The neural control of blood flow to normal, injured and arthritic joints

A thesis submitted to the University of Glasgow in candidature for the degree of Doctor of Philosophy.

By,

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Abstract

An imaging technique (laser Doppler perfusion imaging, LDI), based on measurement of backscattered Doppler-broadened laser radiation, was used to produce two-dimensional images of perfusion in animal and human joints. The advantage of this technique is that it can map the spatial distribution of tissue perfusion in a non-invasive manner. On the downside, however, LDI is unable to provide absolute measures of blood flow and it is incapable of assessing temporal changes in perfusion. The former disadvantage was addressed in this thesis by comparing LDI flux values obtained from rabbit medial collateral ligaments (MCL) with simultaneous absolute measures of blood flow determined by the coloured microsphere technique. These experiments were able to calibrate the LDI system, however, since different tissues have unique optical properties, the calibration factor produced in this study was specific to rabbit MCLs.

Other experiments showed that induction of joint instability in rabbits by transection of the anterior cruciate ligament caused increased blood flow to the ipsilateral MCL during the first 2 weeks post-trauma. Substance P (SP) is a known potent vasodilator in knee joints of rats and its effects in rabbit knees was examined to discover whether it was contributing to the hyperaemia observed following this major articular insult. Topical application of this neuropeptide to the surface of the MCL had no discernible effect on ligamentous blood flow suggesting SP is not vasoactive in the rabbit knee joint. The effect of injury was also investigated in human proximal interphalangeal (PIP) joint. The study centred on former members of the Canadian Olympic volleyball team who had at some time in their career experienced severe PIP joint injury and were therefore

suspected of being in the early stages of osteoarthritis. Assessment of their PIP joints with LDI revealed that basal blood flow to injured joints was not significantly different from normal articular perfusion. A common physiotherapy applied to injured joints is deep friction massage. In normal joints this treatment caused increased blood flow probably by local thermoregulatory mechanisms. However, in traumatised PIP joints deep friction massage had no effect on blood flow suggesting that the normal vasoregulatory mechanisms may have been affected by the pathological changes which are associated with osteoarthritis.

The present project also examined the neural control of blood flow to rat knee joints. Electrical stimulation of the nerves supplying the rat knee joint caused a frequency-dependent vasoconstriction of articular blood vessels. Intra-articular injection of the neurotoxic agent capsaicin had no effect on vasoconstrictor responses which implies that capsaicin is selectively neurotoxic for sensory unmyelinated fibres but not sympathetic postganglionic fibres. Other experiments studied the effect of nerve stimulation in an animal model of chronic inflammation. Synovial inflammation was induced by unilateral intra-articular injection of Freund's complete adjuvant into rat knee joints. One week later, this procedure produced a swelling of the joint with a concomitant fall in basal perfusion. By the third week after adjuvant treatment, knee joint diameter and basal blood flow had returned towards normal. Since articular cartilage is dependent on synovial perfusion, the initial fall in joint blood flow could cause a deterioration in articular integrity which would lead to degenerative changes. The constrictor responses normally associated with electrical stimulation of the articular nerves were abolished in the chronically inflamed model, the abolition persisting throughout the investigation. The

project then tried to elucidate whether nitric oxide (NO) played a rôle in the development of neurogenic inflammation. Intravenous infusion of Nω-nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine (L-NMMA) which inhibit NO production, had very little effect on rat knee joint blood flow. When nerve stimulation was carried out in the presence of these drugs, L-NMMA enhanced vasoconstriction whereas L-NAME had no effect. These findings suggest that NO is not released under basal conditions in the rat knee joint but may be released in response to articular nerve stimulation. As a finale to the NO story, the effects of L-NAME on knee joint protein extravasation in the rat was investigated. When this NO inhibitor was infused intravenously into the rat, there was an increase in knee joint blood vessel permeability which proposes a possible protective rôle for NO in this tissue.

To my darling wife Kirsty, without whom...

And with thanks to my parents
for their continual support.

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Contents

List of Figures

List of Tables

Publications

Refereed papers

Mc Dougall, J.J., Karimian, S.M. and Ferrell, W. R. (1994). Alteration of substance P-mediated vasodilatation and sympathetic vasoconstriction in the rat knee joint by adjuvant-induced inflammation. *Neuroscience Letters*. **174,** 127-129.

Karimian, S.M., **Mc Dougall, J.J.** and Ferrell, W. R. (1995). Neuropeptidergic and autonomic control of the vasculature of the rat knee joint revealed by laser Doppler perfusion imaging. *Experimental Physiology*, **80,** 341-348.

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Mc Dougall, J. J., Bray, R., Forrester, K., Damji A. and Ferrell, W. R. Comparison of coloured microspheres and laser Doppler perfusion imaging for measuring blood flow in rabbit medial collateral ligaments. *Journal of the Autonomic Nervous System. (In Press).*

Refereed Papers (submitted)

Mc Dougall, J.J. and Ferrell, W. R. Inhibition of nitric oxide production during electrical stimulation of the nerves supplying the rat knee joint. *(Submitted to Journal of the Autonomic Nervous System).*

Bray, R., **Mc Dougall, J. J.**, Forrester, K., Damji A. and Ferrell, W. R. Evaluation of laser Doppler imaging to measure blood flow in knee ligaments of adult rabbits. *(Submitted to Journal of Rheumatology).*

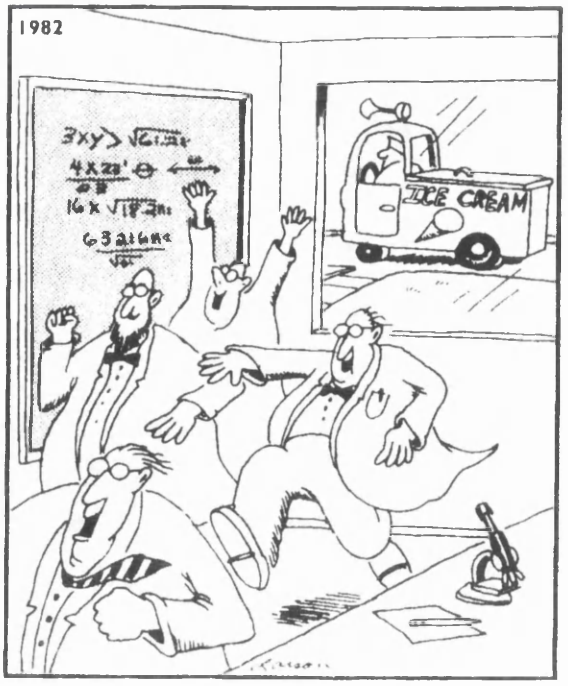
Abstracts

Mc Dougall, J. J., Bray, R., Forrester, K., Damji A. and Ferrell, W. R. Comparison of coloured microspheres and laser Doppler perfusion imaging for measuring blood flow in rabbit medial collateral ligaments. (Oral presentation at a joint meeting of the British Physiological Society and the Nagoya Symposium in Japan) *Japanese Journal of Physiology. (In Press).*

Abbreviations

ACL	Anterior cruciate ligament
ANOVA	Analysis of variance
CGRP	Calcitonin gene-related peptide
CMs	Coloured microspheres
DIP	Distal interphalangeal
EDRF	Endothelium-dependent relaxation factor
EDTA	Ethylenedinitrilotetraacetic acid
FSD	Full scale deflection
HR	Heart rate
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenous
L-NA	Nω-nitro-L-arginine
L-NAME	Nω-nitro-L-arginine methyl ester
L-NIO	N-iminoethyl-L-ornithine
L-NMMA	N^G-monomethyl-L-arginine
LCL	Lateral collateral ligament
LDF	Laser Doppler flowmetry
LDI	Laser Doppler perfusion imager (imaging)
MAN	Medial articular nerve
MAP	Mean arterial pressure
MCL	Medial collateral ligament
n	number of observations
NKA	Neurokinin-A
NO	Nitric oxide
NOS	Nitric oxide synthase

NPY	Neuropeptide Y
OA	Osteoarthritis
PAN	Posterior articular nerve
PCL	Posterior cruciate ligament
PIP	Proximal interphalangeal
RA	Rheumatoid arthritis
S.E.M.	Standard error of the mean
SD	Standard deviation
SP	Substance P
VIP	Vasoactive intestinal polypeptide



THE MICROBE

The Microbe is so very small
You cannot make him out at all,
But many sanguine people hope
To see him through a microscope.
His jointed tongue that lies beneath
A hundred curious rows of teeth;
His seven tufted tails with lots
Of lovely pink and purple spots,
On each of which a pattern stands,
Composed of forty separate bands;
His eyebrows of a tender green;
All these have never yet been seen -
But Scientists, who ought to know,
Assure us that they must be so...
Oh! Let us never, never doubt
What nobody is sure about!

(Hilaire Belloc)

Chapter 1

General Introduction

Gross anatomy and physiology of joints

The structural rigidity and stability of the human body is provided by the bony endoskeleton. The controlled and almost frictionless movement of this framework is achieved by the joints. Joints are most often classified according to the type of movement they perform. Three types are often described:-

1. **Synarthroses-** the immovable joints (*eg.* between the bony plates of the skull).
2. **Amphiarthroses-** slightly moveable joints (*eg.* between the vertebrae).
3. **Diarthroses-** the moveable joints (*eg.* the knee).
   (From Gray, 1973).

The diarthroses constitute the majority of all joints and are most susceptible to disease. As such the present project was centred on this joint type and it is to them that we turn our attention.

Diarthroidal (synovial) joints are typified by their wide range of almost frictionless movement. The articulating bony surfaces have at their ends a thin plate of dense, cortical bone called the articular end-plate. Beneath this lies the spongy or cancellous bone often containing a red marrow filled cavity which is haematopoietic *ie.* erythrocytes are formed there. Tightly adhered to the bony end-plate is the hyaline (glass-like) articular cartilage, a specialised form of connective tissue that serves as the load bearing and gliding surface (Fig. 1.1). The joint cavity is an anatomically discrete space containing only a few millilitres of lubricating synovial fluid. This biological lubricant is secreted from a specialised layer of connective tissue cells within the

joint called the synoviocytes (Mankin and Brandt, 1984; Revell, Mayston, Lalor and Mapp, 1988). Deep to this layer are varying amounts of highly vascular adipose, areolar and fibrous tissue which support the synoviocytes.

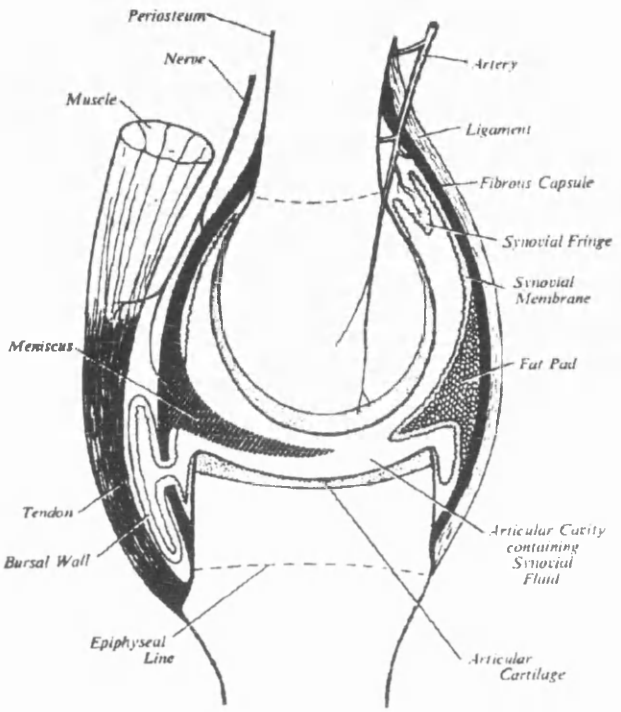


Fig. 1.1. Diagram of a diarthroidal joint (from Barnett, Davies and MacConaill, 1961).

The movement of the cartilaginous surfaces provide mobility, however joints also require a certain degree of stability to prevent movement in abnormal planes or excessive slipping under load. Stability is attained by the bony configuration of the joint, the ligamentous and capsular support systems, and the muscles controlling

the joint. Each joint has a unique configuration that dictates not only the range of movement but the type of movement executed. For example, the knee is a rounded, cam-shaped structure that not only allows flexion and extension but a limited amount of rotation as well. Let us now examine the individual components of synovial joints.

Ligaments and capsule

Ligaments and capsular structures vary considerably in thickness and position depending on the joint studied. Structures range from the thin, redundant capsule of the shoulder joint (Rothman, Marvel and Heppenstall, 1975) to the thick, dense collateral and cruciate ligaments of the knee (Arnoczky, 1983; Ellison, 1985; Girges, Marshall and Monajem, 1975). The insertion of ligaments and capsule into the adjacent bone requires the structures to undergo subtle changes in chemical composition as they approach their insertion points. The parallel bundles of collagen that make up ligaments and capsule first become invested with fibrocartilaginous stroma, and then as they near the bone, become calcified. This gradual transition of ligament to mineralised fibrocartilage and then to bone enhances the ability of the insertions to distribute forces evenly and decrease the likelihood of them "popping out".

As mentioned previously, ligaments and the joint capsule provide stability to joints however, this steadfastness is redundant if the articular muscles are paralysed. Muscles are most important in stabilising large, proximal joints (*eg.* shoulder and hip) whereas smaller joints (*eg.* of the wrist and foot) rely more heavily on the bone configuration and ligamentous connections for their support. In the knee joint, for example, a number of ligaments may be found each

knee joint, for example, a number of ligaments may be found each oriented in a specific way so as to prevent movement in each of the abnormal planes (Fig. 1.2). The medial and lateral collateral ligaments (MCL and LCL) extend from the distal femur to the tibia and provide stability in the coronal plane, whereas the anterior and posterior cruciate ligaments (ACL and PCL) control stability in the saggital plane.

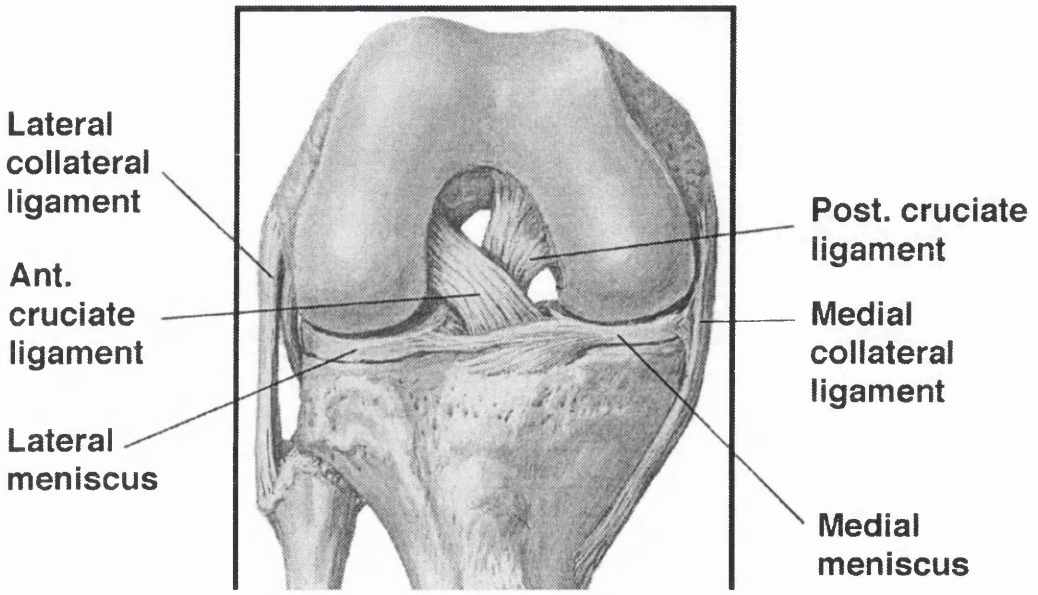


Fig. 1.2. Position of the ligaments associated with the human knee joint. The joint is flexed with the patella and capsule removed to show the cruciate ligaments.

Articular cartilage

These specialised connective tissues are firmly attached to the underlying bone and measure less than 5mm in human joints. Articular cartilage is white, tending to become yellow with age (Van der Korst, Sokoloff and Miller, 1968). Collagen is the most prevalent organic constituent of cartilage. The superficial collagen fibres are arranged in bundles which are parallel to the surface of the cartilage forming a "skin" layer. The water content of cartilage is up to 80% of its weight and is freely exchangeable with synovial fluid. This "weeping" of the cartilage may be an essential feature of a boundary lubricating system.

Articular cartilage is the principal "working" component of a diarthroidal joint and, along with synovial fluid, is responsible for the almost frictionless movement of the articulating surfaces (Charnley, 1969). They represent the load bearing surface of the joint and their structure is able to resist the repetitive deformation this surface is subjected to over the years. The deformable characteristics of articular cartilage allow the largest possible surface contact area when a force is applied to the joint.

The synovial membrane

The synovial membrane is the thin layer of tissue that lines the inner surfaces of joints and tendon sheaths, but not hyaline cartilage. The inner layer of the synovium closest to the joint cavity consists of synoviocytes and is one to three cells deep. This zone overlies a subsynovial layer which comprises of fibroblasts, adipocytes, collagen fibres and proteoglycans. This subsynovial layer is endowed with a

rich plexus of blood vessels which are responsible for the transfer of blood constituents to the joint cavity and the formation of synovial fluid (Adkins and Davies, 1940; Davies and Edwards, 1948).

The ultrastructure of the synovial lining reveals two distinct types of cells termed A and B cells (Barland, Novikoff and Hamerman, 1962). Type A-cells possess endoplasmic vacuoles, filopodia and are phagocytic. They are thought to remove necrotic debris from synovial fluid and may also secrete hyaluronate (Ghadially, 1978). Type B-cells are characterised by a prominent rough endoplasmic reticulum and probably secrete interstitial collagen (Linck and Port, 1981).

To summarise, the normal synovial membrane serves at least three main functions. First, it produces the lubricating fluid that allows the hyaline cartilage covered articular surfaces to glide smoothly over each other with minimum friction; the synovial fluid is largely produced by the type B synoviocytes. The second normal synoviocyte function is the removal of cellular debris and particulate matter from the joint cavity: this is performed by the type A-cells. The third property is the repair of damage to the joint; this involves collagen production and is executed predominantly by the type B-cells.

The gross anatomy of the knee joint in man is not dissimilar to that of the rat (one of the animal models examined in the present study) although minor anatomical differences do occur (Hildebrand, Oqvist, Brax and Tuisku, 1991).

Knee joint vasculature

The anatomy of the arterial supply to diarthroidal joints in general and the knee joint in particular has been studied thoroughly (Davies and Edwards, 1948; Scapinelli, 1968). The main arteries channelling blood to the knee joint are the genicular arteries which arise from the femoral and popliteal arteries (Fig. 1.3). The most important of these is the supreme genicular artery which gives off three branches: (a) the saphenous branch (superficial), (b) the muscular articular branch (deep), and finally (c) the deep oblique branch. These vessels freely divide and anastamose to form a complex network of vessels around the joint. The synovial membrane is considerably more vascular than the periarticular support structures such as the capsule, tendons and ligaments. Articular hyaline cartilage is notably avascular and as such relies on synovial fluid for its nutritional requirements (McKibbin and Maroudas, 1979) which in turn depends on synovial blood flow.

Many studies have examined the vascular anatomy of articular ligaments (Alm and Stromberg, 1974; Arnoczky, Rubin and Marshall, 1979) and tendons (Lundborg, Myrhage and Rydevik, 1977; Myrhage, Lundborg and Rydevik, 1979). The general vascular pattern in ligaments, tendons and capsule is similar consisting of arterio-venous anastamoses which course longitudinally creating a ladder-like effect. The vessels of the ACL and the collateral ligaments in rabbits are most abundant in the epiligamentous layer (Bray, Fisher and Frank, 1990). The same study also showed that a limited number of these epiligamentous vessels give rise to intraligamentous vessels which ramify into the ligament itself. It is interesting to note that the tendinous and ligamentous insertion points into bone (the entheses)

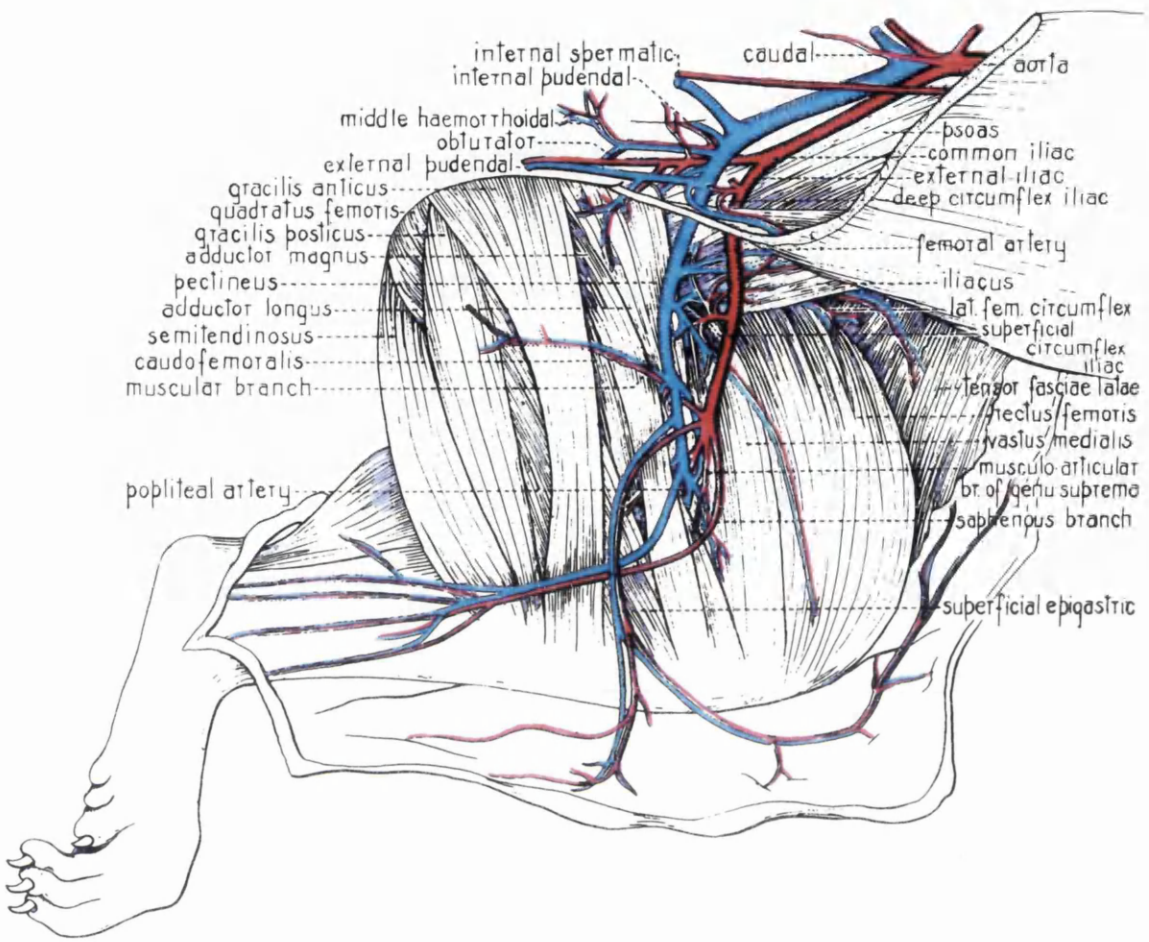


Fig. 1.3. Superficial blood vessels of the medial aspect of the rat thigh (from Greene, 1955).

are avascular and this could affect the repair process of these structures following trauma.

The arteries supplying the synovial membrane enter at the articular fringes, then branch and anastamose regularly to form a wide meshed plexus of vessels. These vessels communicate *ad libitum* with those of the capsule, periosteum and epiphysis (Davies, 1946), possibly influencing flow to these structures. Scapinelli (1968) reported that those parts of the synovial stratum which are subject to the highest degree of mechanical forces are relatively devoid of vessels. Synovial capillary density is highest over the adipose and areolar tissue (67000-83000 capillaries/cm^2) and lowest over the tendons (2000 capillaries/cm^2) (Knight and Levick, 1983). Capillary density gradually increases with depth below the joint lining, peaking at 6-11μm; capillary density rapidly decays with subsequent increases in depth.

Knee joint innervation

Neurologists have been studying the innervation of the knee since Dogiel in 1892. Articular nerves arise from branches descending from main nerve trunks or their muscular, cutaneous and periosteal branches (Polacek, 1966). Over the years it has been shown that synovial joints are innervated by both myelinated and unmyelinated afferent nerve fibres (Samuel, 1952; Skoglund, 1956). Afferent fibres transmit sensory information from peripheral tissue to the central nervous system. The larger myelinated fibres arise from specialised encapsulated structures at the nerve ends (Boyd, 1954) and whose function is primarily proprioceptive (Ferrell, 1992). The unmyelinated and thin myelinated axons with unmyelinated terminals are termed

free nerve endings and account for the majority of all joint afferents (Langford, 1983; Langford and Schmidt, 1983). These free nerve endings are normally found throughout the synovium and have high thresholds to stimuli which could be detrimental to the joint (Ferrell, 1992); as such they probably act as nociceptors (pain receptors). Sensory nerves in the joint were originally classified by their histological appearance (Freeman and Wyke, 1967) but are now classified by their neurophysiological properties (Table 1-1). In addition to the afferent nerve fibres, unmyelinated sympathetic efferent fibres innervate the joint and exert a vasomotor effect on the articular blood vessels producing a resting vasoconstrictor tone (Cobbold and Lewis, 1956).

In the rat knee, the primary nerve emerges from the tibial nerve and courses to the posterior aspect of the joint - this is the posterior articular nerve (PAN). A less prominent medial articular nerve (MAN) comes from the saphenous nerve and extend to the vicinity of the medial collateral ligament (Fig. 1.4). The PAN is composed of about 400 axons, 80% of which are unmyelinated (Hildebrand, Oqvist, Brax and Tuisku, 1991). Approximately 1/3 of the unmyelinated axons are type IV afferents, and the remaining 2/3 are sympathetic efferents. The density of nerve fibres in the rat knee joint is highest in the fibular collateral ligament followed by the tibial collateral ligament, the posterior cruciate ligament, the anterior cruciate ligament and finally the articular capsule (Marinozzi, Ferrante, Gaudio, Ricci and Amenta, 1991). A proportion of the afferent and efferent fibres innervating the joint contain neuropeptides. Since these neurotransmitters are known to be pro-inflammatory, a neuronal rôle in the development of joint inflammation appears feasible and will be discussed later.

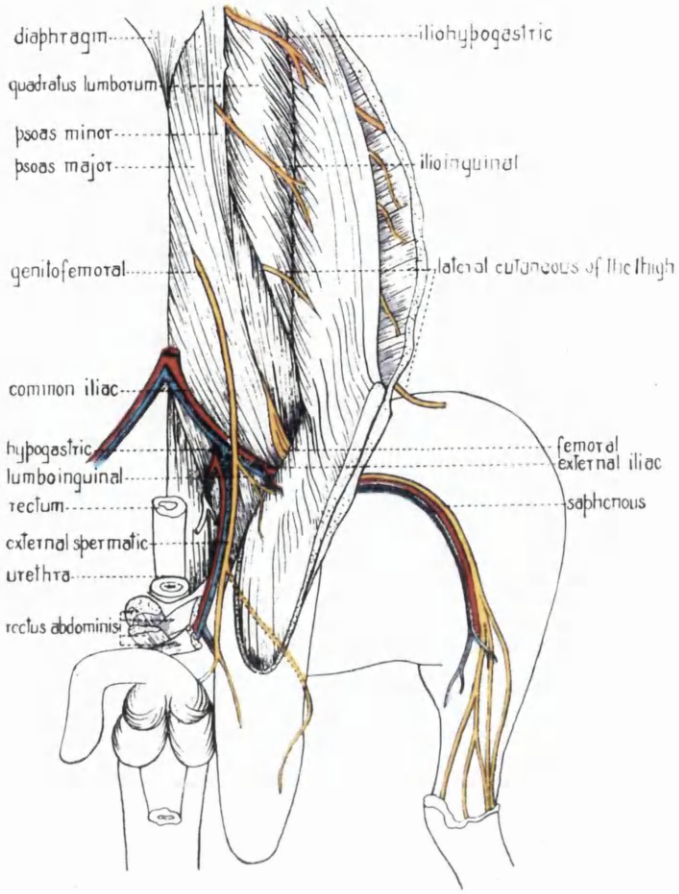


Fig. 1.4. Superficial dissection of branches of left lumbar plexus to show the position of the saphenous nerve (from Greene, 1955).

Histological appearance	Discharge pattern	Afferent fibre [Diam (μm)]	Conduction velocity (m/s)	Afferent fibre classification	Location	Function
Tendon organ	Slowly adapting	Myelinated [10-18]	60-100	I (Aα)	Ligaments	Proprioceptor
Ruffini ending	Slowly adapting	Myelinated [5-12]	20-70	II (Aβ)	Fibrous layer	Proprioceptor
Paciniform corpuscle	Rapidly adapting	Myelinated [5-12]	20-70	II (Aβ)	Fibrous layer	Proprioceptor
Free nerve ending	Non-adapting	Myelinated [1-5]	2.5-20	III (Aδ)	Fibrous layer & synovium	Nociceptor
Free nerve ending	Non-adapting	Unmyelinated [<1]	<2.5	IV(C)	Fibrous layer & synovium	Nociceptor

Table 1-1. Classification of mammalian articular sensory innervation. (Ferrell, 1992).

Epidemiology and aetiology of the rheumatic diseases

Diseases of the musculoskeletal system are among the most common of all human afflictions. Their prevalence is highest among the elderly but, these conditions affect all age groups and are associated with disability, excruciating pain and loss of quality of life. In the United States approximately 33% of adults exhibit musculoskeletal signs of swelling, limitation of movement or pain (Kelsey, 1982). These functional limitations and impairments result in substantial work loss and incur enormous costs in direct medical care and from loss of productivity.

Most musculoskeletal complaints commonly affect the neck, lower back, knee, hip and shoulder. These regional ailments are classed together as the rheumatic diseases. The most common of these disorders include osteoarthritis (OA), bursitis or tendinitis, and lower back pain. Less prevalent of the rheumatic diseases include rheumatoid arthritis (RA), the seronegative arthritides, and crystal associated arthritis such as gout or pseudogout. The present project is mainly concerned with the causal factors involved in OA and RA development so it is to these that we turn our attention.

Rheumatoid Arthritis

RA is a chronic inflammatory arthropathy of unknown cause that can affect most joints. The lack of accurate definition of the disease poses the dilemma to researchers trying to investigate its epidemiology (*ie.* frequency and distribution of the disease in the population). In 1958, the American Rheumatism Association published 11 diagnostic criteria for the disease to improve

communication between researchers (Table 1-2). A patient meeting 7 of the criteria is defined as having *classic* RA; 5, *definite* RA; and 3, *probable* RA.

Women are affected with RA two to three times more often than men, and the disease can occur at any age although its prevalence increases with advancing years. Occasionally multiple family members are affected, and the occurrence in identical twins is greater than expected. This suggests that RA has a genetic predisposition.

Lesions are associated with the disease and are mainly found in the diarthroidal joints but to a lesser extent in the related tissues *eg.* tendons, ligaments and bursae. Since lesions may also be found in other areas of the body (*eg.* kidneys), it may be concluded that RA is a systemic disease and not just confined to joints.

1. Morning stiffness
2. Pain on motion or tenderness in at least one joint
3. Swelling of one joint representing soft tissue or fluid
4. Swelling of at least one other joint
5. Symmetric joint swelling
6. Subcutaneous nodules over bony prominences
7. Typical radiological arthritic changes
8. Positive test for rheumatoid factor in serum
9. Poor mucin precipitate from synovial fluid
10. Characteristic histological changes in synovial membrane
11. Characteristic histopathology of rheumatoid nodules

Table 1-2. Rheumatoid Arthritis Diagnostic Criteria. (Ropes *et al* ., 1958).

Osteoarthritis

OA is considered the most frequent articular disorder amongst man. It affects some joints but spares others. Commonly impaired joints include cervical spine, the lumbosacral spine, the hip, the knees and the first metatarsophalangeal joint in the foot. Notably spared are the ankle, wrist, shoulder and elbow. OA especially involves the weight bearing lower extremity joints and the joints of the hand associated with the pincer grip. With respect to age, OA is uncommon in adults under 40 but is extremely common in those aged 60 and over.

It is important to realise that OA is not a single disease entity but a common reaction pattern to several types of joint injury. It occurs as a result of the degradative and repair processes in cartilage, bone and synovium, and produces secondary components of inflammation. Its cause therefore, involves the interaction of mechanical and biological factors.

Animal models of arthritis

The factors which lead to the development of arthritis are still largely unknown. It is thought that the pathogenesis of the disorder might more easily be understood if convenient animal models were available for laboratory study. These experimental models would also allow the development and screening of drugs which would have value in the treatment of human arthritic diseases. The disadvantage of this approach is that as with all animal experimentation, care must be taken when comparing results obtained using arthritic models with what may be occurring in the human pathological state. However, it must be stressed that a great deal of overlap occurs between the

physiology of humans and that of other animal species, and also the need for an investigative starting point must be appreciated. The arthritic lesions induced in an animal may be short-lasting (acute inflammation) or more longer-lasting (chronic inflammation). The effectiveness of arthritis-induction may be gauged by noting behavioural signs of hyperalgesia such as inactivation of the leg during walking or rapid withdrawal of the leg when the inflamed joint is squeezed. With time, acutely inflamed knees may undergo histopathological changes and eventually become more chronic (Santer, Sriratana and Lowther, 1983). The following is an appraisal of some of the more widely used animal models of arthritis.

Models of osteoarthritis

Numerous animal models of OA have been developed (Adams and Billingham, 1982; Moskowitz, 1984). Small surgically-induced defects do not usually produce OA. However, larger defects produced by deforming the joint contour or direct damage to the articular cartilage by heat/freezing (Simon, Lane and Beller, 1981) or other physical agents may lead to OA.

Isolated rupture of the cruciate ligaments in humans occurs naturally (Kennedy, Weinberg and Wilson, 1974; Wang, Rubin and Marshall, 1975) and the resulting joint laxity causes changes in the joint which resemble OA (Frankel, Burnstein and Brooks, 1971). Cutting the anterior cruciate ligament of the knee in dogs (Pond and Nuki, 1973) also results in laxity of the joint which leads to biochemical and morphological changes resembling those of natural OA (Mc Devitt, Muir and Pond, 1974; Mc Devitt and Muir, 1976; Mc Devitt, Gilbertson and Muir, 1977). In a similar animal model using

rabbits, a partial meniscectomy is performed to generate the osteoarthritic-like condition (Malemud, Goldberg and Moskowitz, 1986; Lanzer and Komenda, 1990).

Compression of articular cartilage causes death of chondrocytes which may lead to the development of OA, possibly by preventing diffusion of interstitial fluid into the cartilage (Hough and Sokoloff, 1989). Joint immobilisation also leads to articular degeneration resembling OA (Tipton, James, Mergner and Tcheng, 1970; Laros Tipton and Cooper, 1971; Amiel, Akeson, Harwood and Frank, 1983; Woo, Gomez, Sites, Newton, Orlando and Akeson, 1987), however, the disadvantage of such a model is that it is difficult to restrict joint motion without altering joint loading (Hough and Sokoloff, 1989). In humans, extensive joint immobilisation produces contracture and ankylosis rather than OA (Enneking and Horrowitz, 1972).

Repetitive impact loading of joints has been employed in animal models to induce degenerative changes akin to the early stages of OA (Radin, 1973). Finally, intra-articular instillation of abrasive particles such as carborundum into the joint induces a low-level synovitis by superficial deterioration of articular cartilage and a foreign body reaction with the synovium.

Arthritis induced by infectious agents

There are a large number of infectious agents which can produce arthritis in animals (for review see Gardner, 1960). Some of the earlier described agents include *Pleuropneumonia*-like organisms, *Streptococci, Erysipelothrix rhusiopathiae, Typhoid bacillus, Streptobacillus moniliformis, Tubercle bacillus* and *Corynebacteria.* More recently, arthritis has been induced by *Diplococcus agalactiae*

(Svartz, 1972), *Mycoplasma arthritides* (Delbarre, Kahan, Amor and Kahn 1964; Delbarre, Kahan and Amor, 1964), and *Salmonella enteritidis* (Volkman and Collins, 1976).

Arthritis induced by intra-articular injection of antigens and antibodies

It is widely known that the immune system plays an important rôle in the development of RA and it therefore follows that a chronic arthritis could be induced by injecting antigens into the joint space. Dumonde and Glynn (1962) produced a chronic arthritis by introducing fibrin into the joints of rabbits previously immunised with fibrin in Freund's complete adjuvant. Other possible antigens include egg albumin (Consden, Doble, Glynn and Nind, 1971), bovine serum albumin (Cooke, Hurd, Ziff and Jasin, 1972) and autologous inflammatory exudates (Phillips, Kaklamanis and Glynn, 1966). The histological appearance of antigen-induced arthritis is very similar to human arthritis (Glynn, 1968) and unlike many other animal models, a single intra-articular injection is sufficient to produce an inflammatory reaction which can last for over a year.

Urate and Kaolin-induced arthritis

Acute, aseptic joint inflammation can be induced by injecting crystals such as urate and kaolin into the articular cavity of rats (Otsuki, Nakahama, Niizuma and Suzuki, 1986), cats (Okuda, Nokahama, Miyakawa and Shima, 1984), dogs (Faires and Mc Carty Jr., 1962; Rosenthale, Kassarich and Schneider, 1966; Van Arman, Carlson, Risley, Thomas, and Nuss, 1970) and man (Faires and Mc

Carty Jr., 1962; Seegmiller, Howell and Malawista, 1962). These injections lead to the synthesis and release of immunocompetent cells which produce a localised oedema (Moncada, Ferreira and Vane, 1979; Sedgwick and Willoughby, 1985; Schiable and Grubb, 1993). In human volunteers and patients, sodium urate produces an inflammatory reaction and pain which is akin to gout (Seegmiller, Howell and Malawista, 1962). Sodium urate crystals have also been injected into rat ankles to give a gout-like inflammatory response within 24 hours (Coderre and Wall, 1987).

Carrageenan-induced arthritis

Carrageenan is a sulphated polysaccharide which can produce acute and chronic inflammatory responses (Di Rosa, 1972). A single intra-articular injection of carrageenan results in an inflammatory response in the joint and is characterised by thickening of the synovial membrane and influx of polymorphonuclear leucocytes into the synovial fluid (Santer, Sriratana and Lowther, 1983). Attendant with the inflammatory response, there is a decrease in the amount of proteoglycan present in the treated cartilage and also a decline in the ability of the cartilage to synthesise this major constituent (Lowther and Gillard, 1976; Gillard and Lowther, 1976). The rate of synthesis of proteoglycan is at its nadir three to seven days after carrageenan treatment and then slowly returns to normal levels four weeks after treatment.

Capsaicin-induced arthritis

Capsaicin (8-methyl-N-vanillyl-6-nonamide) is the naturally occurring constituent of red hot chilli peppers and can exert a dual effect on nerves *viz.* neuroexcitation (Baranowski, Lynn and Pini, 1986; Szolcsanji, 1987; Maggi and Melli, 1988; He, Schepelmann, Schiable and Schmidt, 1990; Rang, Bevan and Dray, 1991) and neurotoxicity (Buck and Burks, 1986; Rang, Bevan and Dray, 1991; Ferrell, Lam and Montgomery, 1992). Capsaicin has been shown to activate polymodal group IV nociceptors in the joint. Close intra-arterial infusion of capsaicin (10^{-4}-10^{-6} M) induces a brief discharge in both group III and IV afferents. The excitatory mechanism of capsaicin involves the selective opening of cationic channels in the afferent neurone (Wood, Winter, James, Rang, Yeats and Bevan, 1988). Consequently, sodium ions are able to enter and potassium ions leave the cell by moving down their concentration gradients, leading to depolarisation of the nerve.

Prolonged application of capsaicin to peripheral nerves or systemic administration of the chemical to adult or neonatal animals results in degeneration of the majority of unmyelinated sensory neurones (Jansco, Jansco-Gabor and Szolcsanji, 1967; Jansco and Kirally, 1981). Intra-articular injection of 1% capsaicin into rat knee joints causes degeneration of unmyelinated nerve fibres one week post-injection (Ferrell, Lam and Montgomery, 1992). The joints subsequently recover two weeks post-injection.

It has also been reported that capsaicin may be beneficial in the relief of arthritic pain. Administration of capsaicin cream to aching knees in patients suffering from RA and OA alleviated pain more

effectively than a placebo control (Deal, Schnitzer, Lipstein, Seibold, Stevens, Levy, Albert and Renold, 1991).

Adjuvant-induced arthritis

The most widely used model of experimental arthritis in the rat is based on the use of Freund's complete adjuvant, a suspension of heat-killed mycobacteria (usually *Mycobacterium tuberuclosum, Mycobacterium phlei* or *Mycobacterium butyricum*) in mineral oil. Stoerk *et al.* (1954) were the first to notice the appearance of a polyarthritis (*ie.* arthritis in a number of different joints) in rats injected with Freund's complete adjuvant. This type of arthritis is immunological in origin whereby an epitope contained on a mycobacterial heat-shock protein (65 kDa) (Van Eden, Hogervorst, Van der Zee, Van Embden, Hensen and Cohen, 1989) is cross-reactive with an antigen on cartilage (Van Eden, Holoshitz, Nevo, Frenkel, Klajman and Cohen, 1985). A high degree of homology exists between the bacterial heat-shock proteins and human cartilage and as such this arthritic model is thought to be best representative of human autoimmune arthritis (Van Eden, Hogervorst, Van der Zee, Van Embden, Hensen and Cohen,1989).

The concentration of mycobacterium in the suspending vehicle is critical for the production of arthritis. Ward and Jones (1962) obtained 100% incidence with 0.6 mg per rat, 80% with 0.3 mg, and 30% with 0.1 mg. When generating a systemic polyarthritis, the most common route of injection is into the footpad or tail of the rat. The tail is often preferred to the footpad mode of administration so as to avoid excessive weight loss in the animal (Winder, Lembke and Stephens, 1969). Alternatively, Koga *et al.* (1976) state that the most effective

route for both a higher incidence and earlier onset of adjuvant arthritis is directly into the inguinal lymph node.

In its most severe form, adjuvant-induced polyarthritis produces a 2-stage inflammatory reaction. The initial stage involves an acute local inflammatory response within the first few hours of administration which subsequently subsides after 3-5 days. During the second week a diffuse inflammatory reaction develops in the distal joints of the limbs. Lesions may also develop at other areas of the body *eg.* eyes, tail genitals and ears (see Pearson, 1963; Billingham and Davies, 1979). A study by Baumgartner *et al.* (1974) described five phases throughout the course of adjuvant arthritis largely in terms of plasma-born mediators of inflammation (see Table 1-3).

A less severe model of chronic inflammation has been developed whereby the adjuvant-mediated arthritis is unilateral *ie.* a monoarthritis is produced (Grubb, McQueen, Iggo, Birell and Dutia, 1988). This model employs lower doses of Freund's complete adjuvant (100-150μg of *Mycobacterium tuberuclosum* or *Mycobacterium butyricum*) which therefore avoids many of the detrimental factors associated with polyarthritic models *ie.* severe and extensive disease, systemic illness and profound weight loss. The duration of the inflammatory lesion is between 2-4 weeks.

Phase	Time after adjuvant inoculation (days)	General characteristics
a	1-4	Acute local inflammation- slight fall in thiol titre and fall in plasma albumin.
b	7-12	Remission of acute inflammation - rise in albumin levels.
c	12-28	Very extensive inflammation - marked fall in thiol titre and albumin level - secondary lesion development.
d	21 onwards	Residual inflammation with osteogenesis.
e	35 onwards	Permanent attendant deformity with minimal burnt out inflammation - albumin levels and thiol titre return to normal values.

