Peripheral vascular studies in sheep with reference to the pathophysiology of laminitis

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Abstract

Altered blood flow to the foot contributes to the syndrome of laminitis, an important cause of lameness in domestic ungulates. Factors such as vasoconstriction, arterio-venous shunting and microthrombosis are believed to reduce perfusion within the hoof leading to ischaemic tissue damage. Endothelium-derived mediators, such as nitric oxide and prostacyclin, have a key role in the modulation of local vascular tone and alterations in the production of these agents could contribute to the vascular derangements observed in laminitis.

In this study, the sheep was used as a model for both equine and bovine laminitis. The responses of isolated ovine digital arteries to vasoconstrictor and vasodilator agents were studied *in vitro* and the effects of endothelium-removal, nitric oxide synthase inhibition and/or cyclooxygenase inhibition were studied. The response to phenylephrine, an α_1-adrenergic agonist, was found to be modulated by vasodilatory prostanoids, whilst relaxation to bradykinin was mediated by a combination of endothelium-derived nitric oxide and prostanoids. Both endotoxin and cytokines have been implicated in the pathogenesis of laminitis, therefore digital artery vascular responses were also examined following a 6 or 16 hour incubation with these agents. Incubation with endotoxin did not significantly alter the dose-response curves to phenylephrine or bradykinin. However, incubation for 6 hours with interferon-γ and tumour necrosis factor-α did significantly increase the maximum relaxation to bradykinin. Furthermore, a 16 hour incubation with interferon-γ, interleukin 1-β and tumour necrosis factor-α significantly increased the EC_{50} of the response to phenylephrine, i.e. decreased the sensitivity to this vasoconstrictor. Thus, the vascular responsiveness of ovine digital arteries may be altered by cytokines *in vitro* and further work is needed to determine the mechanism of these alterations.

The second part of this project sought to evaluate limb blood flow in the live animal, thereby allowing for the influence of the hoof. The shape of the velocity-time waveform of an artery changes in response to alterations in downstream resistance or impedance and such changes in shape can be quantified by calculation of the pulsatility index. In this study, the femoral artery velocity waveform was examined and the response to infusion of vasoactive agents was described in terms of the pulsatility index. Pulsatility index rose in response to infusion of phenylephrine. However, there was also a tendency for pulsatility index to rise, mainly from an increase in reflected velocities, on infusion of the vasodilator sodium nitroprusside. This unexpected result implies that the blood flow response to intense vasodilation is modified in the intact animal, possibly by the hoof.

This work is dedicated to my parents, Tony and Irene, and to Helen, a much loved and much missed friend.

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Declaration

I, Patricia Edith Pawson, do hereby declare that the work carried out in this thesis is original, was carried out by myself or with due acknowledgement, and has not been presented for the award of a degree at any other University.

Publications

Abstracts

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Abbreviations

A	area
ACH	acetylcholine
BK	bradykinin
c	velocity of sound
$CaCl_2$	calcium chloride
cAMP	3',5' cyclic adenosine monophosphate
cGMP	3',5' cyclic guanosine monophosphate
cm	centimetre
CT	computed tomography
d	diameter
EC_{50}	effective concentration 50%
EDHF	endothelium-derived hyperpolarizing factor
EDRF	endothelium-derived relaxing factor
EGF	epidermal growth factor
E_{max}	maximum response
ETYA	5,8,11,14-eicosatetraynoic acid
EU	endotoxin units
f	frequency
f_D	Doppler frequency shift
g	gram
h	hour
IFN-γ	interferon-γ
IL-1β	interleukin-1β
INDO	indomethacin
J_{vc}	net capillary filtration rate
KCl	potassium chloride
kg	kilogram
KH_2PO_4	potassium dihydrogen orthophosphate
K_{fc}	capillary filtration coefficient
kg	kilogram
L	litre
LAL	limulus amoebocyte lysate
LD_{50}	lethal dose 50%
L-NAME	N^G-nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl arginine
L-NOARG	N^G-nitro-L-arginine
log	logarithm

m	metre
M	molar
mg	milligram
$MgSO_4$	magnesium sulphate
MHz	megahertz
min	minute
ml	millilitre
mmHg	millimetres of mercury
MRI	magnetic resonance imaging
NaCl	sodium chloride
NADPH	nicotine adenine dinucleotide phosphate
$NaHCO_3$	sodium hydrogen carbonate
NDGA	nordihydroguiaretic acid
ng	nanogram
NIRS	near infrared spectroscopy
NMDA	N-methyl-D-aspartate
NOS	nitric oxide synthase
p	pressure
p_aCO_2	arterial partial pressure of carbon dioxide
p_aO_2	arterial partial pressure of oxygen
Pc	capillary hydrostatic pressure
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGI_2	prostacyclin
PHE	phenylephrine
PI	pulsatility index
Pt	tissue hydrostatic pressure
R_c	reflection coefficient
RNA	ribonucleic acid
s	second
SD	standard deviation
SEM	standard error of the mean
SNP	sodium nitroprusside
t	time
T_3	triiodothyronine
T_4	thyroxine
^{99}TcMAA	technetium labelled macroaggregated albumin
TNF-α	tumour necrosis factor-α
v	velocity

v_{max}	maximum velocity
v_{mean}	mean velocity
v_{min}	minimum velocity
z	distance
Z	impedance
Z_{pr}	impedance at zero frequency
Z_o	characteristic impedance
α	coefficient of friction (where indicated)
λ	wavelength
μg	microgram
μm	micrometre
πc	capillary osmotic pressure
πt	tissue osmotic pressure
θ	angle at which ultrasound intercepts blood flow
ρ	density
σd	osmotic reflection coefficient
Σ	sum of
Δ	change in

1. Pathophysiology of laminitis

1.1 Introduction

Laminitis is an important disease syndrome, affecting a number of ungulate species. It is well recognised in horses where it can be responsible for a range of clinical signs from an acute shock-type condition to a debilitating chronic lameness (Colles and Jeffcott 1977, Yelle 1986, Baxter 1992). In cattle, although the acute form of the disease is uncommon, subclinical laminitis has been linked to the occurrence of sole ulcers, white line lesions and heel erosions (Chew 1972, Greenough 1985, Bradley *et al.* 1989). As such it is an important cause of bovine lameness and responsible for considerable economic loss (Whitaker *et al.* 1983).

The precise mechanisms and inciting causes for laminitis remain elusive. Much of the relevant research has been conducted in horses, in which laminitis can be reliably induced following carbohydrate overload (Garner 1975b). Good reviews are provided by Coffman and Garner (1972), Garner (1980), Hood and Stephens (1981), Moore *et al.* (1989), Hunt (1991), Funkquist (1992), Moore and Allen (1996) and Weiss (1997). Carbohydrate overload does not consistently induce the disease in cattle (Dougherty *et al.* 1975b), therefore most of the work in this species focuses on examination of clinical cases. Literature pertaining to the condition in cattle has been reviewed by Mgasa (1987), Boosman *et al.* (1991b) and Vermunt (1992).

1.2 Anatomy of the ungulate digit

Laminitis has been defined as an inflammation of the dermal laminae within the hoof, which if severe, leads to separation of the hoof wall and underlying pedal bone. Before considering how these changes arise some understanding of the normal structural and vascular anatomy of the digit is required.

1.2.1 The equine digit

The following account is based on descriptions from anatomy texts (Sisson 1975, Schummer *et al.* 1981a, Dyce *et al.* 1987a) and review papers (Kainer 1989, Pollitt 1992). Anatomically the hoof can be divided into an epidermis, a dermis and a subcutis.

The epidermis forms the outer hoof capsule and comprises a number of regions having different horn structures. The perioplic horn is tubular and has a rubbery consistency. It forms a narrow band at the coronet that widens over the palmar/plantar aspect to cover the bulbs of the heels and merge with the base of the

frog. The horn of the frog is also soft and tubular, being kept moist by fatty secretions from the digital cushion. The horn of the sole is a mixture of tubular and intertubular horn and is therefore more rigid although it is still softer than the horn of the wall. The wall is that part of the hoof that is visible in the standing animal. It can be divided into 3 layers each arising from a different region of the epidermis. The outermost layer, the glossy *stratum externum*, is formed by drying of the perioplic horn as it grows distally. The bulk of the wall is formed by the *stratum medium*, a dense mixture of tubular and intertubular horn which grows towards the weight-bearing surface from the coronary epidermis. Deep to this lies the *stratum internum* which bears primary and secondary horny laminae that interdigitate with the underlying sensitive laminae of the dermis.

The dermis or corium lies deep to the hoof capsule and consists of connective tissue rich in blood vessels and nerves. Its main function is to supply nutrition to the overlying epidermis. It can be divided into regions in the same way as the epidermis, thus there is perioplic dermis, coronary dermis, laminar dermis, dermis of the sole and dermis of the frog. All regions bear papillae except for the laminar dermis, which is composed of primary and secondary dermal laminae. These interdigitate with the horny laminae of the epidermis.

The subcutis is thin and serves to attach the dermis to the underlying structures. The subcutis of the wall and sole forms the periosteum of the pedal bone and the perichondreum of the lateral cartilages. At a number of sites it is thickened and modified to form shock absorbing cushions. The largest of these is the digital cushion which forms the base of the frog. It consists of islands of fat and cartilage embedded in a mesh of elastic fibres and collagen.

Section 3.1.1 describes the gross anatomy of the digital vascular system, however the dermal microcirculation is not discussed. This level of the circulation has been investigated in the horse, using scanning electron microscopy to examine microvascular corrosion casts (Mishra and Leach 1983, Pollitt and Molyneux 1990). The dermis is supplied by an intricate vascular network comprising papillary and laminar units. Each papilla is supplied by a central artery and vein, which are surrounded by a delicate mesh of capillaries. Arteriovenous anastomoses, which link the arterial and venous sides of the circulation, are numerous, especially in the proximal third of the vascular unit. The vascular organisation within the laminae is more complex (Figure 1.2.1). A vertical parietal artery is located at the base of each dermal lamina. This gives rise to a series of axial arteries which are parallel to each other and are linked by slender connecting branches. Further branches give rise to vertically orientated capillaries which ultimately drain into an axial venous

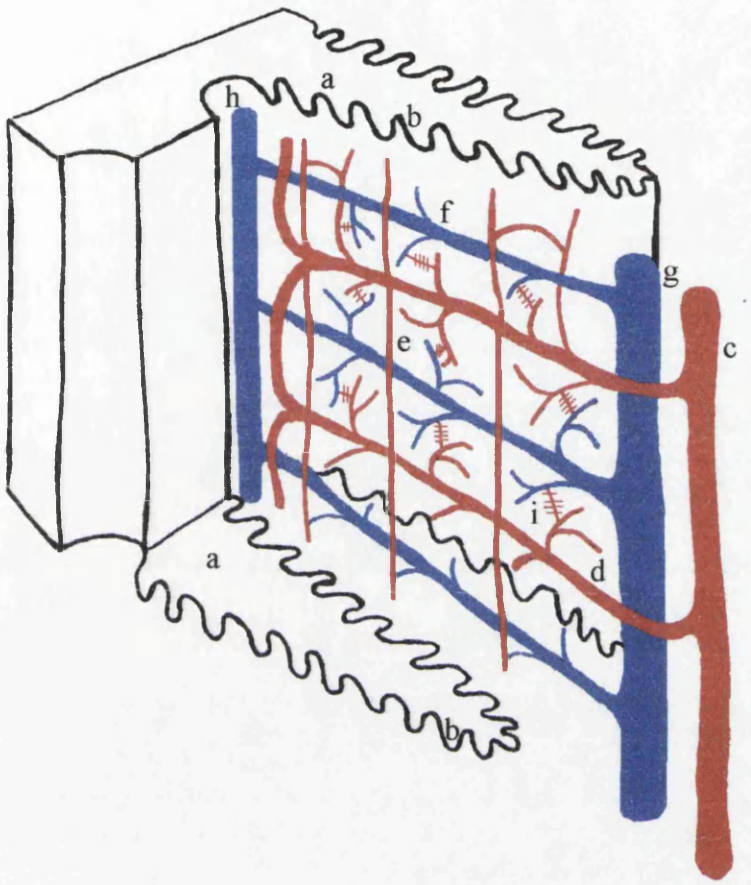


Figure 1.2.1. Schematic diagram of the laminar microcirculation of the equine hoof wall. Adapted from Pollitt (1992).

a - primary epidermal lamina

b - secondary epidermal lamina

c- parietal artery

d - axial artery

e - capillary

f - axial vein

g - parietal vein

h - marginal vein

i - arteriovenous anastomosis

network, comprising interconnected axial and parietal veins. As in the papillae, arteriovenous anastomoses are abundant.

1.2.2 The ruminant digit

This summary of the anatomy of the ruminant digit was compiled using anatomy texts (Schummer *et al.* 1981b, Dyce *et al.* 1987c, Smallwood 1992). Ruminants differ from the horse in that they are arteriodactyls, i.e. even-toed ungulates, each foot consisting of two main and two accessory claws. Despite this obvious difference, the structural organisation of the digit is similar in many respects. Each claw comprises the central supporting structures, i.e. phalanges, sesamoids, ligaments and tendons etc., enclosed by a covering of modified skin. This skin has epidermal, dermal and subcuticular layers.

The epidermis, a highly keratinised stratified squamous epithelium, forms the horny capsule of the claw. This capsule has a convex outer wall and a slightly concave interdigital wall, which meet dorsally to form the toe and posteriorly to form the heel. The ground surface is formed by the sole and the bulb of the heel. The epidermis can be divided into perioplic, coronary, laminar, solar and bulbar segments. The perioplic horn is soft and forms a slender band at the coronet, extending only a short distance distally. The bulk of the wall consists of dense tubular and intertubular horn originating from the coronary epidermis. The horny laminae, which lie deep to this, are relatively short and lack secondary laminae, thus the interdigitation with the laminar dermis is not as strong as in the horse. The extensive bulbar horn and horn of the sole is not as concave as it is in the horse, and it therefore constitutes a greater part of the weight-bearing surface in ruminants.

The claw capsule is moulded around a vascular dermis, which bears papillae except in the laminar segment. As in the epidermis, dermal laminae are non-pennate. The subcutis attaches the dermis to underlying structures and is thickened in the bulbar segment to form the digital cushion.

The dermal microcirculation in the bovine digit has been shown to be similar to that described in the horse (Vermunt and Leach 1992a). Arteriovenous anastomoses were found to be numerous, especially at the base of the dermal papillae and laminae.

1.3 Inhibited keratinisation.

Two theories have been proposed to explain the pathogenesis of laminitis. According to the first, the primary lesion is a metabolic disturbance of the keratinising epidermal cells. This theory was suggested by Obel (as reported by Larsson *et al.* 1956), who described the histopathological changes in biopsies from the hoof and chesnut of normal and laminitic horses. During the early stages of laminitis, he found a decrease in keratin precursors, i.e. onychogenic substance and keratohyalin, in the epidermis of the nonweight-bearing parts of the hoof and chesnut. A lack of onychogenic substance was also evident in the laminar epidermis of the hoof wall and the secondary epidermal laminae had a stretched appearance. Dermal changes, such as oedema, were not evident until the disease progressed further leading Obel to suggest that they occurred secondary to the defective keratinisation. Similar changes have been reported more recently in a case of post-surgical laminitis (Ekfalck *et al.* 1992).

Further support for this theory was provided by Larsson *et al.* (1956) who used radioisotopic techniques to study the process of keratinisation. During keratinisation, sulphur-containing amino acids such as methionine and cystine, are incorporated into the epidermis. The authors were able to demonstrate a decrease in the incorporation of radiolabelled cystine in cases of equine laminitis. They proposed that this occurred as a result of depressed differentiation, cells of the stratum spinosum failing to mature to the level required for the normal incorporation of cystine. Studies using cultured keratinocytes have shown that agents which promote keratinocyte multiplication will also repress differentiation. These agents have been reviewed by Ekfalck *et al.* (1991) and they include epidermal growth factor, corticosteroids and cholera toxin. Epidermal growth factor (EGF), a potent inhibitor of the differentiation of keratinocytes (Rheinwald and Green 1977), has attracted much attention as a potential mediator of the early epidermal changes observed in laminitis. Its presence in the tissues and secretions of both the intestine and the uterus (Hirata and Orth 1979, Simmens *et al.* 1986) provided the initial link with the alimentary and puerperal forms of the disease. Later studies demonstrated specific EGF receptors in the bovine and equine hoof (Ekfalck *et al.* 1988, Grosenbaugh *et al.* 1991), implying a physiological role for epidermal growth factor at that site.

Epidermal growth factor may also mediate the histopathological changes observed in chronic laminitis, particularly dermal and epidermal hyperplasia (Grosenbaugh *et al.* 1991). Epidermal hyperkeratinisation is also a feature at this stage (Roberts *et al.* 1980) and it is unclear if this arises from defective keratinisation or simply proliferation of keratinised cells. Grosenbaugh *et al.* (1990) compared EGF receptor-binding activity in urine from normal and chronically laminitic horses, but

were unable to detect a difference. However in a subsequent study, they found that EGF receptor binding characteristics were altered in chronic laminitis (Grosenbaugh *et al.* 1991) and proposed that this was the mechanism of altered growth. Whilst there is evidence for epidermal growth factor having a role in the pathogenesis of laminitis, this may not be a primary role. It may simply mediate secondary changes.

1.4 Altered digital blood flow

The second more widely held theory is that laminitis arises from a primary vascular disturbance. Factors such as vasoconstriction, microthrombosis, oedema and arteriovenous shunting are thought to reduce laminar perfusion, producing ischaemia of the laminar interdigitation with potential separation of the distal phalanx from the hoof (Moore and Allen 1996). Support for this theory is provided by histopathological studies of the laminar region in horses with laminitis (Roberts *et al.* 1980). Changes such as dermal oedema and epidermal necrosis were considered to be consistent with ischaemic damage.

Despite numerous investigations, the exact nature of the local blood flow changes that occur in laminitis, remains uncertain. Clinical observations of a warm foot and a bounding pulse would suggest an overall increase in digital blood flow. This is supported by experiments using isolated perfused digits from control and laminitic ponies (Robinson *et al.* 1976). The authors measured venous outflow directly and found it to be increased in laminitis. This finding is contradicted by angiographic studies in horses, which show a decrease in the filling of the terminal arch in both acute (Coffman *et al.* 1970) and chronic laminitis (Ackerman *et al.* 1975). Alterations in the vascular pattern have also been observed in studies of the bovine laminitic digit, although dilation and tortuosity of the distal phalangeal artery were the most frequent findings (Maclean 1970, Boosman *et al.* 1989). Hood *et al.* (1994) prepared vascular perfusion casts from the digits of laminitic horses and demonstrated avascular areas, not found in normal horses. Further evidence of reduced perfusion is provided by two scintigraphic studies using technetium-labelled macroaggregated albumin (^{99m}Tc MAA, Hood *et al.* 1978, Galey *et al.* 1990). In both studies the amount of radio-labelled albumin trapped in the capillary bed of the digit was reduced in laminitis, suggesting a decrease in blood flow.

If these findings of increased digital blood flow and decreased laminar perfusion are to be reconciled, redistribution of flow must occur within the hoof. Robinson (1990) suggested that in laminitis flow might be redirected through low resistance arteriovenous anastomoses, therefore bypassing the dorsal laminae. Such arteriovenous shunts have been shown to be numerous in equine and bovine hooves (Talukdar *et al.* 1972, Pollitt and Molyneux 1990, Vermunt and Leach 1992a). This

theory is supported by the work of Hood *et al.* (1978) who demonstrated radio-labelled albumin macroaggregates in the digital veins. These aggregates are of such a size that they normally become trapped in the capillaries and their presence in the venous circulation implies passage through wider diameter bypass vessels. An angiographic study of the equine digit showed that the arteries supplying the dorsal laminae are the last branches of the terminal arch to be perfused (Colles *et al.* 1980). They are therefore particularly vulnerable to ischaemia should blood flow be reduced or redirected.

Not all research confirms the occurrence of reduced laminar perfusion. A scintigraphic study by Trout *et al.* (1990), using ^{99m}Tc MAA demonstrated an increase in laminar blood flow in laminitis. An explanation for this anomalous result has been suggested by Robinson (1990). Macroaaggregated albumin particles range from 10-50 μm in diameter (Hood *et al.* 1978). Arteriovenous anastomoses in the equine hoof are of similar dimensions (Pollitt and Molyneux 1990). Consequently MAA particles may lodge in shunt vessels as well as capillaries. Thus the apparent increase in laminar blood flow may in fact be due to increased shunt flow.

1.4.1 Changes in vascular tone

How are these alterations in blood flow implemented? One suggested explanation is that excessive vasoconstriction within the foot, perhaps resulting from a change in sensitivity to vasoactive mediators, reduces laminar perfusion and diverts blood through the relatively low resistance shunt vessels. Hood *et al.* (1990, 1993) compared laminitis to Raynaud's phenomenon in people, a disease characterised by digital ischaemia and caused by digital artery spasm. Similarities in some aspects of the pathology and pharmacology of the two conditions led the authors to propose that laminitis, like Raynaud's phenomenon, may result from initial vasoconstriction followed by reactive hyperaemia. This is supported by a study in which pre-treatment with the vasodilator prazosin, prevented the development of laminar hypoperfusion in horses with experimentally-induced laminitis (Galey *et al.* 1990).

A number of studies have investigated this hypothesis further through an examination of the vasoactive mediators that regulate the digital circulation. Robinson *et al.* (1975a) used isolated pump-perfused equine digits to characterise the pressure-response to a variety of mediators including acetylcholine, histamine, adrenaline and prostaglandins. Similar studies have been conducted in the bovine digit (Elmes and Eyre 1977). Robinson *et al.* (1976) found these vascular responses to be unaltered in digits from laminitic ponies and horses. This however was not the finding of an *in vitro* study of the reactivity of isolated rings of equine digital artery

and vein (Baxter *et al.* 1989). Agonist-induced contraction was shown to be reduced in vessels from horses with early laminitis. In addition, the reduced response was more marked in arteries than veins, producing a relative increase in venous to arterial contraction ratio. The authors proposed from this, that the ratio of postcapillary to precapillary resistance would be increased, reducing venous drainage sufficiently to increase capillary pressure and produce oedema.

The studies described above addressed the nature of the vascular changes, i.e. was vascular reactivity increased or decreased in laminitis? With the discovery of nitric oxide (Palmer *et al.* 1987), a mechanism through which vascular reactivity might be altered was revealed. Endothelium-derived nitric oxide has been shown to be an important regulator of vascular tone in people (Vallance *et al.* 1989a) and alterations in its production have been implicated in the pathogenesis of other vascular diseases, such as atherosclerosis (Förstermann *et al.* 1988), hypertension (Panza *et al.* 1990) and endotoxic shock (Nava *et al.* 1992). Nitric oxide-mediated relaxation has been demonstrated in the digital veins and arteries of the horse (Elliott *et al.* 1994, Baxter 1995, Cogswell *et al.* 1995). It is therefore feasible that disturbances in nitric oxide production could contribute to the vascular derangements observed in laminitis (Bryant and Elliott 1994, Elliott 1996). A decrease in nitric oxide release with consequent increase in vascular tone has been proposed as a cause of laminar ischaemia (Baxter 1995). This is supported by a study by Hinckley *et al.* (1996a) in which the provision of exogenous nitric oxide, in the form of glyceryl trinitrate, was shown, subjectively, to enhance recovery in ponies with clinical laminitis.

1.4.2 Disturbances in Starling forces

The formation of oedema has been proposed as a second mechanism of laminar ischaemia. Fluid exchange within the digit is governed, as for all vascular beds, by the Starling equation (Hunt 1991). It is a balance between the hydrostatic pressure difference that favours filtration and the net osmotic pressure that favours absorption.

$$J_{vc} = K_{fc} ([Pc - Pt] - \sigma d [\pi c - \pi t])$$

where J_{vc} = net capillary filtration rate
K_{fc} = capillary filtration coefficient
Pc = capillary hydrostatic pressure
Pt = tissue hydrostatic pressure
πc = capillary osmotic pressure
πt = tissue osmotic pressure
σd = osmotic reflection coefficient

Measurement of these forces, using methods outlined by Allen *et al.* (1988b), has revealed major differences between the digit of the horse and that of the dog. Capillary hydrostatic pressure has been estimated to be 34mmHg in the normal equine digit (Korthuis *et al.* 1983). This is greater than that reported in the dog, 10mmHg, (Brace and Guyton 1977) and confirms the earlier finding of high small vein pressure in the equine digit (Robinson *et al.* 1975a). There are no reports of capillary pressure measurement in the digit of other ungulate species, therefore it is unclear if this difference applies to cattle and sheep. The potentially oedemagenic effects of high capillary pressure must be counteracted by adjustments in the other Starling forces. Suggested opposing factors include a low capillary filtration coefficient, a steep gradient of oncotic pressure, drawing fluid back into the circulation, and high tissue pressure (Korthuis *et al.* 1983). Capillary filtration coefficient, which is the product of microvascular surface area and permeability, is indeed low in the equine digit (Korthuis *et al.* 1983). Estimations of capillary permeability, as expressed by the osmotic reflection coefficient, suggest that it is high (Allen *et al.* 1988a) and therefore imply that the surface area available for exchange is low. A further consequence of the high capillary permeability is the failure to maintain a steep gradient of oncotic pressure. This is confirmed by the demonstration of a relatively high lymph protein concentration in the equine digit (Robinson *et al.* 1975b). Perhaps the most significant factor counteracting the high capillary pressure is a raised tissue hydrostatic pressure. This has been estimated at approximately 25mmHg (Allen *et al.* 1988b). It is clear from such studies that fluid exchange is delicately balanced and should any of the parameters alter oedema is a risk.

Measurement of Starling forces in laminitis, induced by either carbohydrate overload (Allen *et al.* 1990) or extract of black walnut (Section 1.5, Eaton *et al.* 1995) demonstrated marked elevations in both capillary and tissue hydrostatic pressures. Capillary hydrostatic pressures were respectively, 55 and 52mmHg in the 2 studies, whilst tissue hydrostatic pressures were 45 and 41mmHg. The high tissue pressure lead the authors to propose that laminitis may be a compartment syndrome. Such syndromes are known to develop when tissue pressure exceeds the critical closing pressure of capillaries, approximately 30mmHg in man (Hargens *et al.* 1977). Increases in postcapillary resistance were interpreted as arising from digital venoconstriction, which the authors suggested may have triggered the initial rise in capillary pressure. No change in capillary permeability was detected in either study (Allen *et al.* 1990, Eaton *et al.* 1995). This supports the earlier conclusion, by Robinson *et al.* (1976), that capillary permeability did not change in laminitis. This earlier study also demonstrated an increase in capillary permeability following intraarterial infusion of histamine and on this basis histamine was rejected as an

important causative factor in laminitis (Chavance 1946). Since then, Robinson and Scott (1981) have failed to demonstrate any change in capillary permeability following intravenous administration of histamine, (a route more analogous to the situation in laminitis), and so have qualified their earlier rejection of a role for histamine.

1.4.3 Altered haemostasis

A third factor reputed to produce laminar ischaemia is microvascular thrombosis. Microthrombi have been demonstrated in the dermal vessels of ponies and horses with experimentally-induced laminitis (Weiss *et al.* 1994, Weiss *et al.* 1995) and cattle with the clinical disease (Nilsson 1963, Maclean 1971). Systemic activation of coagulation has been proposed as a cause, but alterations in coagulation parameters have been difficult to demonstrate. Whilst Hood *et al.* (1979), working with horses, demonstrated mild fluctuations in activated partial thromboplastin times and fibrin split product titres, the majority of studies have failed to demonstrate any change in the coagulation profile during the development of laminitis (Kirker-Head *et al.* 1987, Prasse *et al.* 1990, Weiss 1996). The efficacy of the anticoagulant heparin in treating equine laminitis is controversial (Stashak 1987). Hood (1979) showed that heparin reduced the incidence of lameness in a group of 9 horses fed a laminitis-inducing diet. Although heparin pre-treatment failed to reduce the incidence of laminitis in a retrospective study of horses undergoing surgery for small intestinal disorders (Belknap and Moore 1989). This was however, a fairly select population of animals and the overall incidence of laminitis was low with only 9 of 71 horses developing the disease.

Detection of alterations in platelet aggregation, led Bell *et al.* (1979) to propose that an acquired platelet defect might trigger microthrombosis. Weiss *et al.* (1994) demonstrated accumulation of radio-labelled platelets distal to the coronary band in ponies with experimentally-induced laminitis. Platelet accumulation was detected at at the onset of lameness, leading the authors to suggest that it may have a primary pathogenic role. This was supported by a subsequent study in which decreased platelet survival time, an indicator of platelet consumption, was detected just 6 hours after carbohydrate administration (Weiss *et al.* 1995). Lameness did not develop for a further 22 to 46 hours. However Moore *et al.* (1981), using a larger group of horses, detected no change in platelet numbers until 8 hours after the onset of lameness.

1.5 Systemic disturbances

Alterations in blood flow are not restricted to the digit and more generalised cardiovascular disturbances, including systemic hypertension, have long been

recognised as part of the laminitis syndrome in both horses and cattle (Garner *et al.* 1975a, Andersson and Bergman 1980). The development of an experimental model for equine laminitis, i.e. carbohydrate overload, has permitted more detailed studies of the cardiovascular disturbances involved in this species. Following carbohydrate overload, increases in arterial blood pressure and heart rate were consistent findings (Garner *et al.* 1975b, Garner *et al.* 1977, Harkema *et al.* 1978). Garner *et al.* (1975b) also recorded a fall in central venous pressure, which in the light of concurrent increases in packed cell volume and total protein, implied a depletion of plasma volume. This has since been confirmed (Clarke *et al.* 1982). The increase in heart rate observed preceded the development of lameness and it has been suggested that it occurred as a compensatory response to depleted plasma volume (Garner *et al.* 1977). Arterial pressure rose later, coinciding with the onset of clinical lameness, and it has been proposed that this may reflect a response to pain, mediated by catecholamines (Garner *et al.* 1977, Harkema *et al.* 1978). Catecholamine-mediated vasoconstriction has also been implicated in the local blood flow changes within the foot. There is also evidence that the renin-angiotensin-aldosterone system may be activated by the plasma volume changes. Clarke *et al.* (1982) recorded increases in plasma renin activity and aldosterone concentration during the development of laminitis. Renin is responsible for the generation of angiotensin I, which is further converted to angiotensin II (Garrison and Peach 1990). Angiotensin II is a powerful vasoconstrictor, and the authors suggested that it may have a role in the development of laminar ischaemia in the horse. However this seems unlikely in the light of a later study, which found angiotensin II to be a poor vasoconstrictor of equine digital vessels (Baxter *et al.* 1989).

Haematological changes have also been documented in equine alimentary laminitis. Whilst neutrophils were increased in number, lymphocytes and eosinophils were decreased (Moore *et al.* 1981, Kirker-Head *et al.* 1987). Moore *et al.* (1981) suggested that this blood picture was consistent with increased circulating adrenal glucocorticoids and/or catecholamines, a theory supported by the finding of concurrent hyperglycaemia. Clarke *et al.* (1982) detected an increase in circulating hydrocortisone during the development of laminitis, but this failed to reach statistical significance.

Some of the systemic disturbances detailed above may be specific to carbohydrate overload-induced laminitis. Another method, used experimentally to induce laminitis in horses, is the oral administration of an aqueous extract of black walnut. This substance consistently produces laminitis, accompanied by mild depression and limb oedema, 8-12 hours after administration (Galey *et al.* 1991). Juglone, a toxin present in the bark of black walnut trees, can allegedly cause allergic problems in

people (MacDaniels 1983), but does not consistently induce laminitis when administered to ponies and horses (True and Lowe 1980). The toxic principle responsible for this effect has not been identified but appears to be present, unlike juglone, in an aqueous extract of black walnut shavings (Uhlinger 1989). Severe fluid and electrolyte shifts are not a feature of the laminitis associated with black walnut toxicosis (Galey *et al.* 1991). Whilst evidence for plasma depletion is lacking, tachycardia, neutrophilia with left shift, elevated plasma adrenaline and a prolonged diurnal cortisol peak are features common to both models.

1.6 Predisposing factors

Epidemiological studies have not always agreed on their assessment of risk factors. Dorn *et al.* (1975) found ponies to be at greater risk of developing laminitis than horses. An increased incidence of laminitis was also noted in the summer months and in mares and stallions compared to castrated males. However Slater *et al.* (1995) and Polzer and Slater (1996) failed to demonstrate any significant association between age, sex, breed or seasonality and the occurrence of acute equine laminitis. In cattle, an age predisposition has been observed, with first-calf heifers being at increased risk of disease (Nilsson 1963, Moser and Divers 1987, Greenough and Vermunt 1991). A seasonal peak in the number of laminitis cases, related to housing and/or calving, has also been identified (Nilsson 1963, Maclean 1965, Greenough and Vermunt 1991).

Dietary changes are commonly implicated in the induction of laminitis, especially in cattle (Vermunt and Greenough 1994). Accidental grain overload is an extreme example, which has been used to induce the disease in horses (Garner *et al.* 1975b). In dairy cows, the change from a high fibre-diet during the dry period to a high concentrate ration at calving is believed to increase the risk of laminitis (Maclean 1965, Weaver 1979). Studies by Peterse *et al.* (1984) and Manson and Lever (1988a) demonstrated a higher incidence of laminitis in cows fed a high concentrate diet, but not all studies confirm this association (Smit *et al.* 1986, Frankena *et al.* 1992). Not surprisingly, bolus-feeding of concentrate has also been linked to laminitis (Moser and Divers 1987). The carbohydrate content of a diet is not the only important factor. Whilst, high fibre diets may have a protective effect (Livesey and Fleming 1984), diets rich in protein have been associated with an increased incidence of laminitis (Manson and Leaver 1988b, Bargai *et al.* 1992). The ingestion of lush growing grasses, high in protein and metabolisable energy, also predisposes to the development of laminitis, especially in ponies (Stashak 1987, Vermunt 1992).

Trauma to the digit has been cited as another predisposing factor in both species. In horses, an increased incidence of laminitis has been observed in the contralateral limb of horses with a unilateral lameness (Martin *et al.* 1984, Peloso *et al.* 1996). This is believed to result from excessive loading of the supporting limb. Excessive work on hard surfaces and long periods of standing may have a similar effect (Stashak 1987). In cattle, an increased incidence of laminitis is observed in heifers newly housed on concrete (Vermunt and Greenough 1994) and this has been attributed to the combined effects of repeated concussion on hard floors (Bazeley and Pinsent 1984) and failure to use uncomfortable cubicles (Colam-Ainsworth *et al.* 1989). Bergsten (1988) has demonstrated a positive correlation between the occurrence of laminitis-like lesions and concrete flooring.

Hypothyroidism has been cited as a factor predisposing to laminitis in horses (Colles and Jeffcott 1977). Hood *et al.* (1987) showed that whilst levels of thyroxine (T_4) and triiodothyronine (T_3) were depressed in the acute phase of disease, responses to thyroid stimulating hormone were normal. This implies that the reduced levels of T_3 and T_4 were a response to laminitis and not a cause. Another endocrine disorder that has been associated with an increased risk of laminitis is hyperadrenocorticism (Freestone and Melrose 1995) and this is discussed further in section 1.6.2. Field and Jeffcott (1989) showed that overweight ponies and those with a history of laminitis were relatively insensitive to insulin. Insulin normally reduces platelet production of thromboxane A_2, a pro-aggregatory vasoconstrictor (Qvist and Larkins 1983), therefore levels of this mediator may be raised where there is insulin resistance. Field and Jeffcott (1989) suggested that this increased the tendency for thrombosis in overweight ponies and accounted for the increased incidence of laminitis in this group (Dorn *et al.* 1975).

An increased incidence of laminitis has also been associated with a number of other disease states. Horses with gastrointestinal disorders or a retained placenta appear to be at greater risk (White *et al.* 1987, Blanchard *et al.* 1987), as do cattle with mastitis or post-partum metritis (Nilsson 1963, Maclean 1965). Endotoxaemia is a potential complication of all these conditions, implying that endotoxin is an important predisposing factor for laminitis.

1.6.1 Endotoxin

Endotoxin has been further implicated in the pathogenesis of laminitis by studies examining the series of events that follow a dietary change, such as carbohydrate overload. Garner *et al.* (1978) detected alterations in the bacterial population of the equine caecum following administration of a laminitis-inducing diet. The authors demonstrated an increase in the number of lactic acid-producing bacteria and

recorded a fall in caecal pH, believed to result from increased lactic acid production. At the same time, gram-negative bacteria were found to decline and their destruction, with release of free endotoxin, produced an increase in the level of caecal endotoxin (Moore *et al.* 1979). Studies conducted in cattle and sheep, have shown similar alterations in the microbial population of the rumen following grain overload (Allison *et al.* 1975). Krueger *et al.* (1986) collected caecal biopsies from horses fed a laminitis-inducing diet and examined the caecal epithelium using transmission and scanning electron microscopy. Widespread sloughing of the caecal epithelium was evident and the authors proposed that the damage was sufficient to permit absorption of endotoxin into the portal circulation. This has been confirmed by detection of circulating endotoxin in both ruminants and horses subject to carbohydrate overload (Dougherty *et al.* 1975a, Sprouse *et al.* 1987). In the later study, the endotoxaemia was quantified and values of 2.4 to 81.53ng/L were detected in 11 of the 13 horses.

Many studies have attempted to confirm the involvement of endotoxin, by comparing the lesions that occur in laminitis with those induced by endotoxin. Mishra and Sack (1987) used electron microscopy to illustrate the similarity between endotoxin-induced and laminitis-associated vascular lesions in the equine digit. Similarities have also been noted between the haematological changes observed in laminitis (Moore *et al.* 1981) and those seen in acute *Escherichia coli* endotoxaemia (Burrows 1981). Activation of coagulation, which has been proposed as a possible mechanism of laminitis, can be induced in horses by endotoxin infusion (Duncan *et al.* 1985). Infusion of endotoxin at 1μg/kg/hour for 24 hours caused shortening of the one-stage prothrombin time and the activated partial thromboplastin time, and increased the concentration of circulating fibrin degradation products. Similar alterations have been recorded in horses with laminitis (Hood *et al.* 1979). Endotoxin infusion, various doses, has also been shown to produce a hypercoagulable state in cattle (Boosman *et al.* 1991a). The effects of intravenous endotoxin administration, 0.03μg/kg, on digital haemodynamics have been described by Hunt *et al.* (1990). Using isolated pump-perfused equine digits, they demonstrated an increase in total vascular resistance and a consequent reduction in digital blood flow, findings that are similar to those described in a study using laminitic horses (Allen *et al.* 1990). However, following endotoxin infusion the increased vascular resistance was predominantly precapillary in origin, not postcapillary as shown in the laminitic horses. Ingle-Fehr and Baxter (1996) confirmed that endotoxin infusion, 0.1μg/kg, acutely reduces digital blood flow in conscious horses, using ultrasound to quantify flow in the palmar digital artery.

Studies *in vitro* have yielded more variable results. Baxter *et al.* (1989) detected no change in the contractile responses of digital vessels following a 10 minute incubation with 23ng/L endotoxin. However in a subsequent study, the contractile response to noradrenaline was shown to be increased in arteries from horses treated with 0.1μg/kg endotoxin (Baxter 1995). Contraction to a second vasoconstrictor, serotonin, was reduced, as were the relaxant responses to acetylcholine and bradykinin. The author proposed that this enhanced vasoconstriction was a consequence of reduced nitric oxide production, caused by endotoxin-mediated endothelial damage. He suggested that this also accounted for the decreased digital blood flow observed in response to endotoxin infusion (Hunt *et al.* 1990, Ingle-Fehr and Baxter 1996). These findings are only partly confirmed by a study in which equine digital arteries and veins were incubated overnight in endotoxin (Bailey and Elliott 1996). No change in the response of digital veins to the α_1-adrenergic agonist phenylephrine was noted, whilst serotonin-induced contraction was reduced.

