a lethal intravenous injection of phenobarbitone (Lethobarb, J.M. Loveridge plc, Duphar Veterinary Ltd, Southampton, UK) 0.7 ml kg^{-1} .



CHAPTER 6.0

ELECTROPHYSIOLOGICAL METHODS

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6.1 INTRODUCTION & GENERAL SETUP

Electrophysiological methods for assessing the function of nerve and muscle were first studied in the 19th century (Carpenter, 1837). However, the increased clinical application only came with improvements in electrical design resulting in an improvement in the ability to record small electrical potentials while filtering the background noise.

All electrophysiological tests were performed using a Medelec Sapphire EMG machine (Medelec, Old Woking, UK) in order to stimulate the nerve under study and record the compound muscle action potential (M-wave) in the associated muscle. Figure 6.1.1 is a photograph of the Medelec Sapphire.

A ground electrode was placed on the skin over the biceps femoris muscle and a 6 mm silver / silver chloride disc electrode (Medelec, Old Woking, UK) was placed over the motor point of the extensor digitorum muscle (EDL). A second 6 mm silver / silver chloride disc electrode was placed distal to the first electrode over the tendon of insertion of the EDL. A small amount of electrode cream (Grass Instruments Co., Maine, USA) was placed on the surface of all three electrodes to improve conductivity. Figure 6.1.2 is a photograph of all three electrodes in the correct position.



Figure 6.1.1 **Photograph of the Medelec Sapphire EMG Machine**



Figure 6.1.2 **Photograph of the recording electrodes and ground electrode in the proper position.**

6.2 MAXIMUM CONDUCTION VELOCITY

6.2.1 Introduction

The measurement of maximum conduction velocity (*MCV*) involves calculating the velocity of an impulse traveling through a segment of nerve. measurement of *MCV* provides a useful tool for assessing the recovery of function after nerve repair because it is minimally invasive, can be preformed quickly and repeated at different times without destroying the sample tissue. The reproducibility of results, ease of determining and high degree of inter-examiner reliability has made *MCV* the ‘gold standard’ test in assessing the recovery of function after nerve repair.

Several early studies demonstrated that after peripheral nerve injury the *MCV* did not return to normal levels. It was found that the *MCV* returned to 80% of normal velocity after nerve section and suture (Berry *et al.*, 1944a). While Cragg and Thomas (Cragg & Thomas, 1964) and Erlanger and Schoepfle (Erlanger & Schoepfle, 1946) found that after nerve crush injuries the *MCV* returned to only 76% of normal or below.

It has been shown that the velocity of an impulse varies along the curse of the nerve (Sunderland, 1978) and even at different times by as much as 8 m s^{-1} (Low *et al.*, 1962; Honet *et al.*, 1968) It has also been shown that the conduction velocity in the proximal section of a nerve is faster than in the distal portion of the nerve (Stålberg, 1966).

There are several factors which affect the velocity of the impulse ranging from axon and fibre diameter, schwann cell thickness, schwann cell structure, internodal length. It was observed as early as 1907 (Göthlin, 1907) that the larger the diameter of a nerve the faster it would conduct an impulse. From this observation several authors confirmed this result (Gasser & Erlanger, 1927) (Erlanger, 1927; Matthews, 1927) by demonstrating the conduction velocity changes in different sized nerves. This theory was refined to state that the conduction velocity of a nerve is proportional to the square of its diameter (Erlanger & Gasser, 1937; Zotterman, 1937) while others suggested it was proportional to 0.6 of the diameter (Pumphrey & Young, 1938).

A relationship between myelin sheath thickness and conduction velocity was also observed around the same time (Bishop & Heinbecker, 1930; Bullock, 1945; Sanders & Whitteridge, 1946). The problem with these studies however, is that the variation in thickness of the myelin sheath of similar diameter fibres was not taken into account.

The internodal length has also been postulated to have a causal affect on the conduction velocity (Schmitt & Bear, 1939; Taylor, 1941; Taylor, 1942). The longer the internodal length the faster the conduction velocity. However if the internodal length is too great conduction will stop as the current will decay below the threshold level before it reaches the next exposed segment of nerve.

It is probable that all of these anatomical features of a nerve affect conduction velocity but the mathematical formula has not been developed as of yet. There are also many other factors of the nerve anatomy and physiology that will affect conduction velocity. Some of these might be: the amount of proteins within the axoplasm, ion concentration inside and outside the membrane, and the number and location of the mitochondria within the axon.

To measure the maximum motor conduction velocity, the time for both neuromuscular transmission and generation of the muscle action potential must be removed. By stimulating the nerve at two separate points (S1 and S2) and using simple algebra, it is possible to remove the two time components mentioned above. If the distance between the segment of nerve S1S2 is known and the latency to contraction from stimulating the nerve at S1 and S2 is also known, it is possible to determine the maximum motor conduction velocity using the following formula.

$$ConductionVelocity = \frac{\overline{S1S2}}{L_{s1} - L_{s2}}$$

Figure 6.2.1.1

Where:

S1S2 is the length of the segment

L_{s1} is the latency to muscle contraction when the nerve is stimulated at S1

L_{s2} is the latency to muscle contraction when the nerve is stimulated at S2

6.2.2 Theory

The measurement of conduction velocity is one of the main analytical tools used in assessing the success or failure of peripheral nerve repair. The accuracy in measuring conduction velocity is determined by the accuracy of the M-wave latencies and measuring the distance between the electrodes. The use of conduction velocity as a means of assessing peripheral nerve function was developed by Berry *et al* (Berry *et al.*, 1944b) who demonstrated that the conduction velocity was reduced in regenerating nerve fibres. The main reason for the popularity of this test is the reproducibility of the results and the ease with which they are obtained. However, the error involved in determining the conduction velocity is seldom addressed. With some simple assumptions and a little knowledge of the experimental model, *i.e.* the maximum conduction velocity, one should be able to determine a set of parameters to maintain this error at a minimum level.

The conduction velocity is determined by measuring the difference in evoked potentials or M-wave latencies from a muscle when its nerve is stimulated at two separate locations along its course. By subtracting the M-wave latencies from these points, the time required for the fastest impulse to travel between them is calculated. Since the distance can be measured between these two points and the time the impulse takes to travel between them can be calculated, the velocity of the impulse can be determined and is generally denoted as the conduction velocity (*CV*).

$$CV = \frac{d}{t}$$

Figure 6.2.2.1

Where: d = distance between point S1 and point S2

t = time required for the fastest impulse to travel between S1 and S2

From equation 6.2.2.1, one can see that there are two sources of error when calculating the conduction velocity. The first source of error is in the numerator that is the measured distance between the two points of stimulation. These two points are called S1 and S2 and the segment between them is denoted $\overline{S1S2}$. The second source of error is in the denominator that is the time the impulse takes to travel the segment $\overline{S1S2}$. Looking ahead to figure 6.2.2.2, error is decreased as the distance increases. Given that both sources of error are known and that as the segment length of $\overline{S1S2}$ increases, error decreases, then the question that must be addressed in calculating conduction velocity is:

Knowing the elements of error within the system of conduction velocity, what must the distance be between points S1 and S2 such that the error in the system is at a minimum?

To answer this question the two sources of error must be accounted for. The distance is the measurement between the two stimulating electrodes, point S1 and S2. By convention, this is measured with calipers from stimulating cathode to stimulating cathode. This measurement is then rounded to the nearest millimeter. Since the eye is able to detect distances less than a millimeter, it must be concluded that the error to rounding should not exceed 0.5 mm.

The second source of error is the marking of the M-wave latency. Conduction velocity is a value of the fastest conducting fibres therefore, the latency must be calculated from the first sign of an M-wave. However, this take off point is often difficult to determine especially in the repaired nerve. Electrode placement, visual marking errors and background noise will affect the determination of when the M-wave begins. With the advance of digital computers this value has improved in accuracy but still may produce a ± 0.02 ms source of error, which may be greater in some algorithms.

Given that there are two sources of error in the calculation of conduction velocity, and that these errors can be estimated to a reasonable level, the distance required to minimize error can then be calculated.

The formula for determining the source of error of a system is:

$$Error = \frac{E1 - E2}{(E1 + E2) / 2}$$

Figure 6.2.2.2

Where:

E1 is the correct conduction velocity

E2 is the conduction velocity with some source of error

If this error equation is expressed with respect to conduction velocity, the *E1* will represent the correct conduction velocity, while *E2* will denote the conduction velocity with some source of error. Since the equation for conduction velocity is known, equation 6.2.2.1, an equation for conduction velocity with some source of error can be developed, called *CV'*. If it is assumed that in the equation for *CV'* the error will change the time by *k* ms and the distance by *n* mm. Therefore, formula 6.2.2.2 will become:

$$CV' = \frac{d + n}{t + k}$$

Figure 6.2.2.3

Where:

d = distance

n = error in measuring length

t = time,

k = error in measuring time

If equation 6.2.2.3 is substituted for $E2$ in equation 6.2.2.2 and equation 6.2.2.1 is substituted for $E1$, then the following expression is produced:

$$Error = \frac{\left(\frac{d}{t} - \frac{(d+n)}{(t+k)} \right)}{\left(\frac{\left(\frac{d}{t} + \frac{(d+n)}{(t+k)} \right)}{2} \right)}$$

Figure 6.2.2.4

Since there are two sources of error, k and n , logic dictates that equation 6.2.2.4 can be written as 8 cases. Each case can be solved yielding a distance required to maintain error levels to a minimum. To determine the minimum distance required to produce acceptable results for conduction velocity k and n must be

assigned different signs. Such that, in the determination of conduction velocity the M-wave latency is increased (+ k), while the distance is decreased (- n). The proof of all eight cases and the reason why k and n must have opposite signs is given in appendix A.

If k and n with their respective signs are substituted into figure 6.2.2.4, figure 6.2.2.5 will be produced.

$$Error = \frac{\left(\frac{d}{t} - \frac{(d-n)}{t+k} \right)}{\left(\frac{\left(\frac{d}{t} + \frac{(d-n)}{t+k} \right)}{2} \right)}$$

Figure 6.2.2.5

This can be simplified to:

$$Error = \frac{2dk + 2tn}{2dt + dk - tn}$$

Figure 6.2.2.6

From figure 6.2.2.1 it is known that for a constant conduction velocity, distance is proportional to time. Thus as d increases the error will decrease, given that d is proportional to t , as demonstrated by figure 6.2.2.1.

If it is assumed that the conduction velocity will remain constant for the segment $\overline{S1S2}$, the equation in figure 6.2.2.1 can be rewritten as:

$$t = \frac{d}{CV}$$

Figure 6.2.2.7

Substituting the equation in figure 6.2.2.7 into the equation in figure 6.2.2.6 will produce the following equation:

$$Error = \frac{2dk + 2\frac{dn}{CV}}{2d\left(\frac{d}{CV}\right) + dk - \frac{dn}{CV}}$$

Figure 6.2.2.8

If it is assumed that the maximal conduction velocity is 60 m s^{-1} , $k = \pm 0.02 \text{ ms}$ and that $n = \pm 0.5 \text{ mm}$ then the equation in figure 6.2.2.8 will take the form:

$$Error = \frac{.04d + \frac{d}{60}}{\frac{2d^2}{60} + .02d - \frac{0.5d}{60}}$$

Figure 6.2.2.9

If an acceptable level of error is set at 5%, then the equation in figure 6.2.2.9 can be solved such that:

$$d=3.37 \text{ cm}$$

Figure 6.2.2.10

Therefore, in a system where the maximum conduction velocity is 60 m s^{-1} , $k=\pm 0.02 \text{ ms}$ and $n=\pm 0.5 \text{ mm}$, the minimum length of segment $\overline{S1S2}$ must be greater than 3.37 cm to attain a level of error less than 5%.

There are however, several problems with this line of logic. First, the maximal conduction velocity may be greater than 60 m s^{-1} . Second, the maximal conduction velocity may not be constant between points S1 and S2. Third, is 5% an acceptable level of error in determining conduction velocity? Fourth, nerve fibres seldom lie in a straight line.

With respect to these four concerns, each has been estimated on the conservative side. In animal experiments after peripheral nerve repair, conduction velocity rarely exceeds 60 m s^{-1} . It can be shown from equation 6.2.2.8 that as conduction velocity increases so will error. Thus, by setting the conduction velocity along the upper limit of nerve velocity it will yield the minimal distance required to calculate conduction velocity with a predetermined level of error.

The level of acceptance has been set arbitrarily at 5%. In scientific studies, significant results are observed when the probability of occurrence is less than or

equal to 5%. Although percent error and probability are not directly related, it was felt that the 5% error mark should provide an acceptable standard.

Whether or not the nerve lies in a straight line is a difficult concern to address. It has been shown that nerve fibres in a nerve trunk do not lie in a straight-line (Sunderland, 1978). However, since it is impossible to assess the exact length of individual nerve fibres without destroying the nerve, the length of the nerve trunk is used as an estimation. The value of error used in this calculation is the error in measuring the length of the nerve trunk.

A graphical representation of how error will change with distance is demonstrated below. As the distance increases, variations in the latency and measured distance (error) become less significant. Thus, as distance increases error will decrease. Extrapolation along the y-axis at 5% error will show that the distance should be 3.37 cm, as determined by equation 6.2.2.8.

Thus, in designing an experiment to determine the success of nerve repair where maximal conduction velocity will be an analytical test, the distance between the points *A* and *B* must be greater than 3.37 cm. This will ensure that slight variations in the measurement of distance and M-wave latency determinations are minimized in the calculation of conduction velocity.

6.2.3 Methods

The tibial nerve was divided proximally in the gluteal region as close to the sciatic notch as possible and distal to the femoral artery in the leg. By cutting the tibial nerve at both locations it was possible to minimize the effect of any current spread from the peroneal nerve to the tibial nerve after stimulation. Cross current from the peroneal nerve to the tibial nerve travelling in the orthodromic direction was unable to stimulate the soleus muscle. Similarly, cross current travelling in the antidromic direction was unable to produce F-wave responses in the peroneal nerve. Therefore, the recorded M-wave was the result of stimulating the peroneal nerve only.

6.2.3.1 Electrode setup

A non-traumatic low impedance platinum wire stimulating electrode (Harvard Electronics, USA) was placed under the left peroneal nerve proximal to the graft site (S1). The cathode of this electrode was positioned so that it was in the distal position. A similar stimulating electrode was placed under the peroneal nerve distal to the site of repair (S2) also with the cathode in the distal position. The stimulating electrodes were positioned to avoid both the site of repair and excessive fat tissue that could not be removed. To improve the accuracy of calculating the maximum conduction velocity the distance between the segment S1S2 was always greater than 3.5 cm, see Appendix A for explanation and theory. The S2 electrode was positioned

so that it was 2 cm proximal to the EDL muscle. The reason for this is conduction lag of the slowest fibres when the nerve is stimulated. Figure 6.2.3.1.1 shows the position of the electrodes.

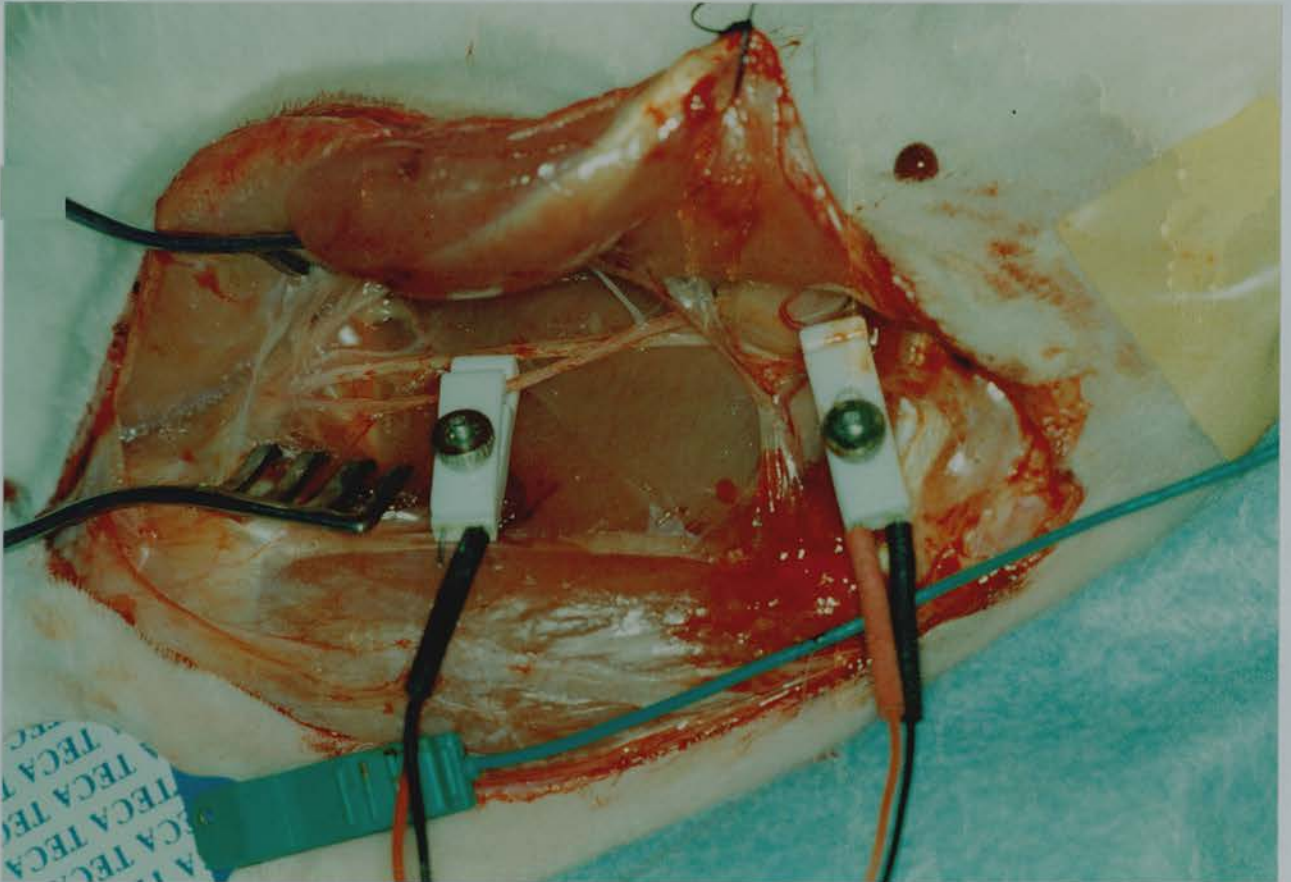


Figure 6.2.3.1.1 Photograph of the electrode position under the nerve.

Surface electrodes were chosen as the recording electrodes for this test instead of needle electrodes because surface electrodes record from all discharging muscle fibres within the pickup range of the electrodes. In addition, with surface electrodes the onset latency of the M-wave indicates the conduction time of the fastest motor fibres whereas with needle electrodes this is not always true.

Two 6 mm silver / silver chloride disc recording electrodes were placed on the skin over the EDL to record the electrical activity of the muscle during contraction. One electrode, the cathode, was placed slightly lateral to motor point of the EDL. This reduced any possible interference from the contracting tibialis anterior muscle during stimulation of the peroneal nerve. A second surface electrode, the anode, was placed on the skin over the tendon of insertion for the EDL muscle. A ground electrode was placed on the skin over the biceps femoris muscle. The electrodes were secured to the skin with tape to prevent unwanted movement of the electrodes during contractions.

6.2.3.2 Recording procedures

The recording and ground electrodes were connected to a preamplifier that was then connected to the amplifier of the Medelec Sapphire II EMG machine (Medelec, Old Woking UK). The stimulating electrode at the S1 site was connected to the B stimulator and the stimulating electrode at the S2 site was connected to the A

stimulator of the EMG machine. The sweep speed of the EMG visual screen was set at 20 ms and the stimulation rate was set at 1 pulse per second. The low cut-off filter, which removes high frequency components, was set at 3 Hz while the high cut-off filter, which removes the low frequency components, was set at 10 kHz.

The peroneal nerve at the S1 site was stimulated with a square wave current of 50 μ s duration. The resulting electrical activity of the EDL muscle due to the contraction was recorded by the surface electrodes. The difference between the cathode and anode was amplified and digitally filtered while the common frequency components recorded from the ground electrode were removed. The resulting data were displayed on the EMG screen and called an M-wave (muscle wave).

The current was increased until a M-wave could be detected on the EMG screen at a gain of 200 mV per division. The M-wave was often observed before any visual confirmation of muscle twitch of the EDL. The current was then increased until there was no further change in the size of the M-wave and was called the maximal stimulation current for S1. The gain on the Medelec Sapphire II EMG machine was adjusted so that the M-wave could be visualized entirely on the EMG screen. The maximal stimulation current for S2 was also determined using the same method. A supramaximal current was used for all stimuli in the experiments, and was defined as 30% above the maximal stimulus current.

Once all parameters were set, the peroneal nerve was stimulated at S1 with supramaximal stimuli. Eight traces were averaged to help remove any interference.

This trace was stored in the EMG memory. The peroneal nerve was then stimulated at S2 with the supramaximal current determined for that site with eight traces being averaged. This trace was also stored in memory. The following values were determined from each trace.

- A. Latency to onset (L) – latency from stimulation to deflection from baseline of the M-wave. (A to 1 in figure 6.2.3.2.1)
- B. Amplitude of the M-wave – The change in voltage from take-off to peak (1 to 2 in figure 6.2.3.2.1)
- C. Area of the M-wave – The area bounded by the positive phase of the M-wave. (The area under the trace 1 to 3 in figure 6.2.3.2.1)
- D. Duration of the M-wave – The latency from beginning of the M-wave till completion. (1 to 5 in figure 6.2.3.2.1)

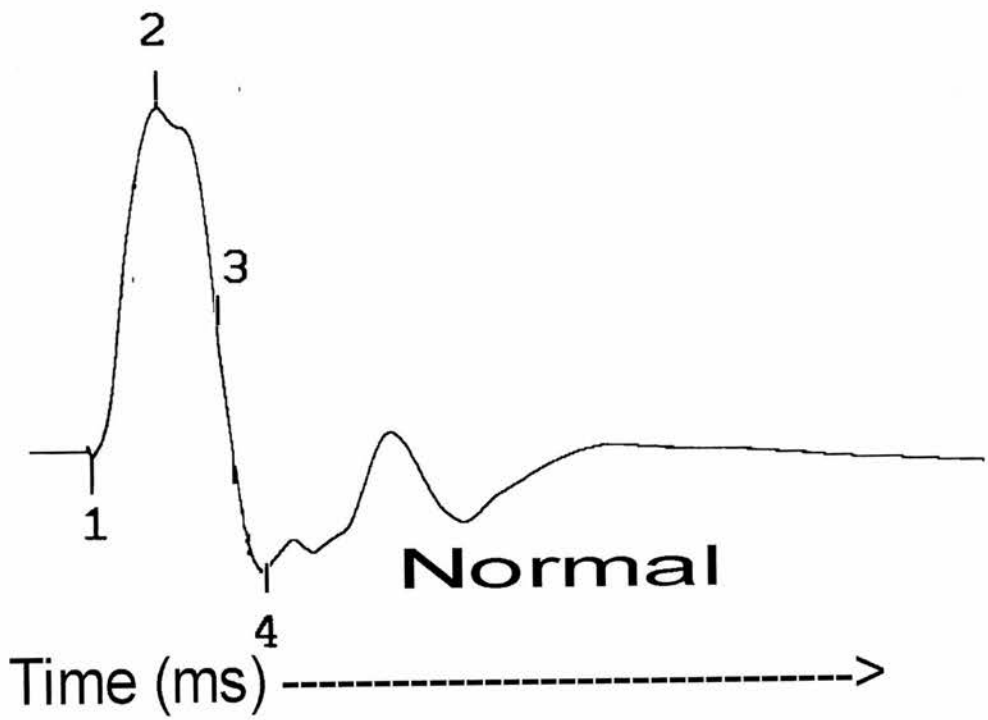
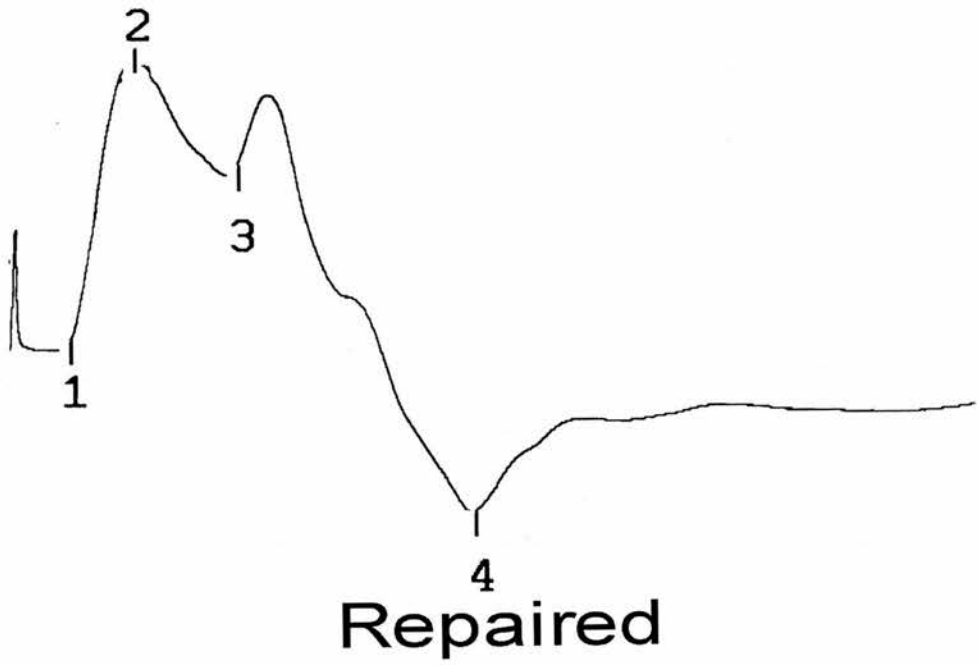


Figure 6.2.3.2.1 Two graphs: one normal and one repair group with each trace labelled accordingly.

The animals in the repair groups demonstrated an altered M-wave shape when compared to the normal group. Where two or more positive peaks were present, the amplitude was taken from the baseline to the largest peak. The point where the M-wave returned to baseline was difficult to determine accurately and caused the results to be erratic.

The shape of the M-wave when the nerve was stimulated at S1 was of lower amplitude and longer duration when compared to the M-wave produced when the peroneal nerve was stimulated at the S2 site. The reason for this is that the impulses of the slower conducting motor fibres will lag progressively behind those of the fastest conducting motor fibres over longer conducting distances. However, it is important to note that this does not affect the onset latency of the fastest conducting fibres. Therefore, when assessing the results in chapters 9-12 only the M-wave produced from stimulating the peroneal nerve at S2 was assessed.

The distance between S1 and S2 was measured next. The maximum conduction velocity was calculated using the latency to M-wave when the nerve was stimulated at S1 and S2 and the distance between segment S1S2 in the equation shown in figure 6.2.1.1

6.3 **MINIMUM CONDUCTION VELOCITY**

6.3.1 **Introduction**

The maximum conduction velocity provides data on the fastest conducting alpha motor axons in a nerve. However, it gives no detailed information of the conduction velocity of the smallest and slowest conducting fibres within a nerve.

The conduction of the slowest conducting motor fibres in a nerve can be determined using a collision technique. There are several different collision techniques employed today however, the technique most widely used and the one used in this thesis was first described by Hopf (1963) and has been used in clinical studies (Hopf *et al.*, 1976). This technique involves stimulating the nerve at two points, a proximal point (S1) and a distal point (S2) at the same time. When a nerve is stimulated at the S2 point with a supramaximal stimulus it will not only elicit an M-wave in the respective muscle but will also give rise to antidromic impulses travelling in the nerve fibres. These impulses prevent the generation of a second M-wave when the nerve is stimulated at S1. This is because the impulses travelling in the orthodromic direction from S1 'collide' with the antidromic impulses from S2. By increasing the time between stimulating the nerve at the S1 and S2 points some of the fastest impulses from S2 will travel past the S1 site. Therefore, when the nerve is stimulated at S1 some of the fibres can elicit an M-wave, provided of course that the nerve is not in the absolute refractory period. As the time interval between S2 and S1 increases more fibres will be stimulated when the nerve is stimulated at S1. This

time interval between stimulating the nerve at S1 and S2 is increased until no further change in a second M-wave is observed. Using the formula shown in figure 6.3.1.1 it is possible to determine the velocity of the slowest conducting motor fibres. A diagrammatic representation of this is provided in figures 6.3.4.2.

$$CV_{\min} = \frac{d}{l_{m2} - l_{m1}}$$

Figure 6.3.1.1

Where:

d = distance between the S1 and S2 electrodes (mm)

t_{m2} = latency of full second M-wave (ms)

t_{m1} = latency of beginning second M-wave (ms)

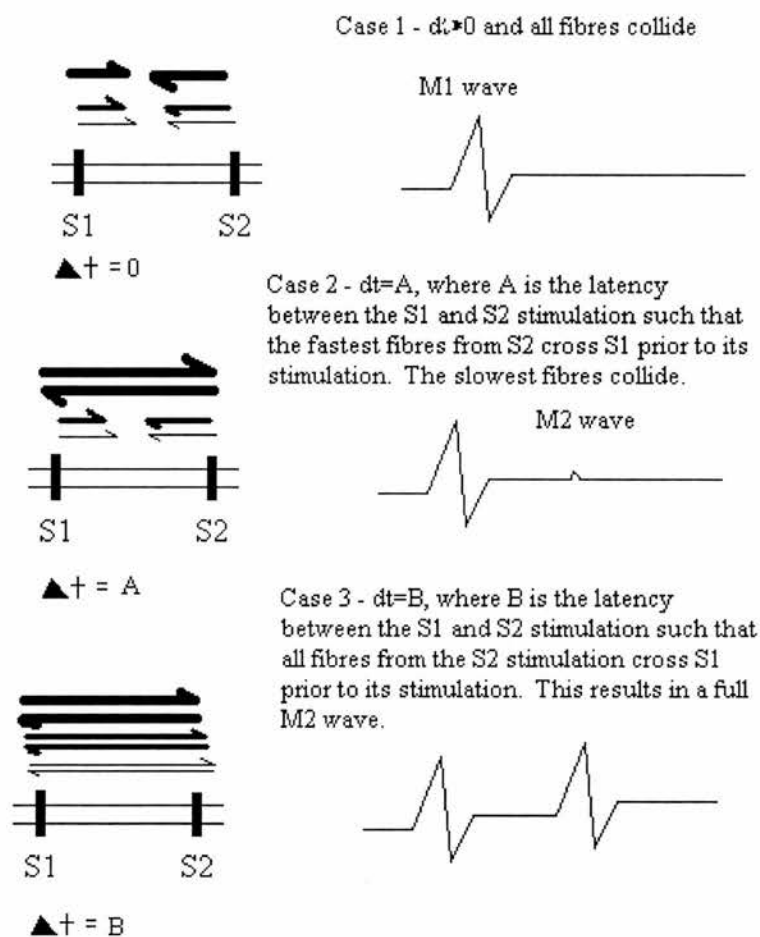


Figure 6.3.1.2 A graphical representation of how the minimum

6.3.2 Methods

The electrode position, filter settings and supramaximal current intensity were kept the same as described in the maximum motor conduction velocity section. The sweep speed of the screen was set to 30 ms to insure that the entire second M-wave would be completely displayed on the EMG screen. All stimulations in this section were of supramaximal intensity for that location.

The peroneal nerve was stimulated at S2. The resulting M-wave was amplified and digitally filtered as described in the section above and then stored in memory 1 of the EMG unit. This was used as a reference trace. Then, the peroneal nerve was stimulated at S2 followed 0.1 ms later by a second stimulus at S1. The resulting M-wave was stored in memory 2. In theory, memory 1 subtracted from memory 2 should be a flat line trace because the antidromic impulses from S2 inhibit the impulses from S1 reaching the muscle. This was confirmed by digitally subtracting memory 2 from memory 1 using the EMG machine. If however, a flat line trace was not observed after the subtraction of memories 1 and 2 the supramaximal current used to stimulate the nerve at S2 was increased 10% further and the process repeated. When a flat base line was observed on the EMG screen, the new current setting was used throughout the remainder of this experiment.

The time interval between the S2 and S1 stimuli was increased by 0.1 ms increments. Each active trace was saved in a unique memory location and then subtracted from the reference trace. The second M-wave was often embedded in the initial M-wave because of the small distances between the S1 and S2 electrodes. Subtracting the reference trace from the active trace removed the initial M-wave that allowed for detection of the second M-wave. The time when the second M-wave was first recorded was denoted t_{m2} . The time when a full second M-wave (an area 95% of the initial M-wave) was detected was t_{m1} . Figure 6.3.1.2 is an example of the this process

The latency between t_{m2} and t_{m1} represented the conduction delay between the fastest and slowest motor fibres. Therefore, the minimal conduction velocity can be calculated by using formula 6.3.1.1.

6.4 REFRACTORY PERIOD

6.4.1 Introduction

The absolute refractory period is defined as the time a nerve becomes inexcitable after stimulation. The nerve will then recover gradually over the next few milliseconds, after this inexcitable period, and may produce another impulse provided the stimulation is of sufficient strength. The refractory period has been studied in animals (Bishop & Heinbecker, 1930; Bergmans, 1973) and in human mixed nerves (Gilliatt R.W., 1963; Betts R.P. *et al.*, 1976). However the clinical usefulness of this technique has not been assessed satisfactorily (Kimura, 1981).

Sodium conductance (g_{Na^+}) is the principle component in the physiologic mechanisms of the refractory period. When a segment of nerve is stimulated, the sodium channels open to allow for the diffusion of sodium in to the axon. After the impulse passes, the sodium channels close. This closure of the sodium channels will allow the nerve segment to repolarize as the potassium (K^+) channels open resulting in potassium exiting the axon. Once the sodium channels close they are inactive for a period of time. The latency the of the inactive sodium channels remain is called the absolute refractory period.

Paintal and others have demonstrated that the refractory period is prolonged in nerves with low temperatures (Paintal, 1965b; Buchthal & Rosenfalck, 1966) or in nerves at slowed conduction velocity (Paintal, 1965a). It has also been shown that

the refractory period is prolonged in experiments where the nerve is demyelinated (Davis, 1972; Low & McLeod, 1977; Smith, 1980a; Smith, 1980b). Owing to these experiments and the relative ease at which the refractory period can be calculated it was determined to measure these values.

6.4.2 Electrode setup

As with the maximum conduction velocity, the tibial nerve was divided proximally as close to the sciatic notch as possible and approximately 1 cm distal to the femoral artery in the leg. By cutting the tibial nerve at both locations it was possible to minimize the effect of any current spread from the peroneal nerve to the tibial nerve after stimulation. Therefore, the recorded M-wave was the result of stimulating the peroneal nerve only.

A non-traumatic low impedance platinum wire stimulating electrode (Harvard Electronics, USA) was placed under the left peroneal nerve proximal to the graft site. As per convention, the cathode of the electrode was positioned so that it was in the distal position. The stimulating electrode was positioned to avoid both the repair site and excessive fat tissue that could not be removed.

Surface electrodes were chosen as the recording electrodes for this test instead of needle electrodes because surface electrodes record from a larger area than needle electrodes. In addition, with surface electrodes the onset latency of the M-wave

indicates the conduction time of the fastest motor fibres and therefore hypothesised to be the largest diameter fibres, whereas with needle electrodes this is not always true.

Two 6 mm silver / silver chloride disc recording electrodes were placed on the skin over the EDL to record the electrical activity of the muscle during contraction. One electrode, the cathode, was placed slightly lateral to motor point of the EDL. This reduced any possible interference from the contracting tibialis anterior muscle during stimulation of the peroneal nerve. A second surface electrode, the anode, was placed on the skin over the tendon of insertion for the EDL muscle. A ground electrode was placed on the skin over the biceps femoris muscle. The electrodes were secured to the skin with tape to prevent unwanted movement of the electrodes during contractions. Figure 6.1.2 shows the position of the surface electrodes with respect to the leg.

The recording and ground electrodes were connected to a preamplifier that was then connected to the amplifier of the Medelec Sapphire II EMG machine (Medelec, Old Woking UK).

6.4.3 Stimulation setup

The stimulating electrode was connected to the 'A' stimulator of the EMG machine. The sweep speed of the EMG visual screen was set at 50 ms and the stimulation rate was set at 1 pulse per second. The low cut-off filter was set at 10 MHz while the high cut-off filter was set at 10 kHz.

The peroneal nerve was stimulated with a square wave current of 50 μ s duration. The resulting contraction of the EDL muscle was recorded by the surface electrodes. The difference between the cathode and anode was amplified and digitally filtered while the common frequency components recorded from the ground electrode were removed. The resulting data were displayed on the EMG screen and called an M-wave (muscle wave).

The current was increased until a M-wave could be detected on the EMG screen at a gain of 200 mV per division. Then the current was increased until there was no further change in the size of the M-wave. The current was increased a further 30% to produce a supramaximal current. The gain on the Medelec Sapphire II EMG machine was adjusted so that the M-wave could be visualized entirely on the EMG screen.

Once all parameters were set, the peroneal nerve was stimulated with a supramaximal stimuli. Eight traces were averaged together to help remove any interference. This trace was stored in the EMG memory and used as the reference trace.

6.4.4 Recording procedures

The absolute refractory period (ARP) was determined using a paired shock technique. Two supramaximal stimuli of equal magnitude (S1 and S2) were delivered to the peroneal nerve separated by a delay of 0.01ms. The resulting M-wave was

recorded and used as the reference trace. The time delay between (S1) and (S2) was then set at 10 ms resulting in two M-waves. The delay between the S1 and S2 stimuli was reduced by 0.01 ms until the second M-wave was no longer observed. Each trace recorded was called the active trace. By subtracting the active trace from the reference trace it was possible to determine the time at which a second M-wave disappeared. This point was called the absolute refractory period (ARP) and took approximately 5 minutes to determine.

6.5 JITTER

6.5.1 Introduction

Developments in peripheral nerve repair over the past four decades have concentrated on mechanical methods of improving the coaptation of the transected nerve. There are several authors presently assessing the use of chemical factors to enhance nerve regeneration. It seems unlikely that any new advances in the mechanism of nerve repair are going to produce quantifiable differences in electrophysiological tests when compared with the nerve graft. If research in this field is to yield clinically useful data, it must be accompanied by the development of objective and relatively non-invasive methods of assessment that allow the clinician to better assess the quality of repair. The use of jitter a SF-EMG test offers a potential for detailed objective quantitative monitoring of the regenerative process at the motor unit.

Transection of a motor peripheral nerve will result in denervation of its muscle. Degeneration of the neuromuscular junction will occur within 48 hours (Birch *et al.*, 1986) after denervation. If the nerve is correctly repaired, reinnervation will occur from the proximal segment into the distal segment. The time to muscular reinnervation will depend on both the distance the regenerating neurites are required to travel and the quality of the repair.

When the regenerating terminal motor axons reach their target muscles, new end plates will form (Hakelius *et al.*, 1975). These new or immature motor units (terminal motor axon, neuromuscular junction and muscle fibre) will have an increased variability in neuromuscular transmission. Once the regenerating motor axon reaches an appropriate motor target it will establish a neuromuscular junction and mature. As the fibre matures the end plate potential becomes more stable (Wiechers, 1990). It is this stability that jitter can measure in order to assess the timing and quality of repair.

Single fibre electromyography (SF-EMG) was developed to assess the microphysiology of the motor unit. The motor unit includes the terminal motor axon, neuromuscular junction and muscle fibre (Stålberg & Trontelj, 1979). Stimulated jitter is a SF-EMG technique used to assess the variability in neuromuscular transmission time. The variation in time between stimulation of the motor axon and recording of the muscle fibre action potential in successive discharges is called jitter. Stålberg and Trontelj (1979) showed that there was little variability in the

transmission of the impulse down the terminal axonal branch in a normal nerve and Trontelj and Stålberg (Trontelj & Stålberg, 1983) demonstrated that variability in the transmission of the impulse down the muscle fibre is minimal in normal muscle.

6.5.2 Theory

Jitter is different from other EMG techniques in that the recording is much more specific. Jitter uses a SF-EMG needle electrode for recording the action potentials from individual muscle fibres. The SF-EMG needle electrode is made from a steel cannula 0.5 mm in diameter with an insulated silver wire in the lumen. A small opening of approximately $25 \mu\text{m}^2$ is made about 3 mm proximal to the tip of the needle to expose an uninsulated area of the silver wire. This side port becomes the active recording electrode. An electrode diameter of 25-30 μm^2 has been shown to be optimal (Ekstedt & Stålberg, 1973) because this is smaller than the diameter of the average muscle fibre. The result is that a reduced shunting effect is produced. The small recording area also increases the probability of recording action potentials from muscle fibres within close proximity to the active electrode.

Filtering further enhances the selectivity of recording. When an action potential is conducted along a muscle fibre the frequency of its different components are changed depending on the location of the recording electrode and the nature of the surrounding tissue. Muscle and connective tissue act as low pass (high cut-off) filters so that action potentials lose their high frequency components faster or in a greater proportion than their low frequency components. Action potentials from distant muscle fibres will have a higher proportion of low frequency components than those from adjacent muscle fibres. Increasing the frequency of the high pass filter will thus attenuate or remove recordings of more distant fibres. Stålberg (1966)

demonstrated that setting the high pass filter at 500 Hz resulted in a reduction of background activity (and distant muscle fibre activity) while only reducing the M-wave amplitude of near fibres by 10%.

Stimulation must be as selective as the recording procedures. The duration of the stimulating pulse should be set at 50 μ s. Previous experiments have shown that this pulse width produces little or no additional jitter at the stimulation point on the axon (Trontelj *et al.*, 1992). The stimulus amplitude should be between 1 mA and 25 mA and should allow for stepwise increments and decrements of 0.1 mA. Both of these will improve the selectivity of stimulating only the required terminal motor axons.

The latency between the stimulus pulse and the muscle fibre action potential is called the inter-potential interval (IPI). Stålberg showed that the terminal motor axon must be stimulated 50 times and the IPI for each pair of events determined to produce a statistical sample of that motor unit (Stålberg, 1966). The jitter for the end plate under study is calculated as the mean consecutive difference of latencies or MCD using the following equation:

$$MCD = \sum \left(\frac{|ipi_n - ipi_{n-1}|}{n} \right)$$

Figure 6.5.2.1

where:

MCD = mean consecutive difference (μ S)

ipi = interpotential interval (ms)

n = number of stimulations

Previous work has shown that for a jitter study to be statistically valid a population of at least 20 muscle fibres per muscle needs to be considered. The average of all the calculated values of MCD is the 'jitter value' for that muscle. A normal value for jitter is taken to be that at which no more than one fibre in the population under study has a value of MCD greater than the 95th percentile of the distribution of the control group and that the 'jitter value' for the muscle under study is within one standard deviation of the measurement of the MCD of the control group.

6.5.3 Methods

On the day of assessment, each animal was anaesthetized as described in chapter 4. The left leg was shaved and the foot was firmly clamped to the table with a non-crushing clamp to prevent any needle movement during stimulation. The EDL was chosen as the muscle to assess because it is supplied solely by the peroneal nerve. Care was used to ensure that the EDL was placed in a relaxed position because it has been shown that tension in a muscle would increase jitter values (Trontelj and Stålberg 1992).

In this thesis, the measurement of jitter is the electrophysiological test most sensitive to changes in temperature. Previous work has shown that jitter increases 2 to 3 μs per degree Celsius in the range of 36°C to 32°C (Stålberg *et al.*, 1971). If the average value for a muscle is 20 μs this represents a change of 10 to 15 percent for every degree Celsius. In contrast, the maximum conduction velocity will increase approximately 5 percent per degree (Lafratta & Zalis, 1973; Kimura, 1979). Therefore, jitter was always the first test performed once the animal was unconscious.

Two monopolar needle electrodes (Medelec MF37) were used as the stimulating cathode and anode. The cathode was inserted through the skin into the EDL approximately 1 cm distal to the knee joint and slightly lateral to the midline. This is where the peroneal nerve enters the EDL and is called the motor point. This location is shown in figure 6.5.3.1.

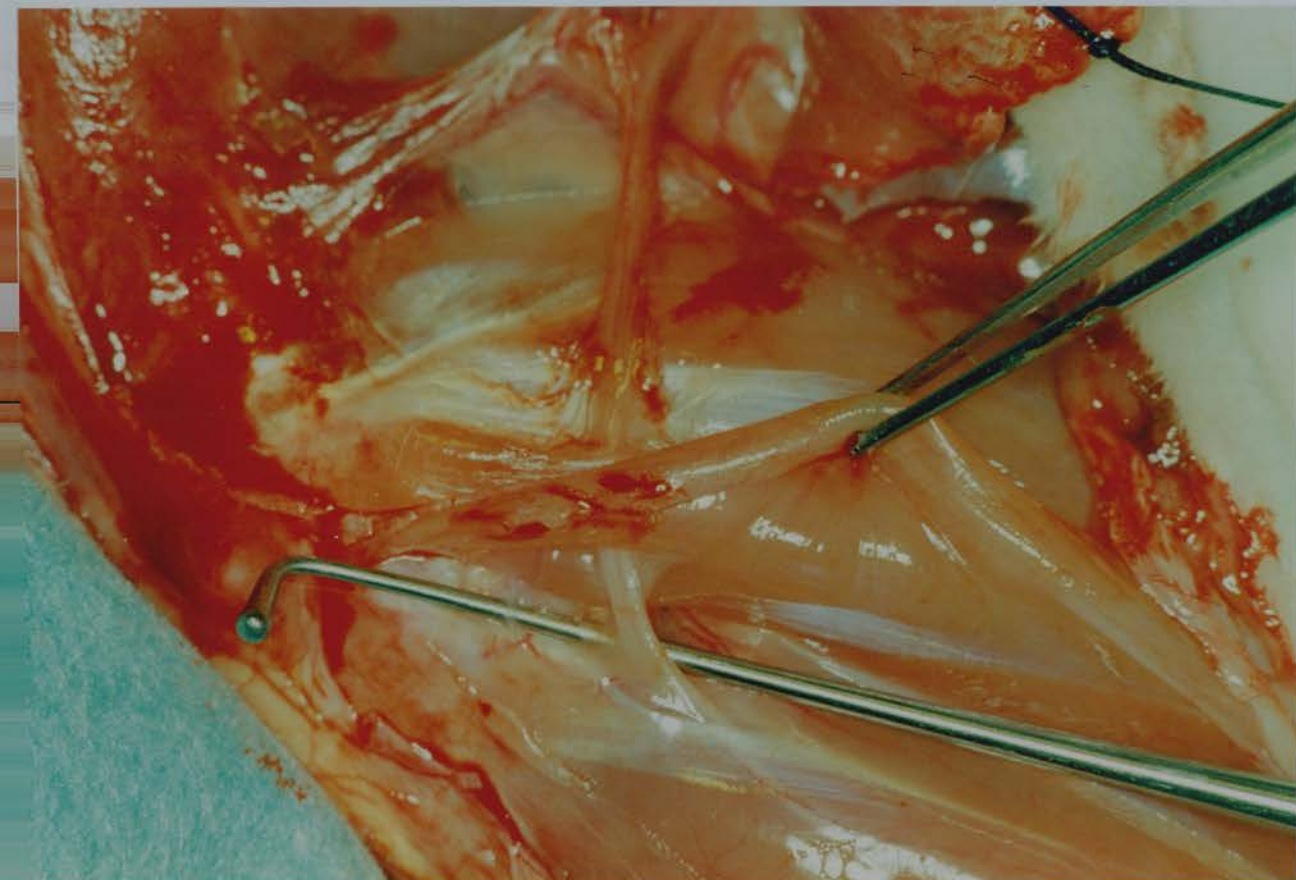


Figure 6.5.3.1. Photograph of the peroneal nerve entering the EDL muscle (the motor point).

The anode was placed 0.5 cm proximal and 0.5 cm lateral to the cathode. Stimulus pulses with a duration of 50 μ s and amplitude of 1 mA were initially used at a frequency of 1 Hz. The high pass filter was set at 10 Hz to allow recording of distant muscle fibres thus acting as a guide to positioning of the electrodes. The current was increased by 0.1 mA increments until small twitches were observed in the EDL. At this stage a SF-EMG needle recording electrode (Medelec SF25) was inserted into the twitching portion of the muscle approximately 2 cm distal to the cathode. It was also important to have the sound of the EMG machine at a high level. Experience showed that as the needle moved closer to a contracting muscle fibre the pitch of the sound increased. This change in sound was the quickest and most accurate methods of determining the location of the stimulated muscle fibres within the muscle. The position of the SF-EMG electrode was adjusted until satisfactory recordings of the muscle action potential could be observed. The order of manipulation of the SF-EMG needle electrode was rotation followed by advancement into the muscle and then rotation again. This pattern ensured that fresh undamaged muscle fibres were recorded. The needle was never moved in a superficial direction because doing so would yield recordings from damaged muscle fibres.

The frequency of the stimulation was then increased from 1 Hz to 10 Hz and the high pass (low-cut-off) filter was increased from 10 Hz to 500 Hz . Increasing

the high pass filter removed background noise caused by the contraction of distant muscle fibres.

The average value of jitter for each animal was calculated from measurement made from 20 different sites in the EDL. To ensure that there was no duplication of muscle fibre recordings the recording electrode was reinserted through the skin into a different position in the EDL after every 4 or 5 recordings. The stimulating electrodes were moved after 8-10 recordings to stimulate different terminal motor axons. The *MCD* at each site was determined from 50 consecutive discharges using the formula shown in figure 6.5.2.1.

If the muscle fibre was stimulated directly a jitter value of less than 5 μ s would be observed and the value discarded and the electrode moved. Similarly, if the terminal motor axon being stimulated resulted in sub-maximal stimulation of an adjacent terminal motor axon, summation of the muscle fibres under study would occur and again, then this trace would be discarded.

Below are examples of jitter for both normal and repaired nerves.