Table 1-3. Phases of adjuvant arthritis (from Baumgartner *et al.*, 1974).

Joint inflammation

The inflammatory response is usually considered a biological adaptation to protect the host from a hostile environment. A prerequisite is that the response occurs without any detrimental effect on the host's own tissues. It is therefore apparent that joint inflammation is either inappropriate or in some way "mismanaged". Since joints are moving structures, they are subject to microtrauma almost constantly and are therefore predisposed to inflammation. In most instances, however, articular inflammation or synovitis is self-limited. After a variable period, the joint returns to its premorbid state, but occasionally the process persists and chronic inflammation and eventual tissue damage ensues.

The initiating factors in acute arthritis are unclear but agents known to initiate an inflammatory response elsewhere include cell death (possibly due to tissue necrosis as a result of loss of blood supply), trauma, pathogens or antibody-antigen complexes. All of these will lead to the release of mediators which give rise to inflammation (q.v.). These mediators may also be released directly *eg.* from nerve endings in the form of neurotransmitters. Inflammation has been classified as acute or chronic, depending on the duration of the process (acute being short-lived; chronic is long-lived).

The acute inflammatory response is characterised by three main stages:-

1. Vasodilatation and increased blood flow to the area.
2. Increased vascular permeability with leakage of plasma from the microcirculation.
3. Migration of phagocytic leucocytes from the microcirculation into the surrounding tissue.

Chronic inflammation is epitomised by tissue damage resulting from an agent which is antigenic leading to an immune response. It is possible for an acute inflammatory response to be an initiating factor in a chronic inflammatory disease such as RA, where the necessary genetic susceptibility exists to allow perpetuation of the disease.

The pathophysiology of joint inflammation remains unclear, although there is increasing evidence that most, if not all, aspects of acute inflammation are due to the activity of host-derived mediators as opposed to the direct action of an offending agent.

Mediators of joint inflammation

A number of inflammatory mediators have been isolated in synovial fluid. They are either produced by tissues within the joint itself and/or released during the inflammatory process. The mediators of joint inflammation may be placed in the following categories:

1. **Cytokines** - these are a group of glycoproteins which are released from mast cells during the defence/repair responses of the body. They include the interleukins, growth factors, tumour necrosis factors and interferons. Interleukin-1 is the most extensively studied of these (for review see Martin and Resch, 1988).

2. **Eicosanoids** - these compounds are produced by the oxygenation of arachidionic, dihommogamma linolenic, and eicosapentaeonic acids. They include the prostaglandins, leukotrienes, thromboxanes and lipoxins (Robinson, 1986).

3. **Kinins** - these are biologically active peptides which can increase vascular permeability. The most important kinin is bradykinin which has been shown to excite and/or sensitise joint afferents (Neugebauer, Schiable and Schmidt, 1989).

4. **Histamine and 5-hydroxytryptamine** - these amines are found in various cell types (such as platelets, basophils and neurones) but most importantly in mast cells. Mast cells are known to contribute to inflammatory responses including those of the joint (Mican and Metcalf, 1990). Intra-articular injection of serotonin and histamine in the rat significantly increases plasma protein extravasation and vasodilatation (Bignold and Lykke, 1975; Lam and Ferrell, 1990).

5. **Neuropeptides** - these peptides include substance-P (SP), calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP), neurokinin A & B and neuropeptide Y (NPY). They are released from the peripheral terminals of afferent nerve fibres in the joint and as such are thought to be involved in neurogenic mechanisms of inflammation.

Neurogenic inflammation

Many clinical observations have described bilateral symmetry in joint inflammation. That is to say that arthritis may initially be manifest in a single joint, however, the contralateral joint soon mirror images the inflammatory response. Kidd *et al.* (1989) put forward an hypothesis that the symmetry of arthritis may be due to neurogenic effects. Evidence for this conjecture comes from the lack of an inflammatory response in the paralysed joint of patients who have RA in addition to upper hemiplegia or poliomyelitis (Thompson and Bywaters, 1962; Glick, 1967).

Recent studies have provided compelling evidence that in addition to the sensory rôle of the small diameter articular nerve fibres (Schiable and Schmidt, 1985), they may also have an efferent function. The involvement of the nervous system in the regulation of inflammation was first suggested by Bayliss (1901) who found that antidromic (*ie.* away from the spinal cord) stimulation of the peripheral sensory nerves caused a localised vasodilatation. In a series of experiments, Lewis (1927; 1936; 1941) showed that skin trauma leads to a discrete sequence of events (*viz.* blanching, wheal formation

and the gradual spread of a reddening effect or flare) and that this triple response was produced by the release of substances from the peripheral afferent terminals. Neurogenic vasodilatation and plasma extravasation (the rapid exudation across blood vessels of immunocompetent cells into a damaged area) have been shown to occur by antidromic electrical stimulation optimum for C fibre activation in both skin (Gasser, 1950; Jansco, Jansco-Gabor and Szolcsanji, 1967; Lembeck and Holzer, 1979; Gamse, Holzer and Lembeck, 1980; Foreman, 1987) and the joint (Levine, Clark, Devor, Helms, Moskowitz and Basbaum, 1984; Ferrell and Russell, 1986; Ferrell and Cant, 1987). Direct activation of C polymodal nociceptive afferents in the skin also elicits an inflammatory response (Kenins, 1981).

Neuropeptides and neurogenic inflammation

Evidence for the involvement of sensory neuropeptides in the development of joint inflammation arises from studies demonstrating an elevation of neuropeptide concentration in synovial fluid aspirated from arthritic joints. Despite a large number of clinical studies performed, the emergent data is often equivocal. For example, higher SP levels have been found in synovial fluid of RA patients (Devillier, Weill, Renoux, Menkes and Pradelles, 1981; Lygren, Østensen, Burhol and Husby, 1986; Marshall, Chiu and Inman, 1989; Marabini, Matucci-Cerinic, Geppetti, Del Bianco, Marchesoni, Tosi, Cagnoni and Partsch, 1991). Conflicting results were obtained by Larsson *et al.* (1989) who were unable to isolate SP from neither normal nor rheumatoid arthritic human knees. The reason for this divergence in results may be that the rate of degradation of different neuropeptides is

variable *eg.* SP is metabolised much faster than CGRP, hence any increased release of SP would not be as easy to detect as increases in CGRP. Other studies have detected CGRP, VIP, NPY, somatostatin and low levels of Neurokinin-A (NKA), secretin and pancreatic polypeptide in normal and inflamed knee joints (Lygren, Østensen, Burhol and Husby, 1986; Larsson, Ekblom, Henriksson, Lundberg, and Theodorsson, (1991). Some of these clinical findings have been simulated in experimental models of monoarthritis where significant increases in SP, NKA, CGRP and NPY were found in inflamed rat knees compared to control knees pre-treated with saline (Bilevicuite, Lundeberg, Ekblom and Theodorsson, (1993).

Virtually all small diameter nerve fibres present in normal synovia are immunoreactive for neuropeptides (Hokfelt, Kellerth, Nilsson and Pernow, 1975; Grönblad, Konntinen, Korkala, Liesi, Hukkanen and Polak, 1988; Mapp, Kidd, Merry, Gibson, Polak and Blake, 1989). The neuropeptides found in these fibres were SP, CGRP and NPY. Nerves containing NPY are exclusively associated with vascular structures. Although SP and CGRP were found in perivascular neurones, many free fibres were also present, with some extending almost as far as the synovial surface (Mapp, Kidd, Merry, Gibson, Polak and Blake, 1989).

In order to ascertain the proportion of joint afferents containing particular neuropeptides, the dorsal root ganglion cells of knee joint afferents were labelled with Fast Blue (a retrogradely transported marker) and then treated with antisera to several neuropeptides. In a study in the rat (O' Brien, Woolf, Fitzgerald, Lindsay and Molander, 1989), the knee joint afferents labelled with Fast Blue were subsequently treated with antineurofilament antibody TR97 which labels large light cells with myelinated axons but not small dark cells

with unmyelinated axons (Lawson, Harper, Harper, Garson and Anderson, 1984). CGRP-like immunoreactivity was found in 78% of the unmyelinated axon cells and 70% of the myelinated axon cells. SP-like immunoreactivity was found in 66% of unmyelinated axon cells and 17% of the myelinated axon cells. None of the labelled neurones contained somatostatin-like immunoreactivity.

The distribution of peptide-containing nerve fibres is radically altered in the pathological state. As we have already seen, neuropeptidergic nerves are normally abundant throughout the joint capsule, however in RA (but not OA) patients nerve fibres are depleted in synovial tissue (Grönblad, Konttinen, Korkala, Liesi, Hukkanen and Polak, 1988; Mapp, Kidd, Gibson, Terry, Revell, Ibrahim, Blake and Polak, 1990; Pereira da Silva and Carmo-Fonseca, 1990). In agreement with these human studies, experimental models of arthritis show a depletion in peptidergic innervation. Capsaicin treatment of rat knee joints leads to a decrease in the number of unmyelinated fibres (Ferrell, Lam and Montgomery, 1992). Adjuvant-induced polyarthritis in rats caused near total disappearance of nerves supplying the synovium as assessed by the neuronal marker, protein gene product 9.5 (PGP 9.5) (Konttinen, Rees, Hukkanen, Grönblad, Tolvanen, Gibson, Polak, and Brewerton, 1990; Hukkanen, Grönblad, Rees, Konttinen, Gibson, Hietanen, Polak, and Brewerton, 1991). More recent studies, however, have described an increase in detectable CGRP- and SP-immunoreactive nerve fibres in acute monoarthritis in cat knees (induced by intra-articular injection of kaolin and carrageenan) and chronic adjuvant-induced inflammation in rat paws (Donnerer, Schuligoi and Stein, 1992; Hanesch, Heppelmann and Schmidt, 1994; Nahin and Byers, 1994). Adjuvant-induced arthritis also produced a rise in SP and CGRP content in cell bodies of the

dorsal root ganglia in the spinal cord (Donnerer, Schuligoi and Stein, 1992; Smith, Harmar, McQueen and Seckl, 1992; Nahin and Byers, 1994). Since sensory nerve fibres project from cell bodies in the dorsal root ganglia, it has been postulated that the increased amount of spinally produced neuropeptide is transported toward the periphery in the axoplasmic flow. This would, therefore, account for the higher levels of neuropeptide found in arthritic joints.

Electrical and chemical stimulation of the nerves supplying the joint causes the release of SP and CGRP from the nerve terminals (Yaksh, 1988; Yaksh, Bailly, Roddy and Harty, 1988; Holzer, 1988). The undecapeptide SP is known to be pro-inflammatory in joints by producing vasodilatation and increased blood vessel leakiness. Topical application of SP onto rat knees causes a potent but transient vasodilatation which is enhanced by prior intra-articular carrageenan injection (Lam and Ferrell, 1993b). Plasma extravasation occurs by either a single intra-articular injection (Lam and Ferrell, 1989a; 1989b; 1990) or intra-articular perfusion (Scott, Lam and Ferrell, 1991) of SP into rat knee joints. This SP-induced protein extravasation is potentiated and longer lasting in rat knees which have undergone prior treatment with carrageenan, thus the level of inflammation in the acutely inflamed model is exacerbated (Scott, Lam and Ferrell, 1992). In addition to its vascular effects, SP can also produce other responses in the joint. For example it has been shown that SP can increase the production of prostaglandins in cultured synoviocytes (Lotz, Carson and Vaughyn, 1987) and it can exert effects on white blood cells.

The 37-amino acid CGRP is known to be a potent vasodilator in skin (Brain, Williams, Tippins, Morris and MacIntyre, 1985) and joints (Lam and Ferrell, 1991b; 1993b), however the long-lasting vasodilatation produced by CGRP may be curtailed by co-injection

with SP (Brain and Williams, 1988; Lam and Ferrell, 1993a). The vasodilator response produced by CGRP is more potent and longer-lasting than that of SP. In addition, carrageenan -induced inflamed rat knees show an enhanced vasodilatation in response to topical CGRP application compared to normal knees (Lam and Ferrell, 1993b). In contrast to SP-mediated responses, some studies have found that intra-articular infusion of CGRP itself does not cause plasma extravasation in rat knees but does cause a potentiation of histamine and SP-induced plasma extravasation (Cambridge and Brain, 1992; Cruwys, Kidd, Mapp, Walsh and Blake, 1992). The problem with these experiments is that the markers used to measure extravasation (*ie.*. Evans blue and ^{125}I-labelled albumin) are relatively insensitive. When the more sensitive micro-turbidimetry technique is used, it was found that CGRP elicited a prolonged protein extravasation into the synovial cavity (Karimian and Ferrell, 1994).

In summary, different proportions of afferent nerve fibres supplying joints stain for various neuropeptides such as SP and CGRP. After the occurrence of an acute injury, these sensory nerves are activated leading to the release of neuropeptides from their terminal varicosities. Accumulation of the neuropeptides in the joint elicits a pro-inflammatory response typified as a localised hyperaemia and increased blood vessel permeability. These effects facilitate the delivery of nutrients and the passage of cells and mediators necessary for the healing process from the blood into the affected area. It is important that the inflammatory reaction be transient as prolonged extravasation of inflammatory mediators into the injured area could be detrimental. In acutely inflamed knees, this self-regulatory mechanism is defective causing the inflammatory response to become sustained. Under these conditions the protracted presence of neuropeptides could

inflict further damage to the injured area instead of exerting a beneficial effect. The rôle of sensory neuropeptides in the perpetuation of neurogenic inflammation, ergo, is unequivocal.

Nitric oxide and neurogenic inflammation

The vascular endothelium plays a vital rôle in the control of the circulation. Furchgott and Zawadzki (1980) demonstrated that the acetylcholine-mediated relaxation of the rabbit aorta was dependent on the presence of the endothelium. This dilator effect was found to be mediated by a humoral factor which Furchgott termed the endothelium-dependent relaxation factor or EDRF. Later studies found that nitric oxide (NO) appeared to have the same biological properties as EDRF and it now commonly regarded that these substances are one and the same (Ignarro, Byrns, Buga and Wood, 1987; Palmer, Ferrige and Moncada, 1987; Furchgott, 1988). NO is synthesised by oxidation of a terminal nitrogen atom of the guanidino moiety of the amino-acid L-arginine. The enzyme responsible for this reaction is called NO synthase (NOS) of which there are several isoforms (for review see Marletta, 1993). The growing family of NOS isoforms generally fall into two categories:

(1) a constitutive form regulated by Ca^{2+} and calmodulin which releases NO for short periods in response to receptor or physical stimulation.

(2) a cytokine-inducible form that is not known to be regulated post-transcriptionally which synthesises NO for longer periods.

The chain of events which lead to the release of NO starts with an endothelial cell or other effector cell being stimulated either chemically (*eg.* by acetylcholine or bradykinin) or mechanically (*eg.* by sheer stress or pulsatile pressure) to cause the enzymatic conversion of L-arginine to NO and L-citrulline. The NO then diffuses to a smooth muscle cell where it activates the cell's guanylate cyclase to increase cyclic guanylate monophosphate concentrations which causes muscular relaxation (see Fig. 1.5).

Experiments performed in the late 1980's showed that the release of NO from endothelial cells in culture could be inhibited by N^G-monomethyl-L-arginine (L-NMMA) by competitively inhibiting the enzymatic activity of NOS (Palmer, Rees, Ashton and Moncada, 1988; Palmer and Moncada, 1989; Mayer, Schmidt, Humbert and Bohme, 1989). This compound is therefore a useful tool in the investigation of the biological significance of the L-arginine: NO pathway in the cardiovascular system. The discovery of L-NMMA led Rees *et al.* (1990) to develop other L-arginine analogues which inhibit NO production in vascular tissue. These inhibitors were Nω-nitro-L-arginine (L-NA), N-iminoethyl-L-ornithine (L-NIO) and Nω-nitro-L-arginine methyl ester (L-NAME). The intravenous infusion of these drugs has been shown to cause a dose-dependent hypertension and bradycardia in rats (Rees, Palmer, Schulz, Hodson, and Moncada, 1990) and rabbits (Rees, Palmer and Moncada, 1989). These compounds appear to have different potencies: L-NIO being approximately five times more potent than the other analogues (Rees, Palmer, Schulz, Hodson, and Moncada, 1990) and the effects of L-NAME being ten times more potent than L-NMMA although its rate

of onset of effect is slower (Gardiner, Compton, Kemp and Bennett, 1991).

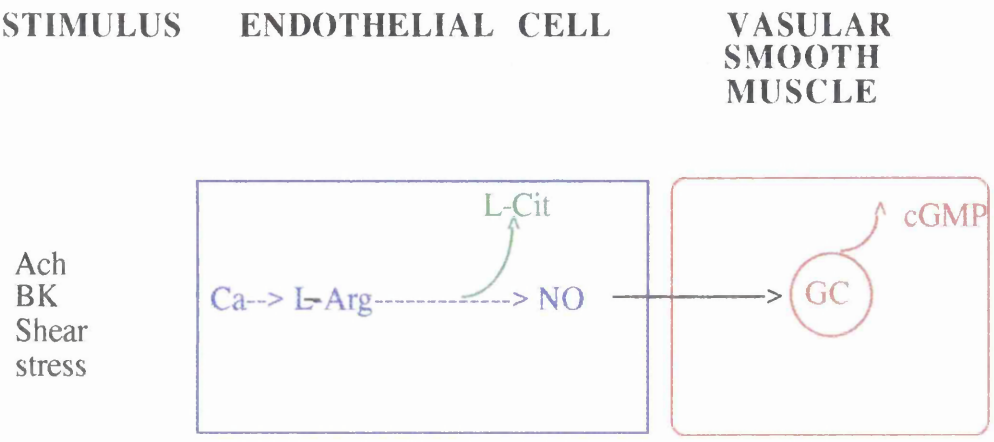


Fig. 1.5. Release of NO from an endothelial cell to induce smooth muscle relaxation (acetylcholine- Ach; bradykinin - BK; L-arginine - L-Arg; L-citrulline - L-Cit; guanylate cyclase - GC; cyclic guanylate monophosphate - cGMP).

EDRF has a half-life of 4-50 seconds depending on how it is measured (Gryglewski, Palmer and Moncada, 1986). In addition to being a potent vasodilator, EDRF also inhibits platelet aggregation (Azuma, Ishikawa and Sekizaki, 1986; Furlong, Henderson, Lewis and Smith, 1987; Radomski, Palmer and Moncada, 1987a) and inhibits platelet adhesion (Radomski, Palmer and Moncada, 1987b; 1987c). NO is also known to be cytotoxic due to its ability to invade microorganisms and tumour cells. It also seems likely that the release of NO has other biological consequences including pathological vasodilatation and tissue damage (for review see Moncada, Palmer and Higgs, 1991).

A neural rôle for NO was first implied by Bredt *et al.* (1990) who found that NOS was present in discrete populations of nerves in the brain, adrenal medulla, intestine and vascular endothelial cells. Further evidence came to light during investigations into the vasodilator responses produced by stimulation of the non-adrenergic non-cholinergic autonomic nerve supply to the Guinea-pig intestine (Wicklund, Leone, Gustaffson, and Moncada, 1993). This study demonstrated release of the NO metabolites NO_2^- and NO_3^- following nerve stimulation which was inhibited by prior treatment with L-NA. Thus nerve stimulation in this model resulted in the release of NO, via activation of NOS, but whose mechanism was unknown. L-NMMA and L-NA are known to potentiate the vasoconstrictor responses induced by transmural electrical stimulation of dog cerebral, mesenteric and temporal arteries (Toda and Okamura, 1990a; 1990b; Toda, Yoshida and Okamura, 1991), which supports the hypothesis for neurally-mediated NO release. It should be noted, however, that further studies are required to ascertain whether NO acts as a secondary messenger modulating the release or action of other vasodilator neurotransmitters, or whether it is itself released from nerve terminals exerting dilator responses directly.

In summary, NO has been shown to be an ubiquitous potent vasodilator and as such could be pro-inflammatory. Strong evidence is accumulating to suggest that NO is released from non-adrenergic non-cholinergic autonomic nerve fibres, and acts as a second messenger in cutaneous afferent nerves in rats (Holzer and Jocic, 1994). The rôle of NO in the development of neurogenic inflammation *in vivo* is probably precipitated by both of these mechanisms. NO has also been implicated in the development of arthritis since elevated levels of NO_2^- was found in serum samples and synovial fluid of RA and OA

patients (Farrell, Blake, Palmer and Moncada, 1992). Other studies have demonstrated increased NO synthesis in adjuvant arthritic rats (Stichtenoth, Gutzki, Tsikas, Selve, Bode-Böge, Böge and Frölich, 1994) and even suppression of experimentally-induced arthritis in rats by treatment with L-NMMA (McCartney-Francis, Allen, Mizel, Albina, Xie, Nathan and Wahl, 1993).

Sympathetic innervation and neurogenic inflammation

In addition to the afferent nerve fibres already discussed, the joint also receives efferent innervation in the form of sympathetic fibres. The presence of these nerves may be established histologically by staining them with the axonally transported label Horseradish Peroxidase. Studies using this technique have shown that large joints are supplied by efferent fibres arising from several sympathetic ganglia. In the knee joint of the cat, sympathetic nerve fibres originate from the paravertebral ganglia L4-L6 of the ipsilateral trunk (Heppelmann and Schiable, 1990) whereas in the monkey they arise from the sympathetic ganglia L3-S3* (Wiberg and Widenfalk, 1991).

The function of the sympathetic nerves *in vivo* is the control of vascular tone in articular blood vessels. This conclusion was arrived at after it was found that blood flow increased following sympathetic denervation and decreased during electrical stimulation of the articular nerves (Cobbold and Lewis, 1956; Sato and Schiable, 1987; Ferrell and Khoshbaten, 1990). In conjunction with this fall in joint perfusion, electrical stimulation of the PAN in the rabbit causes a fall in the partial pressure of oxygen in the synovial fluid (Ferrell and Najafipour, 1992). This constrictor response in the rabbit is

* NB. The "L" and "S" terms refer to the lumbar and sacral segments of the spinal cord respectively.

predominantly mediated via α_2-adrenoceptors since prazosin (an α_1-adrenoceptor antagonist) only slightly reduces the response whereas rauwolscine (an α_2-adrenoceptor antagonist) not only abolishes the response but transforms it into a vasodilatation (Najafipour and Ferrell, 1993a).

The sympathetic postganglionic fibres supplying articular blood vessels maintain a state of tonic vasoconstriction in the dog (Cobbold and Lewis, 1956) and cat (Sato and Schiable, 1987) by producing tonic nervous discharges. These sympathetic nerve discharges occur in bursts and oscillate at low frequencies (Folkow, 1952; Cohen and Goodman, 1970; Hagbarth, Hallin, Hongel, Torebjörk and Wallin, 1972; Camerer, Stroh-Werz, Krienke and Langhorst, 1977; Barman and Gebber, 1980; Gebber and Barman, 1980; Wallin, 1981). This tonic sympathetic outflow may, however, fluctuate reflexly in response to lowering of the systemic blood pressure due to the inhibitory effects of baroreceptors. Excitatory reflexes may also be observed by applying noxious mechanical stimuli to the knee however, innocuous stimuli are ineffective (Sato and Schiable, 1987). The rate of vasoconstriction is also dependent on the pattern of sympathetic nerve discharges. In the rabbit, the rate of vasoconstriction of the vessels in the hindquarters is greatest with modulated impulses of 0.5 and 1 Hz, or bursts of impulses at 10 Hz (Ando, Imaizumi and Takeshita, 1993).

Changes in sympathetic efferent activity have been observed during the progression of joint inflammation. Sympathetic activity in cardiac postganglionic neurones was found to increase during the movement of inflamed cat knee joints whereas noxious stimuli was required to elicit the same response in normal controls (Sato, Schiable and Schmidt, 1985; Sato, Schiable and Schmidt, 1987).

The sympathetic nervous system also appears to influence the development of arthritis. A series of experiments showed that the sympathetic nervous system enhanced the severity of adjuvant-induced polyarthritis in rats (Levine, Dardick, Roizen, Helms and Basbaum, 1986; Levine, Goetzl and Basbaum, 1987; Coderre, Basbaum, Dallman, Helms and Levine, 1990) since:

(a) Treatment with reserpine and guanethidine (*ie.* chemical sympathectomy) reduced the joint inflammation.

(b) Pre-treatment with β_2-adrenergic receptor antagonists retarded the disease onset and also reduced the severity of the inflammation.

However, other studies have not shown an exacerbation of the inflammatory response by sympathetic interaction. Carrageenan-induced acute arthritis in rat knees was unaffected by reserpine as shown by no change in the level of plasma extravasation (Lam and Ferrell, 1989a). Moreover, the vasoconstrictor response to sympathetic nerve stimulation is also altered by carrageenan-induced inflammation. Electrical stimulation of the saphenous nerve in normal rats causes a frequency-dependent vasoconstriction, but this response is greatly attenuated in the carrageenan model (Lam and Ferrell, 1993b).

The explanations put forward for this phenomenon were:

(a) Effectiveness of neurotransmission in inflamed joints is decreased.

(b) Post-synaptic α-adrenoceptors are altered

(c) Co-release of a vasodilator substance such as nitric oxide.

In the rat paw neurogenic mustard oil inflammation and non-neurogenic carrageenan were found to be impervious to chemical sympathectomy (Donnerer, Amann and Lembeck, 1992).

Further involvement of the sympathetic nervous system in the development of inflammation has been postulated as electrical stimulation of articular sympathetic nerves in dogs increases vascular permeability (Linde, Chisolm and Rosell, 1974) and plasma extravasation in cat knee joints is reduced after lumbosacral sympathectomy (Engel, 1941; 1978). Inhibition of plasma extravasation also occurs following chemical sympathectomy with reserpine (Gozsy and Kato, 1966), α-methyl-p-tyrosine or guanethidine (Green, 1974). Other studies have shown that intra-articular infusion of 6-hydroxydopamine, which stimulates release of the contents of sympathetic postganglionic nerve terminals, produces a long-lasting increase in plasma extravasation (Coderre, Basbaum and Levine, 1989). This response was later shown to be unaltered by combined elimination of the primary afferents and mast cells, but was blocked by prior sympathectomy (Basbaum and Levine, 1991). The results of all these experiments show that substances released from the

sympathetic neuro-effector junction cause the induction of plasma extravasation in the rat knee joint.

The afferent and sympathetic efferent nervous systems may well interact to stimulate each other and hence exacerbate the inflammatory response. As we have already seen activation of joint nociceptors during injury will transmit impulses to the spinal cord which, via an axon reflex, will induce the release of inflammatory mediators from C fibre terminals. These inflammatory mediators will aggravate inflammation and further stimulate afferent C fibres. Concurrent with these responses, sympathetic post-ganglionic neurones are excited either by somatosympathetic reflexes or by increased sympathetic nerve discharges. Sympathetic nerve fibres may in turn release pro-inflammatory mediators which would also activate C-nociceptors. The net effect of this cascade of reactions is to set up a positive feedback system which will lead to potentiation of joint pain and inflammation (Fitzgerald, 1989). A diagrammatic representation of this potential model for the interaction of the somatosensory and sympathetic nervous systems is shown in Fig. 1.6.

Evidence is also accumulating that the sympathetic nervous system may be involved in the control of RA. Patients treated with guanethidine to produce a localised sympathetic blockade described less pain and a greater pinch strength. Grip strength, tenderness and morning stiffness, however, were not improved by the treatment (Levine, Fye, Heller, Basbaum and Whiting-O'Keefe, 1986).

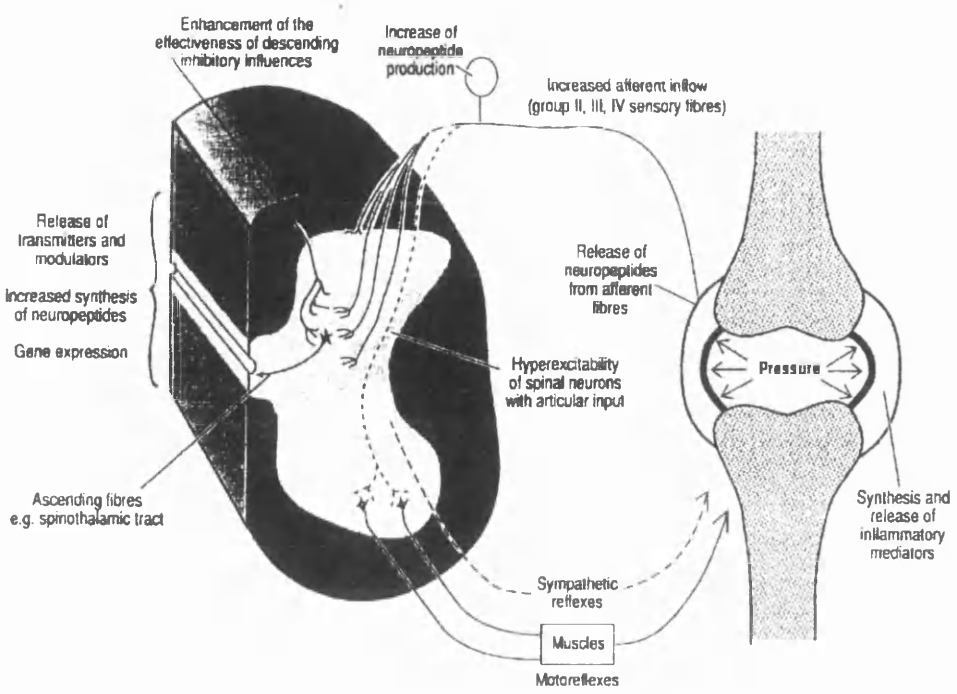


Fig. 1.6. Interaction between the afferent and sympathetic nervous systems in response to an inflammatory reaction in the joint. (From Schiable and Grubb, 1993).

The spinal cord and neurogenic inflammation

In addition to the sensory and sympathetic nervous systems, the spinal cord is gaining recognition as having a rôle in the development of joint inflammation. Spinal neurones show increased excitability following tissue injury and inflammation (Neugebauer and Schiable, 1988; Hylden, Nahin, Traub and Dubner, 1989). This increased firing of spinal neurones may contribute to hyperalgesia in injured tissue and it may even be instrumental in the development of arthritis. Electrical stimulation of PAN in anaesthetised cats has been shown to excite spinal interneurones in the lumbar spinal cord (Gardner, Latimer and Stilwell, 1949; Lundberg, Malmgren and Schomberg, 1978; Harrison and Jankowska, 1985; Schiable, Schmidt and Willis, 1986).

Sensitisation of spinal neurones also occurs in animal models of inflammation. Neugebauer and Schiable (1988; 1990) showed that intra-articular injection of kaolin and carrageenan in cats caused a reduction in threshold in spinal cord neurones. A similar story was found in chronic models of joint inflammation. Anaesthetised rats with unilateral adjuvant-induced chronic arthritis in the ankle show a significant reduction in the mechanical threshold of spinal neurones which have input from the ankle and surrounding tissues (Grubb, Stiller and Schiable, 1992).

The measurement of joint blood flow

The study of the blood supply to diarthroidal joints has been a major concern of man for centuries and has taxed some of the greatest medical minds. Whereas the anatomy of the blood supply to joints has been thoroughly documented, the physiology of articular blood flow is less well understood. The reason for this is that synovial blood flow is difficult to measure because the synovium lacks homogeneity in structure and has a multiplicity of anastamosing vessels supplying it (Liew and Dick, 1981). In order to gain insight into the measurement of joint blood flow we must first have a basic understanding of the physics involved in tissue perfusion.

Basic haemodynamics

Blood flow is the rate of blood displacement with respect to time and is dependent on blood pressure and vascular resistance. The most fundamental law governing the flow of blood through a vessel is based on the following fluid dynamics formula which was empirically derived by Poiseuille:

$$Q = \frac{(P_i - P_o)}{R}$$

where Q is blood flow, P_i and P_o are the inflow and outflow pressures across a vessel respectively, and R is vascular resistance.

The main determinant of vascular resistance is the calibre of the blood vessel. Changes in vessel radius are achieved by contraction of circular smooth muscle in the vessel wall and are principally

associated with the arterial system. The greatest resistance to flow occurs across the arterioles and not the narrower capillaries because there are a lot more capillaries than arterioles resulting in a larger cross-sectional area.

Techniques for measuring blood flow

The earliest measurements of articular blood flow include calorimetric methods (Hollander, Stoner and Brown, 1950) which were later disputed since temperature measurements cannot be transposed into blood flow without knowledge of the temperature of blood entering or leaving the tissue (Greenfield, Shepherd and Whelan, 1951). Around about the same time, other studies into joint blood flow were being performed by Cobbold and Lewis (1956) using anaesthetised dogs. Their method involved measuring the venous outflow from dog knee joints using an electromagnetic flowmeter, however, the technique required ligation of non-articular vessels which would have altered the physiological pattern of blood flow to the joint.

The most popular estimate of synovial blood flow is based on a technique developed by Kety (1949) involving the clearance rate of a radioactive tracer from the joint cavity. Radioisotopes used for such measurements include sodium (Davison and Wisham, 1958), seral protein (Rodnan and MacLachlan, 1960), iodine (Hernborg, 1968), and Xenon (StOnge, Dick, Bell and Boyle, 1968). Radioactive xenon (^{133}Xe) possesses particular properties which make it an ideal tool for such studies.

Xenon clearance technique

^{133}Xe is a biologically inert gas which is a 'soft' gamma emitter. It is also lipid soluble which means it can readily diffuse across cell membranes allowing ease of distribution. The isotope is administered via an intra-articular injection and the wash-out time of the exponentially decaying radioactivity is continuously monitored by a collimated scintillation counter. The advantage of the isotope clearance method is that it provides continuous measures of joint blood flow in absolute units (ml/min/100g of tissue). However, the technique is based on a couple of basic assumptions:

(1) plasma equilibrates fully with synovial fluid in a single transit.

(2) the clearance of ^{133}Xe is purely by the bloodstream and not by diffusion into the surrounding tissues.

The latter assumption is highly tenuous due to the freely diffusive properties of ^{133}Xe. Thus when the gas is injected into the joint it must be distributed through the cartilage, menisci and ligaments as well as the synovial fluid; hence the technique overestimates synovial blood flow (Simkin and Nilson, 1981; Wallis, Simkin, Nelp and Foster, 1985). Care is also required when interpreting the ^{133}Xe clearance method because insertion of the needle into the joint causes tissue trauma which alters the microcirculatory flow (Holloway, 1980; Engelhart, Kristensen and Kristensen, 1983; Staxrud, Kvernebo and Salerud, 1991). In addition, the non-homogeneous nature of the joint

makes acquiring data for the area and thickness of the synovium and cartilage difficult (Levick, 1987).

Microsphere-derived blood flow

A method of tissue perfusion measurement using radiolabelled microspheres (Heyman, Payne, Hoffman, and Rudolph, 1977) has recently been applied to knee joint blood flow determination (Bunger, Hjermind and Bulow, 1983; Bunger, Hjermind, Bach, Bunger and Myhre-Jensen, 1984). This technique involves the slow infusion of radioactively labelled microspheres (approximately 15.5-16.5 μm in diameter) into the left ventricle of the heart. A reference blood sample is withdrawn at a known rate from a cannulated artery just prior to microsphere injection and continued throughout the infusion of the microspheres. As the microspheres enter the general circulation they eventually become lodged in the terminal arterioles of a tissue. Since the level of tissue perfusion is dependent upon the amount of open arterioles, the number of microspheres in a sample will increase with increasing blood flow. At the end of the experiment the animal is sacrificed and the number of microspheres present in the synovial tissue is compared with the number in the reference sample. Since the reference sample is withdrawn at a known rate, it is possible to calculate blood flow to the synovium.

The microsphere technique provides quantitative data about regional blood flow in absolute units (*ie.* ml/min/100g of tissue), however, the method is highly invasive and tells us nothing of the spatial distribution of blood flow within a given tissue. Moreover, the measurements are discontinuous although several evaluations can be generated in a single animal by using different radioactive labels.

Laser Doppler techniques

Laser Doppler flowmetry (LDF) is a technique which has been used extensively in recent years to monitor changes in blood flow in a number of tissues such as skin (Holloway and Watkins, 1977), brain (Busija, Heistad and Marcus, 1981; Haberl, Heizer, Marmarou and Ellis, 1989; Haberl, Heizer and Ellis, 1989), kidney (Stern, Bowen, Parma, Osgood, Bowman and Stein, 1979), bone (Notzli, Swiontkowski, Thaxter, Carpenter and Wyat, 1989), intestinal mucosa (Ahn, Lindhagen and Nilsson, 1985), and knee joints (Geborek, Forslind and Wollheim, 1989; Ferrell, Khoshbaten and Angerson, 1990). LDF is based on the principle that photons from a monochromatic light source undergo frequency (Doppler) shifts by moving erythrocytes which are circulating through a discrete volume of tissue (Stern, 1975; Watkins and Holloway, 1978; Nilsson, Tenland and Öberg, 1980a; 1980b). A more detailed account of the principles involved in this technique shall now be discussed.

The monochromatic light source is derived from a red or near infra-red laser and is directed via an optical fibre onto the tissue under examination. As mentioned earlier, some of the photons are scattered by circulating erythrocytes and undergo Doppler shifts in frequency, the magnitude of which depend upon the velocity of the erythrocytes. The laser light is backscattered towards the optical probe where it is collected by a photodetector which is incident within the probe. The light which enters the probe will be a mixture of Doppler shifted photons and those which have been scattered by stationary structures and as such will have no change in frequency. Analysis of the two types of photons results in the generation of a beat frequency the

amplitude of which will increase with the intensity of the incident light *ie.* the more erythrocytes present in the tissue the greater the amount of light backscattered towards the probe. Because the same beat frequency results from both a positive and negative Doppler shifts, the system is insensitive to the direction of movement.

Monitoring of the collected light by the photodetector will give rise to both DC and AC components of the photodetector current which are analysed to provide a measure of tissue perfusion. The AC component includes any beat frequencies produced by light scattered from moving cells and the power of these frequencies provides an estimate of the number of erythrocytes moving at different velocities; hence, total blood flow can also be estimated. Several adjustments are made to this initial blood flow signal during subsequent processing. One such adjustment compensates for fluctuations in the intensity of light entering the tissue, due for example to changes in laser power. The blood flow signal is therefore normalised to be equivalent to that expected by a constant level of illumination. The DC component of the photodetector current is used in this normalisation process as an indication of the intensity of tissue illumination. However, this DC level is affected by factors other than the incident light intensity such as changes in the amount of light absorbed and scattered within the tissue. Thus, an increase in the number of erythrocytes present in a tissue will cause a rise in absorption and decrease the intensity of backscattered radiation, producing an error in the blood flow signal.

Further adjustments made to the signal include for those made by noise in the photodetector. This photodetector noise adds a DC component to the output which is mixed in with an AC component produced by the moving erythrocytes. The DC component is

compensated for by subtracting an offset calculated from the DC level of the photodetector thereby leaving the requisite AC component.

The signals are centrally processed by the flowmeters and the following outputs are made available to the user:

(1) **Blood flow** - often referred to as the perfusion value, it represents the product of the number of moving erythrocytes and their mean velocity.

(2) **Light intensity** - this is the DC component (level) of the photodetector current which indicates the average intensity of the backscattered light.

(3) **Cell concentration** - this represents the concentration of moving erythrocytes in the tissue studied.

(4) **Cell velocity** - an indication of the mean velocity of the moving erythrocytes.

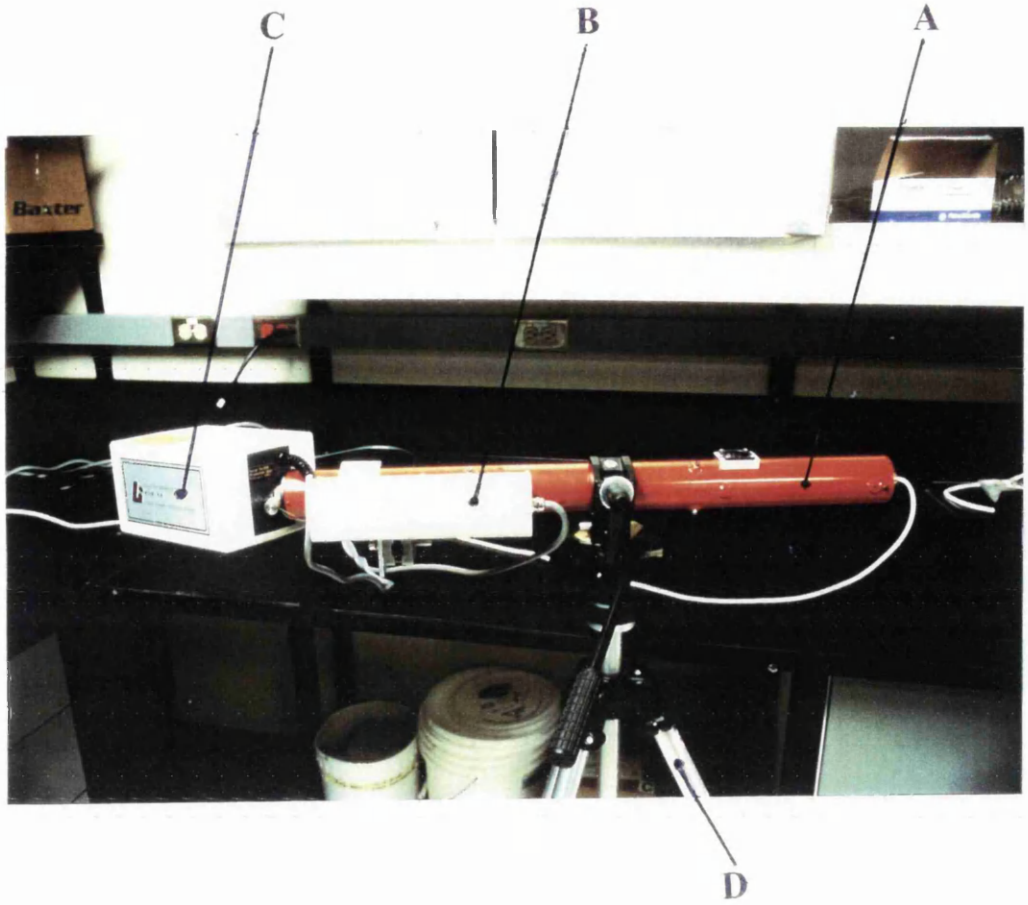
Although LDF provides a continuous non-invasive method of estimating tissue perfusion it can only assess relative changes in blood flow and not absolute measures. Moreover, because of the large spatial variations in tissue perfusion, the single point measurements provided by LDF are unsuitable for mapping blood flow over a tissue area. It was for this reason that an imaging technique was developed to monitor blood flow in 2-dimensions.

Laser Doppler perfusion imaging

Laser Doppler perfusion imaging (LDI) is based on the principles of LDF but allows the 2-dimensional mapping of tissue perfusion (Wårdell, 1992; Wårdell, Jakobson and Nilsson, 1993). The majority of studies using an imager have been used for monitoring skin blood flow (Wårdell, Naver, Nilsson and Wallin, 1993; Harrison, Abbot, Swanson Beck and McCollum, 1993), however its application is becoming more widespread and has recently been used to measure blood flow in joints (Lam and Ferrell, 1993b).