In several species, the deleterious effects of endotoxin have been shown to be mediated by cytokines, such as tumour necrosis factor, (TNF-α, Beutler *et al.* 1985, Tracey *et al.* 1987b, Mathison *et al.* 1988). The importance of this cytokine in horses has been demonstrated by the detection of TNF-α activity in the circulation following endotoxin administration (Morris *et al.* 1990, MacKay *et al.* 1991). Baxter (1994) proposed that TNF-α may mediate the vascular effects of endotoxin and a study in which equine digital arteries were exposed to TNF-α tends to support this view. Incubation with TNF-α enhanced noradrenaline-induced contraction but depressed relaxation to acetylcholine, effects similar to those observed following incubation with endotoxin (Baxter 1995).

Whilst circumstantial evidence suggests that endotoxin has a role in the pathogenesis of laminitis, the clinical disease has only once been induced by the administration of endotoxin. Mortensen *et al.* (1986) were successful in inducing mild signs of laminitis following injection of large doses of endotoxin into the digital arteries of calves. Sprouse and Garner (1982) accounted for this poor success rate by suggesting that laminitis may represent a local Schwartzman reaction, in which case prior sensitisation to endotoxin would be required. This is supported by the demonstration of 2 peaks in circulating endotoxin, following carbohydrate overload in horses (Sprouse *et al.* 1987). Some aspects of the disease have been reproduced by prolonged or repeated infusions of endotoxin. Duncan *et al.* (1985) noted signs of hoof discomfort in 6 out of 7 horses receiving a 24 hour endotoxin infusion. This was accompanied by a decrease in hoof temperature. Boosman *et al.* (1991a) administered endotoxin to cattle, by bolus or infusion, on 3 consecutive days. Whilst no clinical signs of laminitis were observed, histopathology of the dermis of the claw revealed laminitis-like lesions, with white

cell infiltration, congestion and vessel thrombosis. Schwartzman reactions were not evoked in this study.

1.6.2 Corticosteroids

The administration of corticosteroids appears to increase the risk of laminitis in horses (Colles and Jeffcott 1977, Slater *et al.* 1995). Hyperadrenocorticism, in which there is increased production of endogenous corticosteroids, is also associated with an increased incidence of disease (Freestone and Melrose 1995). A number of theories have been proposed to explain this predisposition. Eyre (1982) suggested that corticosteroids may induce laminitis by altering the reactivity of the digital vasculature to endogenous mediators. In support of this theory, the contractile responses to catecholamines were shown to be enhanced in equine digital arteries and veins incubated with corticosteroids (Eyre *et al.* 1979). Similarly, the pressure responses to adrenaline and serotonin were increased in isolated pump-perfused digits following treatment with hydrocortisone (Eyre and Elmes 1980). However, these effects, mediated by inhibition of catecholamine uptake (Rang *et al.* 1995), are not limited to digital vessels and therefore their relevance to laminitis is questionable. Slone *et al.* (1981) demonstrated significant sodium and chloride retention following treatment with dexamethasone and triamcinolone and suggested that these additional effects might favour oedema formation and contribute to hypertension. Other factors that may be involved include corticosteroid-mediated inhibition of keratinocyte differentiation (Ekfalck *et al.* 1991) and corticosteroid-induced insulin resistance (Field and Jeffcott 1989).

1.7 Aims of the project

Despite many years of research, our understanding of the pathogenesis of laminitis is not yet complete. The aim of this project was to investigate the regulation of digital blood flow, with particular reference to factors implicated in the development of laminitis, such as endotoxin and nitric oxide. A further aim was to investigate the impact of the hoof on digital blood flow.

Despite the importance of laminitis in cattle, as a welfare issue (Hassall *et al.* 1993) and a source of economic loss (Whitaker *et al.* 1983), relatively few studies have been conducted in this or any other ruminant species. We therefore chose to use sheep as a model for both bovine and equine laminitis. Although the clinical disease is not common in sheep, laminitis can be induced experimentally by creation of a rumenal lactic acidosis (Morrow *et al.* 1973), a method not dissimilar to that used in the horse. Ovine tissues, for the *in vitro* studies, were readily available from a local abattoir and the relative ease of handling of sheep was advantageous in the *in vivo* studies.

The initial aim of the *in vitro* studies was to determine the role of endothelium-derived nitric oxide and prostanoids in the modulation of digital artery tone. This was accomplished by comparing the tension developed in isolated rings of ovine digital artery maintained under different conditions. Endotoxin has been implicated in the pathogenesis of both clinical (Blanchard *et al.* 1987) and experimental laminitis (Sprouse *et al.* 1987) and many of its deleterious effects are mediated by pro-inflammatory cytokines, such as tumour necrosis factor (Mathison *et al.* 1988). Therefore, a further aim was to investigate the effect of incubation with such agents on the vascular reactivity of the isolated ovine digital artery.

The aim of the *in vivo* studies was to examine the response of the hindlimb vascular bed to local infusion of vasoactive agents in the live animal. In ungulates the distal limb is enclosed by the hoof and it is not known if this has any impact on digital blood flow. The response to infusion of vasoconstrictor and vasodilator agents was assessed by examining changes in the shape of the femoral artery velocity waveform, obtained using Doppler ultrasound.

2. A study of the contractile and relaxant responses of the ovine digital artery *in vitro*

2.1 Introduction

In the past 10-15 years there have been major advances in our understanding of vascular physiology. Central to this advancement was the discovery that the endothelium has an active role in the regulation of vascular tone, through the synthesis and release of endothelium-dependent factors. These factors were also found to be important in the maintenance of an antithrombogenic endothelial surface. Good reviews of the endothelium-dependent factors are provided by Gryglewski *et al.* (1988), Furchgott & Vanhoutte (1989), Rubanyi (1991), Vane *et al.* (1990) and Tolins *et al.* (1991).

2.1.1 Prostacyclin

The first of these factors to be discovered was prostacyclin (Moncada *et al.* 1976). This vasoactive prostaglandin is derived from arachidonic acid which is liberated from membrane phospholipids by activated phospholipase A_2. A haeme-containing cyclooxygenase enzyme catalyses the conversion of arachidonic acid to the prostaglandin endoperoxides prostaglandin G_2 (PGG_2) and then prostaglandin H_2 (PGH_2). The latter is further transformed into prostacyclin (PGI_2) by prostacyclin synthase (Moncada & Vane 1979). Prostaglandin endoperoxides released from platelets and transferred across the endothelial cell membrane form an alternative source of prostacyclin precursors (Bunting *et al.* 1976). Prostacyclin is chemically unstable and once formed, it is rapidly broken down to 6-keto-prostaglandin $F_{1\alpha}$, a more stable but inactive metabolite (Nicholson *et al.* 1985).

Release of prostacyclin, as for other prostaglandins, is thought to be associated with factors disturbing the endothelial cell membrane (Piper & Vane 1971). These may be physical factors such as pulsatile pressure (Quadt *et al.* 1982) or chemical factors such as bradykinin (Gryglewski *et al.* 1986a), thrombin (Weksler *et al.* 1978) and platelet-derived growth factor (Coughlin *et al.* 1980).

Prostacyclin has an anti-aggregatory action on platelets (Moncada *et al.* 1976) and is a potent vasodilator (Bunting *et al.* 1976). It mediates its effects through activation of adenylate cyclase (Gorman *et al.* 1977) with consequent elevation of platelet and vascular smooth muscle cyclic 3',5'-adenosine monophosphate (cAMP, Tateson *et al.* 1977, Ito *et al.* 1980). Prostacyclin is also believed to enhance the activity of cholesteryl ester hydrolases, enzymes that are responsible for clearing cholesteryl

esters from the vascular wall (Hajjar *et al.* 1982). Interference with this action may be important in the development of atherosclerosis (Hajjar 1985).

Other properties of prostacyclin have been identified, including fibrinolytic activity and a cytoprotective effect. Benefits in the treatment of pulmonary thromboembolism have been observed and have been attributed to the combined vasodilatory, anti-platelet and fibrinolytic properties of prostacyclin (Utsunomiya *et al.* 1980). The cytoprotective effects are thought to be achieved through scavenging of oxygen free radicals which may be associated, for example, with post-ischaemic reperfusion damage (Simpson & Lucchesi 1987). These effects may contribute to the proven efficacy of prostacyclin and its analogues in the treatment of peripheral vascular disease, such as arteriosclerosis obliterans (Szczeklik *et al.* 1979) and Raynaud's phenomenon (Yardumian *et al.* 1988). Moncada & Vane (1984) have reviewed the clinical applications of prostacyclin.

2.1.2 Nitric oxide

2.1.2.1 Discovery and identification of endothelium-derived relaxing factor

Furchgott and Zawadzki (1980) discovered that acetylcholine-induced relaxation of rabbit aortic rings and strips only occurred when the endothelium was intact. In the absence of the endothelium, acetylcholine induced vasoconstriction. It was therefore postulated that acetylcholine stimulated the release of an endothelium-derived relaxing factor (EDRF). Release of EDRF has since been shown to occur in response to many different stimuli and in many different vessels from different species. In addition to acetycholine, bradykinin (Cherry *et al.* 1981), calcium ionophore A23187 (Zawadzki *et al.* 1980), adenine nucleotides, thrombin (De Mey *et al.* 1982), noradrenaline, serotonin (Cocks and Angus 1983) and substance P (Zawadzki *et al.* 1981), have all been shown to trigger release of EDRF (for a more complete list see Furchgott and Vanhoutte 1989). Other factors, such as increased blood flow (Rubanyi *et al.* 1986), visible light and electrical field stimulation (Frank and Bevan 1983) are also capable of initiating release. Endothelium-dependent relaxation is not just a feature of arteries, it has also been demonstrated in resistance vessels (Tesfamariam *et al.* 1985) and to a lesser degree in veins (De Mey and Vanhoutte 1982). It has been demonstrated in vessels from numerous species including rat (Van de Vorde and Leusen 1983), dog (De Mey *et al.* 1982), cow (Gruetter and Lemke 1986), pig (Gordon and Martin 1983) and man (Cherry *et al.* 1982).

Many of the early experiments used separate donor and detector systems to characterise the properties of EDRF. Endothelial cells *in situ* (Förstermann *et al.* 1984, Griffith *et al.* 1984, Rubanyi *et al.* 1985) or cultured on the surface of

microcarrier beads (Cocks *et al.* 1985, Gryglewski *et al.* 1986a) were stimulated to release EDRF, the effects of which were detected by a downstream endothelium-free preparation, such as an arterial ring or strip. Such studies confirmed the labile nature of EDRF, its half-life in the bioassay system was just 6 seconds (Gryglewski *et al.* 1986a). EDRF was shown to reduce vascular tone by activating soluble guanylate cyclase, thereby increasing intracellular cyclic guanosine 3',5'-monophosphate (cGMP, Rapoport *et al.* 1983). This was supported by studies showing that endothelium-dependent relaxation was inhibited by methylene blue, an inhibitor of soluble guanylate cyclase (Gruetter *et al.* 1981), but was potentiated by cGMP phosphodiesterase inhibitors (Martin *et al.* 1986a). In addition, haemoglobin was found to inhibit endothelium-dependent relaxation by both inactivating guanylate cyclase and binding EDRF in solution (White *et al.* 1982, Martin *et al.* 1986c). Several reducing agents, such as hydroquinone (Van de Voorde and Leusen 1983) and dithioreitol (Griffith *et al.* 1984), were also found to inhibit the action of EDRF. Moncada *et al.* (1986) suggested that this inhibition was the result of the inactivation of EDRF by superoxide anion (O_2^-), generated by the reduction of molecular oxygen. This accounts for the finding that superoxide dismutase, a free radical scavenger, protects EDRF from breakdown, thus potentiating endothelium-dependent relaxation (Gryglewski *et al.* 1986b, Rubanyi and Vanhoutte 1986).

In addition to its vasodilating properties, EDRF was shown to inhibit the adhesion of platelets to endothelial cells (Radomski *et al.* 1987c) and to inhibit platelet aggregation (Azuma *et al.* 1986). These effects were also shown to be mediated through elevations in cGMP and could be inhibited or potentiated by similar factors to those described above (Radomski *et al.* 1987b).

The endothelium-dependent relaxation observed by Furchgott and Zawadzki (1980) occurred in the presence of the cyclooxygenase inhibitors indomethacin and aspirin, thereby ruling out the possibility that EDRF was a prostaglandin-like substance. Inhibition of lipoxygenase using either 5, 8, 11, 14-eicosatetraynoic acid (ETYA) or nordihydroguiaretic acid (NDGA), did however abolish endothelium-dependent relaxation (Furchgott and Zawadski 1980, Cherry *et al.* 1982, Furchgott *et al.* 1982), leading to the hypothesis that EDRF may be a short-lived lipoxygenase product, possibly a free radical (Furchgott 1984). However, ETYA and NDGA are not selective inhibitors of lipoxygenase and they may also inhibit EDRF through generation of superoxide anions (Förstermann *et al.* 1988a).

The search for the identity of EDRF took a different direction when two laboratories working independently suggested that EDRF may be nitric oxide (Khan and Furchgott 1987, Ignarro *et al.* 1987b). This hypothesis arose from comparison of the relaxant effects of nitrovasodilators and EDRF. Nitrovasodilators, such as

glyceryl trinitrate, have long been used in the treatment of angina (Murrel 1879), however the mechanism by which these agents induce vasodilation was only recently revealed. Nitrovasodilators were shown to activate guanylate cyclase and elevate cGMP (Schultz *et al.* 1977), an action which Katsuki *et al.* (1977) proposed was mediated through generation of nitric oxide. This similarity between the mechanism of EDRF-and nitric oxide-induced vasodilation prompted the assertion that they were one and the same. Comparisons of the actions of nitric oxide and EDRF on vascular smooth muscle and platelets revealed many similarities. As for EDRF, the effects of nitric oxide were potentiated by superoxide dismutase and cGMP phosphodiesterase inhibitors and inhibited by hydroquinone and haemoglobin (Hutchinson *et al.* 1987, Ignarro *et al.* 1987a, Radomski *et al.* 1987b). In addition, both substances produced the same characteristic shift in absorption spectra on combination with haemoglobin (Ignarro *et al.* 1987a). Final confirmation that EDRF is nitric oxide came from a study by Palmer *et al.* (1987), in which nitric oxide release, stimulated by bradykinin, was quantified and found to be of the correct magnitude to account for the relaxation observed in a bioassay strip.

Not all studies support the view that EDRF and nitric oxide are identical. Shikano *et al.* (1987) showed that whilst nitric oxide relaxed guinea pig teniae coli muscle, EDRF did not. A second study found the same to be true of tracheal smooth muscle, i.e. nitric oxide produced relaxation, EDRF did not (Dusting *et al.* 1988). Differences have also been noted in the retention of EDRF and nitric oxide by anion exchange resins (Long *et al.* 1987). Rubanyi (1991) suggested that these anomalous results could be explained if EDRF were a nitric oxide-containing substance rather than free nitric oxide. Myers *et al.* (1990) proposed that the nitrosothiol S-nitro-L-cysteine, which generates nitric oxide on decomposition, may be such a substance and demonstrated similarities between it and EDRF. However a more recent study identified significant differences between the two substances (Feelisch *et al.* 1994).

2.1.2.2 Synthesis of nitric oxide

Nitric oxide is generated from the terminal guanidino nitrogen of the amino acid L-arginine by an oxidation reaction (Palmer *et al.* 1988). Citrulline is produced as a byproduct and can be recycled back to L-arginine by endothelial cells if required (Hecker *et al.* 1990). The generation of nitric oxide by endothelial cells is catalysed by the enzyme nitric oxide synthase (NOS, Palmer and Moncada 1989), the activity of which depends upon a number of cofactors including NADPH and calmodulin (Busse and Mülsch 1990a). There is also a requirement for calcium, which activates the enzyme by binding to calmodulin. Analogues of L-arginine, such as N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine (L-NOARG), have

been shown to inhibit nitric oxide production, an effect that can be reversed by an excess of L-arginine (Palmer *et al.* 1988, Rees *et al.* 1989a, Moore *et al.* 1990).

Endothelial NOS is a constitutive enzyme i.e. it is already present in cells and requires no induction (Palmer and Moncada 1989). Two other isoforms of the enzyme have been described, a neuronal constitutive form and an inducible form (McCall and Vallance 1992). The neuronal enzyme is similar to the endothelial isoform, being NADPH and calcium-calmodulin dependent (Bredt and Snyder 1990). The inducible enzyme is slightly different being independent of calcium and having an additional requirement for the cofactor tetrahydrobiopterin (Tayeh and Marletta 1989). This enzyme is not normally present in unstimulated cells but can be induced by exposure to bacterial toxins and cytokines such as interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ, Busse and Mülsch 1990b, Geller *et al.* 1993). Its expression has been demonstrated in a number of different cell types, including macrophages (Marletta *et al.* 1988), neutrophils (McCall *et al.* 1989), vascular smooth muscle (Busse and Mülsch 1990b) and endothelial cells (Radomski *et al.* 1990). The inducible enzyme is capable of generating greater quantities of nitric oxide than the constitutive isoform and has been shown to be important in cell-mediated bacterial killing and tumour cell cytostasis (Hibbs *et al.* 1987, Stuehr and Nathan 1989).

2.1.2.3 Physiological functions of nitric oxide

The study of nitric oxide has been greatly facilitated by the development of specific inhibitors of NOS and much of the work in this area focuses on the effects of such substances *in vivo*. Rees *et al.* (1989b) demonstrated an increase in arterial blood pressure following the parenteral administration of L-NMMA to rabbits. This finding has been confirmed in a number of species (Aisaka *et al.* 1989, Tolins *et al.* 1990), including sheep (Tresham *et al.* 1991) and suggests that there is a continuous release of nitric oxide from the endothelium that is important in the modulation of blood pressure. This basal release of nitric oxide is believed to be dependent on pulsatile flow and shear stress (Rubanyi *et al.* 1986) and has been shown to regulate blood flow to a number of regional vascular beds, including the kidney (Radermacher *et al.* 1990, Tolins and Raij 1991), the lungs (Stamler *et al.* 1994), the heart (Amezcua *et al.* 1989, Benyo *et al.* 1991, García *et al.* 1992), the gastrointestinal tract (Gardiner *et al.* 1990, Santiago *et al.* 1994) and the brain (Tanaka *et al.* 1991). There is also evidence that nitric oxide modulates limb blood flow. A study by Vallance *et al.* (1989a) demonstrated a significant reduction in forearm blood flow on infusion of L-NMMA into the brachial artery of human volunteers. This finding has been confirmed in other species (Gardiner *et al.* 1990, Kirkebøen *et al.* 1992, Bellan *et al.* 1993, White *et al.* 1993). Agonist-stimulated

release of nitric oxide may also contribute to the regulation of vascular tone. A number of vasoconstrictors stimulate the simultaneous release of nitric oxide, which may serve to modulate the vasoconstrictive response. This has been demonstrated with noradrenaline, which has been shown to stimulate nitric oxide release via α_2-adrenergic receptors (Cocks and Angus 1983). Inhibition of NOS removed this moderating influence and enhanced noradrenaline-induced contractions (Kaneko and Sunano 1993). The vasoconstrictive actions of adenosine triphosphate and serotonin may also be modified in this way (Burnstock and Kennedy 1986, Cappelli-Bigazzi *et al.* 1991).

It has been suggested that the endothelium is maintained in a thromboresistant state by the basal release of both nitric oxide and prostacyclin (Gryglewski *et al.* 1988). Certainly both agents inhibit platelet aggregation and their release can be increased by mediators produced by activated platelets, such as serotonin and adenine nucleotides (Houston *et al.* 1986). A marked synergism has been demonstrated between the anti-aggregatory effects of nitric oxide and prostacyclin (Radomski *et al.* 1987a) and indeed, the receptor-mediated release of both agents has been shown to be coupled (De Nucci *et al.* 1988). Nitric oxide may also inhibit growth factor-mediated proliferation of vascular smooth muscle, thereby restricting the mitogenic response to vascular injury (Garg and Hassid 1989).

The endothelium is not unique in its ability to produce nitric oxide and a neuronal isoform of NOS has been identified in the brain and peripheral nerves (Bredt and Snyder 1990, Bredt *et al.* 1990). In the brain, nitric oxide behaves as a second messenger and has been shown to mediate the increase in cGMP that follows the combination of the excitatory neurotransmitter glutamate with NMDA receptors (Wood *et al.* 1990). There is also evidence that nitric oxide is able to modulate synaptic transmission. It appears to have a role in the development of long-term potentiation, a form of synaptic plasticity that is important in learning and memory (Bohme *et al.* 1993, Holscher 1997). In addition nitric oxide has been implicated in the process of neuronal degeneration. Neurones containing NOS have been shown to be resistant to damage (Koh and Choi 1988, Hope *et al.* 1991) and it has been postulated that these neurones, which do not contain cGMP, are able to produce sufficient nitric oxide to damage neighbouring cells but are themselves unharmed (Speciale 1993). In the peripheral nervous system, nitric oxide functions as a neurotransmitter (Sanders and Ward 1992). Non-adrenergic non-cholinergic nerves, which innervate the gastrointestinal and urogenital tracts, have been shown to relax visceral smooth muscle through the release of nitric oxide (Gillespie *et al.* 1989, Bult *et al.* 1990).

The production of nitric oxide is also an important component of the body's defence against invading pathogens. Activated macrophages have been shown to generate cytotoxic quantities of nitric oxide, via expression of inducible NOS (Hibbs *et al.* 1987). This mechanism is especially important in combating intracellular parasites, such as Leishmania (Green *et al.* 1990) and those which are too large to be phagocytosed (James and Hibbs 1990).

Nitric oxide has a complex role in inflammation (Moilanen and Vapaatal 1995). Increases in leukocyte adhesion and microvascular protein leakage have been recorded following administration of NOS inhibitors, thus constitutive nitric oxide would appear to have an anti-inflammatory action (Kubes *et al.* 1991, Kubes and Granger 1992). However, overproduction of nitric oxide, following expression of the inducible enzyme, has been shown to increase vascular permeability and promote oedema (Ialenti *et al.* 1992, Boughton-Smith *et al.* 1993). Nitric oxide also appears to have a dual role in reperfusion injury, with studies demonstrating both protective (Williams *et al.* 1995) and damaging effects (Schulz and Wambolt 1995). Generation of the free radical peroxynitrite may account for much of the tissue damage associated with overproduction of nitric oxide and is a key feature of a number of inflammatory diseases (Miller *et al.* 1995, Sakurai *et al.* 1995, Grabowski *et al.* 1997).

2.1.2.4 The role of nitric oxide in vascular disease

Alterations in the production of nitric oxide have been implicated in the pathophysiology of a number of vascular diseases. Production may be decreased as in atherosclerosis and hypertension or increased, as in septic shock.

Endothelium-dependent relaxation has been shown to be impaired in hypertensive rats (Konishi and Su 1983, Lüscher *et al.* 1987). Similar changes have been demonstrated in hypertensive people (Linder *et al.* 1990, Panza *et al.* 1990). In most types of hypertension the cause is a reduction in the production of nitric oxide, although release of an endothelium-derived contracting factor has been demonstrated in spontaneous hypertension (Kato *et al.* 1990). There may also be a reduction in the sensitivity to nitric oxide, but this is not a consistent finding (Konishi and Su 1983, Linder *et al.* 1990). Morphological abnormalities of the endothelium develop in hypertension and these may be the cause of the altered function (Haudenschild *et al.* 1980). However, there is evidence that endothelial dysfunction may precede the development of hypertension, thereby implying that it has a pathogenic role (Taddei *et al.* 1992).

Endothelium-dependent relaxations are also attenuated in atherosclerotic arteries (Bossaller *et al.* 1987, Förstermann *et al.* 1988b) and a decrease in the release of

nitric oxide has been demonstrated in a bioassay preparation (Guerra *et al.* 1989). Oxidised low density lipoproteins, known to accumulate in atherosclerosis, may mediate this effect by reducing L-arginine availability (Andrews *et al.* 1987, Tanner *et al.* 1991). This accounts for the enhanced endothelium-dependent relaxations that occur in such vessels following L-arginine supplementation (Creager *et al.* 1992). In addition, low density lipoproteins have been shown to inactivate nitric oxide and this effect may be enhanced by the generation of free radicals (Galle *et al.* 1991). Decreased nitric oxide production may also contribute to the proliferation of vascular smooth muscle that is a feature of both atherosclerosis and hypertension (Schwartz 1984, Garg and Hassid 1989).

Septic shock can be defined as a state of circulatory collapse that occurs secondary to sepsis and/or endotoxaemia (Goodwin and Schaer 1989). It is most commonly associated with gram-negative bacteraemia, although gram-positive bacteria, fungi, rickettsia and viruses have all been recorded as causes. Clinically, septic shock can be divided into two phases, a hyperdynamic or compensatory phase, during which cardiac output increases and a hypodynamic phase characterised by low cardiac output, severe hypotension and inadequate tissue perfusion (Adams and Wingfield 1992). Although bacterial toxins cause some direct cellular damage (Meyrick 1986), most of the clinical signs of septic shock are believed to result from the activation of inflammatory mediators, in particular cytokines (Giroir 1993).

The cytokine TNF-α is a key mediator and is produced by activated macrophages on exposure to endotoxin (Beutler *et al.* 1986, Sariban *et al.* 1988). Plasma levels of TNF-α have been shown to be elevated in patients with experimental and clinical endotoxaemia (Van Deventer *et al.* 1990, MacKay *et al.* 1991, Marks *et al.* 1990). Furthermore, high concentrations have been correlated with lethal outcome (Calandra *et al.* 1990). Many of the clinical and histopathological features of septic shock have been reproduced by administration of TNF-α, thereby emphasising its pivotal role (Tracey *et al.* 1986, Tracey *et al.* 1987b). In addition, anti-TNF-α antibodies have been shown to provide some protection against the lethal effects of endotoxaemia (Beutler *et al.* 1985, Tracey *et al.* 1987c, Mathison *et al.* 1988). Signs of septic shock have also been reproduced by administration of the cytokine IL-1β and this agent has been shown to act synergistically with TNF-α (Okusawa *et al.* 1988).

Tumour necrosis factor-α and IL-1β are pro-inflammatory mediators and their biological effects have been reviewed by Dinarello (1991), Bellomo (1992) and Molloy *et al.* (1993). They induce the production of other cytokines, including the chemotactic factors interleukin-8, interleukin-9 and neutrophil activating protein-1 (Schroeder *et al.* 1990). In addition, they induce the expression of phospholipase A_2

and cyclooxygenase, thereby increasing production of secondary inflammatory mediators such as platelet activating factor (Doebber *et al.* 1985), leucotrienes and prostaglandins (Lefer 1985). Both cytokines activate neutrophils and promote their margination and migration into the tissues through the expression of endothelial cell adhesion molecules (Bevilacqua *et al.* 1985, Shalaby *et al.* 1985, Pohlman *et al.* 1986). They are also powerful pro-coagulants and can induce microvascular thrombosis (Bevilacqua *et al.* 1986). Tumour necrosis factor-α has a number of catabolic actions that serve to redistribute proteins and fat from the periphery to the liver (Fong *et al.* 1989), thereby fuelling synthesis of acute-phase proteins (Moldawer *et al.* 1987). In addition, both TNF-α and IL-1β act as endogenous pyrogens through the stimulation of hypothalamic prostaglandin synthesis (Dinarello *et al.* 1986).

Hypotension is a key feature of end-stage septic shock and has been induced experimentally by administration of endotoxin or cytokines (Tracey *et al.* 1986, Okusawa *et al.* 1988). Okusawa *et al.* (1988) found that pre-treatment with a cyclooxygenase inhibitor attenuated cytokine-induced hypotension, implying that a cyclooxygenase product, such as prostaglandin E, was responsible for the reduction in vascular tone. However this finding was not supported by subsequent studies. Wakabayashi *et al.* (1987) found that arteries from endotoxin-treated rats exhibited diminished contractile responses to vasoconstrictors and this was later shown to be reversed by L-NMMA, an inhibitor of NOS (Julou-Schaeffer *et al.* 1990). Cyclooxygenase inhibition did not prevent the development of vascular hyporesponsiveness. Similarly, incubation with cytokines was shown to inhibit contraction of isolated arteries by a mechanism that increased smooth muscle content of cGMP (Beasley *et al.* 1989, Beasley 1990) and could be prevented by antagonists of NOS (Busse and Mülsch 1990b). It was therefore hypothesised that these effects were mediated through increased production of nitric oxide as a result of induction of the calcium-independent isoform of NOS. Expression of this enzyme has been demonstrated in vascular endothelial and smooth muscle cells exposed to endotoxin and/or cytokines (Radomski *et al.* 1990, Rees *et al.* 1990). This theory also accounted for the earlier finding that urinary secretion of nitrate was increased in septic patients and endotoxin-treated mice (Green *et al.* 1981, Wagner *et al.* 1983, Stuehr and Marletta 1985).

Further evidence that overproduction of nitric oxide contributes to the hypotension of septic shock has been provided by *in vivo* studies. Inhibitors of NOS have been shown to restore arterial blood pressure in endotoxin and cytokine-treated animals (Thiemermann and Vane 1990, Kilbourn *et al.* 1990a). This led to the proposal that agents such as L-NMMA may be of therapeutic value in the treatment of clinically-occurring septic shock (Kilbourn *et al.* 1990b). However, more recent studies have

questioned the benefits of indiscriminate NOS inhibition (Cobb *et al.* 1992). L-NMMA has been shown to reduce cardiac output and this, in combination with increased peripheral resistance, may reduce regional blood flow (Klabunde and Ritger 1991). Henderson *et al.* (1994) confirmed that this was the case in the renal and mesenteric vascular beds and reduced perfusion of the latter may account for the increased incidence of intestinal mucosal injury observed in L-NMMA treated-rats (Hutcheson *et al.* 1990). Nava *et al.* (1991) examined the effects of different doses of L-NMMA in endotoxin-treated rats and found that whilst low doses were beneficial, high doses were associated with increased mortality. Furthermore, mortality was shown to be reduced by simultaneous administration of the nitric oxide donor, S-nitroso-N-acetylpenicillamine (Nava *et al.* 1992). These findings led the authors to suggest that whilst inhibition of inducible NOS may be beneficial, inhibition of the constitutive enzyme, by high doses of L-NMMA, compromises tissue perfusion. This theory was supported by the finding that pre-treatment with dexamethasone, which inhibits the expression of inducible NOS (Rees *et al.* 1990), reduced mortality in endotoxin-treated rabbits, whereas L-NMMA, which inhibits both isoforms did not (Wright *et al.* 1992). As a result of these studies selective inhibitors of inducible NOS have been sought. Aminoguanidine has an increased affinity for the inducible enzyme although some authors dispute the extent of its selectivity (Griffiths *et al.* 1993, Misko *et al.* 1993, Laszlo *et al.* 1995). Reports of its use in experimental endotoxaemia are promising. Wu *et al.* (1995) demonstrated a dramatic increase in the survival rate of endotoxin-treated rats following administration of aminoguanidine. No such effect was observed with N-nitro-L-arginine methyl ester, a non-specific NOS inhibitor. Newer more selective agents, such as N^6-iminoethyl-L-lysine (Moore *et al.* 1994) and 2-amino-4-methylpyridine (Faraci *et al.* 1996) have been developed but their value in the therapy of septic shock has yet to be evaluated.

The biological functions of the different isoforms of NOS have been clarified by the development of gene "knockouts". Mice that lack the gene for inducible NOS have been shown to be resistant to endotoxin-induced mortality (Wei *et al.* 1995). In addition, they exhibit increased susceptibility to bacterial and protozoal infections and are less able to restrain the growth of tumour cells (Wei *et al.* 1995, MacMicking *et al.* 1995). Huang *et al.* (1995) showed that mice lacking endothelial NOS are hypertensive, thus confirming the role of the constitutive enzyme in the regulation of blood pressure.

2.1.3 Endothelium-derived hyperpolarizing factor

Rubanyi and Vanhoutte (1987) proposed that the endothelium may release more than one relaxing factor after demonstrating that acetylcholine-induced relaxation had distinct transient and sustained phases. The transient phase was mediated via activation of M_1-muscarinic receptors, whilst M_2-receptors mediated the sustained response. In addition, the two responses were subject to different inhibitory influences (Rubanyi *et al.* 1985, Rubanyi *et al.* 1987, Chen *et al.* 1988). The transient phase was associated with hyperpolarization of the smooth muscle membrane (Komori and Suzuki 1987) and this was subsequently shown to be mediated by a diffusible factor released from the endothelium, i.e. endothelium-derived hyperpolarizing factor (Félétou and Vanhoutte 1988, Chen *et al.* 1991). Endothelium-dependent hyperpolarizations have since been demonstrated in a variety of vessels, from small resistance arteries to large conducting arteries (Adeagbo and Triggle 1993, Kilpatrick and Cocks 1994).

Endothelium-derived hyperpolarizing factor (EDHF) has been shown to produce hyperpolarization by activation of a variety of potassium channels (Waldron *et al.* 1996) and this in turn causes inactivation of voltage-sensitive calcium channels, thereby inhibiting contraction (Nelson and Quayle 1995). The identity of EDHF has yet to be established (Mombouli and Vanhoutte 1997). A small number of endothelium-dependent hyperpolarizations have been shown to be mediated by nitric oxide (Tare *et al.* 1990) and prostacyclin (Parkington *et al.* 1993), although most are unaffected by inhibitors of NOS and cyclooxygenase (Garland *et al.* 1995). Other potential options include epoxyeicosatrienoic acids formed from arachidonic acid by the action of cytochrome P_{450} monooxygenases (Campbell *et al.* 1996), endogenous cannabinoids (Randall *et al.* 1996), carbon monoxide (Morita *et al.* 1995) and reactive oxygen species, such as hydroxyl radicals (Rosenblum 1987).

2.1.4 Endothelium-derived contracting factors

The endothelium is a source of a variety of vasoactive factors, some of which have been shown to mediate contraction (Vanhoutte *et al.* 1991, Lüscher *et al.* 1992). Endothelium-dependent contraction has been demonstrated in association with a number of stimuli, including anoxia (De Mey and Vanhoutte 1981), increased transmural pressure (Harder *et al.* 1987), mechanical stretch (Katusic *et al.* 1987), cyclooxygenase precursors (Miller and Vanhoutte 1985) and agonists such as acetylcholine and calcium ionophore A23187 (Katusic *et al.* 1988).

A proportion of these contractions can be blocked by cyclooxygenase inhibitors, implying that they are mediated by cyclooxygenase products. Three such mediators have been identified. Thromboxane A_2 has been shown to mediate some of the

endothelium-dependent contractions to acetylcholine and calcium ionophore A23187 (Altiere *et al.* 1986, Katusic *et al.* 1988), whilst acetylcholine-induced contractions in spontaneously hypertensive rats have been attributed to prostaglandin H_2 (Toda *et al.* 1988, Kato *et al.* 1990). A third contracting factor is superoxide anion, generated when cyclooxygenase is activated (Kukreja *et al.* 1986, Katusic and Vanhoutte 1989).

2.1.4.1 Endothelin

Cultured endothelial cells were known to release a vasoconstrictor substance that was not blocked by cyclooxygenase inhibition (Hickey *et al.* 1985). This substance proved to be endothelin, the 21 amino acid peptide characterised by Yanagisawa *et al.* (1988). Three isoforms of the peptide have been identified but endothelial cells appear only to release endothelin-1 (Inoue *et al.* 1989). Endothelin-1 is a potent vasoconstrictor of isolated blood vessels (Hickey *et al.* 1985, Yanagisawa *et al.* 1988) and it produces a sustained increase in blood pressure when administered to experimental animals (Goetz *et al.* 1988, Clarke *et al.* 1989). Endothelin is generated from the precursor peptides preproendothelin and proendothelin (Yanagisawa and Masaki 1989). Production is increased by exposure to a variety of stimuli, including the vasopressor hormones adrenaline and angiotensin II, substances released by aggregating platelets such as transforming growth factor β, thrombin and the cytokine IL-1β (Yanagisawa and Masaki 1989, Moon *et al.* 1989, Yoshizumi *et al.* 1990). Notable amongst the agents that inhibit endothelin production are nitric oxide and prostacyclin (Boulanger and Lüscher 1990, Yokokawa *et al.* 1991). Conversely, endothelin can initiate release of nitric oxide (Warner *et al.* 1989).

The interactions between endothelin and nitric oxide have implications for vascular disease, in particular we might expect decreased release of nitric oxide to promote endothelin production. However, elevated circulating levels of endothelin are an inconsistent finding in hypertension (Davenport *et al.* 1990, Saito *et al.* 1990). Similarly, attempts to demonstrate an altered sensitivity to endothelin in this condition have yielded conflicting results (Tomobe *et al.* 1988, Dohi *et al.* 1991). The finding that phosphoramidon, an endothelin converting enzyme inhibitor, significantly reduced blood pressure in spontaneously hypertensive rats, supports the theory that endothelin contributes to raised vascular resistance (McMahon *et al.* 1991). There is also evidence that endothelin contributes to the vasospastic tendency of atherosclerotic vessels. Circulating levels of endothelin are more consistently raised in this disease (Rainer *et al.* 1990, Lerman *et al.* 1991) and oxidised low density lipoproteins have been shown to induce expression of preproendothelin messenger RNA, thereby increasing endothelin production

(Boulanger *et al.* 1992). Furthermore, endothelin may promote vascular smooth muscle proliferation (Simonson *et al.* 1989).

2.1.5 Aims of the study

Clearly, the endothelium is far more than a simple cellular lining, it has an important paracrine function modulating vascular tone through the synthesis and release of vasoactive mediators. Alterations in this function occur in a number of disease states and may contribute to the development of laminitis in ungulate species (Section 1.4.1). Thus, the aim of this study was to characterise the role of the endothelium and its products in the regulation of ovine digital artery tone *in vitro*. The responses to phenylephrine, bradykinin, acetylcholine and sodium nitroprusside were characterised by studying the effects of endothelium-removal and inhibition of NOS and/or cyclooxygenase.

Endotoxin has been implicated in the pathogenesis of laminitis, as have proinflammmatory cytokines (Section 1.6.1). These agents have been shown to alter the responsiveness of isolated rat arteries to vasoactive mediators (Beasley 1990). Therefore, the second aim of this study was to examine the effect of these agents on the vascular responses of the ovine digital artery. Responses to phenylephrine and bradykinin were examined after incubation of isolated arterial rings with endotoxin and combinations of cytokines.

2.2 Materials and methods

2.2.1 Collection and preparation of tissues

Sheep digital arteries were obtained from a local abattoir. Distal forelimbs, disarticulated at the carpus, were collected from sheep, recently killed by stunning and exsanguination. The skin over the palmar-medial aspect of the metacarpus was incised and the median artery was identified and sectioned. A second incision was made distally across the skin and soft tissues just above the bulbs of the heels. This incision was sufficiently deep to section the palmar proper digital arteries at the level of the proximal interphalangeal joint. The median artery was then cannulated with a 24 gauge teflon catheter (Vygon UK Ltd., Cirencester, Gloucestershire, U.K.) and flushed with approximately 5ml of refrigerated oxygenated Krebs solution.

After transport to the laboratory, the remaining skin of the palmar aspect of the distal limb was reflected to permit identification of the palmar common digital artery, a continuation of the median artery. A 5-7cm section of this artery, from just below the point of catheterisation to the level at which bifurcation into proper digital arteries occurs, was dissected free and placed in refrigerated oxygenated Krebs solution in a petri dish. A fine dissection was then performed to clear away the loose connective tissue surrounding the artery. Arterial rings, 2-3mm long, were prepared from the distal portion of the artery using a No. 11 scalpel blade (Swann Morton, Merck Ltd., Poole, Dorset, U.K.). Where necessary, arterial rings were denuded of endothelium by gently rubbing the internal surface of the artery with a wooden cocktail stick (Miller *et al.* 1984). The efficiency of this procedure was assessed by histological examination of rubbed and unrubbed arteries (Section 2.2.7).