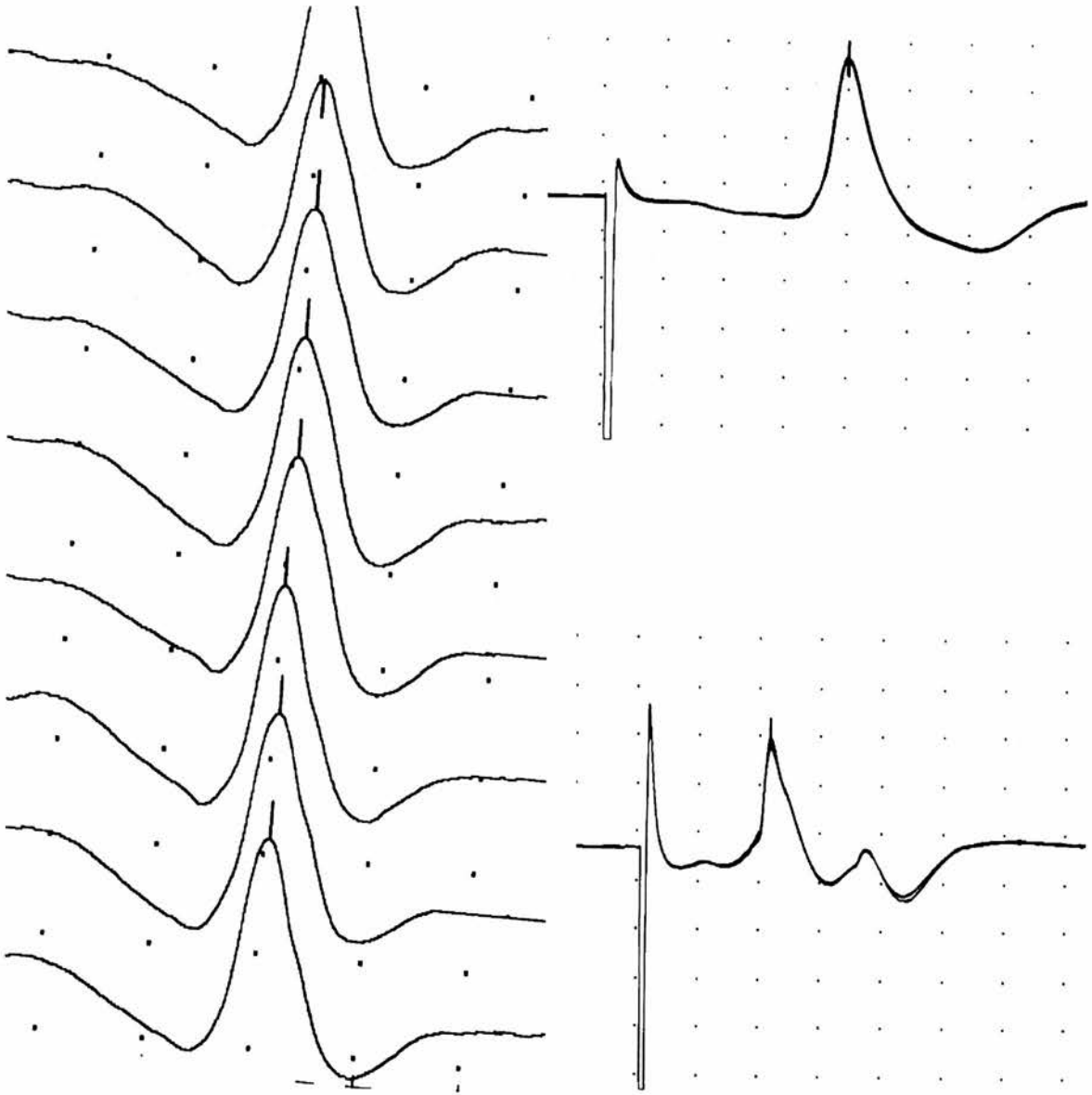


Figure 6.5.3.2 **Jitter in the normal nerve**

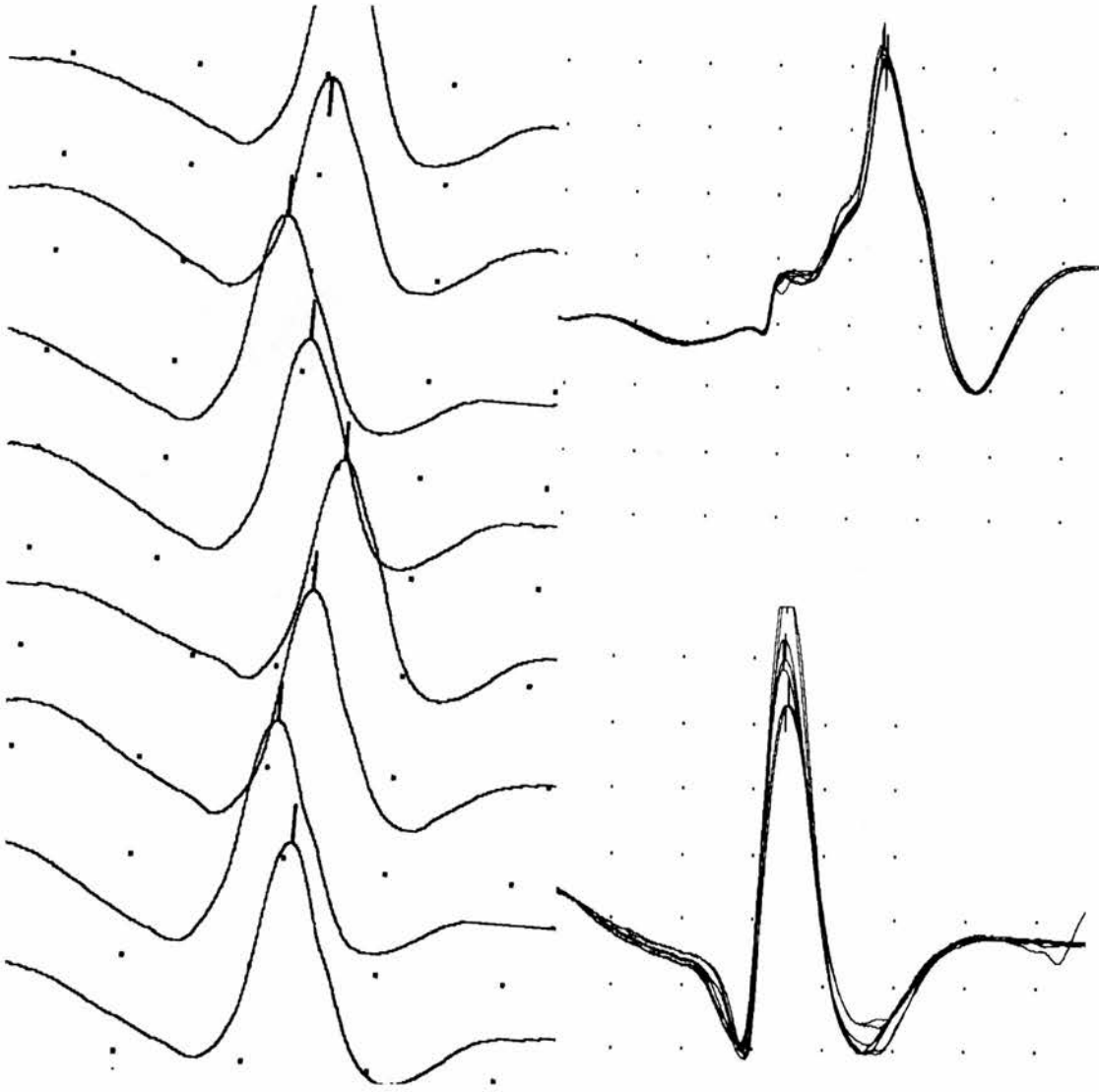


Figure 6.5.3.3

Jitter in the repaired nerve. Note the increase in variability when the traces are superimposed.

CHAPTER 7.0

ISOMETRIC TENSION

| <u>Section</u> | <u>Title</u> |
|-----------------------|----------------------------|
| 7.1 | <u>INTRODUCTION</u> |
| 7.2 | <u>METHODS</u> |

7.1 **INTRODUCTION**

The purpose of measuring the isometric tension or force generated during a muscle contraction is to measure indirectly the number of muscle fibres contracting. Hartree and Hill (Hartree & Hill, 1921) determined in a series of experiments that the force generated in a muscle contraction was approximately proportional to the amount of heat generated. They also determined that the heat produced in a skeletal muscle contraction was proportional to the amount of ATP consumed by that muscle. The ATP consumed was proportional to the number of crossbridges turning over in the muscle fibres. Therefore, in a muscle completely denervated by injury, the force generated after supramaximal stimulation of its nerve provided data on how many muscle fibres were re-innervated after nerve repair. It also provided insight on the quality of repair.

7.2 METHODS

The distal stimulating electrode (S2) was kept in the same position as described in section 6.2. An incision was made in the skin of the leg to extend from the knee to the ankle joint and then re-directed at a 90° angle to extend into the dorsum of the foot. The skin was reflected to expose the anterior compartment of the leg which includes the tibialis anterior (TA), EDL and extensor retinaculum. The extensor retinaculum was cut with a razor lateral to the tibialis anterior tendon and carefully dissected to expose the tendons of the EDL (Figure 7.2.1).



Figure 7.2.1

Photograph of the EDL separated from the TA in the leg.

To prevent recording of any unwanted tension from the TA during contraction, the EDL and TA were carefully separated from the ankle joint to the knee joint. A cotton thread was then tied securely to the tendons of the EDL. (Figure 7.2.2)

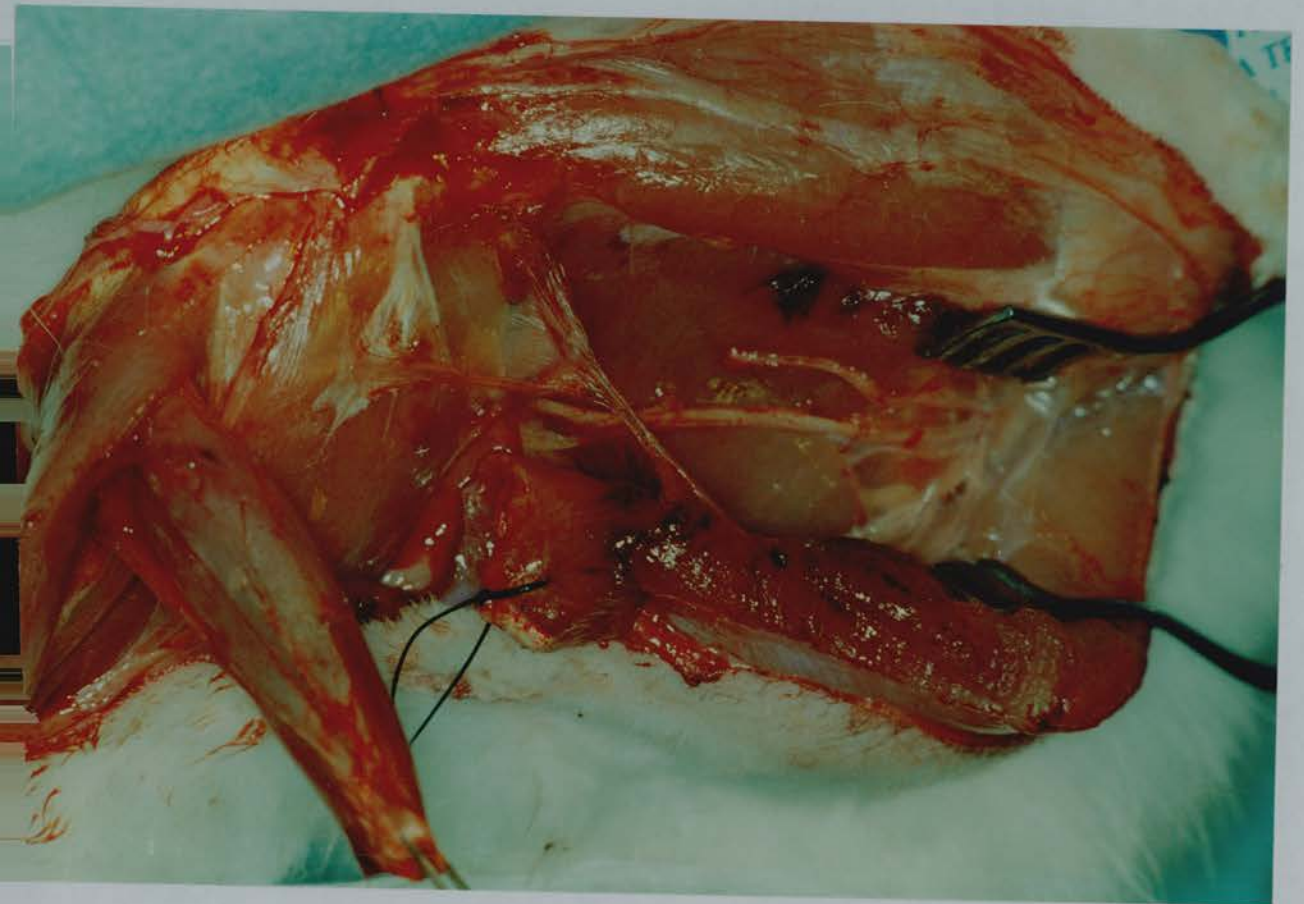


Figure 7.2.2

Photograph of the EDL with a cotton thread attached.

The cotton thread was attached to a steel wire connected to an isometric tension transducer (Harvard Biosciences, USA) which was secured to an anti-vibration table. To minimize movement of the leg during contraction, the knee joint was firmly clamped to the anti-vibration table with a non-crushing vice grip. The output signal from the tension transducer was connected to the Y-input (Channel 3 of 4) of an oscilloscope (Gould, UK). The muscle was then stretched from no tension, recorded as a normal baseline viewed on oscilloscope screen, to a point where the voltage increased one division above the baseline with the gain set at $1 \text{ V division}^{-1}$ or $2.44 \text{ N division}^{-1}$ (Figure 7.2.9). This method of standardizing the tension before assessment assumes that every muscle has similar properties with respect to muscle fibre and connective tissue elasticity.

The RS-232 output from the EMG machine was connected to the trigger input of the oscilloscope with a $75\text{-}\Omega$ BNC cable, thus enabling the EMG machine to trigger the oscilloscope sweep when the stimulation of the nerve was initiated. Figure 7.2.3 is a diagrammatic representation of the connection of machines.

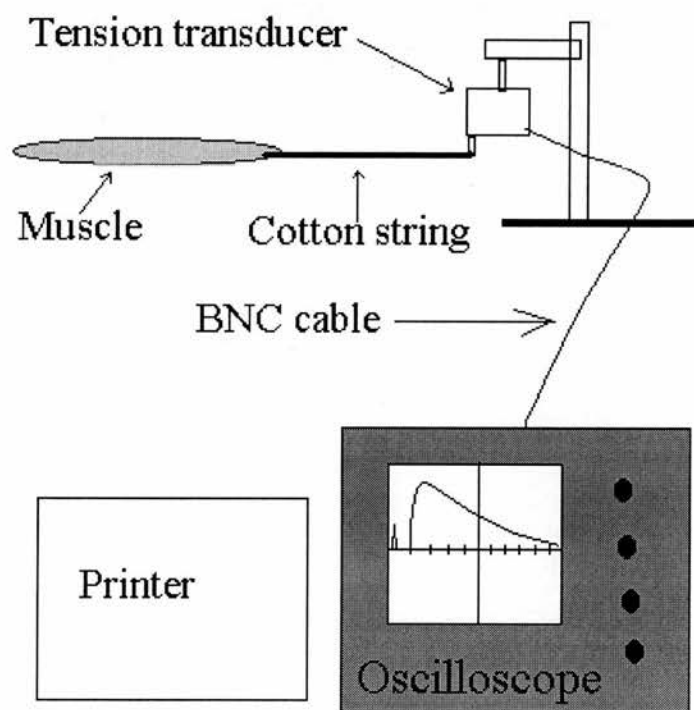


Figure 7.2.3 Diagrammatic representation of the isometric tension machines.

For consistency, the gain for the oscilloscope screen was always set at 1 V division⁻¹ with a sweep speed of 500 ms or 50 ms per division. Experience showed these settings would ensure that the entire muscle twitch was displayed on the oscilloscope screen.

The peroneal nerve was stimulated at S2 with a supramaximal current. The resulting muscle twitch was displayed on the oscilloscope screen. The nerve was stimulated eight times at a frequency of 1 Hz. Each muscle twitch trace was averaged sequentially and then digitally filtered with a 512 MHz filter. Averaging the traces helped remove possible recorded mechanical or electrical interference while the filtering smoothed the shape. This improved locating the points listed below.

The following measurements were determined from the final filtered trace.

- a) **Latency to twitch** (ms) - The latency twitch time is defined as the time from stimulation of the peroneal nerve at S2 to initiation of the muscle twitch. This value is dependent on the distance of the S2 electrode from the EDL muscle and on the conduction velocity of the nerve fibres. Unlike the conduction velocity test where a greater distance between the stimulating electrodes is desired, the distance from the S2 stimulating electrode to the muscle should be at a

minimum. This is because there is a conduction lag in the slower fibres. So there will be a greater gap between the arrival of the impulses travelling in the larger when compared to the smaller fibres. This would not be expected as the distance decreases. To help reduce the effect of conduction lag on the assessment of a muscle twitch the distance from the S2 electrode to the muscle was always 2 cm. The latency to twitch is represented in figure 7.2.11 as the time from A to 1.

- b) **Twitch time or T (ms)** - The twitch time is the time from the initial twitch to the return to a stable baseline. This twitch time was difficult to determine because the point where the trace returned to a stable baseline was difficult to locate. This is represented as the time from 1 to 5 in figure 7.2.11.
- c) **Time to peak tension or T_{peak} (ms)** – The time to peak tension is the time from initial twitch contraction to peak tension. This is represented as the time from 1 to 3 in figure 7.2.11.
- d) **Time to half peak tension or $T_{\frac{1}{2}\text{peak}}$ (ms)** - The time to half peak tension is the time from initial twitch contraction to $\frac{1}{2}$ of the peak tension value in volts. This is represented as 1 to 2 in figure 7.2.11.
- e) **Time to half relaxation or $T_{\frac{1}{2}\text{r}}$ (ms)** – The time to half relaxation is the time from initial twitch contraction to $\frac{1}{2}$ of the peak tension when

measured in volts on the relaxation side of the muscle twitch. This is represented as 1 to 4 in figure 7.2.11.

- f) **Time-tension integral (N)** – This is the area under the muscle twitch curve bounded by the twitch time. It is indirectly proportional to the number of muscle fibres contracting during the twitch and is calculated using the following formula:

$$v = \frac{k}{m} \int_0^T F dt$$

Figure 7.2.4

where: v = velocity of the mass (m s^{-1})

m = mass (kg)

k = constant

F = Tension

T = Twitch time (defined above)

The equation in figure 7.2.4 can be modified to give a value in units of momentum by multiplying each side of the equation by mass. This results in the equation shown in figure 7.2.5.

$$mv = k \int_0^T F dt$$

Figure 7.2.5

where: mv = momentum

- g) Time Tension Index or TTI (N)** – This value is defined as the area under the curve of the muscle twitch from initial deflection to $\frac{1}{2}$ relaxation of the twitch and represents an “average” tension for the contraction. This value was found more consistent between groups because the time to $\frac{1}{2}$ relaxation was easier to determine than the twitch time. The TTI was calculated using the following formula:

$$TTI = \frac{\int_0^{T_{1/2r}} F dt}{T_{1/2r}}$$

Figure 7.2.6

This is represented by the area under the curve bounded by 1 to 4 in figure 7.2.11.

- h) Peak tension or PT (N)** – The peak tension represents the maximal force generated during a contraction as shown by the change in voltage between 1 and 3 in figure 7.2.11.

To convert to Newtons (N), the voltage recorded on the oscilloscope screen from the tension transducer required a calibration graph. To develop a calibration graph, it was necessary to mount the tension transducer vertically and hang weights of different mass from it with fine steel wire. The d.c. voltage change recorded on the oscilloscope from baseline was determined. The tension each weight created while suspended by the steel wire was calculated using equation 7.2.7.

$$T = m \times g$$

Figure 7.2.7

Where: T = Tension in Newtons (N)
 m = mass in kg
 g = the constant of gravitation (9.81 m s^{-2})

The recorded voltage was plotted against the known weight. The graph fitted a linear line equation represented in figure 7.2.8

$$y = m \times x + b.$$

Figure 7.2.8

Where: y = Tension (N)
 x = measured voltage
 m = constant representing the slope of the line
 b = the y-intercept on the graph

The point 0,0 represents the case where there is no weight and therefore no tension being recorded. Therefore, variable b can be removed for equation 7.2.8 to form equation 7.2.9.

$$y = m \times x$$

Figure 7.2.9

After the points were plotted, the slope of the line or constant value m could be determined using simple linear algebra. The constant m was found to be 2.44. Therefore, to calculate the tension generated from the voltage recorded the following equation was used.

$$Y = 2.435 \times X$$

Figure 7.2.10

Where: Y = Tension (N)
 X = Measured voltage

The figure below is a representation of the muscle twitch and points described above.

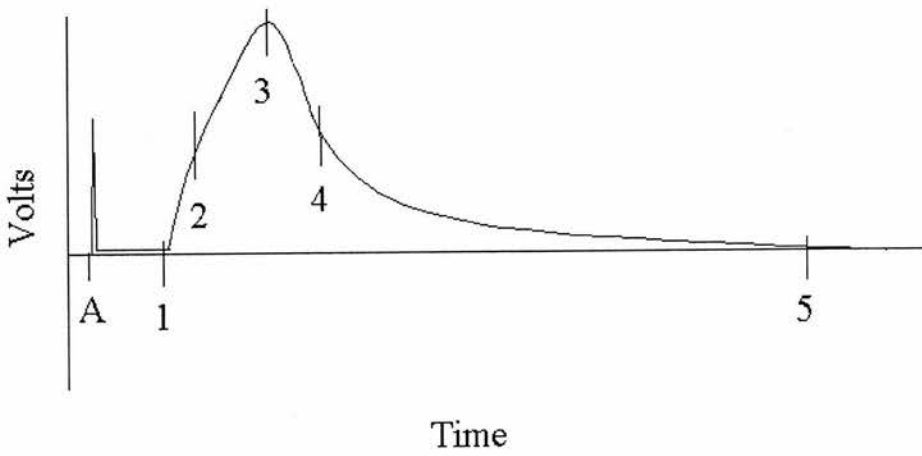


Figure 7.2.11

An illustration of the muscle twitch with associated measurement points.

CHAPTER 8.0

HISTOLOGY

| <u>Section</u> | <u>Title</u> |
|----------------|-------------------------------|
| 8.1 | <u>INTRODUCTION</u> |
| 8.2 | <u>FIXATION</u> |
| 8.3 | <u>EMBEDDING</u> |
| 8.4 | <u>CUTTING</u> |
| 8.5 | <u>STAINING</u> |
| 8.5.1 | <u>Toluidine Blue</u> |
| 8.5.2 | <u>Paraphenylenediamine</u> |
| 8.6 | <u>MORPHOMETRIC ANALYSIS</u> |
| 8.7 | <u>STATISTICAL ASSESSMENT</u> |
| 8.8 | <u>RESULTS</u> |

8.1 INTRODUCTION

Regenerating fibres will increase in diameter if peripheral connections are made with compatible target organs (Weiss *et al.*, 1945; Sanders & Young, 1946; Aitken *et al.*, 1947). If no connection is made in the periphery or if the end organ is incompatible with the nerve fibre, it will remain small and / or die over time. This has been shown by measuring the axon and fibre diameter (Gutmann & Sanders, 1943a).

After the completion of the measurements and presentation in table form the G-ratio was calculated by dividing each axon diameter by the respective fibre diameter. The G-ratio is a measure of nerve fibre maturation (Glasby *et al.*, 1986).

When nerve fibres regenerate they do not regain their normal (Gutmann & Sanders, 1943b). Both axon diameter and myelination are significantly less (Gattuso *et al.*, 1988).

The measurements of axon and fibre diameter, G-ratio and myelin thickness represent variables in a population of nerve fibres that have made functional connections in the periphery and are accepted as a standard index for assessing the quality of repair after peripheral nerve injury.

8.2 FIXATION

Nerve tissue was processed by a standard method to produce 1 μm resin embedded sections (Gschmeissner *et al.*, 1990). When compared to normal nerves, modifications to this technique were required due to the excessive amount of fat and scar tissue.

The peroneal nerve was divided 1 cm proximal to the graft site and at the point where the nerve enters the EDL muscle. This produced a segment of nerve approximately 4 cm long. Excessive fat and fibrotic tissue were trimmed with microsurgical scissors. Vision was aided with an operating microscope.

Trimming the nerve was important to reduce the size of the segment before fixing. It was found that removal of the fibrotic tissue was easier if performed before fixation. In addition, reducing the size of the nerve segment improved the impregnation of the osmium and infiltration of the resin.

After trimming, the nerve segment was immediately placed in a container filled with 4% glutaraldehyde in 0.1M sodium cacodylate buffer and refrigerated at 4°C for 1 hour. This increased the stiffness of the nerve segment, which eased the handling of the tissue. To remove any further fat tissue, the nerve segment was trimmed with the same method described above. A razor blade was used to cut the nerve segment into 1 mm transverse sections. The proximal and distal ends were discarded. The nerve sections were returned to the container with 4% glutaraldehyde for another hour and then washed in 3 or 4 changes of 10% sucrose in 0.1M sodium

cacodylate buffer. After the final wash, the nerve sections were left in the 10% sucrose solution for 18 hours.

The nerve sections were transferred to a cacodylate buffered osmium tetroxide solution for 4 hours. After the allotted time in the osmium tetroxide solution, the nerve sections were washed four times in 10% sucrose solution and then once in 10% alcohol to remove any excess osmium tetroxide. Each wash lasted 15 minutes.

8.3 **EMBEDDING**

The nerve sections were transferred from the 10% alcohol solution into plastic baskets. Care was used to ensure that each basket was not overfilled. Overfilling the baskets could result in damage to the nerve sections and it could increase the chances of the sections being lost in the solution if they floated out of the basket. The plastic baskets were connected to an automatic processor (Lynx, Microscopy Tissue Processor, Australian Biomedical Corporation Ltd., Melbourne, Australia) that was preset with the following settings:

| | | | |
|-----|---------------------|------------|------------------|
| 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°C |
| 12. | Araldite | 12 hours | room temperature |

The nerve sections were removed from the Araldite (step 12) and placed in plastic moulds that would hold two nerve sections placed at opposite ends. Fresh Araldite was poured over the moulds to completely cover the nerve sections which were orientated parallel to the long axis of mould. The moulds were placed in an oven at 60°C to polymerize the Araldite for at least 48 hours. Vision was assisted with a dissecting microscope.

8.4 **CUTTING**

Semithin (1 μ m) slices of the resin embedded nerve sections were produced using a Porter-Blum ultrathin microtome. The embedded nerve section was cut with a glass knife that had a wax mount filled with water placed near the cutting surface. As each nerve section was cut, a slice floated off the knife-edge and into the water. The water helped decrease the probability of the slice being twisted or folded. Four to eight slices were transferred from the water to a slide. Each slide was checked to ensure that no damage or folding of the slices occurred. The slides were allowed to dry in air.

8.5 **STAINING**

The nerve sections were stained by two methods that are described below.

8.5.1 **Toluidine Blue**

A solution of toluidine blue in 1% sodium tetraborate was used to stain the slice. The slides holding the nerve slices were individually placed on a heating plate for a few minutes. After the slide was hot, an 'eye-dropper' was used to place a few drops of the toluidine blue solution over the nerve slices. The slide was left on the hot plate until a light green 'crust' began to form around the edges of the toluidine solution (approximately 30-45 seconds). The slide was then washed with cold water to remove the toluidine blue solution and then allowed to dry in air. One problem

encountered with the toluidine blue stain was, that it stains all structures to some degree and, if the solution was left on too long it was impossible to distinguish myelin from other structures. Through a trial and error process, it was found that a consistent time was not appropriate owing to the varying changes in the temperature of the hot plate, thickness of the section and / or amount of fat tissue in the slice. Therefore, the amount of time the toluidine solution was on the slide was determined by a colour change in the edges of the solution. Although this may not be the best method of standardizing the technique, it proved to be the most reliable.

8.5.2 Paraphenylenediamine

Paraphenylenediamine stains osmiophilic material better than the toluidine blue. This provided a better picture of the myelin but did not show associated structures. The slide was immersed in 1% paraphenylenediamine solution for 40 to 60 minutes and then washed in 96% alcohol. The slide was allowed to dry in air.

8.6 MORPHOMETRIC ANALYSIS

The sections were viewed using a compound microscope (Zeiss, Germany) at a magnification of $1000 \times$ (oil immersion). The fibre diameter and axon diameter were measured directly using a VIDS III computerized image analysis system (Analytical Measuring Systems, Cambridge) which was connected to the compound microscope. A photograph of this system is displayed in figure 8.6.1

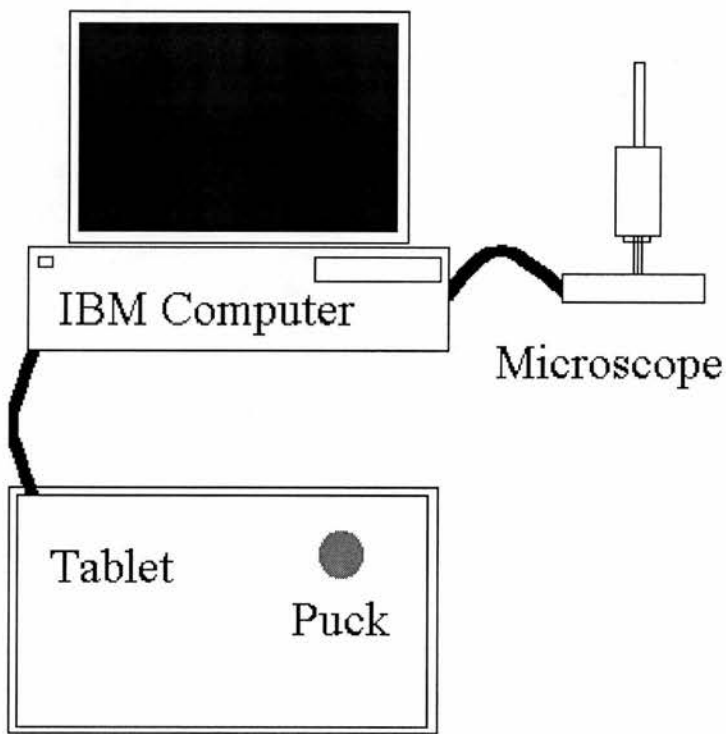


Figure 8.6.1

An illustration of the VIDS III system connected to a compound microscope.

Historical data have shown that there is very little difference of the internal myelin sheath diameter when osmium tetroxide impregnated nerve slices are compared to silver stained sections. It has also been shown that shrinkage of the tissue due to the fixation and dehydration during processing was unlikely to be more than 10% (Boyd & Davey, 1968). Since all the slices were subject to the same preparations, shrinkage should have been similar throughout the groups. It was concluded that accurate and consistent measurements of axon and fibre diameter could be obtained from osmium tetroxide preparations.

A sample of 200 fibres was measured from each section. If the nerve was multi-fascicular an equal number of nerve fibres was measured from each fascicle. For each fascicle, the nerve was divided into equal sized section as shown in figure 8.6.2. The axon and fibre diameter was measured at the shortest vector that crosses through the centre of the fibre. The reason for measuring the diameters using the shortest vector was to compensate for rotation of the fibres when they were cut and would result in the least amount of error. By measuring the all fibres with the shortest vector the variations caused by this rotation were similar through the groups.

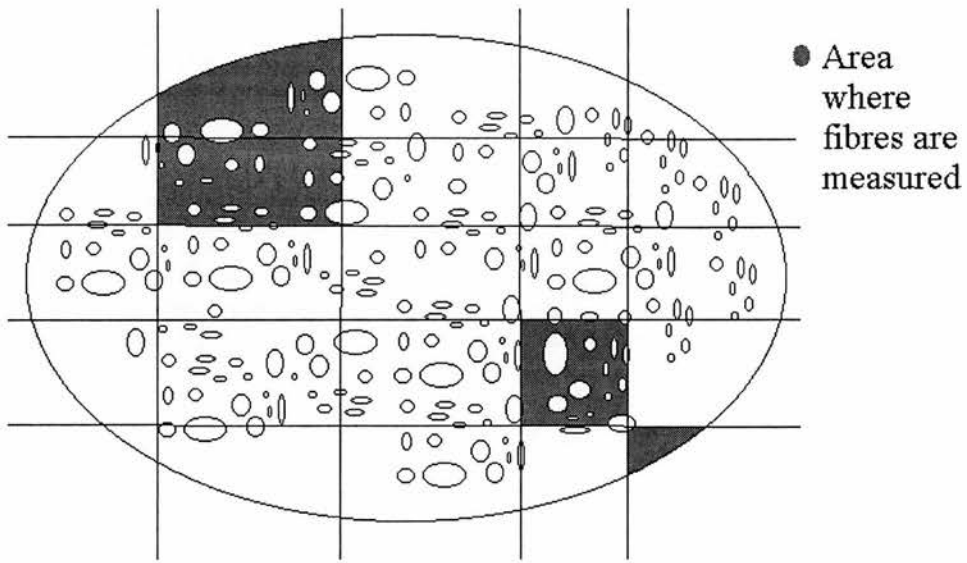


Figure 8.6.2

A diagrammatic representation of the nerve and divisions for morphometric analysis.

All the nerve fibres in a field were measured for axon and fibre diameter. Following the pattern demonstrated in figure 8.6.2, the slice was then moved eight fields from left to right and top to bottom. All the nerve fibres in the second field were measured. This process was repeated until the required number of nerve fibres was measured. This method of sampling eliminated random variation of different parts of the section and produced consistent data when compared to other sections in the same nerve. It has been shown that a count of 200 fibres is a large enough sample to represent the distribution of diameters for the whole nerve (Mayhew, 1990). Therefore, 200 fibres were measured and the data was transferred from the VIDS programme to Lotus 1-2-3 where the myelin thickness and G-ratios were calculated indirectly using the following formulas:

$$MT = \frac{FD - AD}{2}$$

Figure 8.6.3

Where:

MT = Myelin thickness

FD = Fibre diameter (µm)

AD = Axon diameter (µm)

$$G\text{Ratio} = \frac{AD}{FD}$$

Figure 8.6.4

Where:

FD = Fibre diameter (μm)

AD = Axon diameter (μm)

8.7 **STATISTICAL ASSESSMENT**

Sanders and Young (1946) showed that the distribution of fibre sizes in a normal nerve is bimodal. The use of parametric statistical tests, such as Student's t-test, is not indicated in this type of distribution because the assumption necessary to perform a parametric test is a uniform variance around a mean. This does not occur in a bimodal distribution. The data from these experiments were tested and found to be bimodal. Therefore, an alternative non-parametric test was used to compare the different groups. The two tailed Kolmogorov-Smirnov test which has been used in other experiments to compare groups that have bimodal distributions was used to compare the groups in this thesis. (Gilchrist *et al.*, 1998). This test is very tedious to calculate by hand, therefore STATISTICA 5.0 (StatSoft, USA) was used to calculate the data on an IBM PC.

8.8

RESULTS

Six months after nerve repair, all animals demonstrated some degree of nerve regeneration with respect to nerve histology. Specimens from the peroneal nerve distal to the site of repair were compared with normal and FTMG groups. Figures 8.8.1-8.8.14 are photographs of normal and repaired nerves. The presence of fibres in the repair groups is evidence that some degree of regeneration had occurred 6 months after repair.

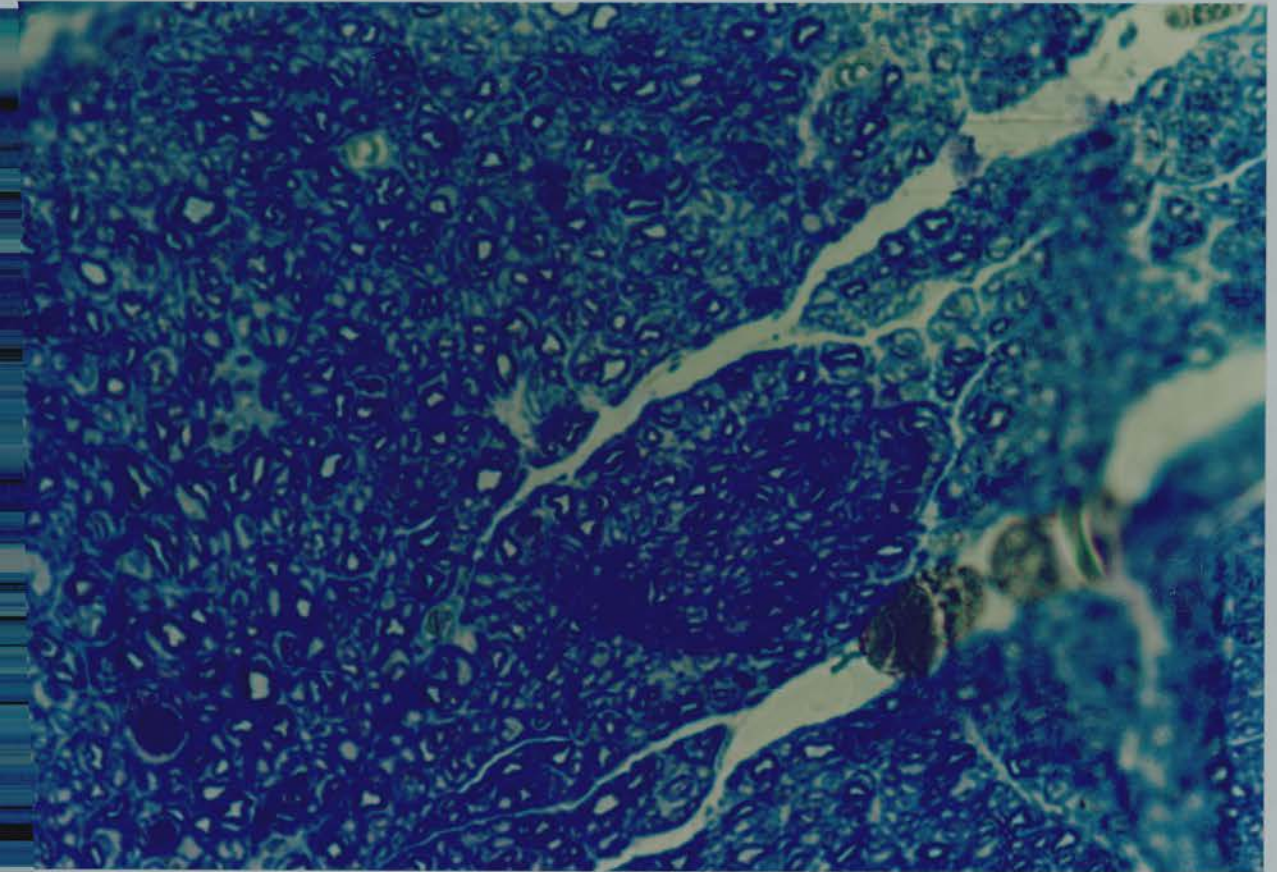


Figure 8.8.1

Photograph of a normal nerve stained with toluidine blue.

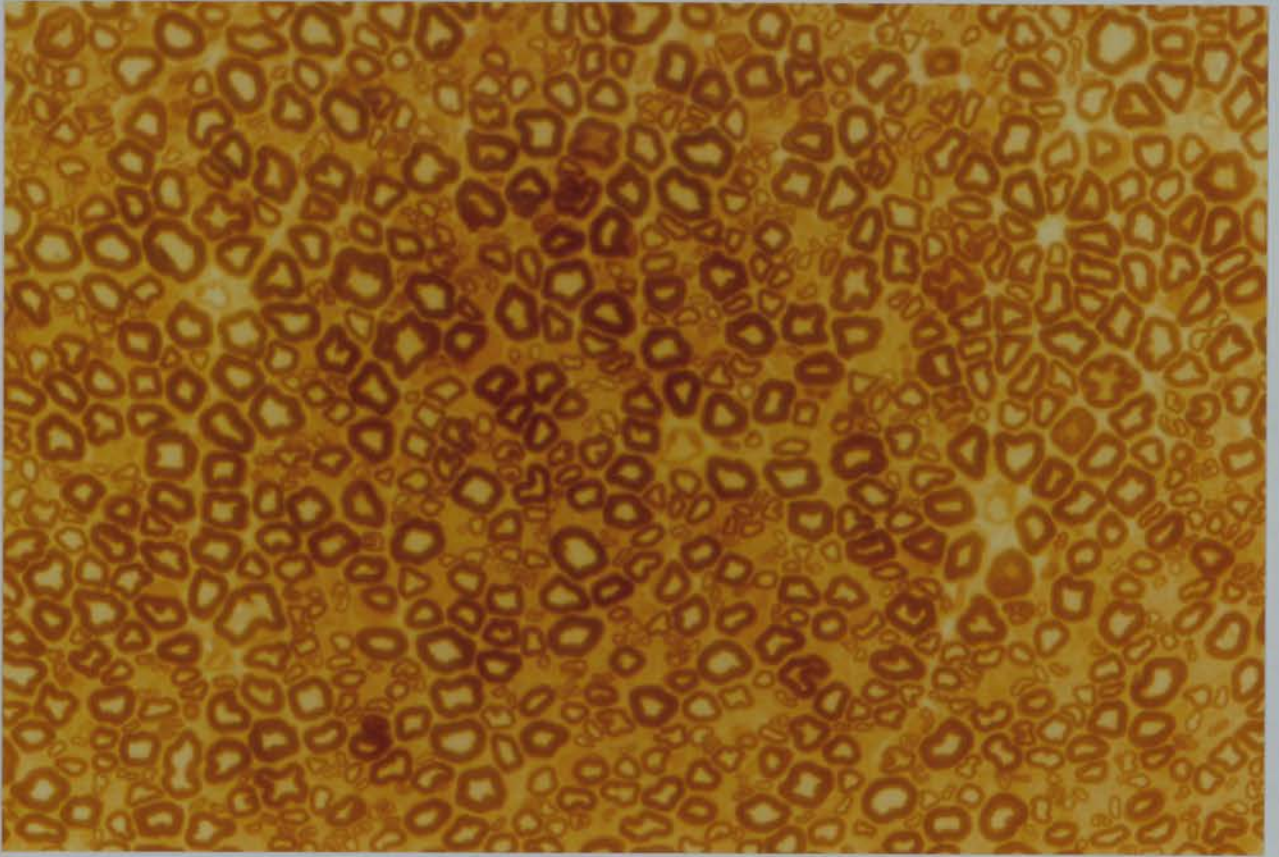


Figure 8.8.2

Photograph of a normal nerve stained with Paraphenylenediamine.

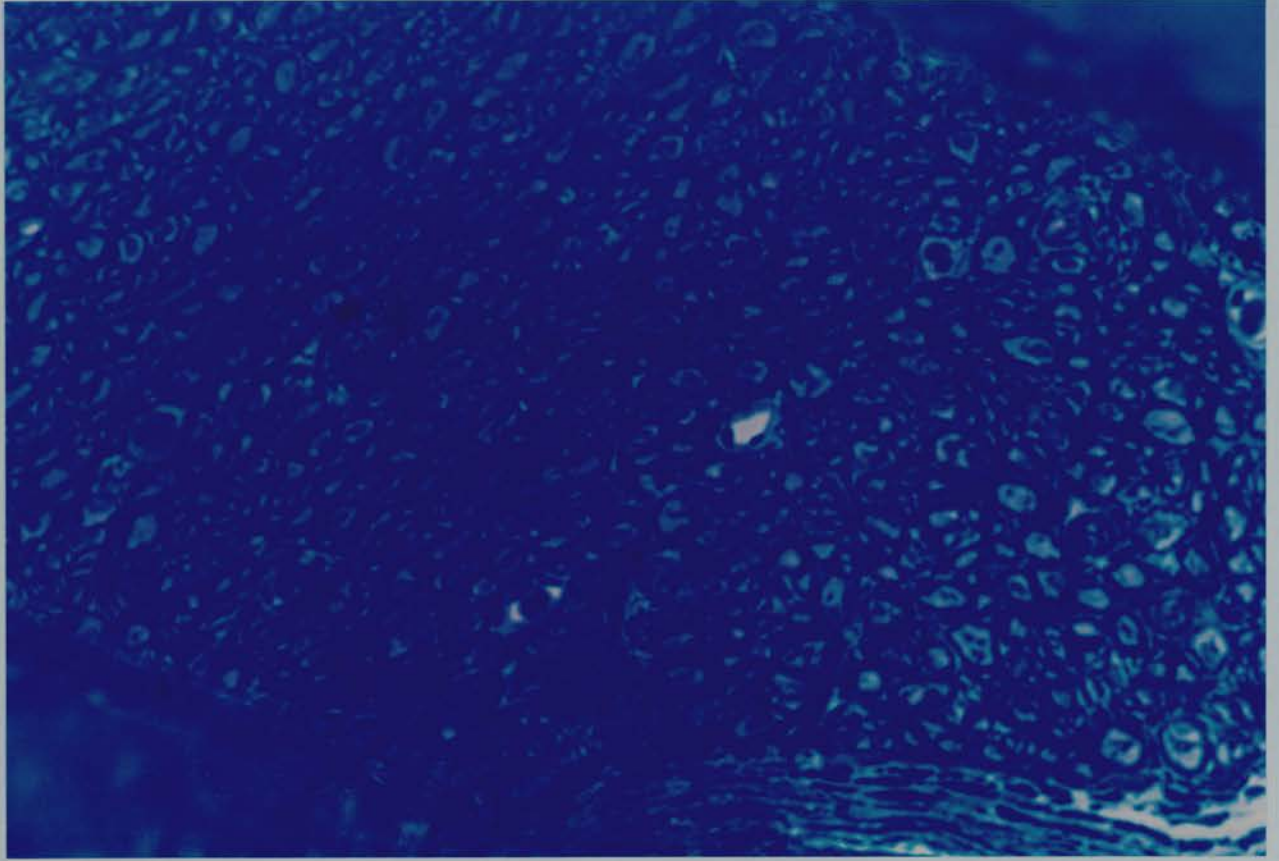


Figure 8.8.3

Photograph of a nerve repaired with a MMG and stained with toluidine blue.

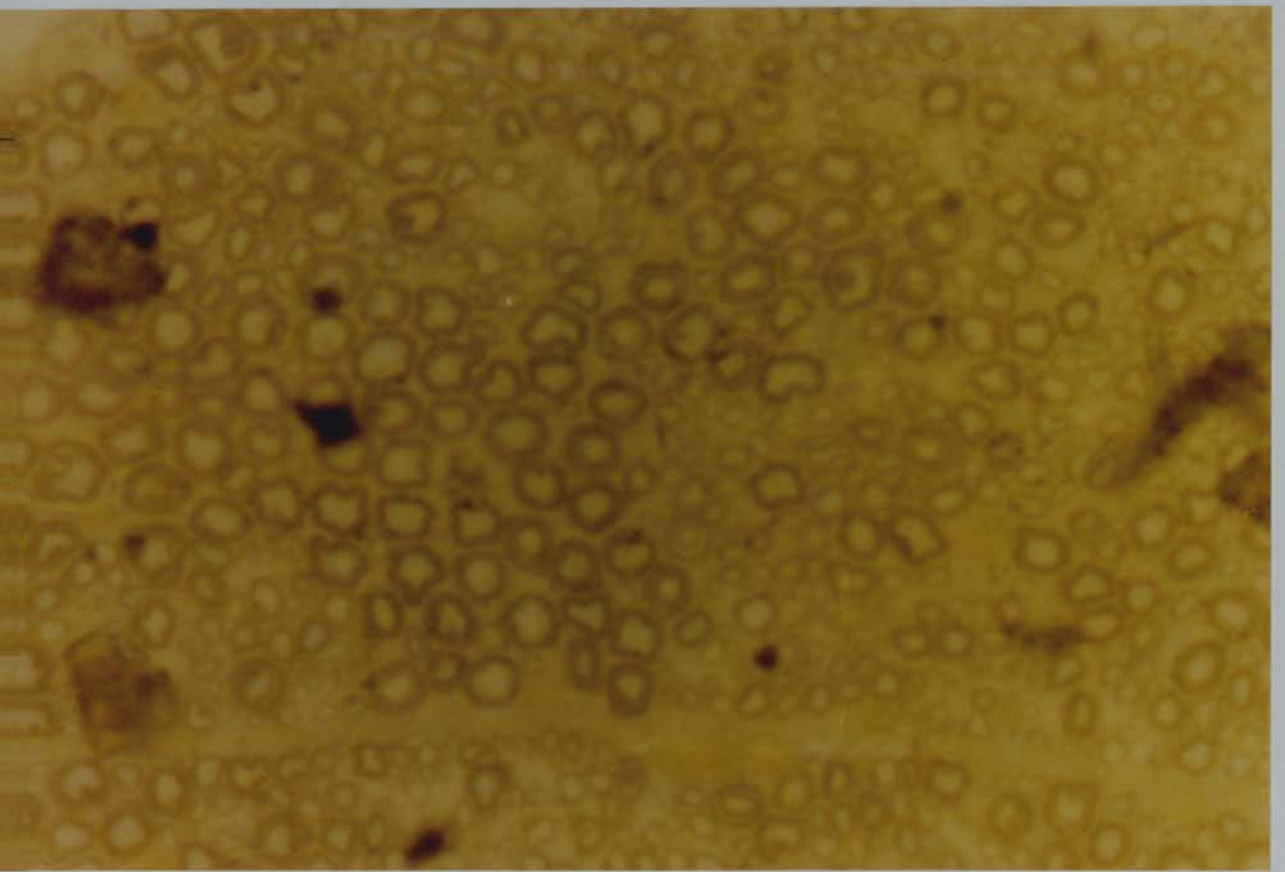


Figure 8.8.4

Photograph of a nerve repaired with a MMG and stained with paraphenylenediamine.

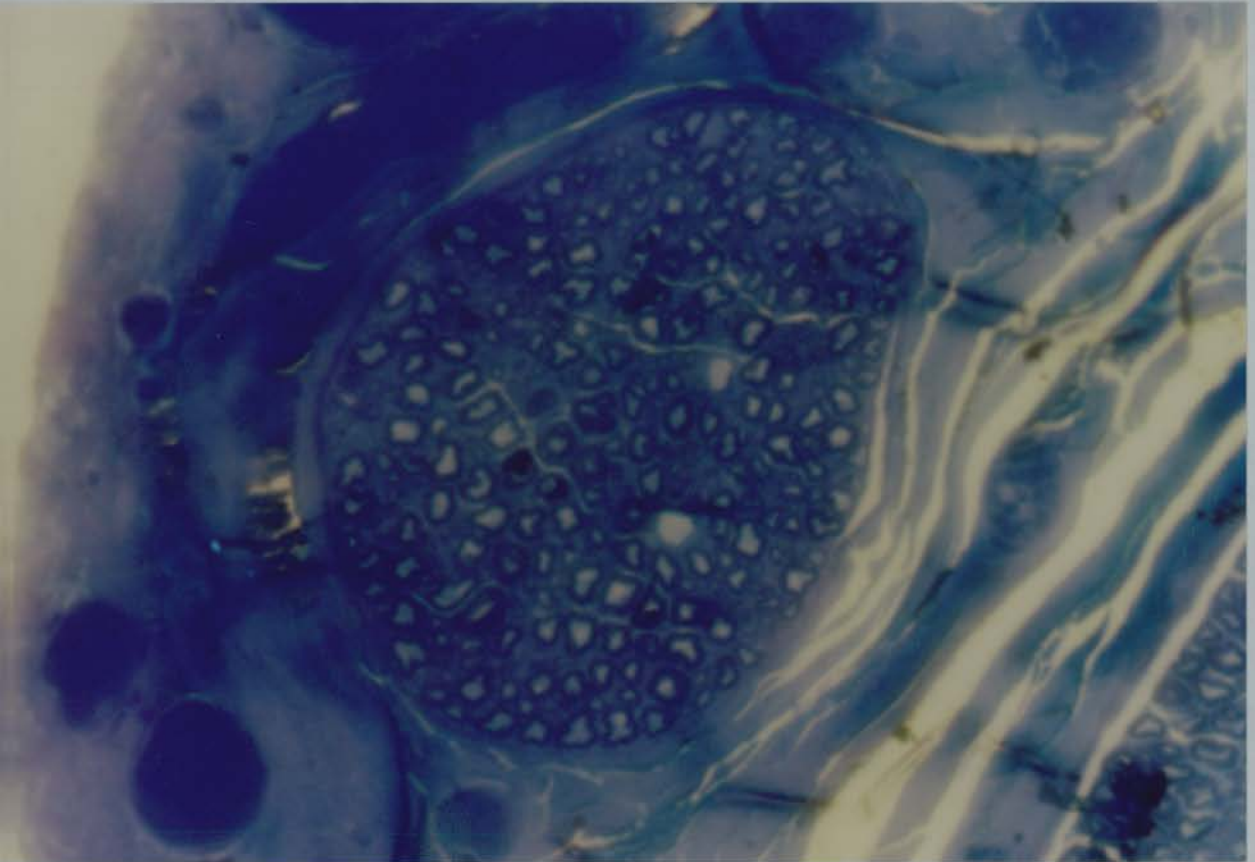


Figure 8.8.5

Photograph of a nerve repaired with a FTMG and stained with toluidine blue.

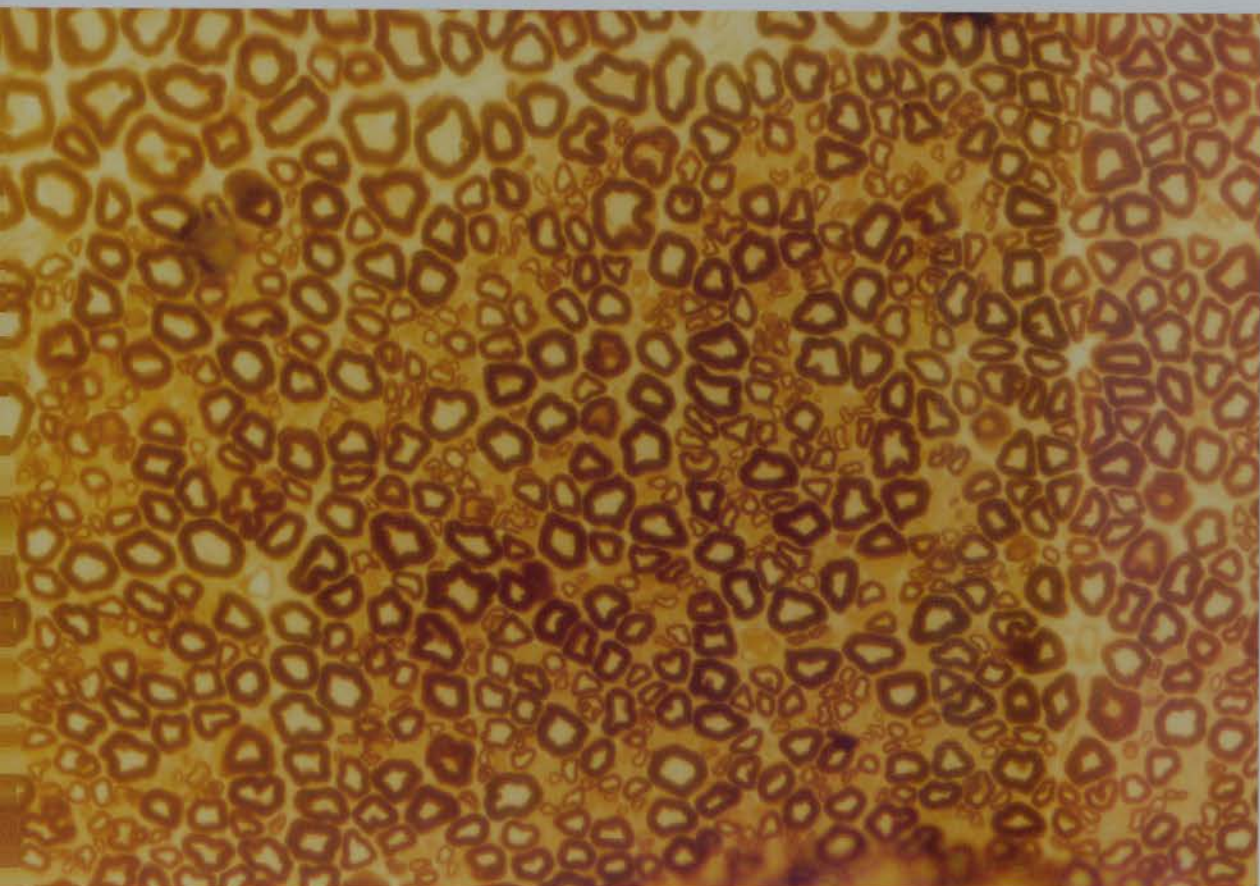


Figure 8.8.6

Photograph of a nerve repaired with a FTMG and stained with paraphenylenediamine.