The imager comprises a scanner head attached to a laser tube which outputs a 2mW red He-Ne laser of wavelength 630nm (Fig. 1.7). A modified imager has recently been developed to incorporate a near infra-red laser diode (wavelength 850nm) which will allow greater depth penetration. The optical scanner head houses two mirrors, the position of which are controlled by two stepping motors (Fig. 1.8). The mirrors direct the laser beam onto the tissue surface and by means of the stepping motors move the beam sequentially over the tissue surface. A full format image covers a tissue area of about 12cm square and the whole scanning procedure takes approximately 4.5 min. At each point in the scan, the laser beam stops briefly and takes a perfusion measurement much in the same way as an LDF. The backscattered radiation is captured by a photodetector in the scanner head which subsequently transforms the beat frequencies into an electrical signal. This signal is fed into an analogue processor which performs computational adjustments to the signal (akin to those carried out during LDF processing). The final output is proportional to tissue perfusion and is stored in a computer for further processing and data analysis. At the end of a scan when all the measurement values

Fig. 1.7. Photograph of the modified laser Doppler perfusion imager with red and near infrared lasers (top) and the experimental set-up (bottom). (A: He-Ne laser tube; B: Infrared laser diode; C: Scanner head; D: Supporting tripod; E: 486 PC computer and monitor; F: Position of rats; G: Electric stimulator.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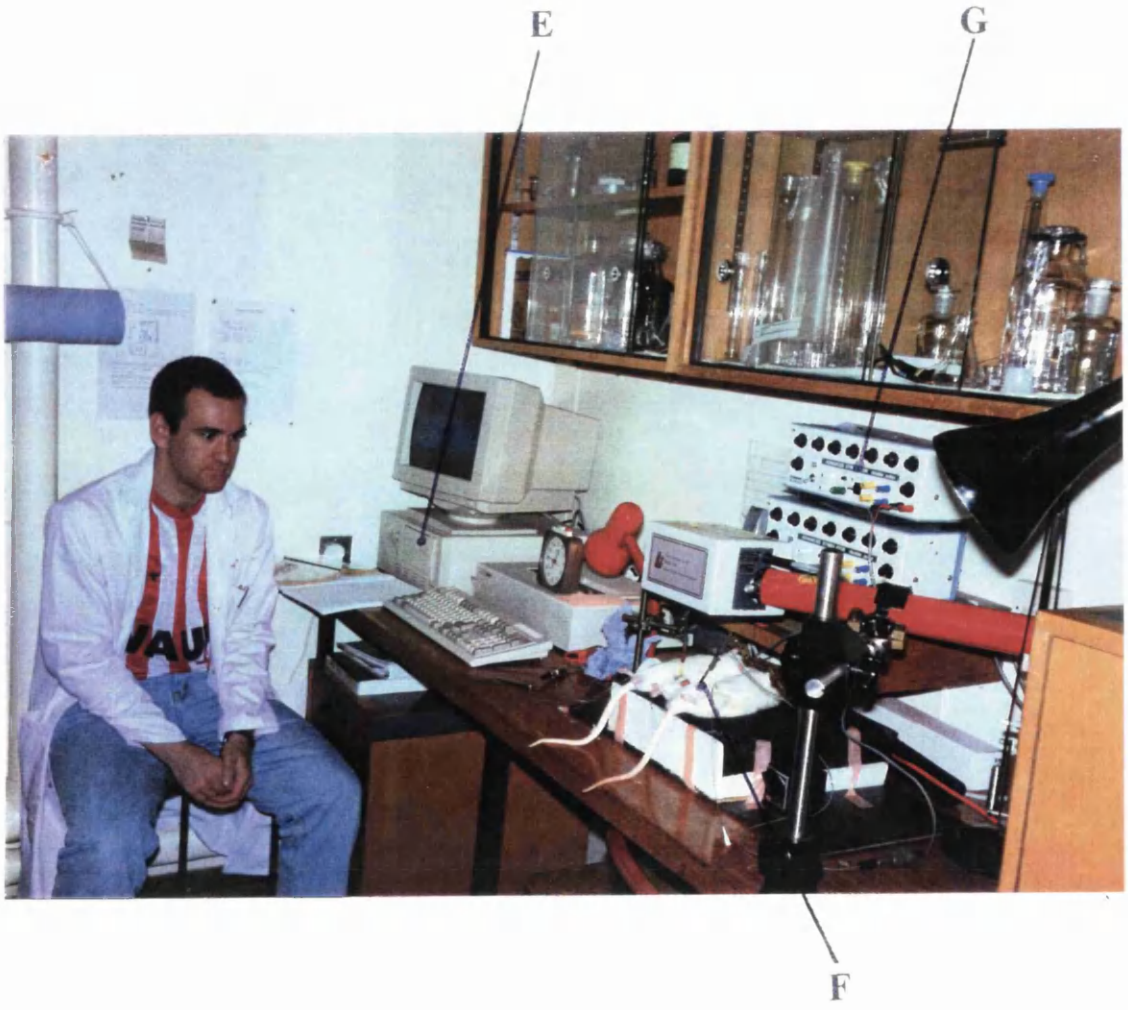
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have been captured and stored, the data are colour-coded to generate a 2-dimensional image of tissue perfusion which can be displayed on a monitor.

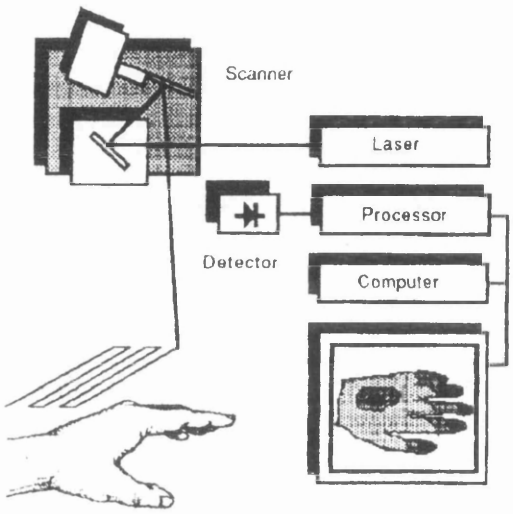


Fig. 1.8. Diagrammatic representation of the laser Doppler perfusion imager (from Wårdell, 1992).

The colour-coded perfusion image displayed on the monitor can be represented in either 'relative' or 'absolute' measures. In 'relative' mode, the highest of the captured perfusion values is set to 100% and all the other values are scaled relative to this maximum value. In 'absolute' mode, however, the user can select the values for the upper and lower limits which are then set to 100% and 0% respectively. An example of a perfusion image is shown in Fig. 1.9.

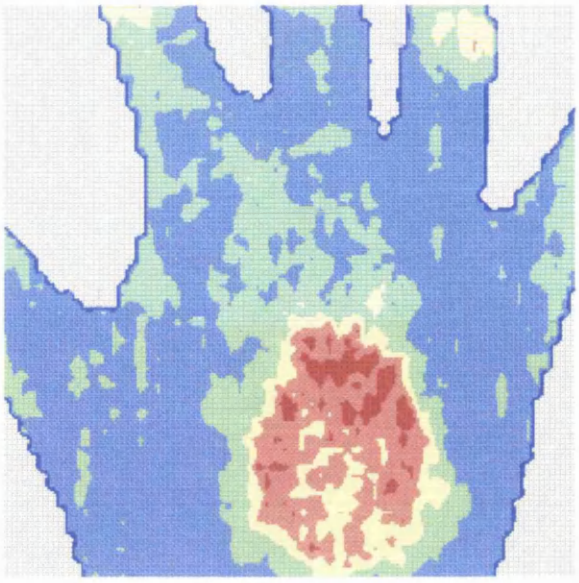


Fig. 1.9. An LDI image of a burn injury to the dorsal side of a hand. The image is colour-coded using a six colour scale where lowest perfusion is coded dark blue followed by light blue, green, yellow, light red and finally a dark red colour which corresponds to regions of highest perfusion (from Wårdell, 1992).

The images generated by the LDI can be imported into a data analysis package such as VIEW which allows quantification and mild statistical testing of the data.

The disadvantages of this technique include that LDI can only produce relative changes in perfusion and not absolute measures of blood flow. Although LDI can resolve the spatial distribution of tissue perfusion, it is unable to monitor blood flow continuously. Another slight disadvantage of this method is that scans must be performed in

the dark since ambient light can saturate the performance of the photodetector.

Recently, a modified version of the LDI has been developed (Niazi, Essex, Papini, Scott, McLean and Black, 1993) which uses a single large mirror instead of the two smaller mirrors seen in the earlier model. Along with a more efficient data collection process, this newer machine can scan a larger area in a shorter time (50x70 cm in 6min).

Chapter 2

General Methods

Animals

The rats used in the present project were adult male Wistars weighing 275-660g. These albino outbred rats are docile and easy to handle (for general information see Table 2-1). Prior to experimentation, the animals were caged and kept in a centralised animal facility. The rats were maintained in a thermoneutral environment (approximately 21°C) and each cage was supplied with sufficient food and water which the rats had access to *ad libitum.*

Other animals used in the project were adult New Zealand white rabbits (4.0-5.5kg in weight) which were housed in a similar facility to the rats and under the same conditions. These lagomorphs are very docile but can easily become stressed if not handled correctly (for general information about the rabbits see Table 2-2).

Weight of adult male	300-400g
Weight of adult female	250-300g
Age at puberty	60-72 days
Heart rate of adult	261-600 beats/min
Heart rate of newborn	81-241 beats/min
Breathing rate	75-115 breaths/min
Daily water requirements	25ml/day
Daily food usage	12-15g/day
Body temp.	37°C

Table 2-1. General information about Wistar rats.

Weight of adult male	4-5kg
Weight of adult female	4.5-5.5kg
Heart rate of adult	123-304 beats/min
Breathing rate	38-60 breaths/min
Daily water requirements	Supplied *ad libitum*
Daily food usage	128.8g/day
Body temp.	37.5 - 40.1°C

Table 2-2. General information about New Zealand White rabbits.

Anaesthesia protocol

(i) *Rat terminal experiments*

All of the blood flow measurements were performed with the rat under deep terminal anaesthesia. For a 300g animal this entailed the intra-peritoneal injection of about 2ml of 25% urethane (Fig. 2.1) with top up doses of 0.25ml whenever the rat became light. Due to the carcinogenic and general toxic properties of urethane it cannnot be used for recovery experiments, However, it's relatively long half-life means that it stays in the body longer providing a deep level of anaesthesia which is fairly easy to maintain.

(ii) *Rat recovery experiments*

This type of anaesthesia was used in studies where the animal underwent surgery for the induction of experimental arthritis. It involved the intra-muscular injection (Fig. 2.1) of 0.1ml of Hypnorm (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml) and intra-peritoneal injection of 0.8ml of diazepam (valium). These values are for a 300g rat and were scaled according to the animal's weight. Hypnorm causes muscle stiffness whereas diazepam is a sedative and muscle relaxant. When administered together these substances induce a deep level of anaesthesia which can last about an hour.

Fig. 2.1. Routes of administration of anaesthetic in the rat: intra-muscular (top) and intra-peritoneal (bottom).

(iii) *Rabbit terminal and recovery experiments*

The rabbits were initially premedicated with an injection of 0.15ml acepromazine maleate into the marginal ear vein. After sedation of the animal a face mask was attached and anaesthesia maintained for the duration of the experiment with gaseous anaesthetics. The inhalation gases consisted of a mixture of 2-5% halothane and 100% oxygen which was set at a flow rate twice the minute volume of the rabbit to ensure complete removal of the exhaled gases from the expiratory tube during the expiratory hiatus. The volume of the expiratory tube is greater than the animal's tidal volume so as to prevent inhalation of atmospheric air.

Rabbits are notoriously difficult to anaesthetise since they rapidly wax and wane between deep and very light levels of unconsciousness. It is for this reason that gaseous anaesthesia is preferred since it can be quickly excreted from the body and the amount delivered may be swiftly adjusted depending upon the state of the animal.

Signs of deep anaesthesia include the abolition of the withdrawal reflex when the hindpaw is squeezed and removal of the corneal blinking reflex which normally occurs when the cornea is touched. Once anaesthetised, the animals are constantly monitored by ensuring that breathing is regular and slow, blood pressure is stable, muscles are relaxed and the pupils constricted.

Preparatory surgery

(i) *Tracheal cannulation*

Once stable anaesthesia was achieved, the animal was placed in dorsal recumbency on a dissecting board and the paws were restrained. The fur around the neck area was then lacquered with mineral oil to minimise hair dispersal (in the rabbit experiments the fur was removed from this region by shaving the skin). A midline incision in the skin covering the throat was made and the large submandibular salivary glands were laterally retracted to expose the sternohyoid muscles. By using a blunt dissection technique with curved forceps, the sternohyoid muscles were longitudinally divided to reveal the trachea. Two loops of saline moistened thread were then placed around the trachea and a small incision was made in the airway between the threads. A cannula of appropriate size was then inserted into the trachea and tied in place with the two loops of thread, one above and one below the incision site.

(ii) *Carotid and jugular cannulation*

The carotid artery was located deep in the neck running parallel to the trachea (Fig. 2.2). The vessel was isolated and a length of moistened surgical thread was wrapped around it, tied off as rostrally as possible and secured to the dissection board with plasticine. A small arterial clip was then fastened as caudally as possible to the carotid and a second thread was placed under the artery for 'tying in' purposes.

With the aid of ophthalmic spring-loaded scissors, a tiny incision was made in the carotid artery as close to the first thread as possible. Fine watchmaker forceps were then employed to hold the upper flap of artery wall so as to keep the incision open. A cannula (diameter 1.02 mm), with an obliquely mitred tip and containing heparinised saline, was inserted into the carotid as far as the arterial clip would allow. The cannula was then tied in with both loops of thread and the clip removed. This cannulation allowed the animal's arterial blood pressure to be monitored (q.v.).

In order to administer drugs intravenously to the animal the external jugular vein had to be cannulated. The jugular is the principal vein in the neck of the rat and may be located in the superficial cervical tissues running just below the skin. The vein was cannulated using the same procedure as the carotid cannulation. The cannula (diameter 0.75mm), also containing heparinised saline, was inserted deep into the vein. Ideally, the cannula should be inserted into the subclavian vein in the thorax which, due to the subatmospheric pressure in this region, has the advantage of reducing the likelihood of collapse of the vessel during blood withdrawal. Finally, the cannula was shortened prior to drug administration to reduce the amount of dead space.

The exposed neck was then covered with a piece of saline-soaked gauze to prevent desiccation of the underlying tissues.

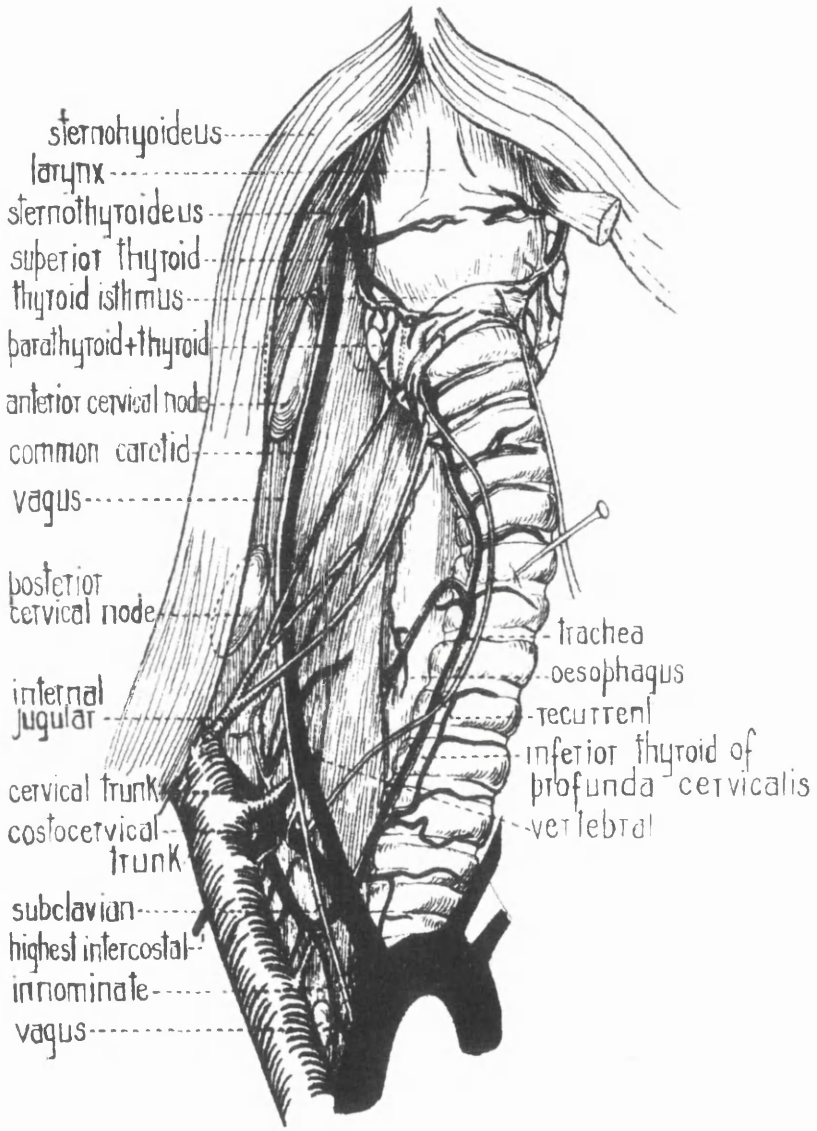


Fig. 2.2. Dissection of the rat neck to show the position of the major blood vessels (from Greene, 1955).

(iii) *Isolation of the saphenous nerve*

The fur covering the hindlimb of the rat was shaved off and a tiny incision (approximately 5 mm in diameter) made in the skin covering the inner thigh. The blunt dissection technique was again employed using blunt-ended Mayo scissors to tease the underlying tissues away from the skin. Removal of the subcutaneous fat pad using a cautery to prevent bleeding, revealed the saphenous nerve which arises from the abdominal cavity and then arcs down towards the paw. Under a dissecting microscope it is possible to distinguish a branch of nerve fibres leaving the saphenous at the level of the knee joint; these are the fibres which supply the vessels of the knee joint and its overlying skin. With the nerves located, the saphenous was peripherally dissected free from the surrounding tissues as far as the great saphenous/superficial epigastric bifurcation. A short length of saline-moistened silk thread was tied around the nerve as proximally as possible, and to prevent centrally-mediated changes in systemic blood pressure during nerve stimulation the saphenous nerve was proximally transected.

A piece of saline-soaked cotton wool was placed on the nerve to prevent it from drying out.

(iv) *Exposure of the knee joint*

To avoid interference of the LDI signal by cutaneous blood flow, it was necessary to excise an ellipse of skin from around the animal's knee (Fig. 2.3). The exposed medial aspect of the knee was prevented from drying out by dousing the joint with physiological saline (0.9% NaCl solution). The saline was warmed (37°C) and applied frequently to the joint to prevent local changes in temperature (*eg.* by evaporation of the saline) which would alter tissue blood flow.

Blood pressure measurement

Systemic blood pressure is measured throughout an experiment so that the investigator can monitor the physiological status of the animal while it is under general anaesthesia. It has greater significance in the present studies to ensure that any changes in joint blood flow are not due to passive mechanisms brought about by changes in arterial blood pressure.

The procedure involved the connection of the carotid cannula to a pressure transducer (Elcomatic EM751) which converted the arterial pulsatile pressure changes into electrical signals. These signals were subsequently amplified by a bridge amplifier (variables set to: Bridge ±5V; Gain A 100; Gain B 20; L.P. Filter 50Hz) and fed into a Multitrace 4 chart recorder (Lectromed Ltd.). The chart recorder had previously been calibrated with a sphygmomanometer to give a full scale deflection equivalent to 200mmHg. During each cardiac cycle there is a maximum and minimum blood pressure; these are the systolic and diastolic pressures respectively (Fig. 2.4). Therefore, an

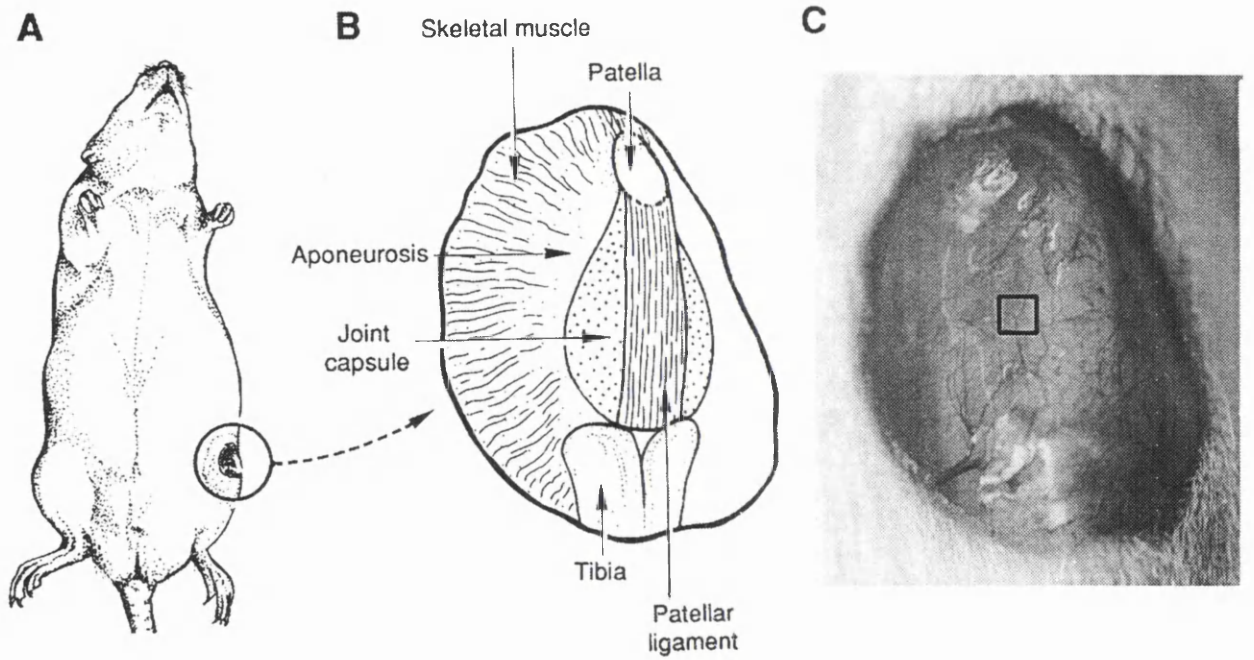


Fig. 2.3. Excision of a piece of skin in order to expose the medial aspect of the rat knee joint. LDI measurements were restricted to the joint capsule by masking the other tissues in black cloth (from Lam and Ferrell, 1993a).

average blood pressure reading is taken for any particular cardiac cycle and is called the mean arterial pressure (MAP) which has the following approximation:

$$\text{MAP} = \text{Diastolic Pressure} + \left(\frac{\text{Systolic Pressure} - \text{Diastolic Pressure}}{3}\right)$$

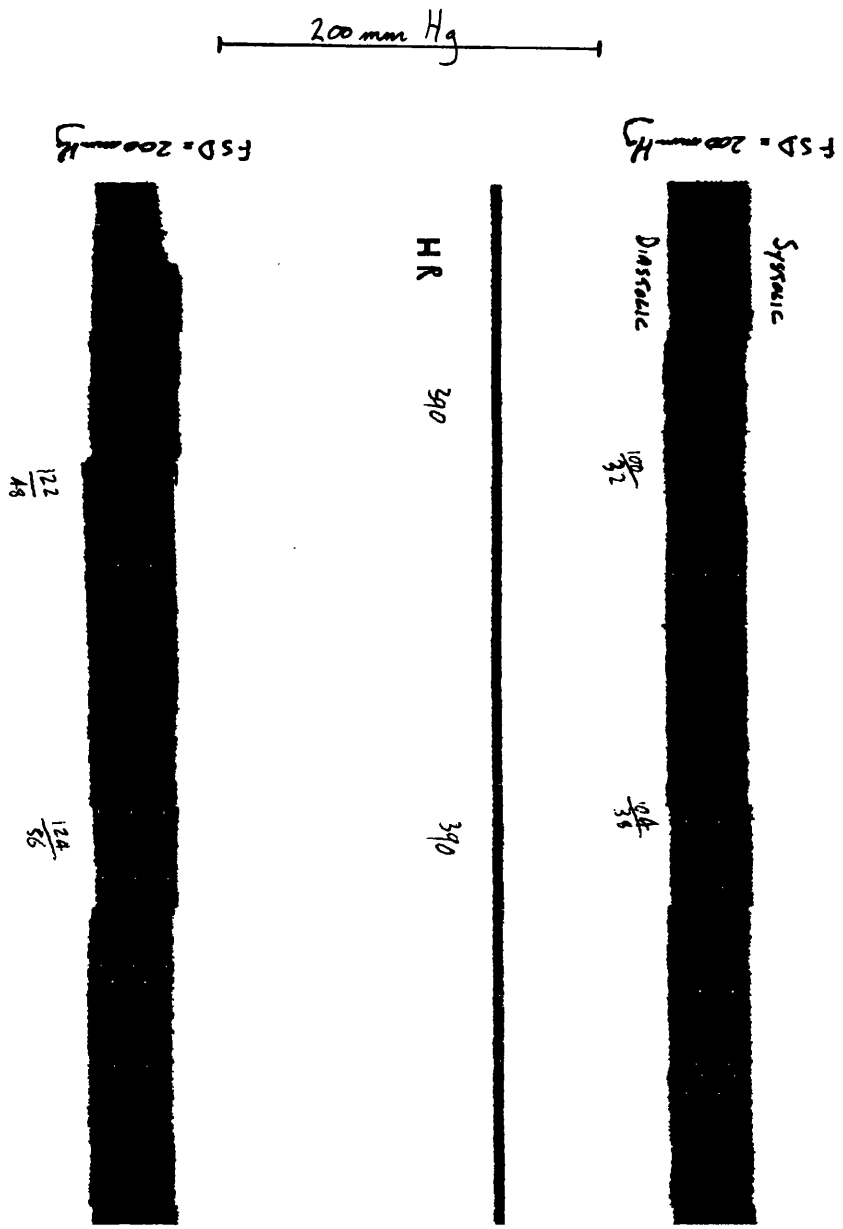


Fig. 2.4. Example of a blood pressure trace showing systolic and diastolic pressures. The traces were calibrated to give a full scale deflection (FSD) equivalent to 200mmHg. The heart rate (HR) trace is also shown.

Electrical stimulation of articular nerve

Electrical nerve stimulation must be applied via a pair of electrodes. It is desirable that these electrodes should not be affected by the passage of small currents through them *ie.* that they should be non-polarisable. For many purposes fine silver wires are adequate, however, slightly better electrodes are made of platinum or from silver wire that has been electrolytically coated with silver chloride. In the present project a set of bipolar silver wire electrodes were used.

The isolated articular nerve was draped over the electrodes in such a way that no part of the nerve nor electrode were in contact with the underlying muscle. This absence of any bridging was tested by delivering a single pulse to the nerve and observing whether the underlying muscle twitched. It is important that the muscle does not contract since any movement of the limb during nerve stimulation will invalidate the LDI measurements. The nerve was then coated in a layer of petroleum jelly to stop it from drying out.

Stimuli were delivered to the nerve via a Harvard dual channel electrical stimulator which was connected in series with the electrodes. This type of stimulator produces 'square' pulses of constant voltage which begin and end abruptly (Young, 1973). If an axon is stimulated with square constant current pulses, it is found that the threshold stimulus intensity rises as the pulse length is lessened. this effect is known as the strength-duration relationship and has the empirical equation (Hill, 1936):

$$\frac{I}{I_0} = \frac{1}{1 - e^{-t/k}}$$

where I is the intensity of the pulse, t is the length of the pulse (the *rheobase*), I_0 is the intensity of the pulse, and k is a constant.

It was for this reason that the stimulator parameters were set at voltage 15V and pulse width 1ms, these parameters being supramaximal for C fibres. Frequency of the impulses was set at 0.5, 1, 2, 5, 10 or 30Hz so that the effect of frequency on joint blood flow could be investigated. The most effective method of stimulation delivery is cathodal *ie.* the electrodes were oriented such that the cathode was closest to the knee.

Recording and processing LDI measurements

Positioning of the animals

After the preparatory surgery, the animals were placed on a stage under the scanner head. The stage was covered in black cloth since the LDI recognises this as background and skips over these areas thereby shortening the scan time. In the later experiments, the joint capsule was isolated by masking the surrounding tissues with black cloth. This had the advantage of reducing the scan time yet further and facilitating data analysis (q.v.).

As we have already seen, during a scan the laser beam sequentially moves across a tissue surface. Each measurement position in the scan corresponds to a specific distance and angle from the photodetector (Fig. 2.5). Consequently, a number of position-dependent coherence areas are generated on the detector surface which are in turn dependent on the system amplification factor to provide measures of blood flow. In order for the LDI to output signals which are proportional to blood flow, it must introduce a correction factor to the amplification component which will compensate for these changes in position. It follows, therefore, that the scanner head must be

positioned at a specified distance from the tissue surface in order for it to function properly. The optimum distance in the present study was found to be 12cm above the tissue.

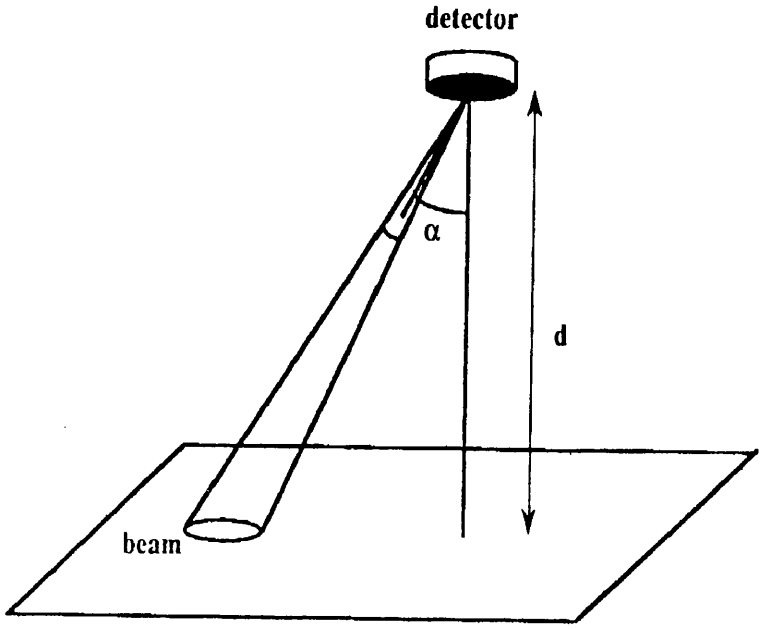


Fig. 2.5. To show that each measurement site in a scan is dependent on its angle (α) and distance (d) from the photodetector (from Wårdell, 1992).

Operating principles

The LDI can generate either high or low resolution images. When operating in low resolution mode the LDI can scan an area up to 12cm square and the measurement points are 2mm apart. However, when the machine is set to high resolution the scanning area is greatly reduced but the sampling points are only 1mm apart. Irrespective of resolution

setting employed, the sampling area of each measurement point is equivalent to the diameter of the laser beam (800μm) and represents a pixel on the final image. For all of the animal experiments the imager was set to high resolution which still allowed two opposite knees from separate animals to be scanned simultaneously. The LDI also has a threshold setting which allows the user to predetermine the background level; for the experiments here this was fixed at 5.81V.

With all the parameters set up and the animals in place the measurement area was marked out and adjusted to the relevant dimensions. Scanning could now take place.

Depth penetration of laser light

The majority of studies examining the depth penetration of laser radiation are based on theoretical predictions. Jakobsson and Nilsson (1991) calculated the median sampling depth of photons from a 633nm laser in skin to be 250μm by using Monte Carlo simulations which are highly involved probability calculations based on the optical properties of the tissue. More practical examinations of light transmission through skin have found that incident light is attenuated to 37% at a depth of 0.56mm with 600nm wavelength and at 1.19mm with 800nm wavelength radiation (Anderson and Parrish, 1981). The penetration of light through skin is limited by the presence of melanin which has significant absorption characteristics of wavelengths up to 1200nm.

These findings are all very well for determining the depth penetration of light, however, laser Doppler techniques rely on both the penetration and return of laser light from a tissue. Nevertheless,

knowledge of the penetration aspect of photons is still very important for laser Doppler studies. Other experiments have placed unperfused tissue slices between an LDF probe and the surface of a perfused tissue (Stern, Lappe, Bowen, Chimosky, Holloway, Keiser and Bowman, 1977; Kiel, Riedel, DiResta and Shepherd, 1985; Kvietsy, Shepherd and Granger, 1985), however, the results of these experiments are not uniform.

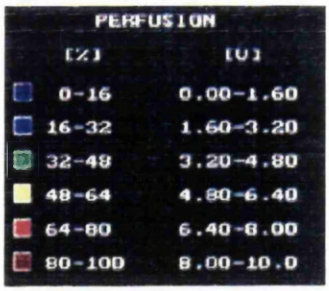
In the present study it was necessary to determine whether the LDI could detect changes in blood flow in synovial vessels of the rat knee joint. To do this scans of the medial aspect of the knee joint were taken before and at intervals after intra-articular injection of adrenaline which vasoconstricts these vessels. As shown in Fig. 2.6C, this produced a rapid reduction of perfusion in the area of the joint capsule, but left surrounding regions unaffected, even 10 minutes after injection. As it has been shown that >90% of capillaries in the joint capsule are located in the thin strip of synovium which lines the synovial cavity, and many of these capillaries are within 20μm of the joint space (Knight and Levick 1983), this result indicates that the red laser penetrates through the capsule to detect blood flow in the deepest layers. As the underlying cartilage is avascular and laser penetration of dense cortical bone is negligible, any changes in blood flow detected by the LDI apparatus exclusively reflect alterations in synovial perfusion.

Fig. 2.6. **A:** colour-coded system for showing perfusion values ranging from dark blue (lowest) to dark red (highest), along with corresponding voltages (0-10V) of the LDI signal. The same range of values were used in all scans.

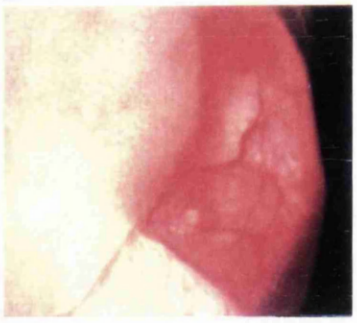
B: Photograph of the exposed medial aspect of a rat left knee joint, showing the high degree of vascularity of the region. The white area lateral to the large branching blood vessel corresponds roughly to the joint capsule.

C: A sequence of perfusion images of the medial aspect of a normal rat left knee joint. The first image is prior to any experimental procedure followed by an image from the same knee 1 minute after intra-articular injection of 10pmol of adrenaline. A reduction in perfusion was found over the capsule region of the joint. The final image was taken 10 minutes after injection of adrenaline, the underperfused area was somewhat larger, but surrounding regions were unaffected, suggesting that there was no diffusion of adrenaline away from the joint.

A

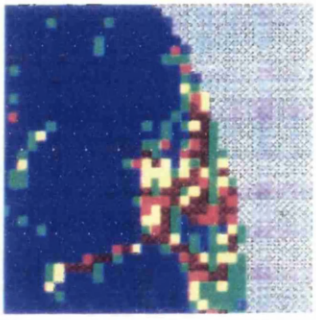


B

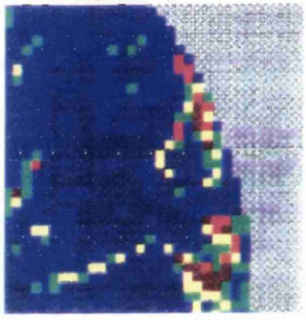


C

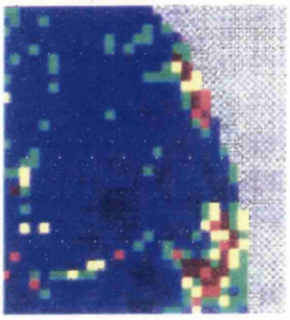
Control



10 pmol adrenaline (1min)



10 pmol adrenaline (10min)



As mentioned earlier, the low sampling depth of red laser light through skin means that the LDI is not ideally suited for transcutaneous imaging. However, it has previously been shown that greater sampling depths can be achieved with near infra-red (780nm) laser Doppler systems than with those using red He-Ne (633nm) lasers (Obeid, Dougherty and Pettinger, 1990). Therefore, modifications were made to an existing LDI to introduce a solid state near infra-red laser source. This dual wavelength LDI allows the user to obtain superimposed Doppler images measured at the two different wavelengths from identical tissue sites. With the aid of the system software, it is possible to separate the combined images into their different components so that they can be compared. As well as obtaining combined images, it is possible to choose either of the two wavelengths to generate single wavelength images.

The dual wavelength LDI consists of a shuttering system which depending on its position, allows only one of the two wavelength laser beams to reach the scanner head. During operation the shutter can rest at one of these two positions or rapidly move between the two points when in dual scanning mode. A beam splitter is present in the system which combines the laser beams from different sources into the same optical pathway before they enter the scanner head.

Biological zero

It has previously been shown that if the arterial input to a tissue is occluded to such an extent that it causes ischaemia, LDF's are still able to measure a residual signal from the tissue (Holloway, 1980; Tenland, Saleru, Nilsson and Oberg, 1983; Caspary, Creutzig and Alexander,

1988; Svensson and Swedman, 1988). This signal is known as the 'Biological Zero' and should be subtracted from all laser Doppler flux readings (Fagrell, 1990; Nilsson, 1990). Thus at the end of all animal experiments, the animal was killed by an overdose of Euthetal (sodium pentobarbitone 120mg/ml; approximately 0.5 ml injected intracardially) and a dead scan performed 5min later with the animal *in situ*. Euthetal was preferred to other drugs to kill the animal (*eg.* concentrated KCl) since it does not induce a state of general tetani which would alter the position of the scanned area, complicating image analysis.

Image analysis

At the end of an experiment, the images were individually analysed using a software package called VIEW (Moor Instruments Ltd.). The program allows 1-4 images to be analysed at once and were displayed by entering their assigned filename. Having chosen the necessary files, the "V" for View button was pressed followed by the "A" for Analysis button. On entering the analysis mode, a cross-haired cursor appeared on each image. The cursor's coordinates and the value of the pixel it covered were presented in the individual image status blocks. The value could either be shown as a percentage or a voltage; the latter was used throughout this project. The cursors could be moved about by pressing the arrow keys which would allow the analysis of each pixel. Each of the individual image's cursor could be moved exclusively of the others by pressing "U" for Unlink. This facility is important if the animal has moved during successive scans since it allows readings to be taken from comparable loci on the images.

Analysing each pixel would have been laborious so statistical ("S") analyses were performed on a chosen rectangular array of pixels. On entering the statistical mode, the cross-hair cursors were replaced by a rectangular cursor (7 x 7 pixels) which was centred over the position of the former cross-hair cursor. The area of the rectangles could be increased in both dimensions either collectively (linked) or individually (unlinked). In order to make comparisons between successive images in a single animal, it was imperative that the rectangles were of the same dimensions and oriented over similar areas of the image. This process was facilitated in later experiments in which the joint was masked with black cloth. Since these images comprised solely of the joint capsule, the analysis rectangle was placed around the whole image.

The "D" for Data button was pressed next which allowed the calculation and display in the status block of the sample size (n), arithmetic mean (AM), standard deviation (SD), median, interquartile range, and skew of the pixel values in the rectangle (NB: the pixels underneath the border of the rectangle cursor were also used in the statistical calculations). Any pixels corresponding to background were ignored and as such took no part in the calculations. For the present project, the median values were used since it was found that they were not significantly different from the AM's.

Data manipulation

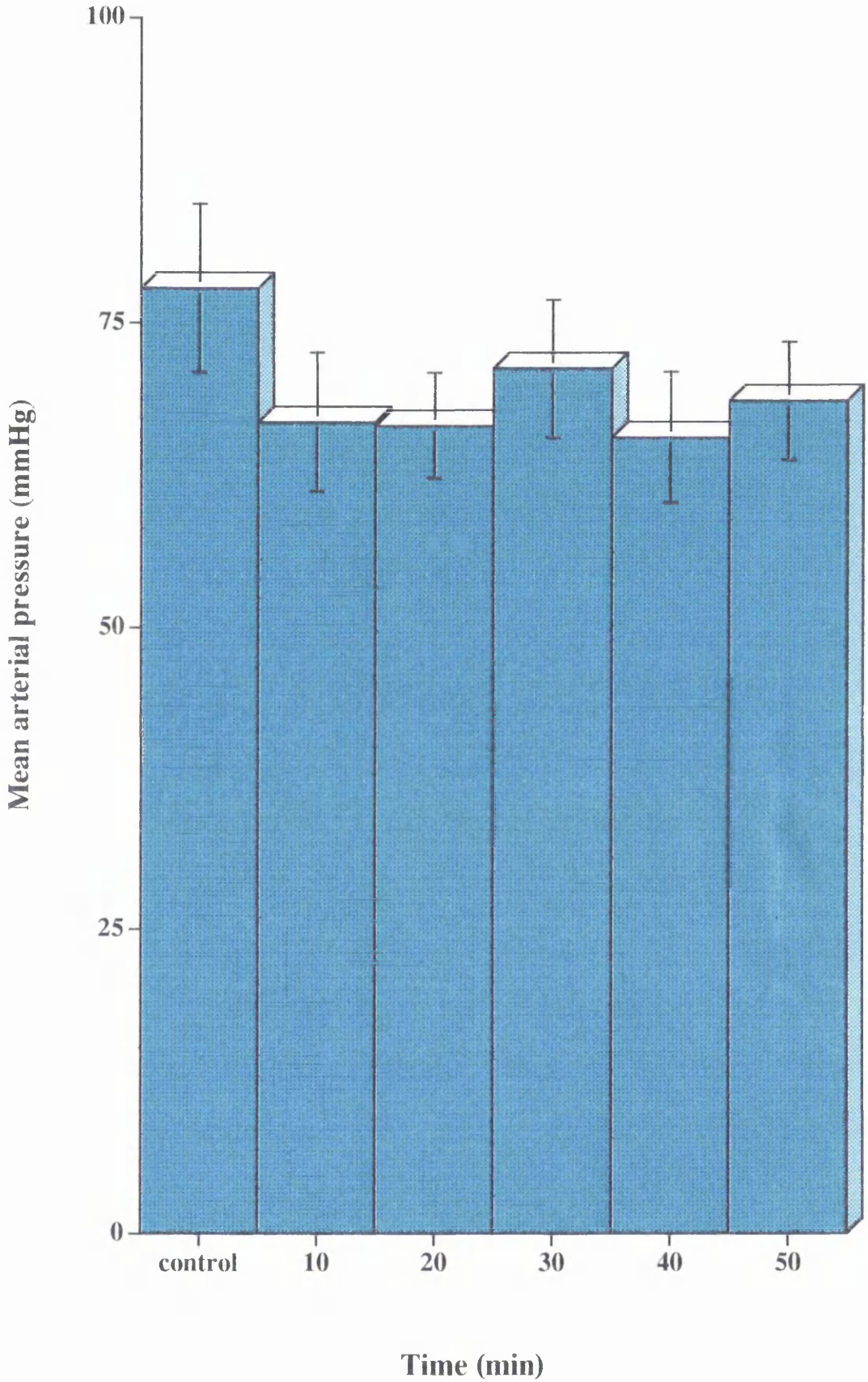
The results were expressed as the percentage change in voltage (± SEM) of a selected rectangular area on the LDI image, comparing images immediately before (control image) with those generated

during experimental manipulation (test image). The subtraction process of test minus control for individual animals precludes the need to express perfusion values per unit area and also eliminates variation due to different area sizes being selected for measurements in different animals. The "biological zero" (image obtained with zero blood flow, q.v.) was subtracted from test and control values prior to calculation of percentage change in blood flow. Subsequent statistical analyses were performed using MINITAB or InStat software and a modified version of the Shapiro-Wilk test examined whether the data were normally distributed. The *n* values in each experiment refer to the number of knees examined or to the number of measurements made.

Reliability of the measurements

Poiseuille's law states that blood flow is directly proportional to changes in blood pressure. Therefore, whenever comparisons are made between blood flow measurements in the same animal (*eg.* the control and test perfusion values described above) it is imperative that they are made behind a background of constant systemic blood pressure. The blood pressures of 12 anaesthetised rats were measured for an hour and it was found that MAP was stable throughout this period (Fig. 2.7).

Fig. 2.7. Changes in mean arterial pressure (± S.E.M., *n*=12) from control of normal anaesthetised rats.