2.2.2 Drugs and solutions

2.2.2.1 Krebs Solution

A stock solution was prepared by dissolving 344.77g of sodium chloride (NaCl), 17.00g of potassium chloride (KCl), 18.67g of calcium chloride 2-hydrate ($CaCl_2.2H_2O$), 8.10g potassium dihydrogen orthophosphate (KH_2PO_4) and 14.66g magnesium sulphate 7-hydrate ($MgSO_4.7H_2O$) in distilled water to give a final volume of 5L. This stock solution was stored at room temperature for up to 8 weeks. Working Krebs solution was prepared by mixing 10.00g of glucose, 10.50g of sodium hydrogen carbonate ($NaHCO_3$) and 500ml of stock solution with sufficient distilled water to give a final volume of 5L. The resulting Krebs solution was stored in a refrigerator at approximately 4°C for a maximum of 48 hours and had the following composition (mM): NaCl 118, KCl 4.57, $CaCl_2$ 1.27, KH_2PO_4 1.19, $MgSO_4$ 1.19, $NaHCO_3$ 25 and glucose 11.1. All ingredients were *BDH*

AnalaR reagents obtained from Merck Ltd. (Poole, Dorset, U.K.). Where necessary, the Krebs was oxygenated by bubbling a mixture of 95% oxygen and 5% carbon dioxide (Special Gases Group, Air Products, Crewe, U.K.) through the solution for at least 10 minutes.

2.2.2.2 Phosphate buffered saline

Phosphate buffered saline was prepared by dissolving 10 phosphate buffered saline tablets (Unipath Ltd., Basingstoke, Hampshire, U.K.) in 1L of distilled water. After preparation, the pH of the solution was checked with a *Kent 7065* digital pH meter (R. & J. Wood Laboratory Supplies, Paisley, Scotland, U.K.) and if necessary, adjusted to pH 7.4 by adding 2M hydrochloric acid or 2M sodium hydroxide (Merck Ltd., Poole, Dorset, U.K.). The solution was then autoclaved.

2.2.2.3 Drugs

Acetylcholine chloride, L-arginine hydrochloride, bradykinin acetate, indomethacin, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), phenylephrine hydrochloride, polymyxin B sulfate, prazosin hydrochloride and sodium nitroprusside dihydrate were all purchased from Sigma Chemical Company (Poole, Dorset, U.K.).

Solutions of acetylcholine, L-NAME, phenylephrine, polymyxin and sodium nitroprusside were freshly prepared on the morning of the experiment by dissolving the drug in sterile phosphate buffered saline. Solutions were stored on ice, prior to, and during the experiment. Aliquots of sodium nitroprusside were wrapped in tin foil to prevent inactivation by sunlight. Stock solutions of the remaining drugs were stored at -20°C and were thawed prior to use. A 10^{-3}M stock solution of bradykinin was prepared in phosphate buffered saline and frozen in 400µl aliquots. Warming was required to prepare a 1M stock solution of L-arginine in phosphate buffered saline. This was also frozen in 400µl aliquots. Prazosin and indomethacin were initially dissolved in 100% ethanol to give 10^{-2}M stock solutions. These were further diluted to 10^{-3}M in phosphate buffered saline before freezing.

2.2.2.4 Endotoxin

Lipopolysaccharide extracted from *Escherichia coli*, serotype 055:B5, was purchased from Sigma Chemical Company (Poole, Dorset, U.K.). Solutions were prepared in phosphate buffered saline on the morning of the experiment and were stored in the refrigerator in polystyrene tubes, prior to use.

2.2.2.5 Cytokines

Human recombinant interferon-γ (IFN-γ), interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) were purchased from Sigma Chemical Company (Poole, Dorset, U.K.). Interferon-γ was reconstituted in sterile phosphate buffered saline to

give a solution of 10μg/ml. Interleukin-1β was reconstituted in phosphate buffered saline containing 1% bovine serum albumin to give a 10μg/ml solution. Tumour necrosis factor-α was supplied as a 10μg/ml solution in phosphate buffered saline containing 0.1% bovine serum albumin. All solution were divided into aliquots and stored at -20°C.

2.2.2.6 Culture medium

Digital arterial rings were incubated in *Medium 199* with Earle's salts, glutamine and 25mM Hepes buffer (GibcoBRL, Life Technologies Ltd., Paisley, Scotland, U.K.) during prolonged exposure to endotoxin and cytokines.

2.2.3 Measurement of isometric tension in isolated digital arteries

The digital arterial rings were mounted between stainless steel wires in 10ml tissue baths containing warm (30°C), oxygenated Krebs (Figure 2.2.1). A temperature of less than 37°C was selected to reduce spontaneous activity of the vascular smooth muscle (Elliott *et al.* 1994). The lower wire was fixed in position, whilst the upper wire was attached to an FT03 force-displacement transducer (Grass Instrument Company, Massachusetts, U.S.A.), which measured isometric tension. The output from each of the transducers was relayed via a pre-amplifier to a 4 channel *Multitrace* pen recorder (Lectromed UK Ltd., Letchworth, Hertfordshire, U.K.). In a small number of experiments a polygraph (*Grass Model 7E*, Grass Instrument Company, Massachusetts, U.S.A.) was used in place of the pre-amplifier and pen recorder. Prior to each experiment the transducers were calibrated at 2 different sensitivities using a selection of standard weights.

Once suspended, the arterial rings were stretched to a tension of 2g and allowed to equilibrate for 2 hours before beginning the experiment. During equilibration, fresh Krebs solution was added every 30 minutes and vessel tension, which tended to fall, was adjusted back to 2g. This degree of passive stretch was chosen on the basis of preliminary experiments, which showed phenylephrine responses to be optimal at a tension of 2g. Increasing stretch to 4g did increase the maximum response but the contractions tended to be unstable. After equilibration, vessel viability was assessed by adding phenylephrine. Those vessels that produced less than 2g of tension in response to 10^{-6}M phenylephrine were not used. The vessels were then washed with fresh Krebs solution to allow tension to fall to baseline before beginning an experiment. On completion of an experiment, all arterial rings were removed from the tissue bath and weighed whilst still wet.

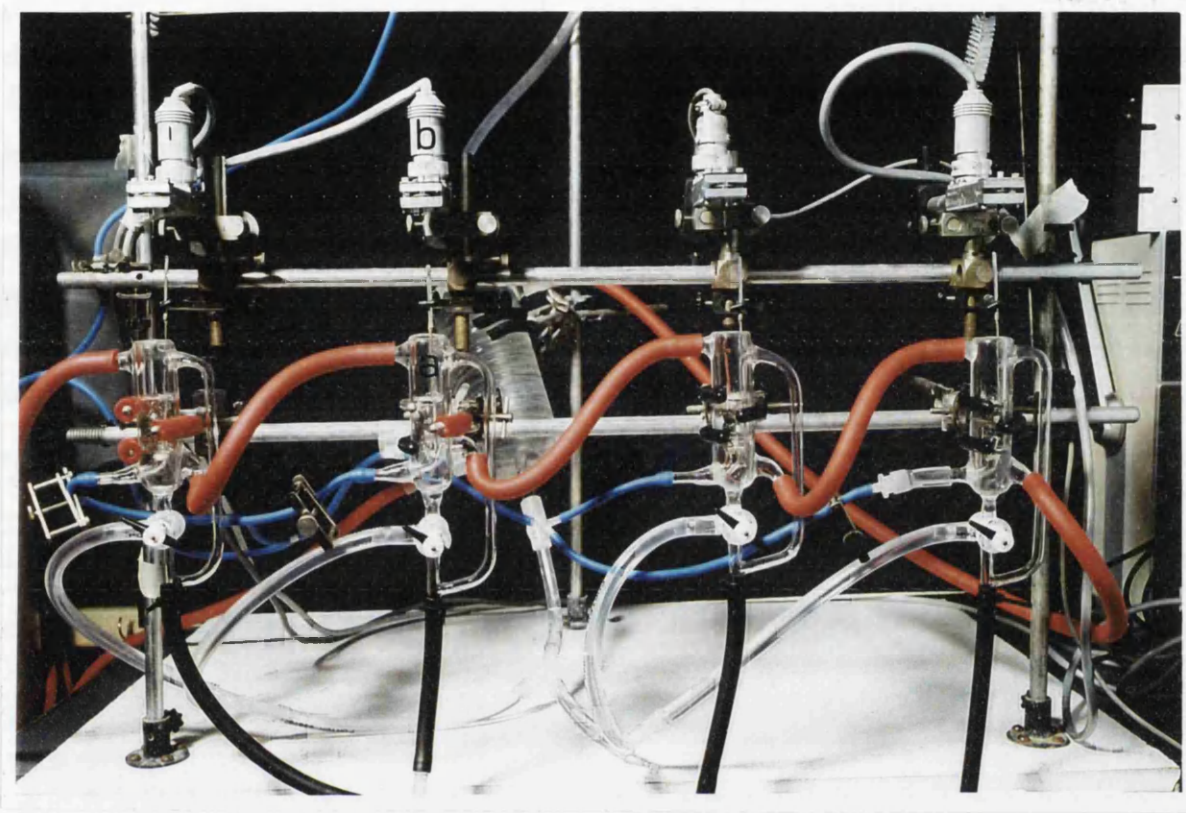


Figure 2.2.1. Photograph of the tissue bath experimental setup. Four glass water-jacketed tissue baths (a), maintained at 30°C, were positioned below 4 force-displacement transducers (b) connected to a pre-amplifier and pen recorder. In each tissue bath an arterial ring was suspended between stainless steel wires in Krebs solution bubbled continuously with 95% oxygen and 5% carbon dioxide.

2.2.4 Experimental Design

2.2.4.1 Effect of endothelium removal

Cumulative concentration-response curves to the vasoconstrictor agent phenylephrine (10^{-9} to $3x10^{-4}$M) were constructed in (i) intact and (ii) endothelium-denuded digital arterial rings. Tension was allowed to stabilise before each new dose of drug was added. Once the dose-response curves were completed the vessels were washed several times with fresh Krebs and tension was allowed to fall to baseline. A second experiment was conducted to confirm that the contraction produced in response to phenylephrine was mediated via α_1-adrenergic receptors. Both intact and endothelium-denuded digital arterial rings were pre-incubated for 20 minutes with (i) no additions, i.e. time-based control, or (ii) the α_1-adrenergic receptor antagonist prazosin (10^{-7}M). All contractile responses were expressed as tension in g/g tissue wet weight.

To investigate vasodilator responses, the arterial rings were first contracted using phenylephrine ($6x10^{-7}$M). This dose gave approximately 50% of the maximum response, as determined above. Once the contraction stabilised, cumulative concentration-response curves to bradykinin (10^{-10} to $3x10^{-6}$M), acetycholine (10^{-9} to 10^{-3}M) and sodium nitroprusside (10^{-9} to $3x10^{-4}$M) were constructed in (i) intact and (ii) endothelium-denuded digital arterial rings. As for phenylephrine, tension was allowed to stabilise before each new dose of drug was given. The relaxant response to bradykinin failed to stabilise and transient relaxation gave way to contraction, making construction of a cumulative dose-response curve difficult. Dose-response curves for bradykinin were therefore repeated in a non-cumulative fashion, each dose of bradykinin being added to a stable contraction produced by $6x10^{-7}$M phenylephrine. All relaxant responses were expressed as the percentage change in tension, i.e. the tension change (g/g) as a percentage of the phenylephrine-induced tension (g/g).

2.2.4.2 Effect of nitric oxide synthase and/or cyclooxygenase inhibition

These experiments were all conducted in intact, i.e. unrubbed, digital arterial rings. As above a contraction of $\geq$ 2g tension in response to 10^{-6}M phenylephrine, was taken to indicate a viable vessel. The integrity of the endothelium was checked using bradykinin (10^{-8}M), only vessels that relaxed by 10% or more were used further.

Arterial rings were pre-incubated for 20 minutes with (i) no additions, (ii) the cyclooxygenase inhibitor indomethacin (10^{-5}M), (iii) the nitric oxide synthase inhibitor L-NAME ($3x10^{-4}$M) or (iv) both indomethacin (10^{-5}M) and L-NAME ($3x10^{-4}$M). Concentration-response curves to phenylephrine (10^{-9} to $3x10^{-4}$M),

bradykinin ($3x10^{-10}$ to 10^{-7}M) and sodium nitroprusside (10^{-9} to $3x10^{-5}$M) were then constructed, as described previously.

In a second experiment, bradykinin dose-response curves were compared in digital arteries pre-incubated with (i) no additions, (ii) L-arginine (10^{-2}M), a substrate for nitric oxide, (iii) L-NAME ($3x10^{-4}$M) or (iv) both L-arginine (10^{-2}M) and L-NAME ($3x10^{-4}$M).

2.2.4.3 Effect of incubating digital arterial rings with endotoxin

In a preliminary experiment, the effect of the endotoxin-binding agent polymyxin on digital artery vascular responses was investigated. Arterial rings were pre-incubated for 20 minutes with (i) no additions or (ii) 10μg/ml polymyxin and dose-response curves to phenylephrine (10^{-9} to $3x10^{-4}$M) and bradykinin (10^{-10} to 10^{-7}M) were determined.

For the main experiment, digital arterial rings were suspended in the tissue bath and bathed in warm (30°C), oxygenated Krebs, containing (i) 10μg/ml polymyxin, i.e. time-based control or (ii) 3μg/ml endotoxin, for 6 hours. During the incubation period, passive tension was maintained at 2g and the Krebs solution ± endotoxin or polymyxin, was changed every 30 minutes. Following incubation, dose-response curves to phenylephrine (10^{-9} to $3x10^{-4}$M) and bradykinin ($3x10^{-10}$ to 10^{-7}M) were obtained. Samples of the Krebs solution were taken from both control (minus polymyxin) and endotoxin-treated tissue baths and were stored at -20°C prior to being assayed for endotoxin content (Section 2.2.6).

In a second experiment, vessels were stored in refrigerated oxygenated Krebs for approximately 7 hours before being transferred to 10ml *Medium 199* containing either (i) endotoxin (10μg/ml) or (ii) polymyxin (10μg/ml). The arterial rings were incubated at 37°C for approximately 16 hours in a cell culture incubator (5% CO_2), before being suspended in the tissue bath and equilibrated as previously described. In this experiment, vessels were not discarded if they failed to produce 2g of tension in response to 10^{-6}M phenylephrine or failed to relax to 10^{-8}M bradykinin. Cumulative concentration-response curves to phenylephrine were constructed in both endotoxin (10μg/ml) and polymyxin-treated (10μg/ml) arteries.

2.2.4.4 Effect of incubating digital arterial rings with cytokines

The 2 experiments used to investigate endotoxin effects were repeated here, substituting cytokines for endotoxin. Thus, dose-response curves to phenylephrine (10^{-9} to $3x10^{-4}$M) and bradykinin ($3x10^{-10}$ to 10^{-7}M) were compared in arterial rings pre-incubated for 6 hours in warm (30°C), oxygenated Krebs with (i) 10μg/ml polymyxin or (ii) IFN-γ (10ng/ml) and TNF-α (5ng/ml). Also, the phenylephrine dose-response curve was examined after a 16 hour incubation in *Medium 199* at

37°C, containing either (i) polymyxin (10μg/ml) or the cytokine mixture IFN-γ (10ng/ml), IL-1β (20ng/ml) and TNF-α (5ng/ml).

2.2.5 Data analysis

Concentration-response curves were constructed by plotting either tension or percentage change in tension against the logarithm of drug concentration. A non-linear curve-fitting package (*Origin 4.1*, Microcal Software Inc., Massachusetts, U.S.A.) was used to fit the curves to the Boltzman equation:

$$y = \frac{A_1 - A_2}{1 + e^{(x-xo)/dx}} + A_2$$

where A_1 = initial y value
A_2 = final y value
x_o = centre x value
dx = width

This permitted derivation of the maximum response (E_{max}), which is equal to A_2 and the EC_{50}, which is given by x_o. Mean E_{max} and mean EC_{50} values were calculated for each treatment group and parallel control and these parameters were compared by one-way analysis of variance (*Minitab for Windows 10.2*, Minitab Inc., Pennsylvania, U.S.A.). A p-value of less than 0.05 was taken to indicate a statistically significant difference. Where necessary, results were further analysed by Tukey's pairwise comparisons (*Minitab for Windows 10.2*).

The results for section 2.3.3.1 are expressed as ranges due to the small sample size. All other results are expressed as mean values ± SEM.

2.2.6 Endotoxin assay

To investigate the effect of endotoxin on vascular reactivity, endotoxin must be absent from the control Krebs solution. Since endotoxin contamination is common it was necessary to estimate the amount of endotoxin in control and treated samples. An *E-Toxate* kit (Sigma Chemical Company, Poole, Dorset, U.K.) was purchased for this purpose. The reagent used in this kit is a lysate of circulating amoebocytes from the horseshoe crab, *Limulus polyphemus*. This lysate (LAL) gels when exposed to endotoxin, allowing detection and semi-quantitation of the amount of endotoxin present.

The reagent is very sensitive and therefore endotoxin-free equipment was required. Glassware was soaked overnight in a 1% solution of an alkaline detergent, *E-Toxa-Clean* (Sigma Chemical Company). It was then thoroughly rinsed in distilled water, dried in a hot-air oven and finally autoclaved.

E-Toxate working solution, i.e. the lysate, was reconstituted using endotoxin free water according to the manufacturers instructions, and was stored on ice prior to use. The endotoxin standard was also reconstituted and was mixed vigorously for at least 2 minutes, using a vortex mixer. From this stock solution, a series of 9 standard dilutions were prepared and stored in the refrigerator.

2.2.5.1 Determination of LAL assay sensitivity

Lysate was added to the standard endotoxin dilutions in order to determine the lowest concentration of endotoxin that gave a positive test result, a positive result being the formation of a hard gel that was not disrupted by complete inversion of the assay tube.

2.2.5.2 Detection and semi-quantitation of endotoxin in samples

Three samples were assayed, a sample of Krebs from a control tissue bath (prior to addition of polymyxin), a sample of Krebs from a tissue bath to which 3μg/ml endotoxin had been added and a sample of distilled water from which the Krebs solution was prepared. Firstly, lysate was added to the undiluted samples to detect any endotoxin present. A positive control, to screen for lysate inhibitors and a negative control, to check for contamination, were also included. The tissue bath samples were serially diluted, 1/2, 1/4, 1/8, 1/16, 1/32 etc. and these dilutions were tested for endotoxin to determine the greatest dilution which still gave a positive result. The endotoxin concentration was then estimated from the following equation:

$$\text{endotoxin (EU/ml)} = \frac{\text{lowest conc. of endotoxin standard to give a +ve result}}{\text{greatest dilution of sample to yield a +ve result}}$$

All samples and dilutions were assayed in duplicate and where one positive and one negative result were obtained a third test was performed. Negative controls, i.e. endotoxin free water plus lysate, were included in each batch of assayed samples.

2.2.7 Histological examination

Rubbed and unrubbed digital arterial rings were fixed by immersion in a 10% solution of formalin for a minimum of 48 hours. The tissues were dehydrated, cleared with xylene and then embedded in paraffin wax. Transverse cross-sections of the arteries were prepared and stained with either haematoxylin and eosin or Verhoeff's haematoxylin, which highlights the internal elastic lamina. In addition, some sections were stained for Von Willebrand's factor, which is associated specifically with endothelial cells. Standard methods were used (Smith and Bruton 1978, Junqueira *et al.* 1995).

2.3 Results

2.3.1 Effect of endothelium removal

2.3.1.1 Phenylephrine

Cumulative dose-response curves to phenylephrine were constructed in intact, i.e. unrubbed, and endothelium-denuded, i.e. rubbed, ovine digital arteries (Figure 2.3.1). Removal of the endothelium slightly increased E_{max} from 2107 ± 76g/g (n = 12) to 2188 ± 129g/g (n = 12), and decreased log EC_{50} from -6.13 ± 0.07 (n = 12) to -6.29 ± 0.05 (n = 12). However, these changes failed to reach statistical significance.

Pre-incubation with prazosin (10^{-7}M), a specific α_1-adrenergic receptor antagonist, shifted the phenylephrine dose-response curves to the right in both rubbed and unrubbed ovine digital arteries (Figure 2.3.2). Prazosin did not significantly alter the E_{max} values, but log EC_{50} was significantly increased from -5.88 ± 0.07 (n = 6) to -3.34 ± 0.13 (n = 6) in unrubbed arteries ($p < 0.001$), and from -5.92 ± 0.03 (n = 6) to -3.49 ± 0.09 (n = 6) in rubbed arteries ($p < 0.001$).

2.3.1.2 Bradykinin

Cumulative dose-response curves to bradykinin were constructed in rubbed and unrubbed ovine digital arteries (Figure 2.3.3). In the presence of an intact endothelium bradykinin induced transient relaxation followed by contraction. This contraction interfered with the construction of a cumulative dose-response curve and necessitated repeating the dose-response curves in a non-cumulative fashion (Figure 2.3.4). Only the non-cumulative data were analysed further. Removal of the endothelium abolished the relaxant response to bradykinin, however contraction still occurred. Thus E_{max} increased from -61.8 ± 7.4% (n = 8) in unrubbed arteries to 76.0 ± 12.1% (n = 8) in rubbed arteries ($p < 0.001$). The log EC_{50} values for the relaxant (-8.68 ± 0.13, n = 8) and contractile responses (-7.91 ± 0.27, n = 8) were also significantly different ($p = 0.022$).

2.3.1.3 Acetylcholine

Cumulative dose-response curves to acetylcholine were constructed in rubbed and unrubbed ovine digital arteries (Figure 2.3.5). Acetylcholine induced a variable relaxant response in unrubbed arteries and curve-fitting was only possible in 15 out of 18 dose-response curves. This yielded an E_{max} of -16.1 ± 12.1% and a log EC_{50} of -5.52 ± 0.24. No response was recorded in arteries from which the endothelium had been removed.

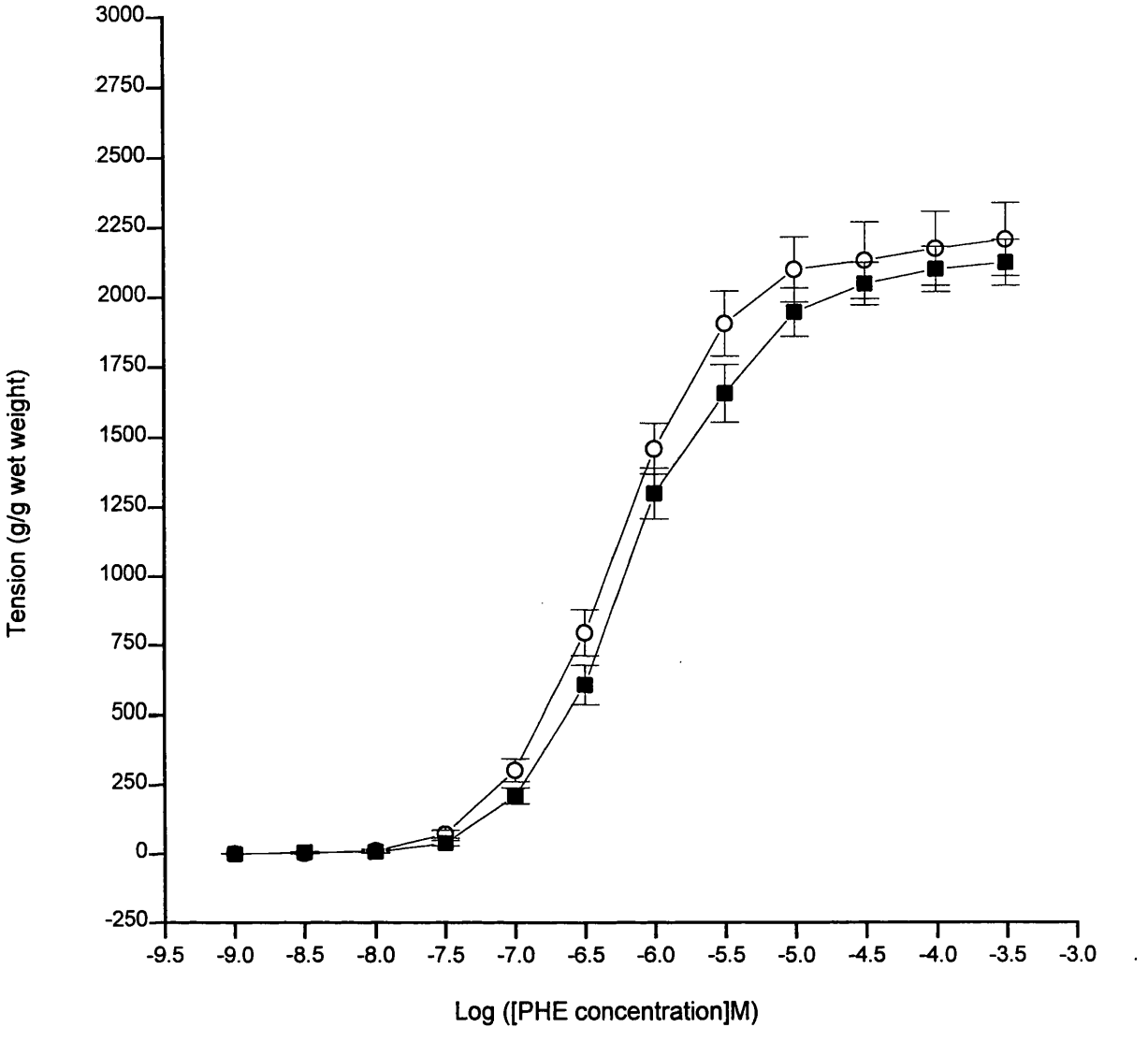


Figure 2.3.1. Cumulative phenylephrine (PHE) dose-response curves in rubbed and unrubbed ovine digital arteries. Each curve represents the mean values (± SEM) of 12 vessels from 6 animals.

—■— unrubbed

—○— rubbed

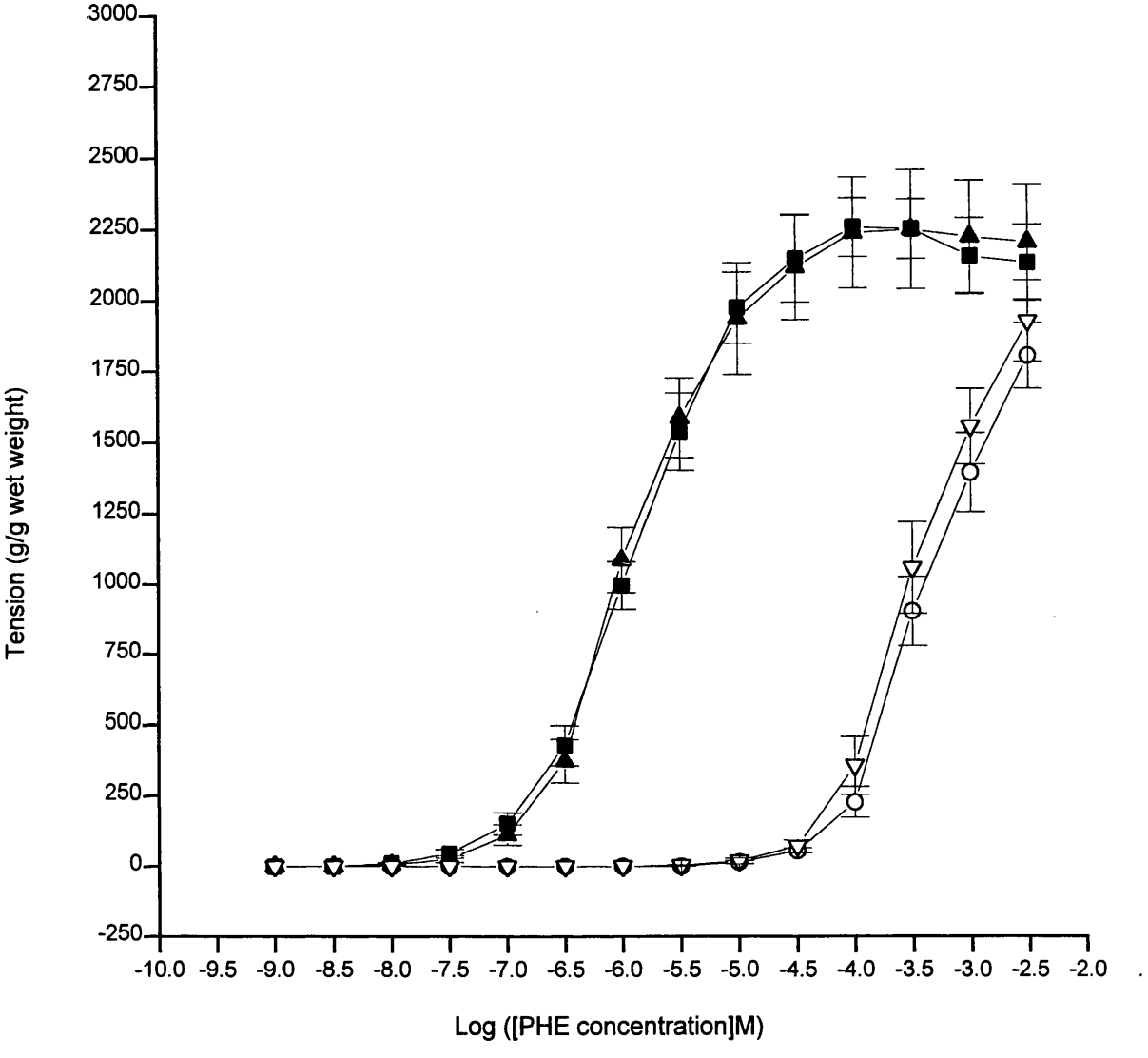


Figure 2.3.2. The effect of pre-incubation with prazosin (10^{-7}M) on the phenylephrine (PHE) dose-response curve in rubbed and unrubbed ovine digital arteries. Each curve represents the mean values (±SEM) for 6 vessels from 6 animals.

—■— unrubbed control

—○— unrubbed + prazosin

—▲— rubbed control

—▽— rubbed + prazosin

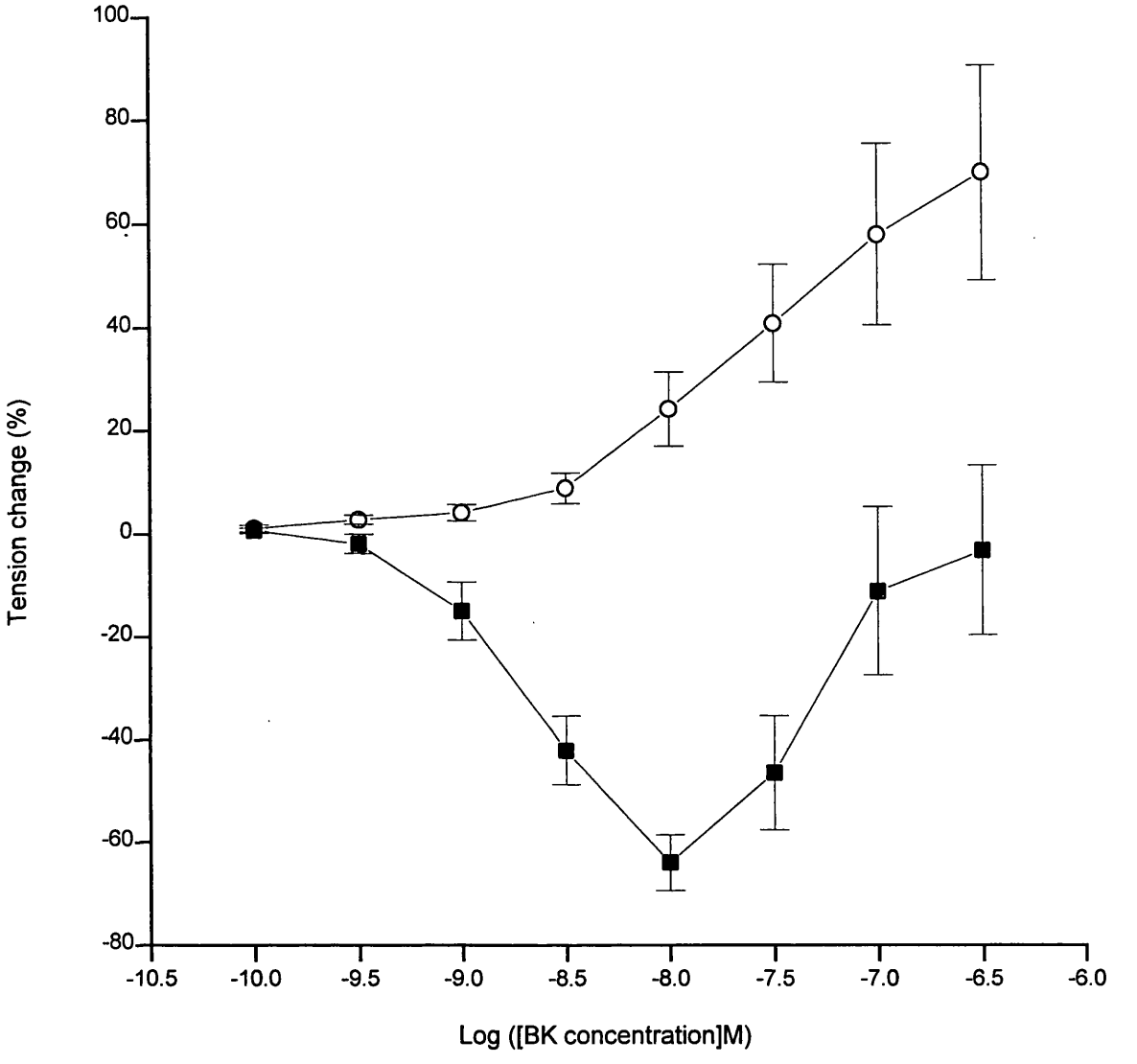


Figure 2.3.3. Cumulative bradykinin (BK) dose-response curves in rubbed and unrubbed ovine digital arteries pre-contracted with $6x10^{-7}M$ phenylephrine. Each curve represents the mean values (±SEM) for 12-13 vessels from 6-7 animals.

—■— unrubbed

—○— rubbed

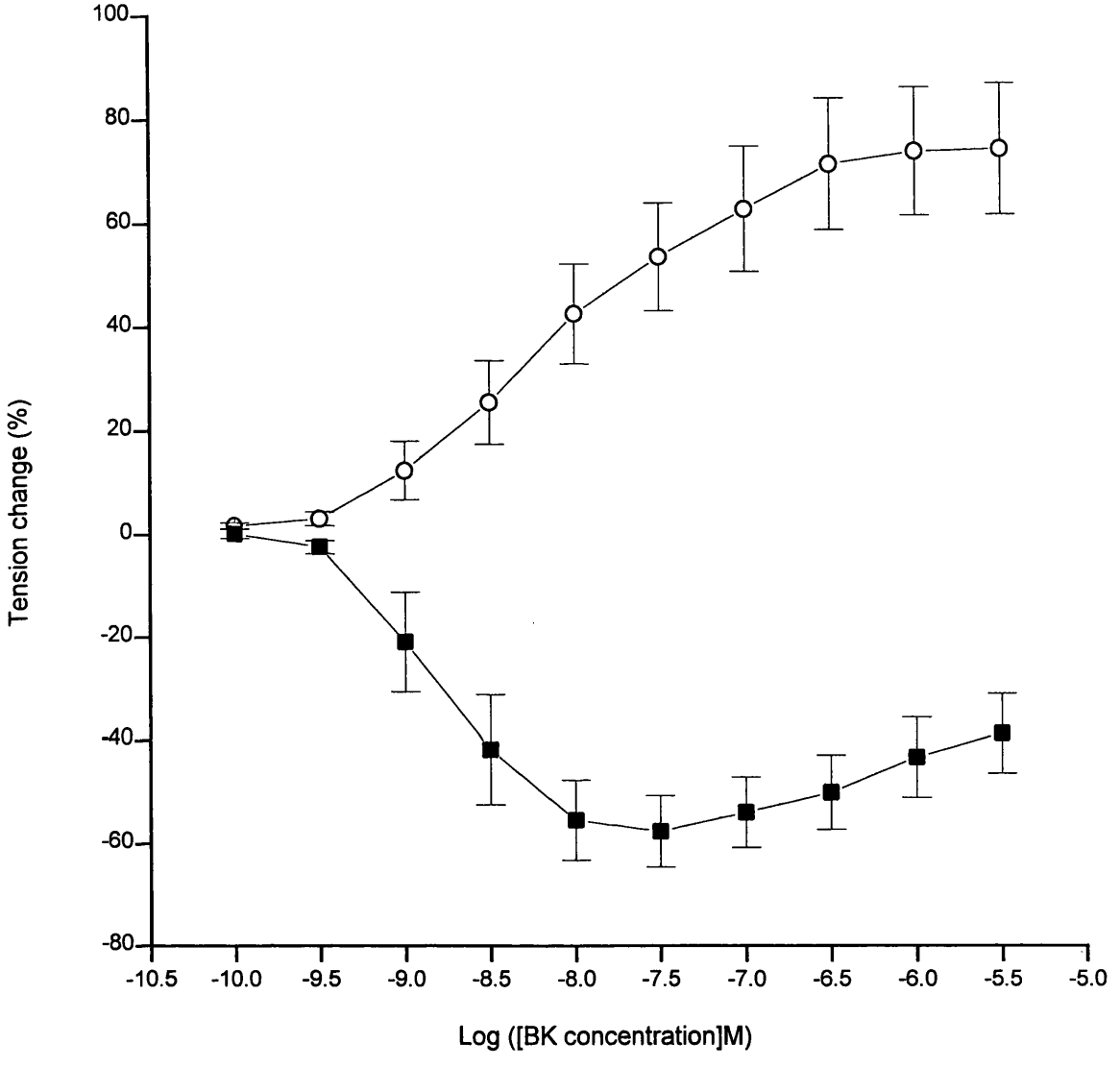


Figure 2.3.4. Non-cumulative bradykinin (BK) dose-response curves in rubbed and unrubbed ovine digital arteries pre-contracted with $6x10^{-7}$M phenylephrine. Each curve represents the mean values (±SEM) for 8 vessels from 8 animals.