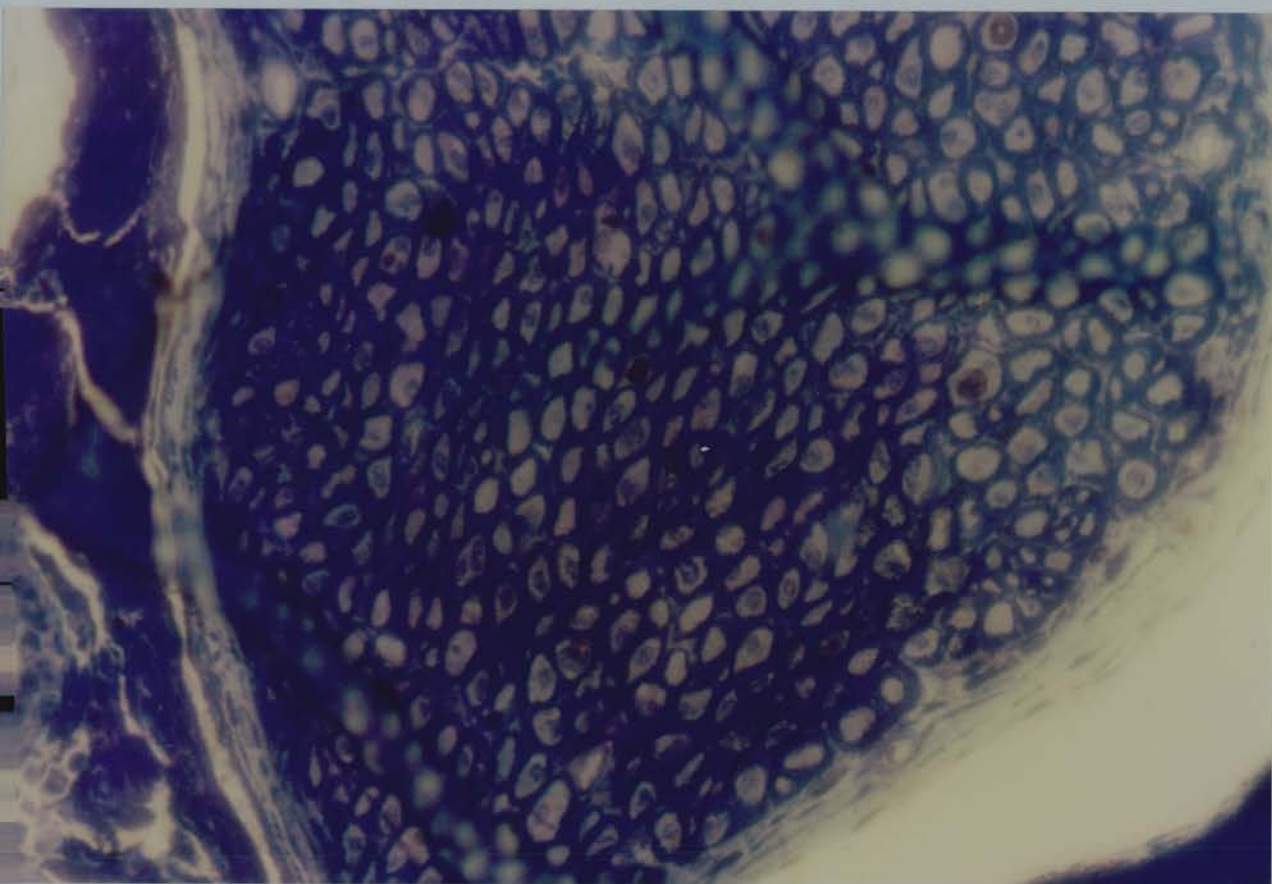


Figure 8.8.7

Photograph of a nerve repaired with a CRG-M and stained with toluidine blue.

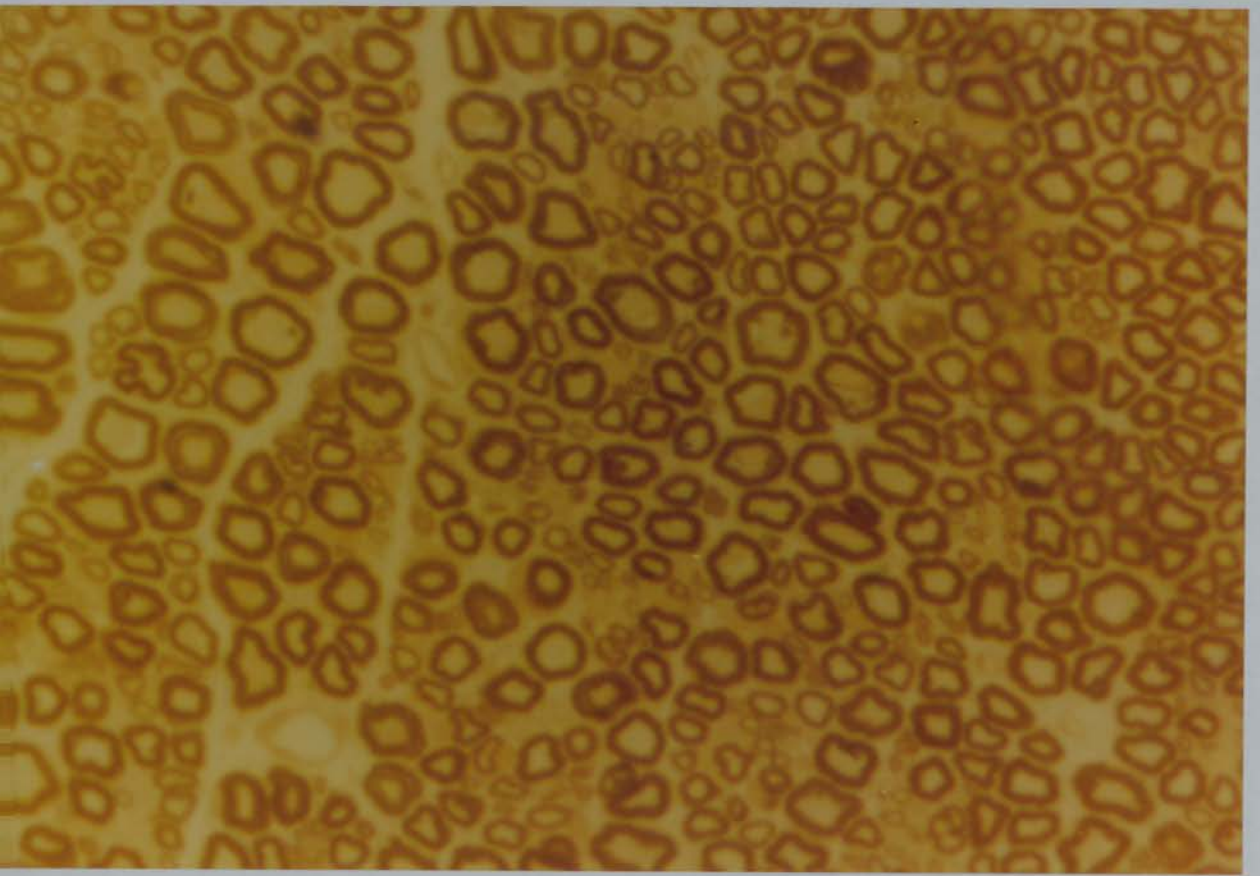


Figure 8.8.8

Photograph of a nerve repaired with a CRG-M and stained with paraphenylenediamine.

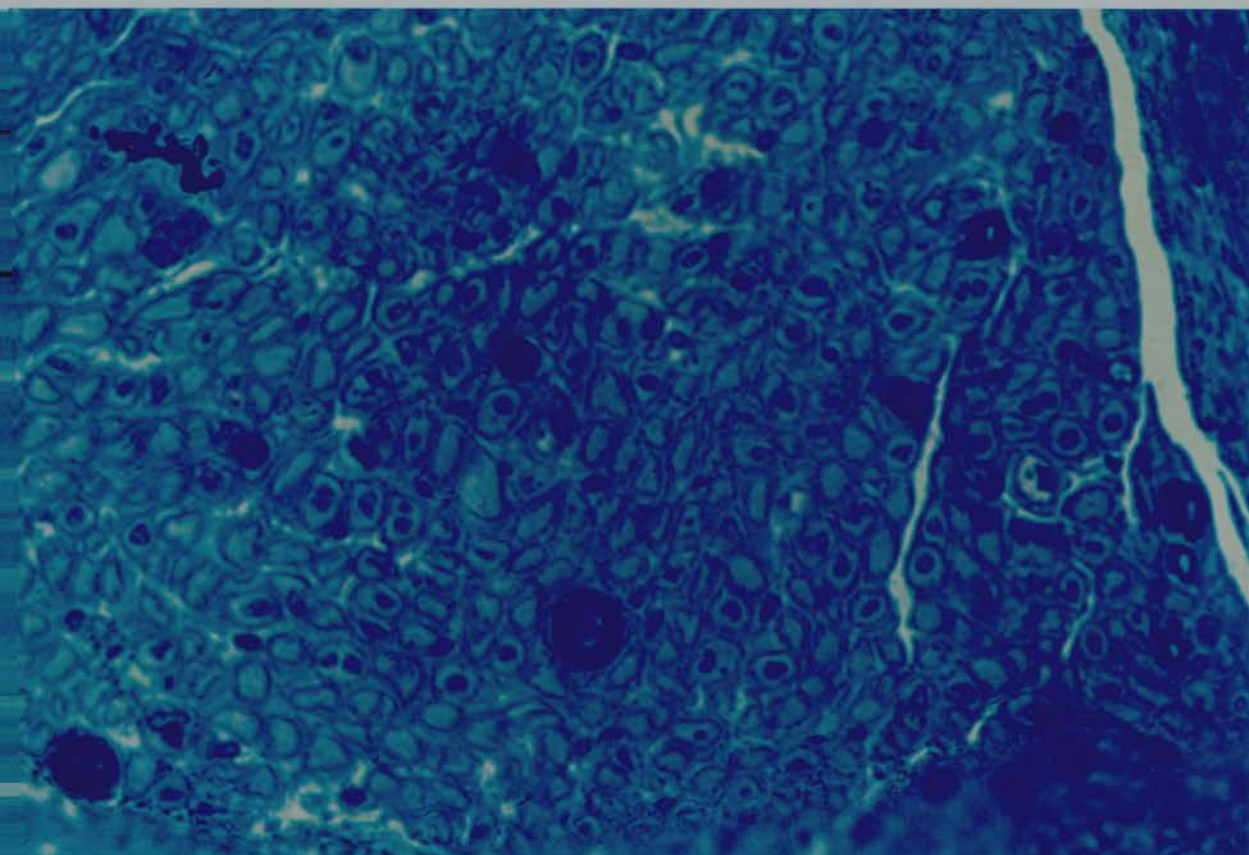


Figure 8.8.9

Photograph of a nerve repaired with a CRG-MN and stained with toluidine blue.

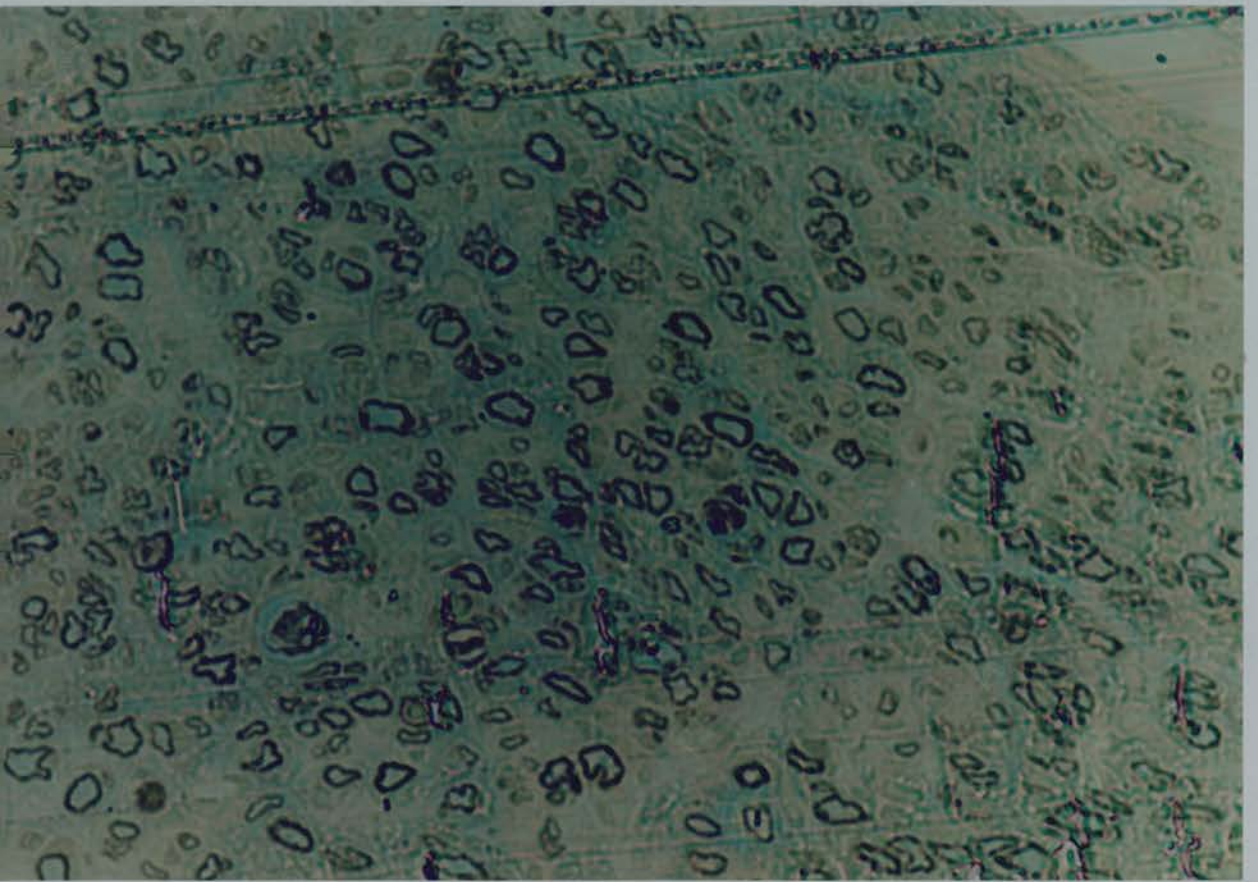


Figure 8.8.10

Photograph of a nerve repaired with a CRG-MN and stained with paraphenylenediamine.

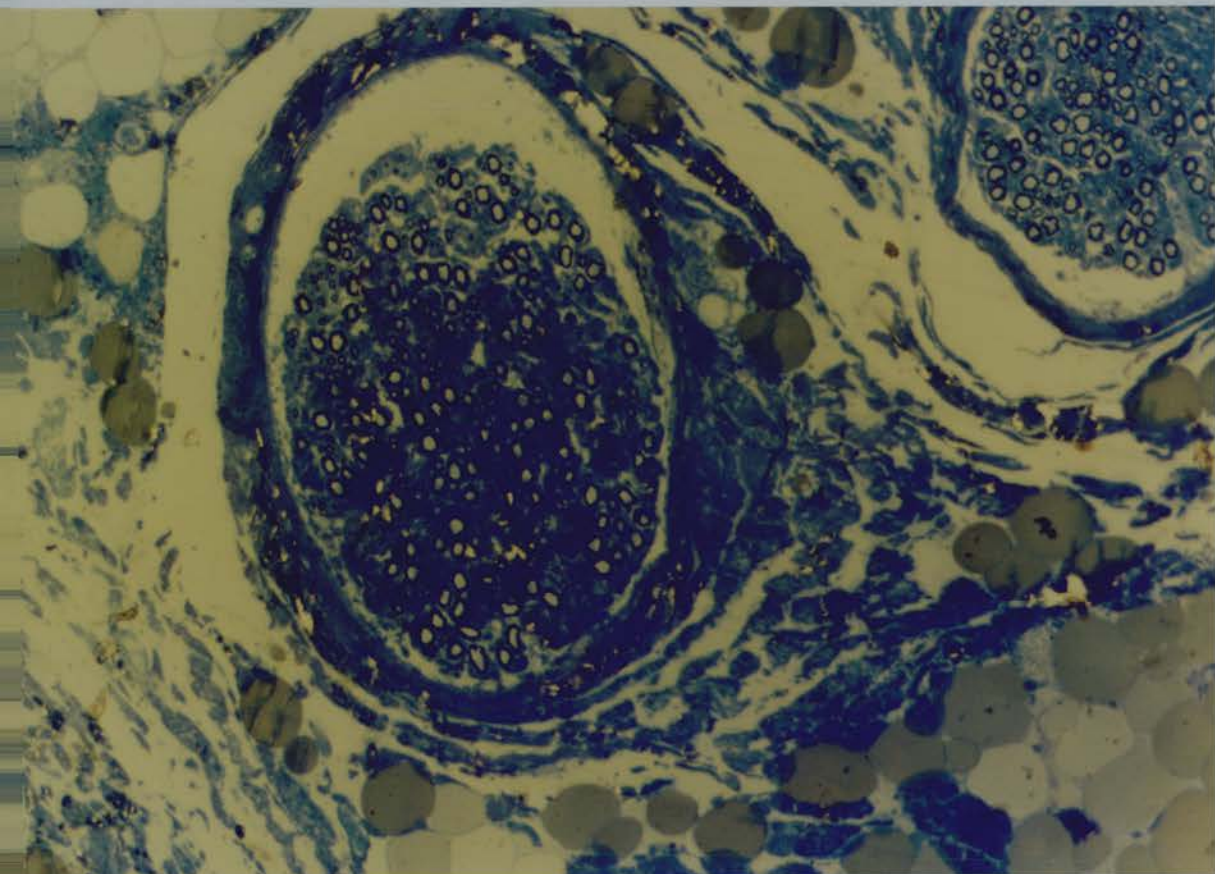


Figure 8.8.11

Photograph of a nerve repaired with a CRG-N and stained with toluidine blue.

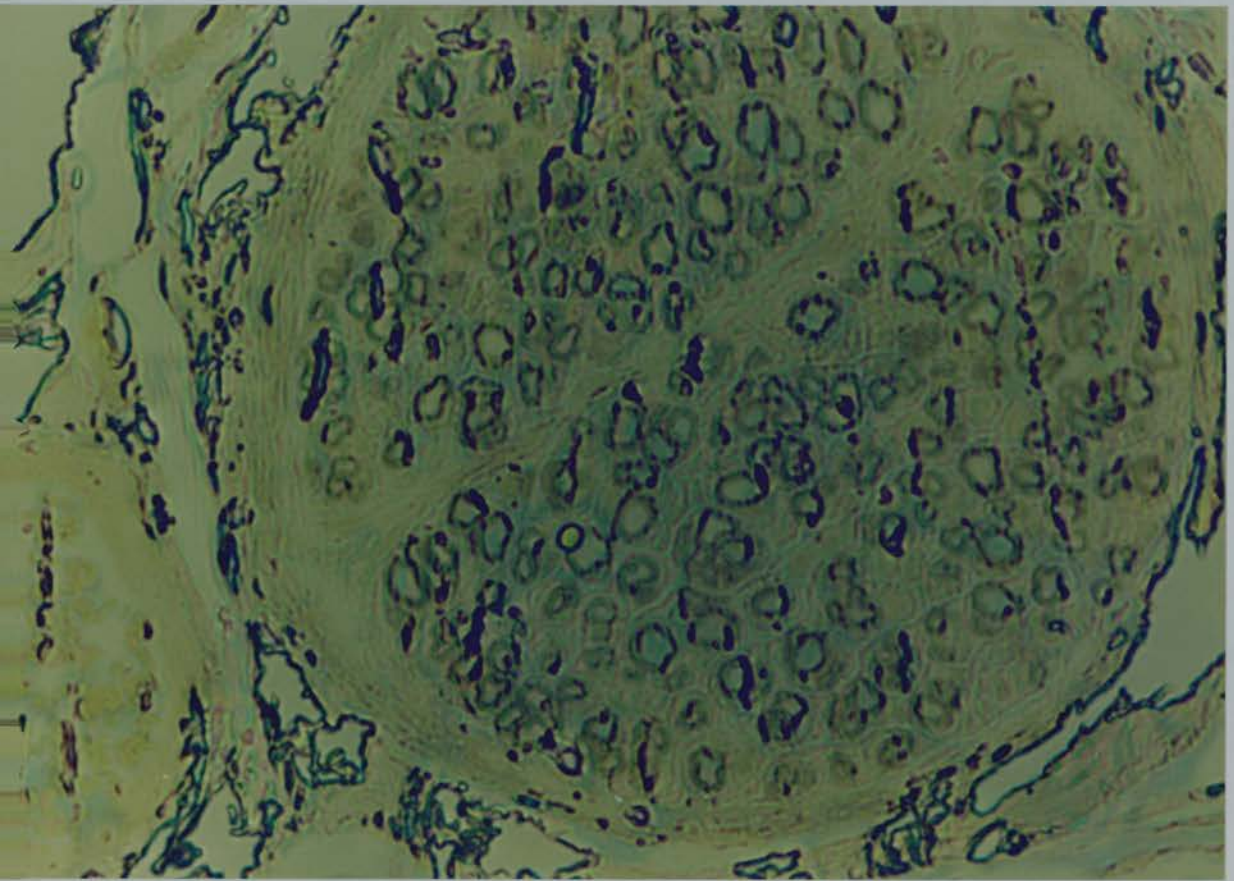


Figure 8.8.12

Photograph of a nerve repaired with a CRG-N and stained with paraphenylenediamine.

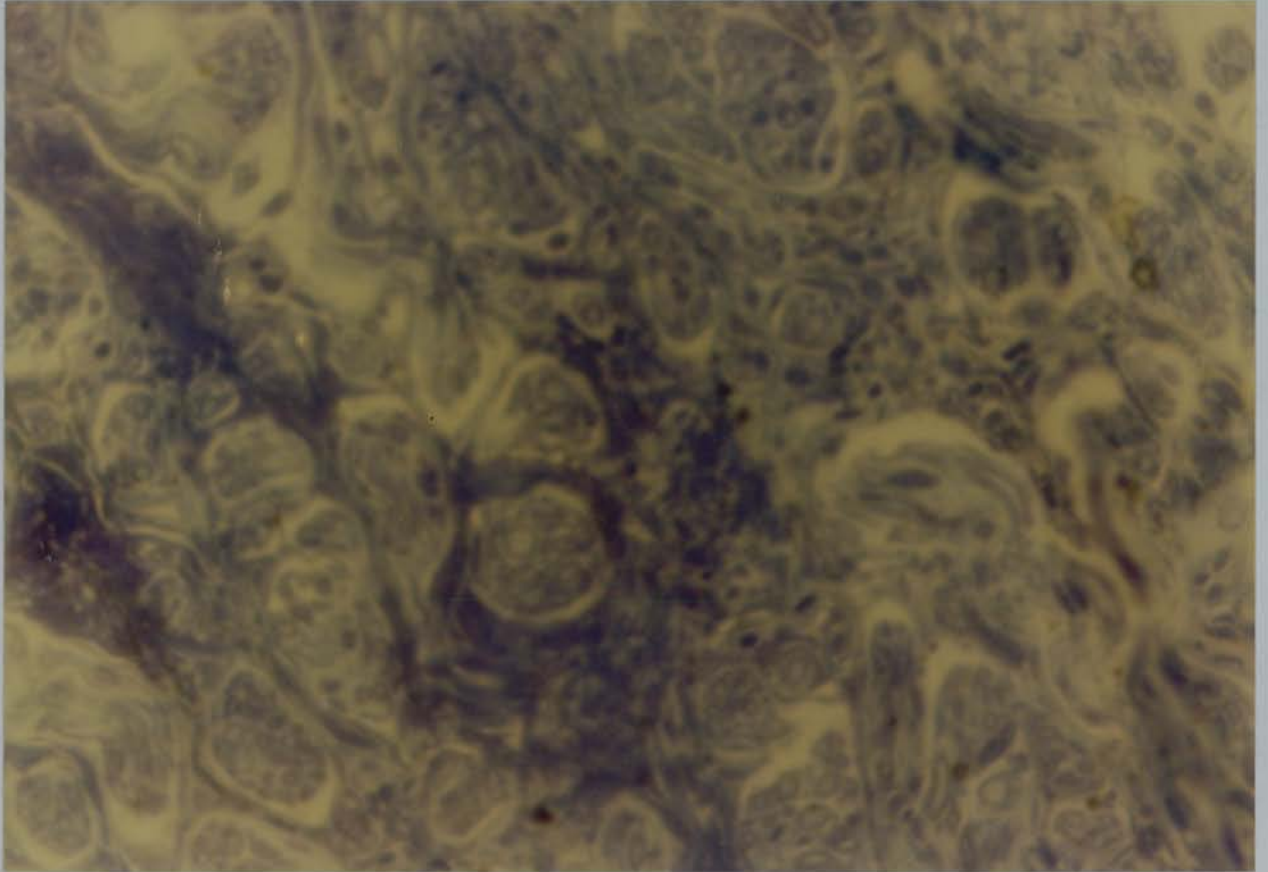


Figure 8.8.13

Photograph of a nerve repaired with a CRG-GAP and stained with toluidine blue.

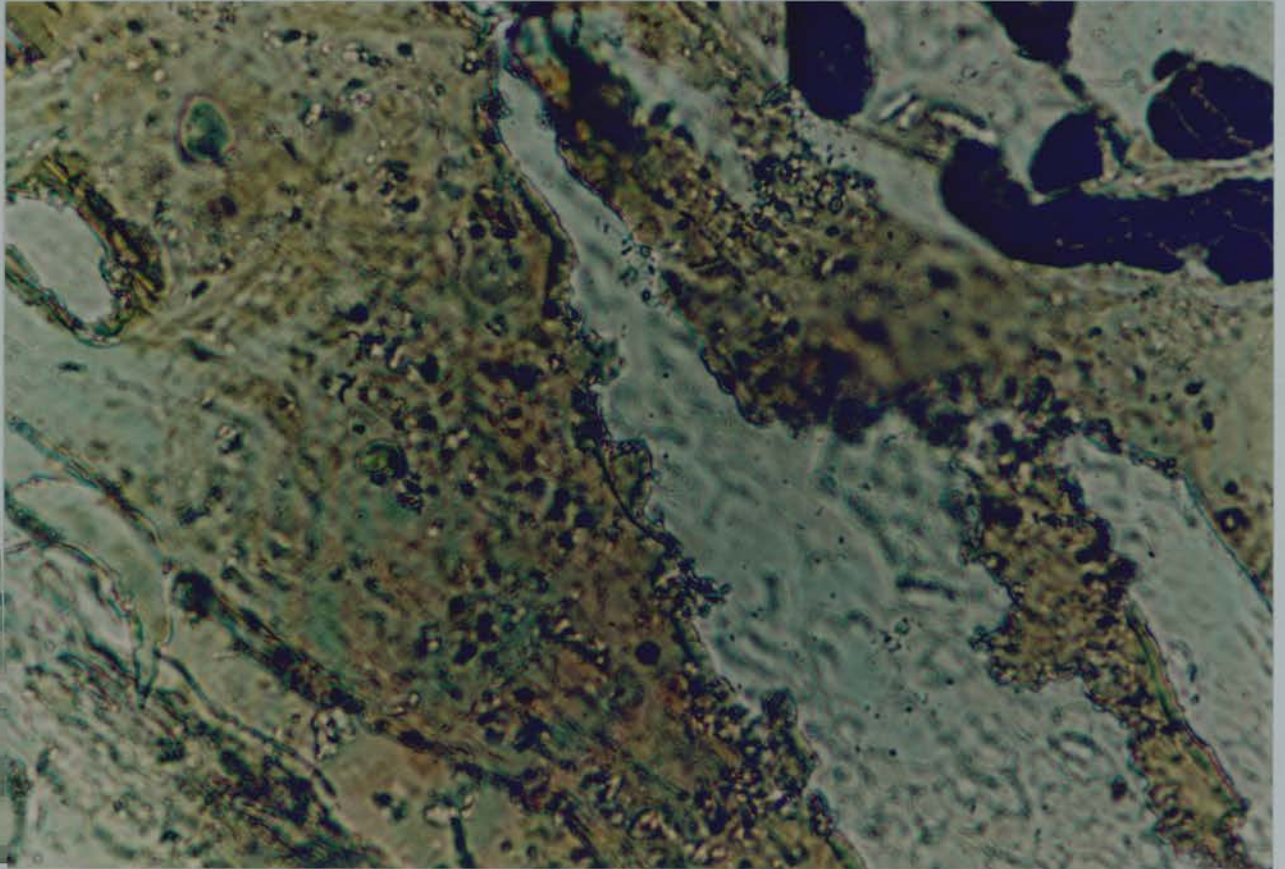


Figure 8.8.14

Photograph of a nerve repaired with a CRG-GAP and stained with paraphenylenediamine.

It became evident early on during measurements that the repair groups had fewer large myelinated fibres. The large fibres that were present were consistently smaller than those in normal nerves. Figure 8.8.15 is a table of the mean data for each group. Figures 8.8.16-8.8.17 are bar plots of axon, fibre and myelin thickness.

| | Normal | FTMG | CRG-M | CRG-MN | CRG-N | CRG-GAP | MMG |
|--|--------|-------|----------|---------|---------|---------|---------|
| Axon diameter (μm) | 4.52b | 1.87a | 2.09a | 1.88a,b | 1.38a | 1.11a,b | 1.66a |
| Fibre diameter (μm) | 9.33b | 4.61a | 4.94a | 4.57a,b | 3.58a | 3.22a,b | 4.37a,b |
| Myelin thickness (μm) | 2.22b | 1.38a | 1.43a | 1.35a,b | 1.10a | 1.05a,b | 1.29a |
| G-ratio | 0.47b | 0.38a | 0.39a | 0.38a,b | 0.36a | 0.33a,b | 0.38a,b |
| 90% confidence interval for fibre diameter (m) | 19.05b | 9.36a | 10.05a,b | 9.28a | 7.29a,b | 6.54a,b | 8.87a,b |

Figure 8.8.15 Table of the mean data for all groups and the 90% confidence interval for fibre diameter. (a = significantly different from normal $p < 0.05$, b = significantly different from FTMG $p < 0.05$)

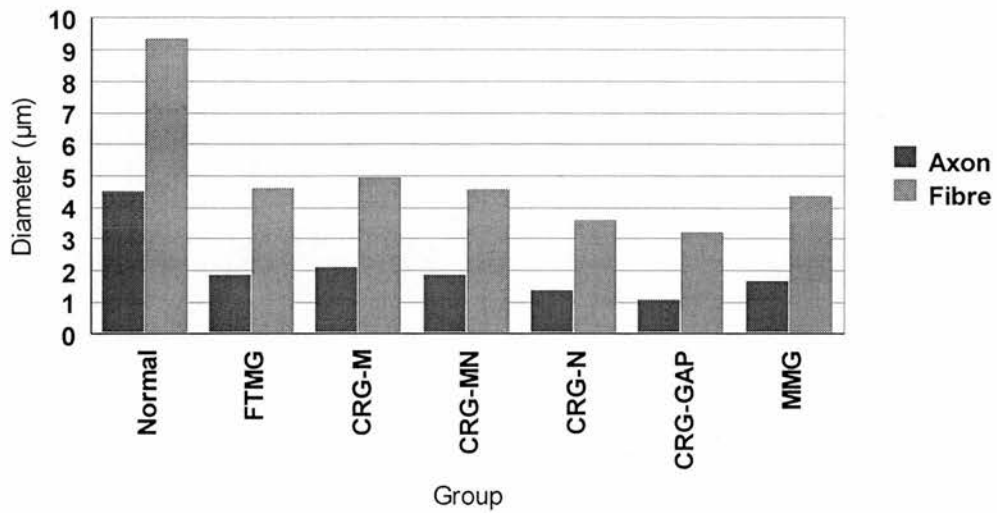


Figure 8.8.16 Bar plot of the axon and fibre diameters for all groups.

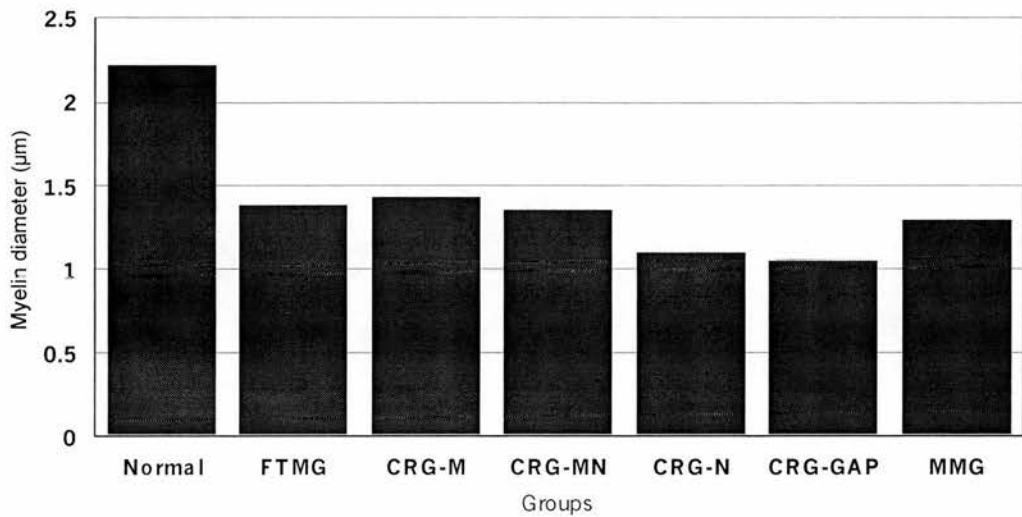


Figure 8.8.17 Bar plot of the myelin thickness for all groups.

THE EXPERIMENTS

CHAPTER 9.0

THE MICROWAVE MUSCLE GRAFT

| <u>Section</u> | <u>Title</u> |
|----------------|---------------------|
| 9.1 | <u>OBJECTIVE</u> |
| 9.2 | <u>INTRODUCTION</u> |
| 9.3 | <u>METHODS</u> |
| 9.4 | <u>RESULTS</u> |
| 9.5 | <u>DISCUSSION</u> |
| 9.6 | <u>CONCLUSION</u> |

9.1 OBJECTIVE

The purpose of this study was to compare muscle grafts prepared by two different methods of denaturing: freezing in liquid nitrogen (FTMG) and heating in a microwave oven (MMG) for the repair of a 1 cm gap in the rabbit peroneal nerve.

The FTMG and MMG were compared with each other and with normal nerves by electrophysiological and histological tests to assess the recovery of function 6 months after repair.

9.2 INTRODUCTION

In 1983 Ide found that regenerating nerves advance on the basal lamina of the schwann cells (Ide *et al.*, 1983). This was suggested previously by Hafteck and Thomas (Haftek & Thomas, 1968) who hypothesized that the regeneration of axons through a nerve graft was guided by the endoneurial tubes *i.e.* the Schwann cell basement membrane. In Ide's experiment a piece of nerve was frozen to kill the Schwann cells and sutured to the proximal stump. The results of this study showed that at 7 days after repair macrophages entered the basal lamina scaffolding and phagocytized the cellular debris while leaving the basal lamina intact. Ide noted that regenerating nerve fibres grew in the space between the Schwann cell basal lamina and plasma membrane. Ide stated that:

“...the regenerating axons were located without exception, within the basal lamina scaffolds. They were always in contact with the inner surface of the basal lamina scaffolds. No axon was found outside the scaffolds” (Ide *et al.*, 1983)

The conclusion of this study was that the basal lamina supported nerve regeneration. However, this conclusion raised more questions than it answered. What was the difference between the inside and outside surface of the basal lamina? What substance was the inside surface of the Schwann cell made of in order to concentrate axonal regeneration at this location? Did this substance have a structural or promoting effect on the regenerating axons or both? Would the basal lamina

scaffolding of the Schwann cell support regeneration over longer distances? Could the basal lamina from other tissues such as muscles, veins or from a synthetic source also support axon regeneration?

Skeletal muscle is made of muscle fibres surrounded by basal lamina. It has been shown that if muscle is denatured so as to kill the myocytes without damaging the basal lamina, it could support nerve regeneration in it. (Glasby *et al.*, 1986a). This observation led to a series of experiments on the feasibility of using denatured muscle grafts instead of nerve grafts to repair gaps in damaged peripheral nerves. (Fawcett & Keynes, 1986; Glasby *et al.*, 1986a; Feneley *et al.*, 1991; Glasby, 1991). Previous studies have shown that denatured muscle grafts produced by freezing demonstrated no significant difference in the recovery of function when compared with nerve autografts of equal diameter in the rat (Glasby *et al.*, 1986a) and marmoset (Glasby *et al.*, 1986b).

A problem associated with the repair of peripheral nerves using freeze-thawed muscle grafts is that the freezing process can produce considerable shrinkage (up to 50%) (Glasby *et al.*, 1986a). Several researchers have attempted to overcome this difficulty by altering the methods by which the muscle is prepared. However, the goal of the denaturing method has not changed, to kill the myocytes, intra-muscular nerves and endothelial cells without destroying the basal lamina.

Some of the different methods of producing a denatured muscle graft include repeated episodes of freeze-thawing at -25°C (Enver & Hall, 1997), the use of

chemicals such as 10mM CaCl₂ in 2 mM imidazole (Feneley *et al.*, 1991) , and heating in distilled water to 60°C (Hall & Enver, 1994; Whitworth *et al.*, 1995)

Whitworth *et al* published a paper where grafts for nerve repair in the rat were prepared by heating muscle in a microwave oven (Whitworth *et al.*, 1995). It was shown that muscle grafts prepared in this maner reduced the amount of shrinkage of the graft when compared to muscle grafts prepared by freezing (Whitworth *et al.*, 1995). Whitworth's paper stated that the MMG provided similar levels of axonal regeneration and Schwann cell penetration when compared with FTMG 60 days after nerve repair in the rat. Based on these results it was concluded that 'MMG provides similar levels of nerve regeneration when compared with FTMG and may have a potential clinical use'.

There were two problems with the logic of Whitworth's conclusion that MMG may have a potential clinical use. First, it has been shown that assessment of a muscle graft 60 days after nerve repair in the rat is not sufficient time to allow for complete and comparable levels of regeneration. (Lenihan D.V. *et al.*, 1998). And second, the MMG has not been compared to the FTMG with respect to any known indices of recovery of function.

The results of comparing the MMG to the FTMG with respect to recovery of function and nerve morphology 6 months after repair are presented. The potential clinical usefulness of the microwave muscle graft will also be discussed.

9.3 METHODS

Fifteen adult female New Zealand White rabbits were separated into three experimental groups of 5 animals. The groups consisted of a control group and groups where the peroneal nerve had been divided and repaired with either a freeze-thawed muscle graft or a microwave muscle graft. Each animal was anaesthetized as described in Chapter 4.

An incision was made in the lateral aspect of the left thigh. The flexor and extensor muscles were reflected to expose the sciatic nerve. The peroneal nerve was separated from the tibial nerve using microsurgical forceps. The peroneal nerve was divided 3 cm below the sciatic notch and, allowing for retraction, an appropriate amount of nerve was removed from the distal segment to create a 1 cm gap. This gap in the peroneal nerve was filled with either a FTMG or a MMG.

The microwave muscle graft (MMG) and freeze thawed muscle graft (FTMG) were prepared as described in Chapter 5.

The animals were assessed 6 months after their repair. Jitter measurement was carried out in the EDL muscle (Trontelj *et al.*, 1988). The intramuscular terminal branches of the motor nerve received supramaximal bipolar stimulation using two monopolar needle electrodes (Medelec, Old Woking, UK). The motor unit action potential (MUAP) was recorded and the inter-potential interval (IPI) between stimulation and the peak of the MUAP was determined. The IPI was calculated for 50 consecutive discharges for the same neuromuscular junction and the mean

consecutive difference (MCD) was determined (Stålberg *et al.*, 1992; Lenihan *et al.*, 1997). The MCD was measured on 20 different neuromuscular junctions and the average was called the jitter.

A supramaximal stimulus was delivered to the peroneal nerve at a point proximal to the graft (S1). The resulting M-wave from the EDL was filtered digitally and amplified before being displayed on the EMG screen. The amplitude, area and latency of the M-wave were measured.

A second non-traumatic stimulating electrode was placed on the peroneal nerve distal to the graft (S2). The peroneal nerve was stimulated at S2 with a similar supramaximal stimulus as above. The amplitude, area and latency of the M-wave of the peroneal nerve when stimulated at point S2 were measured. The difference in latencies from stimulation to the initial deflection of the M-wave when the peroneal nerve was stimulated at points S1 and S2 were used to measure the maximum conduction velocity (CV_{max}) using the formula 6.2.1.1.

The stimulating electrodes were kept in the same position as above to calculate the minimum conduction velocity (CV_{min}) using the collision technique (Hopf, 1963). See section 6.3.

Excitability of the nerve was determined by measuring the absolute refractory period. A supramaximal stimulus was delivered to the peroneal nerve at point S2, followed by a second stimulus of the same intensity 0.01 ms later. This interval was

increased by 0.01 ms increments until a second M-wave could be detected. See section 6.4.

Once all electrophysiological tests were completed, the tendon of the EDL was cut and connected by a steel wire to a tension transducer (Harvard Bioelectronics, USA). The peroneal nerve was stimulated with a supramaximal current at S1. The force generated by the EDL muscle twitch was converted to voltage (V) as described in chapter 6 and the amplitude, area and TTI of the resulting muscle twitch were determined (see Chapter 7).

A 1 cm length of the peroneal nerve was removed 2 cm distal to the graft and processed for morphological analysis as described in Chapter 8.

9.4 RESULTS

The preparation of a muscle graft by heating it in a microwave oven proved very difficult. A large number of trials were required to produce a reliable time and power setting which provided a graft that was heated on all sides between the temperature range of 60°C and 80°C. Variations in the size of the graft produced inconsistent heating and it was therefore very important to ensure that the block of muscle harvested was of a consistent size.

All the animals in the repair groups demonstrated some degree of restriction in their mobility with respect to the operated leg. One animal in the MMG group died on the table during assessment. This reduced the number of animals in the MMG group for electrophysiological assessment to four. The nerve from the animal that died on the table was still used for morphological assessment to complete the group.

Six months after repair all animals had some degree of nerve regeneration. When the FTMG group was compared with the MMG group the following measurements were significantly different ($p < 0.05$): the absolute refractory period, fibre diameter and G-ratio. It was seen in both repair groups when they were compared with normal animals that a significant difference ($p < 0.05$) for all electrophysiological and morphological tests was present.

The mean CV_{max} of the normal nerve, FTMG and MMG was 69.36 m s^{-1} , 40.23 m s^{-1} , and 43.08 m s^{-1} respectively. The corresponding mean values of CV_{min} were 32.98 m s^{-1} , 12.46 m s^{-1} , and 10.13 m s^{-1} . Figure 9.4.1 is a box and whisker plot of

the means and standard errors of the maximum and minimum conduction velocities for all groups. The range of velocities ($CV_{max} - CV_{min}$) was not found to be significantly different ($p>0.05$) when any two group were compared. Figure 9.4.5 is a table which provides the mean values of all the electrophysiological tests for each group.

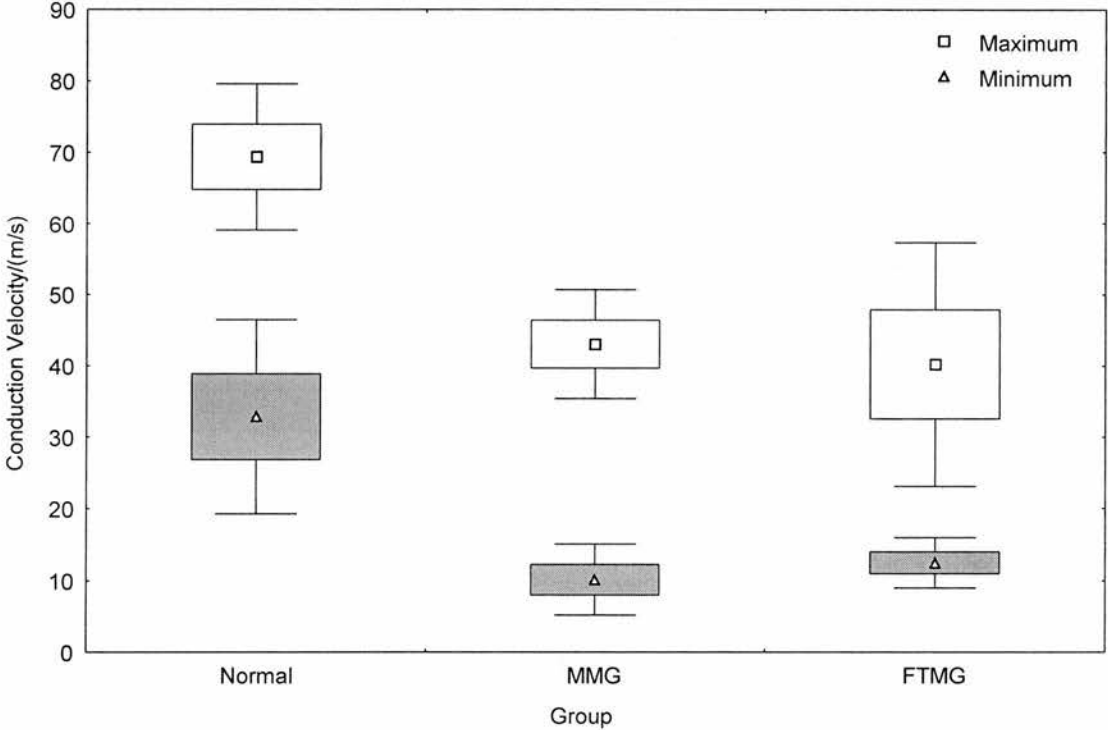


Figure 9.4.1 Box and whisker plot of the means, standard deviations, and standard errors of the maximum and minimum conduction velocity for all groups.

The amplitude and area of the M-wave represent the electrical change in the muscle under the surface electrodes during contraction. The electrical change is approximately proportional to the number of muscle fibres contracting. The amplitude and area of the M-wave were significantly different ($p<0.05$) in the

repaired groups when compared to normal. This indicated that there was a reduction in the number of EDL fibres contracting for the repair groups after nerve stimulation. The mean M-wave amplitudes for the normal, MMG and FTMG were 18.92 mV, 8.67 mV and 8.04 mV respectively. The respective means of the M-wave area for the normal, MMG and FTMG were 30.40 mVs, 14.05 mVs, and 12.76 mVs. Figure 9.4.2 is a box and whisker plot of the means, standard deviations and standard errors for groups.

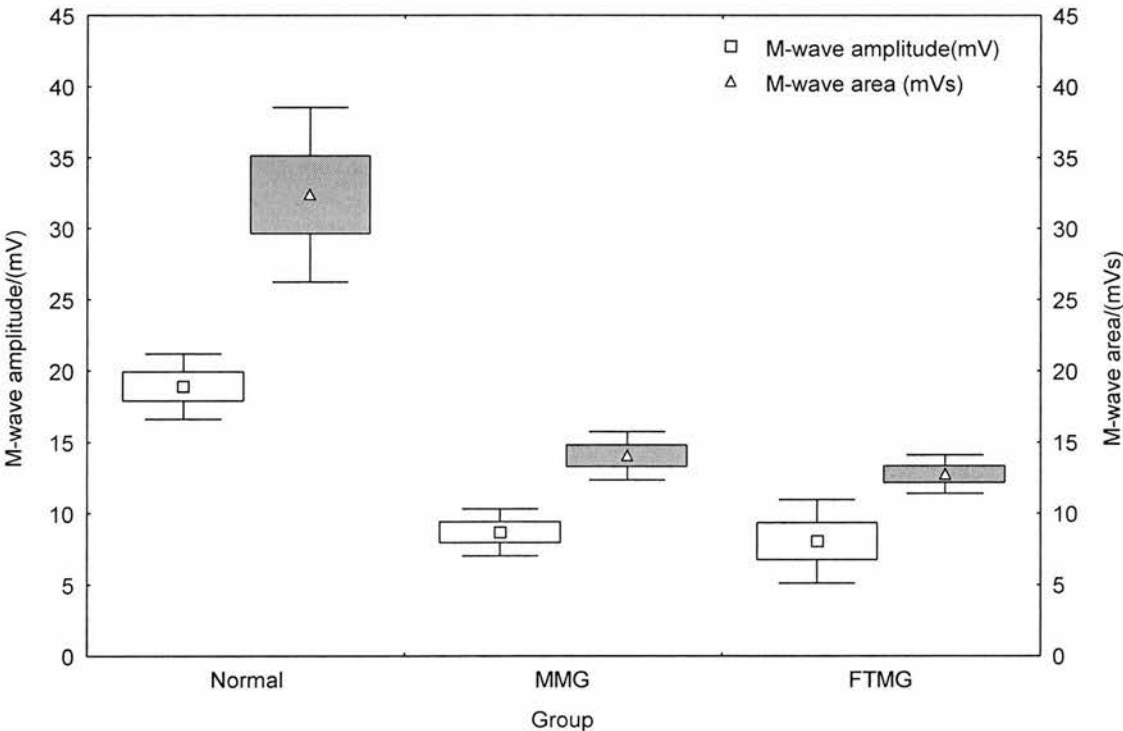


Figure 9.4.2 Box and whisker plot of the means, standard deviations, and standard error for the M-wave area and amplitude for all groups.

The muscle twitch amplitude (Hartree & Hill, 1921) and TTI (Hems & Glasby, 1992) are indirectly proportional to the number of muscle fibres contracting. In both repair groups, the muscle twitch amplitude and TTI were significantly different ($p < 0.05$) when compared with normal. However, there was no significant difference ($p > 0.05$) in the muscle twitch amplitude and TTI between the repair groups. The peak muscle twitch force for the normal, MMG and FTMG groups was 5.22 N, 2.93 N, and 2.62 N respectively. The time tension index (TTI) for the normal, MMG and FTMG group was 6.27 N, 3.93 N, and 3.50 N respectively. These results of the peak muscle twitch amplitude and TTI tests indicate that the repair groups had a reduction in the number of muscle fibres contracting after nerve stimulation when compared to normal. This result was further supported by a similar reduction of the M-wave amplitude and area for the repair groups.

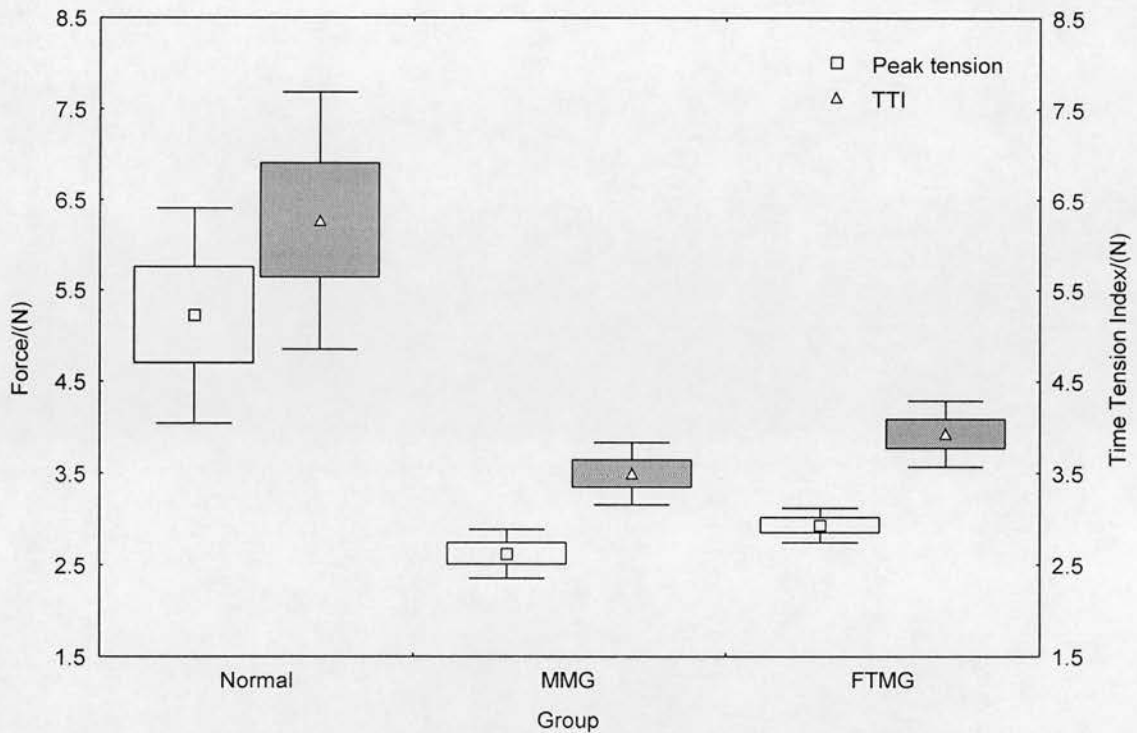


Figure 9.4.3 Box and whisker plot of the means, standard deviations, and standard error for peak muscle twitch tension and TTI for all groups.