100
75
50
25
0
Mean arterial pressure (mmHg)
control
10
20
30
40
50
Time (min)

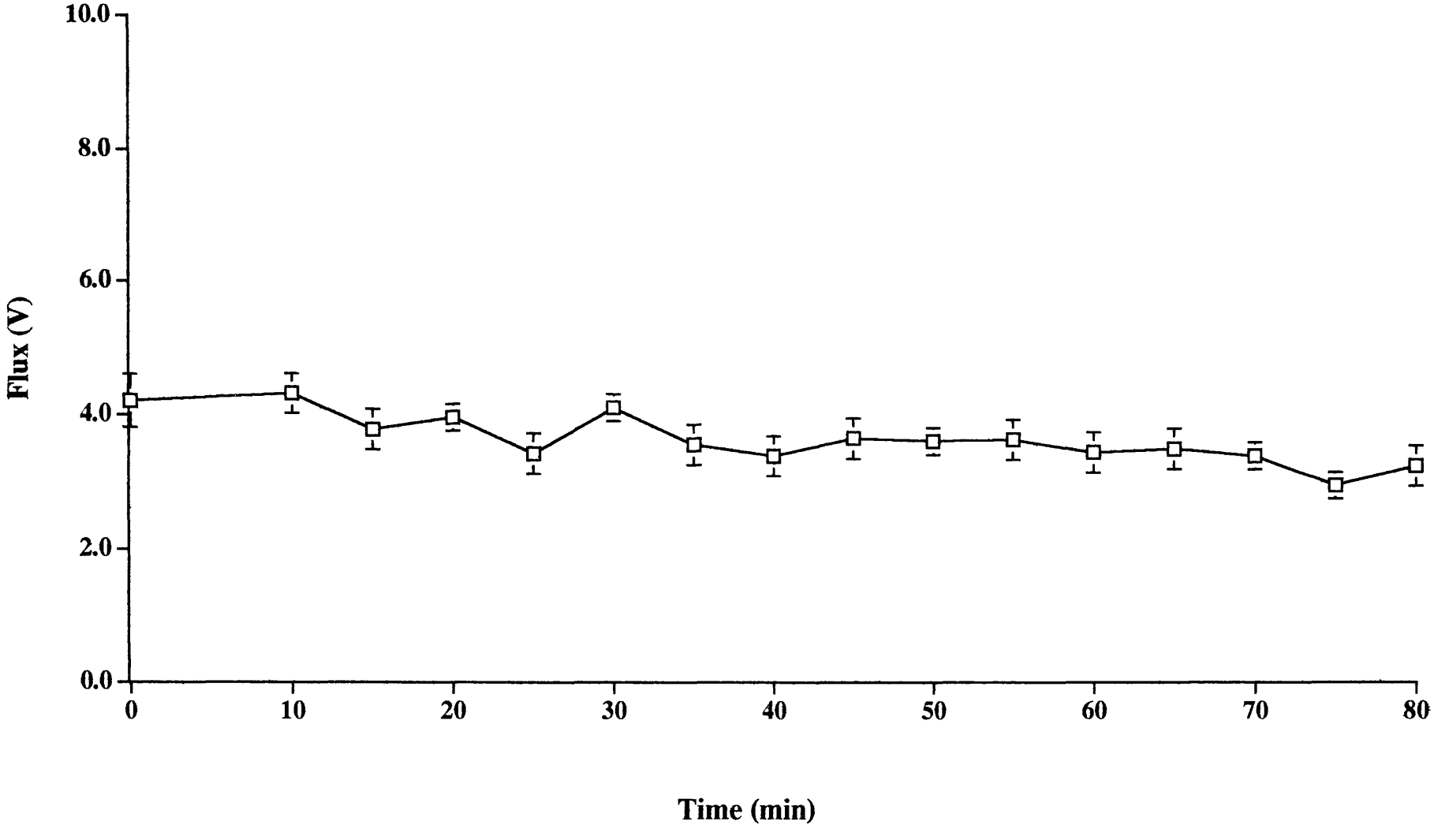
One potential problem in using the LDI technique could be the length of the scan time when examining transient events. In previous work using laser Doppler flowmetry to continuously monitor blood flow, it was found that electrical stimulation of the PAN in rabbits and cats caused vasoconstriction of articular blood vessels which was sustained for 1-2 minutes (Ferrell, Khoshbaten and Angerson, 1990). In experiments where the knee joint is masked, scan times only take about 30 seconds which means that the whole of the constrictor response can be captured by the LDI in these experiments. To ensure that none of the nerve-mediated constrictor response was missed during LDI scans in the rat, images from control animals were examined by placing small analysis rectangles at the upper and lower borders of the knee joint image. As scanning proceeded in a proximal to distal direction, if a significant temporal effect of nerve stimulation was present, it would be expected that the percentage change in perfusion at the upper window would be different from the value for the lower window *ie* vasoconstriction would be greater at the upper border of the knee if the nerve had fatigued by the time the scan reached the lower border. In actual fact, a 11.6±5.9% difference in perfusion between the two areas was found with the *lower* rectangle showing a greater vasoconstrictor response, however this difference proved not to be significant (P=0.065; n=24 paired measurements; paired t-test).

The final consideration is whether joint blood flow in control animals changes as a function of time. If this were to be the case it would make measuring joint blood flow fraught with difficulty and introduce a possible source of error. To test this, control LDI scans were performed on normal anaesthetised rats. Blood flow

measurements were made immediately after excision of the skin overlying the knee joint and then taken every 5 minutes thereafter. The joint was kept moist between scans by topical application of warmed physiological saline. As shown in Fig. 2.8, articular blood flow was initially relatively high (about 4V) probably due to the trauma experienced by excising the skin causing central elevation of systemic blood pressure. 15min later, blood flow subsequently stabilised and remained relatively constant for the next hour. In light of these findings, blood flow measurements in the present series of experiments were carried out at least 15min after exposing the joint and lasted for up to an hour.

In summary, we can be fairly confident that any changes in blood flow produced in the present project are entirely due to the experimental intervention and not as a result of the experimental set up.

Fig. 2.8. Changes in LDI flux signal recorded for 80min after exposing normal rat knee joints (n=6-16).


10.0
8.0
6.0
4.0
2.0
0.0
Flux (V)
0
10
20
30
40
50
60
70
80
Time (min)

Chapter 3

The Effect Of Capsaicin Treatment On Rat Knee Joint Blood Flow

Introduction

Previous experiments have shown that electrical stimulation of the saphenous nerve, which supplies the medial aspect of the rat knee joint, produces frequency-dependent vasoconstriction of blood vessels in this region of the joint capsule (Lam and Ferrell, 1991a). However, this form of stimulation activates not only unmyelinated sympathetic vasoconstrictor efferent fibres but also unmyelinated sensory (C) fibres innervating the joint. As articular C fibres are known to contain neuropeptides such as SP and CGRP (Mapp, Kidd, Gibson, Terry, Revell, Ibrahim, Blake and Polak, 1990) which potently vasodilate articular blood vessels (Lam and Ferrell, 1993a), it is possible that the true magnitude of sympathetic vasoconstriction is masked by concurrent release of vasodilator neuropeptides. Such a hypothesis could explain why nerve-mediated vasoconstrictor responses are attenuated in acutely inflamed joints (Najafipour and Ferrell, 1993b) where excess neuropeptide release could occur. Capsaicin, the pungent principle of green chillies, is known to cause depletion of neuropeptides such as SP from sensory nerve fibres and is neurotoxic when locally applied to nerves (Holzer, 1991). Unmyelinated but not myelinated nerve fibres supplying the rat knee joint are depleted after intra-articular injection of capsaicin (Ferrell. Lam and Montgomery, 1992). Thus, capsaicin pre-treatment of the joint could reveal the extent to which nerve-mediated vasoconstriction is modified by sensory neuropeptides.

Aims of the study

The aim of the present set of experiments was to discover whether nerve-mediated vasoconstrictor responses in rat knee joints were affected by capsaicin treatment. Since capsaicin depletes sensory neuropeptides, the sympathetically-mediated vasoconstrictor response could be examined in isolation without the confounding influence of vasodilator neuropeptides. In addition, this treatment would also reveal whether the neurotoxicity of capsaicin is specific to sensory unmyelinated nerve fibres or whether all unmyelinated fibres, afferent and efferent, are affected. The recently developed technique of LDI, which provides two-dimensional images of tissue perfusion, was used to investigate these neural mechanisms regulating knee joint blood flow.

Methods

Male Wistar Rats (~300 g) were deeply anaesthetised by intra-peritoneal injection of urethane (2g/kg), as judged by the absence of the flexor withdrawal reflex. Relative changes in blood flow were monitored by the technique of LDI which has previously been used to measure non-invasive relative changes in the spatial distribution of blood flow in skin (Wårdell, Naver, Nilsson and Wallin, 1993) but was used here to monitor joint capsule perfusion. The LDI was used to monitor relative changes in knee joint capsule blood flow (the voltage difference between test and control). The term "capsule" is used here to describe the synovium and all overlying areolar and fibrous tissues which invest the joint. An ellipse of skin over the knee was excised, exposing the medial aspect of the knee joint. Perfusion of the exposed

joint capsule was monitored during nerve stimulation to elicit vasoconstriction. This was performed in normal and rat knee joints treated with either 1 or 2 % capsaicin. The scanner head of the imager was placed 12cm above the exposed antero-medial aspect of the joint capsule. A red He-Ne laser beam (λ=633nm; beam diameter=800μm) was scanned back and forth over the joint giving an image covering an area of ~3.5 x 3.5 cm over a period of ~100 seconds. This allowed simultaneous scanning in a rostro-caudal direction of opposite knees of two separate rats placed in close proximity. Backscattered Doppler-broadened photons were detected by a photodetector in the scanner head which converted the instantaneous light intensity into a voltage signal which was proportional to tissue perfusion. Colour-coded perfusion images were generated (Fig. 3.1) which, for display purposes, used six colours to encode perfusion, each colour covering a range of voltages. However, statistical calculations of perfusion within defined areas utilised the actual voltages values at each point in the image.

Images were analysed using VIEW software (Moor Instruments Ltd), which allowed statistical analysis of selected areas of the images. The results were expressed as the percentage change in voltage ($\pm$ SEM) of a selected rectangular area on the LDI image, comparing images immediately before (control image) and during experimental manipulation (test image). The size of the rectangular area selected was chosen to include most of the articular tissue with minimal inclusion of the muscles around the joint. For both the control and test images this area was the same for each animal, but could vary between animals to allow inclusion of the largest possible joint area in knees of different sizes. Background values were excluded from the calculation of voltage values. At the conclusion of the experiment, an intra-

peritoneal injection of Euthatal (pentobarbitone 120mg/ml) was administered and another scan obtained after 15 min to provide the "biological zero" (image obtained with zero blood flow).

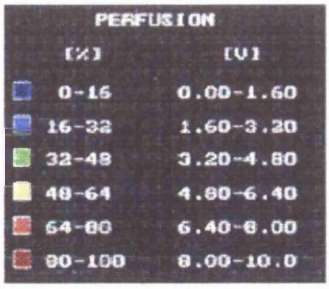
Degeneration of the nerves supplying the knee was induced by intra-articular injection of 0.2ml of 1 or 2% capsaicin into the rat knee 5-7 days prior to the start of experiments. Previous work has shown that this period is sufficient to obtain a significant reduction in unmyelinated fibres (Ferrell, Lam and Montgomery, 1992). The contralateral knee was injected with 0.2ml of the vehicle (ethanol, Cremophor EL in 0.9% saline). These injections were only administered under deep general anaesthesia with Hypnorm (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml: 0.1 ml/300g intramuscularly.) and intraperitoneal injection of 0.8ml of diazepam.

The saphenous nerve was exposed at midthigh, sectioned proximally and placed over bipolar stimulating electrodes. Frequency/response profiles were obtained using stimulus pulse trains of 100s in duration at fixed width and voltage (1ms and 15V respectively, these parameters being supramaximal for C fibres), but varying frequencies (0.5Hz to 30Hz). Warmed (37^{o}C) 0.9% NaCl was regularly applied to the joint surface to keep the tissue hydrated. The left carotid artery was cannulated to allow measurement of arterial blood pressure. The cannula was connected to a pressure transducer (Elcomatic EM751) and the transducer output was monitored on a pen recorder (Lectromed Multitrace 4). After image analysis, subsequent statistical analyses were performed using MINITAB software. A modified version of the Shapiro-Wilk test showed that the data were normally distributed. Comparisons between mean values was by one or two-factor repeated measures ANOVA. All quoted P values are

two-tailed. *n* values refer to the number of knees examined or to the number of measurements made.

Results

Although the images suggest a higher basal blood flow in capsaicin-treated as opposed to vehicle-treated knees (Fig 3.1), the mean voltage signals (± SEM) corresponding to each group (4.95±0.52 and 4.15±0.35 V respectively) did not differ significantly between the two treatments (P=0.21, two sample t-test; n=13 and 15 knees respectively). The heterogeneity of perfusion in the knee joint capsule is clear from the images in Fig. 3.1 and analysis of two separate square areas (an array of 4 by 4 measurement points) in the same knee of control rats, one at the upper border and one at the lower border of the joint, showed significant (P=0.0014; n=47 paired measurements) differences in the LDI signals of 2.2±0.11 V and 2.82±0.16 V respectively.



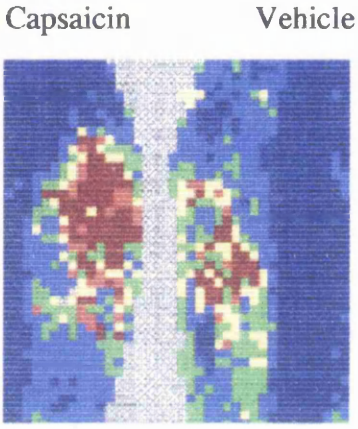


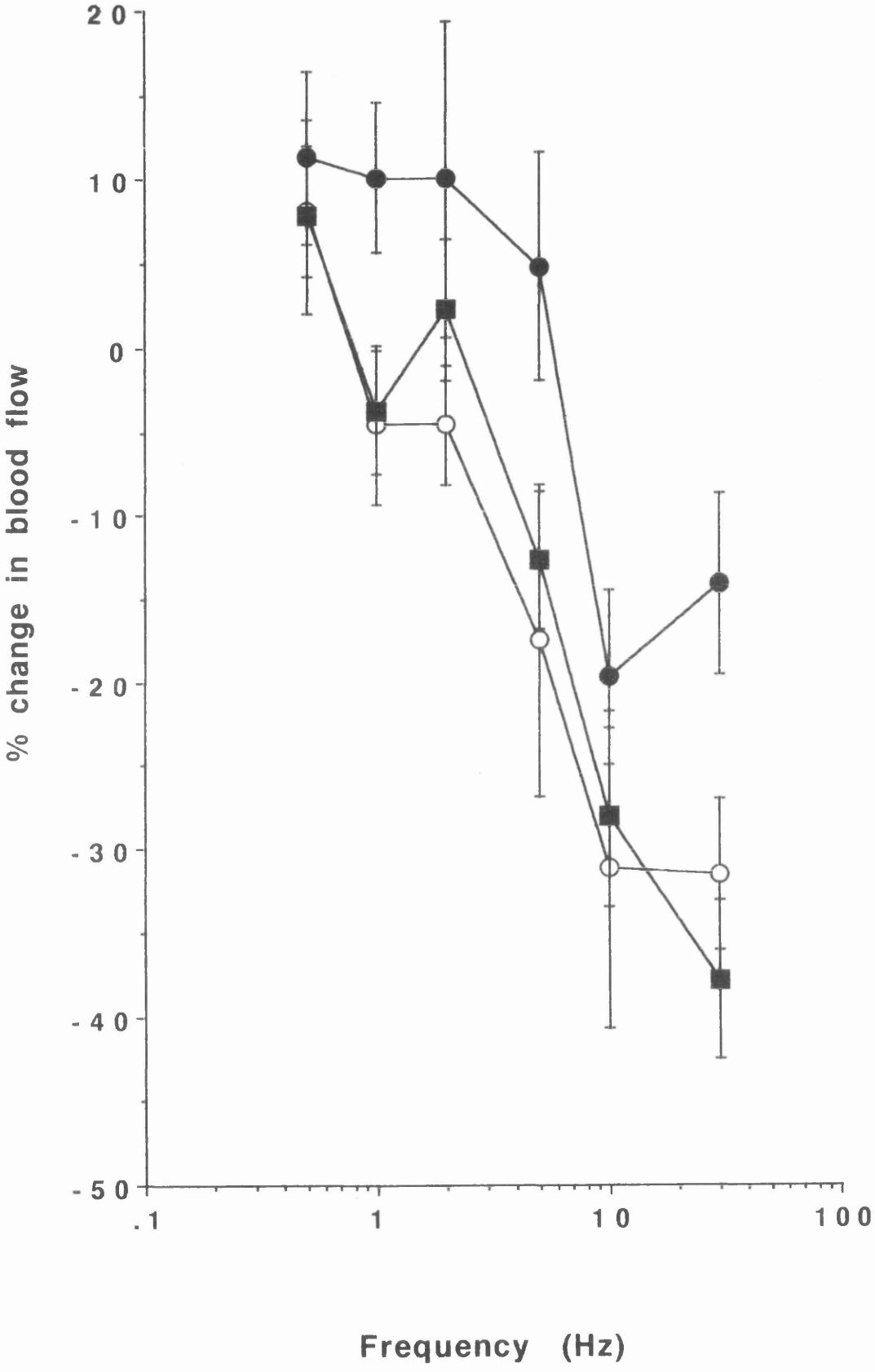
Fig. 3.1. Laser Doppler images of two rat knees, one pre-treated with capsaicin, the other with vehicle. The colour code is shown at the top of the figure.

Nerve-mediated response

Electrical stimulation of the nerve supply to the vehicle-treated knee joint resulted in a frequency-dependent constriction of synovial blood vessels over the range of 1-30Hz. The response was maximal at 10Hz but at 0.5Hz showed a small vasodilatation (Fig. 3.2). A very similar profile across the range of frequencies was found during nerve

stimulation in animals treated with 2% capsaicin. Two-factor ANOVA revealed a significant effect of the frequency of nerve stimulation ($P<0.0001$; $n=14$ knees) but treatment had no significant effect ($P=0.998$). The frequency-response profile in rats treated with 2% capsaicin was not significantly different from that in animals treated with 1% capsaicin ($P=0.064$; one-way ANOVA; n=6 and 14 for 1% and 2% capsaicin respectively). Analysis of two separate areas of the joint during nerve stimulation, using the same windows as described above, revealed a 11.6±5.9% difference in perfusion between the two areas with the lower window showing a greater vasoconstrictor response, but this difference proved not to be significant ($P=0.065$; $n=24$ paired measurements; paired t-test).

Fig. 3.2. Frequency/response relationship (means ± SEM; n=12) to electrical stimulation of the knee joint innervation (1msec width, 15V amplitude) in vehicle-treated knees (○) and knees treated with 1% (●) and 2% (■) capsaicin. Each knee was tested at all frequencies, with the sequence being randomised.


20
10
0
-10
-20
-30
-40
-50
.1
1
10
100
% change in blood flow
Frequency (Hz)

Discussion

The present experiments have revealed that the frequency/response profile produced by stimulation of the saphenous nerve in the rat was not affected by capsaicin treatment, irrespective of dose used. This suggests that unlike sensory unmyelinated (C) fibres, sympathetic postganglionic fibres are resistant to the neurotoxic effects of capsaicin. Previous work has shown that intra-articular injection of 1% capsaicin produces substantial reduction in unmyelinated fibres in articular nerves (Ferrell, Lam and Montgomery, 1992) and the present findings indicate that this reduction is most likely to be due to loss of sensory fibres. Interestingly, vasoconstrictor responses did not seem to be opposed by vasodilator neuropeptides released from sensory fibres during nerve stimulation as capsaicin treatment did not significantly augment vasoconstrictor responses at any frequency. This suggests that with this form of stimulation of afferent and efferent fibres, sensory neuropeptides do not moderate the vasoconstrictor effects of sympathetic efferent fibres in the rat knee joint microvasculature.

The LDI technique provides a convenient means of sampling a large volume of tissue in a relatively short time-frame thereby allowing the determination of the spatial distribution of tissue perfusion. This is particularly useful for analysis of joint capsule perfusion because of the complex geometry of joints, the highly anastomotic blood supply and the heterogeneous nature of the tissues which constitute the capsule. A significant advantage of LDI over LDF is related to the heterogeneity of the responses observed. From the perfusion images (Fig. 3.1) it is obvious that there is considerable variation in perfusion in adjacent areas. By taking measurements over an area of the joint capsule, LDI provides a clear view of the overall

response of the tissue whereas LDF, as it only samples at a single point, is less likely to yield a representative sample. Past experience of LDF has shown that a great deal of sampling is required to find a site on the capsule which gives a similar response to a given drug or nerve stimulation between different animals. Without such standardisation, in a tissue such as the joint capsule, the values obtained with LDF would be very difficult to interpret. By contrast, LDI makes such standardisation unnecessary, and has the added advantage that any variation in the pattern of joint perfusion could be readily detected. This could prove to be useful in examination of the pattern of perfusion in acutely or chronically inflamed joints.

One clear disadvantage of LDI compared to LDF is the poorer temporal resolution of the former. LDF provides a continuous measure of perfusion at a single point whereas LDI to some extent "integrates" the response over time, depending on the area of tissue scanned. In the present experiments, this poorer temporal resolution did not present a problem with the nerve stimulation studies as previous work using LDF has shown that the responses to nerve stimulation tend to be stable for the duration of stimulation (Lam and Ferrell 1991). This hypothesis was substantiated since measurement of perfusion at two smaller regions of the joint did not suggest a temporal effect. Although measurement of perfusion at the upper and lower borders of the joint showed no statistically significant difference, the data suggested that the vasoconstrictor response might well be *greater* at the lower border of the joint. This is the inverse of what would have been expected had the vasoconstrictor response waned during the scan possibly as a result of nerve fatigue or depletion of neurotransmitters. It does however indicate that there could well be significant spatial variations in the magnitude of the vasoconstrictor response. Even if there had been a

significant temporal factor, the result would still be valid as both the vehicle and capsaicin-treated knees were examined under exactly the same conditions with both having similar time courses and thus the response to nerve stimulation must reflect the responsiveness of the articular blood vessels in these knees.

Chapter 4

The Effect Of Adjuvant Arthritis On Rat Knee Joint Blood Flow

Introduction

The mechanisms underlying the formation and development of chronic inflammatory diseases such as rheumatoid arthritis are still uncertain. In order to study the pathogenesis of these disorders a number of experimental models of arthritis have been designed (Gardner, 1960). Of these, injection of Freund's complete adjuvant into the joint capsule induces a unilateral monoarthritis (Grubb, McQueen, Iggo, Birrell, & Dutia, 1988) which is being increasingly used as a model of chronic inflammation. Examination of synovia from adjuvant treated rats shows a reduction in synovial neuropeptidergic nerves when compared to normal samples (Konttinen, Rees, Hukkanen, Grönblad, Tolvanen, Gibson, Polak, & Brewerton, 1990) and these findings were consistent with those seen in patients with rheumatoid arthritis (Grönblad, Konttinen, Korkala, Liesi, Hukkanen, & Polak, 1988; Mapp, Kidd, Gibson, Terry, Revell, Ibrahim, Blake & Polak, 1990). The tachykinin SP is present in afferent nerve terminals (Hockfelt, Kellerth, Nilsson, & Pernow, 1975) and is released in response to electrical antidromic stimulation of their fibres (Yaksh, 1988). The rôle of synovial nerves in the development of various forms of acute inflammation has previously been reported (Lam & Ferrell, 1991) and it is thought that their effects may be mediated by local release of SP which is known to be pro-inflammatory in this region. By using the LDI technique which generates a 2-dimensional representation of tissue perfusion, it has been possible to examine these neurogenic and neuropeptidergic effects on joint blood flow (Lam & Ferrell, 1993b). This study revealed that electrical stimulation of sympathetic postganglionic

nerve fibres elicited a frequency-dependent vasoconstriction of synovial blood vessels in the normal knee joint. Acute joint inflammation, induced by intra-articular injection of carrageenan 24hr previously, attenuated this nerve-mediated vasoconstriction suggesting that the inflammatory process could, in the short term, significantly influence vascular responses.

Aims of the study

The aim of the present study was to ascertain whether the neurogenic control of rat knee joint blood flow is altered by intra-articular injection of Freund's complete adjuvant, an agent known to induce chronic inflammation. The longevity of the inflammatory response was examined by monitoring any changes in joint vasoregulation one and three weeks after inducing adjuvant arthritis.

Methods

Thirty-five male Wistar rats (aged 170 days, in house colony) were used in this study. Twenty of the animals were deeply anaesthetised by Hypnorm (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml: 0.1 ml/300g i.m.) and diazepam (2.5 mg/kg, i.p.). The width of the knee and ankle joints were measured by a digital micrometer (Mtotoyo instruments, Japan) and then the skin over the knee joint was shaved and 0.2 ml of Freund's complete adjuvant (Sigma) was injected into the synovial cavity of the right knee joint. 0.1 ml of the adjuvant was injected into the posterior region and 0.1 into the anterior region so as to maximise the inflammatory reaction throughout the whole of the joint. The

animals were allowed to recover and the inflammatory response was assessed by measurement of knee diameter at one and three weeks post injection. The left knee was also measured at these time points to see if the monoarthritis affected the contralateral side. The diameter of the ankle joints were measured at one and three weeks after adjuvant treatment to ascertain whether the inflammatory agent was having a systemic effect leading to the development of a polyarthritis. Finally, five animals were given a 0.2 ml intra-articular injection of paraffin oil to see if the adjuvant vehicle exerted any effect on knee diameter over the three week period. A two-tail paired t-test was used to compare mean knee diameters during the progression of the chronic inflammatory response; comparison between normal and vehicle treated knees was performed by two-tail unpaired t-test.

For the terminal blood flow experiments, the 30 animals were deeply anaesthetised by i.p. injection of urethane (2g/kg, Sigma). Here, 10 animals were examined at one and three weeks post injection and a further 10 rats were used as controls. The saphenous nerve which supplies the medial aspect of the knee joint was exposed at mid-thigh, sectioned proximally and placed over bipolar stimulating electrodes. The nerve was then covered by a thin layer of paraffin to prevent it from drying out. An ellipse of skin overlying the joint was excised to expose the medial aspect of the knee. Electrical stimuli were delivered to the nerve with a fixed width and voltage (1 msec and 15 Volts respectively, these parameters being supra maximal for C fibres) but at different frequencies (0.5 Hz to 30 Hz) which produced frequency-dependent vasoconstriction of articular blood vessels. Warmed (37°C) physiological saline (0.9% NaCl) was regularly applied to the knee joint surface to prevent

tissue dehydration. Relative changes in synovial blood flow (voltage difference of test minus control) were monitored by a LDI (Lisca developments AB, Sweden). Previous work has shown that this technique can be used to map the spatial distribution of blood flow to the rat knee joint capsule (Lam & Ferrell, 1993b). The term "capsule" is used here to describe the synovial and overlying areolar and fibrous tissues which invest the joint. Perfusion of the exposed joint capsule was monitored during nerve stimulation to elicit vasoconstriction and this was performed in normal animals and in both the adjuvant-treated and its corresponding contralateral rat knee joints. The scanner head of the imager was placed 12 cm above the exposed anteromedial aspect of the joint capsule, as described previously (Lam & Ferrell, 1993b). Scans were taken before (as a control) and during nerve stimulation (test), and these images were subsequently analysed using VIEW software (Moor Instruments Ltd). After image analysis, statistical analysis was performed using MINITAB software and a modified version of the Shapiro-Wilk test showed that the data were normally distributed. Comparison between mean values in the frequency-response profiles was by two-way ANOVA. Data are expressed as percentage change from the control image taken immediately prior to nerve stimulation. At the conclusion of the experiment the animal was given an anaesthetic overdose and after cessation of the heart beat, the "biological zero" was measured and subtracted from all perfusion values. Data are expressed as means ±S.E.M. throughout.

Results

Inflammatory response and basal joint perfusion

Injection of Freund's adjuvant into the rat knee caused a considerable increase in knee diameter at one week post-injection which abated by the third week (Fig. 4.1). At week one, adjuvant-treated knee width increased by 1.797±0.544 mm (mean ±S.E.M.) which compared to week 0 was significantly different (P=0.013, two-tailed paired t-test) from control knees which also showed a small increase in size due to growth of the rats. At the same time the contralateral knee increased by 0.355±0.23 mm and was found to be significantly different (P=0.02; n=8) from the adjuvant arthritic knee but not from normal knees (P=0.378). This suggests that a true monoarthritis was produced in the right knee which did not affect the contralateral joint.

By week three, the inflamed knee had only increased by 1.195 ±0.35 mm from the week 0 level and this was found to be not significantly different (P=0.315; n=8 for adjuvant animals and n =10 for untreated animals) from a normal knee which had grown by 0.744 ±0.11 mm over the three week period. Concurrently, the contralateral knee joint increased by 0.176±0.22 mm and was also found to be not significantly different from normal knees (P>0.05). As shown in Fig. 4.2, intra articular injection of the vehicle had a slight effect on knee diameter compared to normals at week one, however, this change was not significant (P>0.05, two-tailed unpaired t-test; n=5 for vehicle treated knees) and was probably due to slow absorption of the paraffin causing a mechanical distension rather than any real inflammatory response. By the third week knees

injected with vehicle showed an increase in diameter which was comparable to a normal growth rate (P=0.3132 between normal and vehicle treated knees).

The ankle joint ipsilateral to the adjuvant treated knee showed a 0.230 ± 0.19mm increase in diameter one week post-injection (Fig. 4.3) compared to normal ankles which display a negligible fall in ankle width (-0.174 ± 0.17mm). By week three normal ankles have grown by 0.287 ± 0.15mm whereas the ankles of adjuvant animals rise by 0.350 ± 0.15mm. Therefore, Freund's complete adjuvant was found to have no significant effect on ankle diameter over the 3 weeks ($P>0.05$; n =8 and 9 for adjuvant-treated animals at week 1 and 3 respectively; n=10 for normal animals) thereby confirming an absence of a systemic effect of the adjuvant.

One week after induction of arthritis, a marked reduction in joint perfusion occurred as revealed by a fall in the LDI flux values measured at the beginning of each experiment. Perfusion then returned back towards control levels by the third week (Figs. 4.4, 4.5).

Fig. 4.1. Changes in knee joint diameter at one and three weeks in response to intra-articular injection of Freund's adjuvant (blue diagonally shaded columns) compared to the contralateral (red vertically shaded columns) and normal (solid black columns) knees (mean increase ±S.E.M.; n=8 for adjuvant animals and contralateral knees, and n=10 for untreated animals; *P<0.02; NS-not significant, P>0.3).

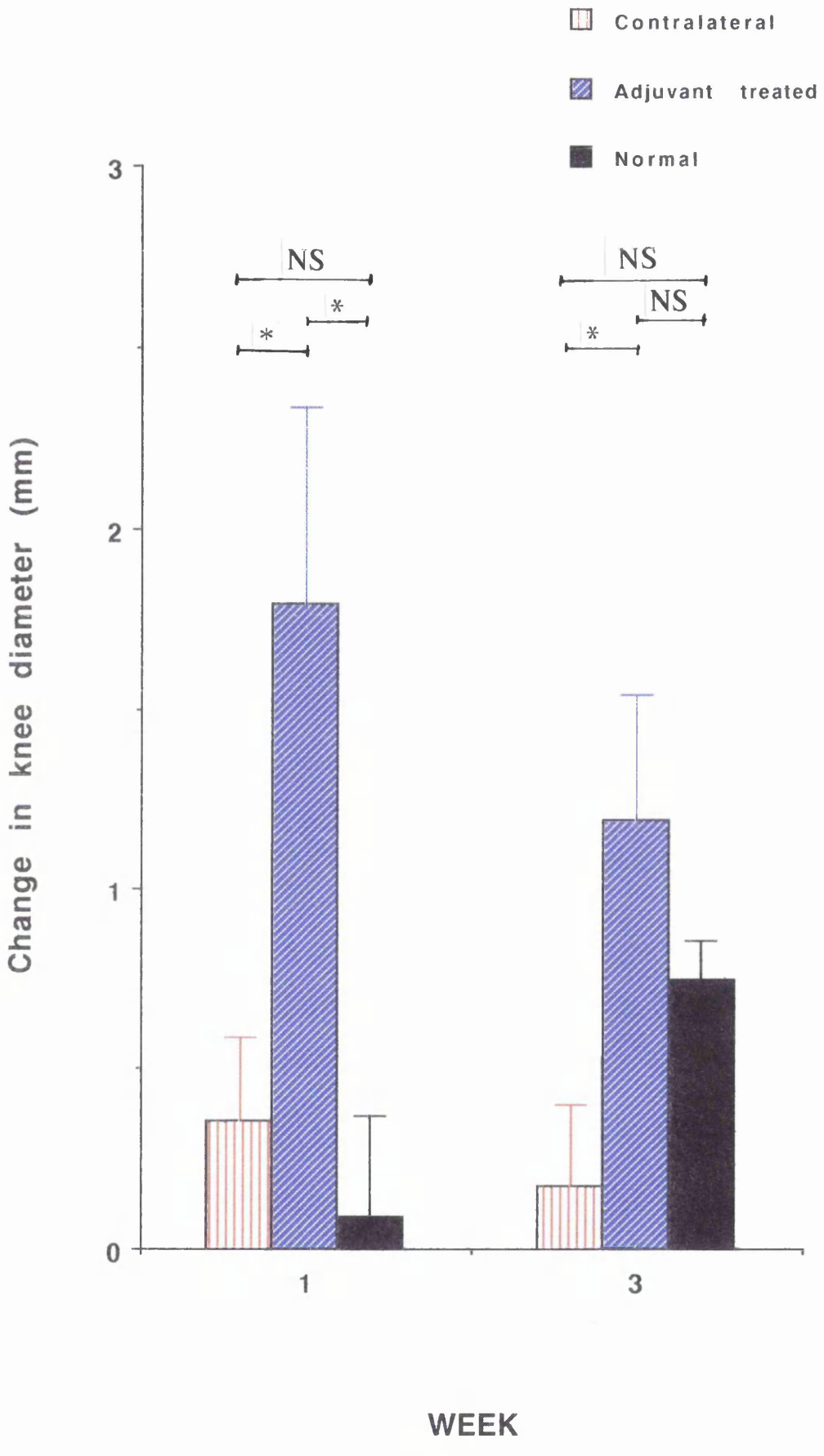

Contralateral
Adjuvant treated
Normal
NS
*
*
NS
NS
*
Change in knee diameter (mm)
3
2
1
0
1
3
WEEK

Fig. 4.2. The effect of vehicle treatment (blue diagonally shaded columns) on rat knee joint diameter compared to normal (solid black columns) at one and three weeks (mean increase ±S.E.M.; n=5 for vehicle treated knees, and n=10 for untreated animals).

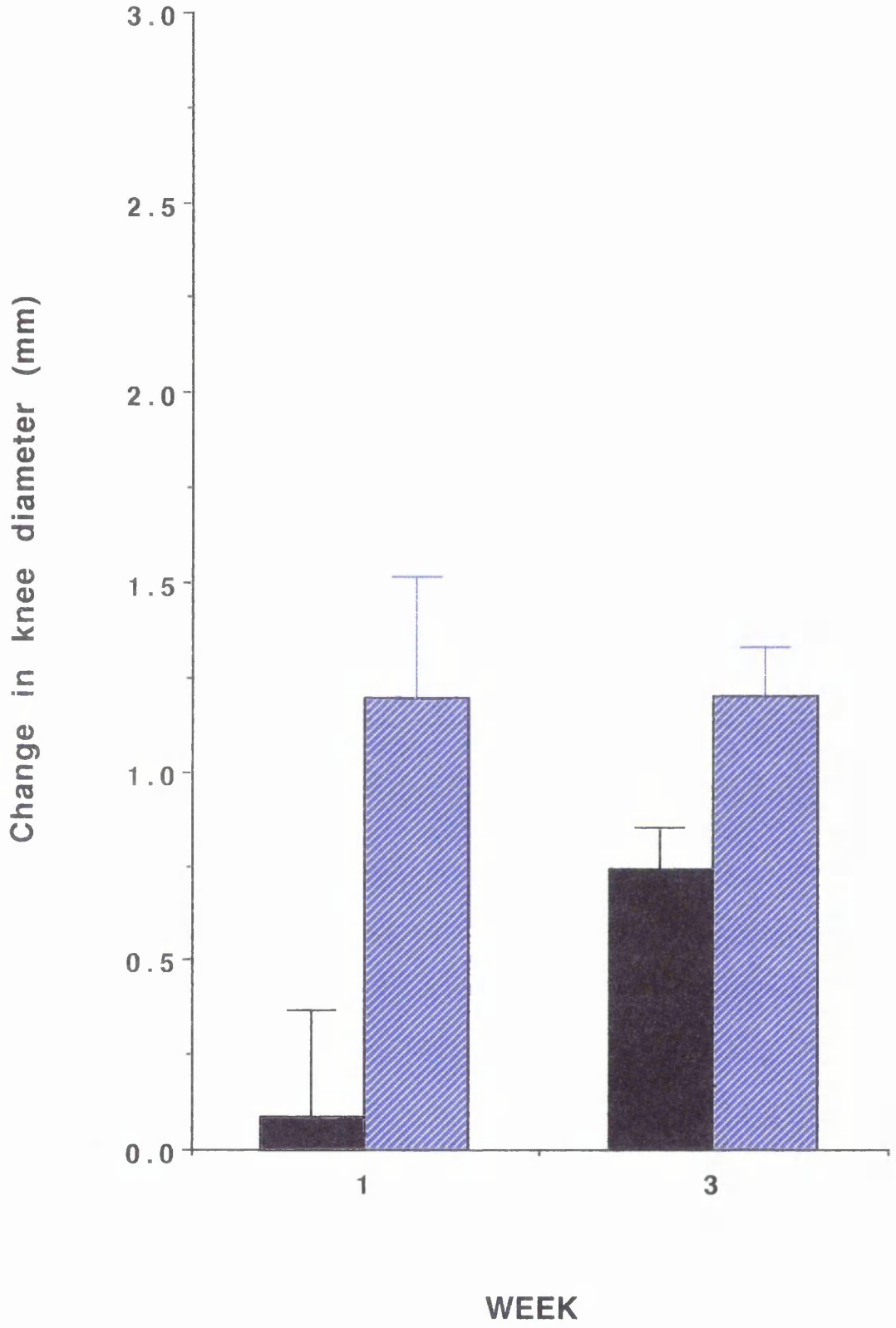

3.0
2.5
2.0
1.5
1.0
0.5
0.0
Change in knee diameter (mm)
1
3
WEEK

Fig. 4.3. The effect of injection of Freund's complete adjuvant into the knee joint on the ipsilateral ankle diameter (blue diagonally shaded columns) compared to ankle diameter in normal rats (solid black columns) at one and three weeks (mean increase ±S.E.M.; n=8 and 9 for the week one and week three adjuvant treated animals respectively, and n=10 for untreated animals).

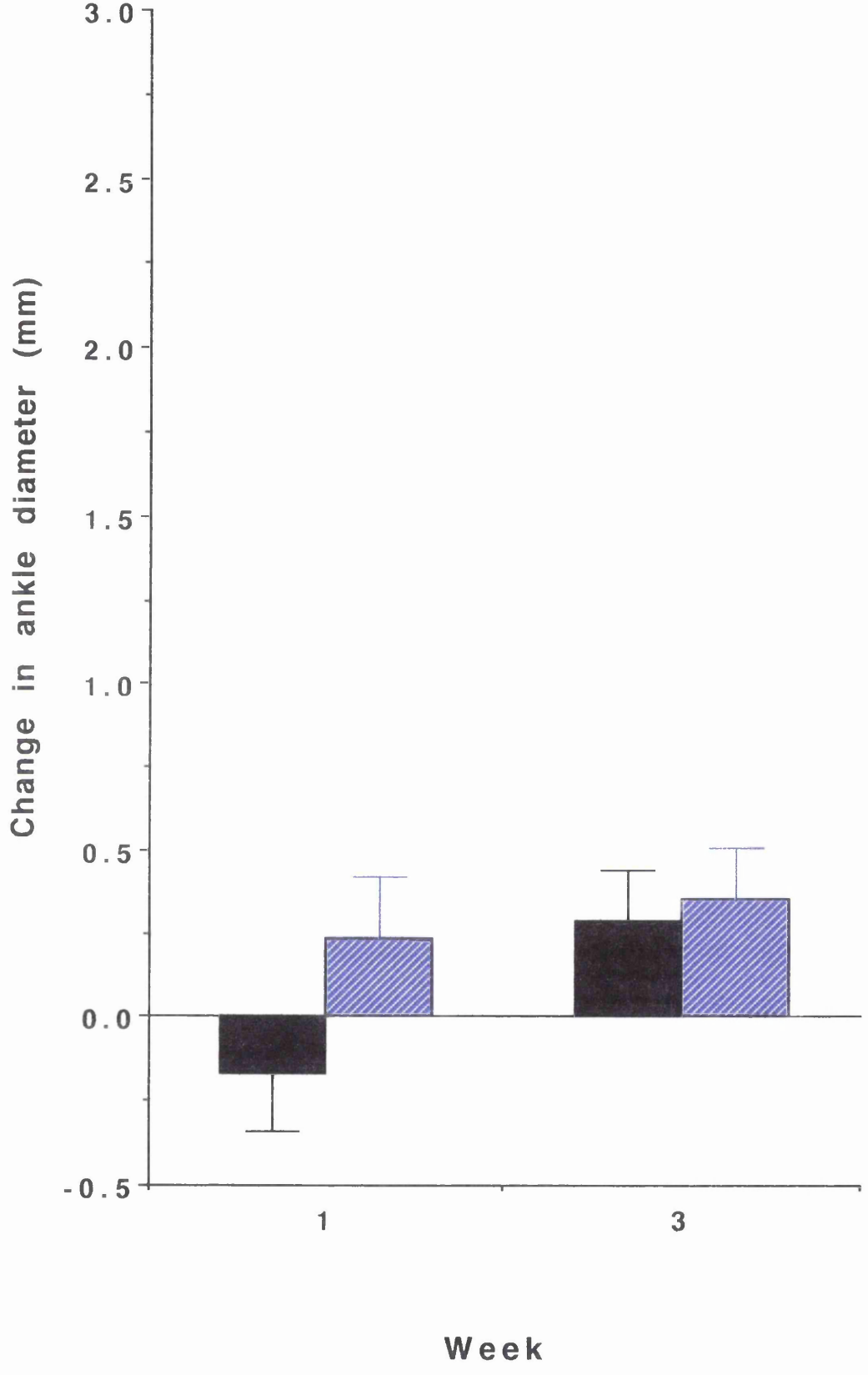

3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
Change in ankle diameter (mm)
1
3
Week

Fig. 4.4. Images showing changes in perfusion of normal and adjuvant-treated knees in response to supramaximal electrical stimulation of the saphenous nerve. Perfusion is encoded using a six colour scale covering 0-10V: lowest perfusion is coded dark blue (0-1.6V), then in ascending order light blue (1.6-3.2V), green (3.2-4.8V), yellow (4.8-6.4V), light red (6.4-8.0V) and a dark red colour corresponding to highest perfusion (8.0-10.0V). Control images indicate a substantial reduction of perfusion in adjuvant treated knees at week 1 which recovers by week 3. However, in adjuvant-treated knees the nerve-mediated vasoconstriction is substantially attenuated.