—■— unrubbed

—○— rubbed

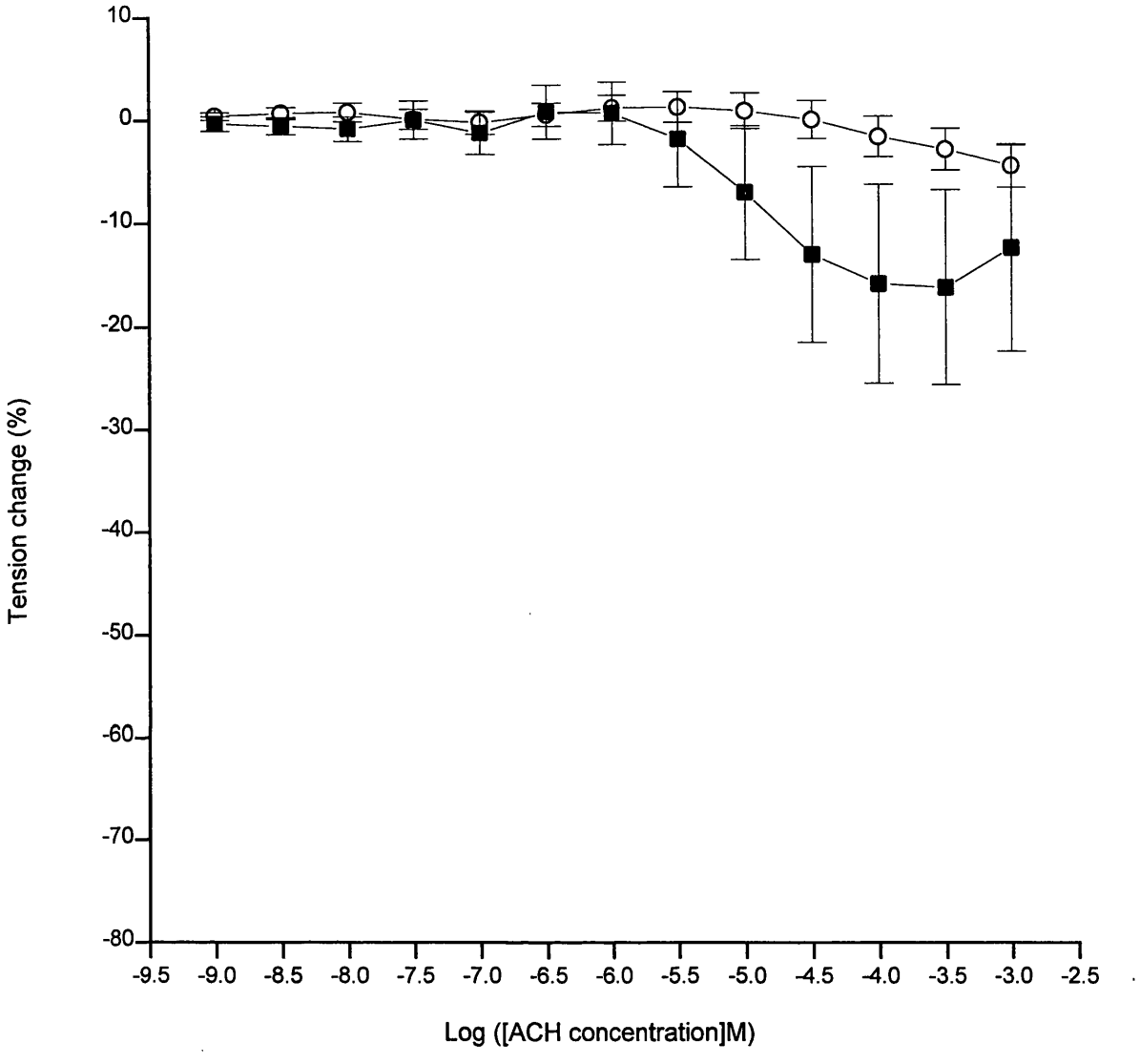


Figure 2.3.5. Cumulative acetylcholine (ACH) dose-response curves in rubbed and unrubbed ovine digital arteries pre-contracted with $6x10^{-7}$M phenylephrine. Each curve represents the mean values (±SEM) of 18-21 vessels from12 animals.

—■— unrubbed

—○— rubbed

2.3.1.4 Sodium nitroprusside

Removal of the endothelium did not significantly alter the dose-response curve to sodium nitroprusside (Figure 2.3.6). Whilst log EC_{50} decreased slightly, from -6.56 ± 0.19 (n = 7) to -6.68 ± 0.15 (n = 7), E_{max} values were similar, i.e. -103.6 ± 1.2% (n = 7) in unrubbed arteries and -103.8 ± 0.5% (n = 7) in rubbed arteries.

2.3.2 Effect of nitric oxide synthase and/or cyclooxygenase inhibition

2.3.2.1 Phenylephrine

Cumulative dose-response curves to phenylephrine were conducted in ovine digital arteries pre-incubated with $3x10^{-4}$M N^G-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor, and/or 10^{-5}M indomethacin, a cyclooxygenase inhibitor (Figure 2.3.7). Dose-response curve parameters were not significantly altered by L-NAME, however, indomethacin both alone and in combination with L-NAME, significantly reduced the log EC_{50} from -6.22 ± 0.08 (n = 8) to -6.55 ± 0.07 (n = 7) and -6.68 ± 0.05 (n = 6) respectively. No significant changes in E_{max} were recorded.

2.3.2.2 Bradykinin

Non-cumulative dose-response curves to bradykinin were constructed in ovine digital arteries pre-incubated with $3x10^{-4}$M L-NAME and/or 10^{-5}M indomethacin (Figure 2.3.8). Treatment with L-NAME significantly decreased the maximum relaxation to bradykinin, reducing E_{max} from -61.7 ± 5.6% (n = 6) to -34.0 ± 2.1% (n = 7). Whilst indomethacin alone had no significant effect on the dose-response curve parameters, when combined with L-NAME it produced a further significant decrease in E_{max} from -34.0 ± 2.1% (n = 7) to -9.6 ± 2.7% (n = 7). No significant changes in log EC_{50} were recorded.

Non-cumulative dose-response curves to bradykinin were also determined following pre-incubation with $3x10^{-4}$M L-NAME and/or 10^{-2}M L-arginine (Figure 2.3.9). Treatment with L-NAME significantly reduced E_{max} from -71.3 ± 6.0% (n = 6) to -30.7 ± 8.0% (n = 7) and increased log EC_{50} from -8.80 ± 0.12 (n = 6) to -8.14 ± 0.13 (n = 7). However, these effects were not observed when L-NAME was combined with L-arginine and the E_{max} (-55.3 ± 4.2%, n = 6) and EC_{50} (-8.49 ± 0.12, n = 6) for the combination were not significantly different from control. L-arginine alone did not significantly alter dose-response curve parameters.

2.3.2.3 Sodium nitroprusside

Pre-incubation with $3x10^{-4}$M L-NAME and/or 10^{-5}M indomethacin did not significantly alter sodium nitroprusside dose-response curves (Figure 2.3.10). There was however a tendency for log EC_{50} to decrease, from -6.46 ± 0.12 (n = 6) in control arteries, to -6.72 ± 0.06 (n = 6) in indomethacin-treated arteries, -6.70 ± 0.15

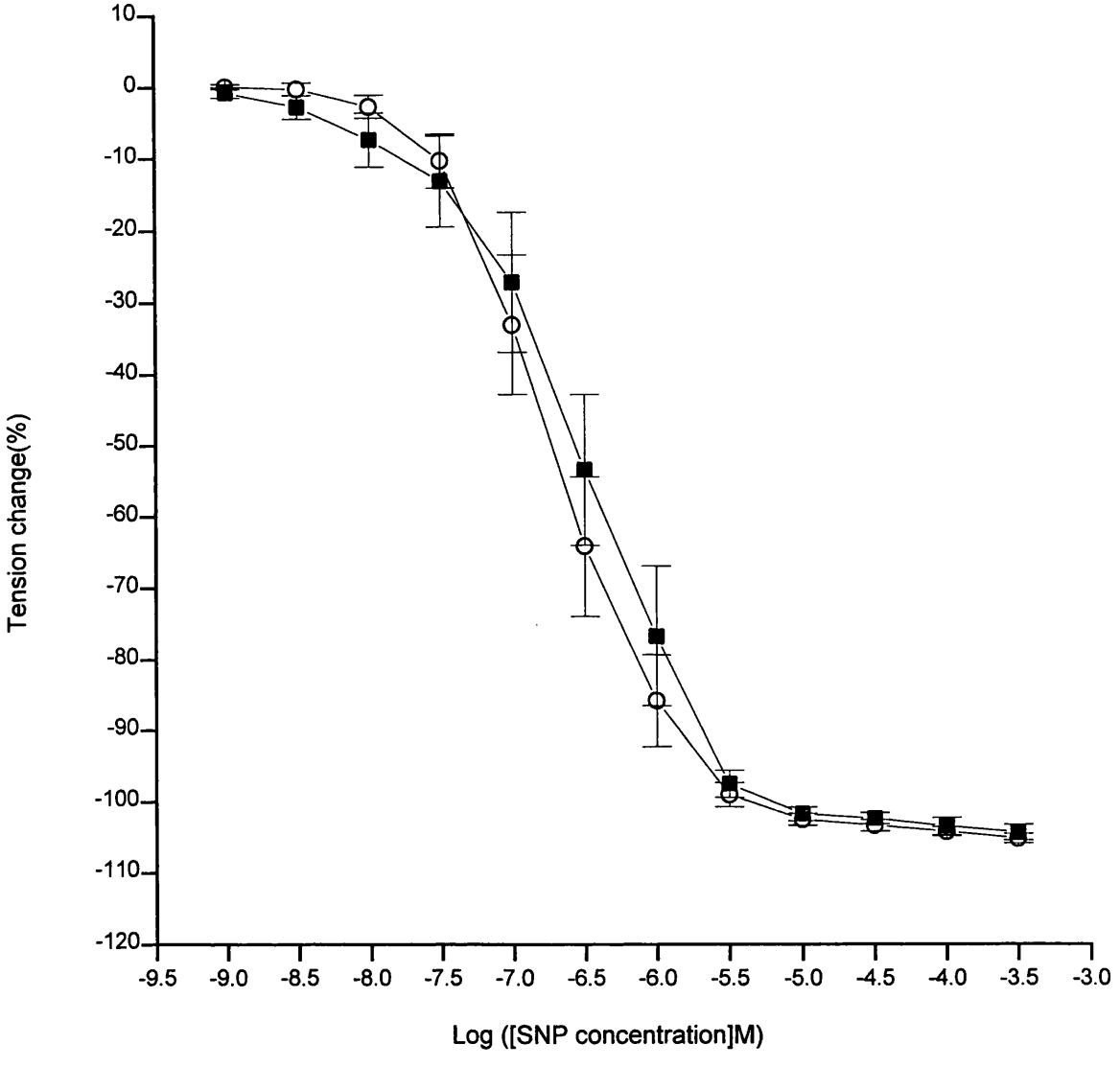


Figure 2.3.6. Cumulative sodium nitroprusside (SNP) dose-response curves in rubbed and unrubbed ovine digital arteries pre-contracted with $6x10^{-7}$M phenylephrine. Each curve represents the mean values (±SEM) of 7 vessels from 7 animals.

—■— unrubbed

—○— rubbed

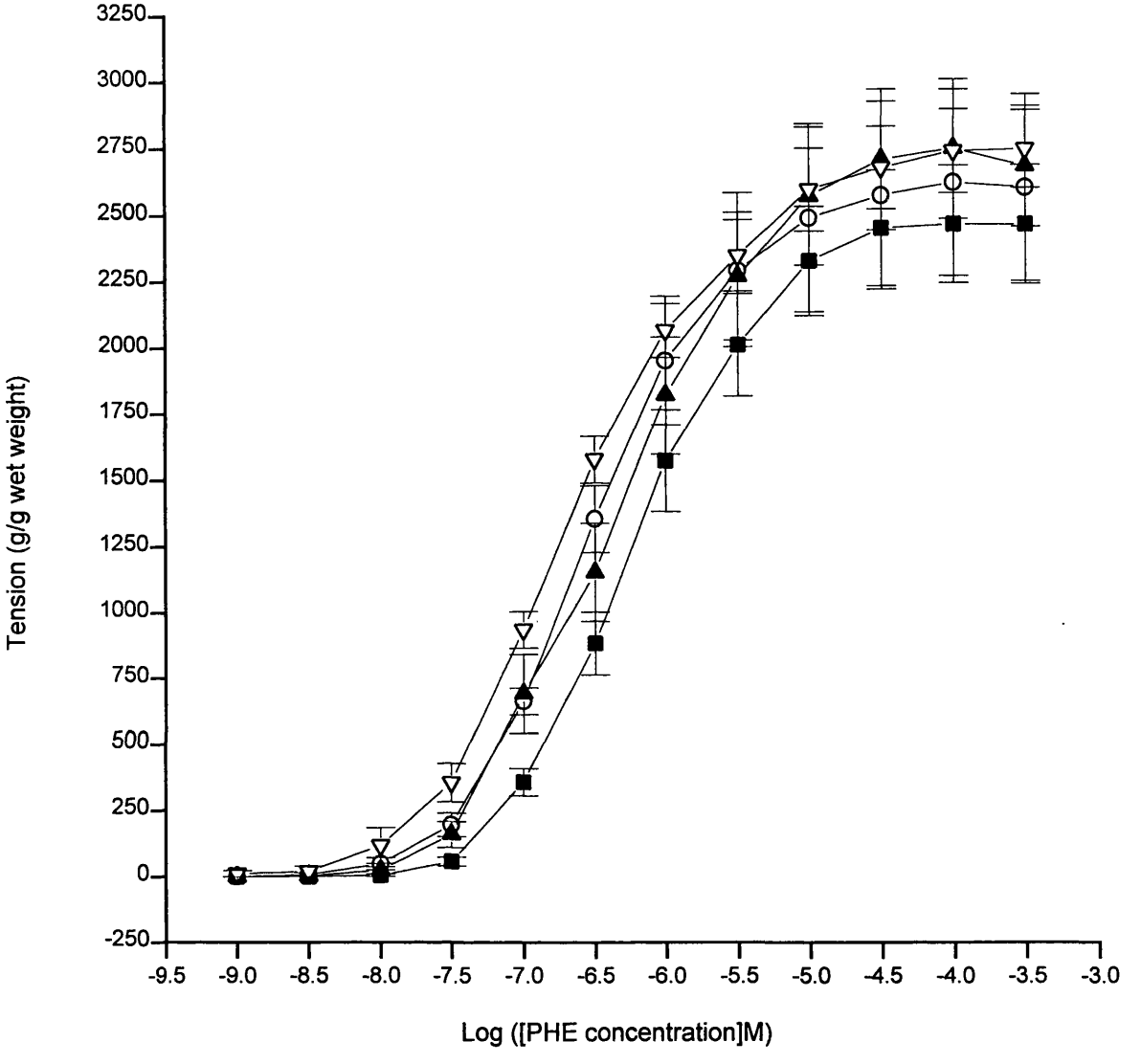


Figure 2.3.7. The effect of pre-incubation with indomethacin (10^{-5}M) and/or N^{G}-nitro-L-arginine methyl ester (L-NAME, $3x10^{-4}$M) on the phenylephrine (PHE) dose-response curve in ovine digital arteries. Each curve represents the mean values (± SEM) of 6-8 vessels from 6-8 animals.

—■— control

—○— indomethacin

—▲— L-NAME

—▽— indomethacin + L-NAME

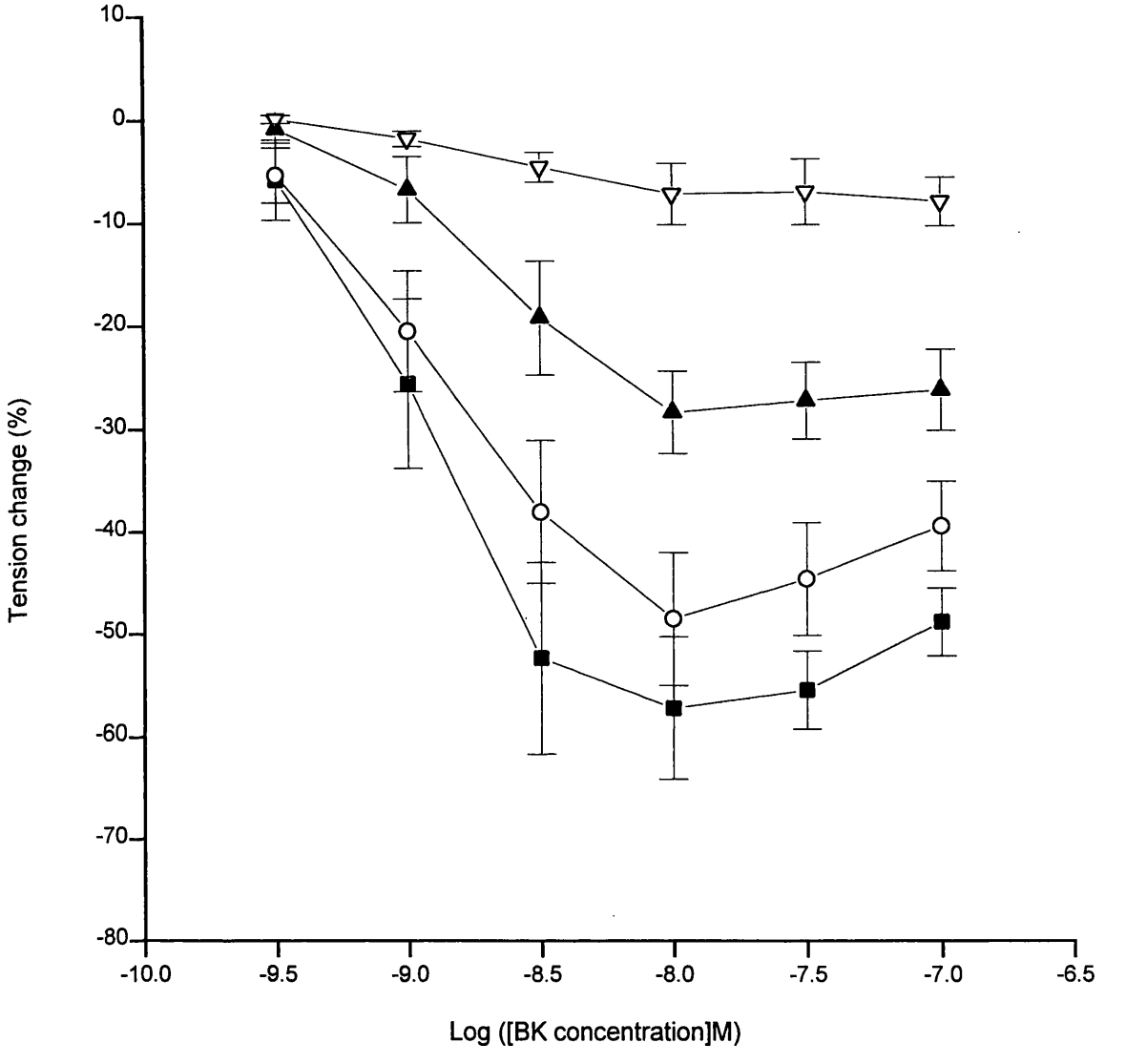


Figure 2.3.8. The effect of pre-incubation with indomethacin (10^{-5}M) and/or N^{G}-nitro-L-arginine methyl ester (L-NAME, $3x10^{-4}$M) on the bradykinin (BK) dose-response curve in ovine digital arteries pre-contracted with $6x10^{-7}$M phenylephrine. Each curve represents the mean values (±SEM) of 6-7 vessels from 6-7 animals.

—■— control

—○— indomethacin

—▲— L-NAME

—▽— indomethacin + L-NAME

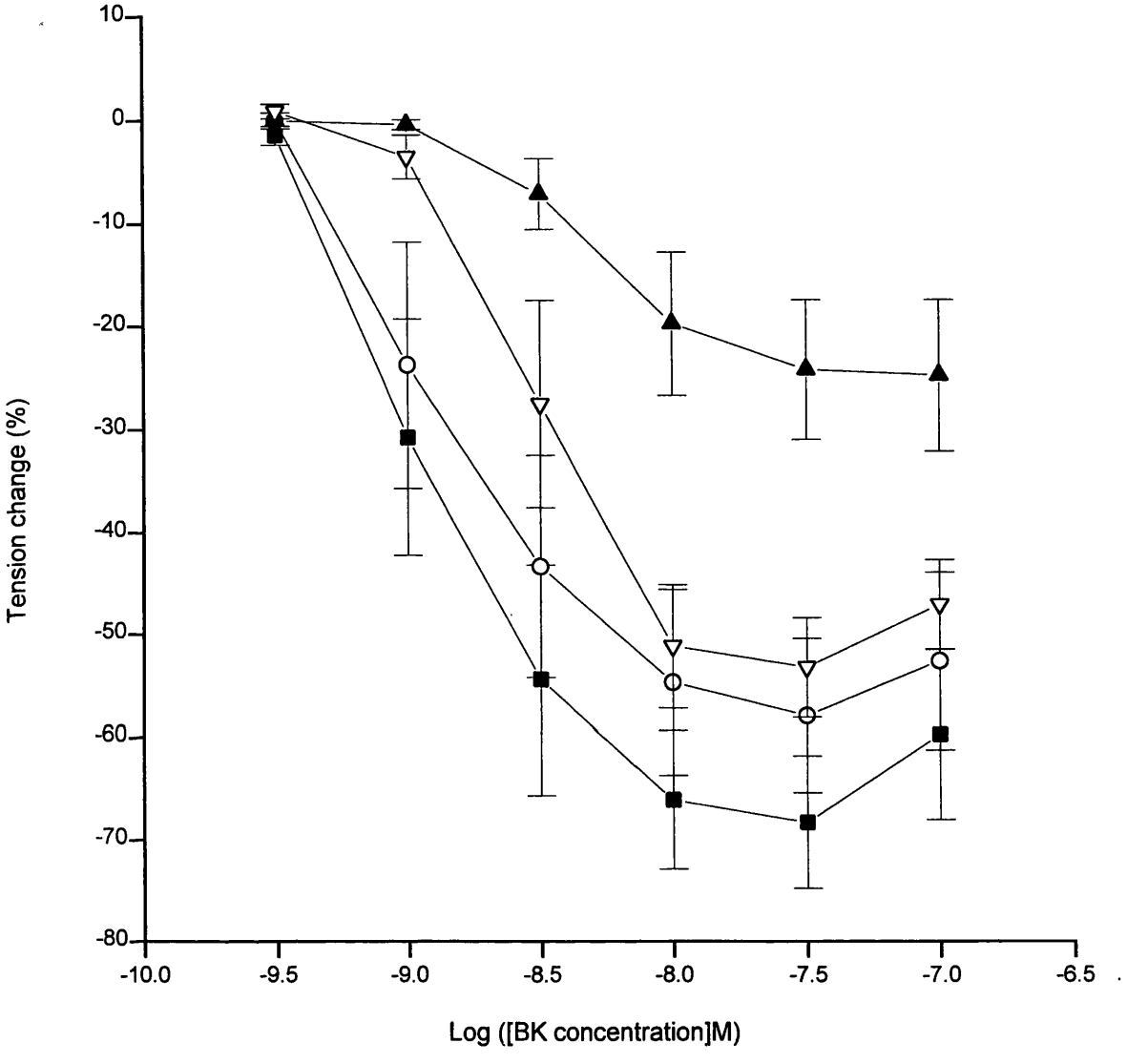


Figure 2.3.9. The effect of pre-incubation with L-arginine (10^{-2}M) and/or N^G-nitro-L-arginine methyl ester (L-NAME, $3x10^{-4}$M) on the bradykinin (BK) dose-response curve in ovine digital arteries pre-contracted with $6x10^{-7}$M phenylephrine. Each curve represents the mean values (±SEM) of 6-7 vessels from 6-7 animals.

—■— control

—○— L-arginine

—▲— L-NAME

—▽— L-arginine + L-NAME

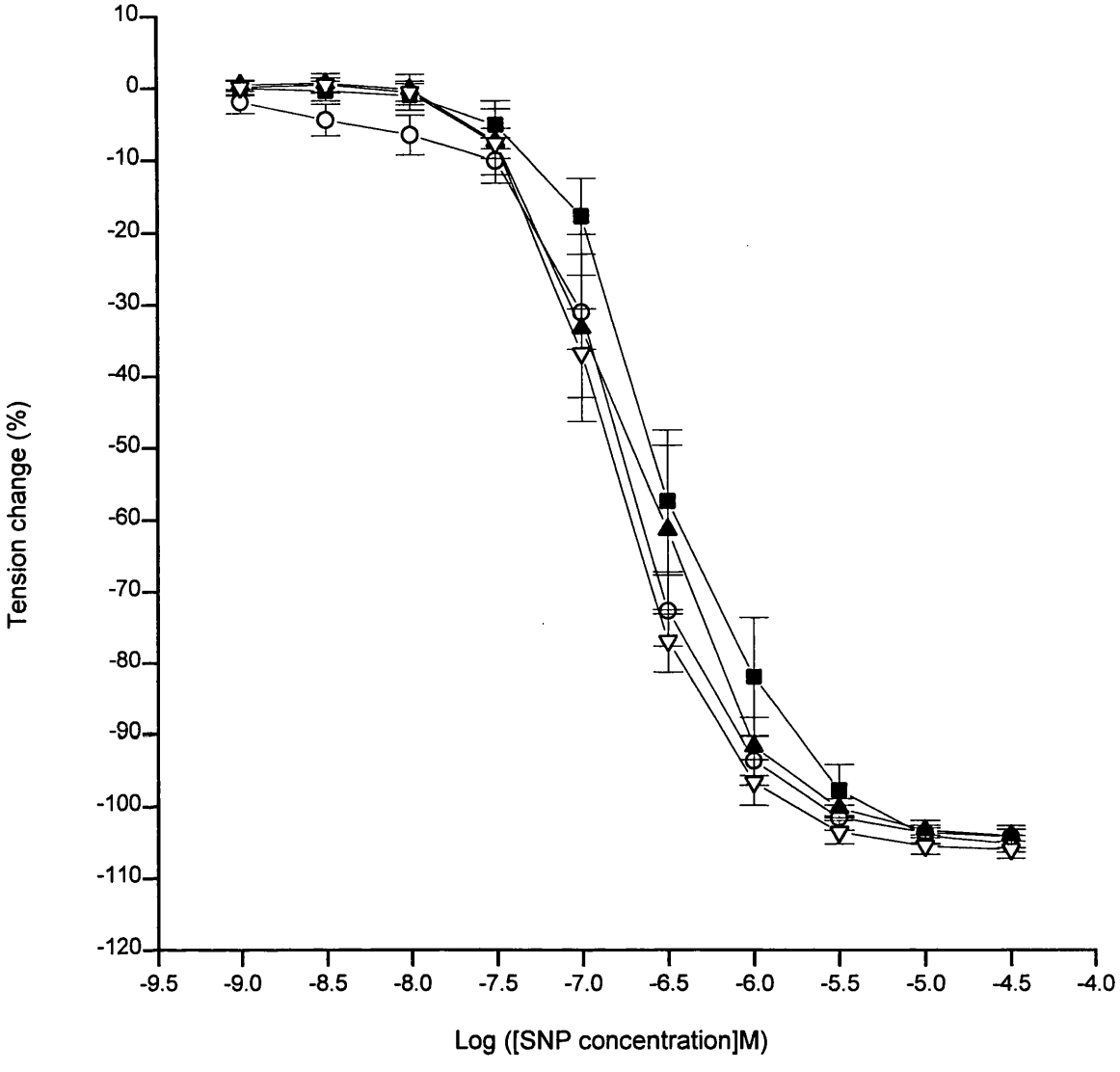


Figure 2.3.10. The effect of pre-incubation with indomethacin (10^{-5}M) and/or N^G-nitro-L-arginine methyl ester (L-NAME, $3x10^{-4}$M) on the sodium nitroprusside (SNP) dose-response curve in ovine digital arteries pre-contracted with $6x10^{-7}$M phenylephrine. Each curve represents the mean values (±SEM) of 6 vessels from 6 animals.

—■— control

—○— indomethacin

—▲— L-NAME

—▽— indomethacin + L-NAME

	PHENYLEPHRINE				
	n	EC_{50} log[PHE(M)]	p	E_{max} g/g	p
UNRUBBED	12	-6.13 ± 0.07	0.087	2107 ± 76	0.591
RUBBED	12	-6.29 ± 0.05		2188 ± 129	
UNRUBBED CONTROL	6	-5.88 ± 0.07	<0.001	2218 ± 108	0.370
UNRUBBED PRAZOSIN	6	-3.34 ± 0.13*		2017 ± 185	
RUBBED CONTROL	6	-5.92 ± 0.03	<0.001	2221 ± 197	0.339
RUBBED PRAZOSIN	6	-3.49 ± 0.09*		1990 ± 117	
CONTROL	8	-6.22 ± 0.08	0.005	2479 ± 213	0.871
INDO	7	-6.55 ± 0.07*		2603 ± 349	
L-NAME	7	-6.37 ± 0.12		2732 ± 251	
INDO + L-NAME	6	-6.68 ± 0.05*		2731 ± 149	

Table 2.3.1. The effects of endothelium removal or treatment with antagonists on phenylephrine (PHE) dose-response curve parameters in ovine digital arteries.
The effects of prazosin (10^{-7}M), indomethacin (INDO, 10^{-5}M) and N^{G}-nitro-L-arginine methyl ester (L-NAME, $3x10^{-4}$M) were studied.
Data are expressed as mean values ± SEM.
P-values were determined by one-way analysis of variance and $p < 0.05$ was considered statistically significant. Where necessary, results were further analysed by Tukey's pairwise comparisons.
* indicates results are significantly different from control group

	BRADYKININ				
	n	EC_{50} log [BK(M)]	p	E_{max} %	p
UNRUBBED	8	-8.68 ± 0.13	0.022	-61.8 ± 7.4	<0.001
RUBBED	8	-7.91 ± 0.27*		76.0 ± 12.1*	
CONTROL	6	-8.89 ± 0.15	0.035	-61.7 ± 5.6	<0.001
INDO	7	-8.92 ± 0.16		-51.0 ± 5.5	
L-NAME	7	-8.56 ± 0.20		-34.0 ± 2.1*	
INDO + L-NAME	7	-8.18 ± 0.22		-9.6 ± 2.8*°	
CONTROL	6	-8.80 ± 0.12	0.004	-71.3 ± 6.0	0.004
L-ARG	6	-8.78 ± 0.15		-61.6 ± 8.9	
L-NAME	7	-8.14 ± 0.13*		-30.7 ± 8.0*	
L-ARG + L-NAME	6	-8.49 ± 0.12		-55.3 ± 4.2	

Table 2.3.2. The effects of endothelium removal or treatment with antagonists on bradykinin (BK) dose-response curve parameters in ovine digital arteries.
The effects of indomethacin (INDO, 10^{-5}M), N^{G}-nitro-L-arginine methyl ester (L-NAME, $3x10^{-4}$M) and L-arginine (L-ARG, 10^{-2}M) were studied.
Data are expressed as mean values ± SEM.
P-values were determined by one-way analysis of variance and $p < 0.05$ was considered statistically significant. Where necessary, results were further analysed by Tukey's pairwise comparisons.
* indicates results are significantly different from unrubbed or control group
° indicates results are significantly different from L-NAME group

	SODIUM NITROPRUSSIDE				
	n	EC_{50} log [SNP(M)]	p	E_{max} %	p
UNRUBBED	7	-6.56 ± 0.19	0.630	-103.6 ± 1.2	0.860
RUBBED	7	-6.68 ± 0.15		-103.8 ± 0.5	
CONTROL	6	-6.46 ± 0.12	0.158	-106.3 ± 0.9	0.273
INDO	6	-6.72 ± 0.06		-104.2 ± 1.7	
L-NAME	6	-6.70 ± 0.15		-103.4 ± 0.6	
INDO + L-NAME	6	-6.80 ± 0.05		-105.8 ± 1.1	

Table 2.3.3. The effects of endothelium removal or treatment with antagonists on sodium nitroprusside (SNP) dose-response curve parameters in ovine digital arteries. The effects of indomethacin (INDO, 10^{-5}M) and N^{G}-nitro-L-arginine methyl ester (L-NAME, $3x10^{-4}$M) were studied.

Data are expressed as mean values ± SEM.

P-values were determined by one-way analysis of variance and $p < 0.05$ was considered statistically significant. Where necessary, results were further analysed by Tukey's pairwise comparisons.

(n = 6) in L-NAME-treated arteries and -6.80 ± 0.05 (n = 6) in arteries incubated with both inhibitors.

The data from sections 2.3.1 and 2.3.2 are summarised in Tables 2.3.1-3.

2.3.3 Effect of incubation with endotoxin

2.3.3.1 Effect of incubation with polymyxin

Dose-response curves to phenylephrine (Figure 2.3.11) and bradykinin (Figure 2.3.12) were constructed in ovine digital arteries incubated for 20 minutes with or without 10μg/ml polymyxin. Phenylephrine E_{max} and log EC_{50} values for control arteries ranged from 2501 to 2561g/g and from -5.89 to -6.09, respectively (n = 2). These parameters were not significantly different in polymyxin-treated arteries (2081 to 2556g/g, -5.61 to -6.28, n = 2). Likewise control (-34.2 to -39.8%, -7.75 to -7.99, n = 2) and polymyxin-treated (-34.4 to -43.9%, -7.51 to -8.37, n = 2) dose-response curves to bradykinin did not differ.

2.3.3.2 Phenylephrine

Cumulative dose-response curves were constructed in ovine digital arteries incubated for 6 hours with 3μg/ml endotoxin or 10μg/ml polymyxin (Figure 2.3.13). Whilst incubation with endotoxin had a minimal effect on log EC_{50} values, E_{max} was increased from 2286 ± 82g/g (n = 7) to 2544 ± 118g/g (n = 7). However this increase did not reach statistical significance (p = 0.097).

Following a 16 hour incubation with endotoxin (Figure 2.3.14) there was a tendency for E_{max} to fall, i.e. from 2066 ± 92g/g (n = 7) to 1698 ± 376g/g (n = 7), but again this failed to reach statistical significance (p = 0.360). The SEM for the endotoxin-incubated arteries was high, reflecting the wide variation in response. Contraction was markedly reduced in 2 out of the 7 arterial rings, whilst the remainder were changed only slightly. In addition, there was an increase in log EC_{50} from -6.49 ± 0.11 (n = 7) to -6.09 ± 0.16 (n = 7), which was close to being significant (p = 0.061).

2.3.3.3 Bradykinin

Non-cumulative dose-response curves were constructed in ovine digital arteries incubated for 6 hours with 3μg/ml endotoxin or 10μg/ml polymyxin (Figure 2.3.15). No significant differences in E_{max} or log EC_{50} were recorded.

2.3.4 Effect of incubation with cytokines

2.3.4.1 Phenylephrine

Cumulative dose-response curves were constructed in ovine digital arteries incubated for 6 hours with 10μg/ml polymyxin or a cytokine mixture containing 10ng/ml IFN-γ and 5ng/ml TNF-α (Figure 2.3.16). Decreases in E_{max}, from 2130 ±

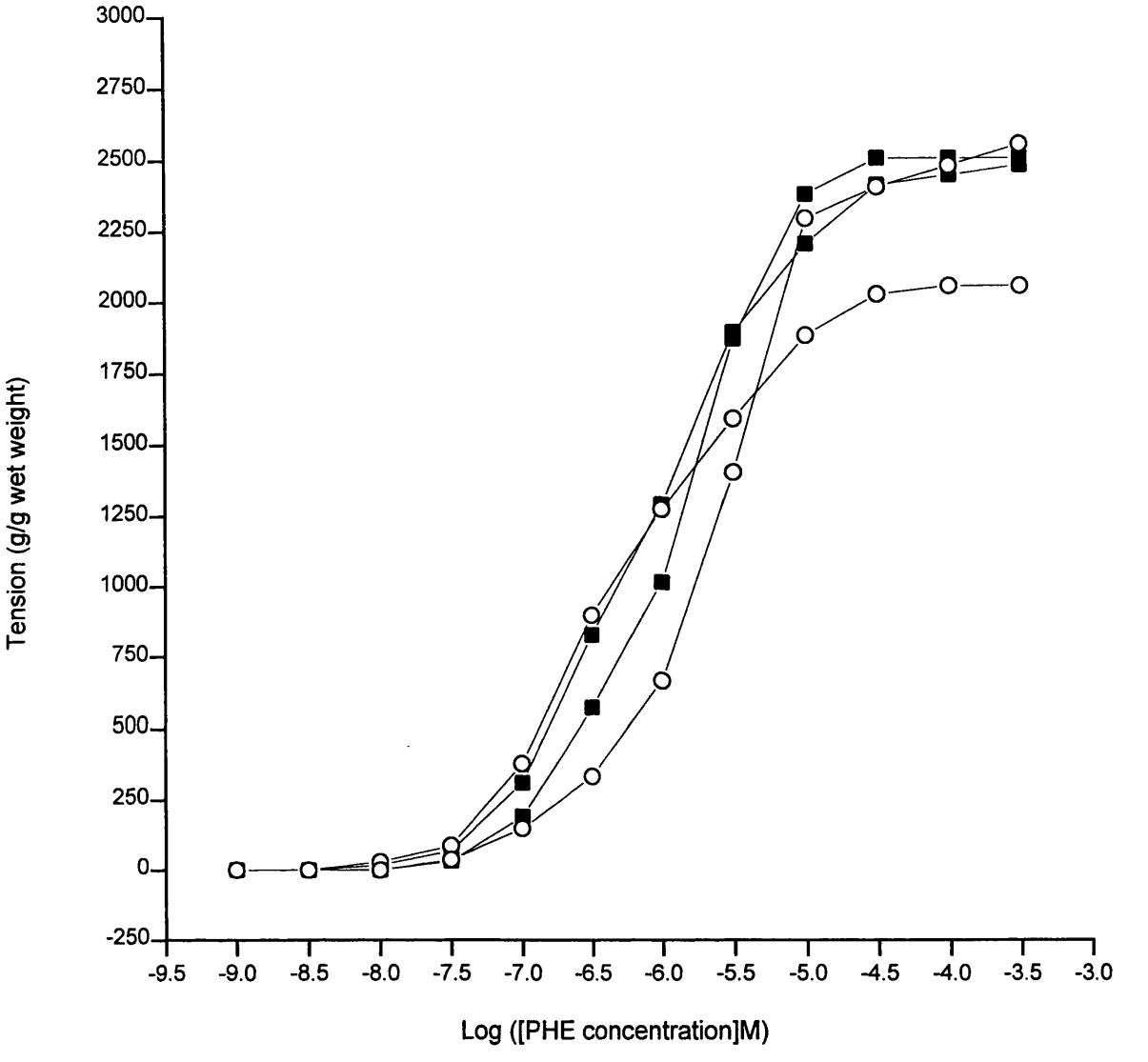


Figure 2.3.11. The effect of a 20 minute incubation with polymyxin (10μg/ml) on the phenylephrine (PHE) dose-response curve in ovine digital arteries. Each curve represents the response of an individual arterial ring. Vessels were obtained from 2 animals.

—■— control

—○— polymyxin

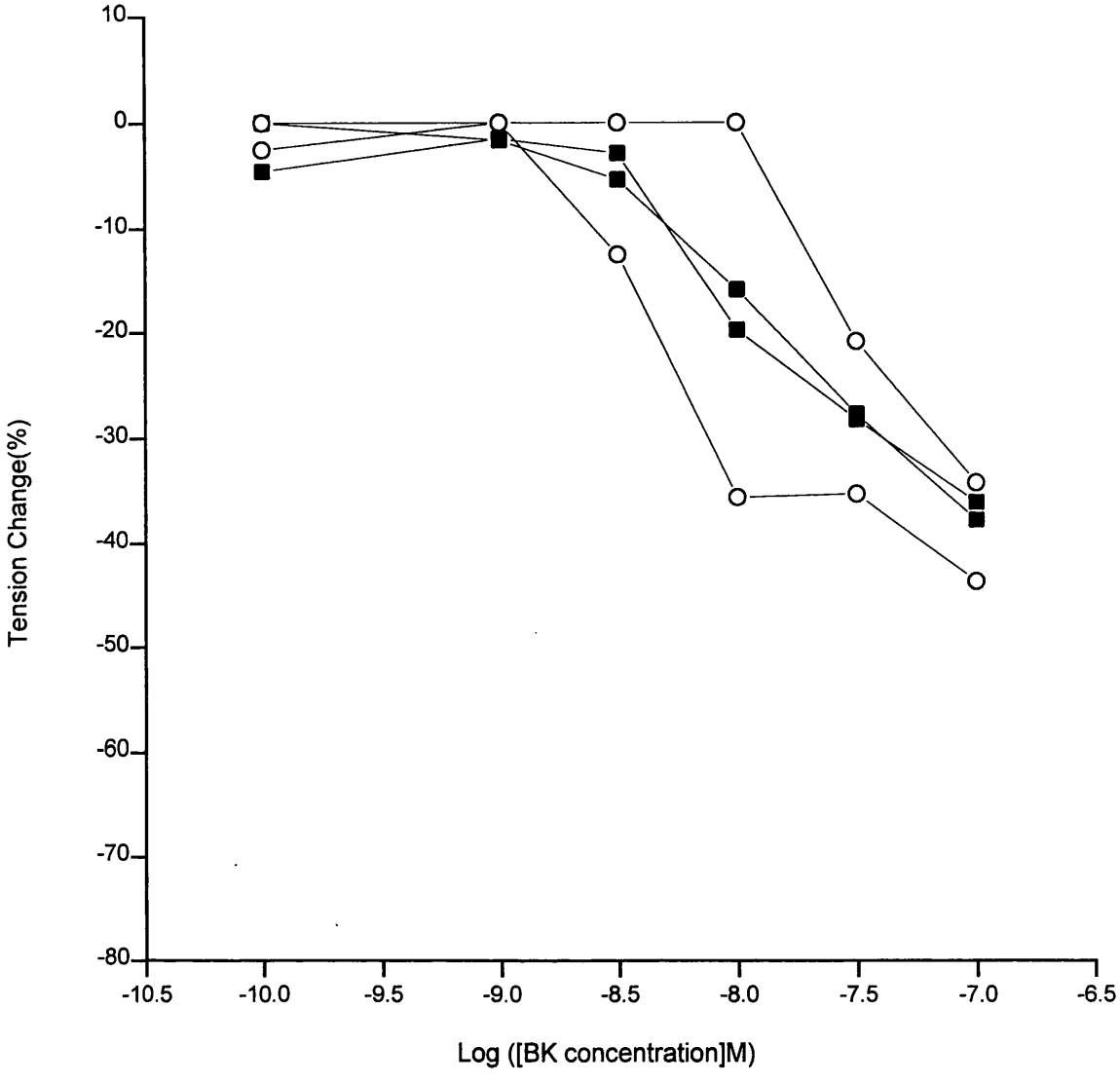


Figure 2.3.12. The effect of a 20 minute incubation with polymyxin (10μg/ml) on the bradykinin (BK) dose-response curve in ovine digital arteries. Each curve represents the response of an individual arterial ring. Vessels were obtained from 2 animals.