The refractory period is related to both the excitability and the maximum frequency at which a nerve can conduct consecutive impulses. The mean absolute refractory period of the normal, FTMG and MMG groups were 0.84 ms, 1.12 ms, and 1.85 ms respectively. It was observed in the repair groups that when they were compared with normal animals, absolute refractory period was significantly different ($p < 0.05$). The absolute refractory period was found to be significantly different ($p < 0.05$) when the two repair groups were compared. These results suggest that nerves repaired with a FTMG are slightly easier to excite and can conduct impulses at a higher frequency when compared to nerve repaired with a MMG.

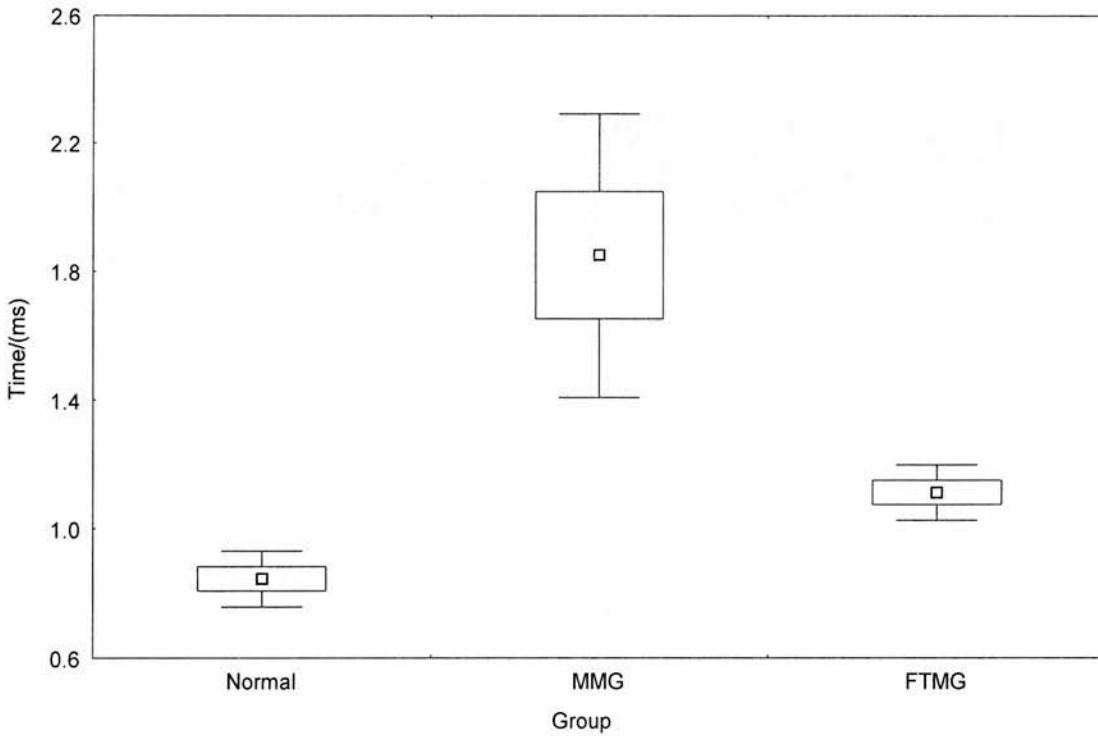


Figure 9.4.4 Box and whisker plot of the means, standard deviations, and standard errors of the absolute refractory periods for all groups.

Jitter measures variability of synaptic transmission at the neuromuscular junction. The mean jitters for the normal, FTMG, MMG groups were 13.48 μ s, 20.68 μ s, and 20.96 μ s respectively. Jitter was found to be significantly different ($p < 0.05$) when the two repair groups were compared to normal. However, there was no significant difference ($p > 0.05$) in jitter between the two repair groups.

| <u>Variable</u> | Normal | <u>FTMG</u> | <u>MMG</u> |
|--|--------|--------------------|-------------------|
| Jitter (μs) | 13.48 | 20.68 | 20.96 |
| M-Wave Amplitude (mV) | 18.92 | 8.04 | 8.67 |
| M-Wave Area (mVs) | 32.40 | 12.76 | 14.05 |
| CV_{Max} (m s^{-1}) | 69.36 | 40.23 | 43.08 |
| CV_{Min} (m s^{-1}) | 32.88 | 12.46 | 10.13 |
| Peak Twitch Amplitude (N) | 5.22 | 2.93 | 2.62 |
| Time Tension Index (N) | 6.27 | 3.93 | 3.50 |
| Absolute Refractory Period (ms) | 0.84 | 1.12 | 1.85 |

Figure 9.4.5 Table of electrophysiological data for all groups.

The mean value for the axon and fibre diameter as well as myelin thickness and G-ratio were reduced when compared to normal and that this difference was significant. There was no difference between the FTMG and MMG with respect to mean axon diameter or myelin thickness. However, the FTMG group did demonstrate a significant increase in fibre diameter (Figure 9.4.6) and G-ratio (Figure 9.4.7) when compared to the MMG. This implies that the FTMG produced a small but significant improvement in myelination and fibre maturation. Figure 9.4.8 is a table of the nerve morphology results for all groups.

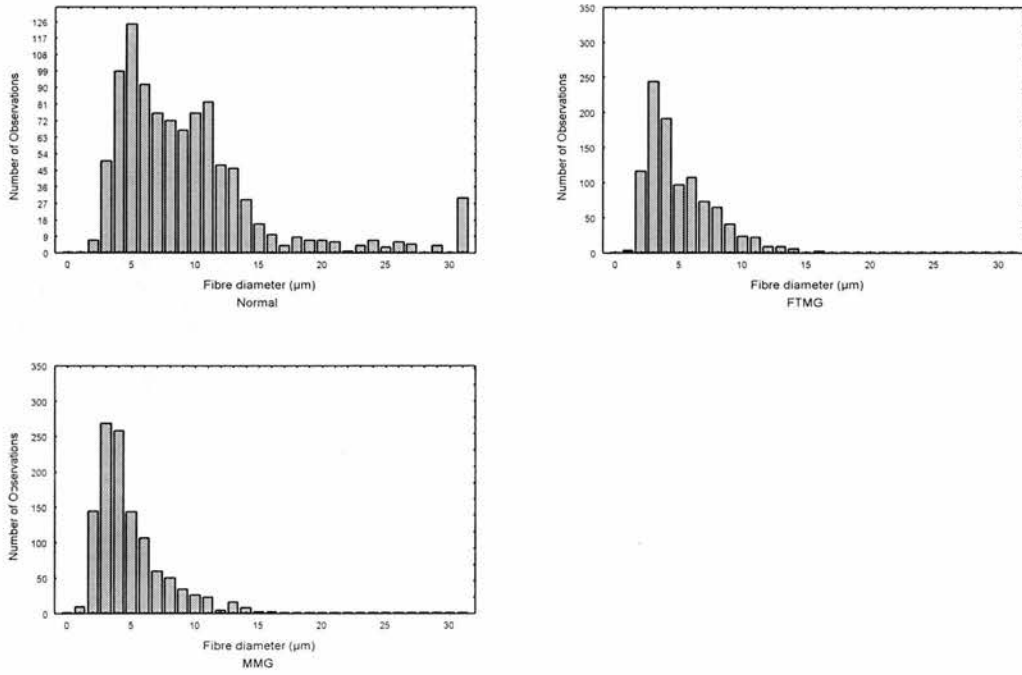


Figure 9.4.6 Histogram of the fibre diameter for all repair groups.

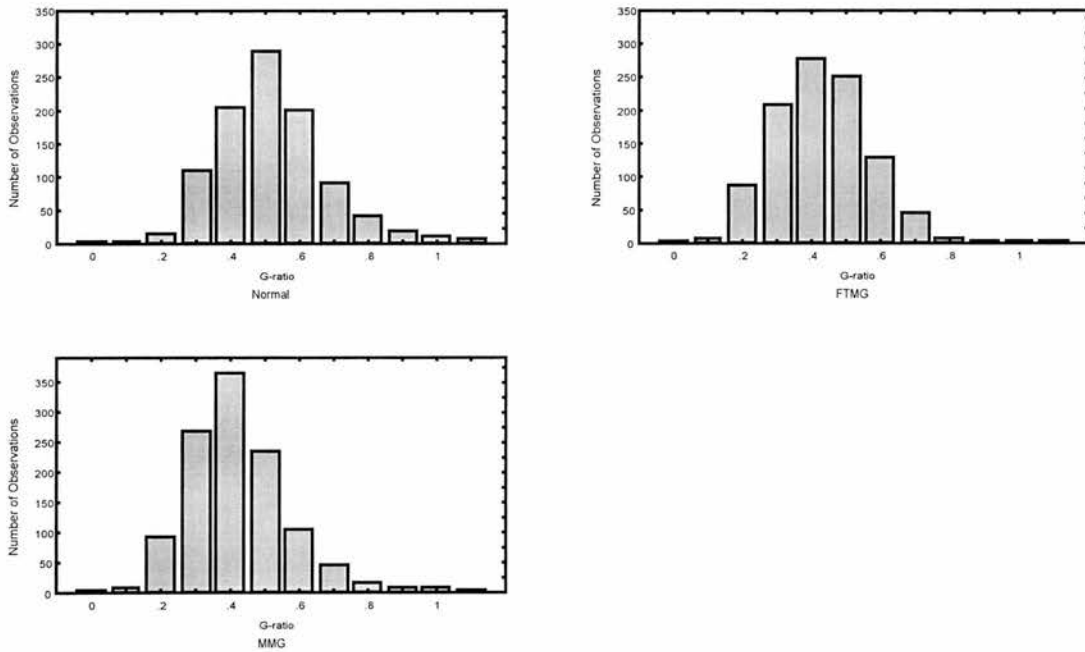


Figure 9.4.7 Histogram of G-ratio for all repair groups.

| <i>Variable</i> | Normal | FTMG | MMG |
|------------------------------|---------------|-------------------|------------|
| Axon diameter (µm) | 4.52 | 1.87 | 1.67 |
| Fibre diameter (µm) | 9.30 | 4.62 ^a | 4.37 |
| Myelin thickness (µm) | 4.44 | 2.77 | 2.57 |
| G-ratio | 0.47 | 0.38 ^a | 0.38 |

^a indicates significant difference ($p < 0.05$) between FTMG and MMG

Figure 9.4.8 **Table of the morphological data for all repair groups.**

9.5 DISCUSSION

Nerves repaired with MMGs did not show significantly different levels of regeneration when compared with nerves repaired with FTMGs. Microwave heating is a suitable and possible alternative method for successfully denaturing muscle grafts and has a potential clinical use. However, the preparation of the MMG proved extremely difficult while the preparation of the freeze thawed muscle graft was considerably easier. It would appear that the FTMG would be much easier to produce than the microwave muscle graft.

A fast rate of regeneration is desirable as it ensures that the regenerating axons reach the target organs quickly so that the muscle does not atrophy as much. (Gutmann & Young, 1944). However, it is more important to have a graft that supports regeneration which allows a maximum number of axons to reach and connect to the correct target. This will result in the best level of recovery of function (Gattuso *et al.*, 1989)

Whitworth *et al* suggested that the MMG produced a 'faster and higher volume of Schwann cells penetration' from the proximal stump when compared to the FTMG (Whitworth *et al.*, 1995). An explanation for this observation was not given. A possible reason why the MMG produced faster Schwann cell penetration is that when the muscle was heated killing the myocytes and associated cells, it also denatured these cells into smaller pieces than are seen after preparing a graft by freezing. This

would allow quicker removal of the cellular debris by the macrophages and may result in faster Schwann cell penetration.

The fibre diameter, G-ratio and absolute refractory periods were all significantly increased ($p < 0.05$) for the FTMG group when compared with the MMG group. These results were unexpected and imply that nerves repaired with a FTMG have improved levels of myelination and can transmit a higher frequency of impulses when compared to nerves repaired with a MMG.

Jitter provides information on the variability of synaptic transmission at the neuromuscular junction. It has been shown that jitter is increased in the early stages of regeneration and then declines with time as regeneration continues (Lenihan *et al.*, 1997). Jitter has been shown to increase in degenerative neuromuscular diseases (Stålberg & Trontelj, 1994) and after nerve repair (Lenihan *et al.*, 1997). This increase in jitter is due to incomplete myelination of the terminal motor axons and / or an elevated end plate threshold potential (Wiechers, 1990). There was no significant difference between the repair groups indicating that the terminal motor axons and the end plate threshold had matured to similar levels.

The M-wave amplitude and area (Kimura, 1989), muscle twitch amplitude (Hartree & Hill, 1921), and TTI (Hems & Glasby, 1992) provide data on the approximate number of muscle fibres contracting during a muscle twitch. The lack of any significant difference ($p > 0.05$) between the FTMG and MMG for these four methods of assessment indicates that there were similar numbers of muscle fibres

contracting after supramaximal stimulation of the peroneal nerve. There was an approximate (40%) reduction in the M-wave amplitude and area, muscle twitch amplitude and TTI for the repair groups when compared with normal. These data provided an estimation to the number of muscle fibres that had been re-innervated after repair.

The CV_{max} was significantly different for both repair groups when compared with normal. The change in the CV_{max} can be explained by the shift observed in the nerve fibre histogram (see figure 9.4.6) and has been described by others (Gutmann & Sanders, 1943; Cragg & Thomas, 1961). Figure 9.4.6 illustrates that the largest and fastest, conducting fibres in the repair groups never achieved normal values.

There has been no study to date relating the change in the CV_{min} to the nerve fibre histogram. The CV_{min} provides information on the smallest slowest conducting fibres within a nerve.

In the present experiment, the change in the CV_{min} was related to the nerve fibre histogram. The CV_{min} for the FTMG and MMG groups were significantly different ($p < 0.05$) from normal by 62.10% and 69.19% respectively. This reduction in the CV_{min} for the repair groups can be explained by the leftward shift of the smallest fibres in the nerve fibre histogram. However, this leftward shift was difficult to visualize from figure 9.4.6. Therefore, the 5% confidence interval of the histogram for each group was determined and the values were 0.46 μm (normal), 0.17 μm (FTMG) and 0.15 μm (MMG). The reduction in the smallest fibre diameters for the repaired

groups compares favourably with the percent reduction in the CV_{min} . The 5% confidence interval demonstrated a 63.04% (FTMG) and 67.39% (MMG) reduction from normal. It would be interesting to expand this assessment to compare the conduction velocity profile with the fibre diameter histogram.

Nerves repaired with the MMG had a similar level of regeneration when compared to nerves repaired with the FTMG. The results of this experiment support the conclusion that the MMG does support nerve regeneration over small gaps in rabbit peripheral nerves. However, the preparation of a MMG was considerably more difficult than the FTMG. It was concluded that the MMG might be useful in experimental studies but provides no advantage over freezing as a method of preparing a denatured muscle graft. The MMG therefore cannot be recommended for use in the clinical setting.

9.6 CONCLUSION

Nerves repaired with the MMG did not show significantly different levels of regeneration when compared with nerves repaired with the FTMG. The results of this experiment support the conclusion that under ideal conditions “microwave heating is suitable as an alternative denaturing method for successful muscle grafts and has a potential clinical use” (Whitworth *et al.*, 1995). However, the preparation of a MMG that supports nerve regeneration was much more difficult than preparation of a FTMG. It is important to note that the FTMG does not function well for larger nerve gaps (greater than 5 cm) and at present, there is no evidence that the MMG will support regeneration over distances greater than 1 cm. It was concluded that the MMG provided no practical advantage over freezing to prepare denatured muscle grafts and therefore, can not be recommended for use in the clinical setting.

CHAPTER 10.0

A COMPARISON OF THE BIODEGRADABLE CONTROLLED RELEASE GLASS TUBE AND THE FREEZE THAWED MUSCLE GRAFT.

| <u>Section</u> | <u>Title</u> |
|----------------|---------------------|
| 10.1 | <u>OBJECTIVE</u> |
| 10.2 | <u>INTRODUCTION</u> |
| 10.3 | <u>METHODS</u> |
| 10.4 | <u>RESULTS</u> |
| 10.5 | <u>DISCUSSION</u> |
| 10.6 | <u>CONCLUSION</u> |

10.1 **OBJECTIVE**

The purpose of the experiments presented in this chapter was to compare recovery of function and nerve morphology in the freeze thawed muscle graft and the controlled release glass tube filled with freeze thawed muscle for the repair of a 1 cm nerve gap in the rabbit peroneal nerve. These two repair groups were then compared with the empty CRG (CRG-GAP) and normal groups. This chapter is also concerned with the ease of application of using a CRG tube in clinical practice.

10.2 **INTRODUCTION**

Repair by entubulation of a peripheral nerve has been studied using many biodegradable and non-biodegradable materials. Some of the materials that have been considered are acrylic copolymers, silicone, polylactates, mesothelial tubes and collagen. There is a growing amount of evidence that repair by entubulation of a peripheral nerve may provide similar levels of recovery of function levels when compared with nerve grafts. The use of entubulation is especially pertinent for clinical situations where direct end to end suture is impossible and where the correct geometric alignment of the fascicles cannot be determined.

Successful clinical application of a tube composed of synthetic materials for peripheral nerve repair must meet the following requirements. 1) the tube must be biodegradable and be completely reabsorbed by normal metabolic pathways. 2) the tube must not provoke an inflammatory reaction. 3) the tube must be nontoxic, nonantigenic and non-carcinogenic. 4) the internal lumen of the tube must be large enough to accommodate the initial swelling of the nerve while allowing the axons to migrate without obstruction. 5) the tube must be completely reabsorbed once the migrating neurites have established appropriate connection in the periphery. 6) the tube must maintain its structural integrity for enough time to allow the slowest neurites to migrate into the distal stump. 7) the nerve should be secured to the tube away from the transected ends to avoid further trauma. 8) the tube should be made of

substances which are amenable to custom fabrication so that the diameter of the lumen, rate of degradation, and permeability can be controlled.

The tubes used in this thesis were made of sodium phosphate polymers annealed to form a biodegradable glass tube (named controlled release glass or CRG). Because the tube was made of inorganic polymers, the solubility rate and size of the tube were easily controlled in the manufacturing stage of production.

The CRG tube has several theoretical advantages over other methods of entubulation used for nerve repair. The CRG tube is removed by dissolving the glass into inorganic ions that do not require a specific metabolic pathway for their removal. Another advantage of the CRG tube is that the solid structure of the tube provides protection from pressure at the site of repair.

The purpose of the experiments presented in this chapter was to compare the CRG tube filled with freeze-thawed muscle (CRG-M) and with a conventional freeze thawed muscle graft (FTMG). These two groups were also compared with controls where a CRG tube was used to enclose the cut ends of a nerve separated by a 1 cm gap.

10.3 **METHODS**

Twenty New Zealand White rabbits were separated into 4 equal sized groups. These groups consisted of one normal and three repair groups. In the repair groups the peroneal nerve was divided and a segment of nerve was removed from the distal end to create a 1 cm gap. This gap was then repaired with either: a 1 cm freeze thawed muscle graft (FTMG), a CRG tube filled 1 cm of randomly arranged freeze thawed skeletal muscle (CRG-M), or a CRG tube with a 1 cm gap between the cut ends of nerve (CRG-GAP).

Anaesthesia and surgical procedures were described in Chapters 4 and 5.

In the CRG-GAP group the proximal and distal ends of the peroneal nerve were placed in opposite ends of the CRG tube. Care was used to ensure that trauma was kept to a minimum and that the nerve ends were placed in the tube without rotation to maintain the correct geometric alignment. The nerve was secured to the tube by placing two interrupted 10/0 sutures through the holes in the tube and in the epineurium of the peroneal nerve 5 mm away from the cut nerve ends.

In the CRG-M group a 2 cm × 2 cm × 1 cm block of parallel muscle fibres was removed from the coccygeofemoris muscle. This block of muscle was wrapped in aluminium foil and submerged in liquid nitrogen. Once the block of muscle was completely frozen it was thawed in distilled water and minced into 5 or 6 tiny pieces. These pieces were stuffed into the CRG tube to fill a distance of 1 cm. Keeping the

muscle fibres in a parallel arrangement was originally attempted. However, owing to the small size of both the CRG tube and the pieces of muscle this proved impossible. Therefore, the freeze thawed muscle was inserted in a random arrangement. The ends of the cut nerve were placed in the tube flush with the freeze thawed muscle inside the tube. The nerves were placed in the tube to maintain the correct geometric alignment and then the nerve was secured to the tube as above.

In the FTMG group a similar sized block of muscle was removed and prepared as in the CRG-M group. However, after the muscle block was thawed it was cut to form a 1 cm × 1 cm × 1 cm graft and sutured in the peroneal nerve gap with 3 to 4 interrupted 10/0 sutures at each end.

Assessment was carried out 6 months after repair by electrophysiological and morphological methods. The sciatic nerve was exposed and the peroneal and tibial divisions separated at the sciatic notch. The tibial nerve was divided at both the sciatic notch and at the knee to prevent any cross-electrical interference during stimulation.

Variability or jitter in the neuromuscular junction was measured first. Two stimulating monopolar needle electrodes were inserted in the EDL muscle at the motor point. These stimulating electrodes were used to provide supramaximal stimulation to the terminal intramuscular axon branches of the peroneal nerve. The resulting motor unit action potential was recorded and the inter-potential interval (IPI) was determined for 50 consecutive discharges. The mean consecutive

difference (MCD) of these 50 IPIs was then calculated {Lenihan et al 1997; Stalberg et al 1994}. The MCD of at least 20 different neuromuscular junctions within the EDL was determined. The average of all the MCD's was the jitter value for that animal.

A non-traumatic platinum wire stimulating electrode (Harvard Electronics, USA) was placed on the left peroneal nerve proximal to the graft site (S1). A second non-traumatic stimulating electrode was placed distal to the graft (S2). To improve accuracy care was taken to ensure that the distance between the electrodes was greater than 3.5 cm (See chapter 6.2). A supramaximal current of intensity 30% above maximal stimulation with a duration of 50 μ s was used for all stimulations in this experiment.

The peroneal nerve was stimulated at the S2 electrode and the resulting M-wave was recorded from the EDL. The area, amplitude and latency from stimulation to the initial deflection of the M-wave were determined. The peroneal nerve was then stimulated at S1 and the latency from stimulation to contraction was determined. The maximum conduction velocity was calculated using equation in figure 6.2.1.1. The minimum conduction velocity was determined using a collision technique (see chapter 6.3) (Hopf, 1963).

After calculating the velocities the tendon of the EDL was cut and connected to a Harvard isometric tension transducer (Harvard Electronics, USA) which was connected to a digital oscilloscope (Gould, UK) The peroneal nerve was stimulated

with a supramaximal current at S1 as described above. The peak tension and the time tension index (TTI) of the EDL twitch were determined as described in chapter 7.

Once all physiological measurements were completed, a 2 cm segment of the peroneal nerve was removed distal to the graft. The nerve specimens were prepared in semithin (1- μ m) sections for light microscopy (Glasby et al 1995). Morphometric analysis of axon and fibre diameter, myelin thickness and G-ratio was carried out on 200 random nerve fibres (Mayhew 1990) using a VIDS III image analysis system (Analytical Measuring Systems Limited, Saffron Walden, UK). "STATISTICA for Windows" was used to perform the statistical assessment for each test between groups. The electrophysiological data were found to be normally distributed and so the means were compared using Student's t-test. The morphological data were found not to be normally distributed therefore, the medians were tested using a Kolmogorov-Smirnoff test.

10.4 **RESULTS**

At the time of assessment, the animals in all three repair groups demonstrated some degree of stiffness in the knee joint and difficulty in hopping. There was no evidence of the CRG tube or infection in any animal. The peroneal nerve distal to the graft in the CRG-M and the FTMG groups was a full thickness 'fatty' nerve while the CRG-GAP did not have the same appearance or girth.

The jitter values for the normal, FTMG, CRG-M and CRG-GAP groups were 13.48 μ s, 20.68 μ s , 19.83 μ s , and 18.48 μ s respectively. There was no significant difference ($p>0.05$) in the jitter value between the repair groups however, they were all statistically different when compared with normal. Figure 10.4.1 is a histogram of the jitter for each group.

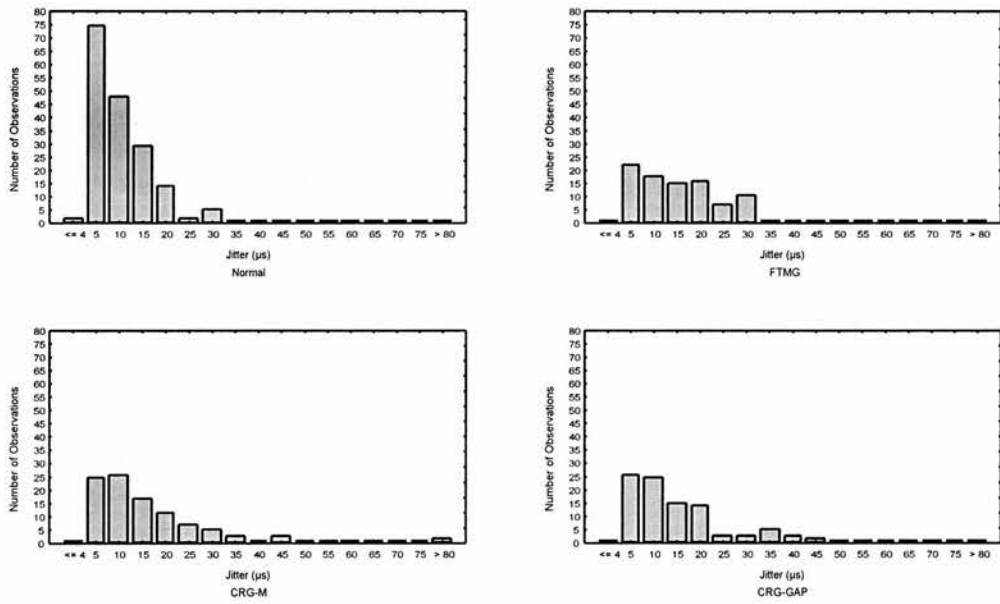


Figure 10.4.1 Histogram of jitter for each repair group

The mean maximum conduction velocity for normal, FTMG, CRG-M and CRG-GAP were 69.36 m s^{-1} , 40.23 m s^{-1} , 47.74 m s^{-1} and 44.79 m s^{-1} respectively. The minimum conduction velocity was reduced from normal by 62.10% (FTMG), 42.15% (CRG-M) and 61.34% (CRG-GAP). Both the maximum and minimum conduction velocities were significantly different from normal ($p < 0.05$). Figure 10.4.2 is a box and whisker plot of the mean, standard deviation, and standard error of the maximum and minimum conduction velocities.

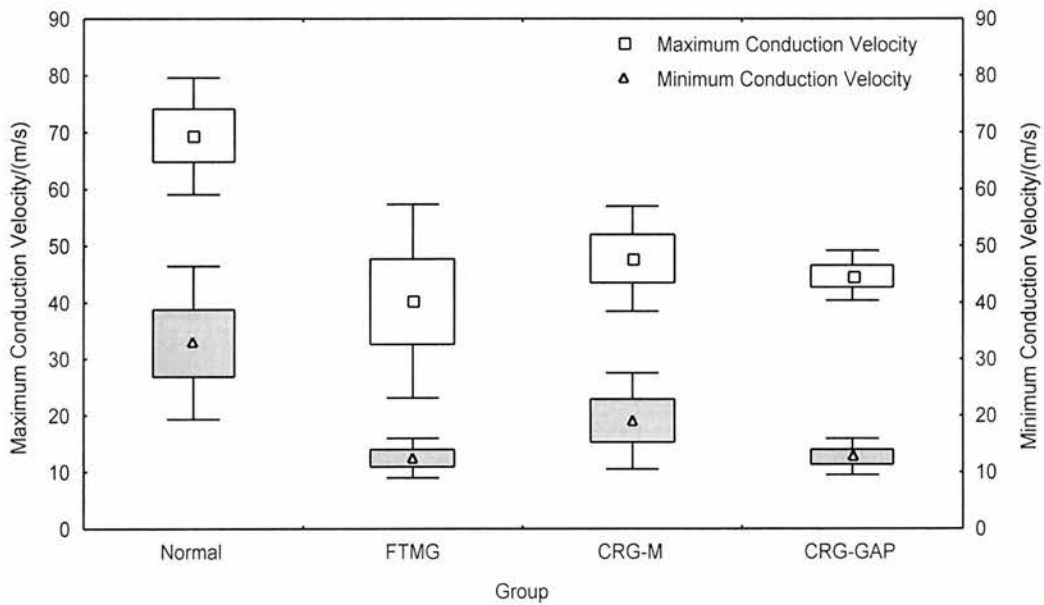


Figure 10.4.2 Box and whisker plot of the mean, standard deviation and standard error of the velocities for each group.

The difference between the maximum conduction velocity and the minimum conduction velocity is defined as the range of conduction velocities. The conduction velocity range for the normal, FTMG, CRG-M and CRG-GAP groups was 36.48 m s^{-1} , 27.77 m s^{-1} , 28.72 m s^{-1} , 32.08 m s^{-1} respectively. There was no statistical

difference ($p>0.05$) in the range of velocities between any of the groups. This indicates that the shift between the fastest and slowest fibres was uniform. Figure 10.4.3 is a box and whisker plot of the mean, standard deviation and standard error for each group with respect to the range of velocities.

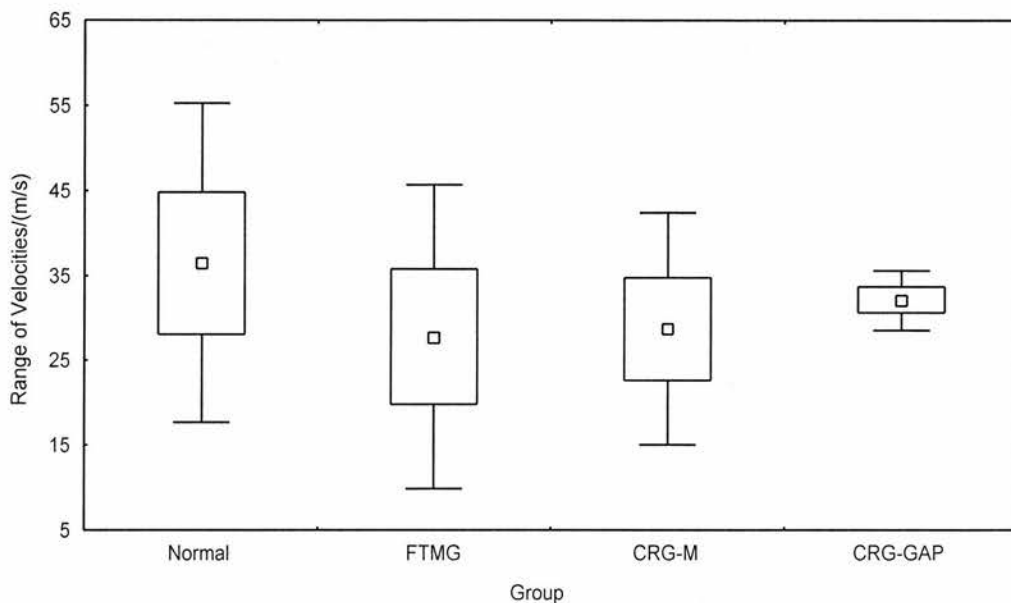


Figure 10.4.3 Box and whisker plot of the conduction velocity range for all groups.

The mean amplitude for the normal, FTMG, CRG-M and CRG-GAP groups were 18.92 mV, 8.04 mV, 7.50 mV, and 7.76 mV respectively. The mean areas for the normal, FTMG, CRG-M and CRG-GAP groups were 32.40 mVs, 12.76 mVs, 14.55 mVs, and 15.47 mVs. All repair groups demonstrated a significant difference ($p<0.05$) from normal for the M-wave area and amplitude while there was no significant difference between the repair groups ($p>0.05$). Figure 10.4.4 is a box and

whisker plot of the mean, standard error and standard deviation of the M-wave amplitude and area for the all groups.

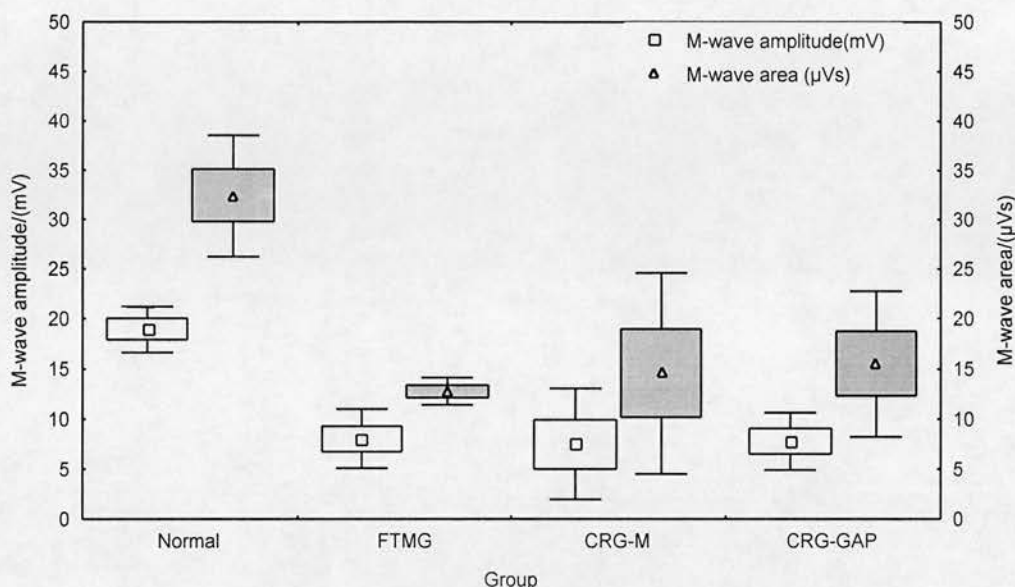


Figure 10.4.4 Box and whisker plot of the M-wave area and M-wave amplitude for all groups.

The mean values of the muscle twitch amplitude and TTI for the CRG-GAP group were significantly ($p < 0.05$) different when compared with normal and with the other two repair groups. The FTMG was significantly different ($p < 0.05$) from normal for both the peak twitch tension and TTI. However, there was no significant difference ($p > 0.05$) when the CRG-M was compared to the normal and the FTMG groups. The muscle twitch amplitude and TTI are indirectly proportional to the number of muscle fibres contracting (Glasby *et al.*, 1992). The results of the muscle twitch amplitude and TTI indicate that the repair groups did not have similar numbers of fibres contracting after supramaximal stimulation of the repaired nerve. The results further showed that the CRG-GAP group did not have as many muscle

fibres re-innervated compared to the other repair groups. Figure 10.4.5 is a box and whisker plot of the peak twitch tension and TTI for all groups.

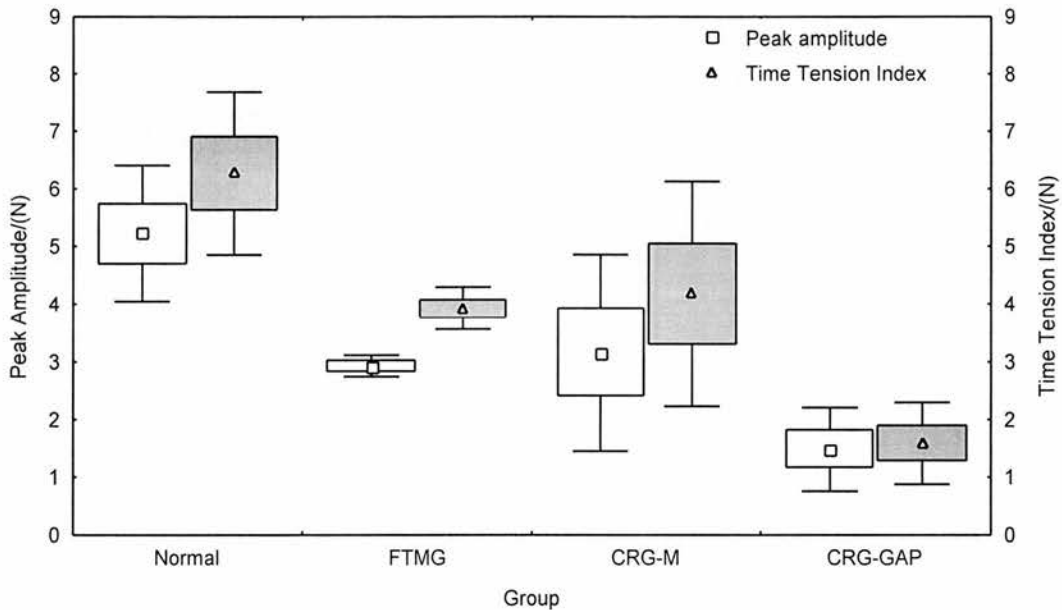


Figure 10.4.5 Box and whisker plot of the peak twitch tension and TTI for all groups.

The absolute refractory period for the normal, FTMG, CRG-M and CRG-GAP groups were 0.84 ms, 1.11 ms, 1.37 ms, and 2.03 ms. There was no statistical difference in the absolute refractory period ($p > 0.05$) when the FTMG and CRG-M were compared. The value of the absolute refractory period of the CRG-GAP group was not significantly different ($p > 0.05$) when compared to the CRG-M group. However, the absolute refractory period of the CRG-GAP group was significantly different ($p < 0.05$) when compared to the FTMG group. All repair groups were significantly different ($p < 0.05$) when compared with normal.

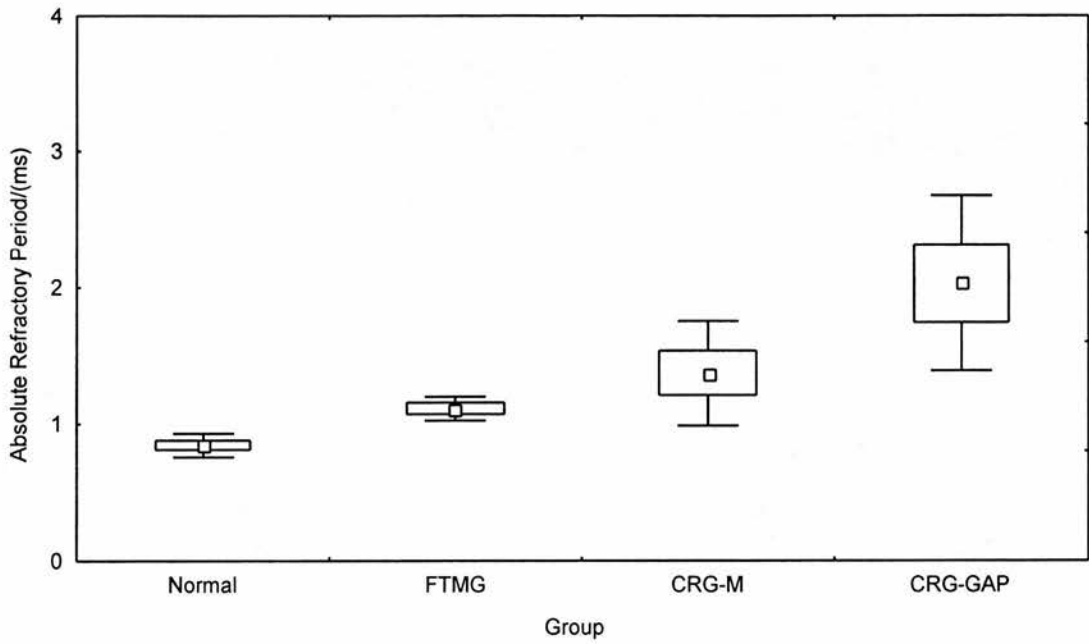


Figure 10.4.6 Box and whisker plot of the absolute refractory period for all groups.

| <u>Test</u> | <u>Normal</u> | <u>FTMG</u> | <u>CRG-M</u> | <u>CRG</u> |
|--|---------------|-------------|--------------|------------|
| Jitter (μs) | 13.48 | 20.68 | 19.83 | 18.48 |
| M-wave amplitude (mV) | 18.92 | 8.04 | 7.50 | 7.76 |
| M-wave area (mVs) | 32.40 | 12.76 | 14.55 | 15.47 |
| Maximal conduction velocity (m s^{-1}) | 69.36 | 40.23 | 47.74 | 44.79 |
| Minimal conduction velocity (m s^{-1}) | 32.88 | 12.46 | 19.02 | 12.71 |
| Range of conduction velocity (m s^{-1}) | 36.48 | 27.77 | 28.72 | 32.08 |
| Peak twitch tension (N) | 5.22 | 2.93 | 3.15 | 1.48 |
| Time tension index (N) | 6.27 | 3.93 | 4.18 | 1.59 |
| Absolute refractory period (ms) | 0.84 | 1.11 | 1.37 | 2.03 |

Figure 10.4.7 Table of the mean data for all electrophysiological tests.

The repair groups were significantly different ($p < 0.05$) from normal for all nerve morphology tests. The CRG-GAP group was significantly different ($p < 0.05$) for all nerve morphology tests when compared to the other repair groups. However, when the FTMG and CRG-M groups were compared there was no significant difference ($p > 0.05$) in the nerve morphology. This result implies that the level of regeneration in the FTMG and CRG-M groups was similar. This was further supported by the electrophysiological results. The results of the nerve morphology assessment showed that the repair groups did not achieve the same level of fibre

maturation or myelination when compared to normal. This was supported by the jitter findings. Figure 10.4.8 is a table of the mean data for each group with respect to the nerve morphology. Figures 10.4.9 – 10.4.12 are histograms of the axon and fibre diameters, myelin thickness and G-ratio. Figure 10.4.13 is a box and whisker plot of the G-ratio mean, standard deviation and standard error for each group.

| <u>Test</u> | <u>Normal</u> | <u>FTMG</u> | <u>CRG-M</u> | <u>CRG-GAP</u> |
|------------------------------------|---------------|-------------|--------------|----------------|
| Axon diameter (μm) | 4.53 | 1.87 | 2.09 | 1.12 |
| Fibre diameter (μm) | 9.33 | 4.62 | 4.94 | 3.23 |
| Myelin Thickness (μm) | 4.44 | 2.75 | 2.85 | 2.10 |
| G-ratio | 0.47 | 0.38 | 0.39 | 0.32 |

Figure 10.4.8 **Table of the nerve morphology tests.**

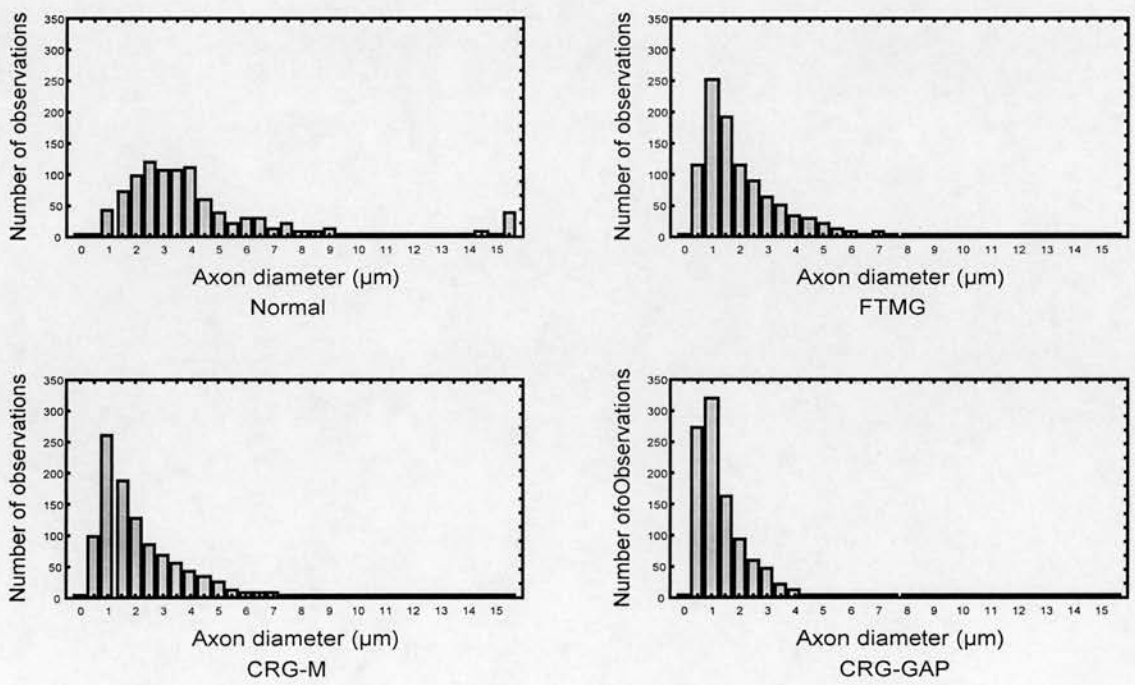


Figure 10.4.9 Histogram of the axon diameter for all groups

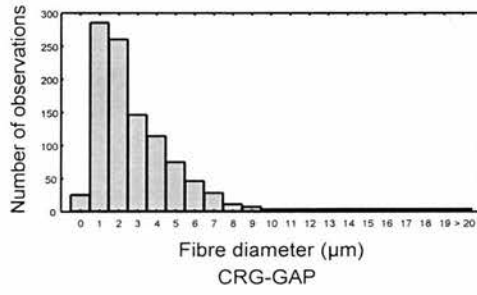
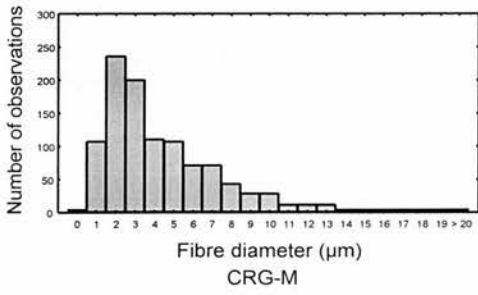
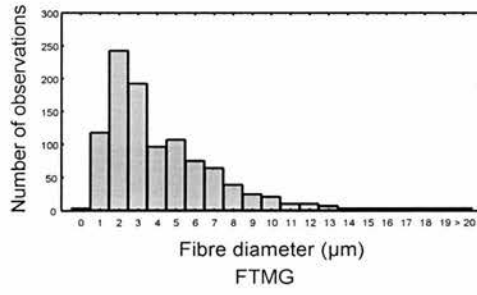
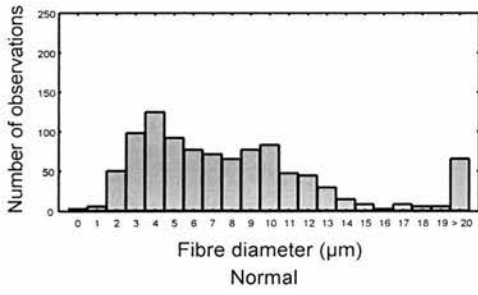


Figure 10.4.10 Histogram of fibre diameter for all groups

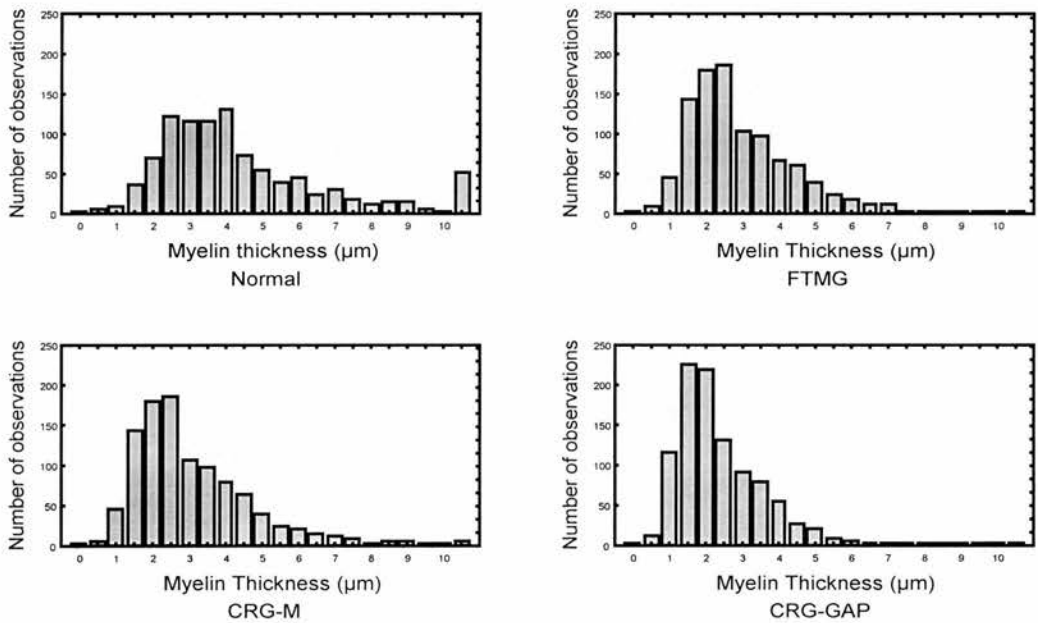


Figure 10.4.11 Histogram of myelin thickness for all groups

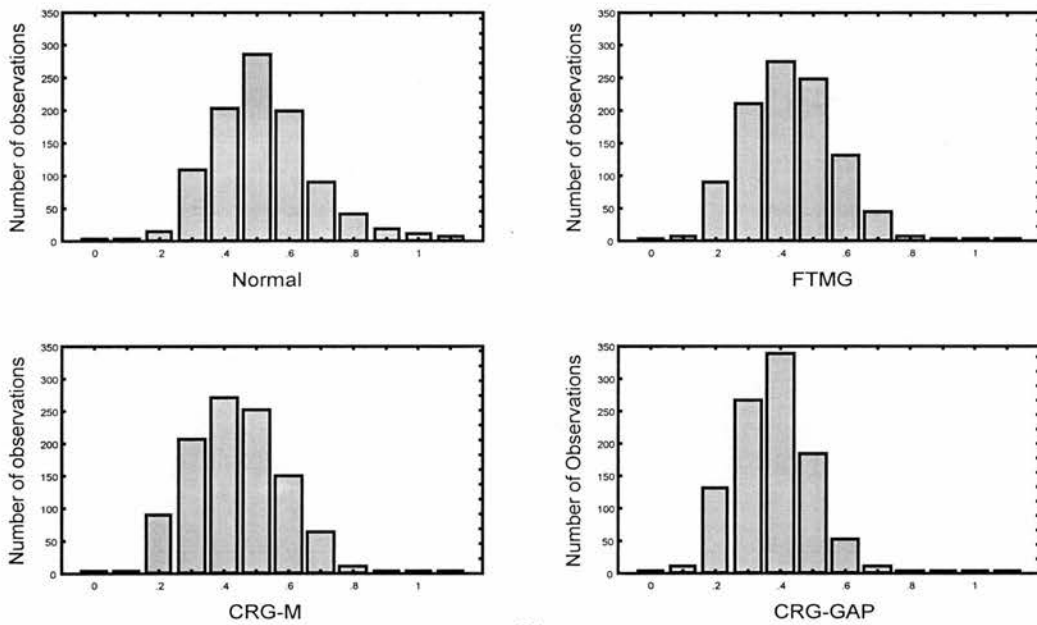


Figure 10.4.12 Histogram of the G-ratio for each group

Figure 10.4.12 Histogram of the G-ratio for each group

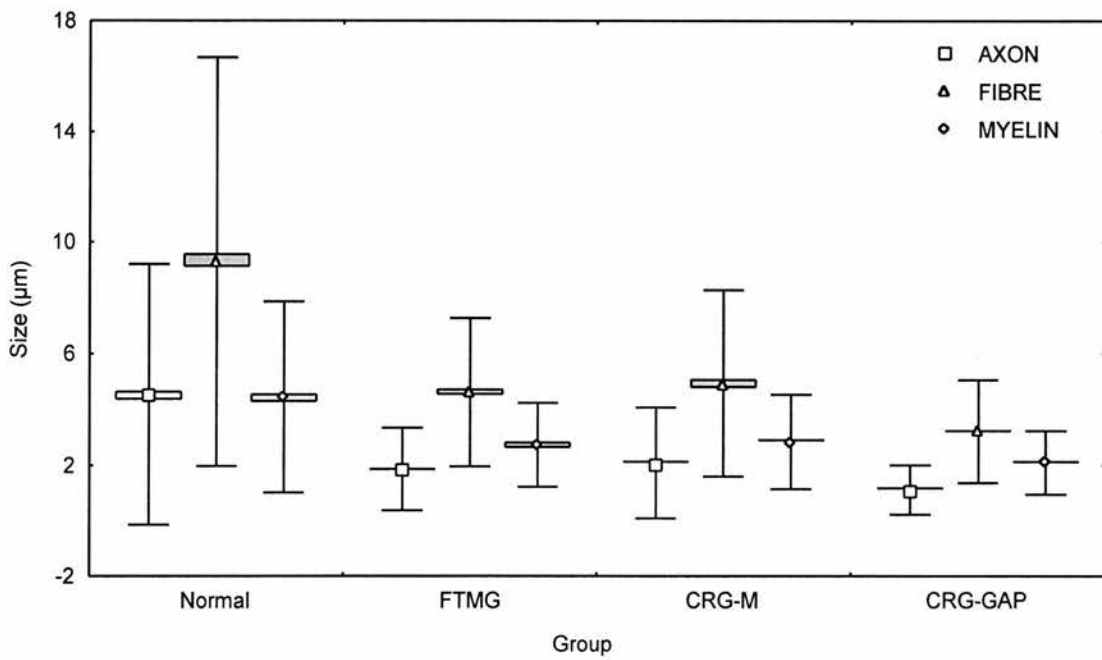


Figure 10.4.13 Box and whisker plot of the axon and fibre diameter and the myelin thickness for all groups.