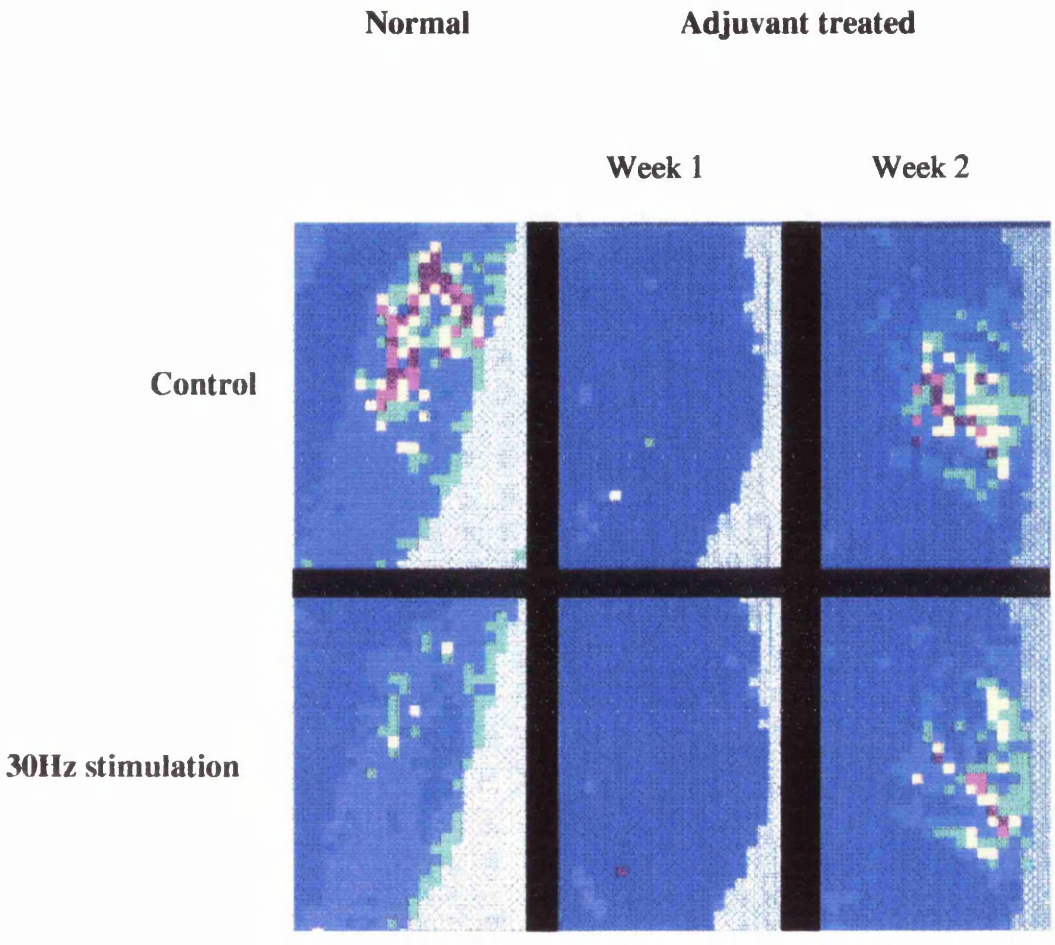
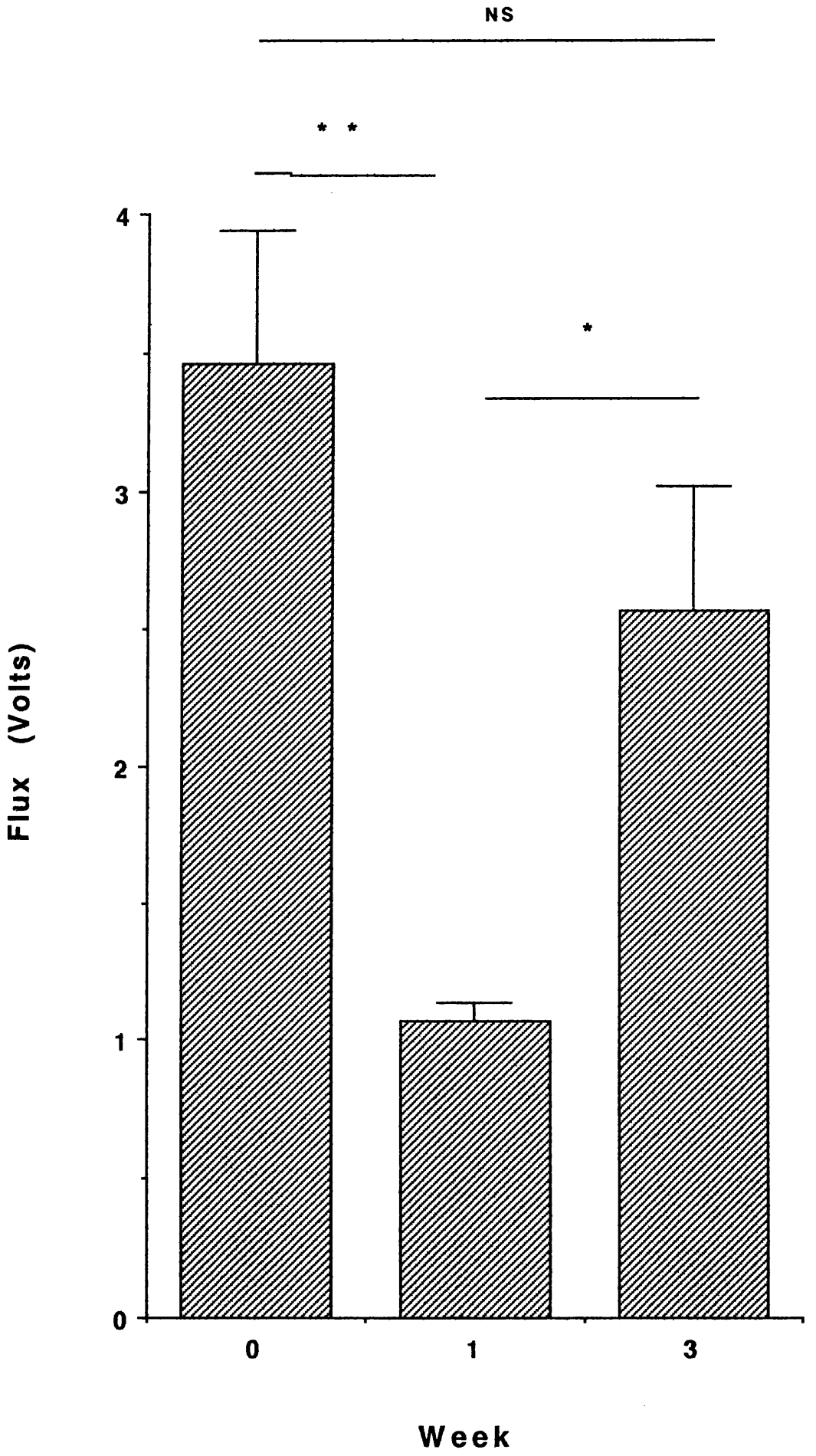

Normal
Adjuvant treated
Week 1
Week 2
Control
30Hz stimulation

Fig. 4.5. Alteration in basal blood flow at one and three weeks following intra-articular injection of Freund's adjuvant (mean ±S.E.M.; n=10 for both control (week 0) and week 1 data and n=9 for the week 3 group). Control and week 1 are significantly different (**=P<0.001) as are week 1 and week 3 (*=P<0.01). Control and week 3 flux values are not significantly different (NS; P=0.342).


NS
* *
*
4
3
2
1
0
Flux (Volts)
0
1
3
Week

Nerve-mediated vasoconstriction

A highly significant frequency-dependent decrease in joint blood flow occurred in normal rats when electrical stimulation of the saphenous nerve was performed ($P<0.001$, repeated measures one-way ANOVA; n =10). The greatest vasoconstriction resulted when 30 Hz stimulation was applied to the nerve eliciting a 37.3.±7.3% fall in perfusion. However, no discernible constriction was apparent at the lower frequencies (0.5-2 Hz). Animals whose knees were treated with Freund's adjuvant showed no significant response to nerve stimulation during weeks 1 and 3 ($P=0.266$ and $P=0.96$ with $n=8$ and 9 respectively) although perfusion consistently fell by about 10% over all frequencies in the week 3 group of rats (Figs. 4.4, 4.6) however, this reduction was not frequency-dependent. The interaction between frequency and treatment showed the chronically inflamed knees to be significantly different from control knees ($P<0.005$).

Three weeks after adjuvant induction, electrical stimulation of the saphenous nerve of the contralateral knee resulted in a frequency-dependent vasoconstriction ($P<0.001$; $n=9$) which was not significantly different from normal knees ($P=0.400$) but different from the adjuvant treated joint ($P<0.01$).

Comparison between normal animals and those which were treated with an intra-articular injection of paraffin showed no significant difference between their frequency-response profiles ($P=0.811$; $n=3$ for the vehicle treated knees). This finding indicates that vehicle treatment has no effect on nerve-mediated vasoconstriction in the joint and reaffirms that the increase in joint diameter it causes is not by an inflammatory reaction.

Fig. 4.6. Alterations in joint blood flow during electrical stimulation of the nerve supply to normal (❑) and chronically inflamed knees at one week (◌) and three weeks (△) (means ± S.E.M.; n =10 for all groups). Stimulation was carried out at various frequencies, the sequence of which was randomised (0.5-30 Hz; 1 msec pulse width; 15 V amplitude).

The frequency/response profile in chronically inflamed knees was found to be significantly different from that in normal knees ($P<0.005$).

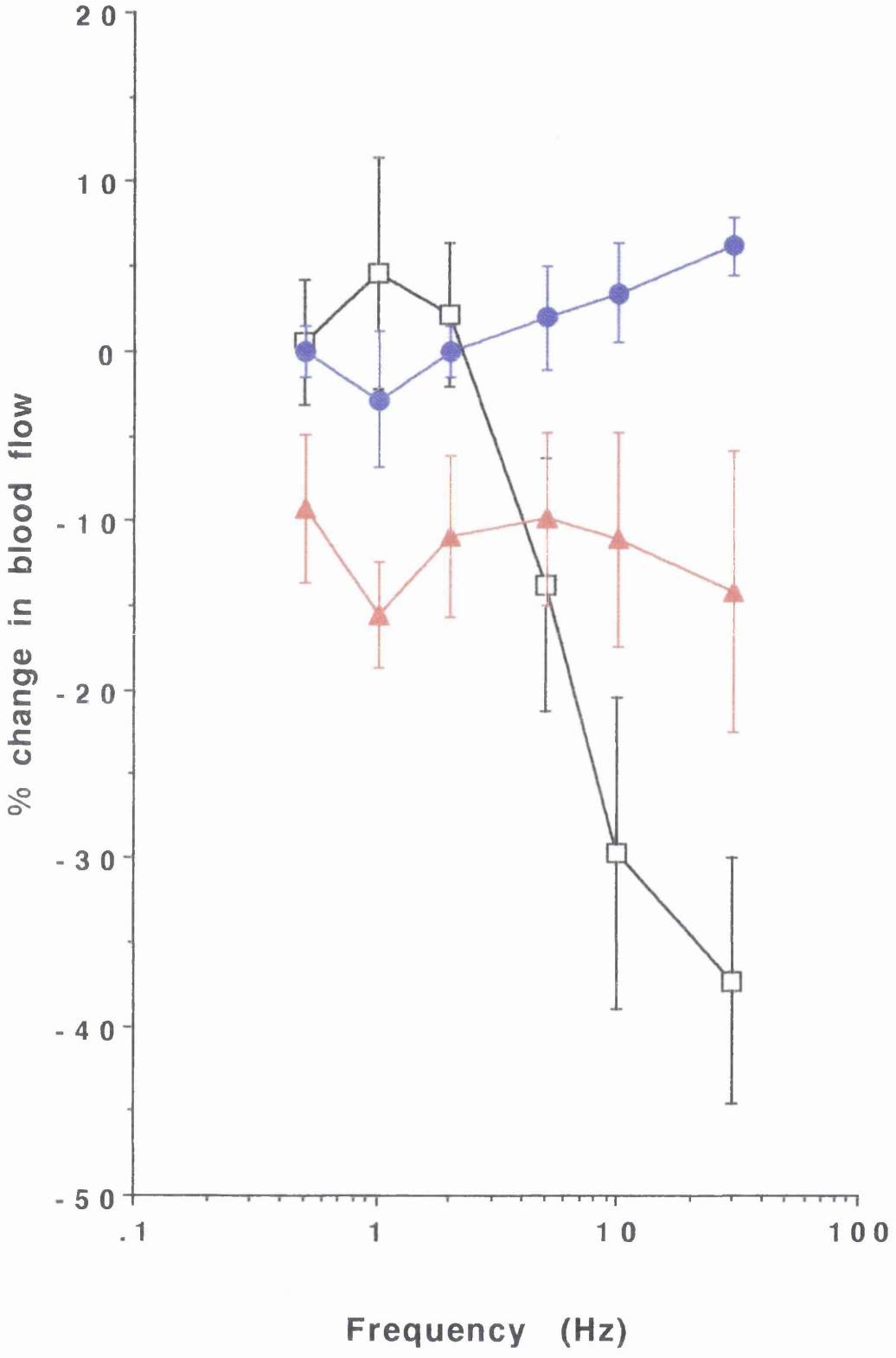
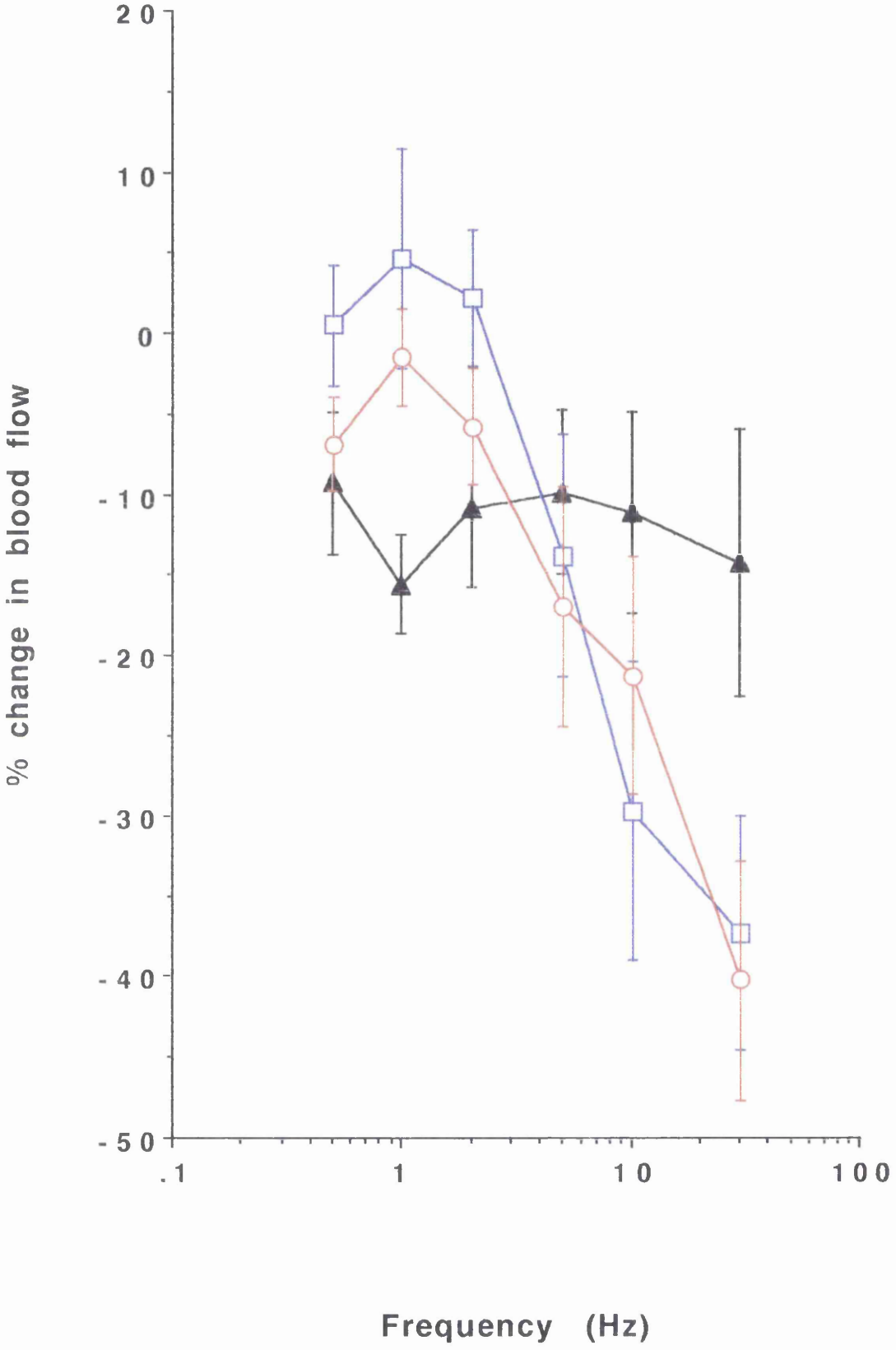

20
10
0
-10
-20
-30
-40
-50
% change in blood flow
.1
1
10
100
Frequency (Hz)

Fig. 4.7. Effect of electrical stimulation of the saphenous nerve in normal (□), week three chronically inflamed (▲) and the contralateral knees (○) (means ± S.E.M.; n =10 for normal and adjuvant knees, and n=9 for contralateral knees). Stimulation was executed at various frequencies (0.5-30 Hz) the sequence of which was randomised.

The frequency/response profile of the contralateral knee was not significantly different (P=0.400) from the normal joint but was significantly different from the chronically inflamed knee (P<0.01).


20
10
0
-10
-20
-30
-40
-50
.1
1
10
100
% change in blood flow
Frequency (Hz)

Discussion

Injection of Freund's complete adjuvant into rat knee joints caused a considerable increase in diameter of the joint and this was used as an index of the magnitude of the inflammatory response. Since no discernible change in ankle diameter occurred in any of the rats, it is clear that inflammation was localised to the knee and hence a polyarthritic model was avoided. Adjuvant treatment was found to have no effect on contralateral knee diameter, suggesting that the inflammatory response was non-symmetrical in this model. Despite this observation, the contralateral knee was not used as a control as it is known that an experimentally-induced monoarthritis causes bilateral changes in peptide content of synovial fluid (Bilevicuite, Lundeberg, Ekblom & Theodorsson, 1993). This effect could result in a modification of basal blood flow in the contralateral knee thereby precluding it as an internal control.

One week after adjuvant treatment, chronically inflamed knees exhibited a fall in basal blood flow which subsequently returned towards normal two weeks later. This initial fall in perfusion is probably real and not due to an inability of the laser to detect blood flow in the enlarged knee. The swelling of the joint is not due to a thickening of the capsule but may be ascribed to accumulation of tissue fluid which does not significantly affect the penetrative power of the laser beam. In addition, blood vessels on the surface of the adjuvant arthritic knee are still visible to the naked eye and therefore should be detectable by the LDI system. The monoarthritis model produces a localised lesion with little systemic disturbance and treated animals are found to be normotensive. Thus, the hypoperfused knee observed one week post adjuvant injection cannot

be ascribed to circulatory disturbances which might occur in the more severe conventional adjuvant polyarthritic model. Microscopic inspection of the arthritic knees suggested that the hypoperfusion might to some extent be the result of localised areas of tissue necrosis and possible blood vessel ablation. These deleterious effects of the adjuvant could lead to a fall in erythrocyte velocity and hence a reduction in the flux values (since LDI flux readings = erythrocyte velocity × erythrocyte concentration).

Differences between the arthritic and control rats could not be ascribed to differences in the reproducibility of measurements as basal flux values at week one in arthritic rats show lower variability than the measurements obtained in control rats or at week three (Fig. 4.5). In addition, flux readings obtained prior to nerve stimulation were found to be stable throughout the experiments. The LDI technique is suitable for comparison of rats at different ages as the scan area can be increased to accommodate the larger knee joint in the older animals. Interestingly, comparison of flux values between normal animals of different ages revealed no significant differences in perfusion, suggesting that there is no alteration of vascularity of the joint as a function of age.

Since articular cartilage is dependent on the synovial perfusion (McKibbin, & Maroudas, 1979) then this initial reduction in joint blood flow could cause the integrity of the joint to deteriorate and lead to degenerative changes. The present study showed that adjuvant-induced inflammation in the rat knee joint abolished sympathetic vasoconstriction at one week. Furthermore, this alteration is not transitory but persists three weeks post-injection of adjuvant, even though the inflammatory process appeared to be abating by the third week. In untreated animals there was a

frequency-dependent vasoconstriction over the range 5-30 Hz but, frequencies of 0.5-2 Hz showed a negligible effect on joint perfusion. Electrical stimulation of the articular nerves in the chronically inflamed model elicited no such constrictor response at week one, however at week three a slight fall in blood flow occurred although this was not frequency-dependent. Elsewhere it has been shown that adjuvant arthritic rats are depleted of nerve terminals and mast cells in the superficial lining and sublining areas of the synovium (Konttinen, Rees, Hukkanen, Grönblad, Tolvanen, Gibson, Polak, & Brewerton, 1990; Hukkanen, Grönblad, Rees, Konttinen, Gibson, Hietanen, Polak, & Brewerton, 1991) and this could account for the absence of a nerve-mediated vasoconstriction in these animals. The present study also showed that electrical stimulation of the nerve supply to the contralateral knee elicited a frequency-dependent vasoconstriction of articular blood vessels, similar in profile to control knees. The magnitude of this constrictor response could be larger than controls due to an offset by the adjuvant-induced bilateral increase in synovial fluid peptide content. However, the control and contralateral frequency-response profiles are so similar that this is unlikely hence the contralateral knee is probably acting like a normal joint.

Acute inflammatory responses induced by intra-articular injection of carrageenan show nerve-mediated vasoconstriction to be attenuated but the dilator effects of SP to be greatly enhanced (Lam & Ferrell, 1993b). Comparing this finding with that of the present study, it would appear that the integrity of sympathetic transmission and SP/receptor activation in rat knee joints declines as inflammation becomes more chronic. Loss of these neurovascular controls could

contribute to degenerative changes which commonly accompany chronic inflammatory joint diseases.

Chapter 5

The Rôle Of Nitric Oxide In The Control Of Rat Knee Joint Vasculature

SECTION I:

The neurogenic rôle of nitric oxide in the control of rat knee joint blood flow

Introduction

The nervous control of joint blood vessels was first investigated by Cobbold and Lewis (1956) who measured blood flow by timing the transit of an air bubble between fixed points of flowmeter tubing inserted into the femoral artery of anaesthetised dogs. They and subsequently other investigators found that electrical stimulation of sympathetic post-ganglionic fibres elicited a pronounced vasoconstriction of articular blood vessels (Khoshbaten and Ferrell, 1990; Ferrell and Najafipour, 1992). The rôle of NO in the control of the peripheral vasculature is well established both in endothelially dependent mechanisms (Najafipour and Ferrell, 1993b) and in nervous control where the transmitter is non-adrenergic and non-cholinergic (for review see Rand, 1992). NO is synthesised from L-arginine under the influence of the enzyme NOS which is localised not only in brain and blood vessel endothelium, but also in the endings of peripheral nerves (Bredt, Hwang and Snyder, 1990). It has recently been shown that stimulation of autonomic nerves in guinea-pig intestine induces the release of NO metabolites suggesting release of NO via activation of NOS (Wicklund, Leone, Gustaffson and Moncada, 1993) The enzymatic activity of NOS may be inhibited by L-arginine analogues such as Nω-nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine (L-NMMA). In anaesthetised rats, the intravenous infusion of these drugs caused a dose-dependent

hypertension and bradycardia (Rees, Palmer, Schulz, Hodson and Moncada, 1990). L-NAME has been shown to reduce basal blood flow in the rabbit knee as measured by laser Doppler flowmetry and caused an increase in the vasoconstrictor response generated by stimulation of the sympathetic nerve supply to the joint (Najafipour and Ferrell, 1993b). Similarly, the hypertensive effect of L-NMMA in conscious rats was accompanied by a concurrent vasoconstriction in a number of vascular beds (including the hindquarters) as measured by pulsed Doppler probes positioned around relevant arteries (Gardiner, Compton, Bennett, Palmer and Moncada, 1990). The haemodynamic profile of L-NMMA was similar to that of L-NAME except that the onset of effect was slower and the potency tenfold less with L-NMMA as compared to L-NAME (Gardiner, Compton, Kemp and Bennett,1991). Other studies using the same technique in urethane-anaesthetised rats have shown that administration of another NOS inhibitor L-N-nitro arginine (L-NA) have also demonstrated increased mean arterial pressure and hindquarter vascular resistance (Lacolley, Lewis and Brody, 1991). Furthermore, they found that if the animals were pre-treated with the sympathetic postganglionic blocker chlorisondamine then the hypertensive effects of L-NA were attenuated. This suggests that normal sympathetic discharge modulates the synthesis or release of NO *in vivo*.

Aims of the study

The aim of the present study was to establish what rôle, if any, endogenous NO plays in the control of basal blood flow in the rat knee joint. Possible release or induction of NO in response to stimulation of

the nerves supplying the knee was also investigated. Blood flow measurements were carried out using the LDI technique.

Methods

Surgical procedures

Experiments were carried out on deeply anaesthetised (urethane 1.5g/kg, i.p.) adult male Wistar rats (405-515 g). Halothane anaesthesia was avoided in this study as it is known to attenuate the vasoconstrictor responses to NOS inhibitors (Meyer, Lentz, Herndon, Nelson, Traber and Traber, 1993). After abolition of the flexor withdrawal reflex, the popliteal fat pad was removed and the saphenous nerve was located and dissected free peripherally as far as the superficial epigastric / great saphenous bifurcation. The nerve was transected as proximally as possible for electrical stimulation with silver electrodes at various frequencies (0.5-30 Hz; voltage: 15V; pulse width: 1ms). Nerve stimulation was executed in the presence and absence of L-NAME (0.1mg/kg/hr) and L-NMMA (10mg/kg/hr) which were infused for 1hr via an intravenous cannula inserted into the jugular vein. These doses of inhibitor were used as they give a similar rise in arterial blood pressure. The effects of L-NAME (0.1, 1, 3 and 10mg/kg/hr, i.v.) and L-NMMA (10mg/kg/hr and 30mg/kg/hr, i.v.) on systemic blood pressure were monitored via a cannula inserted into the carotid artery which was in series with a pressure transducer (Elcomatic EM751) and recorded on a Multitrace 4 chart recorder (Lectromed Ltd.). The skin and underlying fascia covering the knee were removed to expose the medial aspect of the knee joint which was prevented from desiccation by applying warmed 0.9% saline solution

to its surface. The joint area was isolated by placing a piece of black cloth around the perimeter of the joint capsule and relative changes in articular blood flow were measured by LDI (Lisca Developments AB, Sweden).

At the end of each experiment, a scan was performed on the dead animal in order to obtain a voltage value for the absence of blood flow *i.e.* a "biological zero". This value was subsequently subtracted from both control and test data before any calculations were carried out.

Analysis of results

Using an image analysis package (VIEW software, Moor Instruments Ltd.), determination of the blood flow readings was achieved by placing a designated rectangular area around the image and the median voltage reading therein was noted. The voltage readings for images obtained during an experimental procedure were expressed as a percentage change from their control values. With respect to the frequency - response profiles, the control reading was the scan immediately preceding nerve stimulation. The vascular responses were expressed as percentage changes in vascular resistance (calculated as mean arterial blood pressure divided by blood flow). All data were tested for normality and expressed in graphical format showing means ±S.E.M. Statistical analysis took the form of general linear models of analysis of variance with a significance level of less than 5%. All statistics were performed using MINITAB software.

Results

The haemodynamic effects of L-NAME

Intravenous infusion of L-NAME (0.1mg/kg/hr) in deeply anaesthetised rats caused a steady rise in mean arterial blood pressure by 7.07 ± 1.5 mmHg (n=10) probably as a consequence of generalised vasoconstriction elsewhere in the body (Fig. 5.1A). After administering the drug for 5 min, joint vascular resistance increased by 16.38 ± 8.49% (n=10). Resistance subsequently fell by 17.18 ± 6.4% and did not significantly change from this level for the remainder of the experiment (P=0.705, one-way ANOVA). Changes in vascular resistance are shown in Fig. 5.2.

During a 1mg/kg/hr infusion, L-NAME caused the blood pressure to gradually rise by 38.20 ± 3.9 mmHg (n=10) 60 min after infusion onset (Fig. 5.1B). This pressor effect was found to be significantly different from the lowest dose of L-NAME (P<0.0001). Vascular resistance initially increased by 15.13 ± 7.75% during the first 5 min of infusion but then had very little effect on resistance throughout the last 55 min of administration.

Mean arterial pressure rose sharply by 28.89 ±3.21 mmHg during the first 10 min of 3mg/kg/hr L-NAME infusion (n=9) and reached an increase of 42.59 ±13.83 mmHg after an hour's infusion (Fig. 5.1C). This hypertension profile was found to be significantly different from both the 0.1 and 1mg/kg/hr doses of L-NAME (P<0.001).

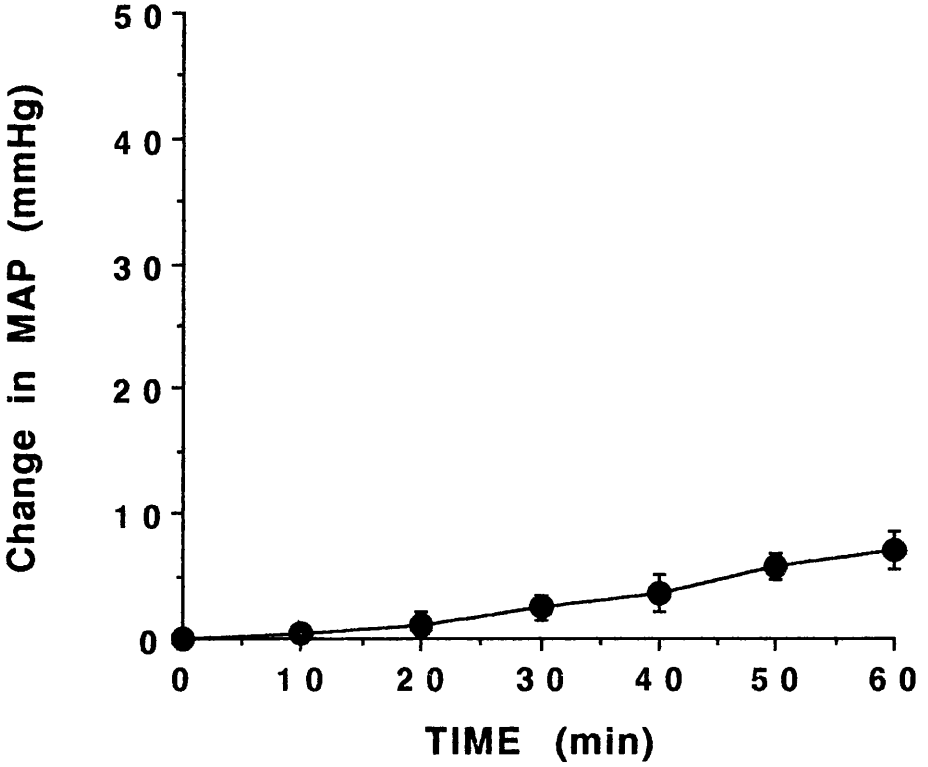
As shown in Fig. 5.1D, 10mg/kg/hr L-NAME infusion caused an initial rise in systemic blood pressure by 43.23 ±5.26 mmHg (n=9) which subsequently fell for the remainder of the experiment probably as a result of homeostatic baroreceptor reflexes. Knee joint vascular

resistance fell by 33.74 ±5.19% 10 min into the infusion of this dose of L-NAME and then induced a slight increase in resistance during the last 40 min of administration. This apparent constrictor response is probably not real and may be attributed to the fall in systemic blood pressure.

Fig. 5.1. A: Effect of intravenous infusion of 0.1mg/kg/hr L-NAME for 1hr on mean arterial blood pressure in the anaesthetised rat (mean ± S.E.M.; *n*=10).

B: Effect of intravenous infusion of 1mg/kg/hr L-NAME for 1hr on mean arterial blood pressure in the anaesthetised rat (mean ± S.E.M.; *n*=10).

A


Change in MAP (mmHg)
50
40
30
20
10
0
0
10
20
30
40
50
60
TIME (min)

B

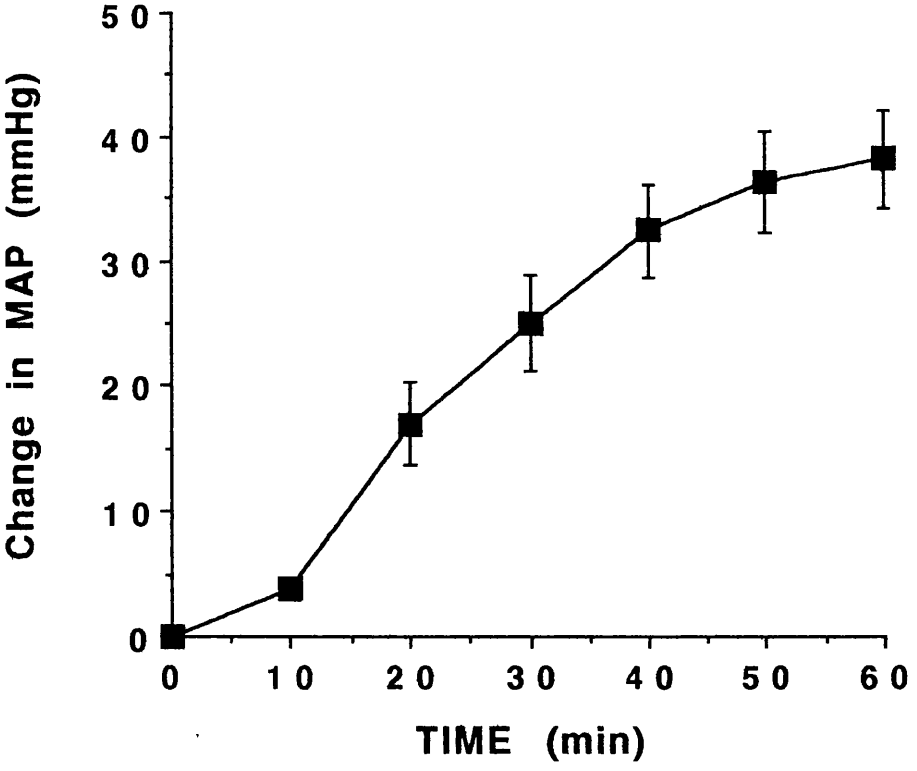
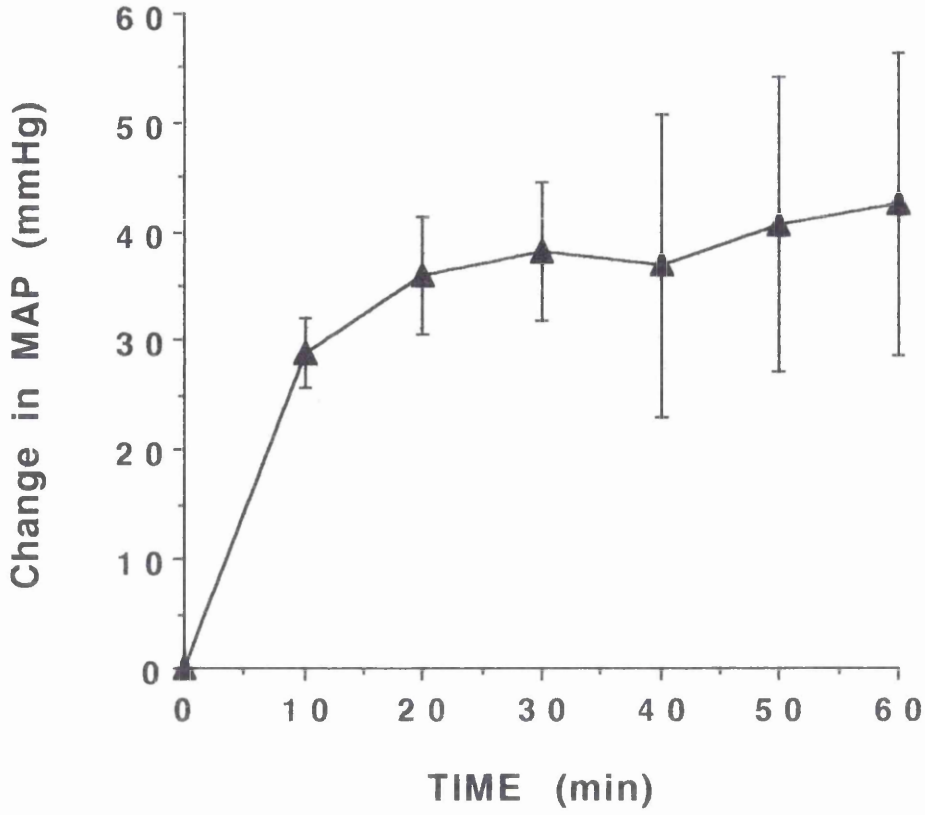

Change in MAP (mmHg)
50
40
30
20
10
0
0
10
20
30
40
50
60
TIME (min)

Fig. 5.1. C: Effect of intravenous infusion of 3mg/kg/hr L-NAME for 1hr on mean arterial blood pressure in the anaesthetised rat (mean ± S.E.M.; *n*=9).

D: Effect of intravenous infusion of 10mg/kg/hr L-NAME for 1hr on mean arterial blood pressure in the anaesthetised rat (mean ± S.E.M.; *n*=9).

C


Change in MAP (mmHg)
60
50
40
30
20
10
0
0
10
20
30
40
50
60
TIME (min)

D

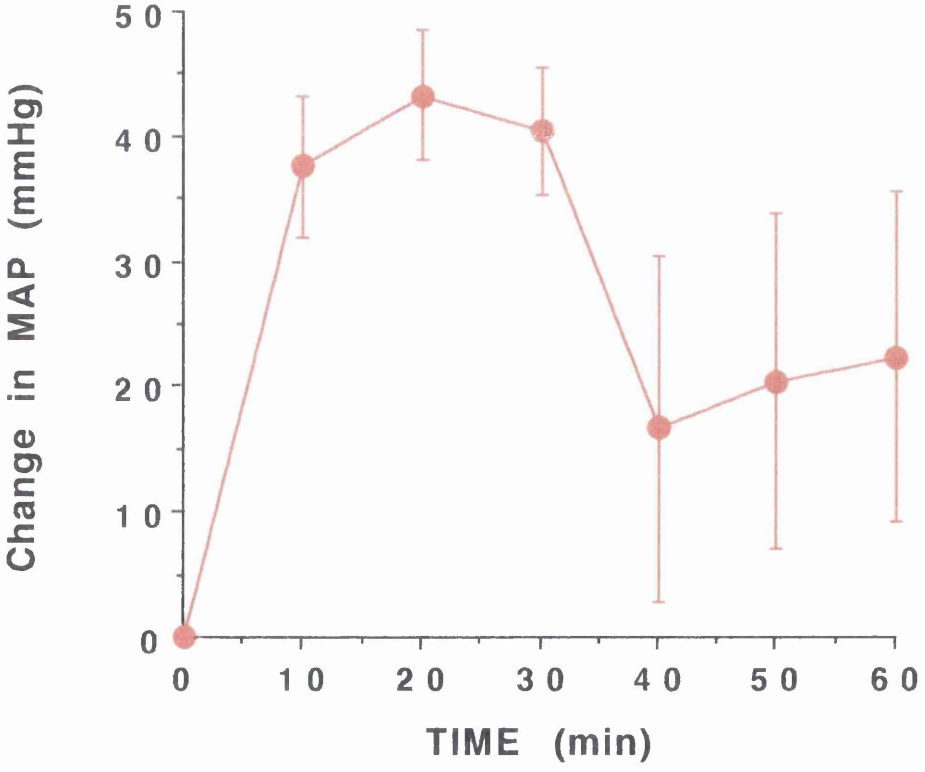
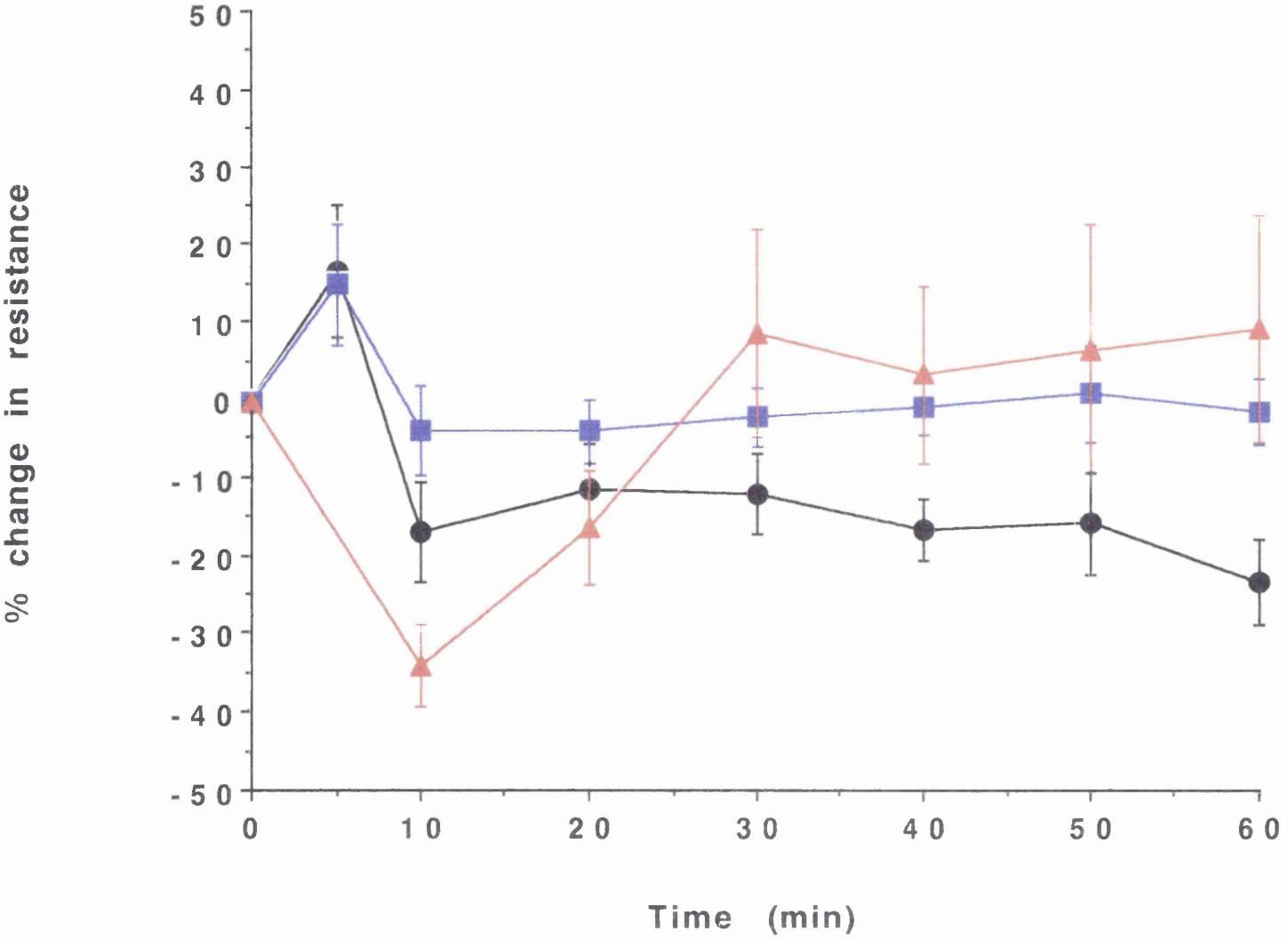

Change in MAP (mmHg)
50
40
30
20
10
0
0
10
20
30
40
50
60
TIME (min)

Fig. 5.2. Changes in vascular resistance in the rat knee joint during intravenous infusion of L-NAME (0.1mg/kg/hr- ●; 1mg/kg/hr- □; 10mg/kg/hr- △) Graph shows means ±S.E.M. *n*=10, 10 and 13 for the three doses of inhibitor respectively.