—■— control

—○— polymyxin

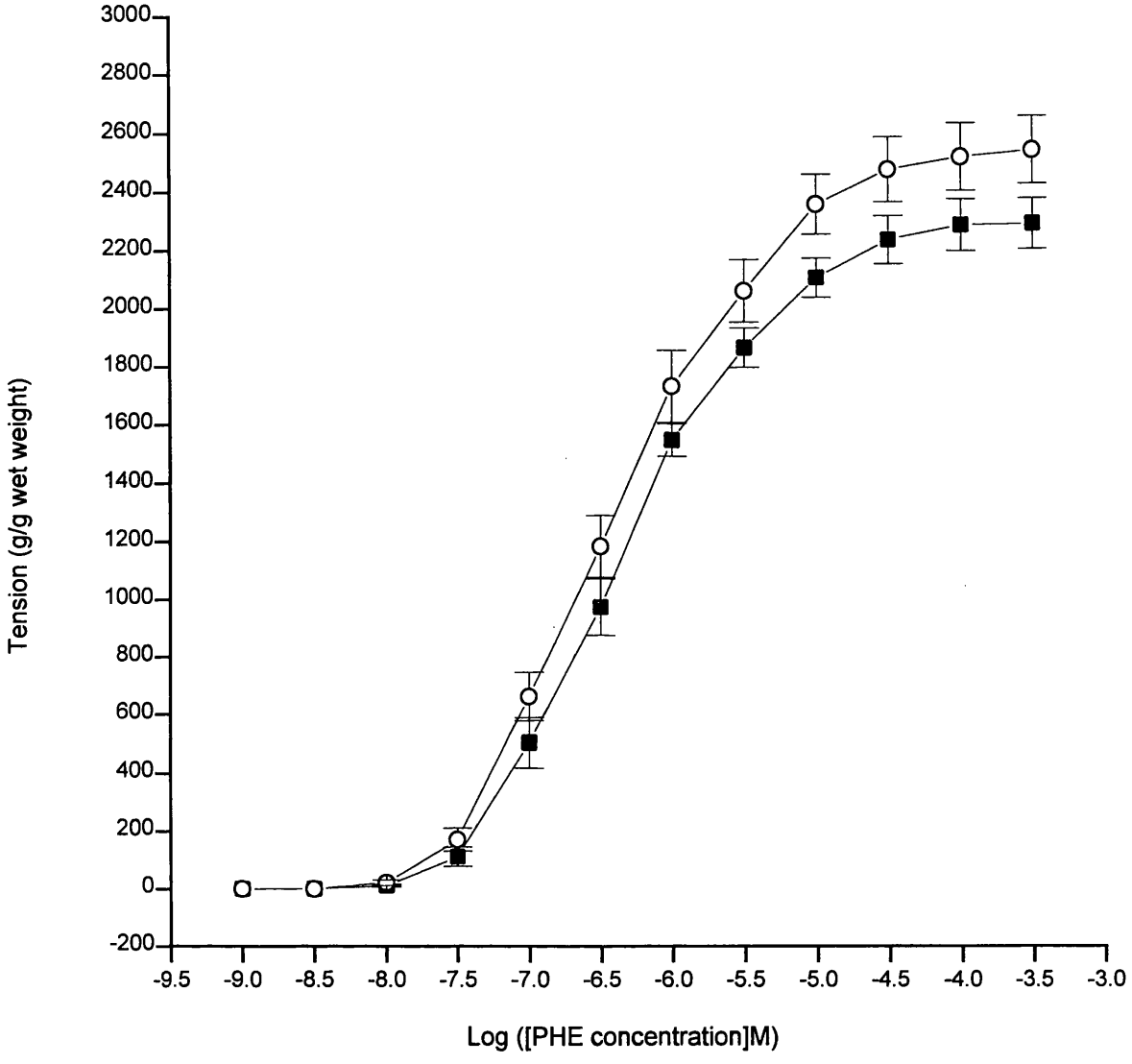


Figure 2.3.13. The effect of a 6 hour incubation with 3μg/ml endotoxin on the dose-response curve to phenylephrine (PHE) in ovine digital arteries. Control arteries were incubated with polymyxin (10μg/ml). Each curve represents the mean values (±SEM) for 7 vessels from 7 animals.

—■— control

—○— endotoxin

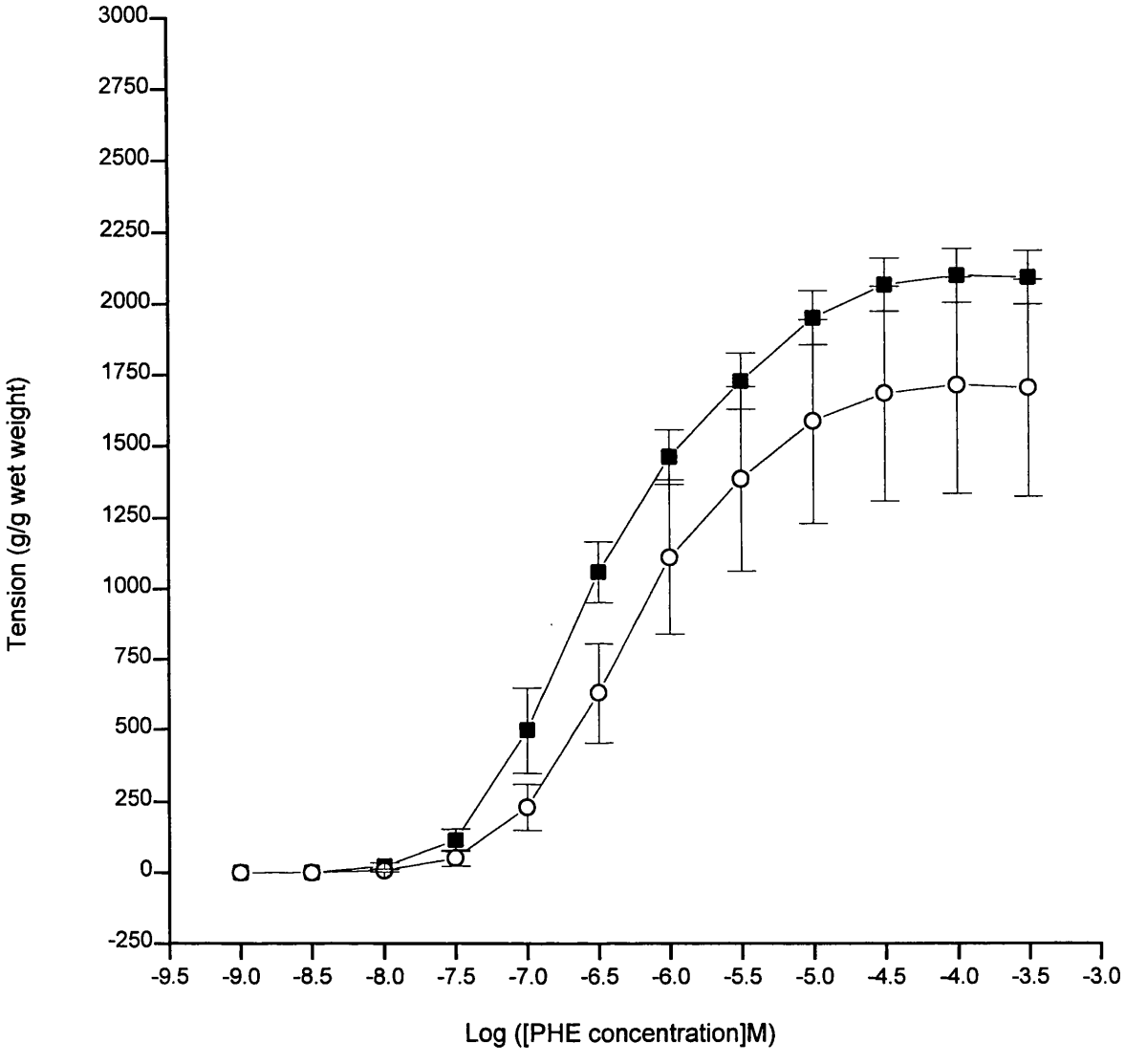


Figure 2.3.14. The effect of a 16 hour incubation with 3μg/ml endotoxin on the dose-response curve to phenylephrine (PHE) in ovine digital arteries. Control arteries were incubated with polymyxin (10μg/ml). Each curve represents the mean values (±SEM) for 7 vessels from 7 animals.

—■— control

—○— endotoxin

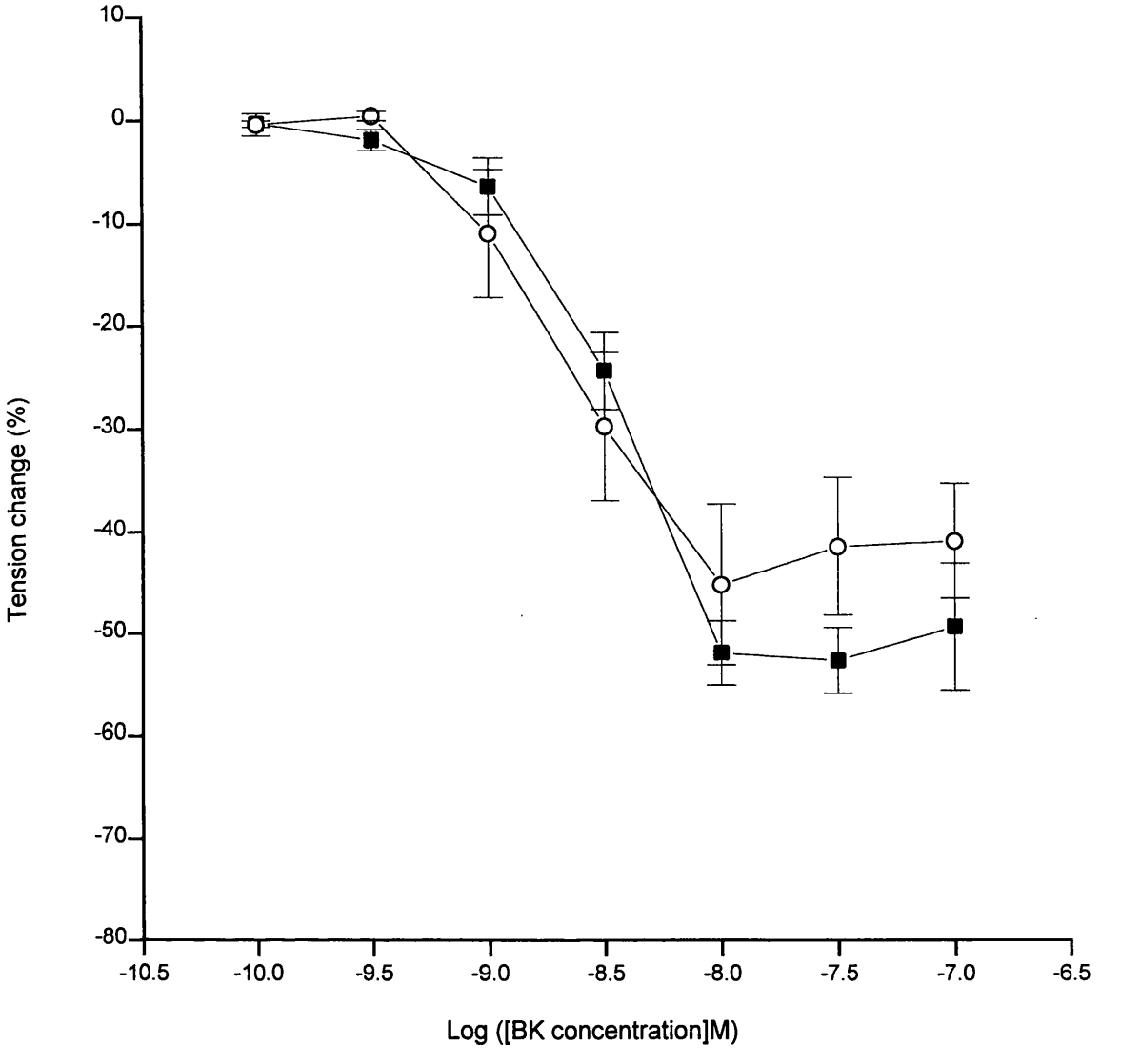


Figure 2.3.15. The effect of a 6 hour incubation with 3μg/ml endotoxin on the dose-response curve to bradykinin (BK) in ovine digital arteries precontracted with $6x10^{-7}$M phenylephrine. Control arteries were incubated with polymyxin (10μg/ml). Each curve represents the mean values (±SEM) for 6 vessels from 6 animals.

—■— control

—○— endotoxin

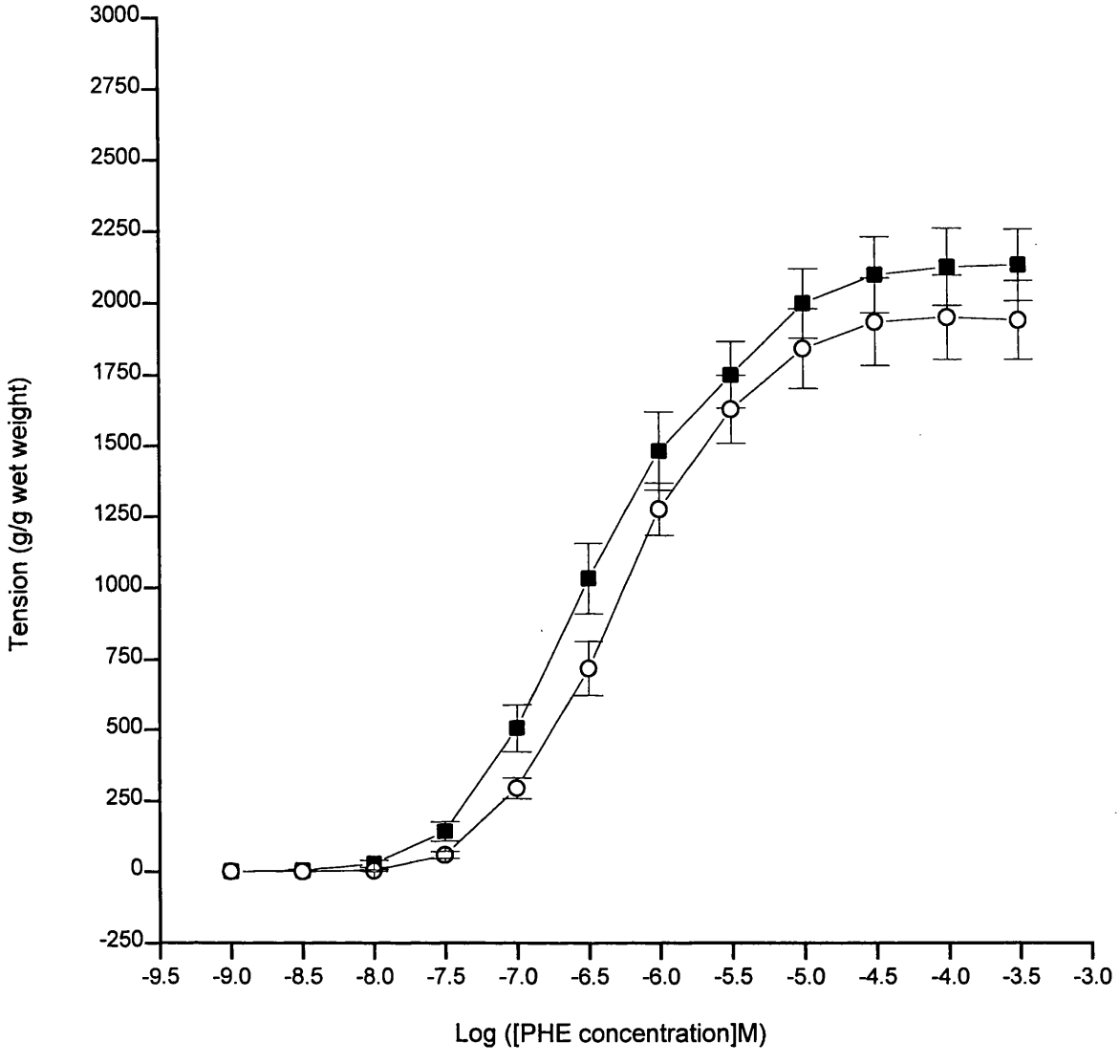


Figure 2.3.16. The effect of a 6 hour incubation with tumour necrosis factor-α (TNF-α, 5ng/ml) and interferon-γ (IFN-γ, 10ng/ml) on the dose-response curve to phenylephrine (PHE) in ovine digital arteries. Control arteries were incubated with polymyxin (10μg/ml). Each curve represents the mean values (±SEM) of 6 vessels from 6 animals.

—■— control

—○— TNF-α + IFN-γ

120 (n = 6) to 1946 ± 145 (n = 6), and increases in log EC_{50}, from -6.43 ±0.09 (n = 6) to -6.29 ± 0.08 (n = 6), failed to reach statistical significance.

Dose-response curves were also constructed in arteries incubated for 16 hours with either 10μg/ml polymyxin or a cytokine mixture containing 10ng/ml IFN-γ, 20ng/ml IL-1β and 5ng/ml TNF-α (Figure 2.3.17). Incubation with cytokines significantly increased log EC_{50}, from -6.44 ± 0.09 (n = 6) to -6.10 ± 0.11 (n = 6, p = 0.033), however the fall in E_{max} from 2318 ±110 (n = 6) to 2134 ± 190 (n = 6) was not significant (p = 0.422).

2.3.4.2 Bradykinin

Non-cumulative dose-response curves were constructed in ovine digital arteries incubated for 6 hours with either 10μg/ml polymyxin or a cytokine mixture containing 10ng/ml IFN-γ and 5ng/ml TNF-α (Figure 2.3.18). Whilst incubation with cytokines did not significantly alter log EC_{50}, the increase in E_{max}, from -33.1 ± 4.0% (n = 6) to -50.9 ± 5.1% (n = 6) was statistically significant (p = 0.021).

The data from sections 2.3.3 and 2.3.4 are summarised in Tables 2.3.4 and 2.3.5.

2.3.5 Endotoxin assay

Three samples were collected for endotoxin analysis, a sample of Krebs from the tissue bath, a sample of Krebs to which 3μg/ml endotoxin had been added and a sample of distilled water from which the Krebs solution was prepared.

First it was necessary to determine the sensitivity of the assay. The most dilute concentration of endotoxin standard to test positive was a solution containing 0.125 endotoxin units/ml (EU/ml). The endotoxin concentrations of the samples were then determined by dividing this value by the greatest sample dilution to give a positive result (section 2.2.6).

2.3.5.1 Krebs solution + 3μg/ml endotoxin

The greatest sample dilution to give a positive result was 1/16384. This gives an endotoxin concentration of 2048EU/ml. The endotoxin used in this study was extracted from *Escherichia coli*, serotype 055:B5 and contained not less than 500EU/μg. Thus, it was estimated that the sample contained not more than 4.10μg/ml of endotoxin. This is in line with the quantity of endotoxin added.

2.3.5.2 Krebs solution

The greatest dilution of this sample giving a positive result was 1/1024, which gives an endotoxin concentration of 128EU/ml. Assuming similar activity to the standard endotoxin, this sample contained not more than 0.26μg/ml endotoxin.

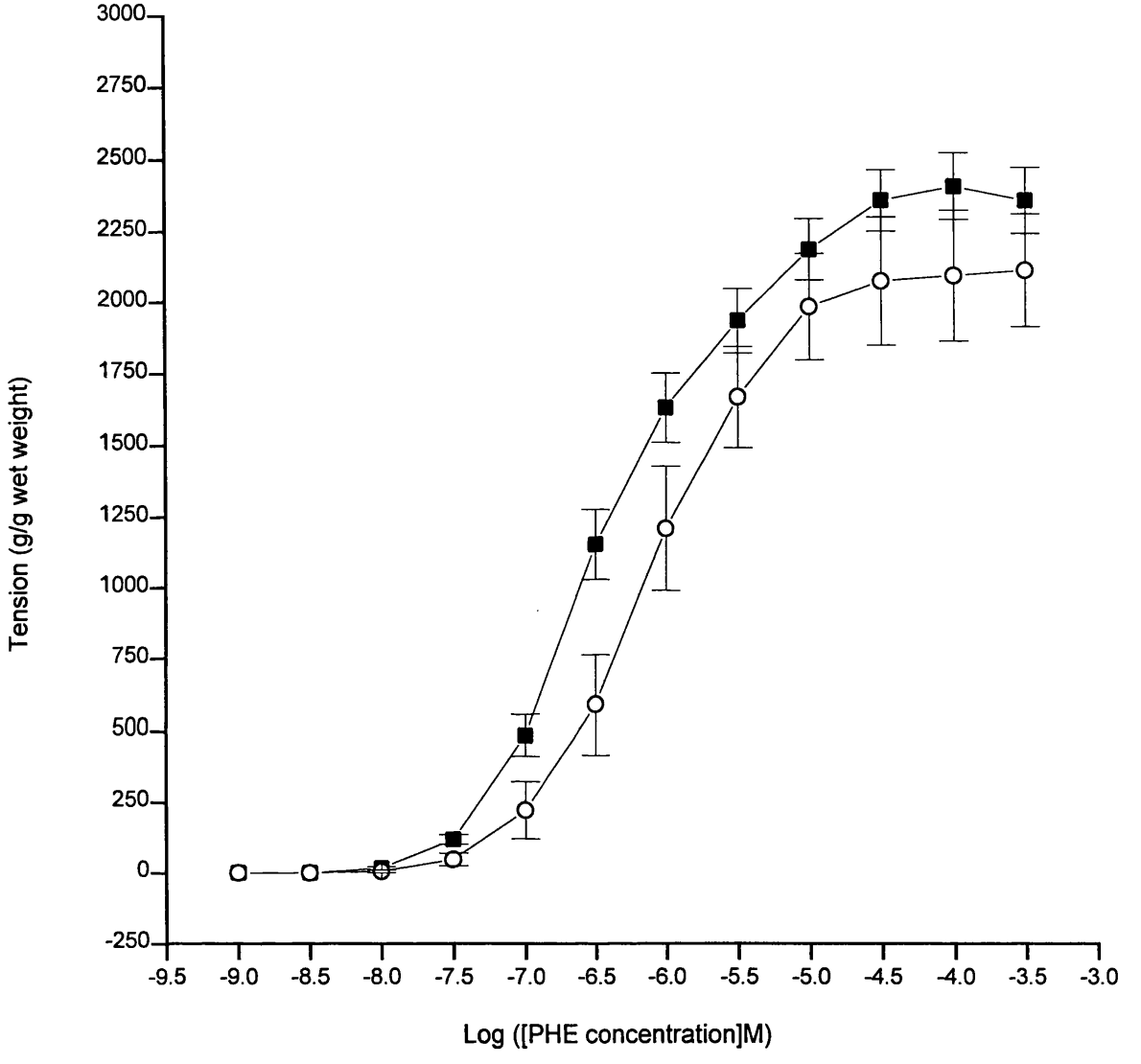


Figure 2.3.17. The effect of a 16 hour incubation with tumour necrosis factor-α (TNF-α, 5ng/ml), interleukin-1β (IL-β, 20ng/ml) and interferon-γ (IFN-γ, 10ng/ml) on the dose-response curve to phenylephrine (PHE) in ovine digital arteries. Control arteries were incubated with polymyxin (10μg/ml). Each curve represents the mean values (±SEM) of 6 vessels from 6 animals.

—■— control

—○— TNF-α + IL-1β + IFN-γ

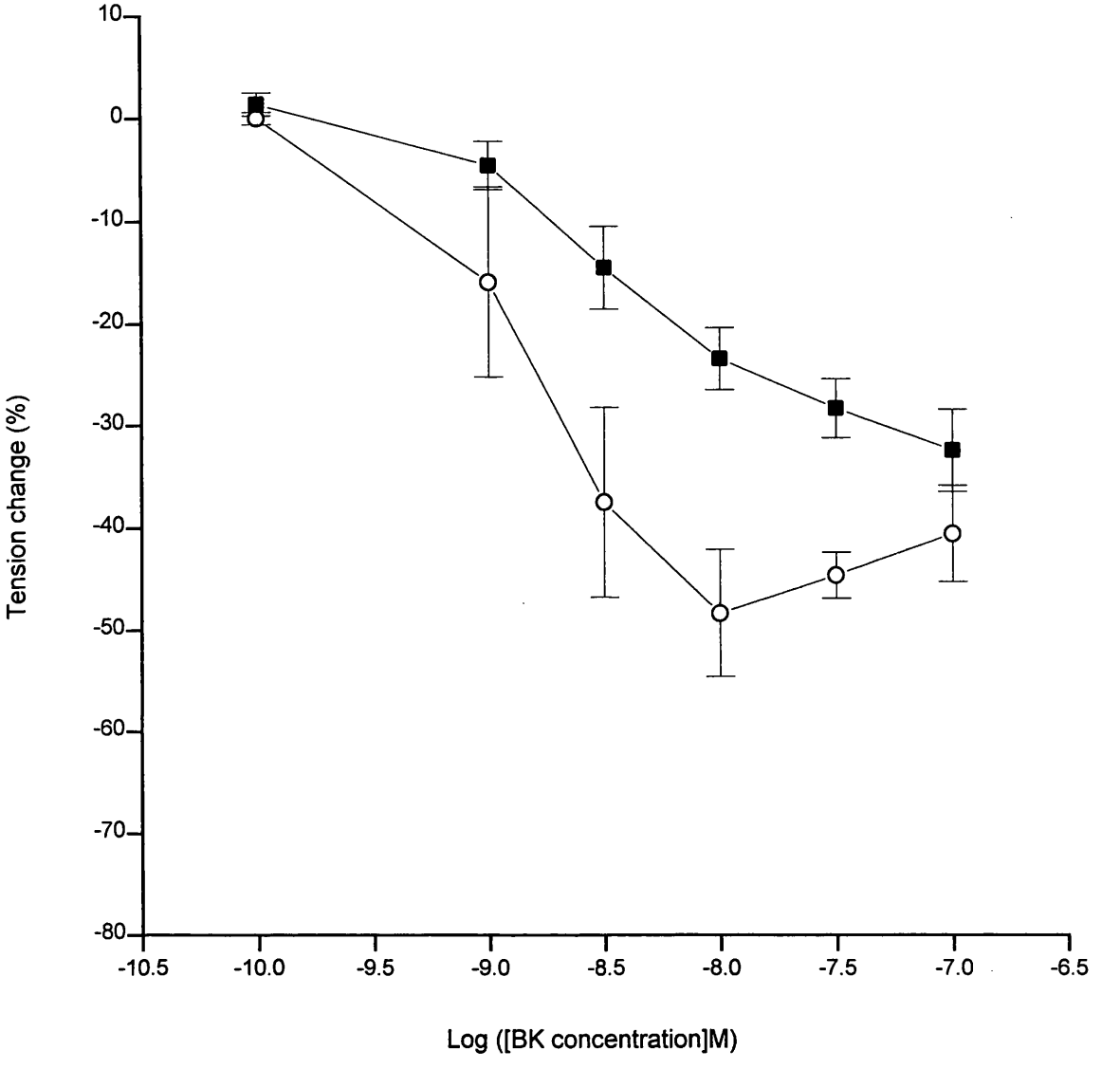


Figure 2.3.18. The effect of a 6 hour incubation with tumour necrosis factor-α (TNF-α, 5ng/ml) and interferon-γ (IFN-γ, 10ng/ml) on the dose-response curve to bradykinin (BK) in ovine digital arteries pre-contracted with $6x10^{-7}$M phenylephrine. Control arteries were incubated with polymyxin (10μg/ml). Each curve represents the mean values (±SEM) for 6 vessels from 6 animals.

—■— control

—○— TNF-α + IFN-γ

	PHENYLEPHRINE				
	n	EC_{50} log[PHE(M)]	p	E_{max} g/g	p
CONTROL	7	-6.37 ± 0.07	0.671	2286 ± 82	0.097
ENDOTOXIN (6h)	7	-6.42 ± 0.08		2544 ± 118	
CONTROL	7	-6.49 ± 0.11	0.061	2066 ± 92	0.360
ENDOTOXIN (16h)	7	-6.09 ± 0.16		1698 ± 376	
CONTROL	6	-6.43 ± 0.09	0.253	2130 ± 120	0.352
IFN-γ + TNF-α (6h)	6	-6.29 ± 0.08		1946 ± 145	
CONTROL	6	-6.44 ± 0.09	0.033	2318 ± 110	0.422
IFN-γ + IL-1β + TNF-α (16hr)	6	-6.10 ± 0.11*		2134 ± 190	

Table 2.3.4. The effects of incubation with endotoxin (3μg/ml) or cytokines on phenylephrine (PHE) dose-response curve parameters in ovine digital arteries.
The cytokines interferon-γ (IFN-γ, 10ng/ml), interleukin-1β (IL-1β, 20ng/ml) and tumour necrosis factor-α (TNF-α, 5ng/ml) were used. Control arteries were incubated with polymyxin (10μg/ml).
Data are expressed as mean values ± SEM.
P-values were determined by one-way analysis of variance and $p < 0.05$ was considered statistically significant. Where necessary, results were further analysed by Tukey's pairwise comparisons.
* indicates results are significantly different from control group

	BRADYKININ				
	n	EC_{50} log[BK(M)]	p	E_{max} %	p
CONTROL	6	-8.51 ± 0.09	0.210	-54.1 ± 2.7	0.183
ENDOTOXIN (6h)	6	-8.70 ±0.11		-44.2 ± 6.4	
CONTROL	6	-8.42 ± 0.17	0.264	-33.1 ± 4.0	0.021
IFN-γ + TNF-α (6h)	6	-8.70 ± 0.17		-50.9 ± 5.1*	

Table 2.3.5. The effect of incubation with endotoxin (3μg/ml) or cytokines on bradykinin (BK) dose-response curve parameters in ovine digital arteries.
The cytokines interferon-γ (IFN-γ, 10ng/ml) and tumour necrosis factor-α (TNF-α, 5ng/ml) were used. Control arteries were incubated with polymyxin (10μg/ml).
Data are expressed as mean values ± SEM.
P-values were determined by one-way analysis of variance and $p < 0.05$ was considered statistically significant. Where necessary, results were further analysed by Tukey's pairwise comparisons.
* indicates results are significantly different from control group

2.3.5.3 Distilled water

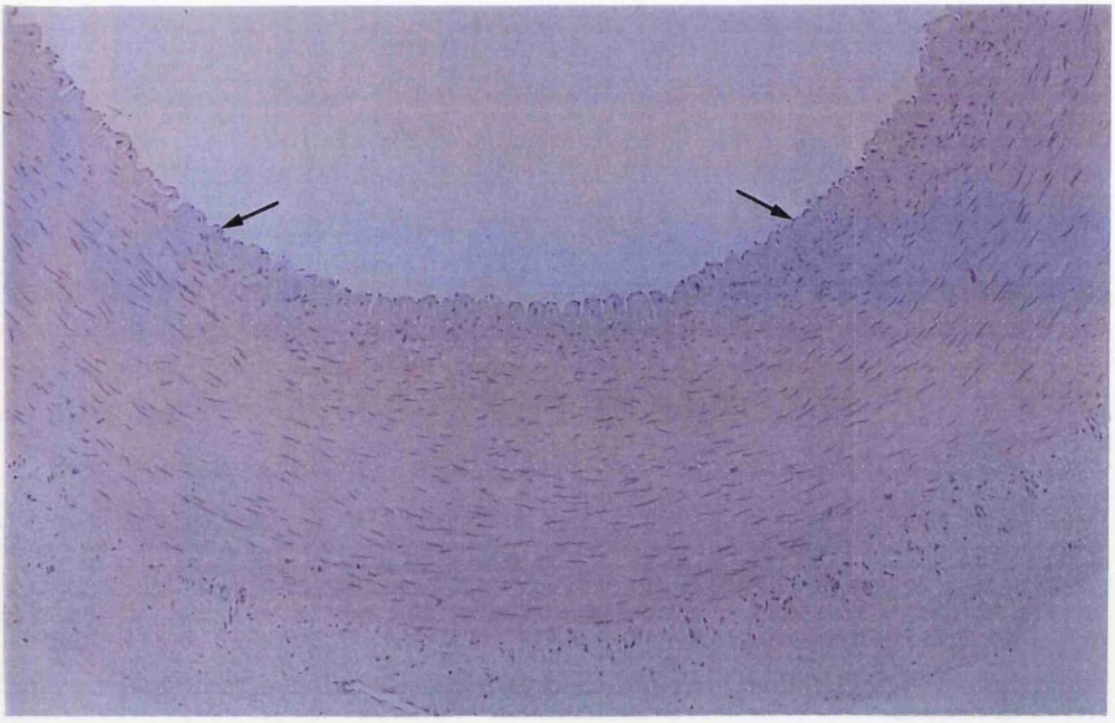
The greatest dilution of distilled water to test positive for endotoxin was a 1/16 dilution. Thus, endotoxin concentration was estimated at 2EU/ml or not more than 0.004μg/ml.

2.3.6 Histological examination

Sections of rubbed and unrubbed digital arterial rings were examined to assess the efficiency of endothelium removal. Endothelial cells could be identified lining the lumen of sections of unrubbed artery stained with haematoxylin and eosin (Figure 2.3.19A) and Verhoeff's haematoxylin (Figure 2.3.20). Staining for Von Willebrand's factor, a more specific technique, confirmed the presence of endothelial cells (Figure 2.3.21A).

In a proportion of vessels the endothelium was removed by gently rubbing the lumenal surface of the artery with a wooden cocktail stick. Examination of sections from rubbed arteries confirmed that the endothelium had been removed (Figures 2.3.19B and 2.3.21B).

(A)



(B)

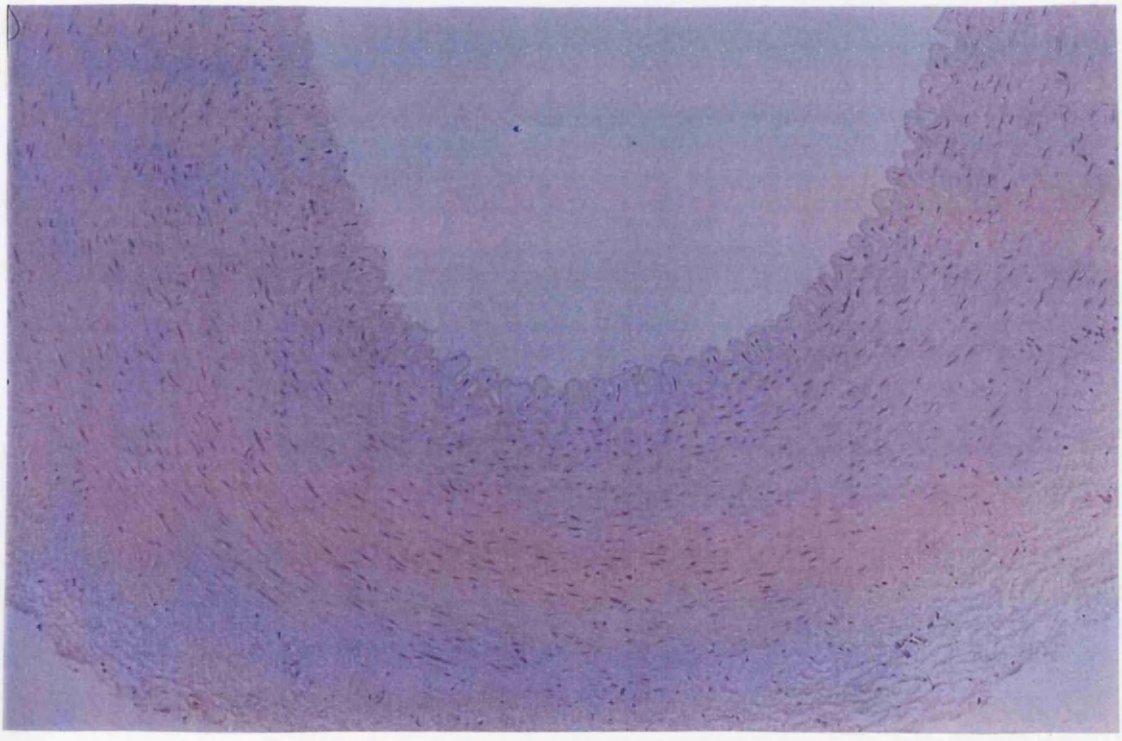


Figure 2.3.19. Transverse sections of unrubbed (A) and rubbed (B) ovine digital artery, stained with haematoxylin and eosin (magnification x10).