10.5 DISCUSSION

The results of this study indicate that entubulation with a CRG tube filled with freeze thawed muscle was at least as good as the FTMG for the repair of a 1 cm nerve gap in the rabbit peroneal nerve. In all three repair groups the recovery of function and nerve morphology were inferior to normal but consistent with previous studies of nerve repair.(Glasby *et al.*, 1992; Lundborg *et al.*, 1997).

It was found that repair of a peripheral nerve by entubulation was easy to perform. The CRG tube when compared with the FTMG was not any different in the level of difficulty. Securing the nerve to the CRG tube required the same number of sutures as the FTMG group and therefore took approximately the same amount of time. It became obvious early in the CRG-M group that the amount of muscle removed was excessive. Approximately 10% of the removed muscle was used to fill the tube. Therefore, one further advantage the CRG-M group provided is that not as much muscle tissue was required.

The CRG-GAP group had much poorer levels of regeneration when compared with the other repair groups. However, there was evidence of some regeneration in all five animals in the CRG-GAP group.

There was no significant difference when the FTMG and CRG-M groups were compared with each other using any test. It has been shown that different grafts or altered orientation of a muscle graft may change the rate of regeneration of the neurites without affecting the quality of recovery of function. When comparing two

groups after nerve repair it is important to be certain that the stage of regeneration is similar otherwise the comparisons are meaningless. Because it was impossible to place the freeze thawed muscle in the tube with the muscle fibres in parallel it was imperative that when the groups were compared the stage of regeneration was similar.

Jitter provides a method of assessing the progression of regeneration by measuring the variability of neuromuscular transmission at the motor end plate (Stålberg & Trontelj, 1994). In all three repair groups, there was no significant difference ($p < 0.05$) for the jitter value indicating that all groups had reached similar stages of regeneration. This was further supported by the nerve morphology results for the CRG-M and FTMG groups. The results for the nerve morphology were consistent with previous work (Gschmeissner *et al.*, 1990). The lack of any significant difference for any nerve morphology test when the CRG-M and FTMG were compared with each other indicates that these they had similar levels of regeneration.

The mean conduction velocity of the normal group was greater than all the repair groups. The conduction velocities were significantly different ($p < 0.05$) for all repair groups when compared with normal.

The tests for peak tension and TTI provide an indirect representation of the number of muscle fibres contracting during a muscle twitch. The CRG-M did not demonstrate a significant difference ($p > 0.05$) for peak twitch tension and TTI when

compared with the normal and FTMG groups. However, the FTMG was significantly different ($p < 0.05$) for both of these tests when compared with normal. These tests were able to differentiate the FTMG and CRG-M groups from the normal group.

This result was not supported by the M-wave area and amplitude. There was no significant difference ($p < 0.05$) among the repair groups for the M-wave area and amplitude. One possible explanation for the lack of correlation between the M-wave and muscle twitch results is the small group size.

10.6 **CONCLUSION**

The purpose of these experiments was to compare the freeze thawed muscle graft and the CRG tube filled with freeze thawed muscle for the repair of a 1 cm nerve gap in the rabbit peroneal nerve.

The results of this experiment indicated that entubulation with a CRG tube filled with freeze thawed muscle was at least as good as the FTMG for the repair of 1 cm nerve gap in the rabbit peroneal nerve.

These results further suggest that the denaturing process of the biodegradable glass does not change the rate or quality of nerve regeneration supporting the view that the CRG tube may be an alternative to nerve repair.

CHAPTER 11.0

ASSESSMENT OF THE BIODEGRADABLE CONTROLLED RELEASE GLASS TUBE USING CHOPPED NERVE AND MUSCLE-NERVE SANDWICH GRAFTS

| <u>SECTION</u> | <u>TITLE</u> |
|----------------|---------------------|
| 11.1 | <u>OBJECTIVE</u> |
| 11.2 | <u>INTRODUCTION</u> |
| 11.3 | <u>METHODS</u> |
| 11.4 | <u>RESULTS</u> |
| 11.5 | <u>DISCUSSION</u> |
| 11.6 | <u>CONCLUSION</u> |

11.1 **OBJECTIVE**

The purpose of this study was to compare the quality of regeneration when the lumen of a biodegradable glass tube (CRG) was filled with either nerve or a muscle–nerve sandwich graft with a FTMG for the repair of a 1 cm deficit in the rabbit peroneal nerve. The results were compared with normal.

The recovery of function was assessed 6 months after repair by electrophysiology and histology of the peroneal nerve.

11.2 INTRODUCTION

The most frequent result of a damaged peripheral nerve that has been cut and not repaired is a mass of disorganized nerve fibres (Sunderland, 1978; Dellon & MacKinnon, 1988). Repair of a transected peripheral nerve is best accomplished by direct end to end suture provided no tension exists at the suture site (Terzis *et al.*, 1975).

If the gap is too long to allow for direct end to end suture without tension, an autologous nerve graft is sutured between the stumps. Preparation of a nerve graft requires a second operative procedure that produces sensory loss, causes scar formation and occasionally leads to a neuroma at the donor site. Therefore, considerable interest has been given to non-neural alternatives that can be used in the repair of peripheral nerve injuries. Non-neural grafts have varied from freeze thawed muscle grafts (Glasby, 1990) to silicone tubes (Lundborg, 1988) to decalcified bone (Weiss, 1944) with varying degrees of success. One problem associated with the application of non-neural grafts is that they are limited by length. It has been shown that the recovery of function declines with increasing graft length (Hems & Glasby, 1992).

When the repair of the an injured nerve requires a short graft (less than 4 cm), the advancing neurite reaches the migrating Schwann cell from the distal segment in the graft and receives the proper support required for regeneration (Ide *et al.*, 1983). However, in the case of a large nerve deficit, the ability of the non-neural graft to

support regeneration is decreased because there is no Schwann cell—neurite interaction in the graft.

Several studies have demonstrated that Schwann cells placed in the centre of a graft improve regeneration compared to similar groups without Schwann cells. These studies state that inserting a peripheral nerve segment in the centre of a silicone tube improved regeneration when compared to an equal length graft made of an empty silicone tube (Bardosi, 1989; Kosaka, 1990; Ikeda *et al.*, 1991).

The most common method of inserting Schwann cells in a non-neural graft is to use of a ‘sandwich graft’. Whitworth *et al* (Whitworth *et al.*, 1995) wrote that a muscle—nerve ‘sandwich graft’ increased the gap distance of the repair of peripheral nerves when compared to a freeze thawed muscle graft of equal length. It has also been stated (Fields & Ellisman, 1986) that autologous nerve interposed with a silicone tube improved regeneration when compared to an equal length graft made of an empty silicone tube.

The most promising sandwich graft is composed of freeze thawed muscle and nerve. It provides both the neurotrophic factors (by way of the Schwann cells) and the structural support (by way of the basement lamina), required to support nerve regeneration. However, one disadvantage of this type of ‘sandwich graft’ is the increased amount of suture material introduced in the repair site.

Different types of entubulization methods for the repair of a cut peripheral nerve have also demonstrated an ability to support recovery of function after nerve

repair. It has been shown by several authors that filling the chamber with various substances can affect the quality and speed of regeneration (Radeva & Taxi, 1975; Rich *et al.*, 1989). Guénard *et al.* (Guenard *et al.*, 1992) demonstrated that increased concentration of Schwann cells in the lumen of a PVC tube improved neurite migration and maturation. Other substances, including fibronectin, laminin, and collagen (Madison *et al.*, 1985) (Millaruelo *et al.*, 1988) inserted in the lumen of tubes have also shown a positive effect on regeneration.

When compared to a FTMG of the same length, Lenihan *et al.* (1998) demonstrated that freeze thawed muscle grafts inserted in the centre of a controlled release glass (CRG) tube produced similar levels in the recovery of function after transection and subsequent repair of the rabbit peroneal nerve. If the lumen of a tube graft had Schwann cells introduced or inserted while also containing a supportive graft, regeneration of the repaired peripheral nerve should be enhanced. An entubulization technique with a muscle—nerve ‘sandwich graft’ inserted in the lumen of the tube would provide both the structure and support required for successful regeneration. This type of entubulized sandwich graft would also remove the potential disadvantage of increased suture material at the repair site. It has been shown that repair of peripheral nerves using a CRG tube as the graft does not produce an immunological reaction in the host and is eventually completely biodegraded (Gilchrist T. *et al.*, 1998).

The purpose of the experiments presented in this chapter was to provide preliminary data on the feasibility of using the CRG tube for the repair of a 1 cm nerve deficit in the rabbit peroneal nerve. The CRG tube filled with a sandwich graft and the CRG tube filled with nerve were compared with a FTMG to assess the recovery of function six months after repair.

11.3 Methods

Twenty New Zealand white rabbits (weight 3.0 - 3.5 kgs) were separated into 4 equal sized groups. These groups consisted of normal and groups in which the left peroneal nerve (P.N.) had been divided and repaired with either: A) a CRG tube filled with a muscle-nerve sandwich graft (CRG-MN), B) a CRG tube filled with chopped nerve (CRG-N), or C) a freeze thawed muscle graft (FTMG).

Each animal was anaesthetized as described in chapter 4 and the sciatic nerve exposed as described in chapter 5. The details provided below are a brief description on the methods of this experiment.

The animal was then placed on an electrically grounded heating blanket to help prevent heat loss during the procedure. Both the rectal temperature and oxygen saturation levels were monitored continuously.

The sciatic nerve was mobilised 3 cm distal to the sciatic notch for a further distance of 3 cm distal. The peroneal and tibial nerves were separated using microsurgical forceps. During repair of the nerves vision was assisted with a continuous variable magnification operating microscope. The P.N. was divided 4 cm below the sciatic notch. A 1 cm deficit in the peroneal nerve was created by removing the appropriate amount of nerve from the distal segment. The P.N. deficit was repaired as described in chapter 5. Care was taken to ensure that tension and trauma to the P.N. were kept to a minimum.

After nerve repair, the biceps femoris and coccygeofemoralis muscles were sutured with 4-6 interrupted 4/0 Vicryl (Ethicon UK Ltd, Edinburgh) to close the opening exposing the sciatic nerve. The skin was then closed with 4/0 Vicryl continuous subcuticular stitch.

The controlled release glass tube (CRG) (Giltech, Ayr, UK) was manufactured as described in chapter 3.

The CRG-MN was prepared as described in chapter 5.2.3 and the CRG-N was prepared as described in chapter 5.2.2. Because the amount of nerve harvested from the distal segment of the P.N. was not sufficient to fill the tube and produce a full sized graft, the nerve was left floating in the lumen with a small gap between the nerve ends and the chopped nerve graft. The proximal and distal nerve ends were then secured to the tube. The FTMG was prepared as described in chapter 5.2.4

Six months after the initial surgery, the animals were assessed by electrophysiology and nerve morphological methods to determine the quality of regeneration through the graft. The animals were prepared, anaesthetized and monitored as described in chapters 4 and 5. A Medelec Sapphire EMG machine (Medelec, Old Woking, UK) was used for all electrophysiologic recordings. A ground electrode was placed on the skin over the biceps femoris muscle.

Electrode placement and the methods for each test are provided here in brief with a more detailed explanation of each test given in the associated chapter for that test. Jitter of the extensor digitorum longus muscle (EDL) was measured first (see

chapter 6.5). Two non polar stimulating electrodes were inserted in the motor point of the EDL. The intra-muscular terminal branches were stimulated with a supra-maximal stimulus and the inter-potential interval was calculated for 50 continuous discharges. The mean consecutive difference was then determined from the 50 discharges. This process was repeated for a minimum of 20 different intra-muscular terminal branches for each animal.

Two 6 mm silver / silver chloride disc electrode (Medelec, Old Woking, UK) were used to record the compound muscle action potential or M-wave after P.N. stimulation. One electrode was placed on the skin over the biceps femoris muscle and used as the cathode. The second electrode was placed on the skin over the tendon of the EDL and used as the anode.

The maximum (CV_{max}) and minimum (CV_{min}) conduction velocity were calculated {see chapter 6}. The area and amplitude of the M-wave were determined from the trace recorded from stimulation of the distal segment of the P.N. when calculating the CV_{max} (see chapter 6.2).

In the next test, two stimuli, S1 and S2, were applied to the peroneal nerve distal to the graft site at the same point with a time delay between the two stimuli of 0.01 ms. The time delay between the S1 stimulus to the S2 stimulus was increased in 0.1 ms increments until a second M-wave was visible. This time delay between the S1 and S2 until a second M-wave was visible was denoted as the absolute refractory period (see chapter 6.4).

A 1 cm segment of peroneal nerve distal to the graft site was removed for histology once all physiological test were completed. The nerve specimens were prepared with the techniques and assessed as described in chapter 8.

All data were compared to determine if groups were similar using “STATISTICA for Windows” (StatSoft Inc., Tulsa, OK, USA). Because the electrophysiological data were normally distributed, it was appropriate to use Student’s t-test to compare groups. The morphological data did not however, fit a normal distribution. Therefore, a non-parametric two-tailed Kolmogorov-Smirnoff test was used to determine if groups were similar. All statistical analysis was assessed as described in the respective chapters.

11.4 RESULTS

The results of this experiment indicate that there was no significant difference in the recovery of function or nerve morphology between the CRG-MN and FTMG groups. However, the data does demonstrate that the CRG-N group did not achieve similar levels of regeneration when compared to the FTMG. All groups were significantly worse than normal.

There was no evidence of rejection of the tube or graft in any animal at the time of assessment. The appearance of the distal segment of the peroneal nerve had a more pale colour, had less tension and was more cylindrical in shape when compared with normal nerve.

The value for jitter in the EDL muscle for the groups in this experiment was 17.94 μ s (CRG-MN), 25.54 μ s (CRG-N), 20.68 μ s (FTMG) and 13.48 μ s (Normal). All groups were significantly different ($P < 0.05$) from normal while the CRG-N group was also significantly different ($P < 0.05$) when compared to the other two repair groups. Figure 11.4.1 is a box and whisker plot of jitter for each group.

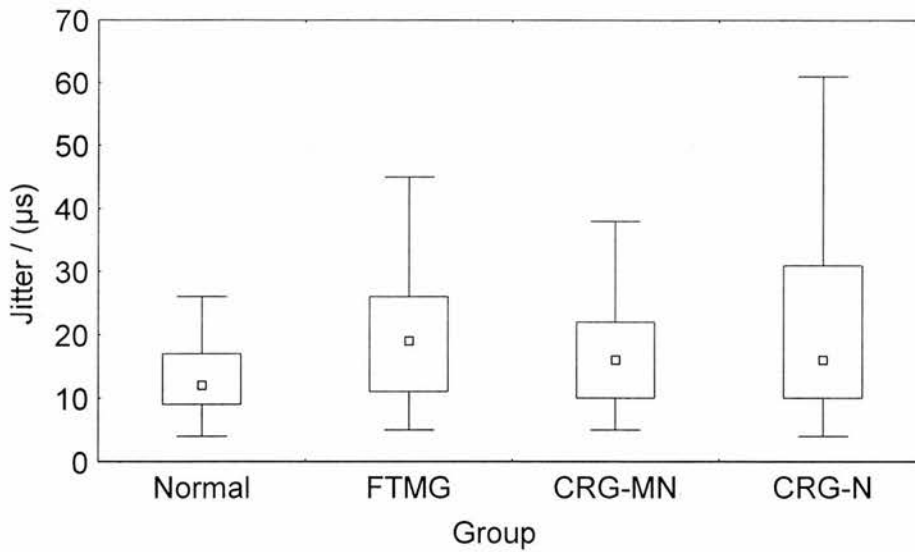


Figure 11.4.1 A box and whisker plot of jitter for all four groups.

The most common physiological measurement to assess the success or failure in the quality of function after peripheral nerve repair is the motor conduction velocity. The CV_{max} measures the speed at which the fastest impulses can travel between two points along the course of a nerve. The mean CV_{max} for the normal, CRG-MN, CRG-N and FTMG groups was 69.36 m s^{-1} , 41.44 m s^{-1} , 44.33 m s^{-1} , and 40.23 m s^{-1} . All repair groups demonstrated a significant difference from normal ($p < 0.05$) with respect to the CV_{max} while there was no significant difference ($P > 0.05$) between any of the repair groups.

The CV_{min} provides an assessment on the slowest conducting myelinated motor fibres within a nerve. The CV_{min} of the P.N. for the normal, CRG-MN, CRG-

N and FTMG groups was 32.88 m s^{-1} , 19.57 m s^{-1} , 20.31 m s^{-1} and 12.46 m s^{-1} respectively. All repair groups were significantly different ($p < 0.05$) from normal while the value of CV_{min} for the CRG-MN and CRG-N groups was significantly different ($p < 0.05$) when compared to the FTMG. Figure 11.4.2 is a box and whisker plot of the means and standard errors of each group with respect to CV_{max} and CV_{min} .

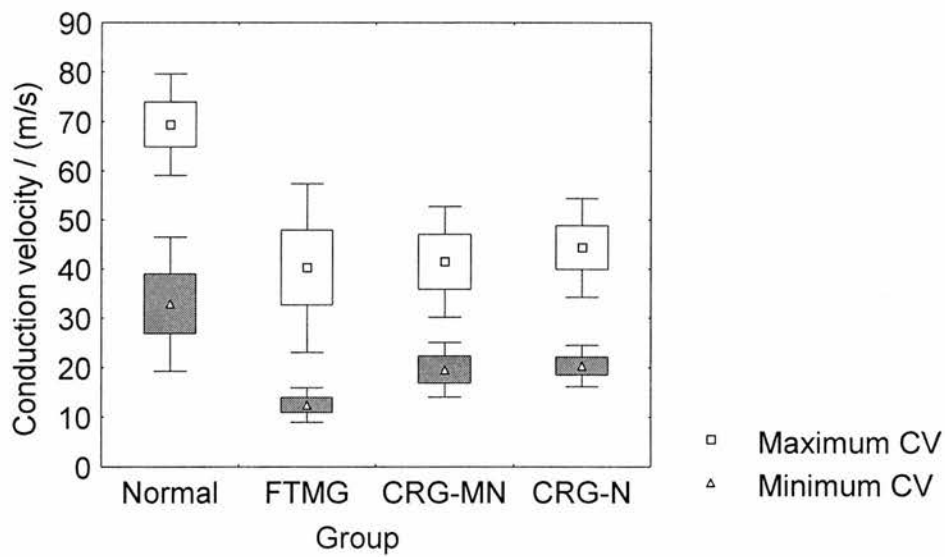


Figure 11.4.2 A box and whisker plot of the means and standard errors of each group with respect to CV_{max} and CV_{min} .

Assessment of the area and amplitude of a M-wave demonstrated a significant reduction ($p < 0.05$) for all repair groups when compared to normal. The amplitude of the M-wave for the CRG-MN, CRG-N, and FTMG was reduced from normal by 61.58%, 80.71% and 57.51% respective. The area of the M-wave also demonstrated a comparable reduction from normal: 72.31% (CRG-MN), 81.48% (CRG-N) and 60.62% (FTMG). The CRG-N showed a significant difference ($p < 0.05$) when compared with the FTMG group for both the M-wave area and amplitude. Figure 11.4.3 is a box and whisker plot of the means and standard errors for each group with respect to the M-wave variables.

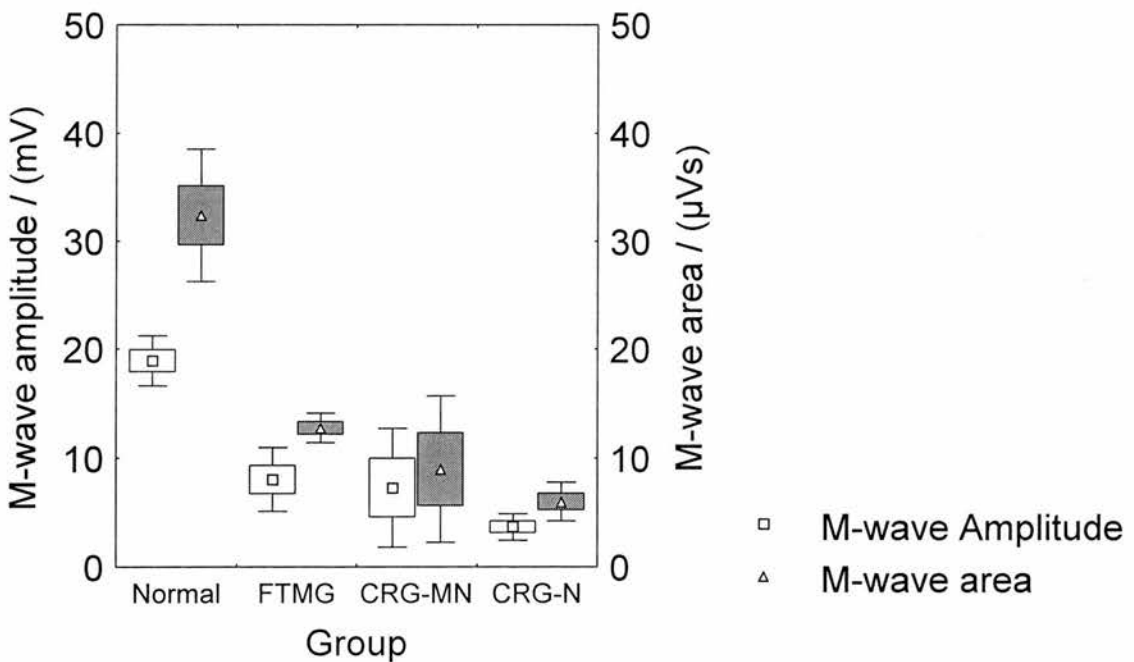


Figure 11.4.3 A box and whisker plot of the means and standard errors for each group with respect to the M-wave variables

The CRG-MN group did not demonstrate a significant difference for the absolute refractory period when compared to the FTMG group ($p>0.05$). However, the CRG-N was significantly different ($p<0.05$) from the FTMG group for the absolute refractory period. All three repair groups were significantly different for the absolute refractory period when compared to normal ($p<0.05$). Figure 11.4.4 is a table that provides the mean data for each group with respect to each electrophysiological test.

| | <u>CRG-MN</u> | <u>CRG-N</u> | <u>FTMG</u> | <u>Normal</u> |
|---|---------------|--------------|-------------|---------------|
| M-wave amplitude (mV) | 7.27 | 3.65 | 8.04 | 18.92 |
| M-wave area (mVs) | 8.97 | 6.00 | 12.76 | 32.40 |
| Maximum conduction velocity ($m\ s^{-1}$) | 41.44 | 44.33 | 40.23 | 69.36 |
| Minimum Conduction velocity ($m\ s^{-1}$) | 19.57 | 20.31 | 12.46 | 32.88 |
| Absolute refractory period (ms) | 1.42 | 1.50 | 1.11 | 0.84 |
| Jitter (μs) | 17.94 | 25.54 | 20.68 | 13.48 |

Figure 11.4.4 A table that provides the mean data for each group with respect to each electrophysiological test.

All the morphological data on the nerve (axon and fibre diameters, myelin thickness and G-ratio) for the CRG-N group were significantly different when compared to any group ($p < 0.05$) and both the CRG-MN and FTMG groups were significantly different for all morphological measurements from normal ($p < 0.05$) but did not differ significantly between each other ($p > 0.05$). These results indicate that the CRG-MN and FTMG groups had superior levels of regeneration with respect to fibre and myelin maturation than the CRG-N group. The mean values for all morphological data for each group are provided in figure 11.4.5.

| | <u>CRG-MN</u> | <u>CRG-N</u> | <u>FTMG</u> | <u>Normal</u> |
|------------------------------------|---------------|--------------|-------------|---------------|
| Axon diameter (μm) | 1.88 | 1.38 | 1.87 | 4.52 |
| Fibre diameter (μm) | 4.57 | 3.58 | 4.62 | 9.33 |
| Myelin thickness (μm) | 2.70 | 2.20 | 2.75 | 4.44 |
| G-ratio | 0.38 | 0.36 | 0.38 | 0.47 |

Figure 11.4.5 **A table showing the morphological data for each group.**

11.5 DISCUSSION

The purpose of these experiments was to study the effect of inserting Schwann cells (by way of chopped nerve) in the lumen of a CRG tube for the repair of a 1.0 cm deficit to the rabbit peroneal nerve. Two type of grafts, CRG-MN and CRG-N, were compared to a FTMG and to normal with respect to nerve morphology and recovery of function.

There was no significant difference between the CRG-MN and FTMG groups for any test, except the CV_{min} , indicating that these two groups produced similar levels of regeneration for the repair of a 1.0 cm deficit in the rabbit peroneal nerve. The results of this study also indicated that inserting Schwann cells into the CRG tube without structural support (by way of FTMG) did not produce a similar level of regeneration when compared to a FTMG with respect to both fibre maturation and recovery of function.

The CRG-N group demonstrated a significant 19.02% increase in jitter when compared to the FTMG group and an even greater 47.22% increase from normal. The increase in jitter indicates either a lack of myelination at the terminal axonal branches or a lower end plate threshold. Both the lack of myelination and lower end plate potential represent a possible reduction in the level of fibre maturation (Lenihan *et al.*, 1997).

The absolute refractory period provides a measurement for the excitability of the largest motor neurones in the nerve. The significantly ($p < 0.05$) increased absolute

refractory period in the CRG-N group compared to the FTMG indicated that the regenerated nerves in the CRG-N group had a lower excitability level. One possible explanation for this result is that the CRG-N did not have the same level of myelination, which resulted in an increased resistance within the nerve fibres.

The hypothesis of less myelination in the CRG-N group was further supported by the nerve morphology where the CRG-N group did not achieve the same level of fibre maturation or myelination compared to the other repair groups. The significantly lower G-ratio for the CRG-N group compared to the other two repair groups further supports the result that the CRG-N group had a reduced level of myelination for the degree of fibre maturation in the regenerated neurons.

The CRG-N showed a significant reduction from the FTMG group ($p < 0.05$) for both the M-wave area and amplitude. The area and amplitude of the M-wave provided a generalized representation on the number of muscle fibres contracting under the electrode after nerve stimulation. Therefore, significantly ($p < 0.05$) lower M-wave amplitude and area indicated that the CRG-N group had fewer regenerating fibres reaching the peripheral terminal motor end organs when compared to the FTMG.

The result that the CRG-N group produced inferior recovery of function and fibre maturation when compared to the FTMG group was unexpected when compared with the results achieved other authors (Jenq & Coggeshall, 1985a; Jenq & Coggeshall, 1985b; Navarro & Kennedy, 1991). These authors stated that a silicon

tube with nerve segments inserted in the lumen to repair a nerve deficit in the rat sciatic nerve produced superior results when compared to an empty tube of similar length with respect to fibre maturation. However, the results from the CRG-N group did not achieve the same level of recovery of function or fibre maturation as the FTMG group. The difference between the present experiment and the other studies may be due to the fact that the luminal diameter in the present experiment was slightly larger when compared to the diameter of nerve to be repaired. Secondly, the total gap length to be repaired was larger in the present study. Thirdly, there may have been a difference between the regenerative ability of the different species.

The CV_{max} represents the fibres which can transmit impulses at the greatest speed within a nerve. All repair groups demonstrated a significant reduction in the CV_{max} when compared to normal. The reduction in the CV_{max} was 40.25% (CRG-MN), 36.07% (CRG-N) and 58.00% (FTMG) from normal which is consistent with previous studies where the rabbit peroneal nerve was used as a model for peripheral nerve repair (Lundborg & Hansson, 1980; Lundborg, 1988; Lawson G.M. & Glasby M.A., 1998). The lack of a significant difference in the CV_{max} for the repair groups suggested that the maturation of the regenerated axons had reached a similar level with respect to the myelination and growth of the fastest conducting fibres.

The results of this study show that the CV_{min} was significantly different from normal after repair for all groups at six months. However, both the CRG-MN and CRG-N groups were significantly ($p < 0.05$) different from the FTMG group. These

results suggest that the maturation of slowest conducting fibres within the rabbit peroneal nerve after repair was improved when a CRG tube was used. There are two possible reasons for this result. One, sutures at the site of repair in the FTMG group may have had a greater influence on the smaller fibres and restricted their growth. Two, the presence of Schwann cells inter—spaced in the CRG tube provided a greater influence on the smaller fibres within the nerve than expected.

The result of the CV_{min} was not supported by nerve. Although the average axon diameter for the CRG-MN group was slightly larger when compared with the FTMG group, there was no statistical difference the two group. A more detailed evaluation of the axon and fibre distribution for each group should be performed in further experiments to see if the lower values of the axon and fibre diameter measurement are indeed similar.

The CRG-MN and FTMG only differed in the CV_{min} measurement indicating the quality of regeneration was similar. These results suggest that Schwann cells inserted in the lumen of a tube, which is used for the repair of a peripheral nerve, must also have the necessary structural support. The results further suggests that a muscle—nerve ‘sandwich graft’ inserted in the lumen of a CRG tube supports regeneration comparable to an established graft for the repair of short nerve deficits. Further studies should be performed to assess the CRG-MN graft in the repair of longer nerve gaps.

11.6 Conclusion

The purpose of this study was to compare the quality of regeneration when the lumen of a biodegradable glass tube was inserted with either nerve or a muscle–nerve sandwich graft with a FTMG for the repair of a 1 cm deficit in the rabbit peroneal nerve.

The results of the experiments presented in this chapter demonstrate that if Schwann cells are inserted without structural support then the recovery of function will be worse for the repair of a 1 cm nerve deficit in the rabbit peroneal nerve.

CHAPTER 12.0

STIMULATED JITTER IN MONITORING OF RECOVERY OF FUNCTION AFTER NERVE REPAIR

| <u>SECTION</u> | <u>TITLE</u> |
|----------------|---------------------|
| 12.1 | <u>OBJECTIVE</u> |
| 12.2 | <u>INTRODUCTION</u> |
| 12.3 | <u>METHODS</u> |
| 12.4 | <u>RESULTS</u> |
| 12.5 | <u>DISCUSSION</u> |
| 12.6 | <u>CONCLUSION</u> |

12.1 **OBJECTIVE**

The recording of stimulated jitter may offer a quantitative and highly sensitive method for assessing the progression of maturation and recovery of function of the neuromuscular junction after peripheral nerve injury. The purpose of the experiment present in this chapter was to determine if jitter could be used to both assess and monitor maturation of the neuromuscular junction after nerve repair.

12.2 INTRODUCTION

Research into peripheral nerve repair has tended to be involved with different mechanical methods of improving the coaptation of the divided nerves and with different types grafts. Research into mechanical methods of coaptation may have reached its limit of sophistication and interest is now turning to the use of chemical factors to enhance nerve regeneration. It is unlikely that enhancement by neurotrophic factors will produce large improvements in regeneration but given the overall results of nerve repair in clinical practice, there would still be a major benefit from small improvements in both the extent and the specificity of nerve regeneration. If research in this field is to yield clinically useful data, it must be accompanied by the development of objective and relatively non-invasive methods of assessment which will better allow the clinician to assess quantitatively the outcome of the treatment. There are few ways at present in which objective and quantitative assessment of nerve repair can be carried out in the clinical setting. Stimulated jitter which specifically measures events occurring in the motor unit offers a potential for detailed objective quantitative monitoring of the progression of nerve regeneration.

Single fibre electromyography (SF-EMG) was developed by Stålberg and Trontelj (1979) to assess the microphysiology of the motor unit. Stimulated jitter, a SF-EMG technique, is a means of measuring the variation in transmission at the neuromuscular junction. It involves the stimulating of a terminal motor axon and the

recording of the associated muscle fibre action potential. The variation in latency between stimulation of the motor axon and the recording of the muscle fibres action potential is called *jitter*.

Transection of a peripheral motor nerve will usually produce a totally denervated muscle. Degeneration of the neuromuscular junction will then occur within 48 hours (Birks *et al.*, 1960) after denervation. If the nerve remains in continuity or if it is correctly repaired, reinnervation will result from sprouting axons growing from the proximal nerve stump. The probability of successful reinnervation and the time it will take for this to occur will depend on both the distance the pioneering axons must travel and the quality of repair.

When regenerating axonal sprouts reach a compatible muscle fibre a new end plate will begin to form. In the immature regenerating motor unit there is a larger variability in the time of transmission owing to the lower and more variable threshold potentials at the end plate (Stålberg & Trontelj, 1979; Stålberg, 1990). As the fibre continues to mature, the endplate threshold begins to rise and stabilize. As this process occurs it should be possible to record these changes in the end plate potential indirectly using a SF-EMG technique such as jitter.

Jitter has been used to monitor reinnervation in a few non-controlled clinical cases, (Wiechers, 1990) however it has not been cited as a method of monitoring reinnervation after peripheral nerve repair. The experiments described in this chapter

are an attempt to assess the use of jitter as a method of monitoring reinnervation after repair of a peripheral nerve.

12.3 Methods

Twenty five female Sprague-Dawley rats each weighing between 400 to 650 g were separated into five equal sized groups. The groups were a control group of normal animals and groups where the sciatic nerve was divided and repaired by direct end to end suture to be assessed at 14, 30, 60, and 90 days.

Anaesthesia was induced in each animal using 5% halothane with gas flow rates of 1.0 l min⁻¹ of O₂ and 0.5 l min⁻¹ of N₂O. Maintenance of anaesthesia required 2% halothane at the same gas flow rates described above. A lateral incision was made in the skin of the left thigh. The skin edges and biceps femoris muscle were reflected to expose the sciatic nerve which was then divided 2 cm distal to the sciatic notch. This was then repaired with 2 to 3 interrupted 10/0 polyamide microsutures (Ethilon, Ethicon, UK). Continuous 3/0 absorbable polyglactin sutures (Vicryl, Ethicon, UK) were used for closure of the muscle and skin.

On the day of assessment each animal was anaesthetized in the same way as described above. The left leg was shaved and the foot was firmly clamped to the table with a non-crushing clamp to prevent any needle movement during stimulation. The muscle chosen for this experiment was the EDL because it is supplied by a single branch arising from the peroneal division of the sciatic nerve. Care was used to place the EDL in a relaxed position because pre-loaded tension in the muscle may increase the interpotential interval (Trontelj & Stålberg, 1992)

The details of measuring jitter are provided in chapter 6.5 and the following is a summary of how the technique was modified in the present experiment.

A Medelec-Sapphire 4ME II EMG unit (Medelec, Old Woking, UK) was used for all stimulating and recording procedures. Two monopolar needle electrodes (Medelec MF37) were used as the stimulating cathode and anode. The cathode was inserted into the EDL at the motor point which is located approximately 0.5 cm distal lateral to the knee. This is the point at which the nerve enters the muscle body and its location was confirmed by dissection of several rats. The anode was placed 0.5 cm proximal lateral to the cathode. Initially, inserting these electrodes in the rat EDL muscle proved difficult owing to the small distance between the EDL and TA overlap. Small variations in placement of the cathode could result in inadvertently stimulating the TA muscle. After several attempts it was possible to determine which muscle was stimulated by the contraction of the foot.

A stimulus pulse of 1 Hz frequency, 50 μ s duration and of an amplitude of 1 mA were used initially. The stimulus amplitude was increased by 0.1 mA increments until small twitches were observed in the EDL. A SF-EMG needle recording electrode (Medelec SF25) was inserted into the twitching portion of the EDL no further than 2 cm distal to the stimulating cathode. The SF-EMG needle electrode was the positioned to produce a motor unit action potential from the muscle fibres. The current was then further increased from threshold to a point 10% greater than maximal. The importance of clamping the foot cannot be over stated during this

process. Because the rat muscle is so small and there is not much mass for the needle electrode to rest in: reducing the movement was more important in this case than in the rabbit or human.

The average value of jitter for each animal was calculated from measurements made from 20 different muscle fibres from at least 4 different sites. In the repaired nerve there were often several recordable muscle fibres that could be distinguished from the SF-EMG recording electrode position. No more than 5 muscle fibres were taken in these instances. The jitter from each muscle fibre was determined from 50 consecutive discharges which were recorded and stored on disk and subsequently used in the calculation for the mean consecutive difference of their latencies (MCD). The mean sorted difference, mean IPI and percentage of blocking (failure to recorded an action potential on stimulation) were also recorded. Recordings where the muscle fibre was stimulated directly were determined by a MCD of less than 5 μ s and discarded.

The results were transferred on disk from the Medelec machine to an IBM compatible computer where they were analysed with CSS STASTICA software (Statsoft Inc. USA). The data was tested to determine if it was normally distributed. Because the data was not normally distributed, group to group statistical comparisons required a non-parametric test. A two tailed Kolmogorov-Smirnoff (non-parametric) test was used to discover the probability that the mean of the experimental groups might have been drawn from the same population.

12.4 Results

The data obtained from the control group are shown in histogram in figure 12.4.1. These data represent the pooled values of the MCD from the 20 different muscle fibres in each of the five rats. Because there was a slight skewing of the data to the right as observed on the histogram the data did not fit a normal distribution curve. However, by re-plotting these data as the abscissa of a log-normal graph it was possible to fit the data to a normal distribution curve – figure 12.4.2. From the log normal curve, the mean MCD from the normal EDL was found to be 18 μs with a standard deviation of 11 μs . The MCD values of individual muscle fibres ranged from 5 μs to 73 μs . The upper normal limit or 95th percentile was 31 μs . Therefore the acceptable range of a jitter study for the normal rat EDL should be below 29 μs with no more than one value being larger than 31 μs .

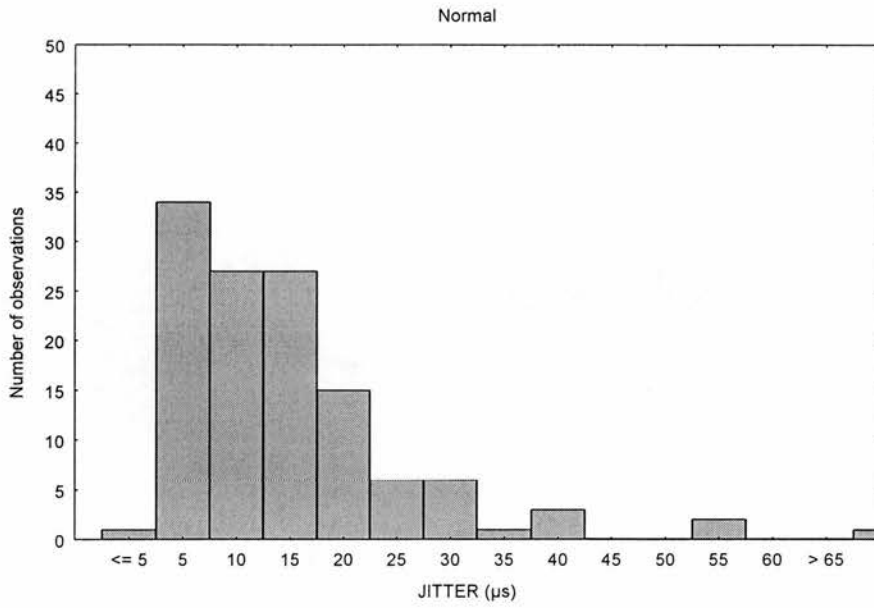


Figure 12.4.1 A histogram of the observed data for jitter in the control group of animals.

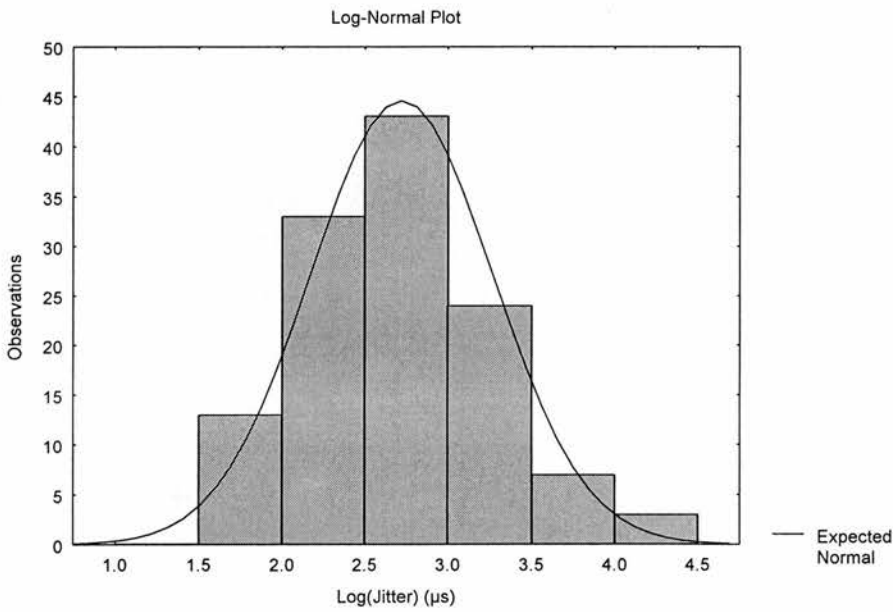


Figure 12.4.2 A histogram of the observed data for jitter, transformed logarithmically

The results obtained for the 14 day group of rats indicated that reinnervation of the EDL had not yet taken place and will not be considered further. This is consistent with previous findings (Glasby *et al.*, 1986)

The mean MCDs for the 30, 60 and 90 day groups were 97 μ s, 32 μ s and 23 μ s, respectively. Histograms for each group were shown in figures 12.4.3 to 12.4.5. These histograms show the MCD shifted to the left as the time to repair increased. No animal in the 30 day group met both the requirements for jitter. One out of five animals in the 60 day group and four out of five animals in the 90 day group demonstrated normal jitter.

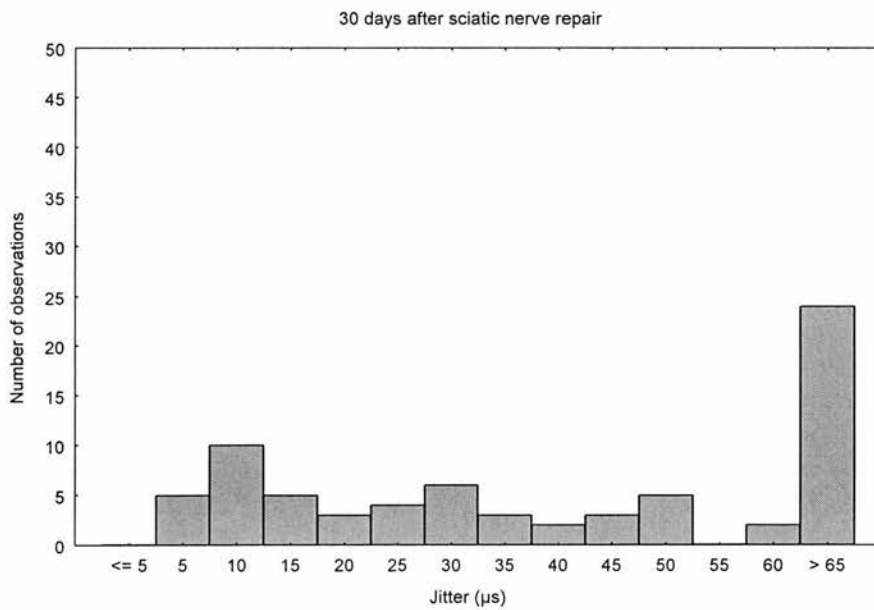


Figure 12.4.3 A histogram of the observed data for jitter in the 30 day group.

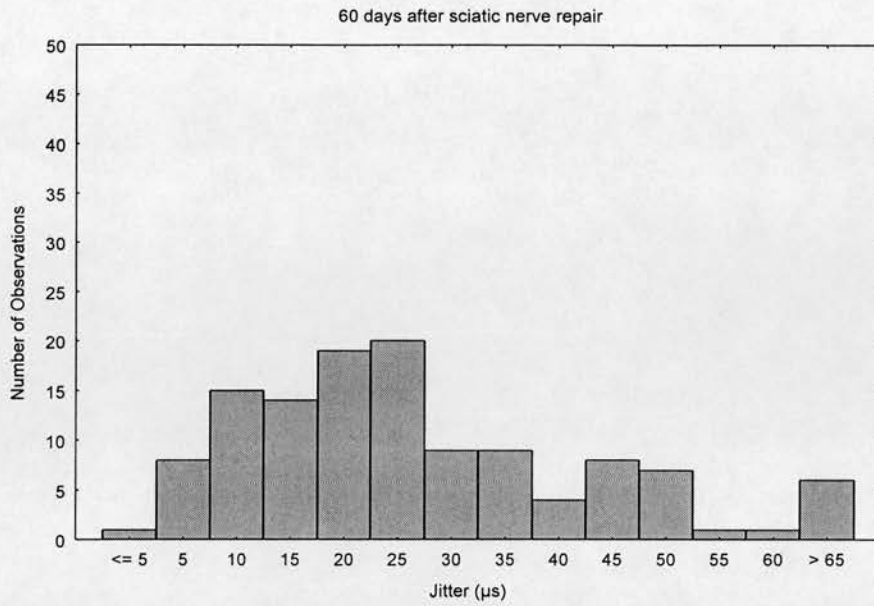


Figure 12.4.4 A histogram of the observed data for jitter in the 60 day group.

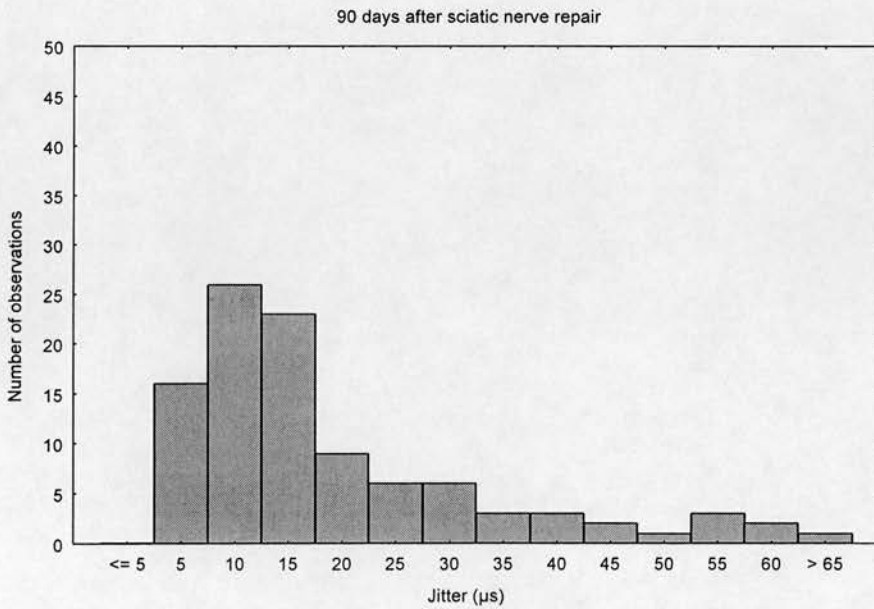


Figure 12.4.5 A histogram of the observed data for jitter in the 90 days group.

The results also showed a relative decrease in the number of fibres with blocking as the time after repair increased. The percentage of fibres which showed blocking decreased from 17% in the 30 day group to 9.9% in the 90 day group. Blocking was strongly correlated with latency (MIPI) in all groups. No blocking was evident in the normal group. The data for all groups are provided in table 1 in figure 12.4.6.

| <u>Variable</u> | <u>30 Days</u> | <u>60 Days</u> | <u>90 Days</u> | <u>Control</u> |
|---|----------------|----------------|----------------|----------------|
| Average jitter (μs) | 97 | 32 | 21 | 18 |
| Maximum Jitter (μs) | 849 | 169 | 160 | 73 |
| Minimum jitter (μs) | 7 | 5 | 6 | 5 |
| Standard deviation (μs) | 141 | 23 | 18 | 11 |
| % of fibres with blocking | 16.7% | 13.1% | 9.9% | 0.0% |
| Mean latency (μs) | 7,757 | 5,397 | 3,930 | 3,703 |

Figure 12.4.6 Mean values of variables.

A statistically significant correlation of increased latency (MIPI) with increased MCD was evident in all groups. This is consistent with results from human studies (Trontelj *et al.*, 1988)

These results demonstrate that jitter decreases with time after repair of a transected peripheral nerve. In the rat, normal values for jitter were attained by 90

days after repair ($P>0.05$). These results show an inverse relationship between the percentage of blocking and MIPI when compared with time after repair.

12.5 DISCUSSION

The purpose of the present study was to investigate whether jitter could be used as a technique to record the progression of reinnervation after the repair of a peripheral nerve. Because jitter is minimally invasive and easy to perform on human subjects, the potential for using it as an objective follow-up study after nerve repair is considerable. The results of the present experiment support this view by demonstrating a significant and easily measurable decline towards normal values as the time after nerve repair increases.

The results demonstrate that in the 90 day group there was no statistical difference between measurements of jitter obtained from the repaired and normal nerves. This result implies that the majority of the end-plates in the EDL have regained their original physiological properties with respect to neuromuscular variability (jitter).

The results obtained in this experiment for the normal EDL did not fit a normal (Gaussian) distribution. They therefore had to undergo log-transformation of the jitter values to fit a normal distribution so that parametric statistics could be applied in calculating the mean MCD range and 95th percentile for the normal. This problem has been identified previously by others in the human (Baker *et al.*, 1987). In Baker's experiment an empirical formula was applied to the data to overcome this problem. The data in this experiment, however, were found to fit a normal

distribution curve after a simple log-transformation, which removed the need for more complex mathematical calculations.

After transection of a peripheral nerve several events take place. The axons degenerate by Wallerian degeneration (Waller, 1850; Langley, 1909) and the neuromuscular junction fails within 36 hours (Birks *et al.*, 1960). The myelin sheaths begin to degenerate within 48 hours (Miledi & Slater, 1968)(Winlow & Usherwood, 1975). The denervation of the muscle fibre causes a lowering of the threshold potential for each fibre (Trontelj & Stålberg, 1983; Kraft, 1990). By one month after adequate nerve repair some neuromuscular junctions become established and calculating jitter by way of axonal stimulation can, in theory, be achieved. This was confirmed in these experiments.

The mean MCD, MIPI and percentage blocking for the 30 day group were all statistically different ($P < 0.01$) when compared with the corresponding values for the 60 day, 90 day and normal groups. The increased jitter in the 30 day group was most likely due to the abnormally low and variable end plate potentials as described by Stålberg and Trontelj (1979), variations of the depolarization threshold in both the motor nerve terminal axon or the muscle fibre (Stålberg & Thiele, 1973) and/or transmission failure in the terminal motor axons. These factors are known to stabilize as maturation of the neuromuscular junction occurs. It is important to state that the method does not allow one to discriminate which one of these factors may be causing the increase in jitter.

It has been shown that jitter along normal axons is minimal (Trontelj *et al.*, 1986). However, in the early regenerated axon there is a greater variability in the steepness of the local potential and this causes a further variability in the take-off point of the action potential. This results in increased jitter.

Blocking of the conduction impulse down the axon is increased in the early stages of reinnervation owing to lack of myelin (Harreveld, 1952). Therefore, during the early stages of reinnervation there are likely to be transmission defects along the course of the nerve which produce conduction block. This has been confirmed in previous studies (Gilliatt, 1966).