% change in resistance
50
40
30
20
10
0
-10
-20
-30
-40
-50
0
10
20
30
40
50
60
Time (min)

The haemodynamic effects of L-NMMA

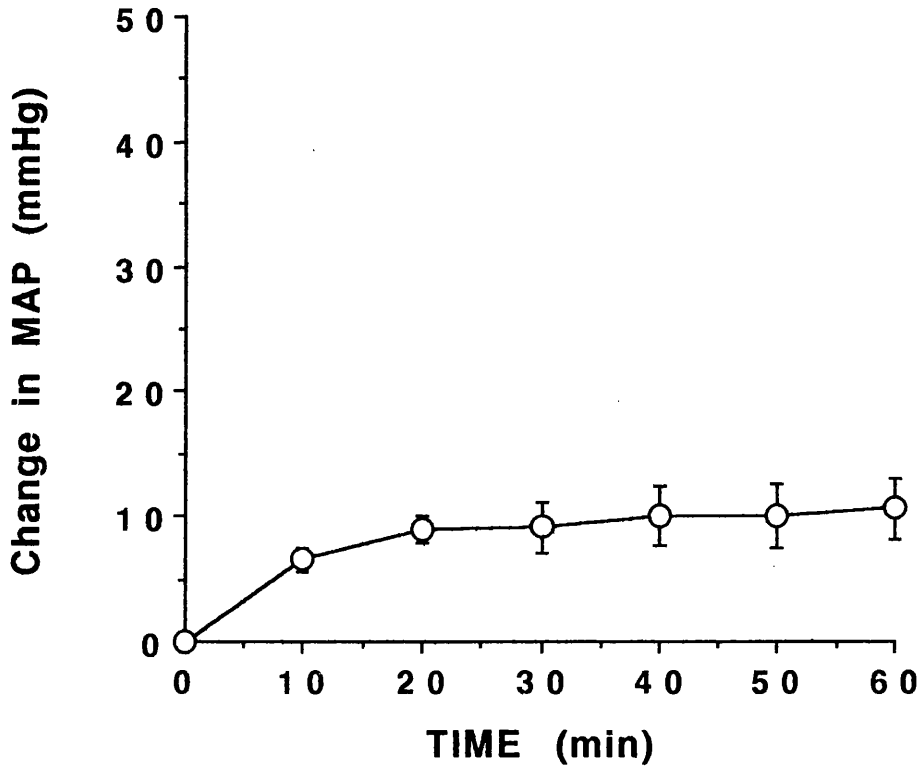
When infused at a rate of 10 mg/kg/hr (i.v.), L-NMMA caused mean arterial pressure to steadily rise by 10.62 ± 2.5 mmHg (Fig. 5.3A). L-NMMA had a negligible effect on vascular resistance for the first 40 min but subsequently fell by 15.0 ± 4.9% (n=15) over the last 20 min of administration (Fig. 5.4).

The higher dose of L-NMMA (30 mg/kg/hr) had a more notable effect on mean arterial pressure causing a rise of 39.11± 3.8 mmHg (n=10) by the end of the infusion period (Fig. 5.3B). This dose of L-NMMA had a complex effect on joint blood vessel resistance. Initially, resistance fell by 8.29 ±5.21% and then gradually increased to about 15% of control levels. During the last 30 min of infusion, L-NMMA caused vascular resistance to decrease again, ending up with an overall fall of about 15% by the end of the hour. Despite this waxing and waning response with the 30mg/kg/hr dose of L-NMMA, it was found to be not significantly different (P=0.917) from the lower dose of the drug suggesting that L-NMMA has very little effect on knee joint vascular resistance. However, since mean arterial pressure rose then L-NMMA affects resistance in other tissues of the body.

Fig. 5.3. A: Effect of intravenous infusion of 10mg/kg/hr L-NMMA for 1hr on mean arterial blood pressure in the anaesthetised rat (mean ± S.E.M.; *n*=15).

B: Effect of intravenous infusion of 30mg/kg/hr L-NMMA for 1hr on mean arterial blood pressure in the anaesthetised rat (mean ± S.E.M.; *n*=10).

A


Change in MAP (mmHg)
50
40
30
20
10
0
0
10
20
30
40
50
60
TIME (min)

B

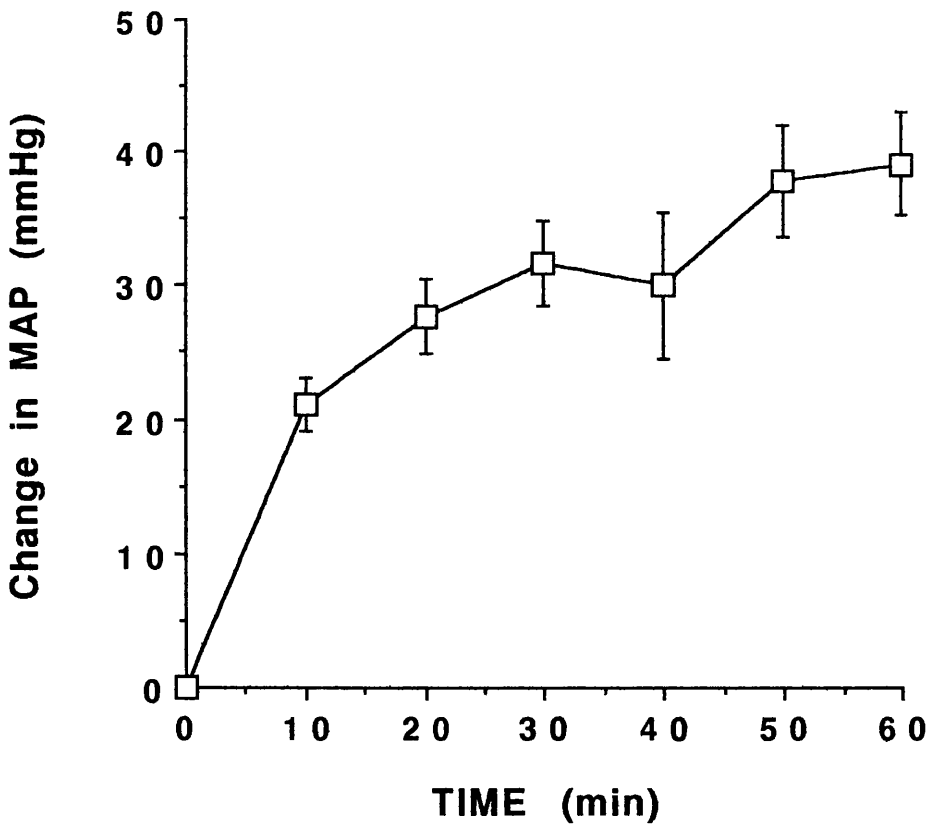
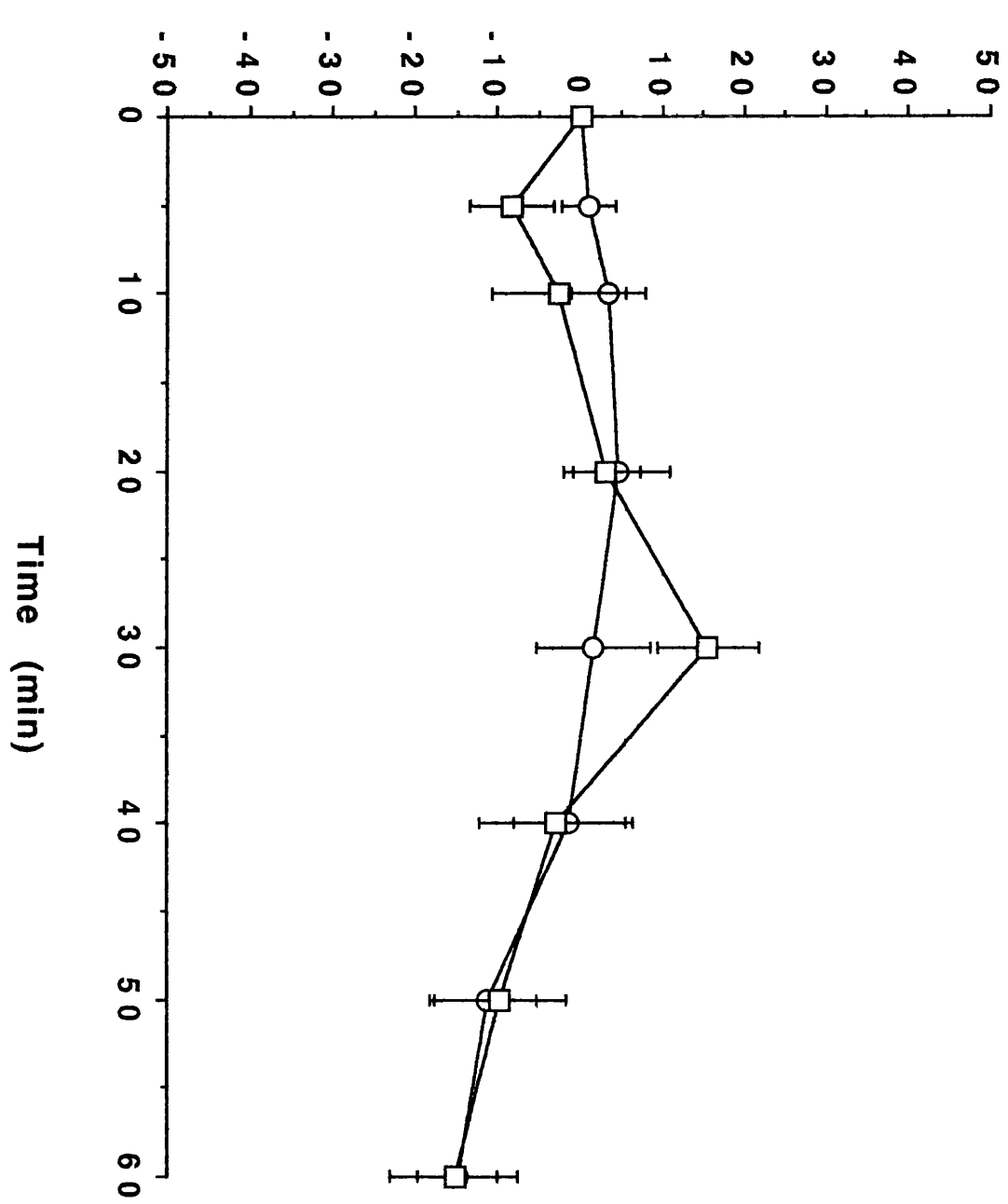

Change in MAP (mmHg)
50
40
30
20
10
0
0
10
20
30
40
50
60
TIME (min)

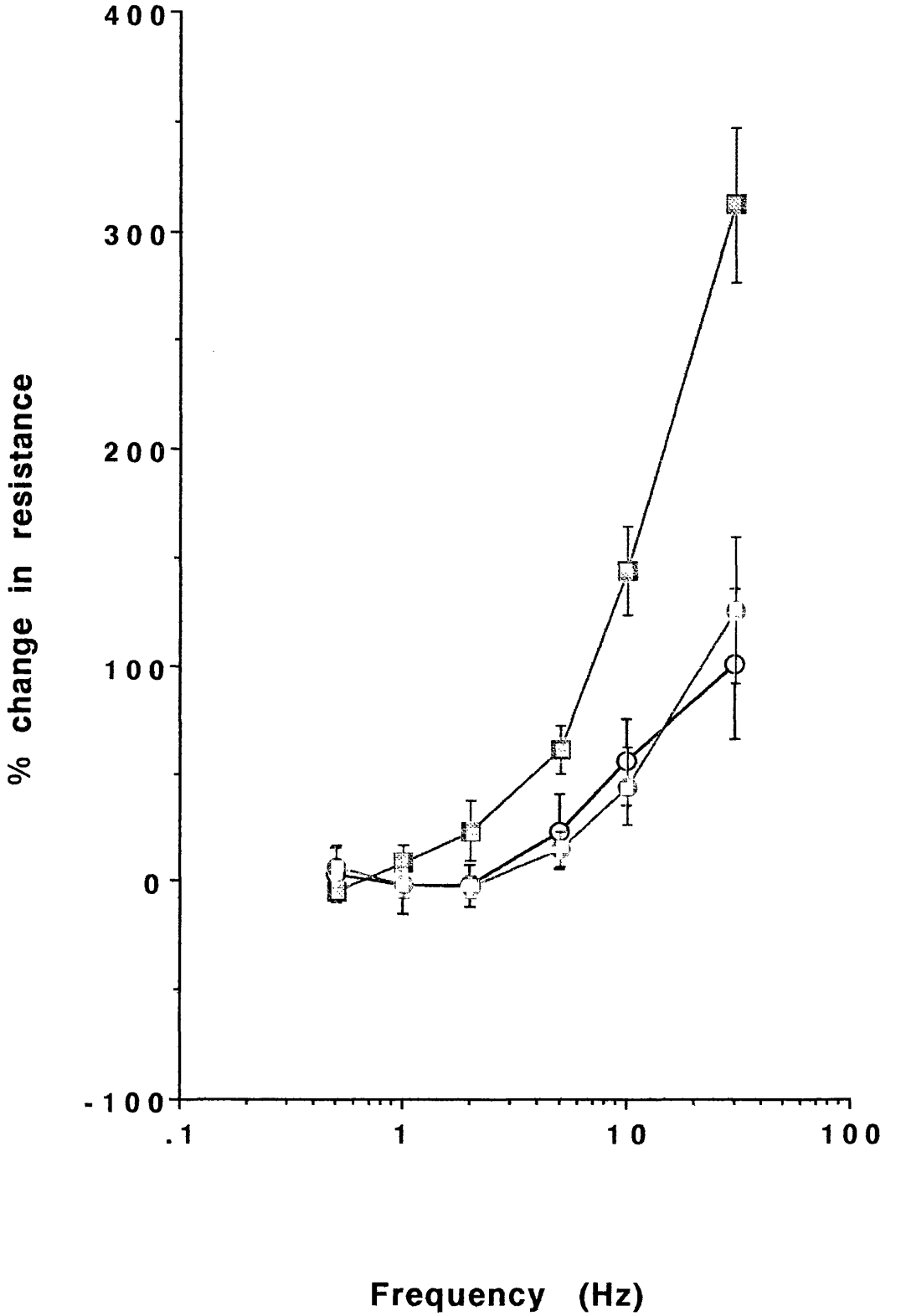
Fig. 5.4. Changes in vascular resistance in the rat knee joint during intravenous infusion of L-NMMA (10mg/kg/hr- ❍; 30mg/kg/hr- ❑). Graph shows means ±S.E.M. *n*=15 and 5 for the two doses of inhibitor respectively.


% change in resistance
-50
-40
-30
-20
-10
0
10
20
30
40
50
0
10
20
30
40
50
60
Time (min)

The effect of nerve stimulation on blood flow in the presence and absence of the NOS inhibitors

In normal animals, electrical stimulation of the saphenous nerve produced a vasoconstriction of joint blood vessels with 5, 10 and 30 Hz (the latter frequency causing the greatest increase in resistance by 100.6 ±35%; n =10). Frequencies of 0.5, 1 and 2 Hz had no discernible effect on knee joint vascular resistance (Fig. 5.5). A similar profile was obtained when stimulation of this nerve was carried out during the infusion of L-NAME, suggesting a lack of a nitrergic component in these particular nerves. A general linear model of variance revealed a significant effect due to frequency of nerve stimulation on vascular resistance ($P<0.002$) but no significant difference ($P=0.911$) was found due to treatment with L-NAME. However, when nerve stimulation was executed in the presence of L-NMMA there was an augmentation of the frequency-response profile which was significantly different from normal and L-NAME treated animals ($P<0.0001$ in both cases). This result provokes a re-evaluation of the previous statement concerning neurogenic NOS activity in this region. Stimulation of the saphenous nerve had no effect on systemic blood pressure which was to be expected as the nerve was transected proximally.

Fig. 5.5. Frequency/response profile of electrical stimulation of the saphenous nerve in the normal (○) rat knee and during the infusion of 0.1mg/kg/hr L-NAME (◌) and 10mg/kg/hr L-NMMA (□). Graphs show mean ± S.E.M. (n=10 for all sets of data).


400
300
200
100
0
-100
% change in resistance
.1
1
10
100
Frequency (Hz)

Discussion

The results of the present study have been able to demonstrate a heterogeneity in the actions of two inhibitors of NOS *viz.* L-NAME and L-NMMA. During the first 5 min of intravenous administration, the former inhibitor had a negligible effect on mean arterial pressure but in most cases caused a concomitant small, dose-independent rise in vascular resistance. As blood pressure subsequently rose, knee joint vascular resistance decreased. The rationale behind this observation is that L-NAME primarily inhibited the formation of NO in the joint effecting a loss of basal dilator tone in the articular vessels. Since the increase in resistance was low, it may be concluded that the amount of NO formed in the joint under resting conditions is sparse. Further systemic infusion of the drug caused inhibition of significant amounts of NO production in other tissues of the body and consequently mean arterial pressure rose. This general hypertension caused mechanical distension of the knee joint vasculature thereby offsetting any minor constrictor response brought about by the effects of L-NAME. In contrast, 10mg/kg/hr L-NMMA had little effect on the resistance of articular blood vessels initially, but caused a fall in resistance 40 min after administration. Mean arterial pressure rose only slightly throughout the experiment and this accounted for the fall in resistance. The higher dose of L-NMMA produced a larger generalised hypertension with a complex effect on knee joint vascular resistance which fluctuated between periods of constriction and dilatation. The inference of these findings is that the basal production of NO in this particular tissue is insubstantial and appears only to be inhibited by L-NAME. Synovial blood vessels appear to differ from those elsewhere in the rat where vascular resistance must have substantially increased

to produce the observed rise in arterial blood pressure. These results differ considerably from those of a previous study in the rabbit where L-NAME administration resulted in a large and sustained increase in vascular resistance of knee joint blood vessels (Ferrell and Najafipour, 1992), suggesting that in this species NO release is continuous and contributes importantly to the maintenance of basal synovial perfusion.

The results of this study are also in contrast to those of Gardiner *et al* (1991) who found that both L-NAME and L-NMMA produced an increase in blood pressure with a sustainable vasoconstrictor response. The effect with L-NAME was found to be ten times more potent than L-NMMA and its rate of onset of effect was slower. Changes in perfusion in their experiments were measured in main arteries supplying certain areas of the body (*eg* the distal abdominal aorta in order to monitor blood flow to the hindquarters), whereas the present study measured blood flow in capillary beds and hence produced a more localised picture of vascular perfusion. The reason for the divergence in inhibition in our study is possibly due to the two inhibitors acting on different isoforms of NOS. It is well known that many isoforms of NOS exist and of which only a few have been purified and characterised extensively (Förstermann, Schmidt, Pollock, Sheng, Mitchell, Warner, Nakane and Murad,1991). It may be that in the vessels supplying the rat knee joint, a unique isoform of NOS occurs whose actions are immediately inhibited by L-NAME but are unaffected by L-NMMA.

Stimulation of the sympathetic nerves supplying the joint confirmed once again a vasoconstrictor response in the anaesthetised rat as measured by LDI (Lam and Ferrell, 1993b). Frequencies of 5, 10 and 30 Hz showed a decrease in blood flow with increasing

frequency, whereas 0.5, 1 and 2 Hz each had a negligible effect. This stimulation profile was unaffected by treatment with L-NAME which might appear to suggest that NO is not released from these nerve terminals in response to electrical stimulation. This finding is in contrast to other studies which have found an enhancement of sympathetic vasoconstrictor responses in the presence of L-NAME (Bucher, Ouedraogo, Tschöpl, Paya and Stoclet, 1992; Vo, Reid and Rand, 1992). In these *in vitro* analyses, however, nerve stimulation was carried out at low frequencies *ie.* 0.4 Hz and 3 Hz respectively. In the present experiments these frequencies would have no discernible effect on joint blood flow. Other investigators have reported that stimulation of sympathetic nerves with a 10 Hz stimulus train whilst administering the NOS inhibitor produced a vasoconstriction which was by and large of the same order as control values thus supporting our findings (Modin, Weitzberg, Höckfelt and Lundberg, 1994). Analysis of joint blood flow in rabbits has shown that nerve-mediated vasoconstrictor responses in the knee are potentiated during L-NAME infusion (Najafipour and Ferrell, 1993b). However, in the rat knee L-NAME was completely ineffective whereas L-NMMA produced a very clear enhancement of vasoconstriction even though it is known to be much less potent than L-NAME. It appears, therefore, that rabbits and rats have distinct isoforms of NOS present in the nerve terminals supplying their joints. The enzymatic activity of the NOS in the rabbit is inhibited by L-NAME whereas the one in the rat is unaffected by this agent but inhibited by L-NMMA. Stimulation of sympathetic nerves in the presence of L-NMMA is known to augment constrictor responses *in vitro* (Toda and Okamura, 1990b). The present study supports this finding *in vivo* with greater constrictor responses occurring at nearly all frequencies tested. It appears, therefore, that

NO is either released from the nerve endings directly or induced secondary to the release of another neurotransmitter. These conclusions can be tested more confidently in the rat as the nerve-mediated vasoconstrictor responses were elicited against a background of minimal change in basal vascular resistance. In the rabbit knee joint basal vascular resistance was substantially elevated by L-NAME and by simply summing with the nerve-mediated increase in vascular resistance, could produce an apparent enhancement of vasoconstrictor responses. The lack of significant change in basal vascular resistance in rat synovial vessels in response to L-NMMA, but its obvious enhancement of nerve-mediated vasoconstriction strongly suggests release or rapid induction of NO in response to nerve stimulation.

In summary, the findings of these experiments suggest that the amount of endogenous NO produced in the rat knee joint is minimal and therefore has only a minor rôle in the maintenance of resting joint blood flow. However, NO appears to be released or induced in response to electrical stimulation of the nerves supplying the joint but the inhibition of its production is executable by L-NMMA but not L-NAME.

SECTION II:

The rôle of nitric oxide in the control of rat knee joint blood vessel permeability

Introduction

In addition to localised hyperaemia, acute inflammatory episodes may also be characterised by increased vascular permeability with a resultant leakage of plasma proteins from the microcirculation. In synovial joints this pathological process manifests itself by the appearance of protein in the synovial fluid (Lam and Ferrell, 1991c; Scott, Lam and Ferrell, 1991; Cruwys, Kidd, Mapp, Walsh and Blake, 1992). The origin of this protein is normally an ultrafiltrate of plasma which is thought to leak through gaps in capillary walls formed by contraction of endothelial cells (Majno, Shea and Leventhal, 1969). Plasma ultrafiltration occurs in response to disturbances in the balance of Starling's forces (Starling, 1896) *viz.* hydrostatic and oncotic pressures (Fig. 5.6). Hydrostatic pressure is dependent on the systemic blood pressure and tends to drive the filtration of protein-free plasma out of the capillaries and into the interstitial fluid. Since the protein concentration of plasma is greater than in interstitial fluid then an osmotic force is generated which counteracts this fluid loss. This force is called the oncotic pressure. When hydrostatic pressure exceeds oncotic pressure *eg.* as a consequence of increased systemic blood pressure, then a net filtration of plasma occurs. If in addition to this pressure imbalance there is extensive capillary injury, then plasma proteins may leak into the interstitium along with fluid. This is the principle of plasma protein extravasation.

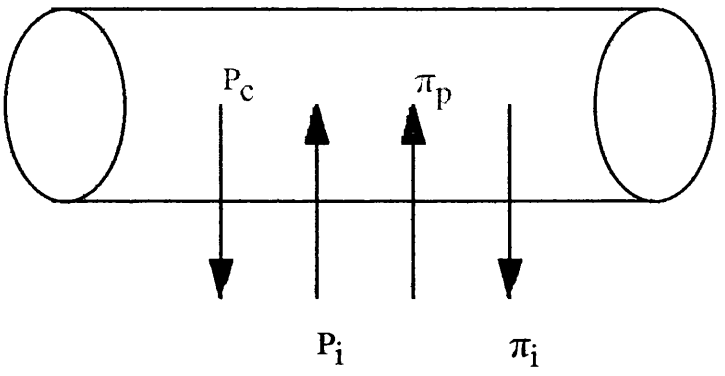


Fig. 5.6. Diagram of Starling's forces determining fluid movement across capillaries. P_C = capillary hydrostatic pressure; P_i = interstitial fluid hydrostatic pressure; π_p = plasma protein oncotic pressure; π_i = interstitial protein oncotic pressure. Net filtration occurs when $(P_c + \pi_i) - (P_i + \pi_c) > 0$

By introducing a suitable marker which labels plasma proteins and subsequently calculating its concentration in synovial fluid, it is possible to indirectly measure synovial blood vessel permeability. Unfortunately the nature of the protein is not identified by this method and therefore, it is not definite that all extravasated protein originates from the circulation. However, a previous study was able to show that a large proportion of these proteins are in fact derived from the microcirculation (Lam and Ferrell, 1991c). Early studies used markers such as Evans blue dye (Lam and Ferrell, 1991c; Cruwys, Kidd, Mapp, Walsh and Blake, 1992) and ^{125}I-labelled albumin (Cambridge and Brain, 1992). However, these techniques are relatively insensitive since only a limited proportion of the plasma protein is labelled and hence the level of extravasated protein is underestimated. The technique known as microturbidimetry has recently been developed

which measures all of the protein in the perfusate aspirated from the joint space (Scott, Lam and Ferrell, 1991) making it a more sensitive method of measuring protein extravasation.

In addition to its potent vasodilator properties, NO inhibits platelet aggregation and adhesion (Azuma, Ishikawa and Sekizaki, 1986; Furlong, Henderson, Lewis and Smith, 1987; Radomski, Palmer and Moncada, 1987a; Radomski, Palmer and Moncada, 1987b; 1987c), and NO is cytotoxic in some cells (Hibbs, Taintor, Vavrin and Rachlin, 1990). Inhibitors of NO have varying degrees of success in affecting vascular permeability in a number of different tissues in anaesthetised rats. Santicoli *et al.* (1993) found that L-NA was unable to induce plasma protein extravasation in the trachea, ureter and urinary bladder. Other studies using L-NAME as the NOS inhibitor found it increased coronary vascular permeability in rats (Filep, Földes-Filep and Sirois, 1993) suggesting that NO may play an important rôle in the regulation of vascular permeability.

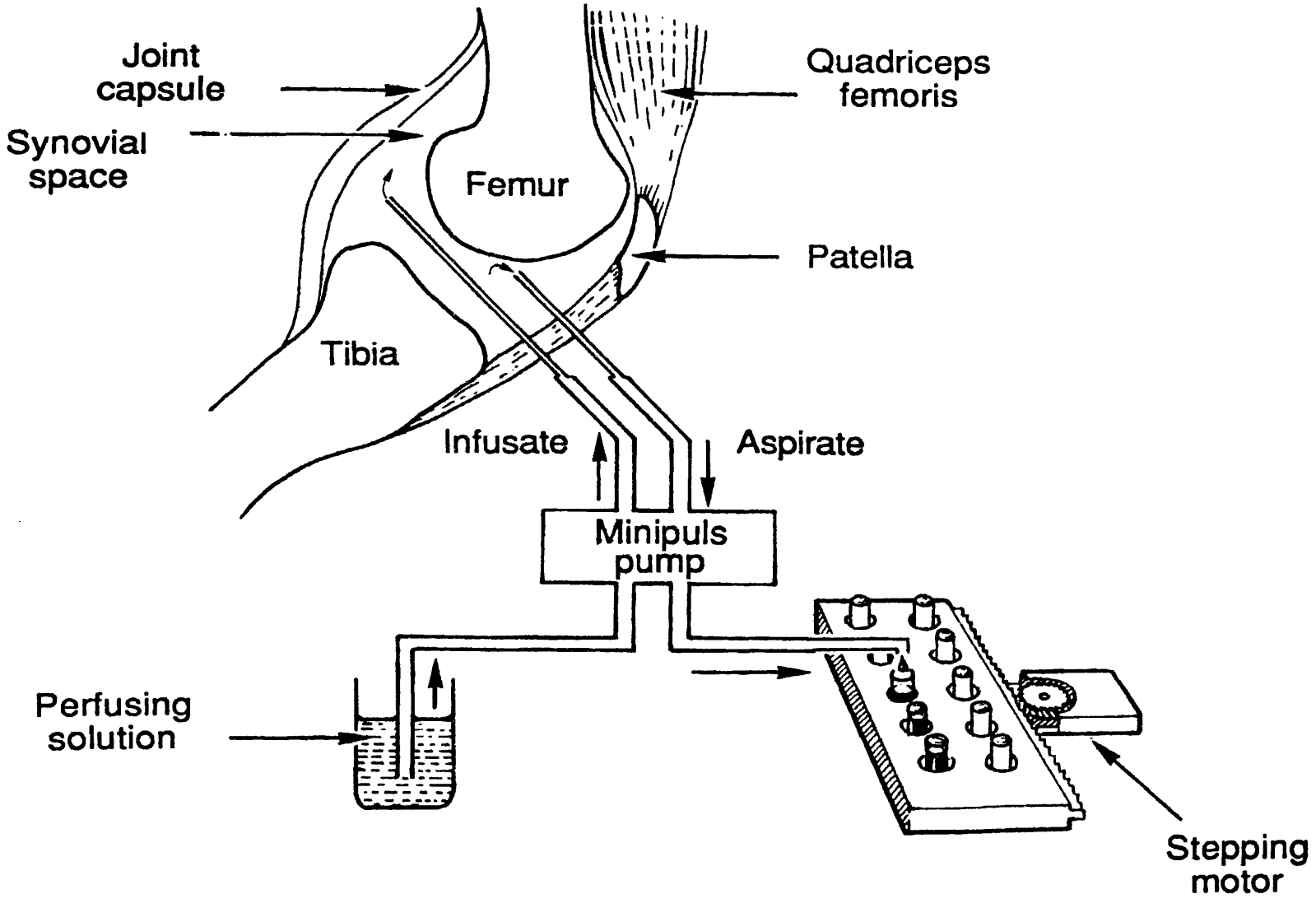
Aims of the study

The aim of the present study was to investigate the rôle of endogenous NO in the regulation of vascular permeability in the rat knee joint. Experiments examined whether NO inhibition by L-NAME could affect synovial microvascular protein extravasation which was measured by the microturbidimetry technique.

Methods

Experiments were performed on male Wistar rats (~400g) which were deeply anaesthetised by intraperitoneal injection of urethane (1.13g/kg). Rats were placed in dorsal recumbency and after abolition of the flexor withdrawal reflex the skin covering the knee was excised. Two 29 gauge needles were then inserted into the synovial cavity. The inflow needle was inserted vertically between the femoral condyles and oriented such that the tip of the needle lay in the posterior region of the joint space (Fig. 5.7). The outflow needle was inserted horizontally through the capsule and just underneath the patellar tendon such that its tip lay in the anterior region of the joint. This orientation of the needles ensured that the whole of the joint was perfused and avoided the possibility of the two needles coming into close proximity of each other causing bypass of the synovial cavity. The needles were connected in series to a peristaltic pump (Gilson minipuls) and physiological saline (0.9%) was perfused into the synovial cavity at a rate of 0.1ml/4min and the effluent was collected over 8 min periods. Saline perfusion was continued for up to an hour after which time the perfusate was changed to one containing various concentrations of L-NAME dissolved in saline. Continuous infusion of 0.9% saline is known to have no effect on protein extravasation in the rat knee joint (Karimian and Ferrell, 1994). Once all the samples had been collected, the protein content of each sample was ascertained by microturbidimetry. This method is based on the finding that in alkaline solution, protein reacts with the quaternary ammonium salt benzyldimethyl{2-[2-(p-1,1,3,3-tetramethylbutylphenoxy)ethoxy] ethyl} ammonium chloride (benzethonium chloride) to produce a turbid suspension (Iwata and Nishikaze, 1979).

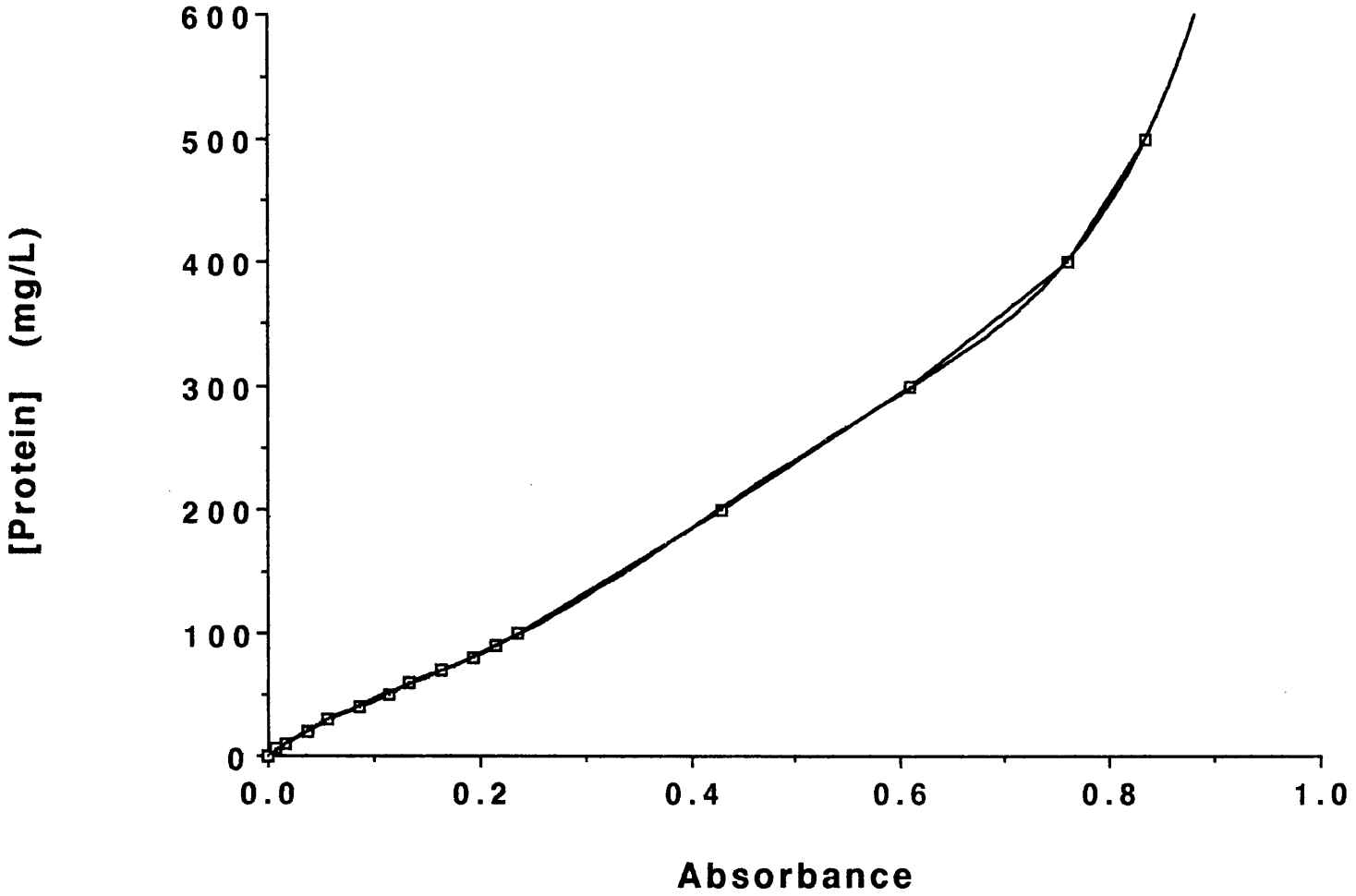
Fig. 5.7. Diagram of the experimental set-up used to measure plasma protein extravasation in the rat knee joint. The tip of the infusate needle is positioned near the posterior region of the synovial cavity whereas the aspirate needle is oriented in the anterior region.


Joint capsule
Synovial space
Femur
Quadriceps femoris
Patella
Tibia
Infusate
Aspirate
Minipuls pump
Perfusing solution
Stepping motor

The 0.2ml samples were mixed with final concentrations of 0.2% benzethonium chloride and 1.6ml of 3.2% NaOH in 1.23% EDTA. The amount of plasma protein amassed was calculated by comparing the absorbance of the samples at 360nm (LKB Ultrospect II) with that of an albumin calibration curve (Fig. 5.8). Samples containing concentrations of L-NAME in saline solution had no effect on absorbance confirming that any increases in absorbance were not due to the presence of L-NAME in the perfusate. All data are expressed as mean protein concentration ±S.E.M. The data were not found to be normally distributed when tested using a modified version of the Shapiro-Wilk test and as such all statistical testing had to be performed using non-parametric tests. All statistical analyses were performed using InStat software. The effect of dose on protein extravasation was tested by the Kruskal-Wallis test and the data at each dose were tested to see if they were significantly different from saline controls using the Mann-Whitney test. Differences between the data were considered significant when $P < 0.05$.

Fig. 5.8. Calibration curve showing the relationship between protein (albumin) concentration and absorbance at 360nm. The 5th order polynomial equation for the relationship is also shown which has a correlation coefficient (r) =1

$$y = 0.40822 + 624.47x - 2538.3x^2 + 1.0330e+4x^3 - 1.5958e+4x^4 + 8604.8x^5$$



Results

Intra-articular infusion of 10^{-4} M L-NAME elicited extravasation of plasma protein into the synovial cavity of the rat knee joint (Fig. 5.9) which was significantly different from a saline control ($P<0.05$; $n=6$). All other doses of L-NAME (*ie.* 10^{-2}, 10^{-3}, 10^{-5}, and 10^{-6} M) had no significant effect on extravasation in this tissue ($P>0.20$). Comparison between doses of L-NAME using the Kruskal-Wallis test showed that they were not significantly different from each other ($P=0.1427$).

Analysis of the time/response relationships showed that the extravasation produced by the 10^{-4}M dose of L-NAME (115 ±25.21mg/L) was transient and only occurred during the first 8min of infusion (Fig.5.10). Following this peak response protein extravasation sharply fell to control levels where it continued to fall for the remainder of the experiment. Infusates containing either 10^{-2}, 10^{-3} or 10^{-5} M L-NAME were unable to effect extravasation of plasma protein into the synovial space at any time during the 48min infusion.

Fig. 5.9. Effect of intra-articular infusion of L-NAME on plasma protein extravasation into the synovial cavity of the rat knee joint (mean ± S.E.M.; *n*=6). Protein concentrations shown are from effusates taken over 8min after commencement of infusion.

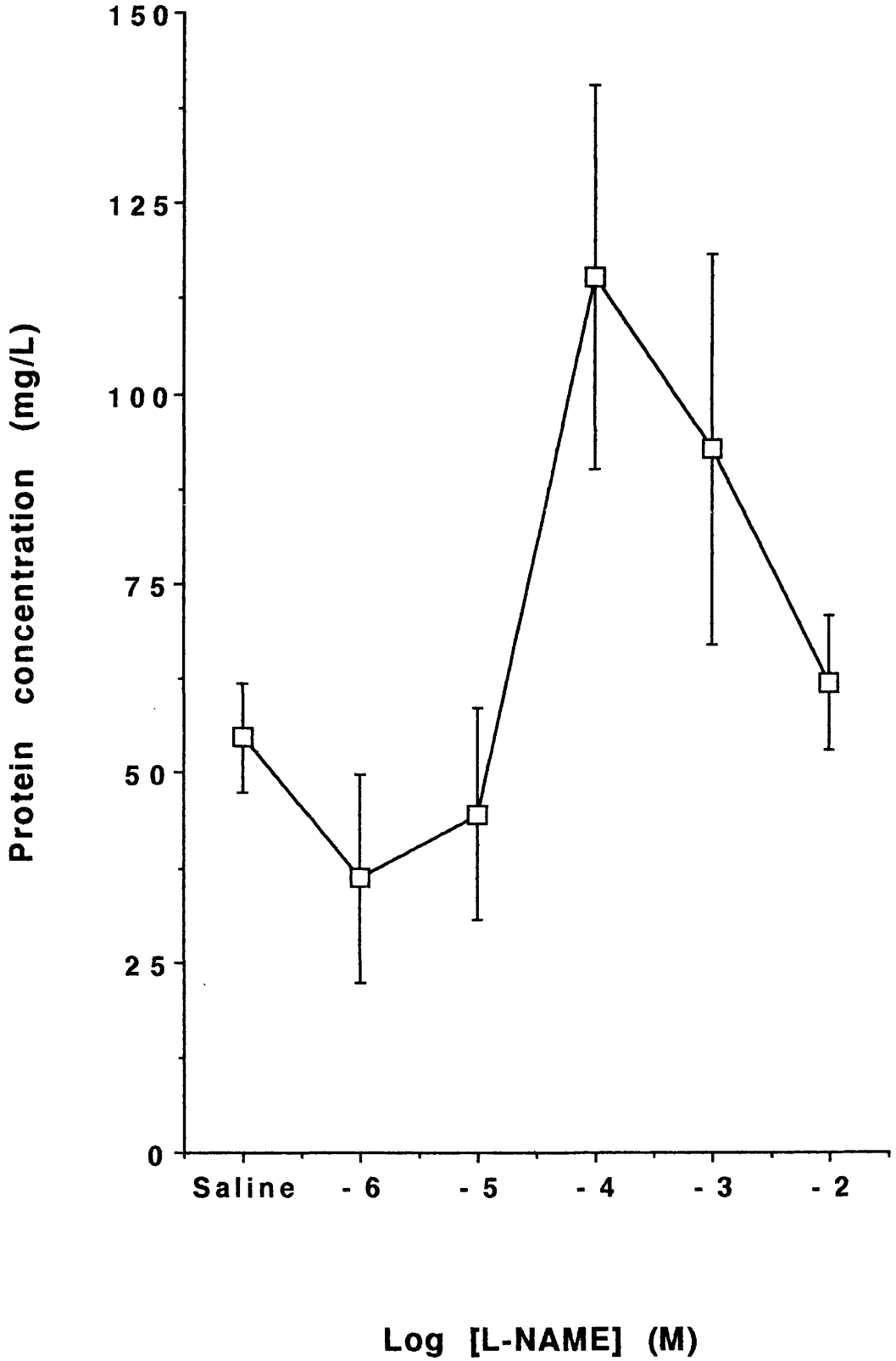
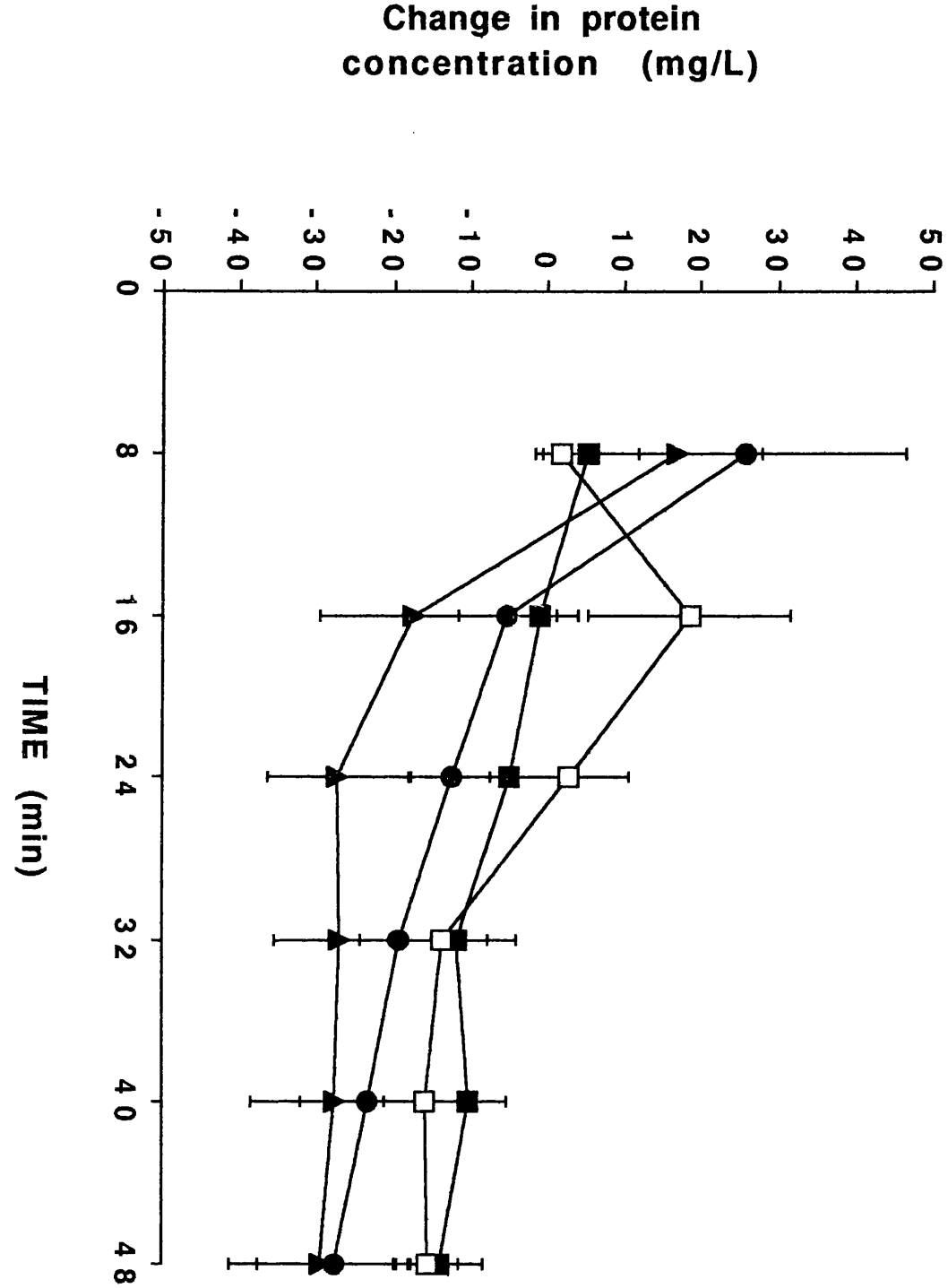

150
125
100
75
50
25
0
Protein concentration (mg/L)
Saline
- 6
- 5
- 4
- 3
- 2
Log [L-NAME] (M)

Fig. 5.10. Time course of changes in protein extravasation into the rat knee joint cavity in response to intra-articular infusion of L-NAME (mean ±S.E.M.). The doses of L-NAME used were 10^{-5}M (■), 10^{-4}M (●), 10^{-3}M (▲) and 10^{-2}M(❑); n=6 for each dose.