↑ endothelium

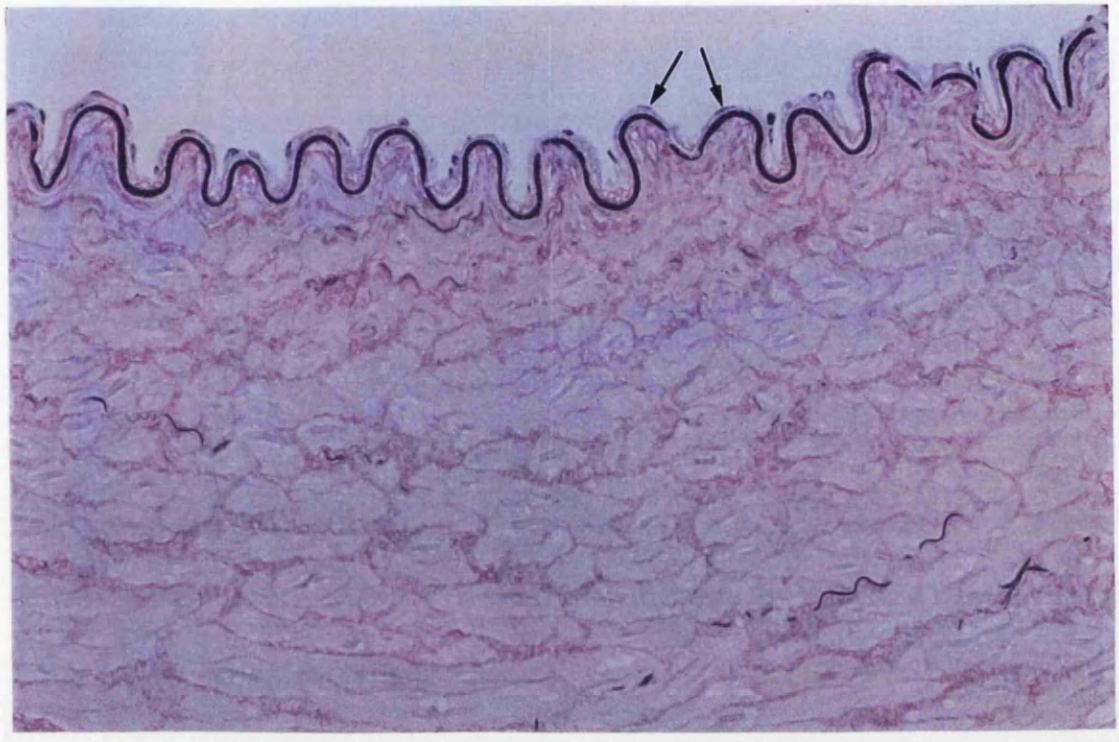
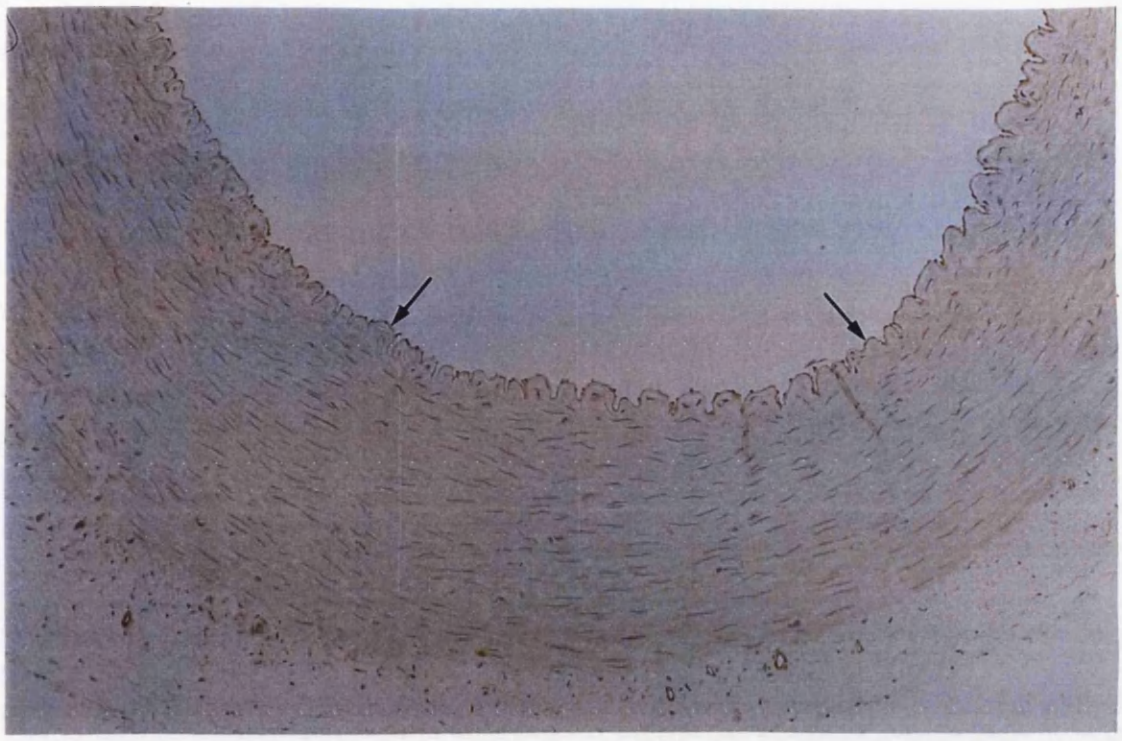


Figure 2.3.20. Transverse section of an unrubbed ovine digital artery, stained with Verhoeff's haematoxylin (magnification x40).
↑ endothelial cells

(A)



(B)

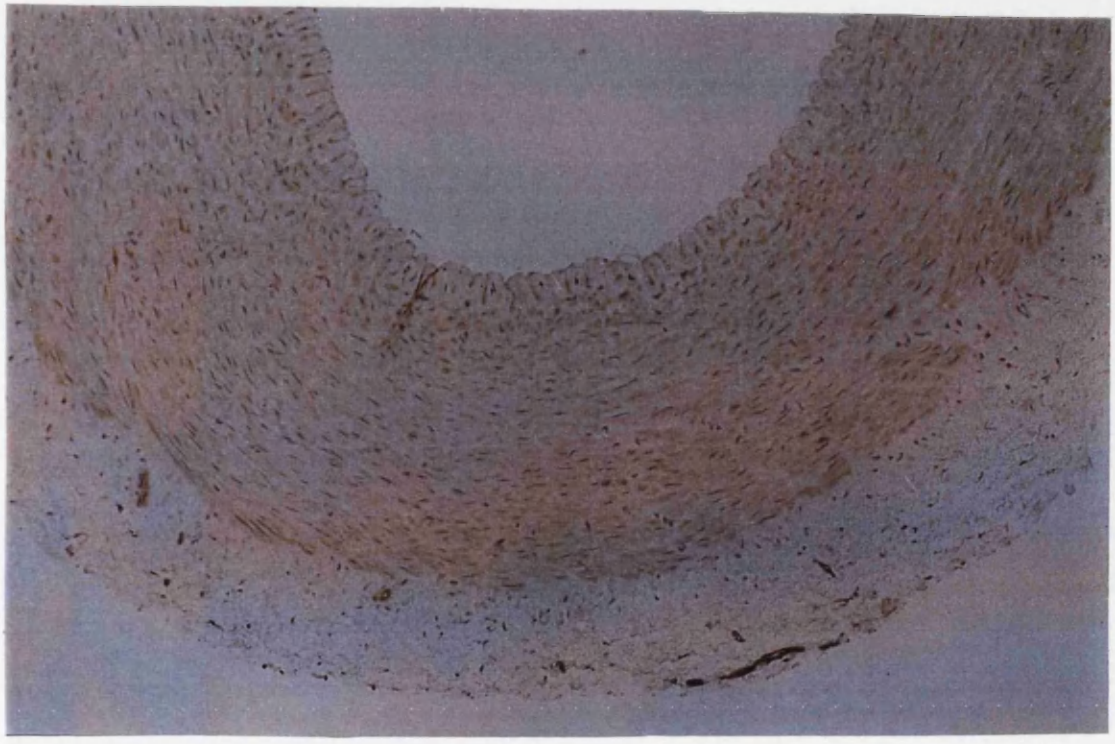


Figure 2.3.21. Transverse sections of unrubbed (A) and rubbed (B) ovine digital artery, stained for Von Willebrand's factor (magnification x10).
↑ endothelium

2.4 Discussion

The initial aim of this study was to assess the role of endothelium-derived nitric oxide and prostanoids in the modulation of digital arterial responses to a number of vasoactive agents. Responses to phenylephrine, bradykinin, sodium nitroprusside, and to a lesser extent acetylcholine, were investigated. After examining the responses of normal digital arteries, responses were studied in arteries incubated with endotoxin or cytokines.

Alpha-adrenergic agonists such as noradrenaline have been shown, in canine and porcine coronary arteries, to stimulate release of endothelium-derived nitric oxide, which modulates the vasoconstrictive response (Cocks and Angus 1983). However, this is not a feature of all vessels and variations both within and between species have been demonstrated (Angus *et al.* 1986, Martin *et al.* 1986b). In addition, contractile responses are opposed by continuous or basal release of nitric oxide from the endothelium (Martin *et al.* 1986b). The extent of this basal release also appears to vary, whilst McGrath *et al.* (1990) found it to be greater in rabbit veins than arteries, Vallance *et al.* (1989b) found the converse to be true in human vessels. Abolition of these influences, by removing the endothelium or incubating vessels with a NOS inhibitor, has been shown to potentiate phenylephrine or noradrenaline-induced contraction (Martin *et al.* 1986b, McGrath *et al.* 1990, Kaneko and Sunano 1993). However in this study, neither removal of the endothelium nor incubation with a concentration of L-NAME, sufficient to inhibit NOS ($3x10^{-4}$M, Elliott *et al.* 1994, Cogswell *et al.* 1995), significantly altered phenylephrine dose-response curve parameters. This implies that in ovine digital arteries neither basal nor α_2-adrenoceptor-mediated release of nitric oxide significantly modify the response to phenylephrine, a finding that is consistent with a study of equine digital arteries (Cogswell *et al.* 1995). Interestingly, log EC_{50} to phenylephrine was significantly decreased by 10^{-5}M indomethacin, a dose that is sufficient to inhibit cyclooxygenase (Cherry *et al.* 1982, Radomski *et al.* 1987b). Thus in ovine digital arteries endogenous vasodilatory prostaglandins appear to modify the α-adrenergic response. This has previously been reported in a number of arteries from the dog, cat and rabbit (Cherry *et al.* 1982).

A biphasic response to bradykinin was observed in ovine digital arteries, transient relaxation being followed by contraction. A similar response has been described in the sheep femoral artery (Félétou *et al.* 1994). The mechanism of bradykinin-induced relaxation appears to vary in vessels from different species (Furchgott 1984). Cherry *et al.* (1982) showed that relaxation to bradykinin in cat and rabbit mesenteric arteries was endothelium-independent and mediated almost entirely by cyclooxygenase products. However, bradykinin-induced relaxation in a variety of

canine and human arteries was abolished by removal of the endothelium and was attributed to release of endothelium-derived relaxing factor (EDRF). In this study, bradykinin-induced relaxation of ovine digital artery proved to be endothelium-dependent and was significantly reduced by L-NAME, an inhibitor of NOS, confirming the role of nitric oxide in this response. This finding is consistent with previous studies of sheep carotid artery (Carroll and Sheehan 1985) and equine digital arteries and veins (Elliott *et al.* 1994, Cogswell *et al.* 1995). In addition, inhibition by L-NAME was reversed by an excess of L-arginine, confirming the competitive nature of L-NAME antagonism. Whilst inhibition of cyclooxygenase alone had no significant effect, it did enhance the inhibition produced by L-NAME. This finding implies a role for cyclooxygenase products in bradykinin-induced relaxation and is consistent with studies demonstrating production of both nitric oxide and prostacyclin in bradykinin-stimulated bovine aortic endothelial cells (De Nucci *et al.* 1988). Interactions between nitric oxide and prostacyclin are complex. De Nucci *et al.* (1988) suggested that the receptor-mediated release of both agents is coupled and that the initial common step is activation of phospholipase C. In contrast, high concentrations of nitric oxide (13-130μM) have been shown to inhibit release of prostacyclin in cultured bovine endothelial cells (Doni *et al.* 1988). However, the concentrations of nitric oxide used were much higher than those required to induce relaxation of isolated blood vessels (4-134nM, Hutchinson *et al.* 1987) and the authors questioned the relevance of this inhibition *in vivo*. An inhibitory interaction between the two mediators has also been described in human saphenous veins (Barker *et al.* 1996). Whilst incubation with either piroxicam, a cyclooxygenase inhibitor, or L-NAME individually did not alter contractions to angiotensin II, contraction was enhanced in the presence of both inhibitors. The authors were able to explain these results by demonstrating an increase in the release of one vasodilator, either nitric oxide or prostacyclin, when production of the other agent was inhibited and suggested that this mechanism may be important in the maintenance of vasodilator tone. An inhibitory interaction could account for the enhanced inhibition of bradykinin-induced relaxation observed in ovine digital arteries incubated with both indomethacin and L-NAME in this study (Table 2.3.2).

Bradykinin-induced contraction of ovine digital arteries was endothelium-independent. Differences in the mechanism of bradykinin-induced contraction have also been described. Contractions of bovine mesenteric veins were shown to be reduced by indomethacin, indicating mediation by a cyclooxygenase product (Wong *et al.* 1977), whilst bradykinin-induced contractions of rabbit aorta (Regoli *et al.* 1977), rabbit jugular vein (Gaudreau *et al.* 1981) and sheep femoral artery (Félétou *et al.* 1994) were unaffected by cyclooxygenase inhibition and were not therefore mediated by vasoconstrictive prostaglandins. Two main types of bradykinin

receptor have been described in vascular smooth muscle, B_1 and B_2 (Hall 1992). Both relaxant and contractile phases of the bradykinin response in sheep femoral arteries were inhibited by the B_2 receptor antagonist, NPC 567 (Félétou *et al.* 1994). However, a second antagonist, Hoe 140, behaved as an agonist at the receptor inducing contraction, leading the authors to propose that 2 B_2 receptor subtypes exist in the sheep femoral artery. Characterisation of the bradykinin receptors in the ovine digital artery was not attempted and further work is needed to determine if the receptor population is similar to that in the femoral artery.

Acetylcholine-induced endothelium dependent relaxation has been demonstrated in vessels from a variety of species, including sheep pulmonary arteries (Abman *et al.* 1991, Toga *et al.* 1996). Kemp *et al.* (1997) found it to be a poor relaxing agent in these vessels, producing only a 30% reduction in serotonin-induced tone. However, this is not consistent with the earlier studies that reported relaxations of 51 and 70%, respectively (Abman *et al.* 1991, Toga *et al.* 1996). Similar levels of relaxation to acetylcholine have been recorded in studies of equine digital arteries (Baxter 1995, Cogswell *et al.* 1995). In this study, acetylcholine-induced relaxation was inconsistent, however it is not clear if this represents a genuine lack of potency or is a consequence of some other factor. This was the first endothelium-dependent response to be studied and lack of experience in the handling of delicate tissues may have resulted in endothelial damage. This is supported by the observation that acetylcholine-induced relaxations were greatest, up to 65%, in the last vessels to be examined. The mechanism of acetylcholine-induced relaxation was not investigated further, due to the inconsistent nature of the response. In other vessels, including equine digital arteries (Cogswell *et al.* 1995) and sheep pulmonary arteries (Toga *et al.* 1996), acetylcholine mediates relaxation through release of nitric oxide and has therefore been widely used to study agonist-stimulated release of this relaxing factor (Furchgott and Zawadzki 1980, Rubanyi *et al.* 1987, McGrath *et al.* 1990).

Sodium nitroprusside is an endothelium-independent vasodilator that relaxes vascular smooth muscle through release of nitric oxide (Schultz *et al.* 1977, Feelisch and Noack 1991). Studies of rat and rabbit arteries and veins have demonstrated enhanced relaxation to nitrovasodilators following removal of the endothelium or inhibition of NOS (Shirasaki and Su 1985, McGrath *et al.* 1990, Moncada *et al.* 1991). It has been suggested that continuous release of nitric oxide from the endothelium desensitises guanylate cyclase and thereby suppresses the response to exogenous nitric oxide. It follows that inhibition of this release, by removing the endothelium or inhibiting NOS, enhances relaxation to nitrovasodilators. However, no such effect was demonstrated in ovine digital arteries and neither endothelium-removal, L-NAME or indomethacin significantly altered the sodium nitroprusside dose-response curve. This finding is consistent with studies of equine digital

arteries and veins (Elliott *et al.* 1994, Cogswell *et al.* 1995) and implies that in these vessels basal release of nitric oxide does not significantly desensitise guanylate cyclase.

In order to study the effect of endotoxin on the vascular responses of isolated digital arteries it was necessary to incubate control arterial rings in an endotoxin-free solution. However as the endotoxin assay showed, Krebs solution and even the distilled water from which it was prepared, contained detectable amounts of endotoxin. To counteract this, control arteries were incubated with polymyxin, which has been shown to bind the lipid A portion of lipopolysaccharide (Morrison and Jacobs 1976). Krebs solution containing polymyxin was not assayed for endotoxin content as part of this project, therefore it was not possible to confirm the efficiency of polymyxin as an endotoxin-binding agent. However, others have shown that concentrations of 1-100μg/ml polymyxin block the effects of endotoxin on vascular smooth muscle (Rees *et al.* 1990, Schott *et al.* 1993, Moritoki *et al.* 1994). An intermediate dose of 10μg/ml was selected for use in this study.

Incubation of ovine digital arterial rings in oxygenated Krebs containing 3μg/ml endotoxin for 6 hours did not significantly alter the dose-response curve parameters to phenylephrine. In a second experiment, the incubation period was extended to 16 hours and the Krebs solution was replaced by Medium 199, an L-arginine supplemented culture medium. Following this incubation, endotoxin caused a slight increase in phenylephrine log EC_{50}, however this decrease in sensitivity was not significant at the 5% level ($p = 0.06$). In contrast, studies of endotoxin-incubated rat aorta have demonstrated far greater depression of the contractile response. Beasley *et al.* (1990) found maximal contractions to be markedly depressed following incubation with 10μg/ml endotoxin for just 3 hours. McKenna (1990) demonstrated a similar effect by incubating rings of rat aorta for a longer period, 16 hours, with much lower concentrations of endotoxin, 1-100ng/ml. This dose of endotoxin was selected to encompass the range of plasma endotoxin concentrations detected in septic people (Levin *et al.* 1970, Brandtzaeg *et al.* 1989). Other studies, employing intermediate endotoxin doses and incubation periods, have also demonstrated inhibition of the contractile responses of rat aorta (Fleming *et al.* 1990, Rees *et al.* 1990).

The effect of endotoxin on isolated vessels from other species has been less consistent. Overnight incubation with endotoxin 100ng/ml failed to alter the response of equine digital veins to phenylephrine, however serotonin-induced contractions were depressed by 51.2 and 41.2% in digital arteries and veins, respectively (Bailey and Elliott 1996). The authors commented that compared to rat blood vessels, those of the horse are less sensitive to the direct effects of endotoxin.

If this is true *in vivo* then the significance of the endotoxin levels measured following carbohydrate overload, 2.4-81.5ng/L, are drawn into question (Sprouse *et al.* 1987). Dixon *et al.* (1990) reported that endotoxin (10-100μg/ml) did not inhibit noradrenaline-induced contraction in porcine carotid arteries, although the 1.5 hour incubation period in this study may have been inadequate. Serotonin-induced contraction in porcine coronary arteries was significantly depressed following a 24 hour incubation with 100μg/ml endotoxin (Shibano and Vanhoutte 1993).

Incubation with 3μg/ml endotoxin for 6 hours also failed to alter the bradykinin dose-response curve in ovine digital arteries. This finding is in contrast to previous studies that have demonstrated impaired endothelium-dependent relaxations in arteries exposed to endotoxin. Beasley *et al.* (1990) found acetycholine-induced relaxation to be significantly reduced in rat aorta incubated *in vitro* with endotoxin. Arteries obtained from endotoxin-treated dogs (Wylam *et al.* 1990) exhibited similar depression of the acetycholine response, whilst depression of bradykinin-induced relaxation has been demonstrated in digital arteries from endotoxin-treated horses (0.1μg/kg, Baxter 1995). Studies of arteries from endotoxin-treated sheep have demonstrated conflicting results, whilst acetycholine-induced relaxation of pulmonary arteries was reduced (Spath *et al.* 1994), the response of femoral arteries was unchanged (Nelson *et al.* 1991). It has been hypothesised that impaired endothelium-dependent relaxation is a consequence of reduced nitric oxide synthesis by the endothelium and there is evidence to support this view. Myers *et al.* (1992) exposed bovine endothelial cells to 0.005-0.5μg/ml endotoxin for 1 hour and after a further hour washout period found nitric oxide production, both basal and bradykinin-stimulated, to be significantly decreased. This is consistent with a study demonstrating reduced expression of constitutive endothelial NOS at the protein and messenger RNA levels in bovine endothelial cells incubated with 2.5μg/ml endotoxin for 8 hours (Lu *et al.* 1996). However, not all studies agree and Salvemini *et al.* (1990) detected immediate release of a nitric oxide-like factor from cultured bovine endothelial cells exposed to 0.5-2μg/ml endotoxin. Increased bradykinin-stimulated release of nitric oxide has also been reported in porcine endothelial cells incubated with endotoxin 10μg/ml and IFN-γ 150U/ml (Radomski *et al.* 1990). This effect, which peaked after 6-12 hours, was attributed to the expression of inducible NOS by the endothelial cells.

Endotoxin is believed to mediate its effects on the vasculature through the generation of cytokines and isolated vessels have been shown to generate significant quantities of IL-1β and TNF-α when incubated with 1-100ng/ml endotoxin (McKenna 1990). Both endothelial and vascular smooth muscle cells have been shown to contribute to cytokine production (Miossec *et al.* 1986, Libby *et al.* 1986). Incubation of ovine digital arteries with the cytokines IFN-γ and TNF-α for 6 hours

did not significantly alter the phenylephrine dose-response curve, however the maximal relaxation to bradykinin was significantly increased. This finding is in contrast to previous studies that have demonstrated inhibition of endothelium-dependent relaxation in arteries exposed to cytokines. Greenberg *et al.* (1993) found that bradykinin-induced relaxation was attenuated in rings of bovine pulmonary artery exposed to various doses of TNF-α (0.0042-1.25μg/ml) for 1 hour and Dekimpe *et al.* (1994b) demonstrated a similar effect in bovine mesenteric arteries incubated with IFN-γ (100U/ml) for 20 hours. In the latter study, endothelium-dependent relaxation was impaired despite an increase in basal nitric oxide production. Reductions in acetylcholine-induced relaxation have also been recorded in cat carotid arteries (Aoki *et al.* 1989), rabbit aorta (Robert *et al.* 1992) and equine digital arteries (Baxter 1994) following *in vitro* incubation with TNF-α. Xie *et al.* (1993) proposed that a selective inhibition of receptor-mediated release of nitric oxide was responsible for the reduction in endothelium-dependent relaxation. However, studies of cultured endothelial cells have suggested that cytokine-induced alterations in the stability of endothelial constitutive NOS messenger RNA may be responsible (Yoshizumi *et al.* 1993, Zhang *et al.* 1997). As for endotoxin, whilst cytokines reduce nitric oxide production by the constitutive enzyme, more prolonged exposure can lead to expression of inducible NOS. This may account for the increased basal production of nitric oxide reported by Dekimpe *et al.* (1994b).

This study has shown that in ovine digital arteries, bradykinin-induced relaxation is mediated via a combination of nitric oxide and prostanoids, the most likely one being prostacyclin (Section 2.3.2.2), therefore a cytokine-induced increase in release or efficacy of either agent could account for the enhanced response to bradykinin. An increase in receptor-mediated release of nitric oxide is unlikely in the light of the studies described above, although vessel or species specific differences are possible (Greenberg *et al.* 1993). Increases in both basal and agonist-stimulated prostacyclin production have been recorded in cultured smooth muscle cells from human pulmonary artery incubated with cytokines (Wen *et al.* 1997a & b). This is not unexpected since cytokines are known to induce expression of enzymes involved in the arachidonic acid cascade, including cyclooxygenase (Carrier *et al.* 1996). Thus increased prostacyclin production could theoretically contribute to the enhanced response to bradykinin. Increasing sensitivity to bradykinin with time, has been demonstrated in a number of isolated vessels, including rabbit aorta (Regoli *et al.* 1977) and rabbit carotid artery (Pruneau and Bélichard 1993). This effect has been attributed to the induction of B_1 bradykinin receptors, a process that can be accelerated by incubation with endotoxin (Regoli *et al.* 1981) or IL-1β (Deblois *et al.* 1988). Therefore, it is feasible that the cytokines used in this study could induce B_1 receptors and thereby amplify the relaxant response. However, it is also possible

that in sheep digital arteries, B_1 receptors mediate contraction not relaxation and therefore diminish the relaxant response rather than amplify it. This effect has been demonstrated in bovine mesenteric arteries (Dekimpe *et al.* 1994a). Incubation with IFN-γ was found to induce production of B_1 receptors thereby converting bradykinin-induced relaxation to contraction. Further studies to identify the bradykinin receptors in the ovine digital artery may help clarify the role of induced receptors in the enhanced relaxant response observed in this study. If bradykinin-induced relaxation is mediated via B_2 receptors, as in the sheep femoral artery (Félétou *et al.* 1994), then a B_1 receptor antagonist could be used to prevent activation of induced B_1 receptors.

In a final experiment the dose-response curve to phenylephrine was examined after incubating ovine digital arteries for 16 hours in a mixture of 3 cytokines, IFN-γ (10ng/ml), TNF-α (5ng/ml) and IL-1β (20ng/ml). Arterial rings exposed to cytokines exhibited reduced sensitivity to phenylephrine, i.e. log EC_{50} values were significantly increased, whilst maximal contraction, E_{max}, was unchanged. This trend is consistent with the findings of previous studies. Depression of contraction has been demonstrated in rings of rat aorta incubated for 3-5 hours with 0.2-50ng/ml IL-1β (Beasley *et al.* 1989, Beasley 1990, French *et al.* 1991). A similar effect has been observed in rabbit aorta incubated for 18 hours with 100U/ml IL-1β (Robert *et al.* 1992) and in porcine coronary arteries incubated with 300ng/ml TNF-α for 24 hours (Shibano and Vanhoutte 1993). However, these studies demonstrated a much greater degree of cytokine-mediated inhibition, with maximal contractions being decreased by as much as 66% (Robert *et al.* 1992).

This endotoxin or cytokine-induced vascular hyporeactivity has been shown to be endothelium-independent (Beasley 1990, Shibano and Vanhoutte 1993) and has been attributed to the expression of inducible NOS in vascular smooth muscle, which in turn increases nitric oxide production (Beasley *et al.* 1991). Thus it can be reversed by agents that bind nitric oxide, such as haemoglobin (Beasley 1990) and by inhibitors of guanylate cyclase (Beasley 1990, Fleming *et al.* 1991) and NOS (French *et al.* 1991, Fleming *et al.* 1991, Robert *et al.* 1992). The poor response to incubation with endotoxin or cytokines observed in this study could reflect a reduced capacity for NOS induction in sheep. There is evidence to support the existence of such species differences, for example, levels of messenger RNA for inducible NOS are significantly lower in cytokine-treated human vascular smooth muscle cells compared to similarly-treated rat vascular smooth muscle cells (Beasley and McGuiggin 1994). Regional differences are also possible. Robert *et al.* (1993) found that whilst IL-1β significantly depressed noradrenaline-induced contractions of rabbit aorta, contraction of rabbit femoral artery was enhanced. Similarly, Suba *et al.* (1992) demonstrated no change in the contractility of

pulmonary arteries isolated from septic pigs, whilst the responses of mesenteric arteries were depressed.

Experimental conditions may be critical in determining the expression and activity of inducible NOS and an alternative explanation for the limited response to endotoxin or cytokines in sheep digital arteries could be that the conditions were unsuitable. Enzyme induction is not an immediate effect and extended exposure to endotoxin or cytokines is required. In rat arteries an incubation period of 3 hours resulted in expression of inducible NOS (Beasley 1990), however in bovine arteries more prolonged exposure, up to 20 hours, may be required (Dekimpe *et al.* 1994b). In this study, the initial incubation period of 6 hours may have been too short to permit enzyme induction. Incubation was therefore extended to 16 hours to ensure adequate exposure to endotoxin or cytokines. In rat aorta, the concentration of endotoxin does not appear to be critical in limiting NOS induction and concentrations from 1ng/ml to 10μg/ml have been used (McKenna 1990, Beasley 1990). It is possible that sheep arteries are relatively insensitive to the effects of endotoxin, however this does not appear to be the case *in vivo*. Administration of a low dose of endotoxin, 1.5μg/kg, to conscious sheep was sufficient to cause significant reductions in systemic vascular resistance and mean arterial pressure, 8-12 hours later (Nelson *et al.* 1991). It therefore seems unlikely that the concentration of endotoxin used in this study, 3μg/ml, was inadequate. Cytokine concentrations may be more critical and doses quoted in previous reports were used. Geller *et al.* (1993) found combinations of cytokines to be more potent inducers of NOS than single agents, therefore 3 cytokines, IL-1β, TNF-α and IFN-γ, were used. Beasley *et al.* (1989) found that IL-1β-induced depression of the contractile response in rat aorta, was concentration-dependent between 0.2-20ng/ml, therefore an optimal dose of 20ng/ml was chosen for use in this study. This is in excess of the levels measured in septic people, up to 300pg/ml (Cannon *et al.* 1990). The concentration of TNF-α used, 5ng/ml, was that used by Baxter (1994). This level was based on the peak concentration of TNF-α measured in the plasma of horses treated with 0.03μg/kg endotoxin (Morris *et al.* 1990). Slightly higher peak concentrations, approximately 12ng/ml, have been recorded in endotoxin-treated calves (1μg/kg, Kenison *et al.* 1991). The concentration of IFN-γ used, 10ng/ml, was derived from the 100U/ml used by Dekimpe *et al.* (1994b). This is slightly higher than the peak plasma level, approximately 4ng/ml, recorded in baboons following administration of a lethal dose of live *Escherichia coli* (Hesse *et al.* 1988). Species differences in the regulation of NOS induction by cytokines have been documented and therefore agents and concentrations that induce NOS in one species may not be effective in sheep. Lamas *et al.* (1992) found that IFN-γ, a potent inducer of NOS in murine macrophages, inhibited TNF-α-stimulated

induction of NOS in cultured bovine endothelial cells. Likewise, Adler *et al.* (1995) were unable to induce NOS in bovine macrophages exposed to 1000U/ml IFN-γ, a concentration that proved effective in murine macrophages. Studies using ovine macrophages incubated for prolonged periods with endotoxin (0.004-4μg/ml) and/or IFN-γ (300U/ml) also failed to demonstrate NOS induction (Bogdan *et al.* 1997). Thus it appears likely that expression of inducible NOS *in vitro* occurs under more restricted conditions in ruminant, compared to rodent cells.

The activity of inducible NOS has been shown to be limited by the availability of the substrate L-arginine and Schott *et al.* (1993) demonstrated that supplementation of the incubation medium with 10^{-4}M L-arginine enhanced endotoxin-mediated depression of the contractile response in rat aorta. A deficiency of L-arginine could account for the minimal effect of endotoxin or cytokines on the digital arteries incubated in Krebs solution, however this does not apply to the arteries incubated in Medium 199, which is supplemented with L-arginine ($3x10^{-4}$M). The 16 hour incubation was performed at 37°C in a humidified atmosphere containing 5% CO_2. Molecular oxygen is also a substrate for nitric oxide synthesis and it is feasible that that a shortage of oxygen could have limited enzyme activity in this environment. However, no such difficulty has been reported by authors using similar incubation conditions (French *et al.* 1991, Robert *et al.* 1992, Dekimpe *et al.* 1994b).

To avoid the problems of *in vitro* incubation, arteries can be obtained from endotoxin-treated animals. Studies of arteries obtained from rats treated with a high dose of endotoxin 20mg/kg confirmed the findings of the *in vitro* studies, i.e. contractile responses were depressed (Wakabayashi *et al.* 1987, Julou-Schaeffer *et al.* 1990, Fleming *et al.* 1991, Gray *et al.* 1991). This response was more difficult to demonstrate in vessels from other species *in vitro* and studies of vessels from other endotoxin-treated animals tend to support this. Arteries removed from endotoxin-treated dogs (5mg/kg) exhibited no significant change in vascular responsiveness to phenylephrine, despite a similar interval, 4-5 hours, between administration of endotoxin and harvesting of vessels (Wylam *et al.* 1990) and noradrenaline-induced contraction was enhanced in digital arteries removed from horses treated with low dose endotoxin 0.1μg/kg, 1-2 hours earlier (Baxter 1995). Whilst lower doses of endotoxin were used in the dog and horse, these species are more sensitive to the effects of endotoxin and have lower lethal dose 50% (LD_{50}) values, 1-5 and 0.1mg/kg respectively, compared to 10mg/kg in the rat (Burrows 1982). It appears likely that more prolonged exposure to endotoxin is required in these species if NOS induction is to occur. Suba *et al.* (1992) employed a longer incubation period, isolating vessels 48 hours after the induction of sepsis in swine and found that the response of mesenteric arteries to noradrenaline was significantly attenuated. In a study of sheep femoral arteries, noradrenaline-induced contraction was reduced 8-12

hours after administration of 1.5μg/kg endotoxin (Nelson *et al.* 1991). The authors found EC_{50} to be increased, but maximal contraction to be unchanged, findings that are consistent with the results of this study. Thus, the moderate response to endotoxin or cytokines recorded *in vitro* is supported by studies of arteries exposed to these agents *in vivo*. However, one piece of evidence argues against this. Examination of the individual dose-response curves for arteries incubated for 16 hours with endotoxin or cytokines reveals not a slight response in all arterial rings but marked depression of contraction in 2 out of 6-7 vessels, hence the high standard errors (Table 2.3.4). This implies that marked depression is possible under some circumstances and its development may after all be limited by environmental conditions. Clearly, further studies are needed to elucidate more fully the effect of endotoxin or cytokines on sheep vascular smooth muscle. The mechanism of vascular hyporesponsiveness, if it occurs, also requires investigation. Nelson *et al.* (1991) were unable to reverse the decrease in ovine femoral artery sensitivity to noradrenaline with methylene blue, a guanylate cyclase inhibitor, and claimed therefore that the effect was not mediated by nitric oxide. However, an *in vivo* study by Landin *et al.* (1994) provided evidence to the contrary. The response to noradrenaline, measured in terms of systemic vascular resistance, was found to be reduced in sheep following infusion of live *Escherichia coli* and this hyporesponsiveness was reversed by administration of a NOS inhibitor.

In summary, both nitric oxide and prostanoids were found to modulate the responses of ovine digital arteries to vasoactive agents. Whilst incubation with endotoxin did not significantly alter the vascular responses of the ovine digital artery, incubation with cytokines enhanced bradykinin-induced relaxation and reduced the sensitivity to phenylephrine. However, the reduction in phenylephrine response was less marked than that recorded in rat aorta and may reflect a genuine difference in the response of ovine digital arteries to endotoxin/cytokines.

3. A Study of the Arteries Which Supply the Ovine Digit

3.1 Introduction

3.1.1 Vascular anatomy of the digit

3.1.1.1 The ruminant digit

Accounts of the limb vasculature in ruminants can be found in standard anatomy texts (Ghoshal 1975, Schummer *et al.* 1981c & d, Dyce *et al.* 1987c & d, Habel 1989a & b, Smallwood 1992). Such descriptions form the basis of the following summary.

The main arterial supply to the forefoot is via the third palmar common digital artery, a continuation of the median artery (Figure 3.1.1). This gives rise to the axial and abaxial palmar proper digital arteries of the third and fourth digits. Dorsal arteries are present but are of less importance. They are linked to the palmar vessels by the interdigital artery which passes through the interdigital cleft.

The major vessel supplying the hind foot is the dorsal metatarsal artery which is a continuation of the cranial tibial artery (Figure 3.1.2). In the ruminant, but not the horse, this vessel maintains a dorsal position on the metatarsus. It is continued beyond the fetlock as the third dorsal common digital artery which then descends into the interdigital space, receiving a contribution from the third plantar common digital artery before dividing into axial plantar proper digital arteries of the third and fourth digits. The plantar arteries of the hind foot are less important. They are derived from the saphenous artery which divides into medial and lateral plantar arteries at the level of the hock. Each plantar artery provides deep and superficial branches. Plantar common digital arteries II and III are derived from the superficial branch of the medial plantar artery whereas plantar common digital artery IV arises from the superficial branch of the lateral plantar artery. Metatarsal arteries II-IV originate from the deep branches and are linked by proximally and distally by deep plantar arches.