Failure of axonal transmission may cause of concomitant blocking. Concomitant blocking is observed when two action potentials block simultaneously. In stimulated jitter the latter results from stimulating proximal to the transmission deficit in an axon which subsequently splits. In the present study this was observed only twice and both instances occurred in the 30 day group. The jitter between the two blocking action potentials in both of the cases was large and therefore considered neurogenic in origin. The literature suggests that in cases of reinnervation, such as multiple sclerosis failure of axonal transmission is common. It was therefore expected to be observed more frequently in the 30 day group than it was. One possible reason for this may be the short distances between the stimulating electrode and muscle fibre. Further study in to this result is suggested.

The myogenic component of jitter will also be increased in the early stages of reinnervation. After severe nerve injury the muscle fibres will atrophy owing to denervation. This results in an altered propagation velocity in the muscle fibre (Stålberg & Trontelj, 1979). The denervated muscle will also have a lower and more variable threshold potential (Thesleff & Ward, 1975). These physiological changes in the muscle fibre will result in an increase in jitter.

Ephaptic transmission is another reason for increased jitter during the early stages of reinnervation. Ephaptic transmission may occur when the action potential occurring in a muscle fibre induces the firing of an action potential in an adjacent muscle fibre with a lower threshold. In the early stages of reinnervation when there is a greater variability and lower thresholds of individual muscle fibres, the probability of ephaptic activity is increased. If a muscle fibre is stimulated by ephaptic transmission there will be an increase in blocking and jitter owing to the fibres being submaximally stimulated. In the present study ephaptic transmission was detected in the 60 and 90 day groups by altering the location of the recording electrode and increasing the current. This did not affect the latency or amplitude of the ephaptic action potential because it was independent of changes in current. Also, if the current was increased sufficiently to stimulate the muscle fibre directly there was a stepwise decrease in the latency of the action potential to 5 μ s or less.

It proved extremely difficult to determine ephaptic transmission in the 30 day group by either method as large variations in jitter and blocking were common. It

was often impossible to distinguish ephaptic transmission from stimulated jitter of the axon.

The end plate is also a source of jitter. In the early stages of reinnervation there is an increase of jitter which is due to the low and variable end plate potentials at both the axon and muscle fibre. The variations in the terminal end bulb of the axon will cause variability in the timing of the acetylcholine quantal release which will cause variability in neuromuscular timing. The muscle fibre end plate will have a more variable end plate threshold which will result in increased jitter owing to the amount of time it takes the muscle fibre to reach threshold. These two phenomena are probably the main reason for the increased jitter during the early stages of reinnervation, if blocking is not considered.

It has been suggested that while blocking is indicative of pathology and will result in increased jitter, it does not necessarily provide any information of the physiology of the end plate (Stålberg *et al.*, 1992). Stålberg showed that blocking will cause a change in the propagation velocity at which an action potential is transmitted down a muscle fibre (Stålberg & Thiele, 1973). The propagation velocity is affected by the inter-discharge interval which will alter the conduction velocity as a function of the property of the muscle termed the velocity recovery function (Stålberg, 1966). The consequence of this is an increased jitter. Although Stålberg stated that blocking does not provide information on the physiology of the end plate it does provide valuable information about the regenerative process. The present

study clearly demonstrated that as time after repair increased the frequency of blocking decreased. In humans electrophysiological studies it has been shown that blocking can exist for many months after reinnervation (Wiechers, 1990).

While the exact reason or reasons for the changes in jitter during nerve reinnervation remain a matter for conjecture, the present study has shown that the recovery of jitter specifically after nerve repair provides a useful way of monitoring regeneration.

12.6 **CONCLUSION**

The purpose of the experiments presented in this chapter was to determine if jitter could be used to assess and monitor the neuromuscular junction maturation after separation and subsequent immediate repair.

It was found that the values of jitter were highest in the early stages of regeneration and declined with time after repair. It was found that the values of jitter were within normal limits 90 days after repair.

It is concluded that jitter measurement may be a useful postoperative measure to assess the recovery of function.

CHAPTER 13.0

STIMULATED JITTER – NEW TECHNIQUE TO ASSESS THE RECOVERY OF FUNCTION.

| <u>SECTION</u> | <u>TITLE</u> |
|----------------|---------------------|
| 13.1 | <u>OBJECTIVE</u> |
| 13.2 | <u>INTRODUCTION</u> |
| 13.3 | <u>METHODS</u> |
| 13.4 | <u>RESULTS</u> |
| 13.5 | <u>DISCUSSION</u> |
| 13.6 | <u>CONCLUSION</u> |

13.1 OBJECTIVE

The recording of stimulated jitter offers a quantitative method for following the recovery of neuromuscular function after peripheral nerve repair.

In groups of rats, electrophysiological recording of jitter was carried out on control animals and on animals 90 days after sciatic nerve division and subsequent repair with either direct end to end suture (NS), nerve graft (NG) or freeze thawed muscle graft (FTMG).

13.2 **INTRODUCTION**

In the clinical setting, anatomical and electrophysiological evidence of regeneration after peripheral nerve injury does not always correlate with the patient's impression of recovery. Providing standardized clinical assessment for treating patients with peripheral nerve injuries is difficult owing to the wide variation in location, severity of injury and method of repair.

Recovery of function after nerve repair depends on the number of fibres reaching and establishing active connections to target sites in the periphery. The time involved for regenerating motor axons to reach the target muscle and develop functional connections depends on the severity and location of the injury as well as on the method of repair used. In the laboratory where severity and location of the injury can be controlled, it has been shown that different methods of repair have different initial rates of regeneration. It has also been shown that grafts offering an increased mechanical resistance to penetrating axons show a reduction in the initial speed of regeneration (Davies et al, 1987).

When regenerating motor axonal sprouts reach their target muscles, new end plates begin to form (Hakelius et al, 1975). These axonal sprouts re-establish neuromuscular junctions. It has been shown that in these immature motor units (terminal motor axon, neuromuscular junction and muscle fibre) there is an increased variability in the latency of neuromuscular transmission which is due to a lower and more variable threshold of the end plate potential (Stålberg and Trontelj, 1979).

Once the axonal motor sprout connects with a target muscle and establishes a neuromuscular junction, the fibre will begin to mature. As fibre maturation continues the threshold end plate potential begins to rise and becomes more stable (Wiechers, 1990). It is possible indirectly to monitor regeneration by assessing the variability in the latency of neuromuscular transmission (Lenihan et al, 1997).

Stimulated jitter is a method of measuring variability in the latency of neuromuscular transmission. The terminal motor axon is stimulated and the associated muscle fibre's action potential is recorded. It has been shown in the normal nerve that most of the variability in latency exists in the process of synaptic transmission and not in the terminal axonal branch (Stålberg and Trontelj, 1979; Trontelj, 1986) or in the muscle fibre (Trontelj et al, 1990) .

If the speed of regeneration seen in the two methods of repair is different, it should be possible to monitor this difference by using the jitter technique. It becomes important when assessing one repair technique against another to ensure that enough time has occurred for all regenerating axons to reach their targets and mature. Chemical growth factors, such as NGF, may initially cause an increase in the number of regenerating axons and possibly affect the initial speed at which these axons grow without affecting the final level of recovery of function. Before these chemical factors can be considered useful they must be compared to a 'gold standard' technique to ensure that sufficient time has passed for adequate regeneration to occur

or at least similar levels of maturation of neuromuscular junctions. Jitter provides a means of assessing neuromuscular maturation.

Direct end to end nerve suture (NS) produces more complete and faster levels of regeneration compared to a grafts (Davies et al, 1987) provided no tension exists at the suture line (Terzis et al, 1975). It is believed that the reason for improved regenerative capacity in the nerve suture group is the improved organization within the repair site. However, it seems unlikely that this would affect the speed of regeneration. Altered initial speeds of regeneration have been noted in differently aligned muscle grafts (Davies et al, 1987). It is thought that the initial reduction of regenerative speed in the non-aligned muscle graft was due to an increased mechanical resistance within the graft. The purpose of the experiments presented in this chapter was to determine if the increased resistance associated with regeneration through nerve grafts (NG) or freeze thawed muscle autografts (FTMG) is measurable by assessing the maturation of the neuromuscular junction. This was tested by recording the stimulated jitter 90 days after different methods of sciatic nerve repair in the rat.

13.3 **METHODS**

A population of 20 female Sprague-Dawley rats each weighing between 400 to 650 g was divided into 4 equal sized groups. These groups consisted of a control group of normal animals and groups in which the sciatic nerve had been repaired with direct end to end nerve suture (NS), a nerve graft (NG), or a freeze thawed muscle autograft (FTMG). The anaesthesia and surgical approach were identical to those in Chapter 12. The control group was the same group as in the previous chapter.

In the NS group the proximal and distal ends of the cut sciatic nerve were sutured together with 2 or 3 interrupted 10/0 polyamide sutures (Ethilon, Ethicon, UK.). In the NG group a 1.0 cm segment was removed from the distal nerve. This segment was reversed about its long axis and sutured into the sciatic nerve gap with 3 or 4 interrupted 10/0 polyamide sutures (Ethilon, Ethicon, UK). In the FTMG group an 0.5 cm segment of nerve was removed from the distal sciatic nerve. Retraction of the sciatic nerve ends produced a 1.0 cm gap. Preparation of the FTMG is described in chapter 5. A 1.0 cm FTMG was sutured in the sciatic nerve gap with 3 or 4 interrupted 10/0 polyamide sutures (Ethilon, Ethicon,UK)

Assessment was carried out 90 days after repair. On the day of assessment each animal was anaesthetized in the same way as before. The left leg was shaved and the foot was firmly clamped to the table with a non-crushing clamp to prevent any needle

movement during stimulation. The muscle chosen for study was extensor digitorum longus (EDL).

The general details of measuring jitter have been discussed in Chapter 6.5. Details proper to the methods used here have been described in Chapter 12.

In calculating jitter, the mean consecutive difference (MCD), the mean sorted difference (MSD) and mean interpotential interval (MIPI) were used after recording from 20 different EDL sites for each animal. The MCD was taken as the jitter value for each site provided the MCD : MSD ratio was 1.25 or below, otherwise the MSD was taken as the jitter (Kimura, 1989; Stålberg and Trontelj, 1979).

The results were transferred on disc from the Medelec machine to an IBM compatible computer where they were analysed with CSS STASTICA software (Statsoft, Inc. USA).

13.4 RESULTS

The data obtained from the control group are shown in figure 12.1 (see previous Chapter). These data did not fit the curve of a normal distribution owing to a slight skewing to the right. It was possible to fit the data to a normal distribution curve by re-plotting the data as a log-normal graph as shown in figure 12.2 (see previous chapter). The normal jitter range and upper limit for the rat EDL was determined from the log-normal plot. The conventionally accepted range of jitter for the normal rat EDL is 7-29 μs with no more than one fibre out of 20 having a value above the 95th percentile — 31 μs .

The means of the mean consecutive differences (MCD) for the NS, NG and FTMG were 23 μs , 22 μs , and 29 μs respectively. Histograms of the MCD for each group are shown in figures 13.4.1-13.4.3. These data represent the pooled results for each group of the 5 rats. Only one animal in the FTMG group and 4 of the 5 animals in both the NS and NG groups demonstrated a normal jitter study by having the mean EDL jitter value between 7-29 μs .

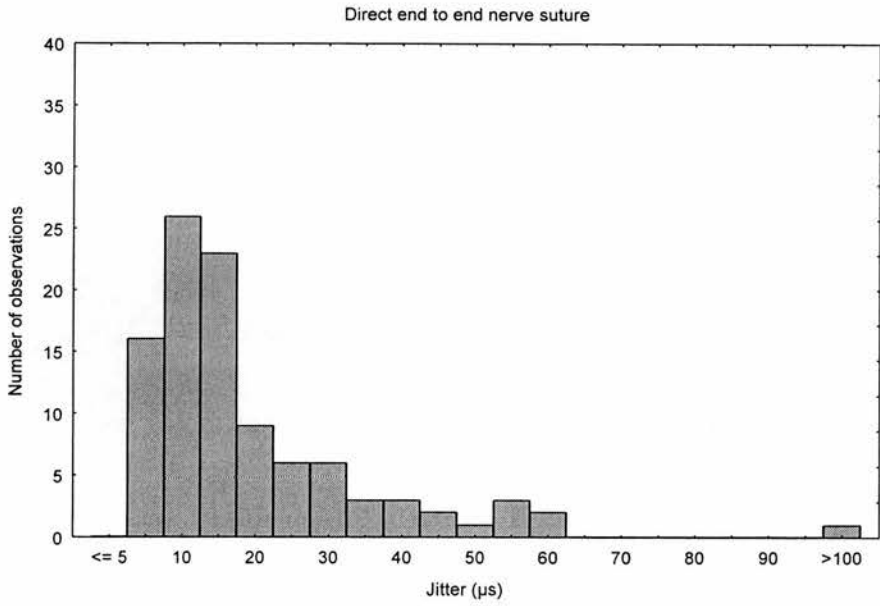


Figure 13.4.1 A histogram of the observed data for jitter in the direct end to end nerve suture group.

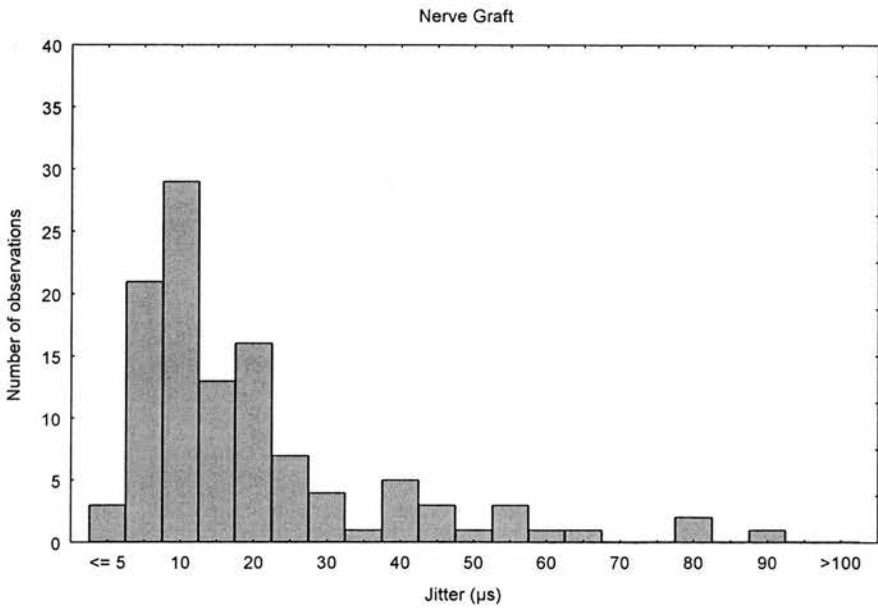


Figure 13.4.2 A histogram of the observed data for jitter in the nerve graft group.

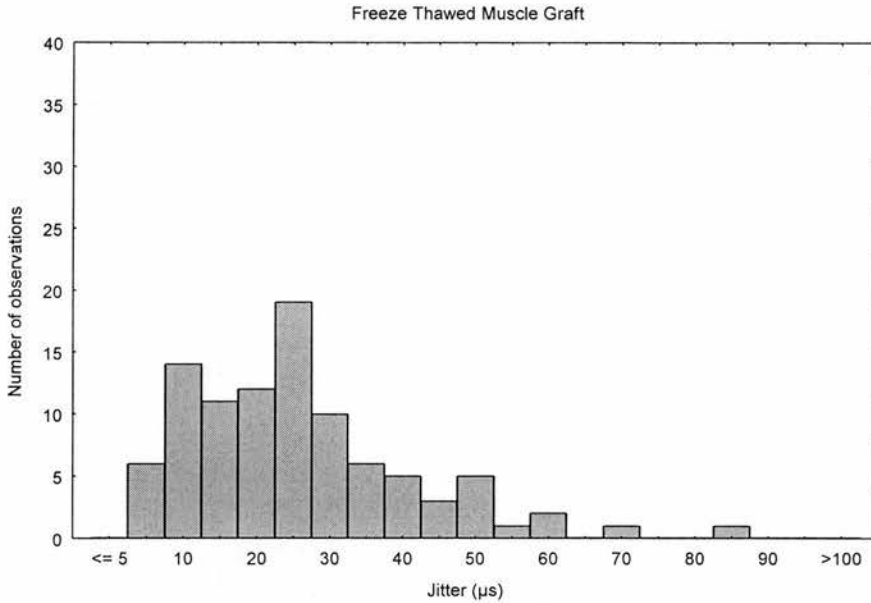


Figure 13.4.3 A histogram of the observed data for jitter in the freeze thawed muscle graft group.

The maximum MCDs for any onset of recordings for the NS, NG and FTMG were 160 μ s, 95 μ s and 86 μ s respectively. The standard deviation of the MCD of the pooled data of the NS, NG and FTMG groups were 18, 19, 15 μ s respectively. These results are displayed in figure 13.4.4.

The results demonstrate that the NS and NG groups develop stable neuromuscular junctions 90 days after sciatic nerve repair but the FTMG group did not have similar levels of neuromuscular junction maturation at 90 days. A matrix of

the statistical differences for jitter (MCD) among the three groups is displayed in figure 13.4.5.

| | <u>Normal</u> | <u>Nerve suture</u> | <u>Nerve graft</u> | <u>Freeze thawed muscle graft</u> |
|-------------------------------|---------------|---------------------|--------------------|-----------------------------------|
| Mean jitter (μ s) | 18 | 23 | 22 | 29 |
| Maximum Jitter (μ s) | 73 | 160 | 95 | 86 |
| Minimum Jitter (μ s) | 5 | 6 | 5 | 6 |
| Standard deviation (μ s) | 11 | 18 | 19 | 15 |
| MIPI (μ s) | 3703 | 3930 | 4040 | 4934 |

Figure 13.4.4 Mean value of variables

| <u>Group</u> | <u>Normal</u> | <u>Nerve suture</u> | <u>Nerve graft</u> |
|-----------------------------|---------------|---------------------|--------------------|
| Normal | — | n.s. | n.s. |
| Nerve suture | n.s. | — | n.s. |
| Nerve graft | n.s. | n.s. | — |
| Freeze thatwed muscle graft | P<0.001 | P<0.001 | P<0.001 |

Figure 13.4.5 Matrix of the probability values for the differences in jitter (MCD)

13.5 DISCUSSION

The purpose of this study was to investigate whether jitter could be used to assess the quality or repair at a specific time with respect to the small differences expected after different repair techniques 90 days after sciatic nerve repair. The results of this experiment imply that the EDL end plates have not regained their original physiological properties with respect to jitter presumably because of an initial delay in the pioneering axons reaching the distal nerve and subsequently the target muscle. This conclusion is supported by an increased jitter value for the FTMG group when compared to the NS and NG groups.

Stålberg and Trontelj, (Stålberg and Trontelj, 1979) demonstrated that immature neuromuscular junctions, as a result of degeneration and subsequent re-innervation, had lower and more variable end plate potentials. In the early stages of re-innervation the depolarization threshold of both the terminal motor axons and the muscle fibre are also lowered (Stålberg, 1990; Wiechers, 1990). These changes in the motor unit produce an elevation in the variability of neuromuscular transmission latency and therefore lead to increase in the value of jitter. As the fibre continues to mature, the threshold potential at the endplate rises and becomes more stable. Lenihan et al (Lenihan, 1997) showed that jitter could be used to monitor the progress of regeneration with respect to time while the present study demonstrated that the speed of regeneration among different repair methods can be assessed using jitter.

Transection of a peripheral motor nerve will produce a denervated muscle. Degeneration of the neuromuscular junction will then occur within a short time after denervation (Birks et al, 1960). If the injury is correctly repaired re-innervation will result from sprouting axons growing down from the proximal end of the transected nerve. The time to muscular re-innervation will depend on both the distance the pioneering axons are required to travel and the quality of the repair.

It has been shown that the quality of the graft can affect the speed of regeneration. When a coaxially aligned muscle graft was compared to an orthogonal muscle graft the initial regeneration rate of the regenerating axons was slowed (Davies et al, 1987). The reduction in initial speed of regeneration was probably due to the increased resistance of the orthogonal graft. It is interesting to note however, that the initial slowing of regeneration did not affect the final recovery of function (Davies *et al.*, 1987).

The present study showed that the MCD and mean inter-potential interval (MIPI) were significantly ($p < 0.001$) different in the FTMG group when compared to the NS, NG and normal groups. The increase in the MCD and MIPI values for the FTMG group was probably due to a lower and more variable end plate potential (Stålberg and Trontelj, 1979) and/or variations in both the motor nerve terminal axon and muscle fibre thresholds (Stålberg & Thiele, 1973) caused by a delay of maturation of the neuromuscular junction.

If the progression of motor re-innervation can be monitored using the jitter technique, it should be possible to assess the speed of regeneration in different methods of nerve repair by measuring the jitter value at specific times. A change in the speed of regeneration through a muscle or nerve graft will affect the timing of neuromuscular maturation when compared to other methods.

It has been shown that quality of peripheral nerve regeneration diminishes if the time latency of reinnervation increases (Sunderland, 1947; Bowden & Scholl, 1950). If one technique greatly increases the time it takes the advancing axons to reach the target muscle the quality of regeneration will decline. The use of jitter to monitor the speed of nerve regeneration among different techniques of nerve repair is based on two assumptions. First, that the speed of the advancing axon in the distal segment is not significantly different among the various groups. Second, that the speed of maturation, once a functional connection is made to a target muscle, is consistent for all repair techniques.

It has been shown that live Schwann cells are required for successful axon regeneration (Feneley *et al.*, 1991) and also that a graft with increased mechanical resistance will reduce the initial rate of regeneration (Davies *et al.*, 1987). The speed of regeneration through a graft may thus be influenced by the rate of Schwann cell migration from the proximal and distal segments of the nerve in the graft.

Mechanical resistance in the graft, in theory, should be lowest in the NS group followed by the NG group and then the FTMG group. Therefore, the pioneering

axons in the NS group should reach the muscle fibre first followed by those in the NG group and then the those in the FTMG group. The mean jitter value was increased in the FTMG group when compared to the NG and NS groups at 90 days after repair. The results of this experiment support the hypothesis that the FTMG has a higher resistance to pioneering axons than the NS or NG. The FTMG group data are comparable to the 2 month NS group in a previous study (Lenihan et al, 1997) indicating that enough time has not elapsed to allow for similar levels or regeneration.

The immature end plate is one of the major contributing factors to increased jitter values in the regenerating nerve. In the early stages of re-innervation there are low and variable end plate potentials which result in a lower safety-factor of neuromuscular transmission (Stålberg and Trontelj, 1979). These low and variable end plate potentials cause variability in the timing of the quantal release of acetylcholine. As the motor unit matures the end plate potentials rise and stabilize. These changes make the use of jitter to monitor the progression of re-innervation ideal.

The study of the way in which chemical factors or new methods of repair help enhance the quality of nerve regeneration may disclose that these are associated with only small (but nevertheless useful) increases in recovery of function. Many of the growth factors will probably be found only to influence the initial rate of regeneration without altering the final level of recovery of function. If assessment is carried out

too early, a false positive result may affect the conclusion. Jitter may by virtue of its sensitivity, provide a minimally invasive and therefore clinically acceptable method of assessing small variations in timing during the regenerative process. If assessment is carried out when the level of jitter has returned to normal it can be assumed that the neuromuscular junctions have had sufficient time to mature.

This was a preliminary study to assess further the feasibility of using jitter to monitor neuromuscular maturation. The next step is to attempt to correlate the jitter values with morphological and electrophysiological results in a serial time study. If these results are positive, stimulated jitter may prove to be a useful technique for monitoring the regeneration after repair. It would be extremely useful in all clinical cases where verbal communication between the patient and doctor is impossible, for example in infants with obstetrical brachial plexus palsy.

13.6 **CONCLUSION**

The results presented in this chapter support the view that the speed of regeneration is initially slower in the FTMG group. The results also support that 90 days after sciatic nerve repair the FTMG group had an increase in the number of immature neuromuscular junctions when compared with the NS and NG groups.

It was found that values for jitter were highest in the FTMG group. The NS and NG groups demonstrated statistically similar jitter values when compared with each other and to the normal group.

Jitter therefore appears to offer a means of detecting small differences in the development of nerve regeneration.

CHAPTER 14.0

CONCLUSIONS

| <u>SECTION</u> | <u>TITLE</u> |
|----------------|--|
| 14.1 | <u>HYPOTHESIS</u> |
| 14.1.1 | <u>The MMG</u> |
| 14.1.2 | <u>The CRG tube</u> |
| 14.1.3 | <u>Jitter</u> |
| 14.2 | <u>FUTURE TRENDS & CLINICAL APPLICATIONS</u> |

14.1 **HYPOTHESIS**

The purpose of the work presented in this thesis was three fold. First, to assess the functional aspects of using the MMG clinically. Second, to determine whether the CRG tube supported peripheral nerve regeneration. Third, could jitter be used as a tool to assess peripheral nerve function after nerve repair? The conclusions to each of these hypotheses are listed below.

14.1.1 **The MMG**

Nerves repaired with microwave muscle grafts had a similar levels of recovery of function when compared with nerves that were repaired with freeze thawed muscle grafts. The results support the conclusion that the MMG supports nerve regeneration over small gaps however, the difficulty in preparation of a MMG made its clinical use doubtful. It was therefore concluded that the MMG might be useful in experimental studies but provided no advantage over freezing as a method of preparing a denatured muscle graft and that the MMG could not be recommended for use in the clinical setting.

14.1.2 **The CRG tube**

The purposes of the experiments involving the CRG tube were two-fold. First, to assess the quality in the recovery of function when comparing the CRG tube with

an established technique and second, to assess the CRG tube with respect to the parameters detailed in Chapter 10.

The results indicate that entubulation with a CRG tube supported regeneration. The results further showed that: 1) the tube was completely reabsorbed at the time of assessment. 2) the tube did not produce any inflammatory reaction. 3) the internal lumen was large enough to support the initial swelling of the nerve and did not interfere with regeneration. 4) the tube maintained its structural integrity for enough time to allow the slowest neurites to migrate into the distal stump. 5) the nerve was able to be secured to the tube away from the transected ends avoiding further trauma. 6) the tube is made of substances that are amenable to custom fabrication so that the diameter of the lumen, rate of degradation, and permeability can be controlled. 7) the tube was easy to install.

These results suggest that the denaturing process of the biodegradable glass does not interfere with the rate or quality of nerve regeneration. This supports the view that the CRG tube may be used as an alternative technique to repair transected peripheral nerves and may be useful clinically.

14.1.3 Jitter

The purpose of this study was to investigate whether jitter was a useful technique in assessing the function of a peripheral nerve after injury and subsequent repair. To determine this, two questions were asked. First, could jitter be used as a

technique to monitor the progression of reinnervation after the repair of a peripheral nerve and second, could jitter be used to assess small differences in the quality of repair?

The answer was *yes* to both questions. Jitter was found to be a useful tool in monitoring the progression of peripheral nerve reinnervation with respect to time. Jitter was also found to be useful in determining small differences in the quality of regeneration.

These two results are very important. One difficulty in peripheral nerve research is that the time after repair is often not enough to allow for complete regeneration and maturation of the fibres. This may result in inaccurate conclusions because one technique may produce slower regeneration but still produce of similar levels of recovery of function after repair. Jitter removes this dilemma because it assesses the physiology of the neuromuscular junction. If the jitter values are similar, it is then possible to assume that the groups being studied have reached similar levels of maturation and can be compared. Thus, jitter can be used to determine if enough time after repair has passed to accurately assess different groups. There are no techniques presently that allow the examiner to determine this.

Because jitter is minimally invasive and easy to perform on human subjects, the potential for using it as an objective follow-up study after nerve repair is considerable. The results of the present experiment support this.

14.2 FUTURE TRENDS & CLINICAL APPLICATIONS

The results presented in this thesis hold many potential clinical applications. The CRG tube is a non-allergenic biodegradable tube that supports regeneration. The CRG tube therefore has many potential applications in both the clinical and experimental setting. At present, many laboratories are studying the use of neurotrophic factors to improve the quality of regeneration. Unfortunately, the results of these studies are often difficult to interpret in the clinical model, or even in the animal model, because the experiments have been done in a petri dish. The costs of extending these experiments from the petri dish to the animal or human are enormous without a proper experimental model. The CRG tube provides an excellent model because the permeability, solubility and biodegradability of the tube can be changed easily at the manufacturing stage. Using the CRG tube allows the examiner to be confident that the expensive neurotrophic factors being studied are delivered at that appropriate site. At present, our laboratory is using the tube as a means of delivering neurotrophic factors at the site of injury in both the facial and median nerve of sheep and found the application of such factors easy when using the CRG tube.. The CRG tube is also being used clinically by Mr. T.E.J. Hems FRCS(Orth) in Glasgow to repair the median nerve of two patients. The results look promising.

The minimum conduction velocity was assessed in this thesis to determine the quality in the recovery of the slowest motor fibres within the repaired nerve. A

direct result of the minimum conduction velocity assessment has been the discovery of a method to assess the entire range of conduction velocities within a peripheral nerve. This technique is called the Distribution of Conduction Velocities (DCV) and provides an exciting new insight into what is actually happening to the spectrum of fibres in a nerve. This is a new technique that should provide a more detailed assessment in the quality of regeneration and on nerve function in general. It is believed that the DCV will also allow an examiners to determine small changes in the quality of repair that are likely to occur with new methods of nerve repair such as neurotrophic factors. Our laboratory is getting ready to embark on a detailed study of nerve repair using the DCV technique which has grown entirely out of the work presented here.

In my opinion, the results of the jitter studies were probably the most beneficial result associated within this thesis. The potential use in both the clinical and experimental setting is exciting. At present, jitter is used almost daily in every NHS electrophysiology laboratory: it is easy to administer and is minimally invasive for the patient and is therefore an ideal technique to be used in assessing peripheral nerve injuries. Jitter is used clinically to assess diseases that affect the neuromuscular junctions however, it is not used to assess nerve function after repair.

Jitter could be used to assess whether regeneration is occurring, at what stage the recovery process is at or even to determine if a motor nerve is in continuity with the periphery. An ideal application of jitter and nerve injury would be in the infant

with brachial plexus palsy to determine if the nerve has lost continuity and to what degree. This could be done without the need for surgery. The potential use of jitter is considerable.

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APPENDIX A

SEGMENT DISTANCE ANALYSIS FOR ERROR IN CONDUCTION VELOCITY

This appendix will go through all five proofs required to calculate the distance needed to minimize error when calculating conduction velocity. To review from Chapter 6, the formula's and constants required to determine the minimum segment distance are listed below.

$$\text{A.1} \quad \% \text{ Error in a system} = \frac{A-B}{\frac{A+B}{2}} * 100$$

$$\text{A.2} \quad CV = \frac{d}{t}$$

Where:

d = distance

t = time

$$\text{A.3} \quad CV' = \frac{d+l}{t+k}$$

Where:

d = distance

t = time

k = error in the measurement of time

l = error in the measurement of distance

CV is the actual conduction velocity and CV' is the conduction velocity with some source of error.

The constants are set at the maximum level of error with l representing error in measurement of the length between the two stimulating electrodes and k representing error in determining the latency from stimulation at one point. The values of these are listed below.

$$k = \pm 0.02$$

$$l = \pm 0.50$$

The conduction velocity is set at a maximum of 60 m s^{-1} .

Case I (+k, l = 0)

$$\text{Error}(0.05) = \frac{\frac{d}{t} - \frac{d}{t+k}}{\frac{d}{t} + \frac{d}{t+k}}$$

$$0.025 = \frac{\frac{(dt+dk) - dt}{t^2+tk}}{\frac{(dt+dk) + dt}{t^2+tk}}$$

$$0.025 = \frac{dk}{2dt+dk}$$

$$0.025 = \frac{0.02d}{2d \frac{d}{CV} + 0.02d}$$

$$0.025 = \frac{0.02d}{\frac{2d^2}{60} + 0.02d}$$

$$d = 23.4 \text{ mm}$$

Case II (+l, k = 0)

$$\text{Error}(0.05) = \frac{\frac{d}{d} - \frac{d+l}{d+l}}{\frac{\frac{t}{d} + \frac{t}{d+l}}{2}}$$

$$0.025 = \frac{\frac{dt - (dt + tl)}{t^2}}{\frac{dt + (dt + tl)}{t^2}}$$

$$0.025 = \frac{-tl}{2dt + tl}$$

$$0.025 = \frac{\frac{-0.5d}{CV}}{\frac{2d^2}{CV} + \frac{0.5d}{CV}}$$

$$0.025 = \frac{\frac{-0.5d}{60}}{\frac{2d^2}{60} + \frac{0.5d}{60}}$$

$$d = 9.75\text{mm}$$

Case III(+k,-l)

$$Error(0.05) = \frac{\frac{d}{t} - \frac{(d-l)}{(t+k)}}{\frac{\frac{d}{t} - \frac{(d-l)}{(t+k)}}{2}}$$

$$0.025 = \frac{\frac{dt+dk}{t^2+tk} - \frac{dt-tl}{t^2+tk}}{\frac{\frac{dt+dk}{t^2+tk} + \frac{dt-tl}{t^2+tk}}$$

$$0.025 = \frac{dk+tl}{2dt+dk-tl}$$

$$0.025 = \frac{0.02d + \frac{0.5d}{CV}}{\frac{2d^2}{CV} + 0.02d - \frac{0.5d}{CV}}$$

$$0.025 = \frac{0.02d + \frac{0.5d}{60}}{\frac{2d^2}{60} + 0.02d - \frac{0.5d}{60}}$$

$$d = 33.7mm$$

Case IV (+l, -k)

$$Error(0.05) = \frac{\frac{d}{t} - \frac{d+l}{t-k}}{\frac{d}{t} + \frac{d+l}{t-k}}$$

$$0.025 = \frac{\frac{dt-dk}{t^2-tk} - \frac{dt+tl}{t^2-tk}}{\frac{dt-dk}{t^2-tk} + \frac{dt+tl}{t^2-tk}}$$

$$0.025 = \frac{-dk-tl}{2dt-dk+tl}$$

$$0.025 = \frac{-0.02d - \frac{0.5d}{CV}}{\frac{2d^2}{CV} - 0.02d + \frac{0.5d}{CV}}$$

$$0.025 = \frac{-0.02d - \frac{0.5d}{60}}{\frac{2d^2}{60} - 0.02d + \frac{0.5d}{60}}$$

$$d = 33.7mm$$

Case $V(+k,+l)$ or $(-k,-l)$

$$\begin{aligned} \text{Error}(0.05) &= \frac{\frac{d}{d} - \frac{d+l}{d+l}}{\frac{t}{t} + \frac{t+k}{t+k}} \\ &= \frac{(dt+dk) - (dt+tl)}{(dt+dk) + (dt+tl)} \\ 0.025 &= \frac{t^2 + tk}{t^2 + tk} \\ 0.025 &= \frac{dk - tl}{2dt + dk + tl} \\ 0.025 &= \frac{0.02d - \frac{0.5d}{CV}}{\frac{2d^2}{CV} + 0.02d + \frac{0.5d}{CV}} \\ 0.025 &= \frac{0.02d - \frac{0.5d}{60}}{\frac{2d^2}{60} + 0.02d + \frac{0.5d}{60}} \end{aligned}$$

$$d = 13.15 \text{ mm}$$

APPENDIX B

Papers published as a direct result of this thesis.

GILCHRIST T., GLASBY M.A., HEALY D.M., KELLY G., LENIHAN D.V., MCDOWALL K.L., MILLER I.A. & MYLES L.M. (1998). In vitro nerve repair - in vivo. The reconstruction of peripheral nerves by entubulation with biodegradable glass tubes - a preliminary report. *British Journal of Plastic Surgery* **51**, 231-237.

LENIHAN, D.V., CARTER, A.J., GILCHRIST, T., HEALY, D.M., MILLAR, I.A., MYLES, L.M., GLASBY, M.A. (1997). Biodegradable controlled release glass in the repair of peripheral nerve injuries. *Journal of Hand Surgery* **23B**, 588-593.

LENIHAN D.V., CARTER A.J. & GLASBY M.A. (1998). An electrophysiological and morphological comparison of the microwave muscle graft and the freeze thawed muscle graft. *British Journal of Plastic Surgery* **51**, 300-306.

LENIHAN D.V., SOJITRA, N.M. & GLASBY, M.A. (1998). Stimulated jitter measurement in the assessment of recovery after different methods of peripheral nerve repair. *Journal of Hand Surgery - British Volume* **23B**, 12-16.

LENIHAN, D.V., SOJITRA, N.M., IKEDA, M., CARTER, A.C. & GLASBY, M.A. (1997). Stimulated jitter measurement in the assessment of recovery after peripheral nerve repair. *Journal of Hand Surgery* **22B**, 772-777.



In vitro nerve repair – in vivo. The reconstruction of peripheral nerves by entubulation with biodegradable glass tubes – a preliminary report

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SUMMARY. Biodegradable “controlled release” inorganic polymer glass tubes can be manufactured to fit the dimensions of any nerve and their rate of solubility can be adjusted to encompass the time taken for nerve regeneration. They have been used in a number of biological applications.

The facial nerve was repaired in a group of five sheep by entubulation with biodegradable glass tubes. The sheep were assessed 10 months after repair and compared with a similar sized group of normal sheep.

It was found that while there was a reduction in the peak velocity of conduction in the repaired nerves and in the range of conduction velocities, the minimum conduction velocity was within normal limits. There was a diminution in all of the measured variables of nerve morphometry but in no case did this reach statistical significance. These findings are consistent with the view that regeneration of the nerves had taken place to a degree at least as effective as that seen in nerves of a similar size repaired by conventional means.

The idea of supporting the site of nerve repair or bridging a nerve defect by means of a non-neural tube is not a new one. Experimental work with animals was first reported by Glück in 1880¹ and again by Vanlair in 1882.² Both of these workers used decalcified bone as a conduit. Since then arteries,³ veins,⁴ amnion,⁵ fascia⁶ and synthetic materials⁷ have been employed – with limited success – in an attempt to find the ideal material. Apart from the absence of a donor site, the use of entubulation over autologous nerve grafting has other theoretical advantages. The avoidance of trauma to the nerve endings by suture and the tissue reaction this might provoke may be beneficial. The tube may also act to confine the nerve endings and stop neurites being lost to tissues other than target organs. Also, the tube may prevent fibroblasts from entering the site of nerve repair and so hampering regeneration. All of these would help to maintain an optimal microenvironment for nerve around the site of repair.

The cellular mechanisms for the establishment of nerve-to-nerve continuity within a silicone rubber tube after neurotmesis have been studied by Schröder et al.⁸ At 3 days after the creation of a 1 cm nerve defect in a rat sciatic nerve, a meshwork of fibrin had been precipitated in the tube bridging the proximal and distal nerve stumps. Contained in this meshwork were erythrocytes, granulocytes, thrombocytes and some macrophages. Fibrin was arranged mainly longitudinally and there was a narrow cleft between the cord of tissue and the wall of the tube. This cleft had increased by 7 days after surgery, owing to retraction of the fibrin clot, and there were bipolar cells arranged longitudinally on the outer part of the cord. Perineural cells closed the gap between nerve stumps at 18 days and

Schwann cells, fibroblasts and blood vessels occupied the centre of the cord. By 3 weeks after creation of the neurotmesis and gap, axons from the proximal nerve stump had reached the distal stump although they were not yet myelinated. It is thought that the perineurium serves as a diffusion barrier from the outside as well as from the inside of the nerve fascicle, thus providing a defined endoneurial environment for the developing nerve.^{9,10} The similarity between the events which take place after repair by entubulation and repair by suture has suggested a usefulness for the former where microsurgical facilities are not available. Moreover with the recent rise in interest in the enhancement of nerve repair by chemical means, entubulation would be expected to be the ideal means of delivery of neurotrophic factors at the site of repair. It has thus become a subject of much current interest.

Many types of natural, bioprosthetic and synthetic tubes have been suggested for nerve repair. Lundborg has a considerable experience of both experimental and clinical nerve repair and is an advocate of silicone rubber as an ideal material for entubulation.¹¹ Yet in clinical practice, two out of three of Lundborg's nerve repairs with silicone tubes have required a second procedure to investigate “irritation” around the site of repair. Although the importance of this has been discounted in Lundborg's experience, it is clearly undesirable to the patient and not cost effective if revision procedures are to be required after nerve entubulation with silicone rubber. Most experts now agree¹² that a biodegradable nerve conduit is the method of choice if entubulation for nerve repair is to be considered.

In 1981 Lundborg¹³ showed that the proximal stump of a rat sciatic nerve would regenerate diagonally

through the rectangular space of a silicone chamber to meet with a distal nerve stump. Since then many authors have published their success with entubulation using a variety of other materials and nerves. Lundborg successfully used pseudosynovial tubes, formed from the tissue reaction around a silicone rod, to bridge a nerve gap. A thin metal spiral was used to prevent collapse of the tube.⁶ Pham,¹⁴ compared the standard sutured epineural repair of the rat peroneal nerve to repair with a polyglycolic acid tube and collagen glue. Assessment of recovery was carried out by studying nerve morphology and electrophysiological properties which showed significantly better conduction in the entubulation group than the sutured group. There was no difference in nerve morphology between groups. Hentz¹⁵ used polyglycolic acid tubes as a pseudo-perineurium in an attempt to isolate the functional intrafascicular tissues from the extrafascicular fibro-reactive process. In this non-human primate study median and ulnar nerves were repaired by the three techniques of epineural and fascicular repair and fascicular tubulization. This showed no significant difference between the three types of repair. Other authors^{16,17} have similarly failed to show a difference either physiologically or histologically between these three types of repair.

One of the most interesting concepts to be considered recently is the possibility that entubulation with the deliberate creation of a small nerve defect of 3–5 mm, might be superior to epineural suture even when the nerve ends could be brought together without tension.¹² This idea has the theoretical advantage of providing an optimal microenvironment for nerve regeneration and repair by concentrating neurotrophic factors in the gap between the nerve stumps.

If the microenvironment at the site of nerve regeneration is indeed important, the permeability of the tube may also have an effect upon outcome. In the assessment of repair of nerves with a 1 cm defect, macropore collagen conduits give better recovery of function than either semipermeable or non-permeable collagen tubes.¹⁸ It has been postulated that the macropore tube allows neurotrophic factors, being macromolecular proteins, to gain access to the nerve repair site. The internal diameter and wall thickness of the conduit also appear to influence nerve regeneration.¹⁹ The tube must be large enough to accommodate any swelling of the nerve and must not itself cause compression of the nerve by swelling when the tube degrades.

From the above evidence it follows that the ideal nerve conduit should be biodegradable over the time-course of nerve regeneration and maturation and should provoke little or no inflammatory response: nor should it collapse or swell during degradation. It should be able to be manufactured in a range of sizes to fit all diameters of nerves. If chemical growth-enhancing factors are to be added, the tube must allow them to be contained or delivered locally in adequate concentration and not permit their dissipation by diffusion away from the site. A material that meets these requirements is controlled release glass (CRG).

Controlled release glasses are a new class of materials that have been developed during the last decade. They

are inorganic polymers, normally based on phosphates of sodium and calcium, which have been converted into a glassy form by melting the constituents at about 1000°C. They dissolve in water completely, leaving no solid residue. The rate of solution can be selected by adjustment of the composition and physical form of the CRG and is constant for as long as any of the material remains. The product can be produced in many physical forms: as a powder or granules, fibre or cloth, tubes, or as cast blocks of various shapes.

Elements other than sodium and calcium, including most metals and their oxides and a limited number of inorganic anions, can feature in the composition of the glass. These elements which may be biologically active, can then be delivered at a constant rate into an ambient aqueous medium as the CRG dissolves. This principle has found application in veterinary medicine as a means of delivering such diverse substances as trace elements, anthelmintics and vaccines.

In the course of developments of this type the biocompatibility and absence of toxicity of CRG based on $\text{Na}_2\text{O}-(\text{Ca},\text{Mg})-\text{O}-\text{P}_2\text{O}_5$ with and without other constituents have been investigated. In applications differing as widely as use in orthodontic devices,²⁰ in nutritional experiments with rats and in controlled supply of Cu, Co and Zn in cattle,²¹ no ill effects were observed. When CRG pellets were implanted subcutaneously, intramuscularly and intraperitoneally in rats, sheep and cattle, reaction at the implant site was limited to a sterile fibrous encapsulation less well developed than that expected from biocompatible surgical materials.²²

Other applications of CRG in the $\text{Na}_2\text{O}-\text{CaO}-\text{P}_2\text{O}_5$ system have been found as potential bone graft adjuncts or substitutes. No sign of cytotoxicity was observed in mouse fibroblasts²³ nor after soft tissue implantation in sheep.²⁴ In further experiments with bone no ill effects nor bio-incompatibility could be detected.²⁵⁻²⁸

Materials and methods

Ten adult female Scottish Black-face sheep each weighing approximately 60 kg were separated into two equal sized groups designated control group and repair group. General anaesthesia in each sheep was induced intravenously using a combination of midazolam (0.5 mg kg⁻¹; Hypnovel; Roche) and etomidate (0.5 mg kg⁻¹; Hypnomidate; Janssen). The sheep were intubated with a cuffed endotracheal tube and their lungs were ventilated with a fresh gas flow of 2 l min⁻¹ of oxygen and 4 l min⁻¹ of nitrous oxide. Anaesthesia was maintained with 1–2% halothane. Neuromuscular blockade was not used.

In all of the sheep with each lying on its right side, the entire area between the ear and snout on the left side was shaved and prepared for surgery. Silver/silver chloride transcutaneous recording electrodes were positioned with the cathode over the motor point of depressor labii maxillaris – a muscle which, in the sheep, acts like a guy-rope to anchor the upper lip and which is uniquely innervated by the buccal branch of the facial nerve. The anode was placed nearby over the

same muscle. A ground electrode was placed over levator nasolabialis – a muscle between the nose and the eye. The position of the buccal branch of the facial nerve was thus identified and mapped out using transcutaneous supramaximal constant-current stimulation (see below) from a hand held stimulator (Medelec Sapphire; Vickers, Woking, UK).

Full sterile procedure was used throughout. In all of the sheep a skin incision was made over the buccal branch of the facial nerve and skin flaps raised for about 2 cm on either side of the nerve. With vision assisted by the use of an operating microscope, the nerve was separated from underlying tissues over a length of about 8 cm. In the control group, nothing further was done so that these constituted "sham-operated" animals and in effect were no more than normal nerves. In the experimental group the nerve, having been exposed as above, was divided, using a Meyer neurotome about 2 cm distal to its emergence from the parotid gland. This left a 6 cm distal stump over which was passed a sterile (γ -irradiated) biodegradable glass tube of length 4 cm and internal diameter 4 mm. The two nerve stumps were brought together and held in place while the glass tube was moved proximally so that its mid-point lay at the site of coaptation of the two nerve stumps. The tube and nerve stumps were held in place by two 10/0 polyamide sutures placed through the epineurium of the nerve and through small holes drilled at each of the two ends of the tube. These sutures relieved any tension in the nerve ends and prevented their separation by retraction (Fig. 1). The site of approximation of the nerve stumps within the tube was marked for future reference by placing a large "Ligaclip" (Ethicon UK) in tissue outside the CRG tube. The wound was closed with 4/0 Vicryl (Ethicon UK Ltd, Edinburgh) interrupted sutures to subcutaneous tissue and a 5/0 Vicryl subcuticular stitch to skin.

The sheep, usually fully mobile within an hour of surgery, were kept in the animal house for 7 days. Thereafter they were returned to their farm without restriction of activity.

Ten months after nerve repair, the sheep were anaesthetised as before, and the sites of repair exposed through the original incisions. It was essential to dissect very carefully around the parotid gland as the release of any parotid secretion into the stimulating or

recording field caused massive depolarisation of the nerve and rendered it inexcitable. All electrophysiological tests were performed using a Medelec Sapphire EMG machine (Medelec, Old Woking, UK) in order to stimulate the facial nerve and record the associated compound muscle action potential (M-wave) in depressor labii maxillaris.

A ground electrode was placed on the skin over the nasal bone. A 6 mm silver/silver chloride disc recording electrode (Medelec, Old Woking, UK) (cathode) was placed over the motor point of the depressor labii maxillaris. A second 6 mm silver/silver chloride disc recording electrode (reference anode) was placed on the skin 1 cm superior to the insertion of the levator nasolabialis muscle over the nasal bone. A small amount of electrode cream (Grass Instruments Co., Maine, USA) was placed on each recording electrode prior to placement on the skin to improve both electrode adhesion to the skin and M-wave recording.

A non-traumatic bipolar platinum wire stimulating electrode (S_1) was placed on the facial nerve distal to the site of repair, approximately 5 mm proximal to the zygomaticus muscle. A square wave stimulus of 50 μ s duration of supra-maximal current intensity (30% above maximal stimulation) was then delivered to the facial nerve. The resulting M-wave recorded from depressor labii maxillaris was filtered digitally and amplified before being displayed on the EMG screen. The change in voltage from the isoelectric baseline to the peak (point of inflexion) of the M-wave was taken as the M-wave amplitude. Where two or more peaks were present in the positive phase of the M-wave, the amplitude was taken as the distance from the baseline to the largest peak. Clinical electrophysiological conventions were followed so that the area of the positive phase of the M-wave was defined as the M-wave area and the latency of the M-wave was the distance along the x-axis from facial nerve stimulation (stimulus artefact) to the return to baseline after muscle depolarisation.

A second bipolar surgical stimulating electrode (S_2) was placed on the facial nerve proximal to the site of repair ensuring that the distance between it and (S_1) was greater than 5 cm. The facial nerve was stimulated with the same intensity as above. The latencies (t_2) and (t_1) from stimulation at (S_2) and (S_1) to the initial deflection of their M-waves were determined and the

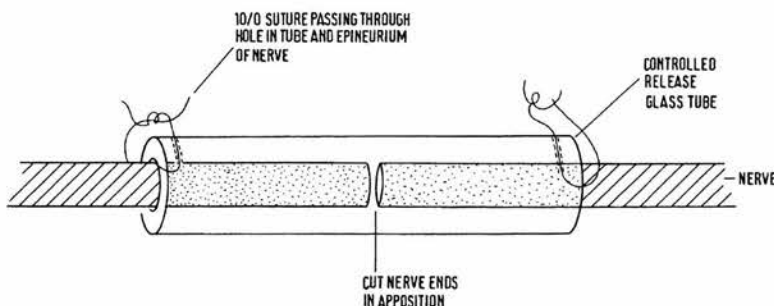


Fig. 1

Figure 1—A diagram to illustrate the placement and fixation of the nerve stumps within the biodegradable glass tube.

distance between the cathodes (S_2) and (S_1) was then determined so that the conduction velocity of the nerve segment between the two stimulation sites could be calculated using the formula:

$$CV_{(\max)} = \frac{(S_2 - S_1)}{(t_2 - t_1)}$$

where:

$$CV_{(\max)} = \text{peak conduction velocity m s}^{-1}$$

$$(S_2 - S_1) = \text{segment length i.e. distance between the stimulating electrodes (mm)}$$

$$(t_2 - t_1) = \text{difference in latencies at } (S_2) \text{ and } (S_1) \text{ (ms)}.$$

The stimulating electrodes and recording electrodes were kept in the same position in order to allow measurement of the minimum conduction velocity. A supra-maximal stimulus of 50 μ s duration was delivered to the facial nerve at the distal stimulating electrode (S_1). After 0.1 ms a second supra-maximal stimulus of 50 μ s duration was applied to the facial nerve at the proximal stimulating electrode (S_2). The time interval between the stimuli applied at (S_1) and (S_2) was increased by 0.1 ms increments until a second M-wave of latency (t_f) was just visible. The time interval between (S_2) and (S_1) was further increased until the second M-wave had increased in area to 95% of the area of the first M-wave. Its latency (t_f) was recorded. The difference between the latencies ($t_f - t_i$) represented the conduction delay between the fastest and slowest nerve fibres. Therefore, the minimal conduction velocity could be calculated by applying the following formula:

$$CV_{(\min)} = \frac{(S_2 - S_1)}{(t_f - t_i)}$$

where:

$$CV_{(\min)} = \text{minimum conduction velocity m s}^{-1}$$

$$(S_2 - S_1) = \text{segment length i.e. distance between the stimulating electrodes (mm)}$$

$$(t_f - t_i) = (\text{latency of full second M-wave}) - (\text{latency of inchoate second M-wave}); \text{ (ms)}$$

By convention, in clinical neurophysiological studies the anode is placed over the tendon of the muscle in question. However, in the present experiment the tendon of the depressor labii maxillaris was under the levator nasolabialis both of which are supplied by the facial nerve. Placement of the anode over the tendon of the depressor labii maxillaris would produce interference from the levator nasolabialis and produce an inaccurate M-wave recording. By moving the anode to a position on the nasal bone the cross-interference from the levator nasolabialis was reduced.