Change in protein concentration (mg/L)
-50
-40
-30
-20
-10
0
10
20
30
40
50
0
8
16
24
32
40
48
TIME (min)

Discussion

The present study was able to show that inhibition of NO synthesis in the rat knee joint by intra-articular infusion of L-NAME induces plasma protein extravasation, but only at 10^{-4}M concentration. Examination of the time course of response revealed that this increased vascular permeability was transient and occurred during the first 8min of infusion. The amount of protein in the effusate subsequently declined over the remainder of the experiment and was probably due to a washout effect since protein concentration has been shown to fall by a similar magnitude during saline infusion (Karimian and Ferrell, 1995). Interestingly the temporal effect of L-NAME on extravasation shown here is similar to the time/response relationship obtained whilst monitoring rat knee joint vascular resistance during intravenous infusion of the drug (see Fig. 5.2). Comparison of the two curves reveals that protein extravasation occurs during the transient phase of increased knee joint blood vessel resistance. This increase in resistance causes a fall in hydrostatic pressure in the joint vasculature and a reduction in capillary surface area available for filtration. Since the combination of these two mechanisms would minimise protein extravasation, it follows that L-NAME exerts its extravasatory effects by altering the joint vasculature directly, probably by endothelial gap formation. This supposition is supported by the fact that continuation of L-NAME administration causes an increase in systemic blood pressure with a resultant fall in knee joint vascular resistance. These haemodynamic changes were unable to promote extravasation into the synovial cavity, substantiating the theory that the mechanism was not via an increase articular hydrostatic pressure. These findings are similar to those of Filep *et al.* (1993) who found that intravenous

injection of L-NAME evoked a two-fold increase in vascular permeability in the coronary circulation. By the same token, the increased protein extravasation in this tissue was probably not related to hypertension since elevation of mean arterial pressure by infusion of noradrenaline did not alter vascular permeability in the coronary circulation.

Mediator-stimulated protein extravasation is thought to be primarily due to formation of inter-endothelial cell gaps exclusively in the venules (Bjork and Smedegard, 1983; Grega, Adamski and Dobbins, 1986). This leads to opening of large pores which allows the transport of macromolecules across the blood vessel wall during inflammatory reactions. The present findings suggest that inhibition of NO by L-NAME activates this transport system especially since the gap formation process is thought to occur independently of changes in hydrostatic pressure.

A major disadvantage of the microturbidimetry technique is the variability in the response. This is probably due to erroneous placement of the inflow/outflow needles such that the needle tips are in close proximity to each other and hence a short circuiting effect ensues. In order to produce a maximal response, the inflow needle should be placed near the posterior region of the joint space and the outflow needle oriented in the anterior region. This arrangement allows the delivery of the infusate to as large an area of the synovium as possible before the outflow needle removes the resulting extravasated protein in the effusate.

Concluding remarks

The endogenous production of NO in the rat knee joint is slight and as such probably only plays a quiescent rôle in the control of joint blood flow. Inhibition of NO production by L-NAME causes a transient increase in knee joint vascular resistance before systemic hypertension takes over causing mechanical distension of articular vessels; henceforth, resistance decreases. During the constriction phase of the L-NAME response there is a concomitant promotion of plasma protein extravasation into the synovial cavity. Since hydrostatic pressure has fallen during this stage, L-NAME probably induces extravasation by causing contraction of vascular endothelial cells leading to the formation of gaps through which plasma proteins can leak.

Electrical stimulation of the nerves supplying the joint causes either the release of NO directly or induces the production of NO by the endothelium. In order to inhibit this effect it is necessary to treat the animal with L-NMMA since L-NAME appears to be ineffective.

Chapter 6

Comparison Of Coloured Microspheres And Laser Doppler Perfusion Imaging For Measuring Blood Flow In Rabbit Medial Collateral Ligaments

Introduction

The stability of diarthroidal joints is essential to prevent movement in abnormal planes. Stability is mainly provided by the ligamentous and capsular support systems of the joint which in turn rely on their blood supply to maintain their structural integrity (Butterwick, Paul, Bray and Tyberg, 1992). Blood flow is necessary to deliver nutrients and remove waste products of metabolism from tissues, but in articular tissues, it may also function in adaptive responses to various states of stress (Bray, Doschak and Tyberg, 1994). Stresses such as joint tissue damage, infection, or inflammation can all lead to acute and possibly chronic vascular responses in joints. Measurement of blood flow in joints has recently been determined by coloured microspheres (CMs) (Bray, Doschak and Tyberg, 1994) and laser Doppler perfusion imaging (LDI) (Lam and Ferrell, 1993b).

Microsphere measurement of tissue blood flow has become an accepted, albeit invasive, standard for determination of *in vivo* tissue blood flow (Heyman, Payne, Hoffman and Rudolph, 1977). Microspheres labelled with a colour dye, as an alternative to the conventionally used radio-labelled microspheres, have recently shown a capacity to measure blood flow quantitatively, particularly for measurements made in low blood flow sites such as articular ligaments (Bray, Doschak and Tyberg, 1994).

Laser Doppler flowmetry (LDF) is a non-invasive method of measuring tissue perfusion and is based on recording the Doppler shifts photons from monochromatic light undergo when scattered by moving erythrocytes in vascularised tissue. LDI is a modification of this technique which is able to generate 2-

moving erythrocytes in vascularised tissue. LDI is a modification of this technique which is able to generate 2-dimensional colour-coded images of tissue perfusion (Wårdell, Jakobsson and Nilsson, 1993). Whereas the CM technique gives absolute measures of blood flow, LDI yields only relative changes in blood flow.

The sensory neuropeptide SP is known to be pro-inflammatory in joints where it exerts such effects as potent vasodilatation (Ferrell and Cant, 1987; Lam and Ferrell, 1993a; 1993b) and increased vascular permeability (Ferrell and Russell, 1986; Lam and Ferrell, 1990; 1991c; Scott, Lam and Ferrell, 1992). Despite this extensive knowledge of SP actions in rats and cats, its vasoregulatory effects in rabbits are relatively unknown.

Aims of the study

Initially, the purpose of the study described here was to confirm whether LDI is able to measure low blood flow to areas such as articular ligaments. Once this was ascertained further aims were to compare blood flow measurements from ligaments using CMs and LDI to establish whether a significant correlation exists between the two techniques. This collation would examine the possibility of calibrating the relative perfusion readings generated by LDI in order to provide absolute measures of tissue perfusion.

In this study, the medial collateral ligament (MCL) of rabbits was studied because of an accumulated database on blood flow in this structure (Paul, Bray and Doschak, 1994), and because prior studies have indicated that injury is a potent stimulus to increasing blood

(Bray, Doschak and Tyberg, 1994). This model was therefore considered appropriate for the CM/LDI comparison as it would broaden the range of measurements.

Finally the effects of SP on MCL blood flow in normal and ACL-deficient rabbits was also examined.

Methods

In vivo MCL blood flow assessment

Experiments were performed on 15 female, one year old, New Zealand White rabbits (4-5 kg). Animals were premedicated with 0.15 ml i.v. acepromazine maleate (Ayerst Laboratories, Montreal) and subsequently anaesthetised with a mixture of halothane (2%-5%) and 100% oxygen (2ml/min). Normal and knee-injured rabbits were used. Injuries were produced in the right knee of 10 rabbits by transecting the ACL (the remaining rabbits were left uninjured). This was carried out by first shaving the knee and then placing the animal in dorsal recumbency in a sterile environment. The joint was covered in iodine to generate a state of asepsis and a longitudinal incision (approximately an inch long) was made in the lateral aspect of the joint capsule. The patellar ligament was reflected and the ACL located deep in the joint space. A pair of curved forceps were hooked under the ligament which was subsequently bisected with a scalpel. The incision was sutured and the animal allowed to recover.

Each rabbit was prepared for coloured microsphere determination of blood flow according to standardised protocols (Kowallik, Schulz, Guth, Schade, Paffhausen, Gross and Heusch,

1991). This version of the CM technique has been modified for determination of blood flow in low blood flow tissues such as articular ligaments and is outlined below.

Dye-Track CMs (Triton Technology Inc., San Diego, California) are polystyrene microspheres labelled with a coloured dye and diluted in a saline-tween solution to prevent aggregation. The CM technique involves the slow infusion of about 10.2 million (15.5μm in diameter) into the left ventricle whilst a reference blood sample is withdrawn at a rate of 3ml/min from a cannulated brachial artery. The CMs enter the general circulation and eventually become lodged in terminal arterioles. After animal sacrifice, the CMs are recovered from the MCL by digestion of the tissue in 7ml of concentrated KOH (at 60°C) followed by microfiltration of the resulting solution. Since the MCL has a low blood flow, the number of microspheres can be counted directly using an inverted Nikon Diaphot-TMD microscope with epi-fluorescent illumination. The number of microspheres present in the reference blood sample, however, is too large to be counted by direct means. In this instance the CMs are placed in 1000 μl of N, N-dimethylformamide, a solvent which is able to leach the dye from the CMs. The concentration of CMs is then determined by measuring the optical density of the solution at 448 nm using a spectrophotometer (Ultraspec 4050 spectrophotometer, LKB Biochrom Ltd., Cambridge, England). By interpolating the linear relationship that exists between absorption and CM number, the absorption units could be converted to CM number. Since the blood sample is withdrawn at a known rate, the blood flow to the MCL can be calculated using the following equation:

$$Q_m = Q_r \times \frac{CM_m}{CM_r}$$

where Q_m is the blood flow to the MCL; Q_r is the withdrawal rate of the reference sample; CM_m is the number of microspheres in the MCL; CM_r is the number of microspheres in the reference sample.

LDI measurements were obtained from exposed MCLs concurrently with CM determinations. The scanner head was placed 15 cm above the exposed ligament which was masked with black velvet cloth to ensure a zero background perfusion reading at the anatomic margins of the MCL. The exposed joint was prevented from drying out by regular application of 0.9% NaCl which had previously been warmed to 37°C. A final LDI scan was carried out following animal sacrifice to obtain a "biological zero" which was subtracted from each LDI measurement.

Blood flow measurements were made in the exposed MCL at several intervals after ACL transection, ranging from 1-14 days. This range in post-injury time periods along with normal control and contralateral MCLs allowed for a wide range of blood flow measurements to be made.

The effect of SP was investigated in 4 normal and 5 injured rabbits. After obtaining basal blood flow readings in these animals, 0.1ml of SP (10^{-7} to 10^{-12} M) was applied as a bolus to the surface of the ligament. An LDI scan was taken immediately after administration of the drug followed by a CM run. A different colour of microspheres was used in this second run to distinguish them from those infused during basal blood flow determination.

Calculations and statistical analysis

Images were analysed using VIEW software (Moor Instruments Ltd.) and the median voltage reading was taken to represent the average blood flow to the ligament. The LDI values were compared with the standardised blood flow values measured by the CMs on a scatter plot where the CM measurements were represented as the independent variable. Statistical analyses were performed using InStat and MINITAB software. The slope and y-intercepts of the graph between the LDI output and the standardised blood flow were determined using linear regression calculations. The significance of the slopes and the difference between injured and contralateral knees were evaluated using an unpaired student t-test. The effect of SP on ligamentous blood flow was tested by one-way ANOVA and the effect of dose tested using a general linear model of variance. All statistics were calculated with a significance level of $P<0.05$.

Results

Effect of injury on MCL basal perfusion

Of the 15 rabbits tested, 10 had injuries induced to their right ACL and 5 were left uninjured. Regardless of the method of blood flow determination, perfusion of the ipsilateral MCL (associated with ACL transection) was significantly higher than that of the contralateral MCL ($P<0.005$ and $P<0.02$ for LDI and CM data respectively; two-tailed paired t-test). LDI-determined blood flow to the ipsilateral MCL increased significantly compared to control ($P<0.01$; two-tailed unpaired t-test), doubling during the first 2 days of recovery (Fig. 6.1) where it remained 14 days after injury induction. Despite an increase in flux at day 1, perfusion of the contralateral MCL was unaffected by the injury and was found to be not significantly different from normal MCL blood flow ($P=0.822$). The initial rise in blood flow to the contralateral MCL is probably due to sudden overuse of the limb whilst weight distribution is concentrated away from the injured side. CM standardised flow to the MCLs associated with ACL transections also showed an increase in perfusion compared to normal MCLs (Fig. 6.2) whereas the contralateral MCL was found to be not significantly different from normal ligaments ($P=0.356$). All data are summarised in Table 6-1.

Rabbit	Knee	Injury Period	Standard Flow (ml/min/100g)	LDI Output (Volts)
1	Right (ipsi)	3 day	4.726	1.15
1	Left (contra)	no injury	-	-
2	Right (ipsi)	5 day	11.161	2.56
2	Left (contra)	no injury	9.422	1.85
3	Right (ipsi)	2 day	4.302	2.78
3	Left (contra)	no injury	2.159	1.69
4	Right (ipsi)	1 day	16.302	2.34
4	Left (contra)	no injury	0.807	2.38
5	Right (ipsi)	4 day	-	2.78
5	Left (contra)	no injury	-	0.24
6	Right (ipsi)	2 week	2.438	2.06
6	Left (contra)	no injury	1.238	0.95
7	Right (ipsi)	2 week	11.241	3.31
7	Left (contra)	no injury	8.096	1.02
8	Right (ipsi)	2 week	5.291	1.79
8	Left (contra)	no injury	1.064	0.66
9	Right (ipsi)	2 week	20.930	4.92
9	Left (contra)	no injury	10.553	1.32
10	Right (ipsi)	2 week	18.155	3.69
10	Left (contra)	no injury	2.738	1.81

Table 6-1. Summary of standard flow and laser Doppler measurements for given injury periods.

Fig. 6.1. Changes in LDI-determined blood flow to rabbit MCLs associated with ACL-deficient knees (green columns) compared to the contralateral knee (blue columns). Graph shows mean blood flow in control knees (n=10) and at day 14 (n=5); n=1 for all other values shown.

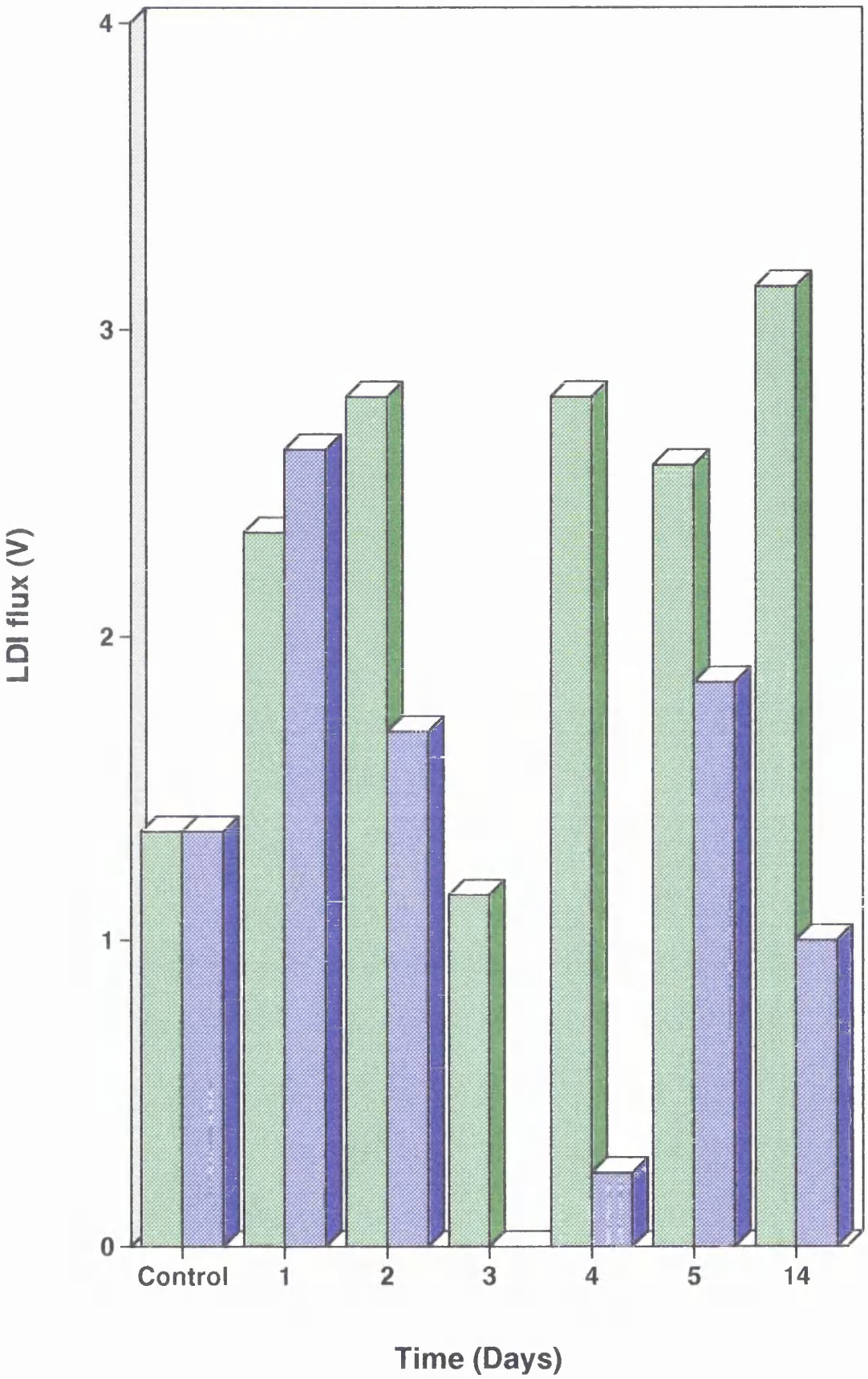

LDI flux (V)
4
3
2
1
0
Control
1
2
3
4
5
14
Time (Days)

Fig. 6.2. Changes in CM-determined standardised blood flow to rabbit MCLs associated with ACL-deficient knees (green columns) compared to the contralateral knee (blue columns). Graph shows mean blood flow in control knees (n=2) and at day 14 (n=5); n=1 for all other values shown.

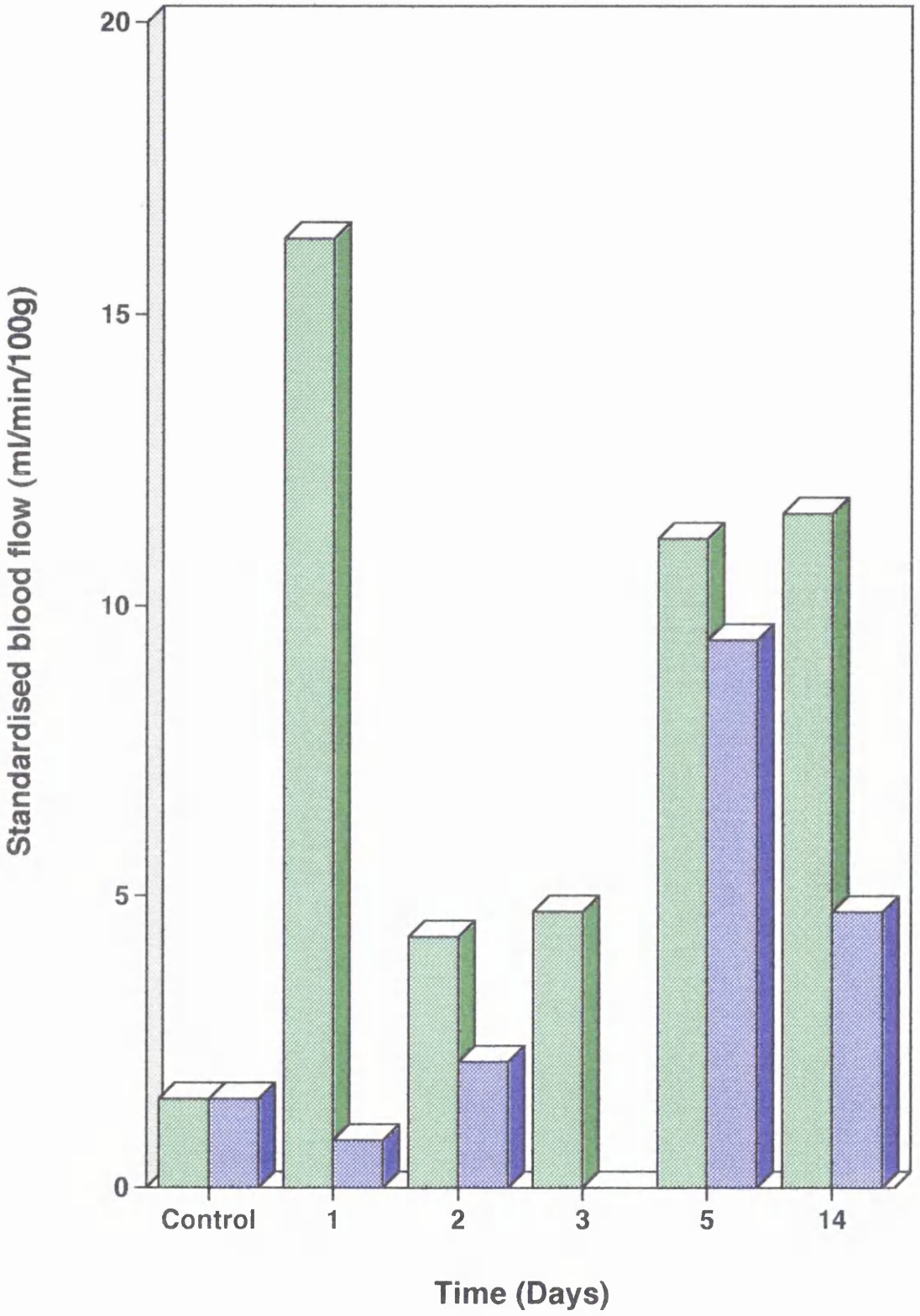
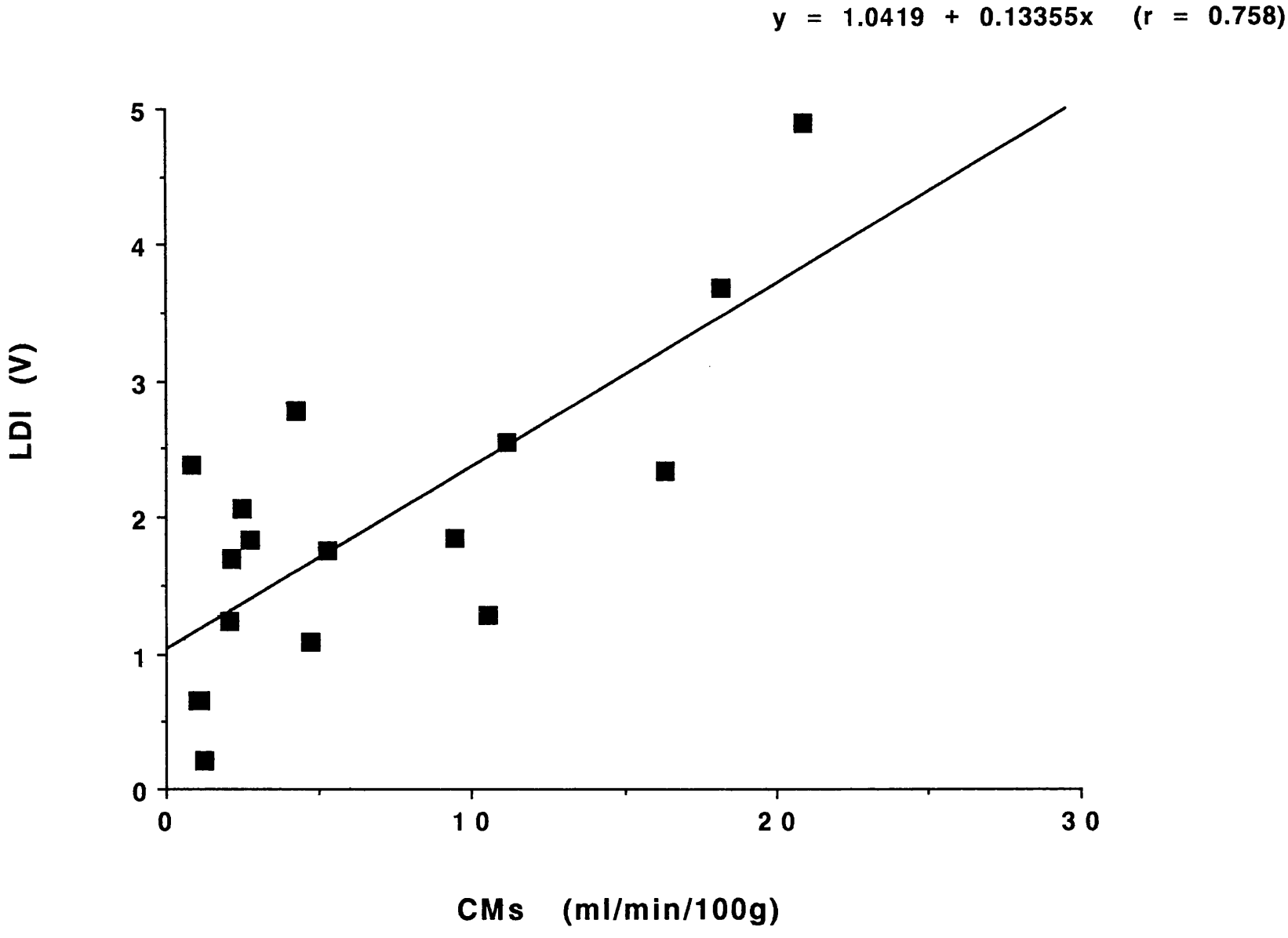

20
15
10
5
0
Standardised blood flow (ml/min/100g)
Control
1
2
3
5
14
Time (Days)

Fig. 6.3 compares the relationship between the LDI output signal with the CM determined standardised blood flow of MCL tissues. The slope of the relationship was determined to be 0.13 with a y-intercept of 1.04 and the data points were significantly correlated ($r = 0.758$; $n = 17$). A linearity test was performed on the slope and the y-intercept, where null hypotheses were proposed that the slope and the intercept could be zero. A highly significant level was obtained for the calculated t values ($P<0.001$) supporting the hypothesis that the LDI output and the standardised blood flow are linearly related with a non-zero y intercept.

Fig. 6.3. Comparison of LDI signal with CMs derived measurements of blood flow to the MCLs of normal and ACL deficient rabbit knee joints. The slope of the relationship is 0.134 with a y-intercept of 1.04. The data points are significantly correlated ($r=0.76$; $n=17$).

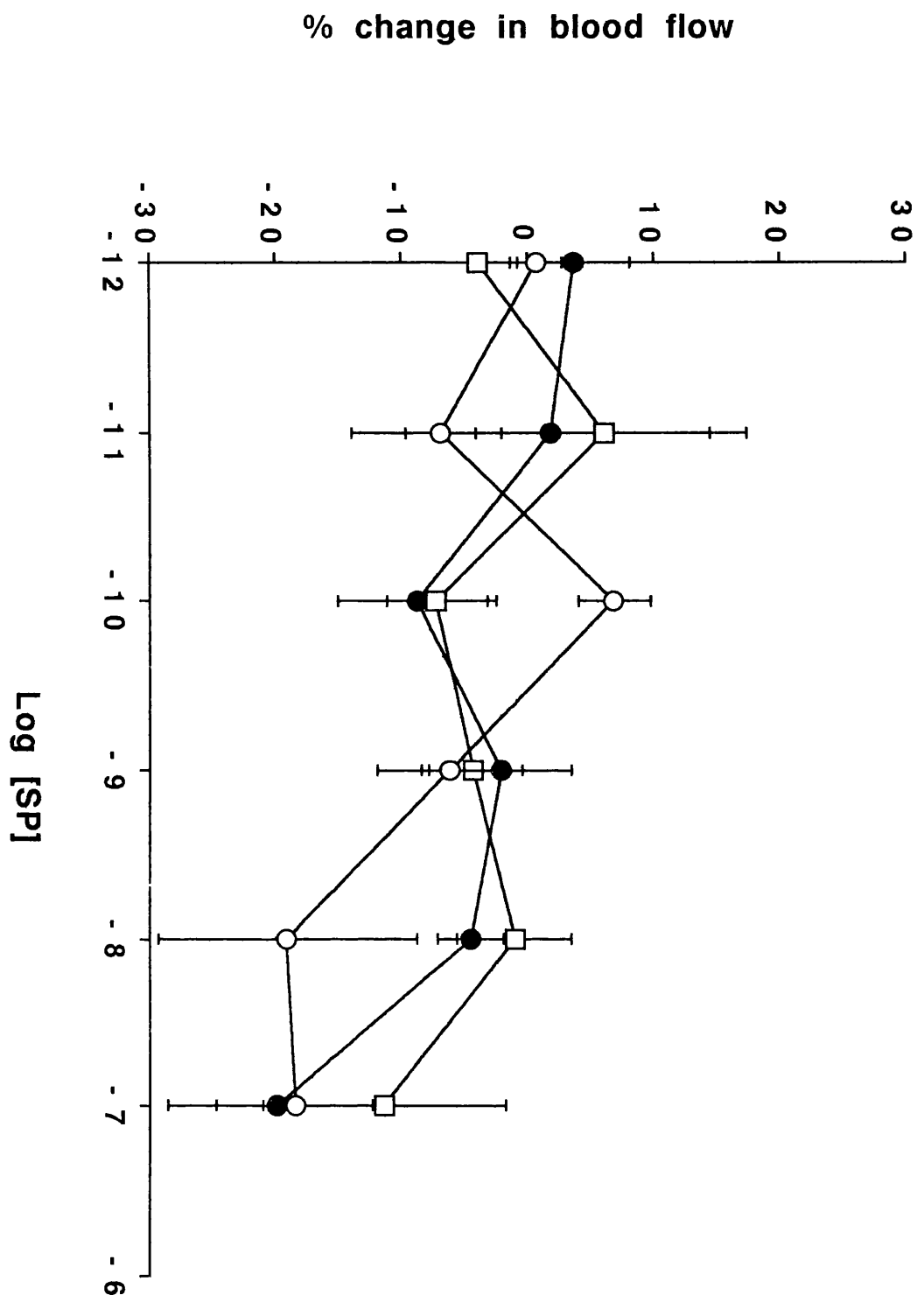

y = 1.0419 + 0.13355x (r = 0.758)
LDI (V)
0
1
2
3
4
5
0
10
20
30
CMs (ml/min/100g)

Effect of substance P on MCL perfusion

The effectiveness of SP on MCL blood flow was tested by constructing a dose-response curve to the neuropeptide. In normal animals, SP was found to have no significant effect on MCL vasculature (P=0.491; n=4 one-way ANOVA) over the range of concentrations tested *ie.* 10^{-12} - 10^{-7} M (Fig. 6.4).

Topical application of SP to the surface of the MCL in ACL deficient and their respective contralateral knees was also found to have no effect and the dose response curves were found to be not significantly different from normal knees (P=0.735 and P=0.596 for injured and contralateral knees respectively).

Fig. 6.4. Dose-response curve (mean values ± S.E.M.) for the topical application of SP to rabbit MCL in normal (●), ACL deficient (❑) and the corresponding contralateral (○) knees. Each knee was tested at all doses the sequence of which was randomised (n=4 for normal animals; n=5 for injured and contralateral knees).


% change in blood flow
-30
-20
-10
0
10
20
30
-12
-11
-10
-9
-8
-7
-6
Log [SP]

Discussion

LDF has been shown to provide reliable assessment of perfusion in low blood flow tissues such as the human Achilles tendon (Åström and Westlin, 1994). The present study extends this finding by showing that LDI is able to measure the spatial variation in perfusion in a low flow tissue, in this case the rabbit MCL.

A strong correlation was found between the LDI flux values and the standard blood flow values produced by CM measurements. It may be possible, therefore, to calibrate the relative perfusion readings generated by LDI with CM determined perfusion to give absolute measures of blood flow. However, since different tissues have unique optical properties, the calibration factor produced in this study is specific to rabbit MCLs. This difference in calibration factor may be illustrated by comparing the slopes of linear correlations derived from various tissues in other studies using LDF (Lindsberg, O' Neill, Paakkari, Hallenbeck and Feuerstein, 1989; Smits, Roman and Lombard, 1986). Such observations indicate that no single calibration factor can be derived to convert LDI units to blood flow values for all tissues. The two dimensional image generated by LDI in reality represents an image of a surface 'layer', since laser Doppler techniques have an associated depth of penetration into the tissue. This depth is determined by a combination of the laser wavelength/power and the optical properties of the tissue bed, which for a rabbit MCL remains unknown. Since it has previously been shown that 75% of the blood flow in the MCL tissue is confined to the surface or epiligamentous layer (Butterwick, Paul,

Bray Tyberg, 1992), it is fair to assume that the red laser penetrates deep enough to provide an accurate estimate of ligamentous blood flow. This premise may account for why LDI output is so closely correlated to CM data in this tissue, however, further study into the depth of penetration of laser light into rabbit MCL would be important to corroborate these findings.

Induction of joint instability in the rabbit by transecting the ACL resulted in an increase in blood flow to the ipsilateral MCL. This model of joint instability would disrupt hindlimb load distribution putting additional stresses on the uninjured ligaments. Therefore, as the work of the MCL increases then its nutritional requirements also rises which is manifest in the hyperaemic response. If the load redistribution in the injured limb is severe enough, then the MCL could itself be injured. Other investigations have shown that MCL vascularity becomes more abundant and chaotic in response to injury (Eng, Rangayyan, Bray, Frank, Anscomb and Veale, 1992) which suggests that the increased MCL blood flow found in the present study could in part be due to healing of the ligament following mild trauma. The contralateral MCL showed no adverse response to the injury except at day one where blood flow showed an increase. This transient effect was probably due to an abrupt enhanced activity of the contralateral ligaments as weight was shifted away from the injured side during saltatorial locomotion. In the early stages of healing, the animals become more inactive and whilst at rest are known to show no alteration in hindlimb loading compared to normal rabbits (Bray, Frank, Shrive, Chimich and Hennenfent, 1990). Hence, MCL activity normalises and blood flow returns back towards control levels.

The present set of experiments also revealed an inability of SP to exert any significant effect on the vasculature of normal, injured and contralateral rabbit MCLs (Fig. 6.4). This finding is in contrast to studies in rat knee joints which show a potent vasodilatation when the neuropeptide is applied to the capsule (Lam and Ferrell, 1993a; 1993b). The ineffectiveness of SP in the rabbit suggests an absence of neurokinin receptors in this tissue and raises major questions as to the mediators of pain transmission and vascular control in the rabbit knee joint.

Chapter 7

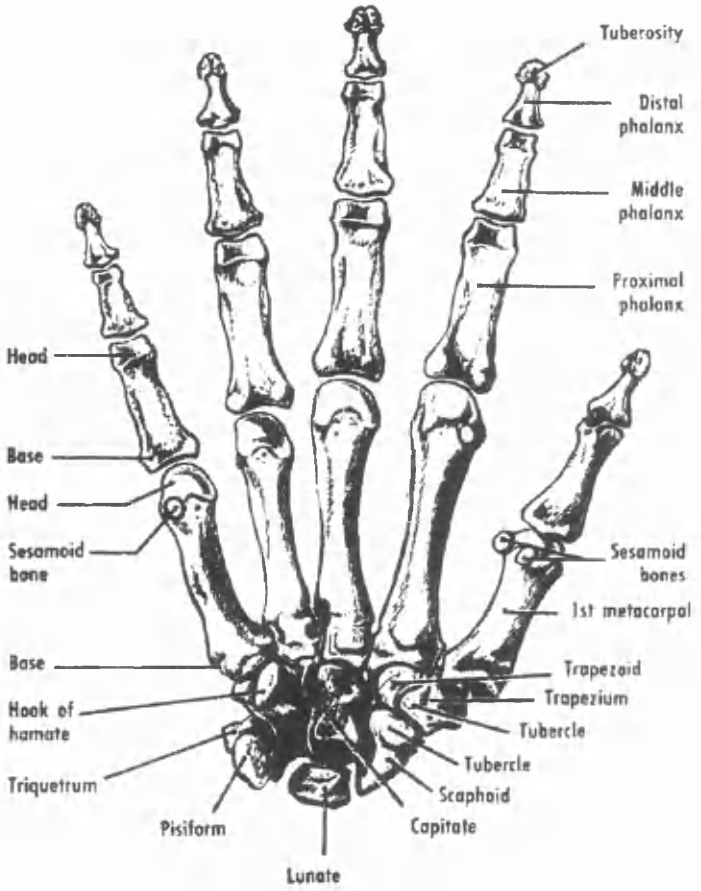
The Effect Of Deep Friction Massage On Blood Flow To Injured Proximal Interphalangeal Joints

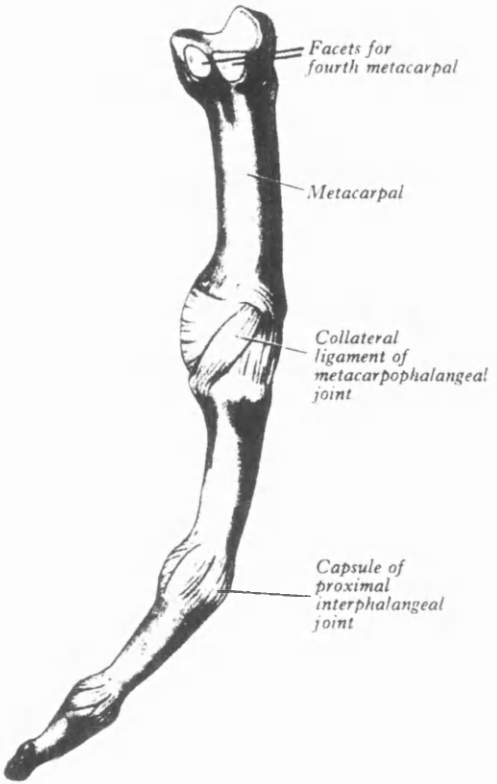
Introduction

Osteoarthritis (OA) is a non-inflammatory disorder of moveable joints characterised by deterioration of articular cartilage and the formation of new bone at the joint surfaces (Gardner, 1965; Sokoloff, 1969; Lee, Rooney, Sturrock, Kennedy and Dick, 1974). Severe joint trauma *eg.* as a result of sport related injuries has been implicated in the development of OA (Burr and Radin, 1990). Various types of joint injury produce altered contours in the joint which cause loads to be focused on these remodelled areas. The end result is a concentration of force transmission with consequential cartilage damage. In hands, OA mainly affects the distal interphalangeal (DIP) and the proximal interphalangeal (PIP) joints (Fig. 7.1) and is clinically manifest by the development of cartilaginous/bony enlargement in the dorsolateral and dorsomedial aspects of the joints; these are called Heberden's nodes (Heberden, 1803) and Bouchard's nodes for the DIP and PIP joints respectively. OA of the finger joints is usually associated with soft tissue damage and often follows trauma such as fracture, dislocation and "jamming" injuries where the condyles are squeezed together as a result of a large, extraneous force being applied to the tips of the fingers.

Fig. 7.1. *(Left)* : Bones of the right human hand, anterior aspect (from Gardner, Gray and O'Rahilly, 1986).

(Right) : Lateral view of the phalanges of right middle finger showing the position of the distal interphalangeal and the proximal interphalangeal joints (from Gray, 1973).


Tuberosity
Distal phalanx
Middle phalanx
Proximal phalanx
Head
Base
Head
Sesamoid bone
Sesamoid bones
1st metacarpal
Base
Trapezoid
Trapezium
Hook of hamate
Tubercle
Tubercle
Triquetrum
Scaphoid
Pisiform
Capitate
Lunate


Facets for fourth metacarpal
Metacarpal
Collateral ligament of metacarpophalangeal joint
Capsule of proximal interphalangeal joint

The earliest treatment of OA was immobilisation of the affected joints but modern therapy includes mild exercise of the joints to prevent them from ankylosing (seizing up) (Bonner, 1969). Heat and cold therapy have also been used to treat joint pain ever since it was found that intra-articular temperature is increased in patients with OA (Hollander and Hovarth, 1949; Hovarth and Hollander, 1949). Application of ultrasound (diathermy) produces a deep heating of tissues which may have an analgesic effect (Feibel and Fast, 1976). Other methods of generating deep heat to alleviate pain include administration of deep friction massage to the joint. Although these physiotherapies have been carried out for a long time with varying degrees of success, their mode of action is unknown.

The anatomical evaluation and subsequent monitoring of arthritides involving the hand has conventionally used radiography. The emergence of nuclear magnetic resonance imaging with its superior soft tissue discrimination, has recently been used to examine changes in articular cartilage, ligaments tendons and synovium as arthritis progresses (Moon, 1983). Monitoring of physiological changes such as synovial blood flow is more difficult due to the invasive nature of the techniques involved. LDF, for example, has only been used to assess human synovial perfusion during arthroscopic investigations where the Doppler probe is inserted intra-articularly (Geborek, Forslind and Woolheim, 1989). LDI on the other hand is able to measure blood flow non-invasively (Wårdell, 1992). The use of the LDI instrument to measure interphalangeal blood flow is problematic since the median sampling depth of the red He-Ne laser (633nm) through skin is about 250μm (Jakobsson and Nilsson, 1991) whilst the skin thickness over the joint is about 1.5mm (Cruikshank, Dodds and Gardner, 1968) (see Fig. 7.2). Transmittance studies have shown that

incident red 633nm laser light is attenuated by 1/e (37%) at a depth of about 550µm in Caucasian skin (Anderson and Parrish, 1981; Kolari, 1985). However, these same studies found that light at 800nm wavelength is attenuated to 1/e at the greater depth of 1200µm. The introduction of near infrared laser sources into LDFs has proved successful in sampling larger volumes of tissue with greater depth penetration compared to red He-Ne lasers (Obeid, Dougherty and Pettinger, 1990).