The axial proper digital arteries give rise to a series of branches which are similar in both forefoot and the hindfoot. These branches, which are analogous to those in the horse, supply the bulbs of the heels, the coronary band and the phalanges (Figure 3.1.3). Each proper digital artery is continued as a distal phalangeal artery which enters the ungular canal of the distal phalanx forming a terminal arch by anastomosing with the bulbar and abaxial palmar proper digital arteries. Branches

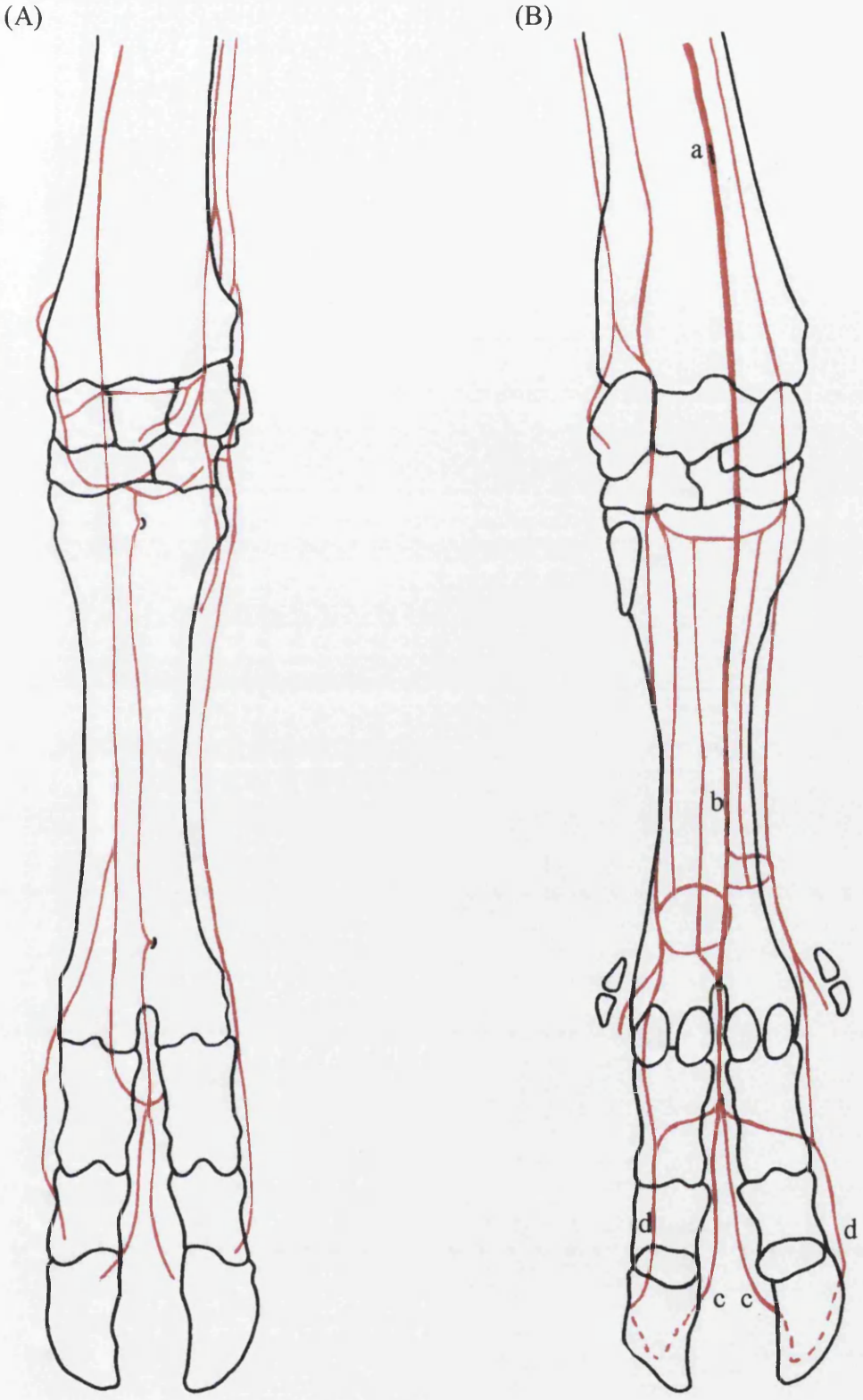


Figure 3.1.1. Dorsal (A) and palmar (B) arteries of the bovine forelimb. Adapted from Schummer *et al.* (1981c).

a - median artery

b - palmar common digital artery III

c - axial palmar proper digital arteries

d - abaxial palmar proper digital arteries

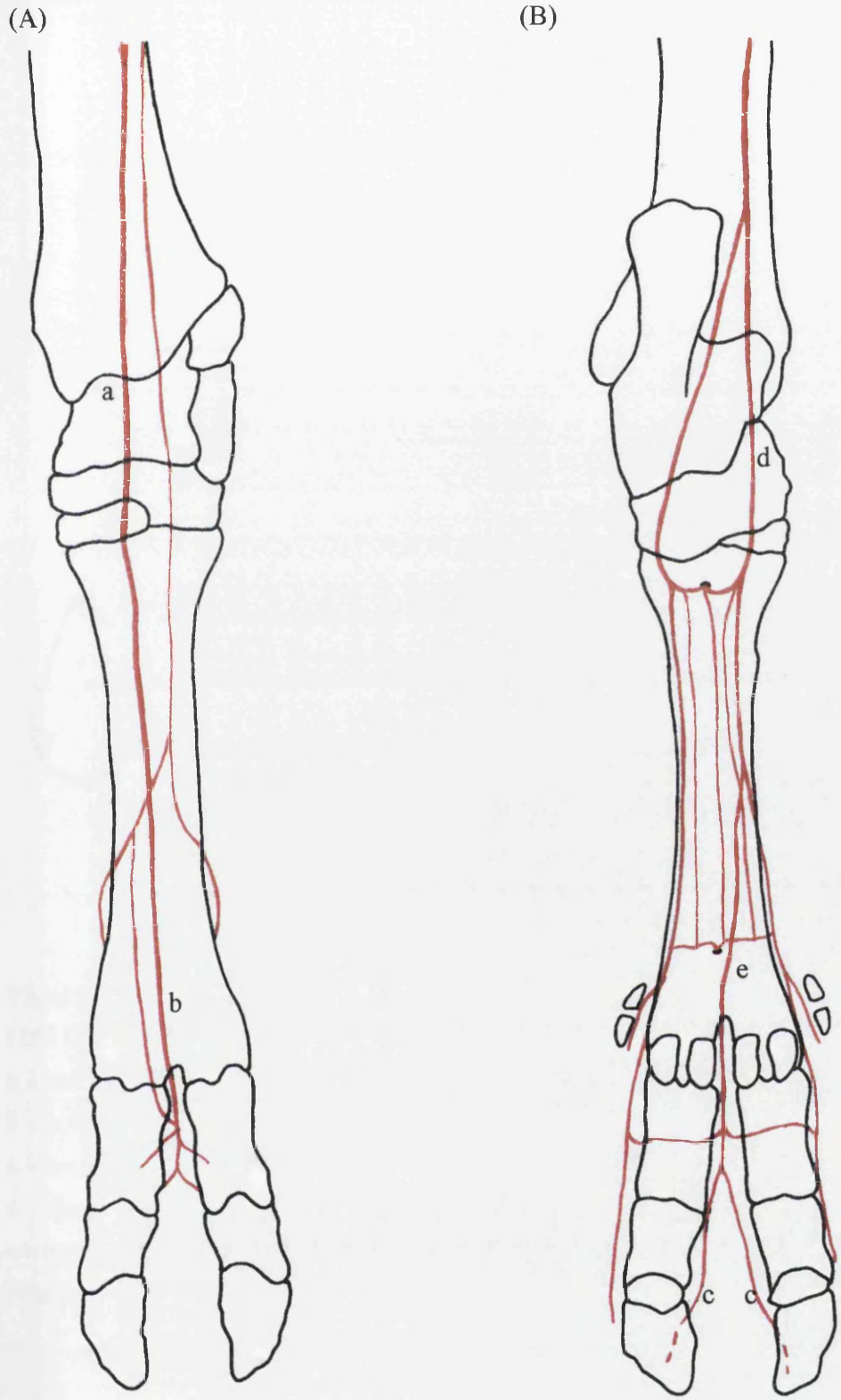


Figure 3.1.2. Dorsal (A) and plantar (B) arteries of the bovine hindlimb. Adapted from Schummer *et al.* (1981d).

a - dorsal metatarsal artery

b - dorsal common digital artery III

c - axial plantar proper digital arteries

d - saphenous artery

e - plantar common digital artery III

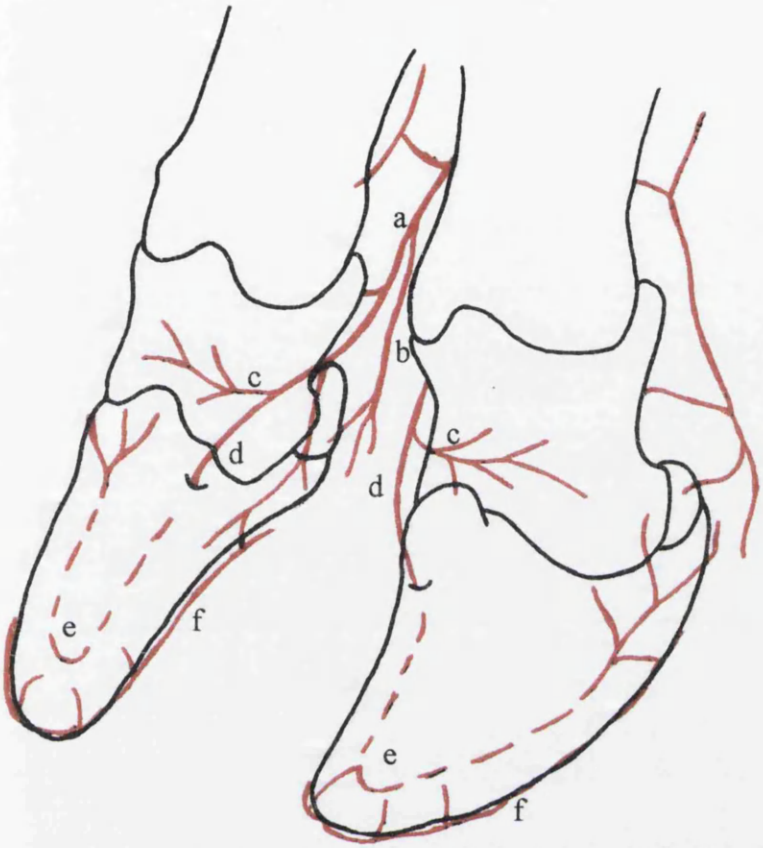


Figure 3.1.3. The arterial supply to the bovine digit. Adapted from Schummer *et al.* (1981b).

a - axial proper digital artery

b - bulbar branch

c - coronal branch

d - distal phalangeal artery

e - terminal arch

f - artery of the margin of the sole

arise from the terminal arch to supply the dermis of walls and sole. Some of these branches anastomose to form the artery of the margin of the sole.

The digit is drained by a dense system of venous plexuses. Five networks are recognised which drain the distal phalanx and the dermis of the coronary region, wall, sole and heel. Each claw has one dorsal and two plantar proper digital veins into which these plexuses empty.

The microcirculation of the ruminant digit has not been extensively studied. Schummer *et al.* (1981b) suggested that arterio-venous anastomoses are likely to occur throughout the dermal circulation of the claw. This has been confirmed in the bovine by scanning electron microscopy of corrosion casts (Vermunt and Leach 1992a).

3.1.1.2 The equine digit

Much of the research pertaining to laminitis has been conducted in the horse and it is in this species that the structure of the digital microcirculation was first described (Mishra and Leach 1983, Pollitt and Molyneux 1990). Descriptions of the vascular anatomy of the equine digit are abundant in the literature. The following summary is based on the accounts of Schummer *et* al. (1981c & d), Dyce *et al.* (1987a & b), Kainer (1989) and Pollitt (1992). The arterial blood supply to the digit is essentially the same in both fore and hindlimbs. It is derived from branches of the medial and lateral digital arteries. In the forelimb, the digital arteries originate just above the fetlock by division of the medial palmar artery which is a continuation of the median artery (Figure 3.1.4). In the hindlimb, the digital arteries are derived from plantar vessels which originate from the cranial tibial and saphenous arteries (Figure 3.1.5).

The digital arteries descend on either side of the flexor tendons to terminate within the hoof (Figure 3.1.6). The first important branch is the bulbar artery which is given off at the level of the proximal interphalangeal joint. This supplies the digital cushion and the dermis of the caudal aspect of the hoof including the frog. The next two branches, the coronal artery and the dorsal artery of the middle phalanx, supply the perioplic and coronary dermis. They arise level with the middle of the second phalanx and anastomose both with each other and with the artery of the opposite side to form an arterial circle around the coronary band sometimes called the coronary circumflex artery. The last major branch of the digital artery is the dorsal artery of the distal phalanx. This divides into medial, dorsal and palmar vessels which supply the frog and digital cushion, the dorsal hoof and the heels.

Just distal to this point the medial and lateral digital arteries enter the solar canal of the distal phalanx (pedal bone) where they unite to form the terminal arch.

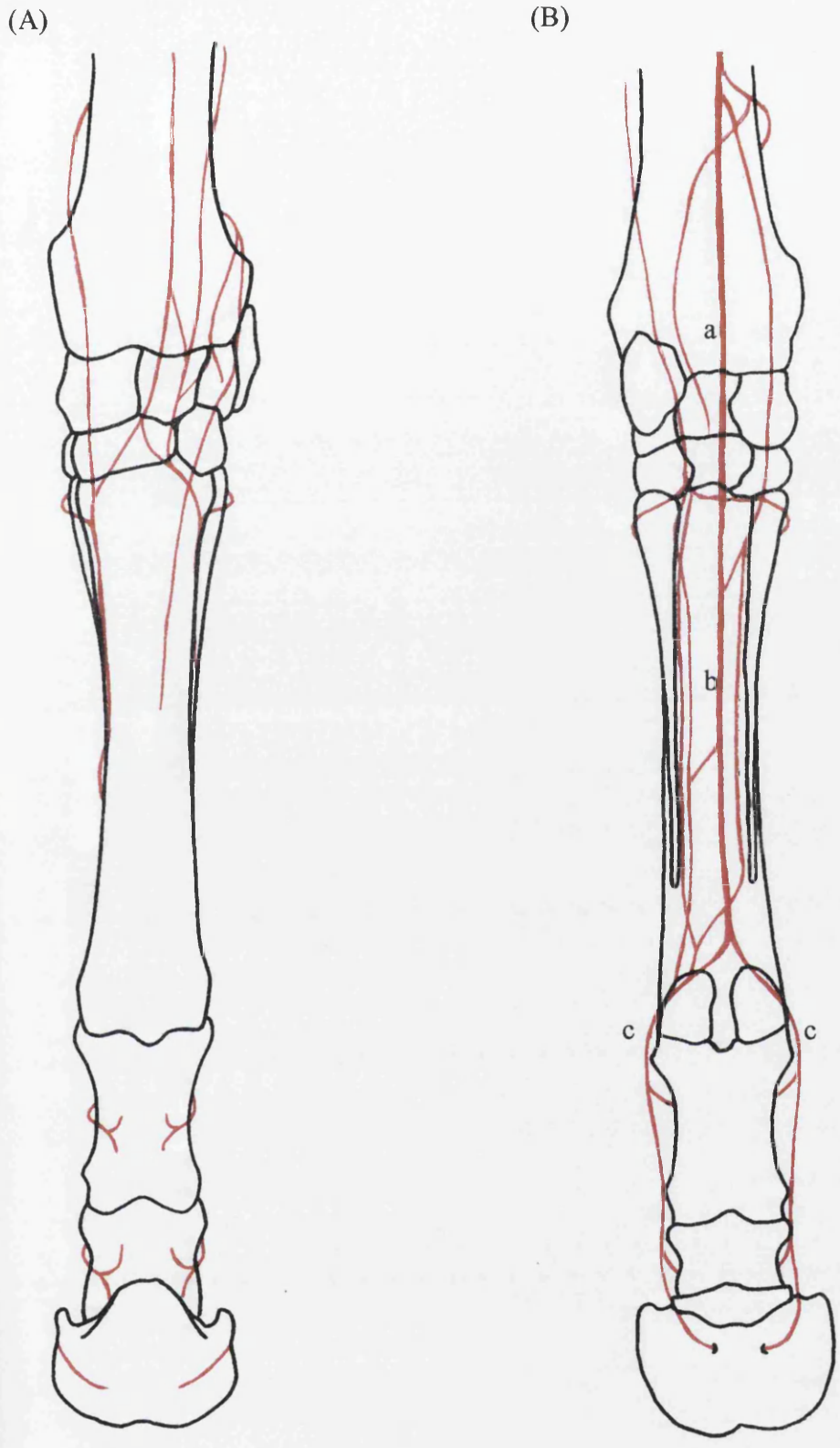


Figure 3.1.4. Dorsal (A) and palmar (B) arteries of the equine forelimb. Adapted from Schummer *et al.* (1981c).

a - median artery

b - medial palmar artery

c - medial and lateral digital arteries

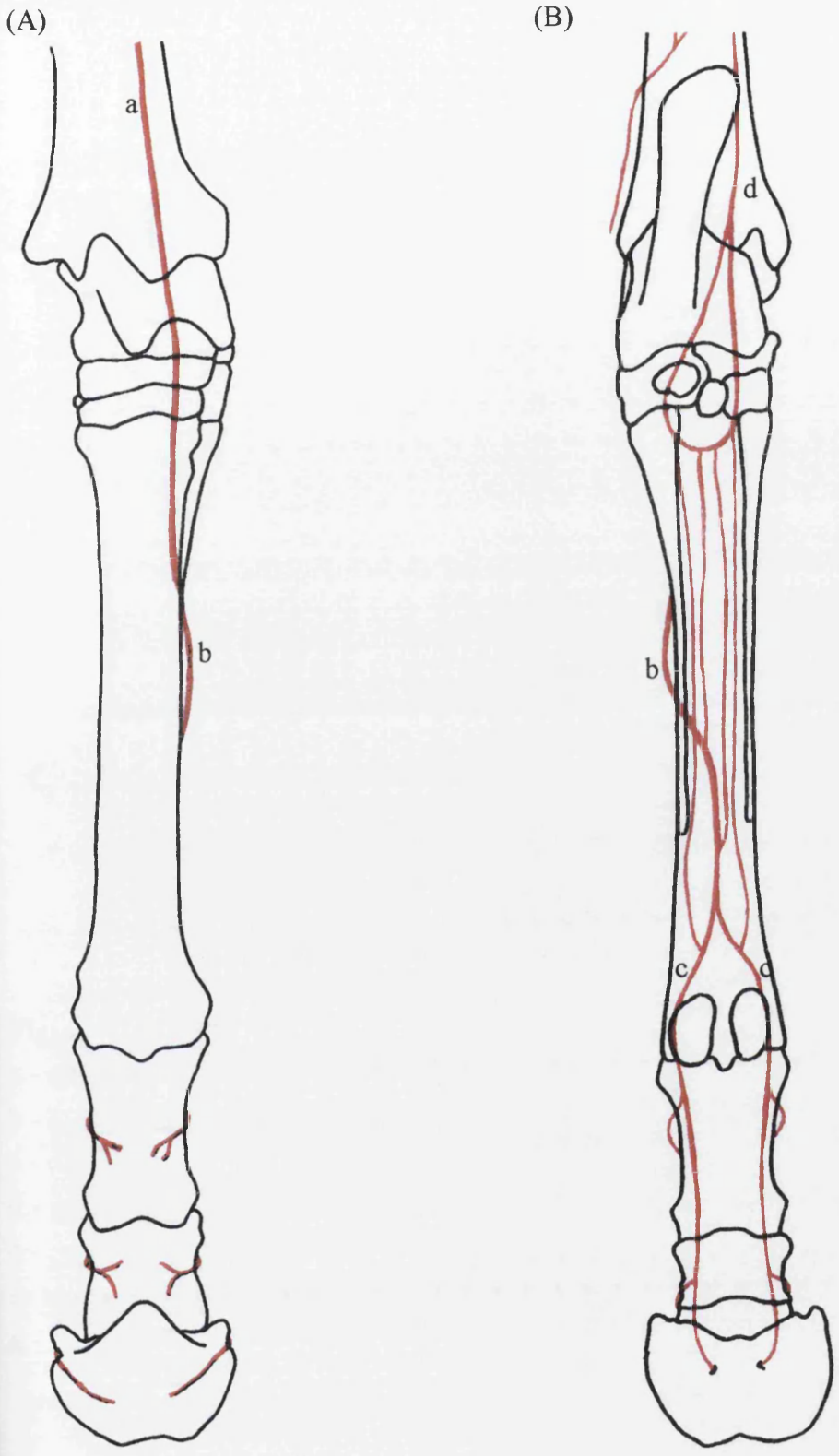


Figure 3.1.5. Dorsal (A) and plantar (B) arteries of the equine hindlimb. Adapted from Schummer *et al.* (1981d).

a - cranial tibial artery

b - dorsal metatarsal artery

c - medial and lateral digital arteries

d - saphenous artery

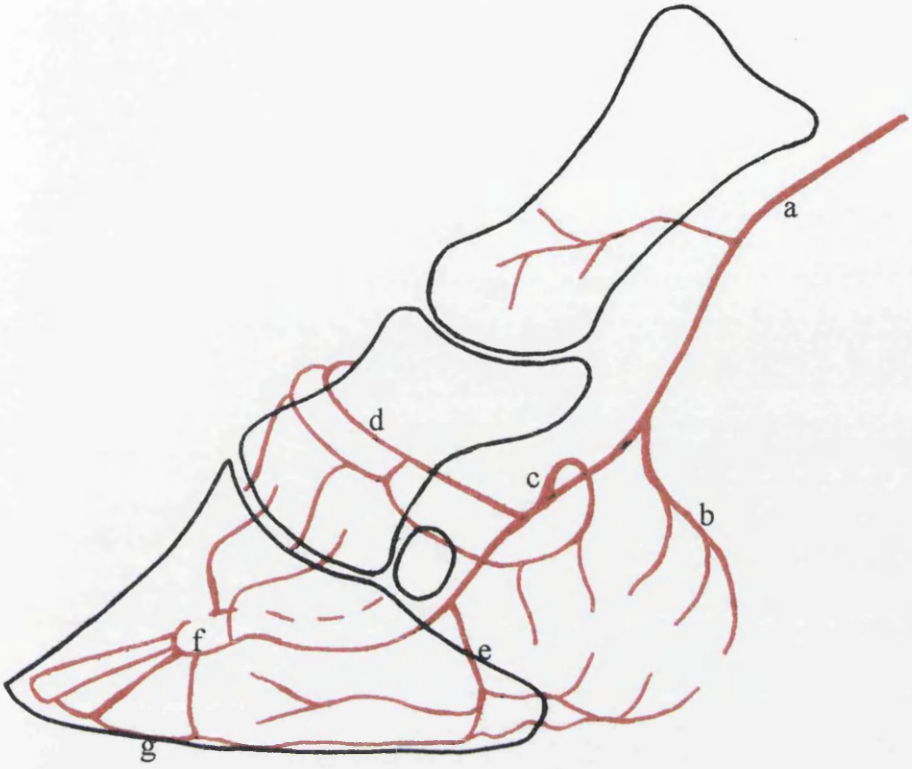


Figure 3.1.6. The arterial supply to the equine digit. Adapted from Pollitt (1992).

a - medial or lateral digital artery

b - bulbar branch

c - coronal artery

d - dorsal artery of the middle phalanx

e - dorsal artery of the distal phalanx

f - terminal arch

g - circumflex artery of the sole

Branches radiate out from this arch and exit the bone through foramina in its dorsal surface. Four-to-five of these vessels emerge in the mid dorsal region and form a network which supplies the laminar dermis. The remainder emerge closer to the solar border where they unite to form the circumflex artery of the sole. This provides many branches which pass under the margin of the distal phalanx to supply the dermis of the sole.

Venous drainage of the dermis is achieved by a system of valveless interconnected venous plexuses; the dorsal or parietal plexus drains the laminar dermis, the coronary plexus drains the coronary dermis and the palmar (plantar) or solar plexus drains the dermis of the sole and frog. The deep structures of the digit are drained by two parallel veins which lie within the solar canal. These anastomose to form a single vessel shortly after exiting the pedal bone. This vessel continues proximally and after receiving drainage from the venous plexuses it becomes the medial or lateral digital vein.

Veno-venous and arterio-venous anastomoses are widespread in the dermis of the hoof (Talukdar *et al* 1972). Recent work using electron microscopy to examine vascular corrosion casts has revealed the structural details of the microcirculation (Mishra and Leach 1983, Pollitt and Molyneux 1990).

3.1.2 Aim of the study

This work was carried out as a prelude to the ultrasound studies in Chapter 4. These subsequent investigations required the placement of intraarterial catheters and infusion of drugs into the digital vascular bed. The aim of the current study was therefore to examine the vasculature of the ovine limb and confirm which arteries supplied the digit and which were accessible for catheterisation.

3.2 Materials and methods

3.2.1 Animals

Tissues were obtained from 3 greyface ewes (Border Leicester x Scottish Blackface), aged between 4 and 6 years. The sheep were purchased from a commercial flock and were due to be culled because of poor dentition.

3.2.2 Euthanasia

Sheep were first sedated with an intramuscular injection of 0.2mg/kg xylazine, (*Rompun 2%*, Bayer plc., Bury St Edmunds, Suffolk, U.K.). Euthanasia was achieved by injection of 50ml of 20% pentobarbitone sodium (*Euthatal*, Rhone Merieux Ltd., Harlow, Essex, U.K.) into the jugular vein. Immediately after euthanasia, the carotid arteries and jugular veins were severed to permit bleeding out.

3.2.3 Solutions

3.2.3.1 Heparinised saline

Heparinised saline was prepared by adding 1ml of heparin sodium (Leo Laboratories Ltd., Princes Risborough, Buckinghamshire, U.K.), i.e. 1000 units, to 500ml of 0.9% saline (*Aqupharm No. 1*, Animalcare Ltd., York, U.K.), giving a concentration of 2 heparin units/ml.

3.2.3.2 Latex solution

Latex solution (*Adhesive 7791*, Scottish Adhesives Company Ltd, Glasgow, Scotland), was coloured with red acetone-soluble dye before use.

3.2.3.3 Methyl methacrylate

Acrylic solution was prepared from *Tensol No. 70* (Evode Speciality Adhesives, Leicester, U.K.). Components A and B were combined, 20 parts A to 1 part B, and coloured with red acetone-soluble dye.

3.2.3.4 Radioopaque solution

One 11g sachet of gelatin (Davis gelatine, Leamington Spa, U.K.) was added to 500ml of a 3 volumes to 1 volume mixture of barium sulphate (*Micropaque*, Nicholas Laboratories Ltd., Slough, U.K.) and water. This mixture was then heated in a waterbath to approximately 50°C until all the gelatin was dissolved.

3.2.3.5 Formalin

A 10% solution of formalin (3.7% formaldehyde) was prepared by diluting 1 part formalin (37% formaldehyde, Sigma, Poole, Dorset, U.K.) with 9 parts water.

3.2.3.6 Potassium hydroxide

A 10% solution of potassium hydroxide was prepared by dissolving 100g of potassium hydroxide (Merk Ltd., Poole, Dorset, U.K.) in 1L of water.

3.2.4 Preparation of casts

The axillary artery was located medial to the scapula and was catheterised with a shortened dog urinary catheter, 6 french, (Rocket Medical, Watford, U.K.). The external iliac artery was identified within the caudal abdomen and was catheterised just proximal to the point at which it left the abdomen and became the femoral artery. Both catheters were secured with linen thread and flushed with heparinised saline.

3.2.4.1 Latex casts

One sheep was used in the preparation of latex vascular casts. Liquid latex, 100-120ml, was injected into the arteries supplying the limb via the preplaced catheters. Catheters were removed and the arteries tied off. After 24 hours in the cold store the skin was removed and the superficial vessels were revealed by shallow dissection. The limbs were preserved in a solution of 10% formalin.

3.2.4.2 Methyl methacrylate casts

Acrylic vascular casts were prepared from the limbs of a sheep. Methyl methacrylate solution, 40-50ml, was injected into the fore and hindlimb arteries via the preplaced catheters. After injection, the catheters were removed and the arteries were tied off using linen thread. The limbs were stored at room temperature for 4-6 hours to allow the casts to set. They were then sectioned just proximal to the carpus/tarsus and placed in a bath of 10% potassium hydroxide. A period of 10-14 days was sufficient to dissolve the majority of the soft tissues. The resulting casts were then rinsed thoroughly in running water before being air-dried at room temperature.

3.2.4.3 Radiographic contrast studies

One sheep was used for the radiographic studies. Radioopaque solution, 100-120ml, was injected into the axillary and external iliac arteries. Catheters were removed and the arteries tied off. After 24 hours in the cold store, radiographs were taken using a *Heliophos 4S* x-ray unit (Siemens, Bracknell, Berkshire, U.K.). Lateral and cranio-caudal radiographs were taken using *Cronex 10s* film (Dupont [UK] Ltd., Stevenage, Hertfordshire, U.K.) and *Cronex Quanta Detail* screens (Dupont [UK] Ltd.). Films were developed by a *Cronex CX-130* automatic processor (Dupont [UK] Ltd.). The exposure settings used are given in Table 3.2.1.

LIMB	VIEW	kV	mAs	mA
FORELIMB	Lateral limb	55	5	160
	Lateral distal limb	55	5	80
	Cranio-caudal limb	55	5	160
	Cranio-caudal distal limb	57	5	80
HINDLIMB	Lateral limb	57	5	160
	Lateral distal limb	57	5	80
	Cranio-caudal limb	57	5	160
	Cranio-caudal distal limb	60	5	80

Table 3.2.1. Exposure factors used to obtain radiographs of the ovine limb after intraarterial injection of 100-120ml contrast agent.

Once casts and radiographs were prepared, the vessels were identified on the basis of their location by consultation with standard anatomy texts (Ghoshal 1975, Schummer *et al.* 1981c & d, Dyce *et al* 1987c & d, Habel 1989a & b, Smallwood 1992).

3.3 Results

3.3.1 Latex casts

Removal of the skin from both fore and hindlimbs revealed a number of superficial vessels containing latex, the majority of which were veins. In the forelimb, the median artery was superficial where it crossed the medial aspect of the carpus and palmar-medial aspect of the metacarpus (Figure 3.3.1A).

In the hindlimb only the saphenous artery, which traverses the muscles of the medial thigh was visible after removal of the skin (Figure 3.3.1B). Deeper dissection was required to reveal the dorsal metatarsal artery, which lay deep to the long digital extensor tendons on the dorsal aspect of the metatarsus (Figure 3.3.2).

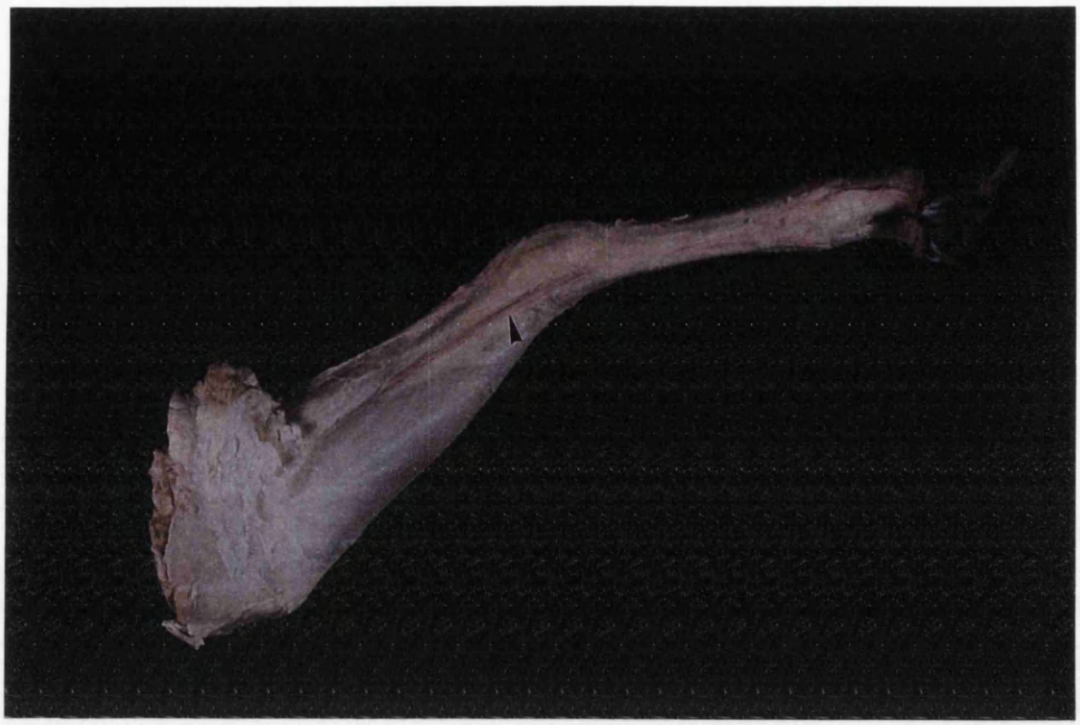
3.3.2 Methyl methacrylate casts and radiographs

The methyl methacrylate casts and contrast radiographs confirmed that the median artery provides the main arterial supply to the forelimb, as described in Section 3.1.1.1 (Figures 3.3.3 & 3.3.4). It descended on the palmar-medial aspect of the metacarpus, before deviating towards midline as the third palmar common digital artery, at the level of the proximal interphalangeal joint. It then ran a short distance in the interdigital space before dividing into third and fourth axial palmar proper digital arteries. Abaxial arteries could not be identified.

The casts and radiographs indicated that the dorsal metatarsal artery, a continuation of the cranial tibial artery, is the largest artery of the hindlimb (Figures 3.3.5 & 3.3.6). It descended the dorsolateral aspect of the metatarsus before moving to a more midline position at the level of the fetlock. Now termed the third dorsal common digital artery, it entered the interdigital space and bifurcated into third and fourth plantar proper digital arteries. Smaller arteries were identified on the plantar aspect of the hindlimb. The largest of these was the third plantar common digital artery, which originated from the saphenous artery (Figure 3.3.6). A short interdigital artery connected dorsal and plantar vessels within the interdigital space. Additional communication was provided by perforating branches in the proximal and distal metatarsus.

Only the contrast radiographs were useful in determining the most distal arterial branches supplying the digit (Figures 3.3.7 & 3.3.8). The angiogram of the right hind provided the clearest picture, as minimal contrast had reached the venous side of the circulation, however the main branches of the digital arteries could also be identified in the forefoot.

(A)



(B)

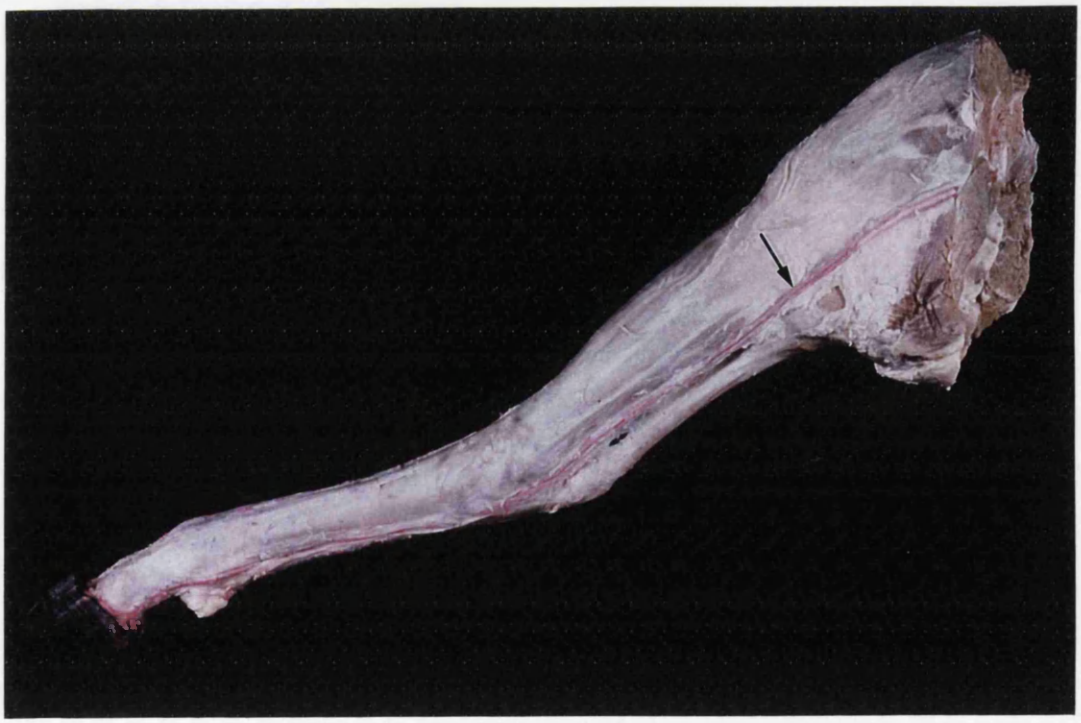


Figure 3.3.1. Latex vascular casts of the sheep medial forelimb (A) and hindlimb (B), preserved in 10% formalin *in situ*.

▲ median artery

↑ saphenous artery

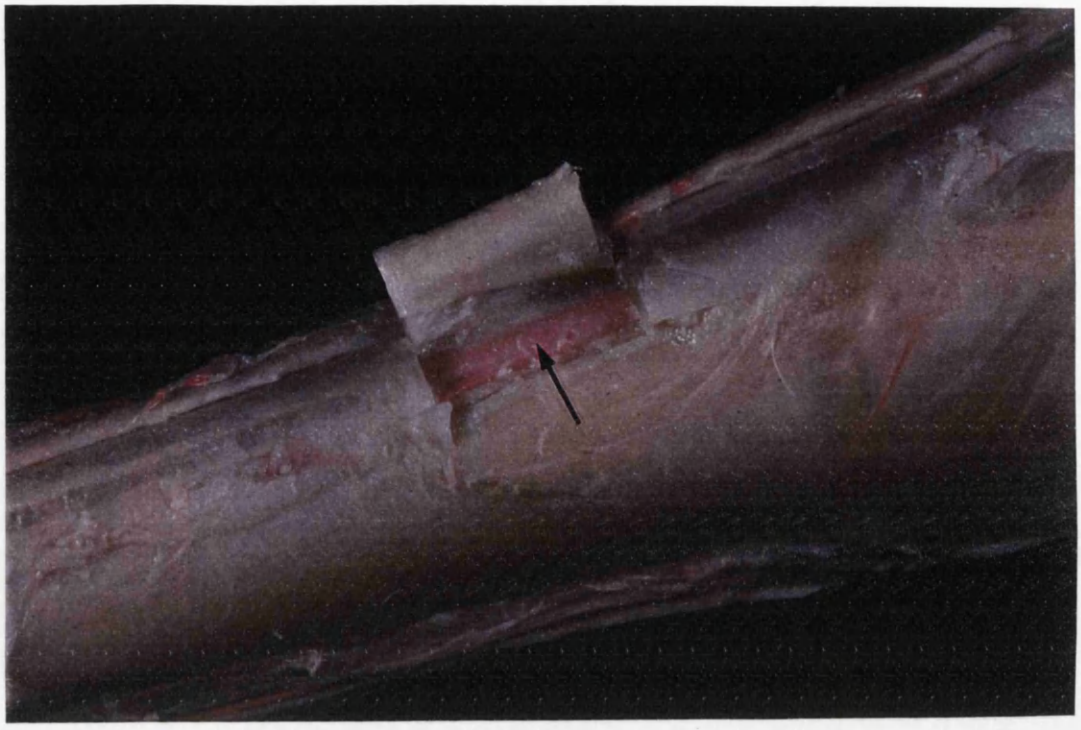
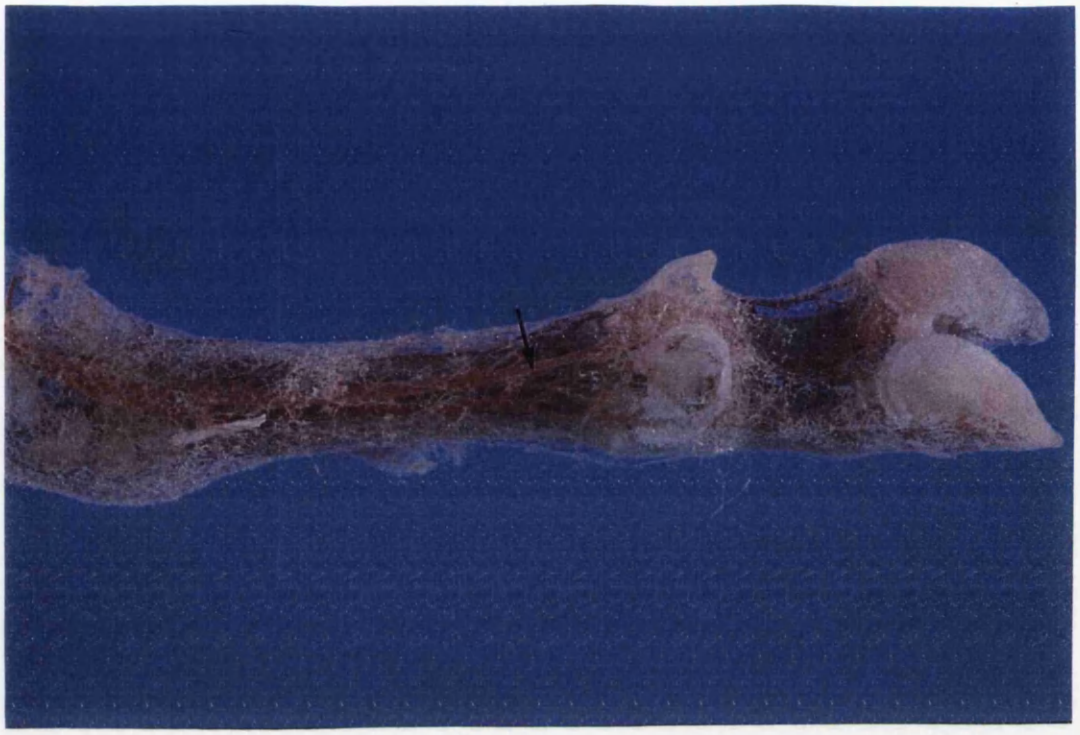


Figure 3.3.2. Latex vascular cast of the sheep hindlimb, preserved with 10% formalin *in situ*. A section of the long digital extensor tendon has been reflected to reveal the dorsal metatarsal artery.