When physiological measurements were complete, samples of nerve tissue distal to the site of the repair were taken. Nerve tissue from the corresponding sites of the control animals was also harvested.

Specimens were fixed initially in cacodylate buffered glutaraldehyde before being trimmed and cut into 0.5–1.0 mm slices. These were then left in 1% cacodylate buffered osmium tetroxide for 24 h. Adequate

osmication is essential for myelin preservation. Excess osmium was removed by washing in buffered 10% sucrose followed by 10% alcohol. At this stage, specimens were transferred to an automatic processor for dehydration in graded concentrations of alcohol and subsequent infiltration with Araldite after which they were embedded in fresh Araldite.

Semi-thin (1 μ m) sections for light microscopy were cut using a Porter-Blum ultrathin microtome and floated onto glass slides. The specimens were then stained with either toluidine blue which stains all structures to a varying degree or paraphenylenediamine which is a more specific stain for osmiophilic material – in this case the myelin sheaths.

The sections were viewed using a compound microscope (Zeiss Oberkochen) at magnifications of 400 \times and 1000 \times . Morphometric analysis (axon diameter, fibre diameter and myelin sheath thickness) was carried out using a VIDS III computerized image analysis system (Analytical Measuring Systems Ltd, Cambridge). Measurements were made using a cursor and digitising tablet. The system was calibrated against a standard 1 mm graticule. Using a random sampling technique²⁹ the axon and fibre diameters of 200 nerve fibres were measured from each section. Half-normal plots were constructed to test for outliers which were rejected. The normality of the data was tested using the normal plot method for small samples of electrophysiological data and a one tailed Kolmogorov-Smirnoff and χ -squared test for the large samples of morphometric data. All data were found to be normally distributed. The entire cohort of data was then tested for any difference between groups by means of an F-test and since differences were found, two-sided *t*-tests were used to determine where these differences lay and the probability that groups were likely or unlikely to have been drawn from the same population. Data handling was carried out using an IBM computer interfaced to the VIDS III system. Statistical analysis and graphics were performed using Lotus spreadsheets, (Lotus Corporation, USA) and "CSS: STATISTICA", (StatSoft, Tulsa, USA).

Results

No significant complications of surgery were seen. One animal in the repair group died of an unrelated illness and was thus excluded from the study. The resulting small sample size necessitates a degree of caution when interpreting the results.

At the time of assessment all of the glass tubes were found to have dissolved completely and all of the repaired buccal nerves were in continuity. There was no evidence of constriction of the nerve which had a uniform diameter along its whole length.

Electrophysiological assessment of the four remaining sheep in the repair group demonstrated a level of functional recovery comparable to that which has been seen with other methods of nerve repair.³⁰ As shown in Table 1, the mean maximum conduction velocity was significantly reduced ($P = 0.01$) when the repair group was compared with the control group (i.e. normal animals). This finding is common to all forms

of nerve repair and is probably attributable to the failure of fibre diameter and inter-nodal length to recover to normal levels.^{30,31} There was no significant difference between the minimum conduction velocities of the control and repair groups: a finding consistent with the idea that it is the large diameter, fast conducting fibres which fail to recover after nerve repair. There was a clear and significant ($P = 0.006$) diminution in the range of conduction velocities found in the repaired nerves. This is a reflection of the above morphological changes and is to be expected after any form of nerve repair.

From Table 1 it can be seen that the mean fibre diameter, axon diameter and myelin sheath thickness were all reduced in the repair group when compared with the normal control group, however none of these results reached statistical significance. The results are displayed graphically in Figure 2 and Figure 3.

Discussion

The object of the present experiments was to test entubulation with controlled release glass as a method of repairing nerves. There appears to have been no adverse reaction due to the presence of the glass in either its solid or dissolved form. In all cases there was successful reconnection and maturation of the regenerated nerves. The properties of the repaired nerves were different from normal controls and this is to be expected in any regenerated nerve regardless of the method of repair. No direct comparison with other methods of repair has been made here but it is worth noting in passing that the reduction in mean peak conduction velocity of 32.20% compares very favourably with that of 35.67% seen at an identical site after repair by means of a short freeze-thawed muscle autograft.³⁰ There are no available data for repair by direct

Table 1 Mean \pm standard error of the mean of all of the variables which were measured in the study. The significance levels for the difference between the mean values of the repair group and the control group for each variable are also given.

| Variable | Control group Mean \pm SEM | Repair group Mean \pm SEM | Significance for difference (P) (control group v repair group) |
|--|---------------------------------|--------------------------------|---|
| Fibre diameter (μm) | 7.81 \pm 0.32 | 6.75 \pm 1.11 | 0.34 |
| Axon diameter (μm) | 4.17 \pm 0.18 | 4.14 \pm 0.61 | 0.96 |
| Myelin sheath thickness (μm) | 1.82 \pm 0.07 | 2.17 \pm 1.03 | 0.71 |
| Maximum conduction velocity (m s^{-1}) | 63.32 \pm 4.67 | 42.93 \pm 2.92 | 0.01 |
| Minimum conduction velocity (m s^{-1}) | 30.31 \pm 3.74 | 30.59 \pm 3.49 | 0.96 |
| Range of conduction velocities (m s^{-1}) | 33.01 \pm 4.60 | 12.33 \pm 1.79 | 0.006 |

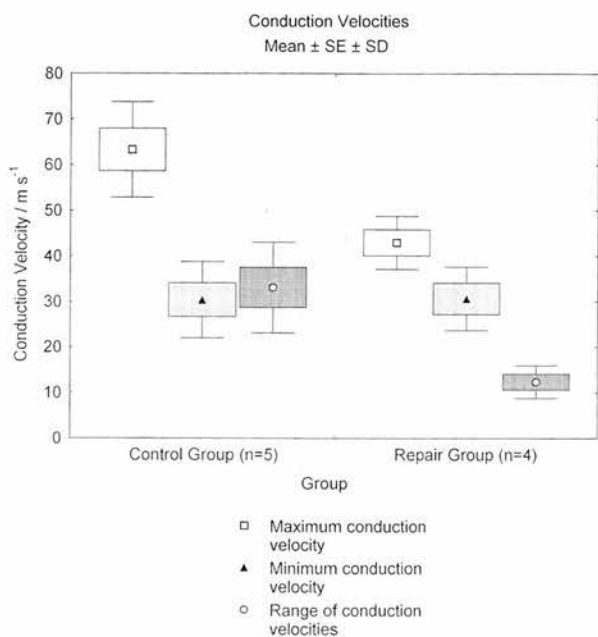


Fig. 2

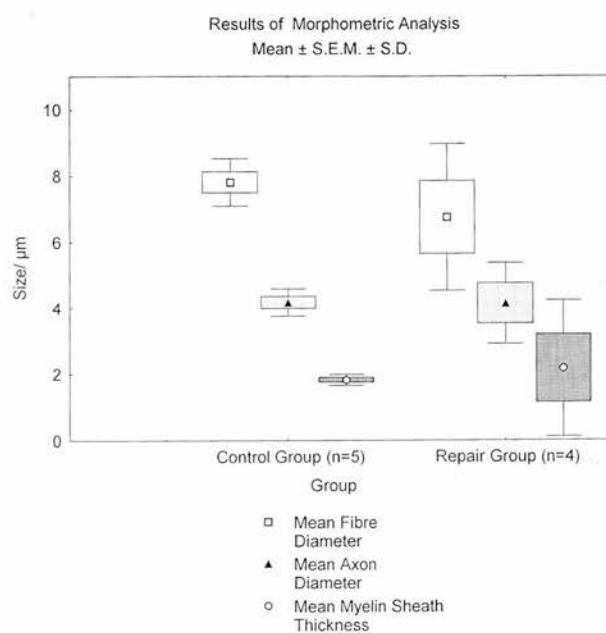


Fig. 3

Figure 2—A graphical representation of the mean (point) \pm standard error of the mean (box) \pm standard deviation (whisker) for the maximum conduction velocity, minimum conduction velocity and range of conduction velocities measured in each of the experimental groups.

Figure 3—A graphical representation of the mean (point) \pm standard error of the mean (box) \pm standard deviation (whisker) for the nerve axon diameter, nerve fibre diameter and myelin sheath thickness measured in each of the experimental groups.

epineurial suture (neurorrhaphy) at this site – that is the subject of current work in our laboratory – however a study by Fullarton¹² in the rat showed a decrease of mean peak conduction velocity of 19% from control values after simple epineurial neurorrhaphy. It is to be expected that results in rats would be superior to those in larger animals and that repair by direct suture of the nerve would result in better regeneration of large fibres than repair by means of any sort of graft. Results in an entirely motor nerve would be expected to be better than those in a mixed nerve. The results seen here, with the use of a biodegradable glass tube appear, therefore, to be of the order of what might be expected for direct repair in a large animal model. This being the case, it is probably fair to say that the technique which has been used here would be entirely acceptable as a means of repairing nerves and as an alternative to direct end-to-end suture.

The advantages of the technique used here fall into three categories. First, where the *matériel* for the microsurgical repair of nerves is unavailable or where the skill required for such repair is absent, the use of a glass tube is a simple expedient which obviates the need for either. Second, if it comes to pass as has been suggested recently, that the preservation of a small gap without suture is the optimal method of repairing nerves,¹² then the use of a biodegradable glass tube provides such a means without the currently unacceptable need for removal of a constricting tube at a later stage. Third, and perhaps most interestingly, if future improvement in the surgical repair of nerves is to be brought about by the chemical enhancement of the regenerative process, then for the first time, the biodegradable glass tube offers an appropriate means of temporarily isolating the site of repair for the localised delivery of the chemical enhancing agent. This agent may be contained in a gel enclosed in the tube or may be bound to the glass itself or delivered into the tube from an implanted osmotic pump. All of these options, which are currently being assessed in our laboratory, allow variation in the rate and time-period of delivery to be made whilst confining the delivery to the site of repair. Additionally the tube, after its rôle as a support and delivery system is over, can be relied upon to dissolve completely and not to constrict the nerve as maturation of the regenerated fibres takes place.

From the present experiments, which must be considered a preliminary report, it nevertheless appears that the entubulation method of repair using controlled release glass may be of use and value both in the clinical field and in the laboratory especially in the elucidation of the effects of neurotrophic factors. It will now be necessary to make a more formal comparison with conventional methods of repair and to examine how this substance may be used in the future.

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BIODEGRADABLE CONTROLLED RELEASE GLASS IN THE REPAIR OF PERIPHERAL NERVE INJURIES

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The experiments in this paper were concerned with the recovery of function and ease of application of an entubulation technique using a biodegradable, controlled release glass tube (CRG) for the repair of a transected peripheral nerve. The peroneal nerves of 15 New Zealand White rabbits were repaired with either a CRG tube filled with freeze-thawed muscle, or a conventional freeze thawed muscle graft (FTMG). These were compared with controls in which a CRG was used to enclose the cut ends of a nerve separated by a 1 cm gap. Electrophysiological and morphometric assessment was carried out 6 months after repair. No statistical difference was found in any test between the FTMG and the CRG tube filled with freeze thawed muscle. The CRG tube and 1 cm gap produced inferior levels of recovery of function when compared with the other two repair groups.

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INTRODUCTION

Repair by entubulation of a peripheral nerve has been studied with limited success by several authors. Some of the materials which have been considered include decalcified bone (Weiss, 1944), artery (Huber, 1920; Weiss and Davis, 1943), vein (Wang et al, 1993) and non-biodegradable synthetic materials such as silicone rubber tubes (Lundborg and Hansson, 1980). Most of these techniques have not been adopted clinically because of poor levels of recovery or because they were inappropriate in clinical practice.

The technique has several theoretical advantages: it is simple to perform and the anchorage of the nerve stumps to the tube is a less delicate procedure than epineurial or fascicular suture. It may even be that a microscope is not required. In addition, and perhaps most importantly for the future, the isolation of the site of repair within a tube may allow for chemical manipulation of the regenerative process by means of, for example, neurotrophic factors. However it could also be argued that the presence of a tube may offer mechanical inhibition to nerve revascularization.

Lundborg et al (1982) have suggested that the maintenance of a very small gap (6 mm) between the nerve ends allows for the accumulation of naturally occurring growth factors which may improve regeneration and which are restricted from access to the nerve ends when the latter are closely approximated by suturing. There is some clinical evidence to support this view (Lundborg et al, 1991) but the continuing presence of the silicone rubber tubes which have been used in the published studies has not been without its problems.

There is therefore a most compelling argument for finding a biodegradable material which can be used for entubulation repair. A rigid, non-porous, non-tissue-reactive tube which maintains its structural integrity throughout its existence is the obvious ideal. In the past, several substances have been used (Archibald et al, 1991; Hentz et al, 1991) but most these depart from the ideal model by being porous or fibrogenic or both.

For the successful clinical application of a biodegradable synthetic material for peripheral nerve repair, several criteria must be met. First, the tube must not provoke an inflammatory reaction and should ideally prevent the ingrowth of fibrotic scar tissue. The internal lumen must be large enough to accommodate the initial swelling of the nerve while allowing the axons to migrate without obstruction. The tube must be completely reabsorbed once the migrating neurites have established a connection in the periphery. The correct original geometric alignment of the nerve should ideally be maintained and the nerve should be secured to the tube away from the transected ends to avoid any possible trauma to the nerve stumps at the suture site. Finally, the tube must maintain its structural integrity for the time required for the migration of the slowest neurites into the distal stump.

The tube used in the present study is made of biodegradable glass made of inorganic polymers based on phosphates of sodium (Burnie et al, 1983). The solubility rate of the controlled release glass (CRG) can be modified by altering the composition and physical form of the glass constituents.

The present study was designed to compare a CRG tube filled with freeze-thawed muscle, or a conventional freeze thawed muscle graft (FTMG without a tube) with controls in which a CRG was used to enclose the ends of a nerve which had been cut and separated by a 1 cm gap.

The purpose of the present experiments was to consider the practicality of the entubulation technique for future clinical use and to assess the potential use of the tube as a means of confining cellular or humoral materials at the site of repair.

METHODS

Four groups, each of five New Zealand White rabbits (weight 3.0-3.5 kg), were used. The groups were a group of normal animals and groups in which the left peroneal nerve had been divided and repaired with: a 1 cm freeze thawed muscle autograft (FTMG); a CRG tube filled

with 1 cm length of randomly arranged freeze thawed skeletal muscle (CRG-FTMG); and a CRG tube with a 1 cm gap between the divided ends of the nerve (CRG-GAP).

Anaesthesia was induced using an I.P. injection of Hypnovel (fentanyl and fluanisone, Janssen Pharmaceuticals Ltd) 3 ml/kg and an IM injection of Hypnorm (midazolam, Roche Products Ltd) 2 mg/kg and maintained using 2% halothane (May & Baker, Rhone-Poulenc Group, UK) with a gas flow mixture rate of O₂ 1 l/min and N₂O 500 ml/min delivered through a mask.

All surgery was performed using conventional sterile procedures. The skin was shaved on the lateral aspect of the left thigh and an incision was made from the sciatic notch to the knee. The skin was then mobilized and reflected and the fascial plane between the hip flexors and extensors was divided to expose the sciatic nerve. Using an operating microscope to assist vision, the tibial and peroneal divisions of the sciatic nerve were separated for a distance of 4 cm directly proximal to the knee. The peroneal nerve was then divided. A segment of the peroneal nerve was removed to create a 1 cm deficit and this was repaired by one of the methods described above. The subcutaneous tissue was closed with four to six interrupted 4/0 Vicryl (Ethicon UK Ltd, Edinburgh) sutures and the skin was closed with a 4/0 Vicryl continuous subcuticular stitch.

The CRG tubes (Giltech, Ayr, UK) were 4 cm in length with an internal diameter of 4 mm. Two small holes at each end of the tube were drilled approximately 5 mm from the edge and 180° apart from each other. The tubes were then individually packaged and γ -irradiated for sterilization.

In the CRG-GAP group the proximal and distal segments of the nerve were placed in opposite ends of a CRG tube. This was carried out without any rotation of the nerve so as to maintain the correct alignment of the two ends. Two interrupted 10/0 polyamide (Ethicon UK Ltd, Edinburgh) sutures were placed approximately 5 mm from each end of the nerve segment and secured through the holes in the CRG tube (Fig 1). This removed tension from the nerve and supported it so that a 1 cm gap could be maintained within the tube and between the two stumps.

In the FTMG group a 2 × 2 × 1 cm block of parallel muscle fibres was removed from the coccygeofemoris muscle. An FTMG was prepared (Glasby et al, 1986) and sutured to the left peroneal nerve with three to four interrupted 10/0 sutures at each end.

A similar sized block of muscle was removed for the CRG-FMTG group and prepared as described above and once the muscle had thawed, it was minced. These pieces were placed in the centre of the CRG tube ensuring the tube was full for a distance of 1 cm. The ends of the divided nerve were placed in the tube so that they were flush with the freeze thawed muscle. The nerve was secured to the tube at each end using two interrupted

10/0 sutures as before. The animals were then returned to a holding pen.

Assessment was carried out 6 months after repair with each animal being anaesthetized and having the left sciatic nerve exposed as described above. The left peroneal nerve was separated from the left tibial nerve at both the sciatic notch and directly proximal to the knee. The skin over the extensor digitorum longus muscle (EDL) was shaved and cleaned with chlorhexidine. The left tibial nerve was divided at the sciatic notch and at the knee to prevent any electrical cross-interference.

All electrophysiological tests were performed using a Medelec Sapphire EMG machine (Medelec, Old Woking, UK). Single fibre electromyography recording of jitter was carried out in the EDL muscle (Trontelj and Stålberg, 1992). The intramuscular terminal branches of the motor nerve received supramaximal bipolar stimulation using two monopolar needle electrodes (Medelec, Old Woking, UK). The resulting motor unit action potential (MUAP) was recorded and the latency between stimulation to the peak of the MUAP was measured and called the interpotential interval (IPI). Fifty consecutive IPIs were measured and the mean consecutive difference (MCD) between the IPIs was calculated (Lenihan et al, 1997, Stålberg and Trontelj, 1994). The MCD or jitter was measured on 20 different intramuscular terminal branches.

A ground electrode was then placed on the skin over the biceps femoris and a 6 mm silver/silver chloride disc electrode (Medelec, Old Woking, UK) was placed over the motor point of the EDL and used as the cathode. A second 6 mm silver/silver chloride disc electrode was placed distal to the first electrode over the tendon of insertion of the EDL. A small amount of electrode cream (Grass Instruments Co., Maine, USA) was placed on each recording electrode before placement on the skin to improve the contact. Each electrode was then secured with tape to prevent any movement during stimulation.

A non-traumatic platinum wire stimulating electrode (S1) (Harvard Electronics, USA) was placed on the left peroneal nerve distal to the site of repair. A second non-traumatic stimulating electrode (S2) was placed on the left peroneal nerve proximal to the site of repair. Care was taken to ensure that the distance between the electrodes was greater than 3.5 cm. A square wave stimulus

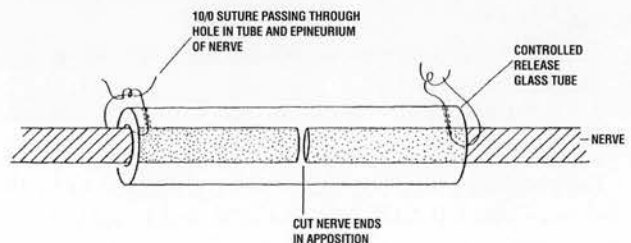


Fig 1 A diagram of the method of anchorage of the nerve ends within the CRG tube.

of 50 μ s duration and of supramaximal current intensity (30% above maximal stimulation) was used for all stimulations in this experiment.

The peroneal nerve was stimulated at the S1 electrode. The resulting compound muscle action potential or M-wave was recorded from the EDL, filtered digitally and amplified before being displayed on the EMG screen. The change in voltage from the baseline to the peak of the M-wave was taken as the M-wave amplitude. When two or more peaks were present in the positive phase of the M-wave the amplitude was taken as the change in voltage from the baseline to the largest peak. The area of the positive phase of the M-wave was defined as the M-wave area. The time from stimulation to the initial M-wave deflection was taken as the latency.

A stimulus was then applied to the peroneal nerve at S2. The latency as defined above was recorded. The maximum conduction velocity was then calculated using the following formula:

$$CV_{\max} = \frac{d_2 - d_1}{t_2 - t_1}$$

Where:

$d_2 - d_1$ = the segment length between the S2 and S1 electrodes (mm)

$t_2 - t_1$ = the latency difference from the S2 and S1 stimuli (ms).

The minimum conduction velocity was measured by using a collision technique (Hopf, 1963). A stimulus was applied to the peroneal nerve at S1 and S2 simultaneously and the resulting M-wave was recorded. A time delay from the S1 stimulus to the S2 stimulus was created in 0.1 ms increments until a second M-wave was visible. The time delay between S1 and S2 is the time required for the fastest impulses to travel between S1 and S2 and is denoted l_1 . The latency between S1 and S2 was further increased until a full second M-wave (95% of the original positive M-wave area) was recorded. This latency represents the time it takes the slowest impulse to travel between S1 and S2 and was called l_2 . The difference between the latencies of l_1 and l_2 represents the conduction delay between the fastest and slowest nerve fibres. Therefore the minimum conduction velocity was calculated using the following formula:

$$CV_{\min} = \frac{d_2 - d_1}{l_2 - l_1}$$

Where:

$d_2 - d_1$ = the segment length between the S1 and S2 electrodes (mm)

$l_2 - l_1$ = (latency of the full second M-wave) - (latency of the initial second M-wave); (ms).

On completion of these electrophysiological tests, the tendon of the left EDL was cut and tied to a steel wire which was connected to a Harvard isometric tension transducer (Harvard Electronics, USA). The tension

transducer was connected to an digital oscilloscope (Gould, UK). The Sapphire EMG was also connected to the oscilloscope by a co-axial 75 Ω cable to synchronize stimulation of the peroneal nerve and subsequent recording of the EDL muscle twitch. A stimulus was applied to the peroneal nerve by means of the Sapphire EMG at S2 as described above and the resulting EDL twitch was displayed on the oscilloscope screen.

The peak twitch tension was determined by comparing the output voltage from the isometric tension transducer, which were recorded on the oscilloscope, to a calibration graph of voltage (V) against force (N). The peak twitch-tension is proportional to the heat produced during an isometric twitch (Hartree and Hill, 1921).

The time tension index (*TTI*) represents an "average" tension for the muscle twitch contraction (Hems and Glasby, 1992a; 1992b). The *TTI* is a variation of Hartree and Hill's observation that the area under the curve of a muscle twitch is proportional to the amount of ATP consumed (Hartree and Hill, 1921). The end-point of the muscle twitch is difficult to locate owing to the slow return to baseline. Therefore, the latency from contraction to half-relaxation is used to represent an "average" tension for the muscle twitch. The *TTI* is calculated using the following formula:

$$TTI = \frac{\int_0^{t_{1/2r}} F dt}{t_{1/2r}}$$

Where:

TTI = time tension index (N)

F = tension (N)

$t_{1/2r}$ = time to half-relaxation (ms).

Once all physiological measurements were completed, the peroneal nerve distal to the site of repair was removed. Similarly, after all electrophysiological tests, nerve tissue from control animals at corresponding sites was also harvested. The nerve specimens were prepared in semi-thin (1 μ m) sections for light microscopy (Glasby et al, 1995). Morphometric analysis of axon and fibre diameter was carried out using a VIDS III image analysis system (Analytical Measuring Systems Ltd, Saffron Walden, UK) on 200 nerve fibres using a random sampling technique (Mayhew, 1990).

Statistical assessment of all data was performed using "STATISTICA for Windows" (StatSoft Inc., Tulsa, OK, USA). The electrophysiological data were found to be normally distributed and the means were compared using Student's *t*-test. Because the morphological data were not normally distributed, the use of medians rather than means was more appropriate to test whether the groups were similar. Therefore a non-parametric two tailed Kolmogorov-Smirnoff test was used.

RESULTS

At the time of assessment there was no evidence of the CRG tube in the CRG-FTMG and CRG-GAP groups. The animals in all three repair groups demonstrated some minor degree of difficulty in walking and stiffness in the knee joint. There was no macroscopic evidence of infection in any animal. In the CRG-GAP group there was evidence in all animals of some tenuous regeneration across the gap. In contrast to the animals in the other groups, the regeneration in the CRG-GAP group never had the appearance of a full-thickness nerve.

The value of jitter provides information on the stability of the neuromuscular junction. Jitter is increased in reinnervation because of the growth of unmyelinated nerve twigs and the formation of new immature end plates in the regenerative process results in a lowering of the threshold for both the end plate and terminal motor nerve fibres (Stålberg and Trontelj, 1994). The jitter values for the normal, FTMG, CRG-FTMG and CRG-GAP groups were 13.5 μ s, 20.7 μ s, 19.8 μ s, and 18.5 μ s respectively. All repaired groups demonstrated a significant elevation ($P < 0.05$) from normal with respect to jitter. However, there was no significant difference ($P > 0.05$) between the jitter in the repaired groups. Figure 2 is a plot of the means and standard errors for all groups with respect to jitter.

The mean CV_{max} values for the normal, FTMG, CRG-FTMG and CRG-GAP groups are shown in Table 1. The CV_{max} in the three repaired groups was significantly reduced ($P < 0.05$) from normal.

The minimum conduction velocities for the FTMG, CRG-FTMG and CRG-GAP groups are given in Table 1.

The range of conduction velocities (CV_{range}) represents the difference between the CV_{max} and CV_{min} . There was no significant difference ($P > 0.05$) in the range of velocities between any of the repaired groups or between the

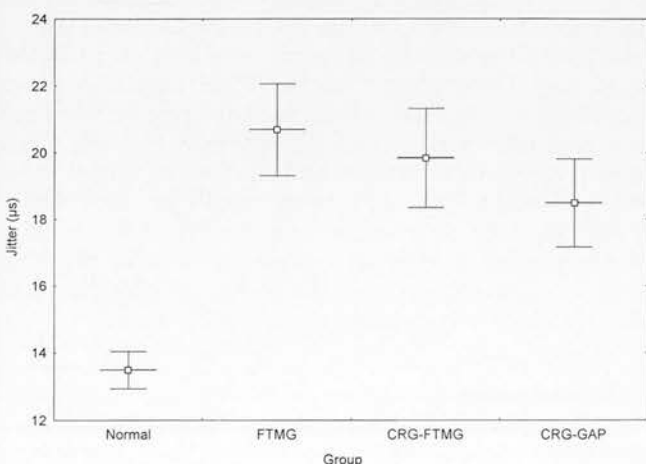


Fig 2 A plot of the means and standard errors for all groups with respect to jitter.

repaired groups and normal. Figure 3 is a box and whisker plot of the means and standard errors of each group with respect to CV_{range} .

Assessment of the M-wave demonstrated a significant reduction in the area and amplitude from normal in the repaired groups ($P < 0.05$) (Table 1). There was no significant difference ($P > 0.05$) between the FTMG, CRG-FTMG and CRG-GAP groups for either variable.

The CRG-FTMG did not demonstrate a significant difference for peak twitch tension and TTI when compared with both the normal and FTMG groups. The FTMG did however, demonstrate a significant reduction ($P < 0.05$) from normal for both of these tests. The muscle twitch amplitude and TTI for the CRG-GAP group was significantly reduced when compared with any other group.

Axon and fibre diameters for the FTMG and CRG-FTMG groups did not differ significantly from each other ($P > 0.05$) whereas in both groups values for these variables were significantly ($P < 0.05$) reduced from normal. These results indicate that the FTMG and CRG-FTMG groups had similar levels of regeneration and fibre maturation. This result is further supported by the values of jitter. When compared with any group, the values for axon and fibre diameter for CRG-GAP were significantly reduced ($P < 0.05$) (Table 2).

DISCUSSION

The results of this study indicate that where a short nerve gap was repaired by means of entubulation with a CRG tube containing conventional graft material recovery was at least as good as the levels obtained with the conventional FTMG. When the entubulation was associated with persistence of the nerve gap, the results were much poorer. In the rabbit there was some evidence of tenuous reconnection which would not be expected to occur in the human. In all cases in which the nerve recovered, the indices of nerve function and of nerve morphology were inferior to those seen in normal nerve but consistent with the levels seen after repair in other studies. Repair by entubulation proved to be a simple technique to perform.

Madison et al (1985) demonstrated that peripheral nerve regeneration was enhanced when the lumen of a biodegradable tube made of glycolic and lactic acids polyester polymers was filled with laminin gel. However, their study used an empty tube as a control and provided no comparison to an established technique for the repair of a transected peripheral nerve. The study also compared gaps of 4 to 5 mm. It has been shown by Lundborg et al (1982) that peripheral nerve regeneration across gaps less than 6 mm produced similar regeneration to direct nerve suture.

When assessing the recovery of function after peripheral nerve repair in the laboratory, it is imperative that the groups to be compared are at similar stages of regeneration. It has been shown that different grafts may alter the rate of regeneration of the neurites without affecting

Table 1—Mean values of the measured electrophysiological values

| Test | Normal | FTMG | CRG-FTMG | CRG-GAP |
|------------------------------------|--------|------|----------|---------|
| M-wave amplitude (mV) | 18.9 | 8.0 | 7.5 | 7.8 |
| M-wave area (μ Vs) | 32.4 | 12.8 | 14.6 | 15.5 |
| Maximal conduction velocity (m/s) | 69.4 | 40.2 | 47.7 | 44.8 |
| Minimal conduction velocity (m/s) | 32.9 | 12.5 | 19.0 | 12.7 |
| Range of conduction velocity (m/s) | 36.5 | 27.8 | 28.7 | 32.1 |
| Peak twitch tension (N) | 5.2 | 2.9 | 3.1 | 1.5 |
| Time tension index (N) | 6.3 | 3.9 | 4.2 | 1.6 |
| Jitter (μ s) | 13.5 | 20.7 | 19.8 | 18.5 |

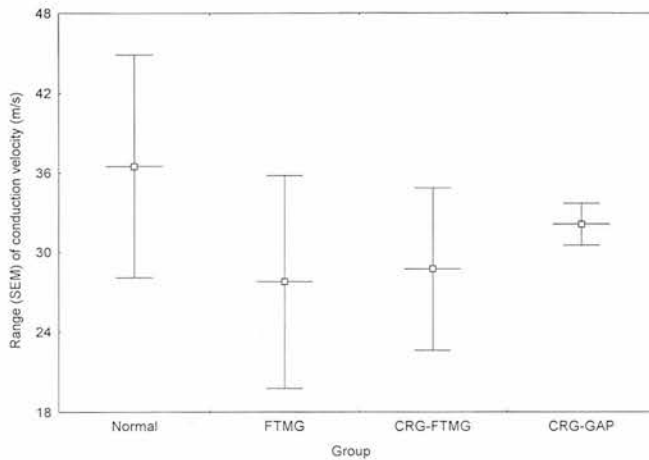


Fig 3 A plot of the means (points) and standard errors (whiskers) for the range (m/s) of conduction velocity for each group.

the quality of recovery of function (Davies et al, 1987). It has also been shown that jitter provides a method of assessing the progression of regeneration by measuring the variability of neuromuscular transmission at the motor end plate (Lenihan et al, 1997). When the migrating neurite reaches the muscle, provided the neurite is of the correct type, a neuromuscular junction will form. The fibre will then mature and this results in an increase in fibre size, of myelin formation around the nerve fibre and an elevation of the end plate potential. All of these result in a lower and more stable jitter value for the neuromuscular junction. By measuring jitter it was possible to evaluate the stage of regeneration.

This finding was further supported by the results of axon and fibre diameter measurement for the FTMG and CRG-FTMG groups. The data obtained for axon and fibre diameter in this study were consistent with previous work (Hems and Glasby, 1992b). The lack of any significant difference between the FTMG and CRG-FTMG groups for axon and fibre diameter indicated that these groups had achieved similar levels of regeneration.

The range of conduction velocity is the difference between the maximum and minimum conduction

Table 2—Mean values of the morphological variables

| Test | Normal | FTMG | CRG-FTMG | CRG-GAP |
|---------------------------|--------|------|----------|---------|
| Axon diameter (μ m) | 4.5 | 1.9 | 2.1 | 1.1 |
| Fibre diameter (μ m) | 9.3 | 4.6 | 4.9 | 3.2 |

velocity. The measurements made in this study appear to indicate that there is a consistent reduction in the velocity of the impulses carried by the fastest and slowest conducting components of the nerve after repair. This would correlate with the leftward shift of the axon and fibre diameter profiles.

The tests for peak tension and *TTI* proved to be the most sensitive in discriminating between the groups studied here. In particular these tests were able to separate the FTMG and CRG-FTMG groups from the normal group. These are difficult tests to perform on humans and large animals but this nevertheless highlights the fact that discriminatory tests do exist and emphasizes the need for further studies to find more of them.

The CRG entubulation technique may have some advantages over other grafting or entubulation techniques. The sutures to secure the nerve ends are not placed at the transected ends which reduces neurite misdirection and death caused by suture trauma. As an alternative the ends of the nerve may be secured in the tube with fibrin glue: thus the CRG may also prove beneficial in situations where microsurgical facilities are not available. A particular advantage is that the CRG tube is easily manufactured and can have its rate of solubility altered to provide a more rapidly or more slowly dissolving tube. A potential benefit of the CRG tube in the laboratory may be to provide a model for studying different grafting methods and to test possible chemical factors which may enhance nerve regeneration.

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An electrophysiological and morphological comparison of the microwave muscle graft and the freeze-thawed muscle graft

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SUMMARY. The purpose of this study was to assess the feasibility of using a microwave oven to produce denatured muscle grafts for the repair of 1 cm deficits in the rabbit peroneal nerve. Recovery of function was assessed after 6 months by electrophysiological and histological examination of the peroneal nerve and target muscles. The results were compared to normal rabbits and to a group whose nerves were repaired with freeze-thawed muscle grafts (FTMG). The results of this study showed that heating muscle in a microwave oven does produce a graft which may be used as an alternative to the FTMG. However, the technical difficulties in preparing a microwave muscle graft make the use of this technique in the clinical setting unlikely.

Introduction

One of the problems associated with the repair of nerves using the freeze-thawed muscle graft (FTMG) is that the freezing process produces considerable shrinkage (up to 50%).¹ Several researchers have attempted to overcome this difficulty by altering the methods by which the muscle is prepared. These methods include heating the muscle in distilled water to 60°C,² freezing the muscle to -25°C followed by immersion in distilled water³ and heating the muscle in a microwave oven at 300 W for 120 s.⁴

Freezing the muscle in liquid N₂ or heating the muscle above 60°C but not above 80°C produce similar changes. There is acute necrosis of myocytes, intramuscular nerves and endothelial cells. Macrophages quickly remove the debris creating evacuated basement membrane tubes which are laminin positive and so provide a path for the regenerating axons.⁵

The production of a functional muscle graft by heating it in a microwave oven is potentially interesting for clinical use because of the availability of microwave ovens. Whitworth et al⁴ demonstrated 60 days after nerve repair in the rat, that a muscle graft (MMG) produced by heating the muscle in a microwave oven provided a substrate for similar levels of axonal regeneration and Schwann cell penetration when compared to the FTMG.

Although the results of experiments by Whitworth et al⁴ suggest a role for the MMG in the clinical setting, these authors did not assess the repair in terms of any indices of nerve function or target organ morphology. They reviewed the results at 60 days, a time which work in our laboratory suggests to be too soon for the attainment of the completion of regeneration.⁶ It seems appropriate now to make a further investigation before recommending the use of MMGs to clinicians.

In this paper the results of comparing the MMG to the FTMG with respect to recovery of function and

morphometric indices of recovery of the regenerated nerve and target muscles 6 months after repair are presented. The experimental animal was the rabbit. The possible use of microwave ovens to create denatured muscle grafts in the clinical setting will also be discussed.

Methods

Fifteen adult female New Zealand white rabbits each weighing between 2.75 and 3.25 kg were separated into three equal sized experimental groups. These were a control group and groups receiving either an FTMG or MMG. Each animal was anaesthetised with an i.p. injection of Hypnovel (midazolam, Roche Products Ltd.) 2 mg kg⁻¹ and an i.m. injection of Hypnorm (fentanyl and fluanisone, Janssen Pharmaceuticals Ltd.) 3 ml kg⁻¹. General anaesthesia was then maintained with a Bain circuit using a mixture of O₂ 1 l min⁻¹, N₂O 0.5 l min⁻¹ and 2% halothane (May & Barker, Rhone-Poulenc Group, UK). The animal was placed on a heating blanket. The rectal temperature was recorded continuously and blood oxygen saturation levels were monitored continuously using a pulse oximeter.

All surgery was carried out in sterile conditions. An incision was made in the lateral aspect of the left thigh. The coccygeofemoralis and biceps femoris muscles were reflected to expose the sciatic nerve. The peroneal nerve (PN) was separated from the tibial nerve (TN) using microsurgical forceps and ensuring that tension and trauma were kept to a minimum. The PN was then divided 3 cm below the sciatic notch and a length of 0.5 cm was removed from the distal segment. This removal, along with retraction of the stumps, produced a 1 cm gap which was either filled with an FTMG or an MMG using 3-4 interrupted 10/0 Ethilon sutures (Ethicon Ltd, Edinburgh, UK).

Care was taken to avoid any tension at the suture line and to maintain the correct rotational alignment of the nerve and graft.

The MMG was prepared by removing a 2 cm × 2 cm × 1 cm block of parallel fibres from the coccygeo-femoralis muscle. This piece of muscle was placed in a sterile container and heated in a domestic microwave for 100 s on 'medium' (350 W). The MMG was then cooled for 2 min, cut to size and sutured into the PN defect. While it is recognised that more accurate microwave ovens exist, the probability of their purchase by Hospital Trusts for use in occasional nerve grafting procedures seems unlikely on account of the expense. The use of a domestic microwave oven thus seemed more realistic in a study which was designed to be applicable to the clinical situation.

The FTMG was prepared by removing a 2 cm × 2 cm × 1 cm block of parallel fibres from the coccygeo-femoralis muscle. This muscle block was wrapped in aluminium foil and submerged in liquid nitrogen until thermal equilibrium was reached. The muscle was then removed and thawed in distilled water, cut to size and sutured into the PN defect.

Regeneration of the PN through the graft was assessed by electrophysiological and morphological methods after 6 months. The rabbits were anaesthetised and monitored as before. The PN was exposed as described above; it was isolated from the muscle upon which it lay by means of an insulating plastic sheet. Moreover, the electrodes were insulated upon the side away from the nerve. Liquid paraffin was not used to provide electrical insulation as this can interfere with subsequent histological processing of the tissue samples. All electrophysiological tests were performed using a Medelec Sapphire EMG machine (Medelec, Old Woking, UK) for stimulating the PN and for recording the associated compound muscle action potential (M-wave). Each physiological result represents the mean of ten observations from which clear outliers were discarded.

The amplitude and area of the M-wave were measured first. Six millimetres of silver/silver chloride disc recording electrodes (Medelec, Old Woking, UK) were placed on the skin over the extensor digitorum longus muscle (EDL). A non-traumatic bipolar stimulating electrode was placed on the peroneal nerve proximal to the graft. The tibial nerve was cut. A square wave constant current stimulus (50 μs) of supramaximal intensity (30% above maximal stimulation) was delivered to the PN. The resulting M-wave was filtered digitally and amplified before being displayed on the EMG display. The change in voltage from take-off to peak (numbers 1 and 2 in Fig. 1) was the M-wave amplitude. The area bounded by the curve 1-3 in Figure 1 was the M-wave area. The duration of the M-wave was the time from 1-5 in Figure 1. These measurements follow the conventions used in clinical neurophysiological studies.

A second non-traumatic stimulating electrode was placed on the PN distal to the graft ensuring that the distance between the electrodes was greater than 3.5 cm. The peroneal nerve was stimulated with the same intensity as described above. The latency from the stimulus artefact to the initial deflection of the

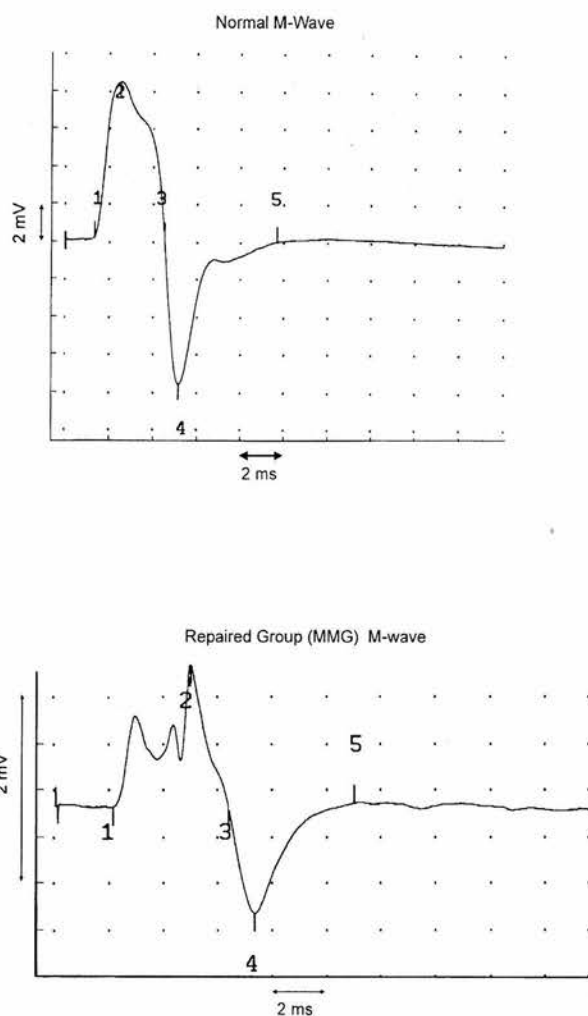


Fig. 1

Figure 1—Example M-waves for normal animals and animals in which the peroneal nerve was repaired with an MMG. The appearance of the M-wave after repair with an FTMG was essentially similar.

M-wave was determined for both proximal and distal stimulation sites. The distance between the stimulating cathodes was then determined so that conduction velocity of the nerve segment between the two stimulation sites could be calculated.

$$CV_{max} = \frac{d}{t_{cv}}$$

where:

d = distance between the stimulating electrodes (mm)

t_{cv} = latency (ms).

The minimal conduction velocity was determined by the use of the collision technique developed by Hopf et al.⁷ The stimulating electrodes and recording electrodes were kept in the same position. A supra-maximal stimulus of 50 μs duration was applied to the PN distal to the graft followed 0.1 ms later by a 50 μs supra-maximal stimulus to the PN proximal to the graft. The resulting M-wave was displayed by the Sapphire EMG machine. The time interval between

the distal and proximal stimuli was increased by 0.1 ms increments until a second M-wave was recorded (t_{m1}). The time interval was further increased until the second M-wave had attained an area of 95% of the first M-wave (t_{m2}). The time between t_{m2} and t_{m1} represented the conduction delay between the fastest and slowest motor fibres. Therefore, the minimal conduction velocity can be calculated by the following formula:

$$CV_{min} = \frac{d}{t_{m2} - t_{m1}}$$

where:

d = distance between the electrodes (mm)

t_{m2} = latency of full second M-wave (ms)

t_{m1} = latency of incipient second M-wave (ms).

The isometric twitch tension for EDL was determined by cutting the tendon of the EDL and attaching it to a Harvard isometric tension transducer (Harvard Electronics, USA) which was connected to a digital oscilloscope (Gould, UK). The PN was stimulated by the Sapphire EMG at the proximal electrode at a supramaximal intensity for a duration of 50 μ s. The Sapphire EMG was connected to the digital oscilloscope through an RS232 port to synchronise stimulation and recording. The amplitude and time tension index (TTI)⁸ of the EDL muscle twitch were calculated with the cursors of the digital oscilloscope.

A 1 cm length of the PN was removed 2 cm distal to the graft and processed for morphometric analysis.⁹ Two hundred fibre and axon diameters were measured at 100 \times magnification with oil immersion from each section using a V.I.D.S. III image analysis system (Analytical Measuring Systems Ltd, Cambridge, UK).

A target muscle supplied exclusively by the peroneal nerve is extensor digitorum longus (EDL). In each of the experimental and control animals this muscle was removed and a thick section taken at the mid-point of the muscle belly and frozen in isopentane which had first been cooled in liquid nitrogen. This method of preparation ensures rapid freezing of the specimen at about -150°C . Frozen specimens were stored at -70°C until analysed. Sections for analysis were cut on a cryostat at -25°C and mounted on glass slides. For estimation of connective tissue content they were stained with Masson's trichrome stain. The proportion of connective tissue in each of these stained sections was estimated by the 'point-counting' technique¹⁰ in which relative proportions rather than absolute amounts or numbers of any type of tissue or cell may be estimated and compared at a number of sampling sites defined by a grid which is used to overlay the section. Where estimation of Type I (slow fibre) and Type II (fast fibre) fibre diameters was required, myofibrillar adenosine triphosphatase staining was carried out at three different values of pH (pH = 4.35, pH = 4.60 and pH = 10.20). Using this technique,¹⁰ the Type I muscle fibres stain more intensely at acid pH and the Type II fibres more intensely at alkaline pH. After staining, the 'narrow diameters' of the stained fibres were measured. Narrow diameter is defined as 'the maximum diameter across the lesser aspect of the

muscle fibre.^{10,19} By the use of this convention, error resulting from any obliquity of the sectioning, is minimised. In each experimental group a sample of 200 fibres was measured. Statistical analysis on all data was performed using STATISTICA 5.0 (StatSoft, USA).

Results

Preparation of the MMG proved difficult. A large number of trials were required to produce a reliable time and power ('wattage') setting to provide a graft that was heated above 60°C but not above 80°C . Variations in the thickness of the muscle reduced the reliability of these settings and made it difficult to ensure that the centre of the graft was heated sufficiently or that the outer edges were not overheated. This is a problem well recognised in the domestic kitchen. It was found that a particular size of muscle block had to be provided consistently to ensure useful results. Even so, 'microwaving' the muscle produced slight charring on the sides of the graft. The MMG did not shrink as much as the FTMG but removal of the charred edges did result in a reduction in the size of the MMG.

Six months after repair, all animals demonstrated some degree of nerve regeneration. There was no difference in the results of any electrophysiological test when the FTMG and MMG were compared. Both repaired groups, when they were compared to normal nerves at the same site, showed a significant reduction ($P < 0.05$) for all electrophysiological variables except M-wave duration.

The mean CV_{max} of the normal nerve, FTMG and MMG were 69.36 m s^{-1} , 40.23 m s^{-1} and 43.08 m s^{-1} , respectively. The corresponding mean values of CV_{min} were 32.98 m s^{-1} , 12.46 m s^{-1} and 10.13 m s^{-1} (Table). Figures 2 and 3 are box and whisker plots of the means and standard errors of the maximum and minimum conduction velocities for all groups.

The animals in the FTMG and MMG groups demonstrated an altered M-wave shape when compared to the normal. The amplitude was reduced and several peaks in the positive phase of the M-wave were often produced. Examples of the repaired group and normal group M-wave shapes are given in Figure 1. Where two or more positive peaks were present, the amplitude was taken from the baseline to the largest peak. The mean M-wave amplitudes for the normal,

Table Mean values of the measured variables

| Variable | Normal | FTMG | MMG |
|------------------------------------|--------|-------|-------|
| M-wave amplitude (mV) | 18.92 | 8.04 | 8.67 |
| M-wave area (mVs) | 32.40 | 12.76 | 14.05 |
| Maximum CV (ms^{-1}) | 69.36 | 40.23 | 43.08 |
| Minimum CV (ms^{-1}) | 32.98 | 12.46 | 10.13 |
| Twitch amplitude (N) | 5.22 | 2.93 | 2.62 |
| Time tension index (N) | 6.27 | 3.93 | 3.50 |
| Axon diameter (μm) | 4.52 | 1.87 | 1.67 |
| Fibre diameter (μm)** | 9.30 | 4.62 | 4.37 |

** indicates significant difference ($P < 0.05$) between FTMG and MMG.

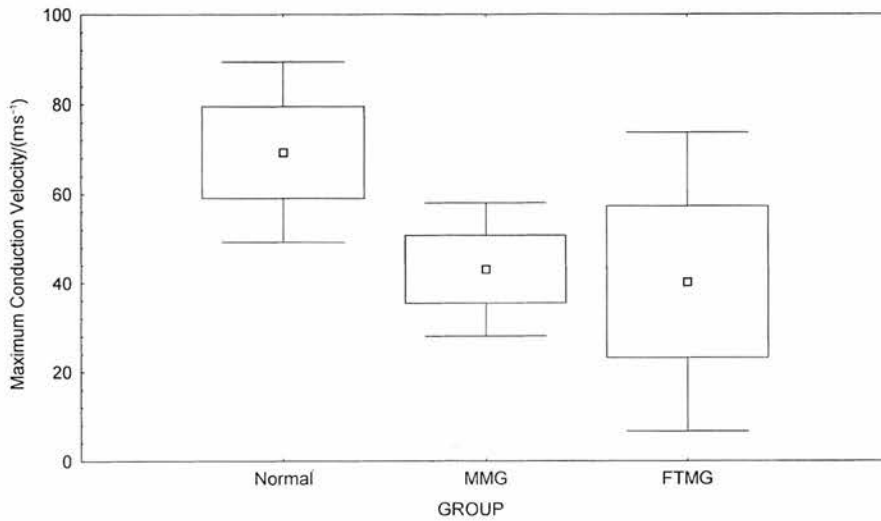


Fig. 2

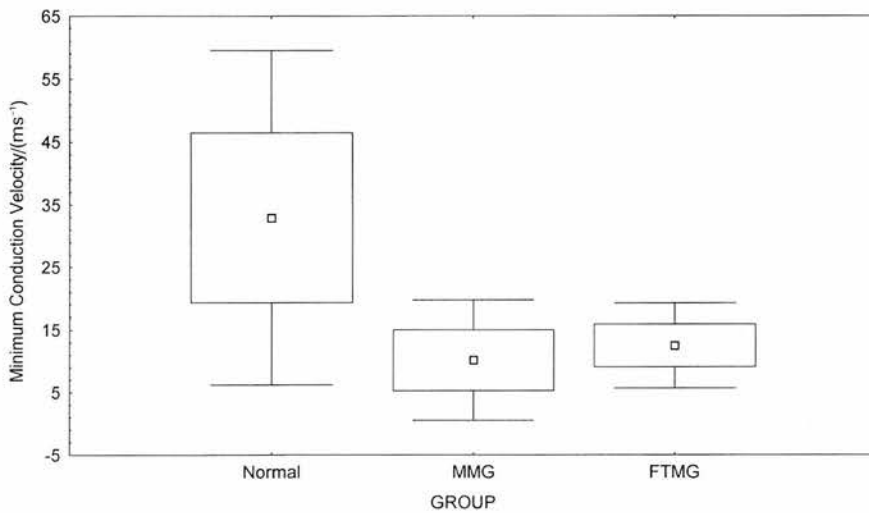


Fig. 3

Figure 2—Mean values (central points), of CV_{max} for each of the experimental groups. The large boxes represent the standard error of the mean and the ‘whiskers’ the standard deviation.

Figure 3—Mean values (central points), of CV_{min} for each of the experimental groups. The large boxes represent the standard error of the mean and the ‘whiskers’ the standard deviation.

FTMG and MMG were 18.92 mV, 8.04 mV and 8.67 mV. There was a corresponding change in the area of the M-wave when the normal and the repaired groups were compared. The mean M-wave areas for the normal, FTMG and MMG were 32.40 mVs, 12.76 mVs and 14.05 mVs. Box and whisker plots showing the means and standard deviations of the M-wave amplitude and area for all groups are shown in Figures 4 and 5.

Muscle twitch amplitude and TTI were also significantly reduced in the animal with repaired nerves. There was no difference between the FTMG and MMG for either of these tests ($P > 0.05$). The Table shows the mean for each test and significant differences where present.

The FTMG and MMG groups demonstrated a significant reduction for both axon and fibre diameter when compared to the normal. There was no difference between the FTMG and MMG with respect to mean axon diameter. However, in the FTMG group, the mean fibre diameter was significantly greater than that of the MMG group. This result implies that the FTMG group had better myelination than the MMG group. The mean values for these measurements are given in the Table.

Muscle connective tissue content was, not surprisingly, always found to be more extensive in muscles supplied by repaired nerves than in normal controls. The relative amounts of connective tissue in the MMG and the FTMG were not found to be significantly different.