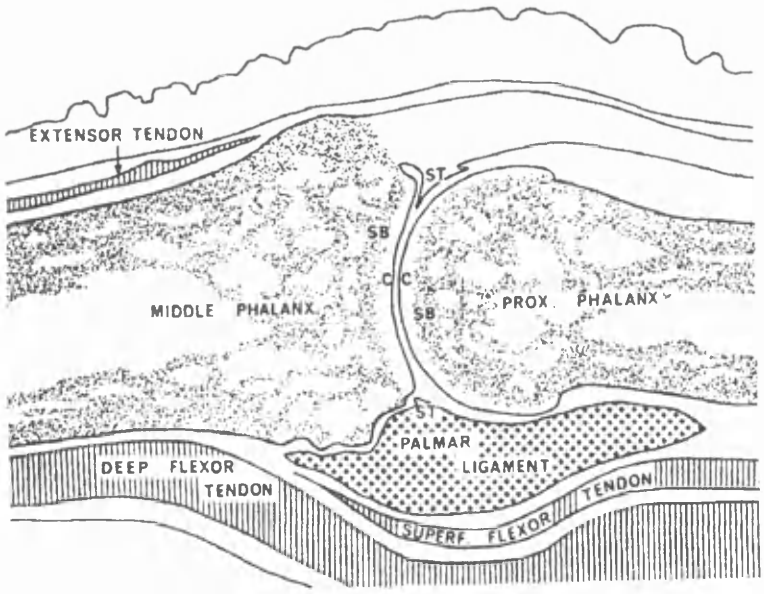


Fig. 7.2. Transverse view of a human finger showing the depth laser light must penetrate to reach the proximal interphalangeal joint.

Aims of the study

The first aim of this investigation is to assess the effectiveness of a dual wavelength LDI to transcutaneously measure blood flow to the PIP joint of the human hand. The study will also compare the basal blood flow to normal and injured PIP joints. Each subject will then have deep friction massage applied to their joint to see if the therapeutic effect of this technique is via an alteration in blood flow to the area.

Methods

Assessment of blood flow was performed in 22 male subjects. Twelve of the subjects had never experienced hand joint trauma and as such comprised the normal control group. The remaining ten subjects were former members of the Canadian Olympic volleyball team who had at some time in their career suffered from severe PIP injury. A brief case history was completed by each of the volleyball players covering such aspects as type of injury, number of times the joint was injured and subsequent physiotherapy received (See Table 7-1).

Blood flow measurements were carried out on normal, injured and non-injured contralateral PIP joints. The major veins supplying PIPs is shown in Fig. 7.3. Scans were taken before (control) and immediately after application of deep friction massage to the joint. This technique was performed by a trained physiotherapist and involved firmly squeezing the lateral aspects of the joint in a pincer-like grip and massaging the area with minute movements for 1 min.

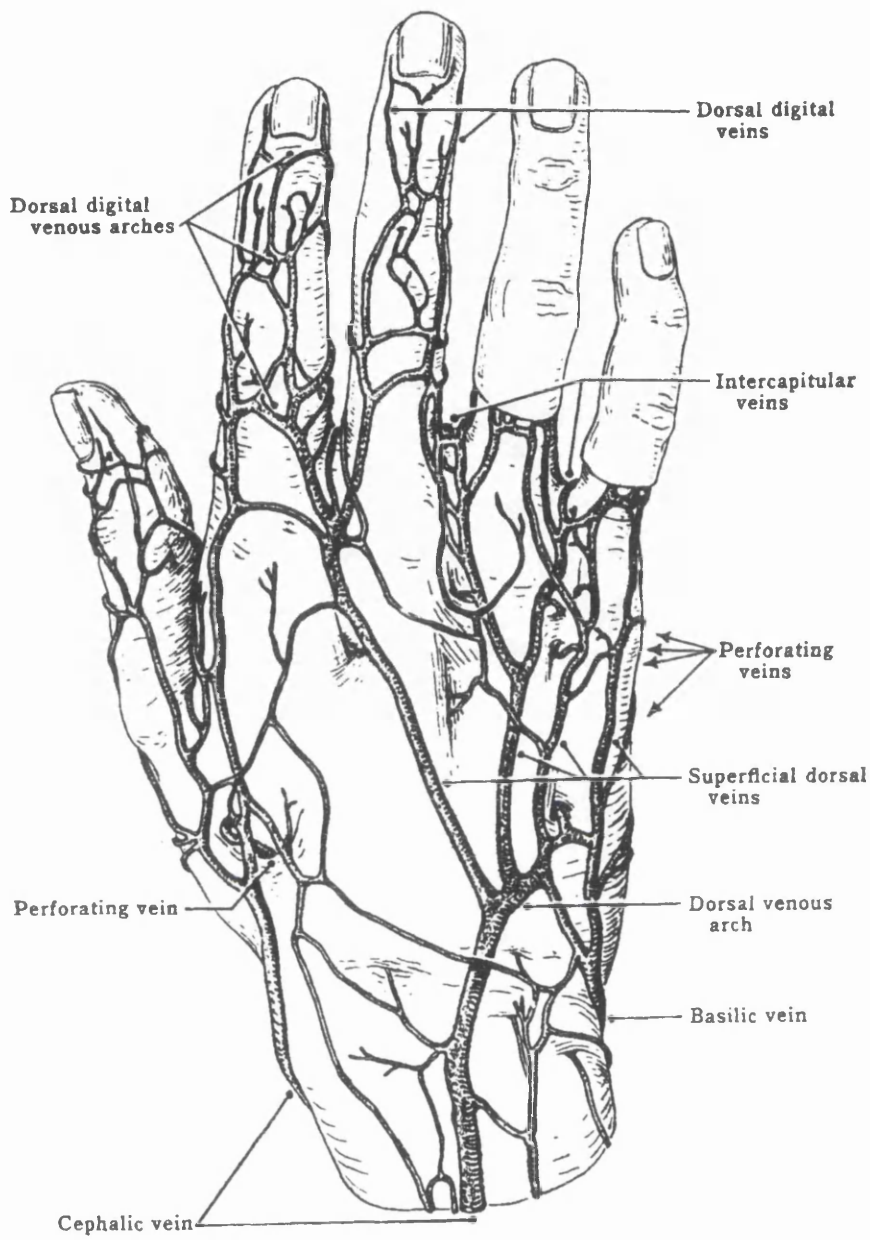


Fig. 7.3. The major veins supplying the posterior aspect of the right human hand.

The instrument used to monitor blood flow was a modified LDI which incorporated a near-infrared laser diode (wavelength =850nm) in addition to the standard red He-Ne laser (wavelength =633nm). By means of a shuttering mechanism this dual wavelength LDI system rapidly alternates between the two laser sources generating two readings at each measurement site thereby constructing two perfusion images, one for each wavelength. The scanner head of the LDI was placed 15cm above the subject's hand which was oriented with the palm face down. The laser beams scanned sequentially over the surface of the hand to give an image covering approximately 9 x 9cm which was sufficient to allow the inclusion of about three digits. Scans typically took about 2-3 minutes to complete.

After scanning, images were analysed using VIEW software. The placement of the analysis rectangles was fairly subjective, the area chosen being the best approximation to the PIP region. The size of the rectangle was kept constant with each individual hand so as to minimise error in rectangle position. Statistical analysis was by Student t-tests and one-way ANOVA performed using InStat software. Groups of data were considered significantly different from each other when $P<0.05$. Data are presented as mean LDI flux values (Volts) ± S.E.M.

Results

The results of the patient case histories are shown in Table 7-1.

Patient	Type of injury	Number of times joint was injured	Previous physiotherapy
ds	Dislocation of little finger	2-3	Ice, splinting, deep friction.
te	Dislocation of little finger	3	Ice, ultrasound, acupuncture, deep friction, exercise.
rb	"Jammed" middle finger	6	Ice, deep friction.
ac	"Jammed" ring finger	4	Ice, splinting, ultrasound.
dh	"Jammed" index finger	3-4	Ice, splinting.
gp	"Jammed" middle finger	4	Ice, ultrasound.
ma	Dislocation of ring finger	10	Ice, ultrasound, deep friction.
dj	"Jammed" little finger	1	Ice, ultrasound
bk	"Jammed" middle finger	3	Ice, ultrasound
rw	"Jammed" index finger	2	Ice, deep friction.

Table 7-1. Brief case history of Canadian Olympic volleyball team subjects who had suffered severe PIP joint injuries.

Comparison of red and near infrared images shows that the 850nm laser generates significantly higher flux values than the 633nm He-Ne laser ($P<0.0001$; paired two-tailed t-test). This is due to the greater penetrative power of the 850nm laser sampling a larger volume of tissue (Figs. 7.4 and 7.5).

Mean basal flux values of normal PIP joints was 0.92 ±0.14V with the red laser and 2.89 ±0.22V in the near infrared images (n=12 for both groups). Irrespective of wavelength of laser employed, the basal perfusion of injured and non-injured contralateral PIP joints was found to be not significantly different from normal (P=0.348 and P=0.812 for red and near infrared images respectively; one-way ANOVA, n=10 for injured and non-injured groups - Fig. 7.5).

Deep friction massage applied to the PIP joints of normal hands caused blood flow to rise by 20.48 ±3.85% (red images) and 12.65 ±4.21% (near infrared images) which was found to be significantly different from control (P=0.0036 and P=0.005 for red and near infrared respectively). As shown in Figs. 7.6 and 7.7, the physiotherapeutic technique was found to have no effect on blood flow to injured (P=0.0949 and P=0.4902 for red and near infrared scans respectively) or contralateral non-injured (P=0.5682 for red and P=0.8390 for near infrared) PIP joints.

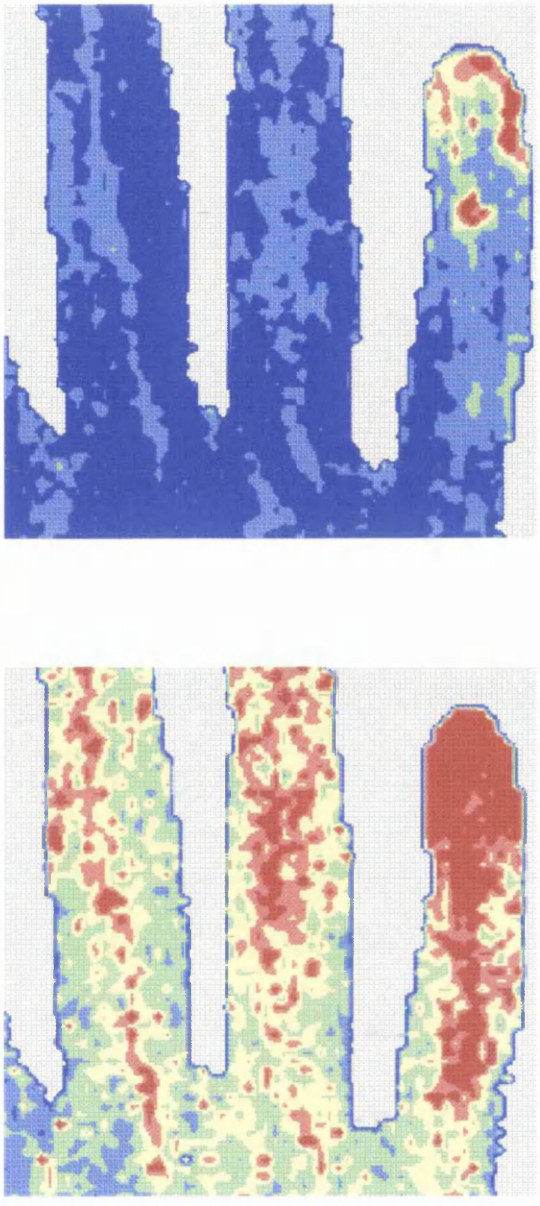


Fig. 7.4. LDI images of a normal right hand performed using a red 633nm (top) and an infrared 850nm (bottom) laser. Images have undergone interpolation and are plotted on a 0-5V scale.

Fig. 7.5. Changes in basal blood flow to injured and non-injured contralateral PIP joints compared to normal controls (n =12 for normal joints and n=10 for injured and non-injured groups). The figure shows scans performed with a red 633nm laser (turquoise columns) and with a near infrared 850nm laser diode (red columns).

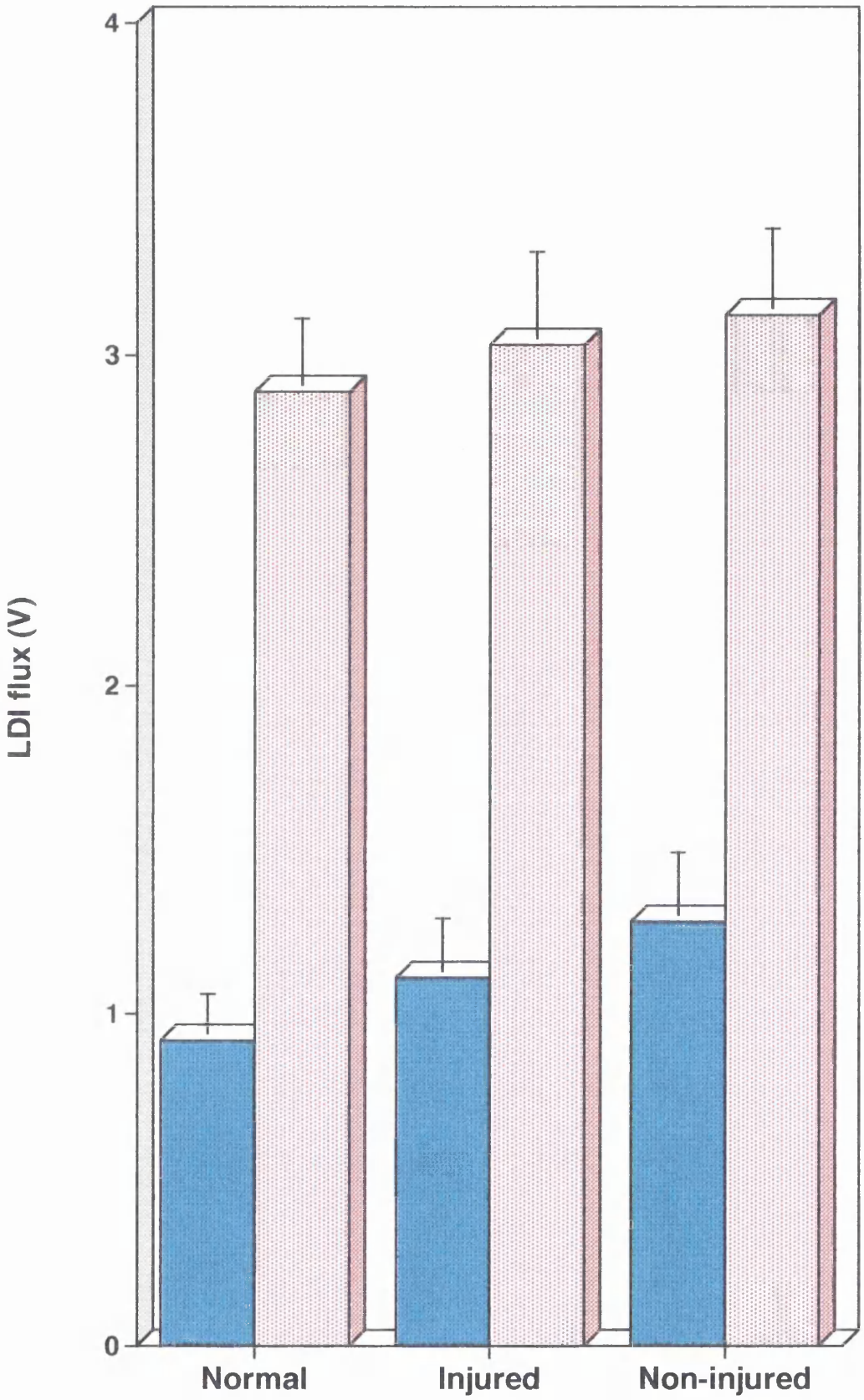

LDI flux (V)
4
3
2
1
0
Normal
Injured
Non-injured

Fig. 7.6. The effect of deep friction massage on blood flow to normal (n=12), injured (n=10) and non-injured contralateral (n=10) PIP joints. Graph shows mean flux values ± S.E.M. for control scans (green columns) and scans taken post-friction (blue columns) with a red 633nm laser.

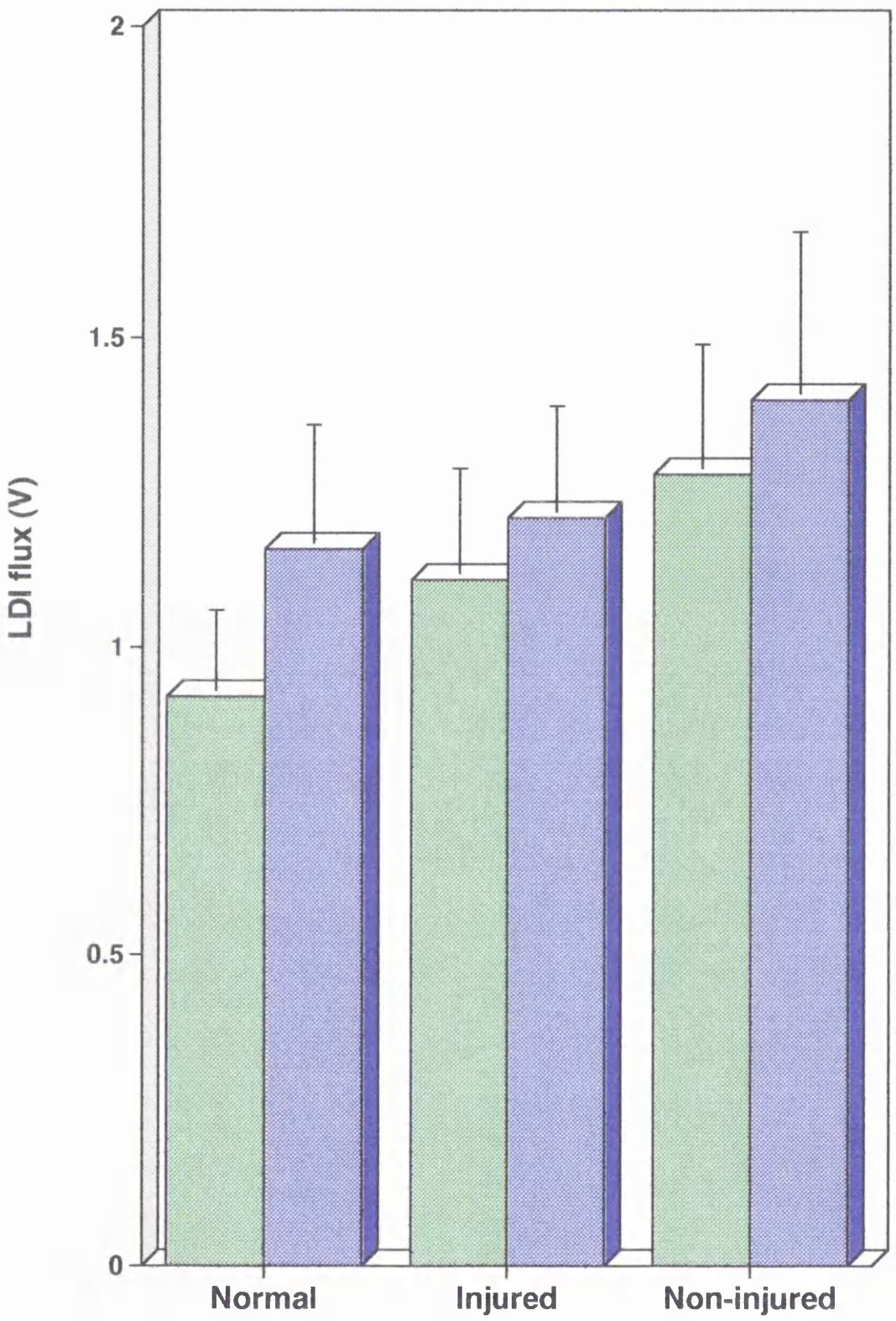
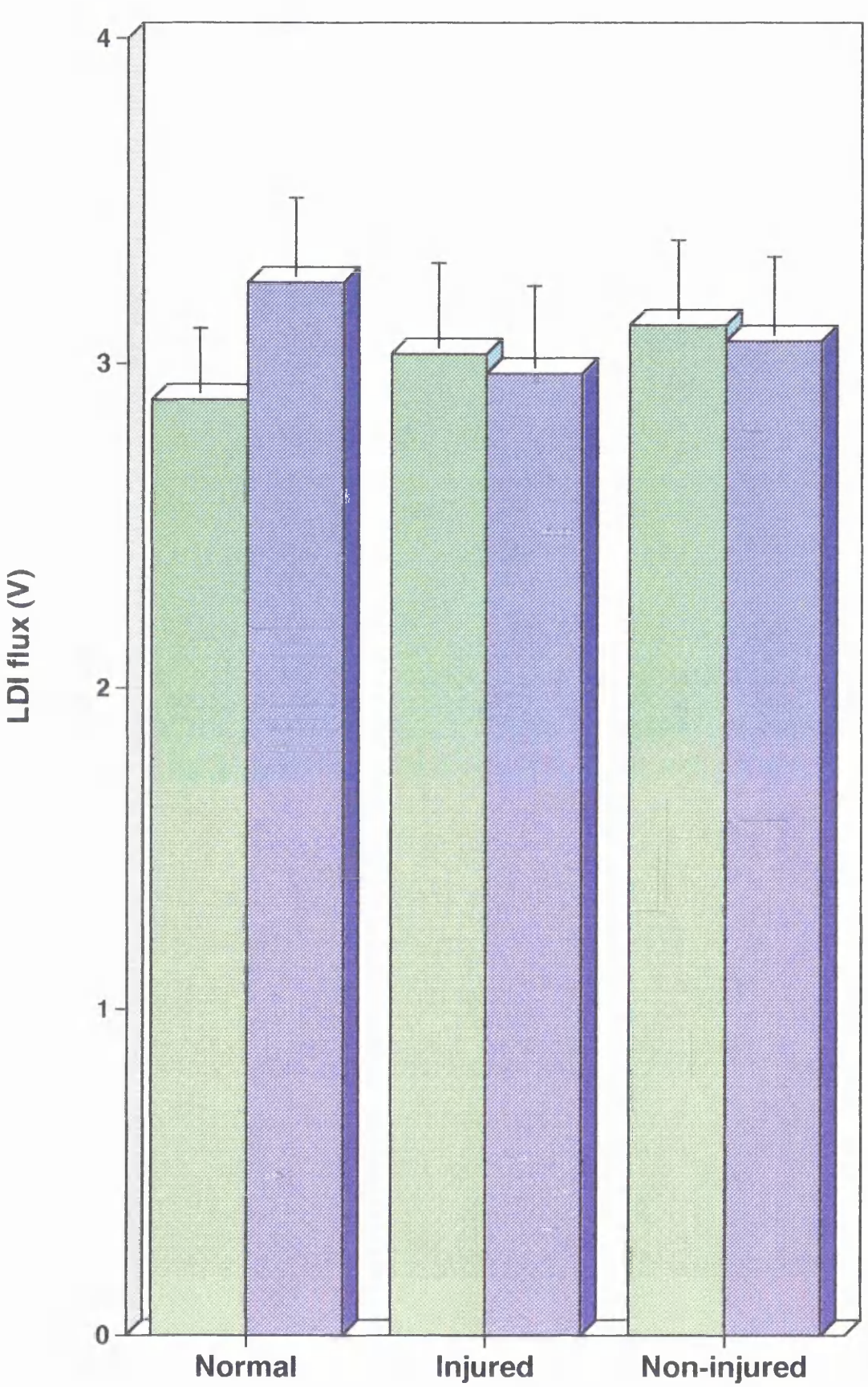

LDI flux (V)
2
1.5
1
0.5
0
Normal
Injured
Non-injured

Fig. 7.7. The effect of deep friction massage on blood flow to normal (n=12), injured (n=10) and non-injured contralateral (n=10) PIP joints. Graph shows mean flux values ± S.E.M. for control scans (green columns) and scans taken post-friction (blue columns) with a near infrared 850nm laser.


LDI flux (V)
4
3
2
1
0
Normal
Injured
Non-injured

Discussion

A previous study by Obied *et al.* (1990) showed that LDF flow signals obtained from a near infrared laser were consistently higher than those recorded from a red He-Ne laser. The present study confirms this finding using a dual wavelength LDI incorporating 633nm and 850nm lasers. The reason for the difference between the two readings is that the near infrared laser samples a larger volume of tissue than the red laser due to the greater penetrability of longer wavelength light. Red 630nm lasers are known to have a median depth penetration through skin of about 250μm (Jakobsson and Nilsson, 1991). Since melanin in skin attenuates the penetration of laser light and the skin overlying the PIP joint is about 1.5mm (Cruikshank, Dodds and Gardner, 1968), then the 850nm infrared laser is probably producing the best estimation of synovial blood flow in the PIP joint.

Development of the degenerative joint disorder OA may be brought about by single or repetitive malformation of a joint by injuries such as fracture, subluxation or "jamming". The processes involved in the progression from trauma to joint degeneration are largely unknown but may include synovial inflammation (Evans, Mazzocchi, Nelson and Rubach, 1984; Lindblad and Hedfors, 1987; Jasin, 1989; Schumacher, 1989). The present study used a group of subjects who had at some time severely injured their PIP joint and as such may be in the early stages of developing OA. Measurement of blood flow to these joints by LDI revealed that perfusion was not significantly different from normal PIPs suggesting that basal blood flow is unaltered in subjects who may have OA. This finding also suggests that the mediators involved in the development of OA are

non-inflammatory but may activate sensory nerve fibres leading to nociception.

Treatment for injured and OA joints includes a number of physiotherapeutic techniques which aim to alter intra-articular temperature. One such treatment is the application of deep friction massage to the joint which produces a deep heating of the underlying tissues. In normal hands this technique caused an increase in articular perfusion probably as a thermoregulatory response. We may be fairly confident that the LDI is not just measuring changes in skin blood flow as the massage comprised of only small movements applied to the lateral aspects of the joint. In contrast, injured and non-injured contralateral PIP joints showed no change in perfusion in response to deep friction massage. It appears, therefore, that the thermoregulatory mechanism in these joints is affected in some way and this alteration in inherent vasoregulation could be linked to the pathogenesis of OA. The fact that contralateral uninjured joints were also unable to vasoregulate implies a neurogenic component to OA giving rise to a possible symmetry to the disease. The therapeutic effects of deep friction massage appear not to be due to increased provision of nutrients brought about by changes in articular blood flow, but may be connected to the release of certain paracrines which act to desensitise afferent nerve fibres.

To summarise, blood flow to PIP joints is unaffected by previous joint trauma, at least at the time of measurement. Deep friction massage, a physiotherapeutic technique commonly applied to injured joints to help alleviate pain, does not exert its rehabilitative effect by altering joint perfusion. Single or recurrent joint injury may have a detrimental effect on PIP vasoregulation and these changes may be one of the causative factors in the early stages of OA.

Chapter 8

Conclusions

Laser Doppler perfusion imaging

The measurement of tissue perfusion is important in assessing the effect of disease on the microcirculation. In joints this would be useful to monitor changes in blood flow during the inflammatory reactions which occur during the pathogenesis of rheumatic diseases. In order to carry out these investigations, a suitable method for measuring blood flow is required which is reproducible, easy to use and preferably non-invasive. Some of the most widely used techniques in blood flow determination satisfy some but not all of these criteria. Radioactive clearancing, for example, gives meaningful measures of perfusion to a variety of different tissue beds but is highly invasive and it is difficult to get consistent values. One of the greatest problems when trying to quantify synovial blood flow is the multiplicity and chaotic nature of the blood vessels that supply the area.

The work carried out in this thesis shows that LDI is a suitable method for measuring articular blood flow in animal models and, due to its non-invasive nature, is ideal for clinical studies in humans. The addition of a near infrared laser (850nm) allows the LDI to sample a greater volume of tissue by increasing its level of depth penetration. This modification to the system is vital when undertaking transcutaneous measurement of blood flow since melanin absorbs the majority of red (633nm) light (Anderson and Parrish, 1981; Kolari, 1985) rendering photons from He-Ne lasers inadequate to penetrate and return from moving elements deep within a tissue. In the animal model experiments, however, the skin was excised and scans performed on exposed joints. Under these conditions the red laser was sufficient to map out synovial blood flow.

One set of experiments in this study has shown that it is possible to calibrate the LDI system by comparing flux readings with colour microsphere derived standard blood flow. The tissue used here was the rabbit MCL and the relationship between the two techniques was found to be:

$$\mathbf{Flux = 0.134(CM_f) + 1.042}$$

where LDI flux is measured in Volts and CM_f is coloured microsphere derived blood flow measured in ml/min/100g of tissue.

Due to the diversity in optical properties of different tissues, this relationship is specific for rabbit MCL. Therefore, in order to calibrate the LDI for an alternative tissue the comparison between the two techniques must be carried out *de novo.*

The neurogenic control of blood flow to diarthroidal joints

The human knee is one of the most complex systems in the realm of physiology. The structure is required to withstand forces that often occur near the limits of biological tissue capacity and as such is often prone to disease and injury. The salubrity of a joint relies on its anastomotic blood supply to provide nutrients and remove metabolites (McKibbin and Maroudas, 1979), hence, any alteration in normal vasoregulation may challenge the integrity of the joint rendering it susceptible to disease.

The anterior aspect of the rat knee joint is innervated by branches of the saphenous nerve which is composed of afferent and sympathetic efferent fibres. Electrical stimulation of this nerve at levels which

activate both afferent and efferent fibres caused a frequency-dependent vasoconstriction of articular blood vessels. Maximal vasoconstriction occurred when 30Hz stimulation was applied to the nerve causing knee joint blood flow to fall by about 40%. Having established this, the study then aimed to elucidate what effect treating the knee with capsaicin would have on joint vasoregulation. Capsaicin, the active ingredient in chillies, is known to deplete unmyelinated sensory nerve fibres supplying the rat knee (Ferrell, Lam and Montgomery, 1992); the question was whether this neurotoxin would affect unmyelinated sympathetic efferents as well? The answer was rather surprising in as much as nerve-mediated vasoconstriction was unaffected by capsaicin treatment suggesting that sympathetic postganglionic fibres were resistant to its neurotoxic effects. The next line of inquiry was to find out whether these doughty nerve fibres would be so intransigent when faced with disease.

Chronic inflammation may be produced in a single rat knee joint by intra-articular injection of Freund's complete adjuvant (Grubb, McQueen, Iggo, Birell and Dutia, 1988). In the present project this treatment caused a monoarthritis with a concomitant fall in basal blood flow to the joint. This hypoperfusion would make the knee hypoxic leading to degeneration of articular structures thereby exacerbating the disease process. This chronic inflammatory model also abolished nerve-mediated constrictor responses in the knee which may have arisen either by nerve degeneration or by the development of neuro-effector complications. The fact that the contralateral knee was unaffected by adjuvant treatment confirmed the model produced a true monoarthritis.

Capsaicin treatment has been shown to attenuate adjuvant arthritis in rats (Colpaert, Donnerer and Lembeck, 1983; Levine, Dardick,

Roizen, Helms and Basbaum, 1986) and topical application of the substance in cream form reduces inflammation in patients with arthritis (Deal, Schnitzer, Lipstein, Seibold, Stevens, Levy, Albert and Renold, 1991; McCarthy and McCarthy, 1992). It would therefore be interesting to investigate whether the adjuvant-induced changes in rat knee joint vasoregulation presented in this thesis would be affected by prior treatment of the animals with capsaicin. Furthermore, the joints could be surgically denervated so see if this intervention was more effective at alleviating the deleterious effects of adjuvant arthritis.

The project also aimed to elucidate some of the neurotransmitters responsible for the development of neurogenic inflammation and specifically examined the possible release of NO from articular nerve terminals. NO is a ubiquitous vasodilatatory molecule which is released from blood vessel endothelium in response to chemical and mechanical stimuli. Other sources of NO are from immunocompetent cells (*eg.* macrophages and mast cells) and from autonomic postganglionic nerve terminals. The synthesis of this vasodilator is under the influence of the enzyme NOS whose activity may be competitively inhibited by the actions of L-NMMA and L-NAME. The work outlined in this thesis showed that the basal production of NO by the rat knee joint is minimal and its main function *in vivo* is probably to prevent endothelial gap formation. These conclusions were arrived at by the observation that intravenous infusion of L-NAME in a normal animal causes only a transient rise in knee joint vascular resistance and protein extravasation. A neural rôle appears to be the more salient feature of NO-mediated vasoregulation in this tissue since a more pronounced vasoconstriction was observed when the articular nerves were electrically stimulated in the presence of L-NMMA. This finding implies that NO is either released directly from

rat knee joint nerve terminals or its synthesis is induced following nerve stimulation *ie.* it is acting as a second messenger. Interestingly this enhancement of nerve-mediated vasoconstriction was only inhibited by L-NMMA and not L-NAME suggesting an inhibitor specific isomer of NOS. This study could be extended to examine the effects of other NOS inhibitors (*eg.* L-NIO) on basal and neurogenic vasoregulation in the rat knee. Moreover, experiments could be performed to see if any of these responses are altered by different models of inflammation. For example in adjuvant arthritis NO production may be upregulated to counteract sympathetic vasoconstriction. This theory could be investigated by measuring knee joint blood flow in adjuvant arthritic rats during intravenous infusion of 10mg/kg/hr L-NMMA.

The processes involved in the development of OA following injury to the joint earlier in life are unknown and an attempt was made in this thesis to address this problem. Although articular cartilage is a hardy tissue which was designed to withstand the stresses of life, it is constantly weakened by the rigours applied to it until finally the cartilage succumbs to mechanical injury. Any individual who has had repeated minor trauma or a significant articular insult is clearly at risk of the slow process of joint degeneration, believed to lead to the widespread disease of the elderly, OA. This disease is the most prevalent of all the arthritides in the realm of rheumatology and its clinical picture is very well documented (Moskowitz, Howell, Goldberg and Mankin, 1984). In general, OA is suspected on the basis of past history, presumptively diagnosed by physical examination, positively identified by radiography, and "confirmed" by arthroscopy (Linblad and Hedfors, 1987). Many definitions for OA have been suggested but a more universal one describe it as a slowly progressive

monoarticular (or less commonly polyarticular) disease of unknown cause (Mankin, Brandt and Shulman, 1986). The condition occurs late in life and mainly affects the hands and large weight bearing joints such as the knees. Pathologically the disease is characterised by focal erosive lesions, cartilage destruction, and cyst and osteophyte formation at the margins of the joint. As the disease becomes more advanced, cartilaginous deterioration is progressively more severe; while structural aberrations in the underlying bone and less commonly synovitis are usually milder and thought to be secondary.

Experiments presented here showed that in an animal model of knee joint instability blood flow to the injured joint increased during the first 14 days post-trauma. This hyperaemia was probably a physiological response to aid healing in the damaged knee. Other experiments were performed on human PIP joints which had over a number of years encountered multiple traumata such as fractures and misalignments. In this study these subjects were *suspected* of suffering from the early stages of OA and it was found that blood flow to these joints was normal. In addition, these joints were unaffected by deep tissue heating generated by deep friction massage. The fact that normal PIP joints showed an increase in blood flow in response to the treatment (probably via thermoregulatory mechanisms), implies that normal vasoregulation is impaired in injured joints. The conclusion from these experiments is that immediately after injury joint blood flow increases to supply the mediators necessary to assist repair. During the subsequent years following trauma, articular blood flow returns back to normal despite degeneration of hyaline cartilage in the joint and consequently the development of OA lesions. It appears, therefore, that the body is unable to sustain joint hyperaemia at the very time when articular structures need it to support their slow

healing process. Inflammation only occurs after fully blown OA has developed possibly as a secondary response to joint pain, but by then it is too late.

In conclusion, this thesis has attempted to unravel some of the mysteries which surround the arthritic diseases. The pathology of these disorders is now well understood, however, the underlying mechanisms which are responsible for their development are less so. While people suffer the pain and inconvenience of arthritis and pray for a miracle cure, the search into a better understanding of the pathogenesis of arthritis must go on...

Appendix A: Statistics

Measures of central tendency

When a collection of data are collected from a common source, it is impractical to memorise all the values within that data set. What is required is a single value which is representative of the set of data as a whole; this value is the measure of central tendency. The most common measure of central tendency is the **arithmetic mean.** It is calculated by adding all the values in a data set and dividing the total by the number of values that were summed *ie.* :

$$\bar{x} = \frac{\sum \bar{x}}{n}$$

where Σx is the sum of all the data values and n is the number of data values.

Another oft used measure of central tendency is the **median** which is the value above which half the data values lie and below which the other half lie. If the number of values is even, then the median is equal to the mean of the two middle values.

Measures of dispersion

Once the mean of a data set has been calculated, we then need to know the extent to which the values differ from this mean. One such measure of dispersion is the **standard deviation** which is defined as:

$$SD = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}}$$

The **standard error of the mean (S.E.M.)** measures the variability among sample means and is equal to $\frac{SD}{\sqrt{n}}$

Hypothesis testing

Throughout this thesis statistics have been calculated to give a "P" or probability value. The P value is a number between 0 and 1 which corresponds to the likelihood of a particular event occurring. The closer the number is to 1 the more likely the event and conversely the closer the number to 0 the less likely the event.

If we observed a difference between a number of data sets we have to have a way of testing whether the difference was real or just a coincidence of random sampling *ie.* whether the difference is **significant** or **not significant**. Before we can decide if the difference is significant we need to discuss hypothesis testing. All statistical tests have a **null hypothesis** which is a statement that a difference does not exist and is usually the opposite of the hypothesis you are trying to test. We then use the P value to test the null hypothesis where the lower the P value the less likely it is that chance alone caused the difference you observed. P values are often compared with an

arbitrary threshold value, usually set to 0.05. If the P value is less than 0.05 then the result is statistically significant and we can reject the null hypothesis. If, however, the P value is greater than 0.05 then the results are said to be not statistically significant and the null hypothesis is not rejected. In other words the P value tests whether groups of data come from the same overall population such that if $P>0.05$ then the chances are that the data do come from the same population; if $P<0.05$ then the data sets are significantly different from each other and the chances are that they came from different populations.

The normal distribution

When we plot the frequency a value occurs in a data set we generate a frequency distribution. The curve associated with the **normal or Gaussian distribution** is a symmetrical bell shape with the mean and median coinciding at the most frequent value. No set of measurement conforms exactly to the specifications of the normal distribution, however, many are approximately normally distributed. In such cases the normal distribution is very useful when we try to answer practical questions regarding these data.

Parametric *v* non-parametric tests

Parametric tests are based on the assumption that the data set is sampled from a population that follows a normal bell-shaped distribution. If you know that your data come from a normal distribution then you should definitely choose a parametric test as these are somewhat more powerful. If, however, you know that the

data do not come from a normal distribution then you should choose a non-parametric test as a parametric test would yield misleading results.

Analysing two columns of data

The student t-test

The t-test compares the means of two columns of data. The null hypothesis is that the two population means are equal. If the P value is low, we may conclude that the data are unlikely to be sampled from populations with equal means. The t-test assumes that the data are randomly sampled from larger populations and that they are normally distributed. The t-test may be either paired or unpaired, and one or two tailed. The paired t-test assumes that the data on each row of two columns of data are repeated measures on the same subject or on subjects matched *eg.* for age and gender. The unpaired t-test assumes that the two columns of data were sampled from independent populations which are totally unrelated. One-tailed t-tests are only appropriate when you can state with certainty (and before you collect any data) that the association between variables will go in one specified direction. If you cannot specify the direction of any association with certainty then a two-tailed P value is more appropriate. If in doubt, always select a two-tailed t-test. the t-test statistic is defined as:

$$t = \frac{\bar{x} - \mu}{\text{S.E.M.}}$$

where μ is the mean of the whole population.

The Mann-Whitney test

The Mann-Whitney is a non-parametric test to compare the median of two unpaired columns. The null hypothesis is that the two population medians are equal. The test statistic is defined as either:

$$U = nm + \frac{n(n+1)}{2} - Rn$$

or

$$U = mn + \frac{m(m+1)}{2} - R_m$$

where R_n and R_m are the rank sums of the respective samples of size n and m.

Analysing three or more columns of data

One-way ANOVA

One-way analysis of variance (ANOVA) compares the means of three or more groups. The null hypothesis is that all columns means are equal. This test assumes that the data are randomly sampled from a larger population that follows a normal distribution. The data are classified into groups of a single property and one-way ANOVA tests whether the groups are significantly different from each other based on this property.

Two-way ANOVA and the general linear model of variance

Two factor ANOVA is an extension of one-way ANOVA but here we can consider two variables of classification or groupings. Therefore, as well as being able to test any difference between column means, we can also test the row means for a second classification. The general linear model of variance is the same as two-way ANOVA but is used when there are different n values within each group or classification.

The Kruskal-Wallis test

The Kruskal-Wallis test is a non-parametric method of comparing the medians of three or more unpaired groups and as such is the non-parametric version of one-way ANOVA. The null hypothesis is that the column medians are equal. The test statistic is defined as:

$$H = \frac{12}{n(n+1)}\left(\sum \frac{\sum R_j^2}{n_j}\right) - 3(n+1)$$

where n_j = the number of observations in group j

n = the total number of observations

R_j = the sum of the ranks of group j

Linear correlation and regression

If a change in one variable is matched by a similar proportional change, then the technique used to measure the degree of association is called **linear correlation**. The strength of the linear relationship between two variables is measured by the population correlation coefficient (r). r can assume values between -1 and +1 inclusive. When r = +1, there is a perfect direct linear correlation between the two variables. When r = -1, there is a perfect inverse linear correlation. An r value of 0 indicates that the two variables are not linearly related.

Linear regression calculates a best fit line through all of the data points, describing the slope and intercepts of the line with the axes. A post-test is often conducted which examines whether the slope and the y-intercept are equal to 0. This usually takes the form of a one sample t-test where the slope and intercept are compared to 0. If the P value is low, then we may conclude that the data are unlikely to be sampled from a population in which the slope and intercept is 0.

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The information for this section was taken from Phillips, 1978; Ball and Buckwell, 1986; Daniel and Terrell,1989; Motulsky, 1993.

Appendix B: Drugs and reagents

Acepromazine maleate (Ayerst Laboratories, Montreal)

Benzethonium chloride (benzyldimethyl{2-[2-(p-1,1,3,3-tetramethyl-butylphenoxy) ethoxy] ethyl} ammonium chloride; Sigma); 0.2% in saline

Capsaicin (8-methyl-N-vanillyl-6-nonamide; Fluka, Switzerland)

Cremaphore (Sigma)

Diazepam (vallium; Roche)

Ethylenedinitrilotetraacetic acid (EDTA; Sigma); 1.23%.

Euthatal (sodium pentobarbitone; Rhône, Mérieux)

Fluothane (halothane; ICI)

Freund's complete adjuvant (*Mycobacterium tuberculosum* in paraffin oil and mannide monooleate; Sigma)

Heparin (sodium heparin in mucous, Leo Laboratories)

Hypnorm (fentanyl citrate/fluanisone; Janssen)

N-N-Dimethylformamide (Bayer AG, Germany)

N^G-monomethyl-L-arginine (L-NMMA; Calbiochem)

Nω-nitro-L-arginine methyl ester (L-NAME; Sigma)

Substance P (ICI)

Urethane (ethyl carbamate; Sigma)

Appendix C: Equipment

Coloured microspheres (Triton Technology Inc., USA)

Cordless cautery (Warecrest, England)

Digital balance (AE50, Europe Instruments, England)

Digital micrometer (Mtotoyo Instruments, Japan)

Dissecting microscope (Wild Heerbrugg M650, Switzerland)

Electric stimulator (Harvard Advance Stimulator, USA)

Epi-fluorescent microscope (Nikon Diaphot-TMD, Japan)

Infusion pump (Gilson Minipuls, France)

Laser Doppler perfusion imager (Lisca Developments AB, Sweden)

Micropipette (Labsystems, Finland)

Multitrace 4 chart recorder (Lectromed Ltd., England)

Pressure transducer (Elcomatic EM751, Scotland)

Spectrophotometer (Ultraspec 4050, LKB Biochrom Ltd, England)

Water bath (Grant Instruments Ltd., England)

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