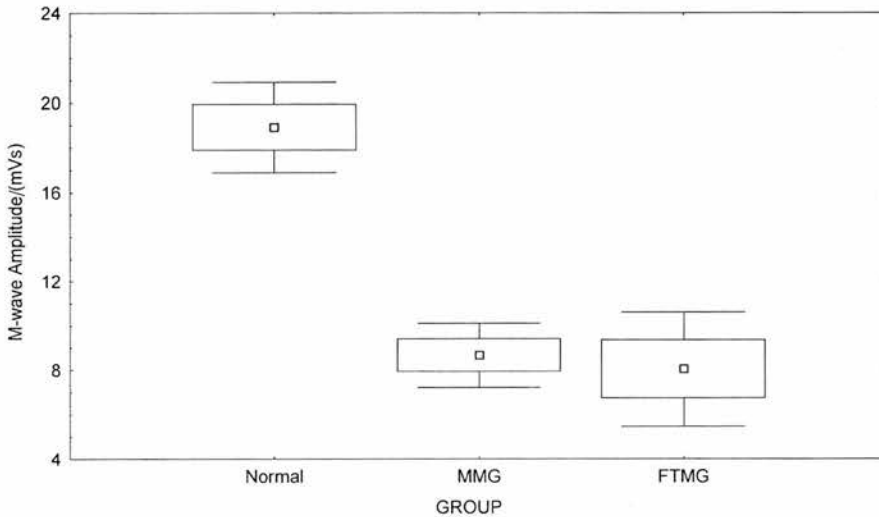


Fig. 4

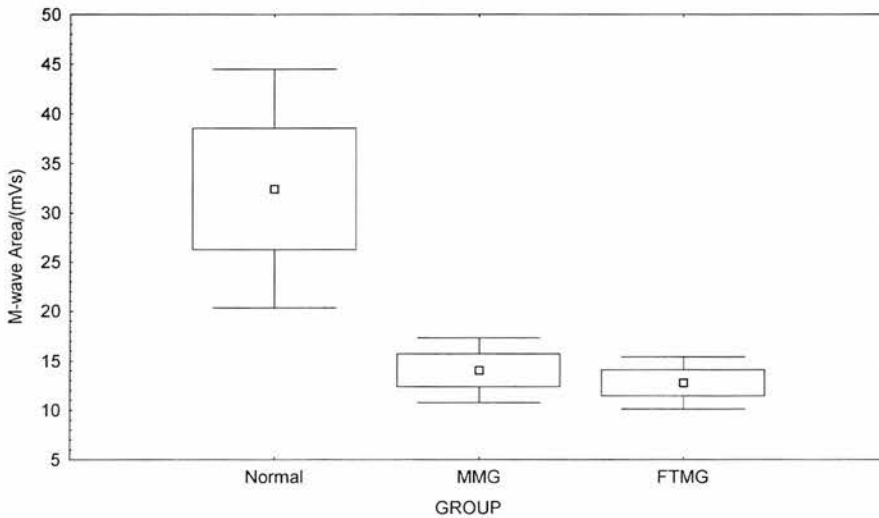


Fig. 5

Figure 4—A box and whisker plot of the M-wave amplitude for all groups. **Figure 5**—A box and whisker plot of the M-wave area for all groups.

When ATPase staining was carried out, it was found that for both Type I and Type II fibres, there was no significant difference in the mean fibre diameters measured in either the MMG or the FTMG groups. Where Type I fibres were concerned there was no significant difference in fibre diameter when normal controls were compared with either MMG or FTMG. However, for Type II muscle fibres, the diameters measured in both MMG (mean = 35.3 μm ; $P < 0.0005$) and FTMG (mean = 34.1 μm ; $P < 0.0001$) were significantly smaller than the diameters of Type II fibres in the controls (mean = 52.4 μm).

Discussion

Nerves repaired with the MMG did not show significantly different levels of regeneration when compared to nerves repaired with FTMG. These results suggest

that heating muscle in a microwave oven may be a suitable method of producing a functioning muscle graft for the repair of 1 cm nerve defects in the rabbit peroneal nerve. However, the preparation of a satisfactory MMG was technically much more difficult than may be inferred from the work of Whitworth et al.⁴ It was also more difficult to prepare an MMG than an FTMG. It has been shown that muscle must be heated above 60°C to produce a useful denatured muscle graft.² However, the muscle must not be heated above 80°C or the laminin will no longer support neurite outgrowth.¹¹ Heating a piece of muscle thoroughly within this narrow temperature range with a microwave oven is inconsistent and calibration is time consuming because of the lack of precise control available in domestic microwave ovens. Settings of 'low', 'medium' and 'high' are all that are usually available and it is therefore not possible for the power in W to be set directly. The only completely adjustable

variable is time. Any differences in the size of the graft require adjustments of the time to ensure that the graft is heated properly and calibration is, in consequence, a matter of trial and error. This problem was removed in our experiment by having a standard size: something which is unlikely to occur in the clinical setting.

This problem does not occur with the FTMG. The graft is submerged in liquid N₂ until thermal equilibrium is reached. Size of graft does not matter as trimming is carried out later. With the freezing process the attainment of thermal equilibrium is absolute and easy to see when all bubbling stops. The latent heat of vaporisation of N₂ and the thermal capacity of muscle are constants and there is thus no question that when the entire block is at -196°C a constant level of thermal insult will have been achieved. Although, the MMG does help in resolving the shrinkage problem associated with the FTMG, the assurance that freezing provided over heating—that the graft was correctly denatured each time—would seem to be more important.

Some confusion exists about how best to prepare an FTMG with 'cracking' or 'shattering' of the graft having been described.^{12,13} Our early papers using small FTMGs in rats recommended pinning the muscle out under tension.^{1,14} We have since abandoned this process and it may be that those who continue to use it find problems with larger blocks of muscle. Our current technique as recommended for clinical use is as follows. A block of muscle is removed and it must be at least 50% larger all round than the anticipated graft. Fat etc. is trimmed off and the muscle is wrapped in sterile foil like a boiled sweet. A ligature is tied round one end of the foil and the free end of the thread passed by the scrub nurse to a 'non-sterile' nurse who freezes the package in liquid N₂ whilst continuing to hold the free end of the thread. When freezing is complete the package is held by the non-sterile nurse above a beaker of sterile distilled water on the scrub nurse's trolley. The scrub nurse cuts the ligature at the level of the package which drops into the water. It is left there to thaw gradually and the package is easy to unwrap in the water with forceps. The graft remains in water until needed when it is dried on a swab and cut to size.

The measurement of maximum conduction velocity CV_{max} provides information on the fastest conducting fibres contained in a nerve. The FTMG and MMG groups showed a respective 42.00% and 37.89% reduction of CV_{max} from normal. CV_{min} provides information about the slowest conducting fibres contained in a nerve.^{7,15,16} Both the repaired groups showed a reduction in CV_{min} with respect to normal. However, there was no significant difference between the FTMG and MMG graft groups in respect of CV_{min} . The implication is that repair of the nerve and subsequent regeneration results in a leftward shift of the velocity profile of the nerve which corresponds to a similar shift in fibre diameter distribution. This shift is maintained even after the completion of the regenerative process and explains why repaired nerves never exhibit identical properties to normal nerves.^{1,6,8,9,12-14}

The area and amplitude of the M-wave approximately parallel the amount of muscle being depolarised

under the recording electrodes.¹⁷ Electrode placement, site of stimulation, cross-talk between other muscles or nerves and/or synchronisation of muscle fibre contraction can have a profound effect on the area and amplitude of the M-wave.¹⁸ To reduce the likelihood of false M-wave recordings, consistency was important. The recording cathode was placed on the skin over the superior lateral belly of the EDL. The recording anode was placed over the tendon of the EDL. The electrode placement and cutting of the tibial nerve helped to minimise any cross-talk from the tibialis anterior or peroneal muscles. The nerve was stimulated directly, proximal to the site of repair. In this situation the area and amplitude should give an approximate representation of the number of fibres being depolarised under the recording cathode. The repaired groups showed a significant reduction from normal but there was no statistical difference between M-wave area and amplitude in the repaired groups. This is consistent with the change in morphology seen after nerve repair.

The amplitude and TTI of a muscle twitch indirectly represent the number of fibres contracting in the muscle.⁸ There was no statistical difference between the FTMG and MMG groups which indicates that they both had an approximately similar number of EDL fibres contracting in response to supramaximal stimulation of the peroneal nerve. The strong correlation between the amplitude and area of the M-wave and the TTI of the muscle twitch is in agreement with this. These findings are supported by the demonstrated increase of connective tissue content at the expense of muscle fibres in muscles supplied by repaired nerves.

There was no significant difference in axon diameter in the repaired groups. The fibre diameter was the only test to demonstrate a significant difference between the FTMG and MMG groups. This represents better myelination in the FTMG group but the reason for this is not clear.

The changes which occurred in the target muscles did not suggest any particular advantage in using either an MMG or an FTMG. The increase in connective tissue in a muscle which has undergone partial degeneration is to be expected and the failure to find any significant difference in the diameters of Type I and Type II fibres when the two grafted groups were compared implies that the repair method adversely affected both target muscles in the same way. The interesting finding, when controls were compared to grafted groups, that while Type I fibres were unaffected, the repair process resulted, in both experimental groups, in a reduction of Type II muscle fibre diameter, probably reflects the supposed greater fragility of these fast fibres which has been described by Dubowitz.¹⁹

Whitworth et al¹ came to the conclusion that 'microwave heating is suitable as an alternative denaturing method for successful muscle grafts and has a potential clinical use'. Our results support the same conclusion under ideal conditions. However, the preparation of freeze-thawed muscle grafts under any conditions would appear to be much easier to achieve than the use of the microwave technique. It is important

to note that the FTMG does not function well if longer than about 5 cm²⁰ and at present there is no evidence that the MMG will support regeneration over distances greater than 1 cm.

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STIMULATED JITTER MEASUREMENT IN THE ASSESSMENT OF RECOVERY AFTER DIFFERENT METHODS OF PERIPHERAL NERVE REPAIR

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The recording of stimulated jitter offers a quantitative method for following the recovery of neuromuscular function after peripheral nerve repair. In groups of rats, electrophysiological recording of jitter was carried out on control animals and on animals 90 days after sciatic nerve division and subsequent repair with either direct end-to-end suture (NS), nerve graft (NG) or freeze thawed muscle graft (FTMG). It was found that values for jitter were highest in the FTMG group. The NS and NG groups demonstrated statistically similar jitter values when compared with each other and with the normal. It was concluded that the speed of nerve regeneration is slower in the FTMG group, at least initially, and that 90 days after sciatic nerve repair the FTMG group had an increase in the number of immature neuromuscular junctions when compared with the NS or NG groups. Jitter measurement would appear to offer a means of detecting small differences in nerve regeneration. The value of this in future developments in nerve repair is discussed.

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In the clinical setting, anatomical and electrophysiological evidence of regeneration after peripheral nerve injury does not always correlate with the patient's impression of recovery. Providing standardized clinical assessment for treating patients with peripheral nerve injuries is difficult, owing to the wide variation in location, severity of injury and method of repair.

Recovery of function after nerve repair depends on the number of fibres reaching and establishing active connections to target sites in the periphery. The time involved for regenerating motor axons to reach the target muscle and develop functional connections depends on the severity and location of the injury, as well as on the method of repair used. In the laboratory, where severity and location of the injury can be controlled, it has been shown that different methods of repair have different initial rates of regeneration. It has also been shown that grafts offering an increased mechanical resistance to penetrating axons show a reduction in the initial speed of regeneration (Davies et al, 1987).

When regenerating motor axonal sprouts reach their target muscles, new end plates begin to form (Hakelius et al, 1975). These axonal sprouts re-establish neuromuscular junctions. It has been shown that, in these immature motor units (terminal motor axon, neuromuscular junction and muscle fibre), there is an increased variability in the latency of neuromuscular transmission, which is due to a lower and more variable threshold of the end plate potential (Stålberg and Trontelj, 1994). Once the axonal motor sprout connects with a target muscle and establishes a neuromuscular junction, the fibre will begin to mature. As fibre maturation continues, the threshold end plate potential begins to rise and becomes more stable (Wiechers, 1990). It is possible to indirectly monitor regeneration by assessing the variability in the latency of neuromuscular transmission (Lenihan et al, 1997).

Stimulated jitter is a method of measuring variability in neuromuscular transmission latency. The terminal

motor axon is stimulated and the associated muscle fibre's action potential is recorded. It has been shown in the normal nerve that most of the variability in latency exists in the process of synaptic transmission and not in the terminal axonal branch (Stålberg and Trontelj, 1994; Trontelj et al, 1986) or in the muscle fibre (Trontelj et al, 1990).

If the speed of regeneration seen in the two methods of repair is different, it should be possible to monitor this difference by using the jitter technique. It becomes important, when assessing one repair technique against another, to ensure that enough time has occurred for all regenerating axons to reach their targets and mature. Chemical growth factors, such as NGF, may initially cause an increase in the number of regenerating axons and possibly affect the initial speed at which these axons grow, without affecting the final level of recovery of function. Before these chemical factors can be considered to be of any use in clinical practice, their effects must be studied over a sufficient time to ensure that the regenerative process is complete, and all of the "dying back" of pioneering axons unsuccessful in making distal connections has occurred. Jitter provides a means of assessing the neuromuscular maturation which follows this process.

Direct end-to-end nerve suture (NS) produces more complete and faster levels of regeneration compared with grafts (Davies et al, 1987), provided no tension exists at the suture line (Terzis et al, 1975). It is believed that the reason for improved regenerative capacity in the nerve suture group is the improved organization within the repair site. However, it seems unlikely that this would affect the speed of regeneration. Altered initial speeds of regeneration have been noted in differently aligned muscle grafts (Davies et al, 1987). It is thought that the initial reduction of regenerative speed in the non-aligned muscle graft is due to an increased mechanical resistance within the graft. The purpose of the present study was to

determine if the increased resistance associated with the nerve graft (NG) or freeze thawed muscle autograft (FTMG) is measurable by assessing the maturation of the neuromuscular junction. This was tested by recording the stimulated jitter 90 days after different methods of sciatic nerve repair in the rat.

METHODS

A population of 20 female Sprague-Dawley rats, each weighing between 400 g and 650 g, was divided into four equal-sized groups. These groups consisted of a control group of normal animals and groups in which the sciatic nerve had been repaired with direct end-to-end nerve suture (NS), a nerve graft (NG), or a freeze thawed muscle autograft (FTMG). The anaesthesia and surgical approach were identical to those presented in the first paper in this series (Lenihan et al, 1997)

In the NS group, the proximal and distal ends of the cut sciatic nerve were sutured together with two or three interrupted 10/0 polyamide sutures (Ethilon, Ethicon, UK). In the NG group, a 1 cm segment was removed from the distal nerve. This segment was reversed about its long axis and sutured into the sciatic nerve gap with three or four interrupted 10/0 polyamide sutures (Ethilon, Ethicon, UK). In the FTMG group, a 0.5 cm segment of nerve was removed from the distal sciatic nerve. Retraction of the sciatic nerve ends produced a 1 cm gap. Preparation of the FTMG is described elsewhere (Glasby et al, 1986a; Glasby et al, 1986b). A 1 cm FTMG was sutured in the sciatic nerve gap with three or four interrupted 10/0 polyamide sutures (Ethilon, Ethicon, UK).

Assessment was carried out 90 days after repair. On the day of assessment, each animal was anaesthetized in the same way as before (Lenihan et al, 1997). The left leg was shaved and the foot was firmly clamped to the table with a non-crushing clamp to prevent any needle movement during stimulation. The muscle chosen for study was extensor digitorum longus (EDL).

The general details of measuring jitter have been discussed extensively elsewhere (Trontelj et al, 1986; 1988; Trontelj and Stålberg, 1992) and the methods used here have been described in an earlier paper, in which was described the change in jitter with time after simple nerve repair (Lenihan et al, 1997). In calculating jitter, the mean consecutive difference (MCD), the mean sorted difference (MSD) and the mean interpotential interval (MIPI) were used, after recording from 20 different EDL sites for each animal. The MCD was taken as the jitter value for each site, provided the MCD:MSD ratio was 1:25 or below, otherwise the MSD was taken as the jitter (Kimura, 1989; Stålberg and Trontelj, 1994).

The results were transferred on disc from the Medelec machine to an IBM compatible computer where they were analysed with CSS STASTICA software (Statsoft, Inc. USA). The statistical analysis used is described elsewhere (Lenihan et al, 1997).

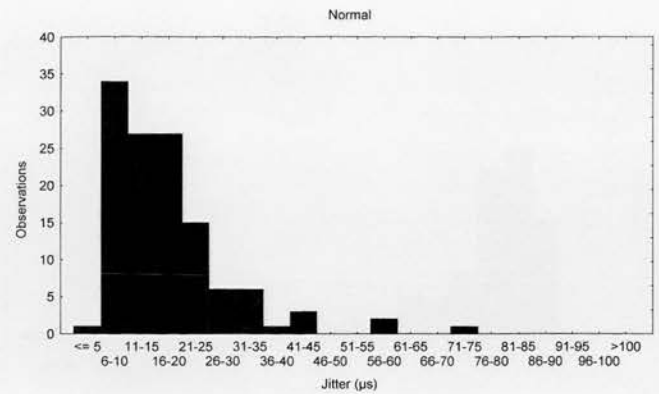


Fig 1 A distribution histogram of the observed data for jitter in the control group of animals.

RESULTS

The data obtained from the control group are shown in Figure 1. These data did not fit the curve of a normal distribution, owing to a slight skewing to the right. It was possible to fit the data to a normal distribution curve by re-plotting the data as a log-normal graph. The normal jitter range and upper limit for the rat EDL was determined from the log-normal plot. The conventionally accepted range of jitter for the normal rat EDL is 7 to 29 μ s with no more than one fibre out of 20 having a value above the 95th percentile – 31 μ s.

The means of the mean consecutive differences (MCD) for the NS, NG and FTMG were 23 μ s, 22 μ s, and 29 μ s respectively. Histograms of the MCD for each group are shown in Figures 2, 3 and 4. These data represent the pooled results for each group of five rats. Only one animal in the FTMG group and four of the five animals in both the NS and NG groups demonstrated a normal jitter study, by having the mean EDL jitter value between 7 and 29 μ s.

The maximum MCDs for any one recording for the NS, NG and FTMG and the standard deviations of the MCDs of the pooled data of the three groups are displayed in Table 1.

The results demonstrate that the NS and NG groups develop stable neuromuscular junctions 90 days after sciatic nerve repair, but the FTMG group did not have similar levels of neuromuscular junction maturation at 90 days. A matrix of the statistical differences for jitter (MCD) among the three groups is displayed in Table 2.

DISCUSSION

Jitter is a technique used to assess the physiology of the motor unit, which consists of terminal axonal branch, neuromuscular junction and muscle fibre. The measurement of jitter involves calculating the variability in neuromuscular transmission latency. It has been shown that this variability in latency becomes elevated in the early

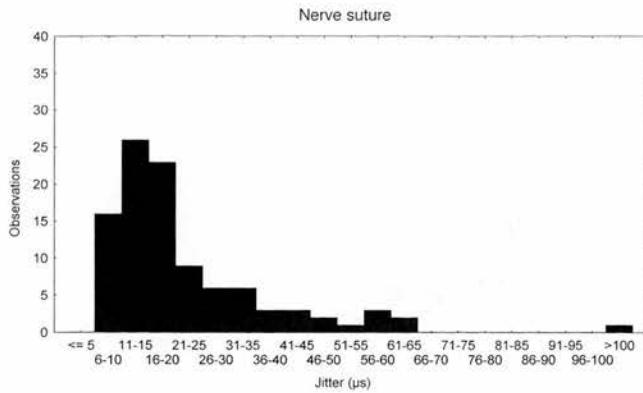


Fig 2 A distribution histogram of the observed data for jitter in the NS group of animals.

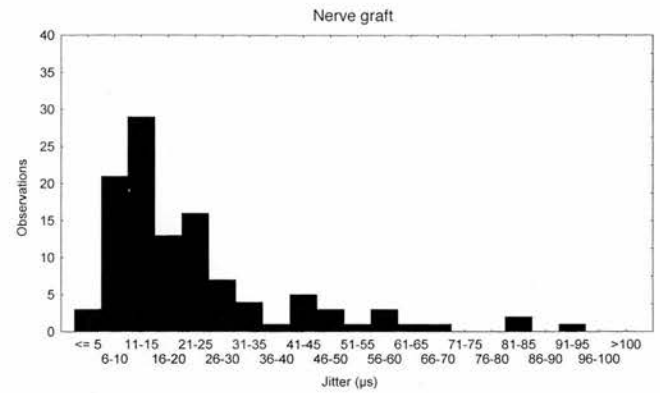


Fig 3 A distribution histogram of the observed data for jitter in the NG group of animals.

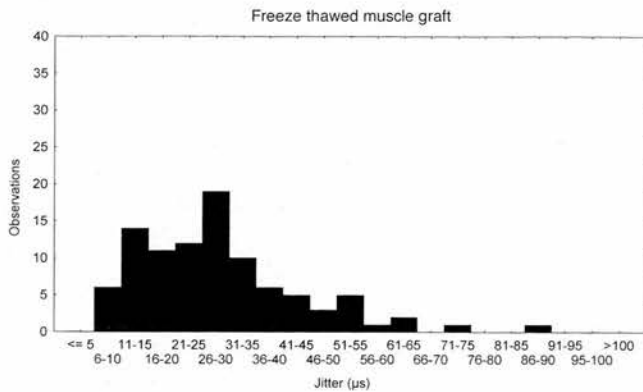


Fig 4 A distribution histogram of the observed data for jitter in the FTMG group of animals.

Table 1—Mean values of the measured variables

| Variable | Normal | NS | NG | FTMG |
|-----------------------|---------|---------|---------|---------|
| Mean jitter (SD) (µs) | 18 (11) | 23 (18) | 22 (19) | 29 (15) |
| Maximum jitter (µs) | 73 | 160 | 95 | 86 |
| Minimum jitter (µs) | 5 | 6 | 5 | 6 |
| MIPI (µs) | 3703 | 3930 | 4040 | 4934 |

Table 2—A matrix of the probability values for the differences in jitter (MCD) when the different experimental groups were compared

| Group | Normal | NS | NG |
|--------|---------|---------|----------|
| Normal | — | n.s. | n.s. |
| NS | n.s. | — | n.s. |
| NG | n.s. | n.s. | — |
| FTMG | P<0.001 | P<0.001 | P<0.0001 |

n.s. Not statistically significant

stages of re-innervation (Stålberg, 1990; Wiechers, 1990). It has also been shown that jitter can be used to monitor this progression of re-innervation after peripheral nerve repair (Lenihan et al, 1997).

The purpose of this study was to investigate whether jitter could be used to assess the quality of repair at a specific time, with respect to the small differences expected after different repair techniques, 90 days after sciatic nerve repair. The results of this experiment imply that the EDL end plates have not regained their original physiological properties with respect to jitter, presumably because of an initial delay in the pioneering axons reaching the distal nerve and subsequently the target muscle. This conclusion is supported by an elevated jitter value for the FTMG group when compared with the NS and NG groups.

Stålberg and Trontelj (1994) demonstrated that immature neuromuscular junctions, as a result of degeneration and subsequent re-innervation, had lower and more variable end plate potentials. In the early stages of re-innervation, the depolarization threshold of both the terminal motor axons and the muscle fibre are also lowered

(Stålberg, 1990; Wiechers, 1990). These changes in the motor unit produce an elevation in the variability of neuromuscular transmission latency, and therefore lead to elevated jitter values. As the fibre continues to mature, the threshold potential at the end plate rises and becomes more stable. Lenihan et al (1997) showed that jitter could be used to monitor the progress of regeneration with respect to time, while the present study demonstrated that the speed of regeneration among different repair methods can be assessed using jitter.

Transection of a peripheral motor nerve will produce a denervated muscle. Degeneration of the neuromuscular junction will then occur within a short time after denervation (Birks et al, 1960). If the injury is correctly repaired, re-innervation will result from sprouting axons growing down from the proximal end of the transected nerve. The time to muscular re-innervation will depend on both the distance the pioneering axons are required to travel and the quality of the repair.

It has been shown that the quality of the graft can affect the speed of regeneration. When a coaxially

aligned muscle graft was compared with an orthogonal muscle graft, the initial regeneration rate of the regenerating axons was slowed (Davies et al, 1987). The reduction in initial speed of regeneration was probably due to the increased resistance of the orthogonal graft. It is interesting to note, however, that the initial slowing of regeneration did not affect the final recovery of function (Davies et al, 1987).

The present study showed that the MCD and mean inter-potential interval (MIPI) were significantly ($P < 0.001$) elevated in the FTMG group when compared with the NS, NG and normal groups. The increase in the MCD and MIPI values for the FTMG group was probably due to a lower and more variable end plate potential (Stålberg and Trontelj, 1994) and/or variations in both the motor nerve terminal axon and muscle fibre thresholds (Stålberg and Thiele, 1973), caused by a delay of maturation of the neuromuscular junction.

If the progression of motor re-innervation can be monitored using the jitter technique, it should be possible to assess the speed of regeneration in different methods of nerve repair by measuring the jitter value at specific times. A change in the speed of regeneration through a muscle or nerve graft will affect the timing of neuromuscular maturation when compared with other methods.

It has been shown that the process of peripheral nerve regeneration diminishes over the whole period of recovery with respect to time (Bowden and Sholl, 1950; Sunderland, 1947). If one technique greatly increases the time the advancing axons take to reach the target muscle, the quality of regeneration will decline. The use of jitter to monitor the speed of nerve regeneration among different nerve repair techniques is based on two assumptions. First, that the speed of the advancing axon in the distal segment is not significantly different among the various groups. Secondly, that the speed of maturation, once a functional connection is made to a target muscle, is consistent for all repair techniques.

It has been shown that live Schwann cells are required for successful axon regeneration (Feneley et al, 1991), and also that a graft with increased mechanical resistance will reduce the initial rate of regeneration (Davies et al, 1987). The speed of regeneration through a graft may thus be influenced by the rate of Schwann cell migration from the proximal and distal segments of the nerve in the graft.

Mechanical resistance in the graft should in theory, be lowest in the NS group, followed by the NG group and then the FTMG group. Therefore, the NS group of pioneering axons should reach the muscle fibre first, followed by the NG group and then the FTMG group. The FTMG had an elevated jitter value, when compared with the NG and NS groups at 90 days after repair. The results of this experiment support the hypothesis that the FTMG has a higher resistance level to pioneering axons than the NS or NG. The FTMG group data are comparable to the 2-month NS group in a previous study

(Lenihan et al, 1997), indicating that enough time has not elapsed to allow for similar levels of regeneration.

The immature end plate is one of the major contributing factors to elevated jitter values in the regenerating nerve. In the early stages of re-innervation there are low and variable end plate potentials, which result in a lower safety-factor of neuromuscular transmission (Stålberg and Trontelj, 1994). These low and variable end plate potentials cause variability in the timing of the quantal release of acetylcholine. As the motor unit matures, the end plate potentials rise and stabilize. These changes make the use of jitter to monitor the progression of re-innervation ideal.

The study of the way in which chemical factors or new methods of repair help enhance the quality of nerve regeneration may disclose that these are associated with only small (but nevertheless useful) increases in recovery of function. Many of the growth factors will probably be found only to influence the initial rate of regeneration, without altering the final level of recovery of function. If assessment is carried out too early, a false positive result may affect the conclusion. Jitter may, by virtue of its sensitivity, provide a minimally invasive and therefore clinically acceptable method of assessing small variations in timing during the regenerative process. If assessment is carried out when the level of jitter has returned to normal, it can be assumed that the neuromuscular junctions have had sufficient time to mature.

This was a preliminary study to further assess the feasibility of using jitter to monitor neuromuscular maturation. The next step is to attempt to correlate the jitter values with morphological and electrophysiological results in a serial time study. If these results are positive, stimulated jitter may prove to be a **single** useful technique for monitoring the regeneration after repair. This would be extremely useful in clinical cases in which verbal communication between the patient and doctor is impossible, for example, in infants with obstetric brachial plexus palsy.

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STIMULATED JITTER MEASUREMENTS IN THE ASSESSMENT OF RECOVERY AFTER PERIPHERAL NERVE REPAIR

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The recording of stimulated jitter may offer a highly sensitive, quantitative method for following the recovery of neuromuscular function after peripheral nerve repair. In groups of rats, electrophysiological recording of jitter was carried out on control animals and animals which had had the sciatic nerves divided and repaired 14, 30, 60 and 90 days previously. It was found that values for jitter were highest in the early stages of regeneration and declined with time so that they were within normal limits by 90 days after repair. It is concluded that jitter measurement may be helpful as a test for the postoperative recovery of function in repaired nerves.

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Progress in peripheral nerve repair has, in recent years, tended to concentrate upon mechanical methods of improving the coaptation of the divided nerve ends or graft. It seems likely that development of such techniques has reached its limit of sophistication and interest is now turning to the use of chemical factors to enhance nerve regeneration either totally or selectively. Realistically, it is unlikely that enhancement by neurotrophic factors will produce large improvements in regeneration but, given the overall results of nerve repair in clinical practice, there would still be a major benefit from small improvements in both the extent and the specificity of nerve regeneration. If research in this field is to yield clinically useful data, it must be accompanied by the development of objective and relatively non-invasive methods of assessment which will better allow the clinician to assess quantitatively the outcome of the treatment. At present there are few ways in which objective and quantitative assessment of nerve repair can be carried out. The use of stimulated jitter which specifically measures events occurring in the motor unit offers a potential for detailed objective quantitative monitoring of a process central to the useful regeneration of motor nerves.

Single fibre electromyography (SFEMG) was developed to assess the microphysiology of the motor unit: terminal motor axon, neuromuscular junction and muscle fibre (Stålberg and Trontelj, 1979). Stimulated jitter, an SFEMG technique, is a means of measuring the variation in transmission at the neuromuscular junction. It involves the stimulating of a terminal motor axon and the recording of the associated muscle fibre's action potential. The variation in time between stimulation of the motor axon and recording the muscle fibre action potential in successive discharges is called *jitter*. It has been shown that normally there is little variability in the transmission of the impulse in the axon (Stålberg and Trontelj, 1979; Trontelj et al, 1986) or muscle fibre (Trontelj and Stålberg, 1983) during the jitter analysis. It has therefore been postulated that the site of variability in neuromuscular transmission is at the end plate.

Transection of a peripheral motor nerve will usually produce a totally denervated muscle. Degeneration of the neuromuscular junction will then occur within a

short time after denervation. If the nerve remains in continuity or if it is correctly repaired, reinnervation will result from sprouting axons growing down from the end of the proximal nerve stump. The time to muscular reinnervation will depend on both the distance the pioneering axons are required to travel and the quality of the repair.

When regenerating sprouts reach their target muscle new end plates begin to form and they compete to re-establish each neuromuscular junction. In the immature regenerating motor unit there is a larger variability in the time of transmission owing to the lower and more variable threshold potentials seen at the end plate (Stålberg, 1990; Stålberg and Trontelj, 1979). As the fibre continues to mature, the threshold potential at the endplate rises and becomes more stable. As this process takes place, it might be expected that the variations in neuromuscular transmission which are occurring might be recordable using an SFEMG technique such as jitter measurement.

SFEMG has been used to monitor reinnervation in a few non-controlled clinical cases (Wiechers, 1990) but it has not, hitherto, been cited as a means of monitoring or assessing reinnervation after peripheral nerve repair. The experiments described here are an attempt to assess the feasibility of using jitter as a method for monitoring reinnervation after repair of a peripheral nerve.

THEORY

Jitter and SFEMG in general are different from other EMG techniques in that the recording is much more specific. Jitter uses an SFEMG needle electrode for recording action potentials from small, discrete loci within the muscle fibre population. The SFEMG needle is made from a steel cannula 0.5 mm in diameter with an insulated silver wire in the lumen. A small opening of approximately 25 μm^2 , which exposes an uninsulated area of the silver wire, is made in the side of the needle: this side port becomes the active recording electrode and is situated about 3 mm proximal to the tip of the needle. In this position recording from muscle fibres damaged when the needle is inserted into the muscle will be minimized. This small recording area increases the probability of recording single action potentials from muscle fibres of the same motor unit.

The selectivity of recording can be further enhanced by filtering. Since muscle tissue acts as a low pass (high cut-off) filter, it will remove the high frequencies originating from more distant muscle fibres. Thus, recording from fibres situated near the active electrode will contain a relatively higher proportion of high frequency components. If the high pass (low cut-off) filter is set at 500 Hz there will be filtering of a majority of the impulses from distant fibres whilst affecting the recorded action potentials from nearby, only by a factor of 10% (Stålberg and Trontelj, 1979).

Stimulation must be as selective as possible. The duration of the stimulating pulse should be set at 50 μ s. It has been shown that this pulse-width produces little or no additional jitter at the stimulation point on the axon (Trontelj and Stålberg, 1992). The stimulus amplitude should be between 1 mA and 25 mA and should allow for stepwise increments and decrements of 0.1 mA to control the selectivity of stimulation.

The criteria for jitter require that the recorded action potential be of an amplitude > 0.5 mV with a rise-time of < 200 μ s and a duration of 1 to 2 ms.

The latency between the stimulus pulse and the muscle fibre action potential is called the interpotential interval (IPI). Each axon is stimulated at least 50 times and the IPI for each event is determined. The jitter (MCD) for the end plate being studied is calculated as a mean consecutive difference of the latencies (MCD) using the following equation:

$$MCD = \Sigma \left(\frac{|ipi_n - ipi_{n-1}|}{n} \right)$$

where:

MCD = mean consecutive difference (μ s)

ipi = interpotential interval (ms)

n = number of stimulations

Previous work has shown that for a jitter study to be meaningful, a population of at least 20 muscle fibres per muscle needs to be considered. The above equation must therefore be applied to the recording from each muscle fibre. The mean of all of the calculated values of MCD is the "jitter value" for that muscle. A normal value for jitter is taken to be within one standard deviation of the measurement for the control group. Also, no more than one fibre in the population under study should have a value of MCD greater than the 95th percentile of the control group distribution (DeLisa et al, 1994; Gilchrist, 1992).

METHODS

A population of 25 female Sprague-Dawley rats each weighing between 400 to 650 g was divided into five equal sized groups. These groups consisted of a control group of normal animals and groups to be assessed at 14, 30, 60 and 90 days after operation. Anaesthesia was induced in each animal using 5% halothane with gas

flow rates of 1.0l/min of O₂ and 0.5l/min of N₂O. Maintenance of anaesthesia required 2% halothane at the same gas flow rates using a face mask. A lateral incision was made in the skin of the left thigh. The skin edges and biceps femoris muscle were reflected to expose the sciatic nerve which was then divided 2 cm distal to the sciatic notch and subsequently repaired with 2 to 3 interrupted 10/0 polyamide microsutures (Ethilon, Ethicon, UK). An operating microscope was used throughout to assist vision. Continuous 3/0 absorbable polyglactin sutures (Vicryl, Ethicon, UK) were used for closure of the muscle and skin.

On the day of assessment each animal was anaesthetized in the same way as described above. The left leg was shaved and the foot was firmly clamped to the table with a non-crushing clamp to prevent any needle movement during stimulation. The muscle chosen for study was extensor digitorum longus (EDL). This is supplied by a single branch arising from the peroneal division of the sciatic nerve. Care was also taken to ensure that the EDL was placed in a relaxed position since stretching the muscle may increase the IPI (Trontelj and Stålberg, 1992).

The details of measuring jitter have been discussed extensively elsewhere (Trontelj et al, 1992; Trontelj and Stålberg, 1992) and the following is a summary of how the technique was used and modified in the present experiments.

A Medelec Sapphire 4ME II EMG unit (Medelec, Old Woking, UK) was used for all stimulating and recording procedures. Two monopolar needles (Medelec MF37) were used as the stimulating cathode and anode. The cathode was inserted into the EDL at the motor point. This is the point at which the nerve enters the muscle body and its whereabouts had been established previously by dissection of rats and by surface electrode studies. It is situated approximately 1 cm distal to the origin of EDL. The anode was placed 0.5 cm proximal and 0.5 cm lateral to the cathode. Stimulus pulses of 1 Hz frequency, 50 μ s duration, and of an amplitude of 1 mA were used initially. The stimulus amplitude could be adjusted with a step variability of 0.1 mA. The current was increased by 0.1 mA increments until small twitches were observed in the EDL. At this stage an SFEMG needle recording electrode (Medelec SF25) was inserted into the twitching portion of the muscle approximately 2 cm distal to the cathode. The position of the SFEMG needle electrode was adjusted until satisfactory recordings of muscle action potentials could be observed.

The frequency of stimulation was then increased from 1 Hz to 10 Hz and the high pass (low-cut-off) filter was set at 500 Hz. The current was then further increased from threshold to a point 10% greater than maximal, which was identified as the stimulus amplitude at which there was no further decrease in interpotential interval with further increases in the current.

The average value of jitter for each animal was calculated from measurements made at 20 different sites in

EDL. The jitter at each site was determined from 50 consecutive discharges which were recorded and stored on disc and subsequently used in the calculation of the mean consecutive difference of their latencies (MCD — see equation above) (Stålberg and Trontelj, 1979). The mean sorted difference (MSD), mean interpotential interval (MIPI) and percentage of blocking (failure to record an action potential on stimulation — see below) were also recorded.

If the muscle fibre was stimulated directly, as evidenced by a jitter value $< 5 \mu\text{s}$ (Stålberg et al, 1992; Trontelj et al, 1990), or if an adjacent motor axon was inadvertently stimulated to produce summation with the action potential under study, the recording was discarded.

The results were transferred on disk from the Medelec machine to an IBM compatible computer where they were analysed with CSS STASTICA software (Statsoft Inc., USA). This software allowed for the data to be fitted against the theoretical curve of a normal distribution with the same mean and variance. Using a one-tailed Kolmogorov-Smirnoff and a Chi-squared test, the fit of the theoretical curve to the data was tested. For group-to-group statistical comparisons a two-tailed Kolmogorov-Smirnoff (non-parametric) test was used to discover the probability that the means of experimentally determined variables might have been drawn from the same population.

RESULTS

The data obtained from the control group are shown as a histogram in Figure 1. These data represent the pooled values of MCD from 20 (EDL) sites in each of five rats. There was a slight skewing to the right of this histogram which when tested as above did not fit a normal distribution curve. By re-plotting these data as the abscissa of a log-normal graph it was possible to fit the data to a normal distribution curve (Fig 2). From the log-normal curve, the mean MCD for the normal EDL was found to be $18 \mu\text{s}$ with a standard deviation of $11 \mu\text{s}$. The MCD

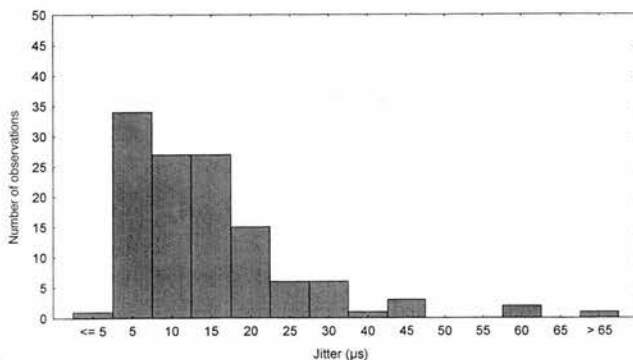


Fig 1 A histogram of the observed data for jitter in the control group of animals.

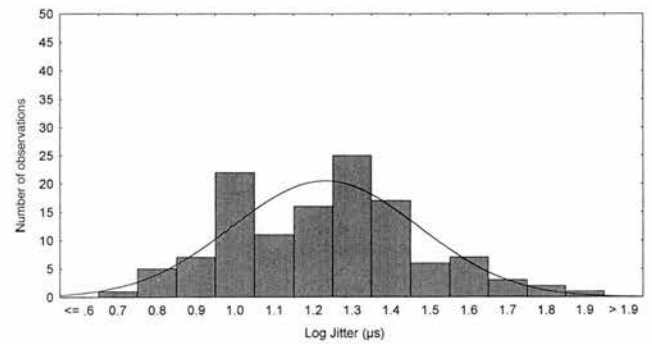


Fig 2 A histogram of the observed data for jitter, transformed logarithmically to enable the data points to be fitted to a normal distribution.

values of individual muscle fibres ranged from $5 \mu\text{s}$ to $73 \mu\text{s}$ with a value for the upper normal limit — the 95th percentile — being $31 \mu\text{s}$. The acceptable range of a jitter study for the normal rat EDL should therefore be 7 to $29 \mu\text{s}$.

The results obtained for the 14 day group of rats indicated that reinnervation of the EDL had not yet taken place. This is consistent with previous findings (Glasby et al, 1986). These results were, therefore, not considered further.

The mean MCDs for the 30, 60 and 90 day groups were $97 \mu\text{s}$, $32 \mu\text{s}$ and $23 \mu\text{s}$, respectively. Histograms for each group are shown in Figures 3 to 5. These histograms show that the MCD shifted to the left as the time after repair increased. No animals in the 30 day group met both the requirements for a normal jitter study. One out of five animals in the 60 day group and four out of five animals in the 90 day group demonstrated normal jitter.

The results also showed a relative decrease in the number of fibres with blocking as the time after repair increased. The percentage of fibres which showed blocking decreased from 17% in the 30 day to 9.9% in the 90 day group. Blocking, when seen, was strongly correlated with latency (MIPI) in all groups. No blocking was evident in the normal group. These results are displayed in Table 1.

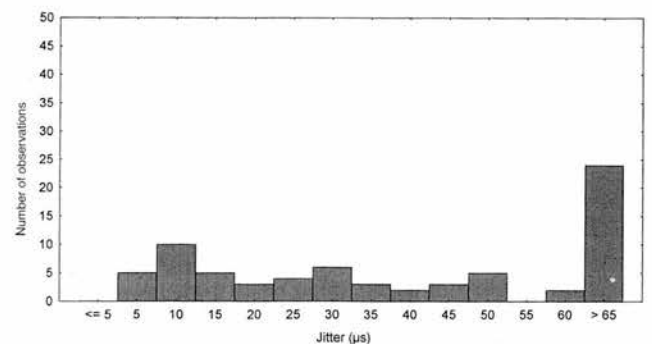


Fig 3 A histogram of the observed data for jitter in the 30 day group of animals.

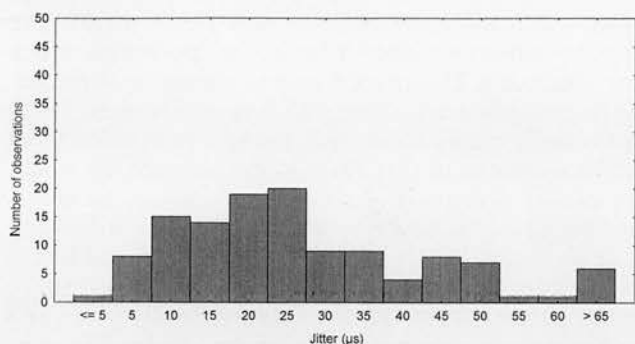


Fig 4 A histogram of the observed data for jitter in the 60 day group of animals.

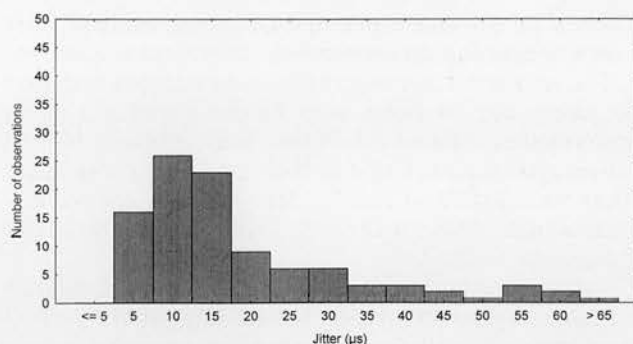


Fig 5 A histogram of the observed data for jitter in the 90 day group of animals.

Table 1—Mean values of variables

| Variable | 30 Days | 60 Days | 90 Days | Control |
|---------------------------|---------|---------|---------|---------|
| Average jitter (µs) | 97 | 32 | 21 | 18 |
| Maximum jitter (µs) | 849 | 169 | 160 | 73 |
| Minimum jitter (µs) | 7 | 5 | 6 | 5 |
| Standard deviation (µs) | 141 | 23 | 18 | 11 |
| % of fibres with blocking | 16.7% | 13.1% | 9.9% | 0.0% |
| Mean latency (µs) | 7,757 | 5,397 | 3,930 | 3,703 |

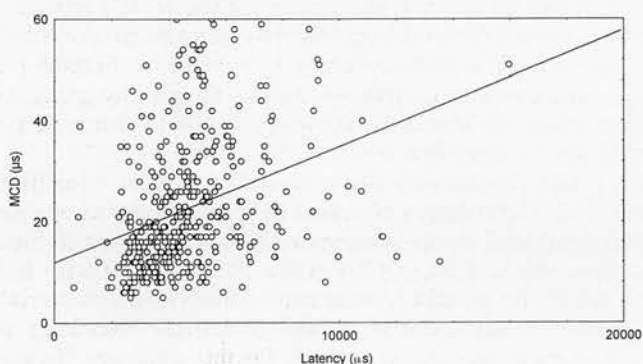


Fig 6 A scatterplot of the observed values of mean consecutive difference (MCD) against latency.

A statistically significant correlation of increased latency (MIPI) with increased MCD was evident in all groups and this is demonstrated in Figure 6. This finding is consistent with results from human and animal studies (Trontelj et al, 1988).

These results demonstrate that jitter decreases as the time after repair of a transected peripheral nerve increases. In the rat, normal values for jitter were attained by 90 days. The results also show an inverse relationship between time after repair, the percentage of blocking fibres and the MIPI.

DISCUSSION

Jitter is a technique used to assess the physiology of the motor unit: specifically the variability of neuromuscular transmission. It has been shown that in a normal stimulated jitter study, variability within the terminal axonal branch and muscle fibre is minimal. It has also been shown that in reinnervation, after trauma or in a neuromuscular disease process, jitter becomes elevated as a result of the altered microphysiology of the motor unit. This has been suggested in previous clinical studies (Wiechers, 1990) and after free muscle transplants (Hakelius and Stålberg, 1974; Hakelius et al, 1975), but has not been studied quantitatively in a controlled environment as a technique to monitor nerve repair.

The purpose of the present study was to investigate whether jitter could be used as a technique to record the progression of reinnervation after the repair of a peripheral nerve. As it is a minimally invasive technique which is easy to perform on human subjects, the potential for using such a test as an objective follow-up after nerve repair is considerable. The results of the present experiment support this view by demonstrating a significant and easily measurable decline towards normal values in the average value of jitter with time after repair.

The results further indicated that in the group studied at 90 days after repair there was no statistical difference between measurements of jitter obtained from the repaired and the normal nerves. This implies that the

majority of the end-plates in EDL have regained their original physiological properties with respect to jitter.

The results of this experiment also demonstrate that the percentage of fibres with blocking and the mean interpotential interval (MIPI) are both inversely related to time after repair. The standard deviation of the mean MCD declined from the 30 day group to the 90 day group which indicates that the variability of the MCD had also declined.

The results obtained here for the normal EDL did not fit a normal (Gaussian) distribution. They therefore had to undergo log-transformation to fit a normal distribution in order that parametric statistics could be applied in calculating the mean MCD range and 95th percentile for the study. This situation has been identified previously by other workers in the human extensor digitorum communis muscle (Baker et al, 1987). These workers obtained an empirical formula which could be applied to overcome this problem. Our data, however, were found to fit a normal distribution curve after simple log-transformation which thus obviated the need to apply a more complex empirical formula.

After transection of a peripheral nerve several events take place. The axons degenerate by Wallerian degeneration (Langley, 1909; Waller, 1851) and the neuromuscular junction fails within 36 hours (Birks et al, 1960). The myelin sheaths begin to degenerate within 48 hours (Miledi and Slater, 1970; Winlow and Usherwood, 1975). The denervation of the muscle fibre causes a lowering of the threshold potential for each fibre (Kraft, 1990; Trontelj and Stålberg, 1983). By one month after adequate nerve repair, neuromuscular junctions start to become established and calculating jitter by way of axonal stimulation can, in theory, be achieved. This was confirmed in the present study.

The mean MCD, MIPI and percentage blocking for the 30 day group were all statistically elevated ($P < 0.01$) above the corresponding values for the 60 day, 90 day and normal groups. The increased jitter was probably due to the abnormally low or variable end plate potentials (Stålberg and Trontelj, 1979), variation of the depolarization threshold in both the motor nerve terminal axon or muscle fibre (Stålberg and Thiele, 1972) and/or transmission failure in the terminal motor axons. These factors are known to stabilize as maturation ensues.

It has been shown that jitter along normal axons is minimal (Trontelj et al, 1986). However, in the early regenerated axon there is a greater variability in the steepness of the local potential and this causes a further variability in the take-off point of the action potential. The result is an increased degree of jitter.

Blocking is increased in the initial stages of reinnervation when the terminal axonal branches are not yet myelinated (Harreveld, 1952). At this early stage there are likely to be transmission defects along the course of the nerve which produce the conduction block (Gilliatt, 1966). It has been shown that in the early regenerative process there is an increase in blocking as evidenced by

concomitant blocking (Stålberg and Thiele, 1972). The latter is observed when two action potentials block simultaneously. This process may be the result of recording from a split muscle fibre (Stålberg and Trontelj, 1979) and is easily recognizable since the split muscle fibre will exhibit a jitter value (MCD) of $< 5 \mu\text{s}$ between the blocking action potentials. Axonal transmission failure is another cause of concomitant blocking. In stimulated jitter the latter results from stimulating proximal to the transmission deficit in an axon which subsequently splits. In the present study this was observed only twice and both instances occurred in the 30 day group. The jitter between the two blocking action potentials in both of the observed cases was large and therefore thought to be of neurogenic origin.

The myogenic component of jitter will also be elevated in the early stages of reinnervation. After severe nerve injury the muscle fibres will atrophy owing to denervation. This results in an altered propagation velocity in the muscle fibre (Stålberg and Trontelj, 1979). The denervated muscle will also have a lower and more variable threshold potential (Thesleff and Ward, 1975). These physiological changes produce an increase of the myogenic component of jitter.

Ephaptic transmission may occur when the action potential occurring in a muscle fibre induces the firing of an action potential in an adjacent muscle fibre with a lower threshold. In the early stages of reinnervation when there is greater variability in the thresholds of individual muscle fibres, the probability of ephaptic activity is increased. If a fibre under study is being fired ephaptically, there will be an increase in blocking and increased jitter owing to liminal stimulation. In the present study, ephaptic transmission was detected in the 60 day and 90 day groups by altering the stimulating current. This did not affect the latency or amplitude of the ephaptic action potential because it was independent of changes in current. Also, if the current was increased sufficiently to stimulate the muscle fibre directly there was a stepwise decrease in latency of the action potential. However, it proved extremely difficult to determine ephaptic transmission in the 30 day group by either method as large variations in jitter and blocking were common and it was often impossible to distinguish this from axonally stimulated jitter.

The end plate is also a source of jitter. In the early stages of reinnervation there is an increase of jitter which is due to low or variable end plate potentials and which results in a lower safety factor of transmission (Stålberg and Trontelj, 1979). The variations at the terminal end-bulb of the axon will cause variability in the timing of acetylcholine quantal release while changes in the muscle fibre end plate will result in variability with respect to the time it takes the muscle fibre to reach threshold. These two phenomena are probably the main reason for the increase in jitter in the early regenerative stages, if blocking is not considered.

It has been shown in the muscles of human patients

that a significant correlation exists between the magnitude of the jitter (MCD) and the latency recorded (Trontelj et al, 1988). In the regenerating nerve the three reasons why this is so are (a) a slower conduction velocity along the nerve; (b) a longer neuromuscular transmission time and (c) slowing of conduction in the muscle fibre.

It has been suggested that while blocking is indicative of pathology and will result in increased jitter, it does not necessarily provide any information on the physiology of the end plate (Stålberg et al, 1992). Stålberg has shown that blocking will cause a change in the propagation velocity at which an action potential is transmitted down a muscle fibre (Stålberg and Thiele, 1972). The propagation velocity is affected by the inter-discharge interval—time between successive discharges—which, as it changes, will alter the conduction velocity as a function of a property of the muscle termed the *velocity recovery function* (Stålberg, 1966). The consequence of this process will be in an increase in the value of the jitter (MCD).

Although blocking may not provide insight into the details of end-plate physiology, it does provide valuable information about the regenerative process. The present study demonstrated clearly that as time after repair increased the percentage of blocking decreased. It has been shown that blocking can exist for many months after reinnervation (Wiechers, 1990) and may last indefinitely.

While the exact reason for the changes in jitter seen during nerve reinnervation remain a matter for conjecture, the present study has shown that the recovery of jitter specifically after nerve repair provides a useful way of monitoring the regenerative process. Work is currently under way in our laboratory to extend the present study to a large animal (sheep) model where the pattern of nerve regeneration is more akin to that of a human.

Because of the large number of data recorded in a jitter study and the way in which they are handled, the degree of sensitivity is considerable. If chemical enhancement of nerve repair is to be the next stage in the surgery of nerve repair then it is important to have an objective method of assessment which will resolve small improvements. Our early results suggest that jitter measurement is a more sensitive way of following recovery in motor nerves or in the motor component of mixed nerves than the commonly used nerve conduction velocity and collision studies.

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