↑ dorsal metatarsal artery

(A)



(B)

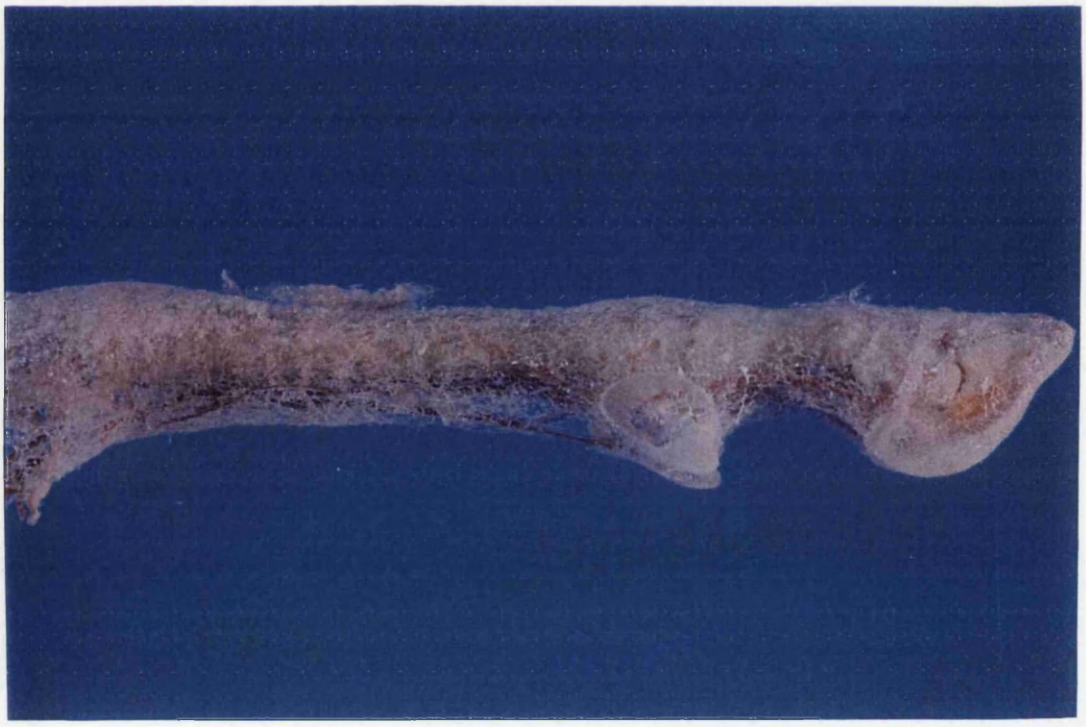


Figure 3.3.3. Palmar (A) and lateral (B) views of a methyl methacrylate corrosion cast of the vasculature of the sheep distal forelimb.
↑ palmar common digital artery III

(A) (B)

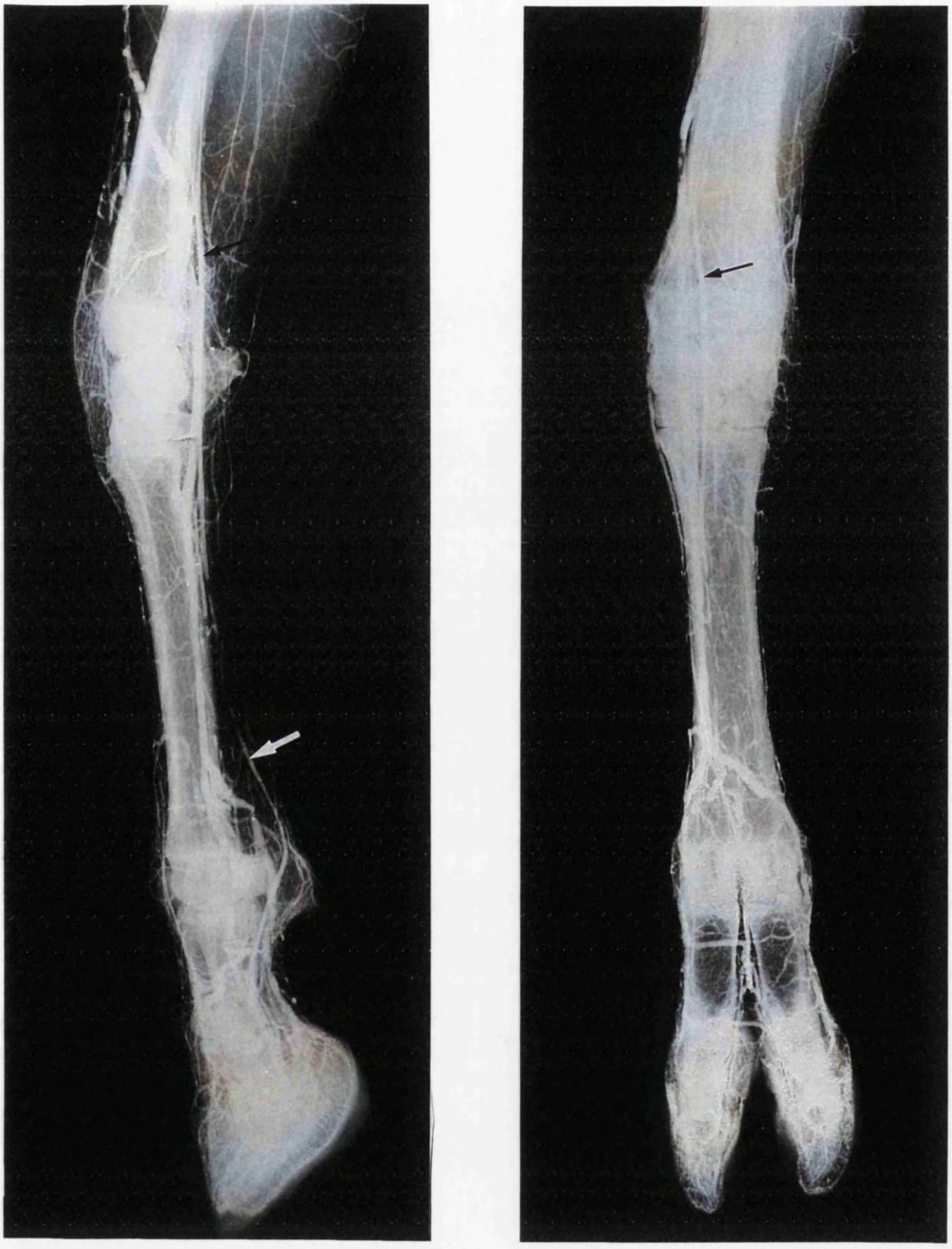
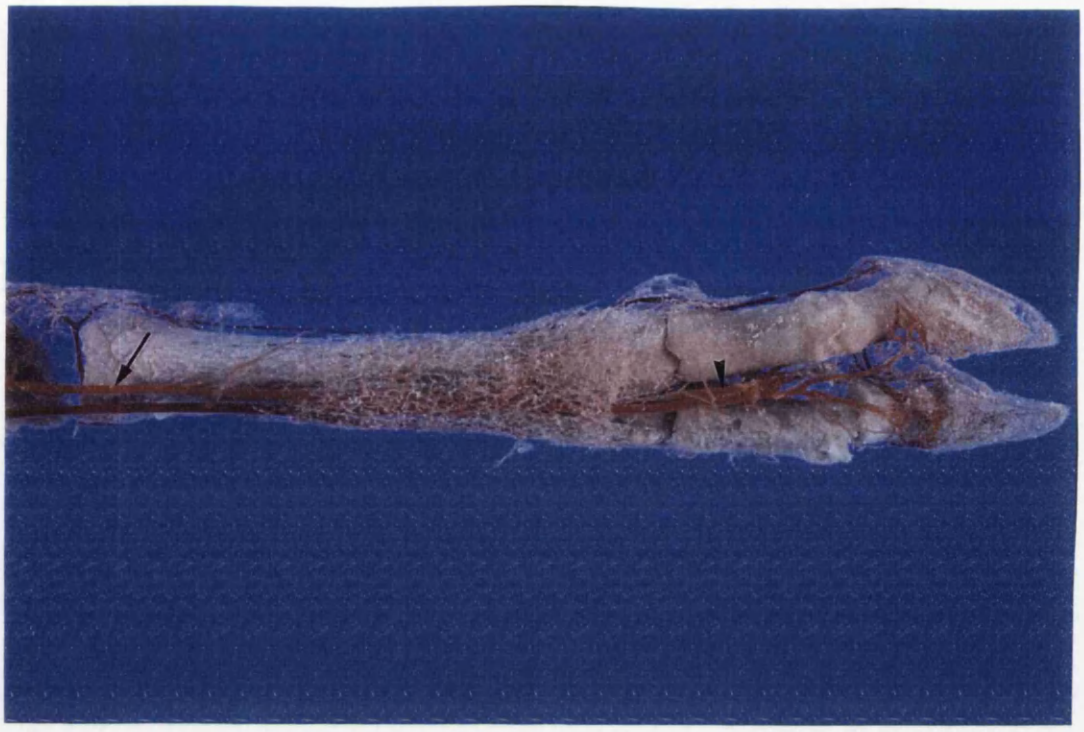


Figure 3.3.4. Lateral (A) and craniocaudal (B) angiograms of the ovine forelimb.
↑ median artery
⇑ palmar common digital artery III

(A)



(B)

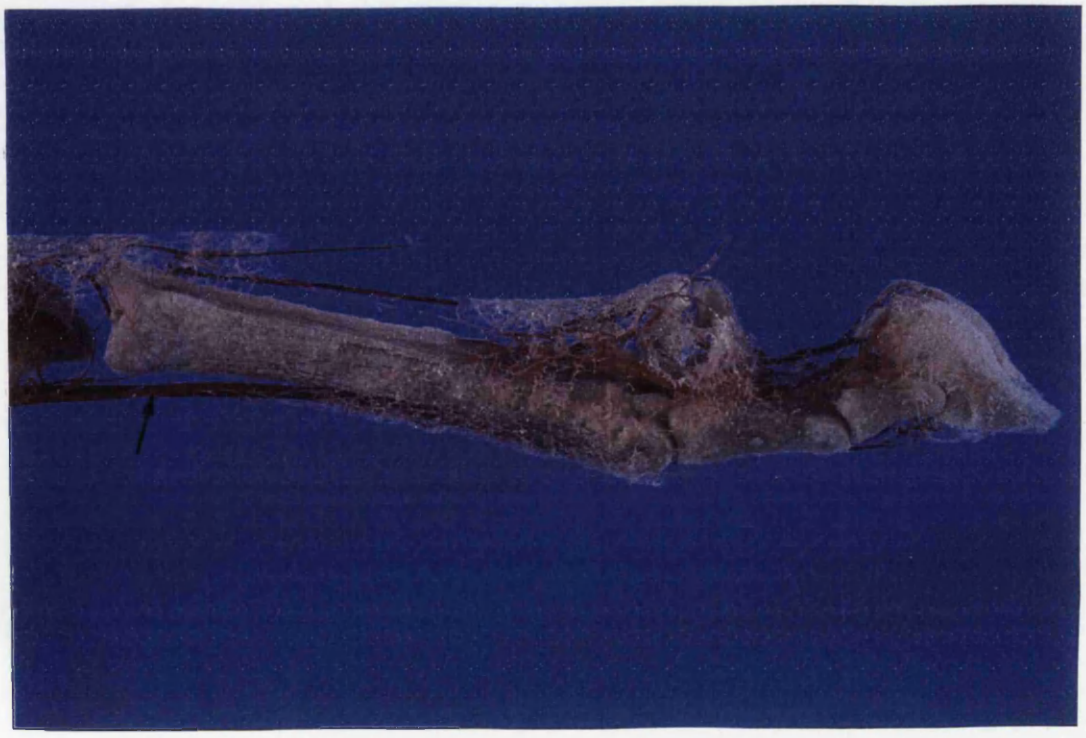


Figure 3.3.5. Dorsal (A) and lateral (B) views of a methyl methacrylate corrosion cast of the vasculature of the sheep distal hindlimb.

↑ dorsal metatarsal artery

▲ dorsal common digital artery III

(A) (B)

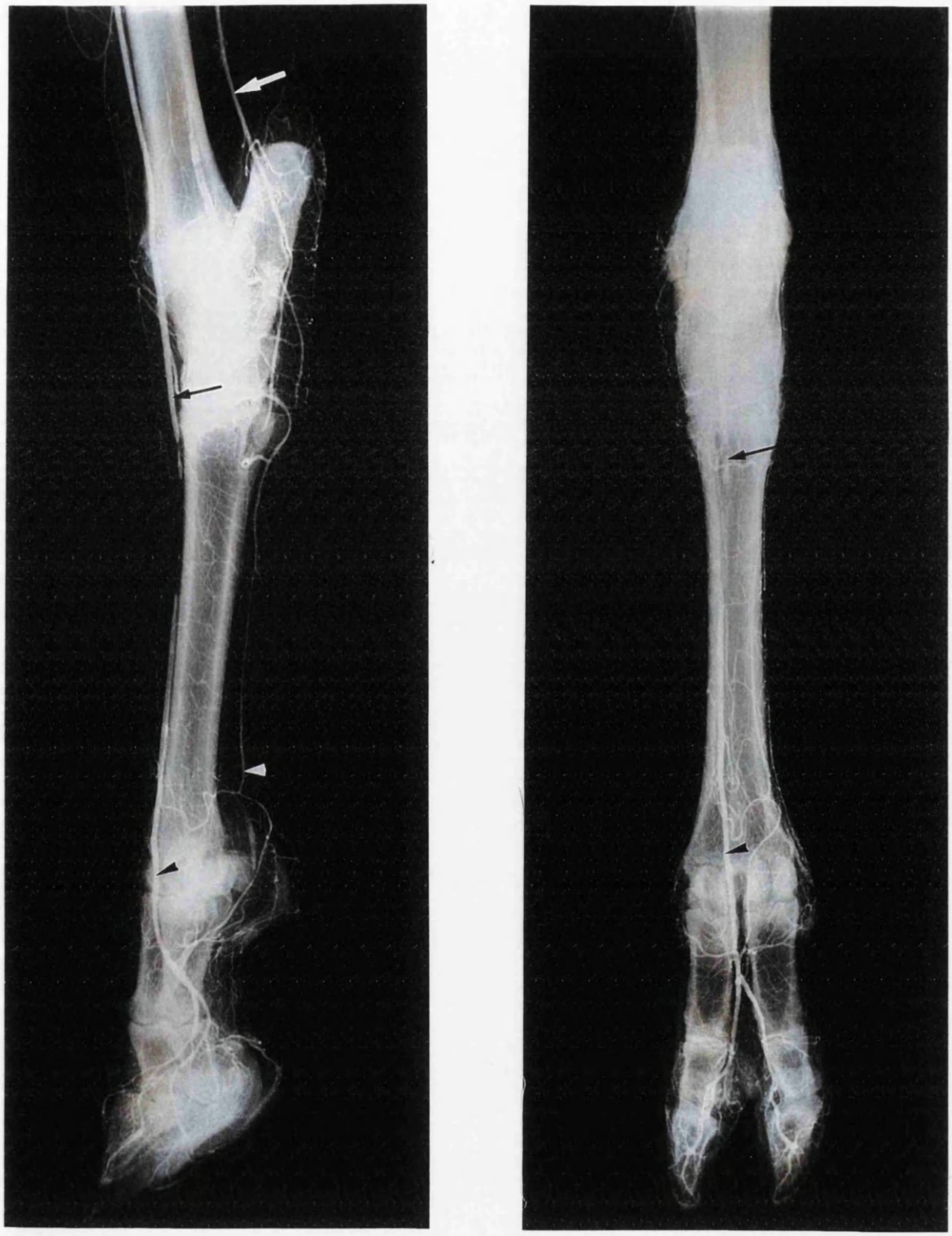


Figure 3.3.6. Lateral (A) and craniocaudal (B) angiograms of the ovine hindlimb.

↑ dorsal metatarsal artery

▲ dorsal common digital artery III

⇑ saphenous artery

Δ plantar common digital artery III

(A) (B)

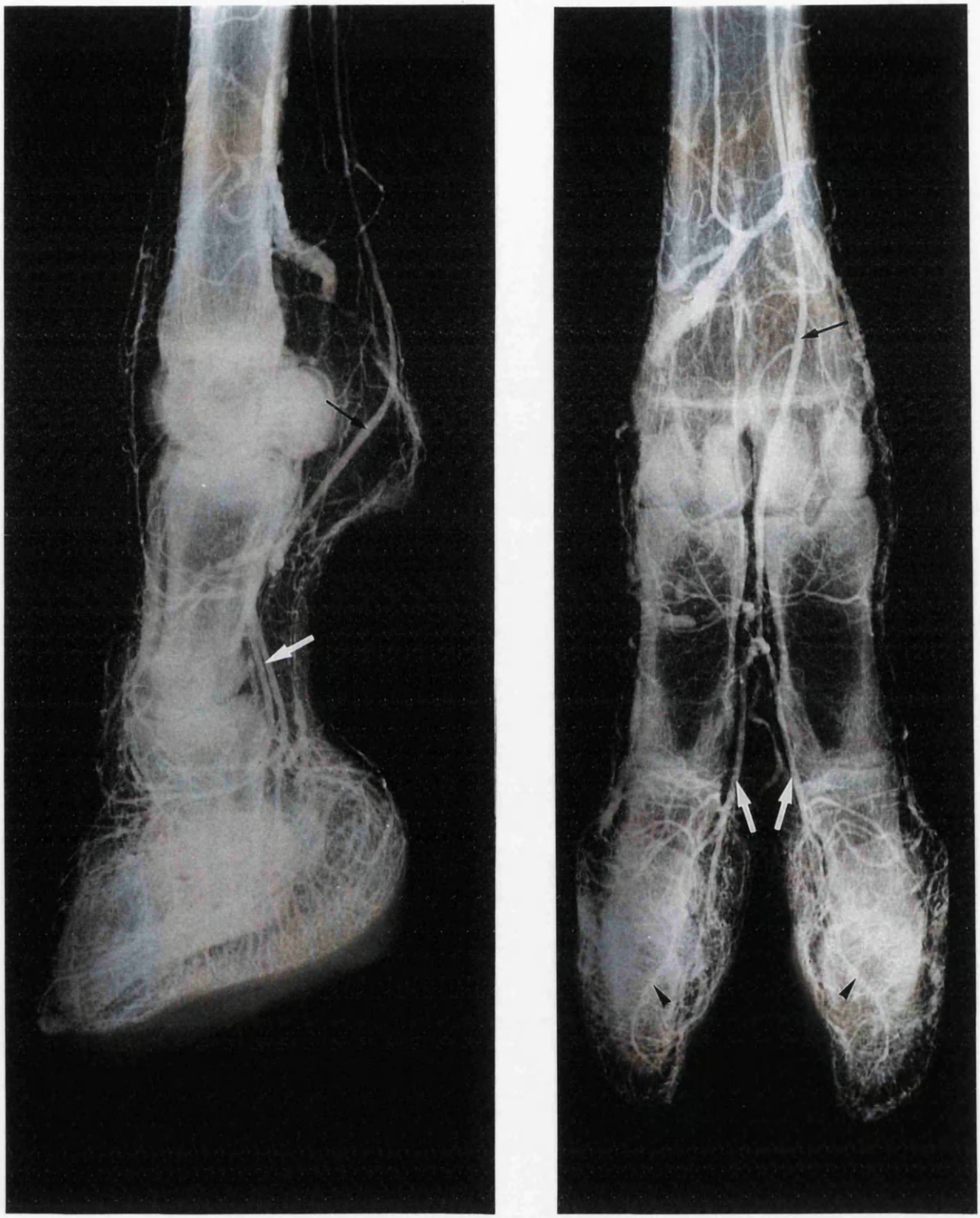


Figure 3.3.7. Lateral (A) and craniocaudal (B) angiograms of the ovine distal forelimb.
↑ palmar common digital artery III
⇑ axial palmar proper digital arteries
▲ distal phalangeal artery

(A) (B)

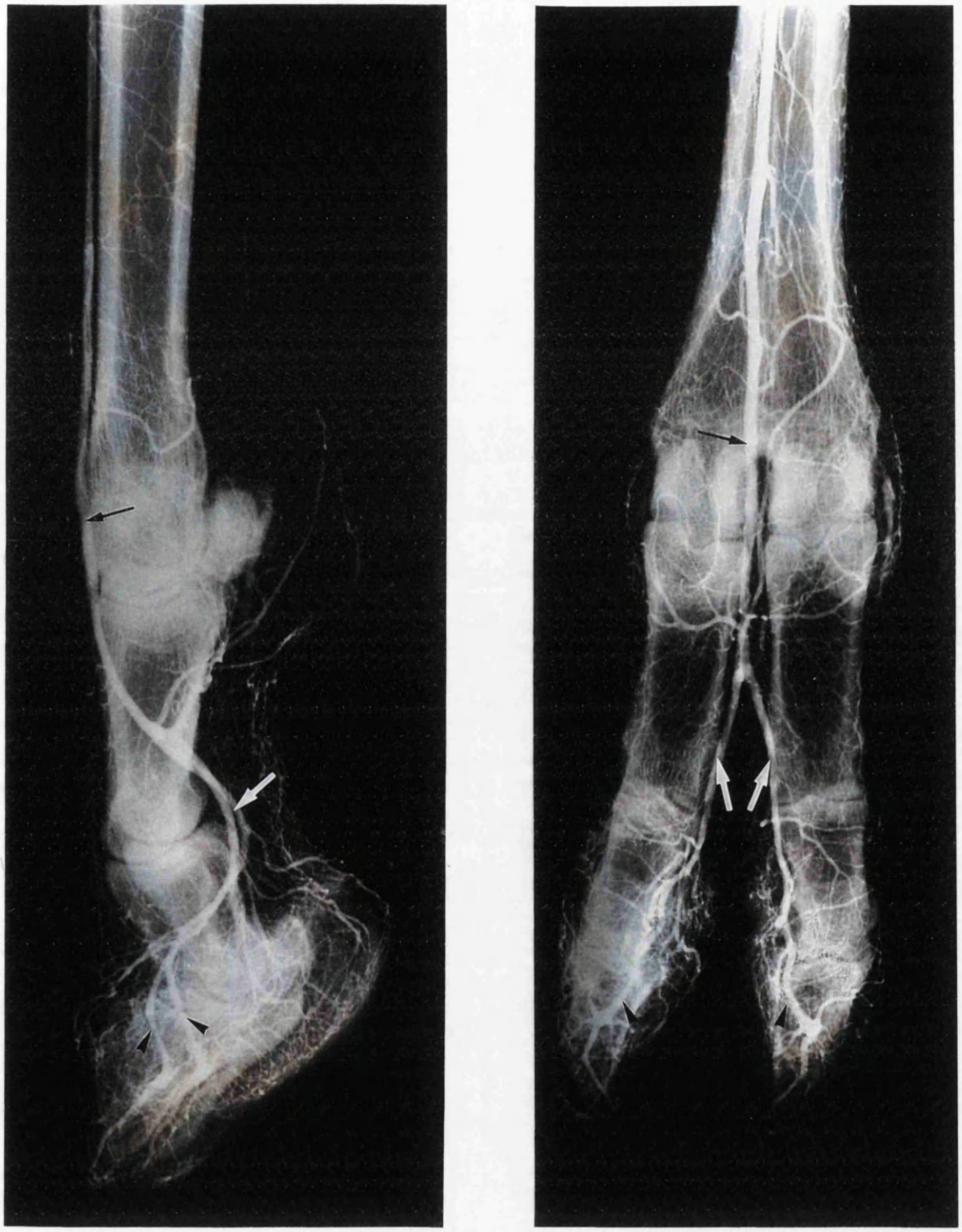


Figure 3.3.8. Lateral (A) and craniocaudal (B) angiograms of the ovine distal hindlimb.
↑ dorsal common digital artery III
⇑ axial palmar proper digital arteries
▲ distal phalangeal artery

Each proper digital artery supplied a branch to the bulbs of the heels, just distal to the proximal interphalangeal joint. This was followed by a palmar/plantar branch. The continuation of the proper digital artery, i.e. the distal phalangeal artery, then changed direction and curved dorsodistally in the interdigital space producing branches to the coronary and perioplic regions. It then entered the bony canal of the distal phalanx, close to the extensor process and was directed towards the palmar/plantar surface of the bone. The dorsoplantar radiograph revealed a rather limited terminal arch. The distal phalangeal artery formed the axial limb which terminated in a number of branches. The major branch continued to the apex of the toe whilst a more slender vessel was deflected along the abaxial border to complete the arch. Fine vessels could be seen arising from this to supply the laminar dermis. No distinct artery of the rim of the sole could be identified, although branches from the terminal arch appeared to form a fine anastomosing network.

3.4 Discussion

There is very little published material relating specifically to the arterial system of the ovine limb. Descriptions of the limb vasculature in ruminants can be found in standard anatomy texts (Ghoshal 1975, Schummer *et al.* 1981c & d, Dyce *et al.* 1987c & d, Habel 1989a & b and Smallwood 1992) but these are based on the ox and make, with the possible exception of Ghoshal (1975), only fleeting reference to the sheep. An investigation of the ovine vasculature was therefore indicated to confirm the above accounts and provide the anatomical knowledge required for the subsequent ultrasound investigations. A limited study based on only 3 sheep was considered sufficient for this purpose. However further work is needed to establish the incidence of individual variation.

Corrosion cast and angiographic techniques have been used to examine the digital vascular bed in cattle (Prentice and Wyn-Jones 1973; Gogoi *et al.* 1982; Vermunt and Leach 1992a & 1992b) and horses (Mishra and Leach 1983; Pollitt and Molyneux 1990). However, there are no reports of such use in sheep. In this study a combination of 2 cast techniques and angiography were used. The methyl methacrylate corrosion casts provided the basic three dimensional model of the vasculature in relation to the bony structures of the limb. However, the casting material was brittle and the finer vessels were easily disrupted and lost. Pollitt and Molyneux (1990) report that the use of a modified plastic, composed of methyl methacrylate diluted with Batson's No. 17 compound, produced softer more flexible casts which were less liable to fracture. This technique was unfamiliar to our technical staff but would perhaps be more appropriate for use in future studies. As a consequence of their delicacy the acrylic casts did not permit good visualisation of the most distal branches of the digital artery. This information was provided by angiography which permitted quite detailed inspection of even fine vessels, including those enclosed within the bony structures of the limb. It was also necessary to determine which vessels could be catheterised for the infusion of vasoactive substances. This required visualisation of the superficial course of the arteries in relation to the soft tissues, information provided by the latex casts. Thus all 3 techniques contributed to the overall picture of the ovine vasculature required for subsequent ultrasound studies.

Another problem encountered in this study was the difficulty in estimating the amount of cast material or contrast agent required to fill the arterial system. In almost all cases this was overestimated leading to additional venous filling. Consequently arteries and veins had to be differentiated and this was done by identifying vessels according to their location. In future angiographic studies this problem could be overcome by using image intensification to visualise arterial filling during the injection of contrast. Assessment of arterial filling during the

injection of cast material is more problematic. Pollitt and Molyneux (1990) and Vermunt and Leach (1992b) produced complete casts of the vascular network and then used scanning electron microscopy to distinguish the surface morphology of arteries and veins. Such techniques require the use of the modified methacrylate plastic described above and were not considered justified in view of the aim of this work.

In the horse, the major blood vessels are located on the flexor aspect of the joints, therefore in the hindlimb the dorsal metatarsal artery passes under the distal end of the lateral splint bone to reach the plantar surface of the metatarsus (Schummer *et al.* 1981d, Dyce *et al.* 1987b). This is not the case in the ox. The major vessel of the hindlimb, the dorsal metatarsal artery, continues as the dorsal common digital artery III over the dorsal and therefore extensor surface of the fetlock joint (Ghoshal 1975, Schummer *et al.* 1981d). The study appeared to confirm that this is also the case in the hindlimb of the sheep.

Accounts of the plantar arteries of the ruminant hindfoot vary. Schummer *et al.* (1981d) suggest that plantar common digital arteries II and III arise by division of the medial plantar artery and plantar common digital artery IV originates from the lateral plantar artery. Ghoshal (1975) refers specifically to small ruminants and proposes that all 3 plantar common digital arteries arise from the medial plantar artery, the lateral plantar artery contributing only to the metatarsal arteries and deep plantar arches. The angiograms support the latter description. In addition they confirm that the dorsal and plantar vessels communicate at a number of levels, namely the proximal perforating branch at the tarsometatarsal junction, the distal perforating branch just proximal to the fetlock and the interdigital artery in the interdigital space. In the light of these connections it seems reasonable to assume that a drug could be infused into the digital vascular bed by either the dorsal or plantar route in the hindlimb.

Distally a terminal arch, which unites axial and abaxial proper digital arteries within the distal phalanx, has been described in the ox (Schummer *et al.* 1981b). Vermunt and Leach (1992a), using corrosion cast techniques, found this union to be incomplete in cattle and the angiographic results from this study would suggest that this is also the case in sheep. The abaxial limb of the terminal arch was not well-defined in either forelimb or hindlimb angiograms. As in the ox, the artery of the margin of the sole comprised a fine anastomosing network rather than a distinct vessel.

The results of this limited study correlate well with the descriptions in the literature and confirm the general similarity between the limb arteries in cattle and sheep.

Minor differences were observed in the origin of the plantar arteries of the hindlimb and the form of the terminal arch. However these variations do not significantly alter the supply of blood to the digit. In addition the latex casts showed that the median artery was the most accessible forelimb artery, having a superficial course over the medial aspect of the metacarpus. In the hindlimb the saphenous artery was the most superficial but the larger dorsal metatarsal artery could also be catheterised if a deeper dissection was performed.

4. A study of the Doppler waveform of the ovine femoral artery *in vivo*

4.1 Introduction

4.1.1 Measurement of peripheral blood flow

Numerous different techniques have been used in an attempt to quantify blood flow. In 1963 Greenfield *et al.* reviewed those applicable to peripheral use, including venous occlusion plethysmography, indicator dilution and thermal devices. Since then new imaging methods have contributed greatly to advances in blood flow measurement, notably ultrasound, magnetic resonance imaging (MRI) and computed tomography (CT). These advances have been reviewed by Kajiya (1989). Rushmer *et al.* (1966) suggested a system for classifying the many techniques, allocating them to 1 of 4 categories:

(i) volume sensors

(ii) energy sensors

(iii) indirect measurements

(iv) external energy sources

Flow can be defined as a volume change per unit of time, thus techniques that measure changes in volume provide a direct measure of flow. Venous occlusion plethysmography is an example of a volume sensor that has been used to measure limb blood flow in man (Brodie and Russell 1905, Landowne and Katz 1942, Vallance *et al.* 1989). An occluding cuff applied proximally to the limb halts venous drainage without interrupting the arterial supply. Thus the initial rate of swelling of the limb can be equated to arterial inflow.

Energy sensors determine the energy content of moving liquids by measuring inertial forces or pressure gradients. Greenfield and Fry (1962) described a technique which estimated instantaneous velocity (v), from the pressure drop (Δp) measured along the length of a vessel. They used the following equation, which relates the inertial ($\rho \cdot \delta v/\delta t$) and frictional components (αv) of the pressure gradient:

$$-\frac{\Delta p}{\Delta z} = \rho \cdot \frac{\delta v}{\delta t} + \alpha v$$

where Δz = distance over which Δp occurred
t = time
ρ = blood density
α = coefficient of blood friction

Indirect methods use tracers or indicator substances. Measurement of changes in indicator concentration, arising from dilution, uptake or clearance, can be used to estimate flow. An example is the measurement of bromsulphalein clearance to quantify hepatic blood flow (Selkurt 1953). Many of these methods rely on the Fick principle, which states that the quantity of metabolically inert substance taken up by tissue is equal to the quantity brought to the tissue by arterial blood minus the quantity taken away by venous blood (Sharp 1994). A number of different indicators have been used. Dyes such as bromsulphalein (Selkurt 1953) and indocyanine green (Caesar *et al.* 1961) have largely been replaced by radioisotopes (Sharp 1994), such as ^{133}Xenon, which has been used to study the cerebral circulation (Obrist and Wilkinson 1990). Methods for measuring the concentration of radioactive tracers include probe detectors, gamma cameras and computed tomography techniques, such as single-photon-emission computed tomography and positron emission tomography (Porenta 1994).

The indirect methods described so far measure changes in tracer concentration. An alternative approach is to measure the static distribution of tracer within an organ or tissue. This can be achieved by administration of radio-labelled particles or microspheres that are of such a size they lodge in the capillaries. Blood flow to that tissue can then be quantified by measuring its radioactivity. This technique has contributed much to our knowledge of regional perfusion and its theoretical basis has been discussed by Holman *et al.* (1975). It has been successfully applied to studies of the peripheral vasculature including measurement of digital arterial blood flow in the horse (Hood *et al.* 1978).

The final group of techniques contains those methods that utilise external energy sources. Thermal flowmeters estimate blood flow by measuring the rate at which heat is transported away from a source (Mellander and Rushmer 1960). This can also be classed as an indirect method with thermal energy assuming the role of an indicator substance. Electromagnetic and ultrasonic flowmeters are also examples of this type of technique. They transmit wave energies which are then modified by moving blood cells. Detection and analysis of the returning signals can yield information on blood flow velocity. The earliest flowmeters were placed in direct contact with the blood vessel (Franklin *et al.* 1962, Greenfield *et al.* 1962) and so their use was limited to invasive procedures. Rushmer *et al.* (1966) developed a transcutaneous ultrasonic device that introduced the concept of non-invasive blood flow monitoring. This device was not direction-sensitive and was subject to many errors. However modern systems have been improved to the point where accurate quantification of blood flow is possible (Griffith and Henry 1978, Gill 1979, Uematsu 1981). Laser Doppler flowmetry is a recent development which uses laser

light, in place of ultrasound, to measure microvascular perfusion (Öberg 1990). Magnetic resonance imaging can also be included in this group. Mobile tissue protons are aligned by applying a strong magnetic field. They exhibit their own natural spin and at the same time move around the axis of the magnetic field, i.e. they precess. If radiowaves are pulsed into the tissue this alignment and precession is disturbed. On re-alignment radiowaves are emitted and this magnetic resonance signal can be converted into an image (Dennis 1993). The signal is altered by blood flow, a finding that has permitted the quantification of flow velocity. Rutt and Napel (1991) and Firmin *et al.* (1991) have reviewed the use of MRI in vascular studies. An additional technique is the use of an MRI contrast agent as a tracer substance to give an indirect measure of flow (Neil 1991).

In this study transcutaneous Doppler ultrasound was chosen as the means of evaluating blood flow for several reasons. The non-invasive nature of the technique was an important consideration since a method that could potentially be used to examine clinical cases of laminitis was considered optimal. In addition, a non-invasive procedure was less likely to induce a response to measurement itself. The high sampling rate, which permitted the detection of changes in flow from one cardiac cycle to the next, was also valuable. Other advantages of a more practical nature included the availability of suitable equipment, its portability and its versatility in being applicable to many different measurement conditions.

4.1.2 Principles of Doppler ultrasound

4.1.2.1 Properties of ultrasound waves

An understanding of the basic properties of ultrasound waves is required before considering how ultrasound may be utilised to investigate blood flow (Wells 1982, Goddard 1995).

Ultrasound is a high frequency acoustic wave composed of a series of compressions and rarefactions that represent areas of high and low pressure. The wave moves through a tissue with a propagation velocity (c), which depends upon properties of the tissue, i.e. its elastic modulus and density. Although each tissue has its own characteristic velocity, for practical purposes an average value of 1540m/s is often quoted. An ultrasound wave also has a characteristic frequency (f), which represents the number of pressure peaks per second, and wavelength (λ), which is the distance between pressure peaks. These properties are related by the following equation:

$$c = f\lambda$$

The resolution of an ultrasound system is its ability to distinguish two closely apposed structures. It is determined by the wavelength of the ultrasound used and is greatest when wavelength is small. Since c remains relatively constant it follows that high frequency waves are of short wavelength and consequently are of high resolution.

An acoustic wave continues to travel through a medium until all of its energy has been dissipated. This loss of energy or attenuation increases with increasing distance from the source. It is also greater for high frequency waves, thus lower frequencies are more suited to the imaging of deep structures. If during propagation the ultrasound wave encounters an interface with a different tissue, a proportion of the wave energy will be reflected back to the source. The extent of this reflection depends upon the difference between the acoustic properties of the two media or tissues and is termed the relative impedance. The type of reflection depends upon the nature of the interface. Specular or mirror-like reflection occurs at a large smooth boundary whereas a wave will be scattered in all directions if it meets a small object such as a red blood cell. Detection of these reflected wave energies forms the basis of ultrasonic imaging.

4.1.2.2 The Doppler effect

The physics of Doppler ultrasound have been reviewed by Nelson and Pretorius (1988) and Waggoner and Pérez (1990). The Doppler principle states that if a sound wave is reflected by a moving object the reflected sound undergoes a shift in frequency which is related to the velocity of the object. Thus, if a wave of ultrasound of frequency (f) is directed through a blood vessel, the blood cells will scatter a portion of the beam back to the transducer and measurement of the frequency shift (f_D) can be used to determine the velocity of blood flow (v).

$$f_D = 2v(cos\theta)f/c$$

where c = velocity of ultrasound in tissue

θ = angle at which ultrasound intercepts blood flow

Most modern Doppler systems measure θ and apply an angle correction automatically. However, since accurate measurement of angulation is difficult, θ remains an important source of error in velocity estimations, especially when the angle is large. Large angles are also associated with smaller frequency shifts since cosine θ approaches zero for angles close to 90°.

4.1.2.3 Generation and detection of ultrasound

Ultrasonic transducers depend on the piezoelectric effect to both generate and detect ultrasound (Wells 1982). A number of natural and synthetic crystals are able to

convert electrical energy to mechanical energy and *vice versa*. Therefore when an electrical potential is applied to a piezoelectric crystal, ultrasound is generated. If sound waves are reflected back to the crystal they are converted into electrical potentials that can be measured.

There are basically two types of ultrasonic transducer. Continuous-wave transducers have 2 separate crystals, one for transmitting and one for receiving (Barnes 1985). They can therefore transmit signals continuously. The disadvantage of such a system is that no information can be obtained on the position of a reflector within the isonated tissue. Pulsed wave transducers differ in that they use the same crystal to both generate and detect acoustic waves (Beach and Strandness 1985). Since the time delay between sending out a pulse and receiving the returning echo can be measured, these transducers do provide information on the depth of the reflector. By range-gating the returning signal, Doppler frequencies can be separated and selected from a specified depth or sample volume.

Duplex systems combine two-dimensional imaging with pulsed-wave Doppler. This allows visualisation of the vessel of interest and accurate placement of the sample volume within the vessel lumen. A more recent development is colour-flow mapping which superimposes a colour image on the two-dimensional scan (Switzer and Nanda 1985, Zwiebel 1988). Each point of colour represents the mean velocity of blood at that point, thus variations in colour and brightness can provide qualitative information on flow.

4.1.3 Doppler measurement of volume flow

Ultrasound can be used to quantify blood flow. Gill (1985) described the 3 approaches to measurement and reviewed their theoretical basis.

4.1.3.1 Velocity profile measurement

In this case, the spatial distribution of velocities across the vessel is first determined. The flow through each component of the vessel cross section is then calculated from the product of its area (ΔA_i) and the vector velocity at that point (v_i). The total rate of blood flow is given by the summation of all these contributions.

$$\text{Flow} = \Sigma(v_i \cdot \Delta A_i)$$

This approach requires high spatial resolution and so pulsed Doppler is necessary. Most frequently a multi-gated pulsed Doppler unit is used (Loisance *et al.* 1973, Keller *et al.* 1976). The need for high resolution dictates that sample volume must be small relative to vessel diameter and so this technique is most useful for large blood vessels, (Baker *et al.* 1978).

4.1.3.2 Uniform isonification

This method first calculates a mean velocity (v_{mean}) over the diameter of the vessel (A). This is then multiplied by the cross sectional area to determine the total blood flow.

$$\text{Flow} = v_{mean} \cdot A$$

For the normal Doppler equation to apply, relating mean shift frequency to mean velocity, the sample volume must span the vessel cross section and be uniform. Both pulsed (Baker *et al.* 1976, Gill 1979) and continuous wave Doppler systems have been used.

4.1.3.3 Assumed velocity profile

The third approach assumes a known velocity profile. It is the simplest method but is only valid in specific circumstances. A flat velocity profile can be assumed where blood is subject to high acceleration such as flow through the aortic arch or heart valves, (McDonald 1974). In this situation a velocity measurement from any point in the vessel will give the correct velocity value. This can then be multiplied by the area to give the total blood flow. This technique has been used to calculate cardiac output (Fisher *et al.* 1983, Huntsman *et al.* 1983).

4.1.3.4 Sources of error

The accuracy of these methods has been reviewed, (Baker *et al.* 1978, Gill 1982, Gill 1985, Vieli 1988) and the potential sources of error identified. The primary sources of error are associated with measurement of the following parameters:

- cross-sectional area
- angle of approach

The cross-sectional area (A) is assumed to be circular and is calculated from the measured diameter (d):

$$A = \pi \cdot d^2/4$$

The spatial resolution of the ultrasound device is critical since the percentage error in measuring d will be squared in calculating A. In addition to measurement inaccuracies, errors arise if the vessel is elliptical or if, as is often the case with both arteries and veins, its area varies with time. The angle between the ultrasound beam and the direction of blood flow is another important factor. Errors here affect not only the velocity but also the area measurements. Velocity depends on the cosine of θ. Since cosine θ changes slowly when θ approaches zero, accuracy is greatest when the ultrasound beam is parallel to the direction of blood flow. This is only

applicable to certain situations such as measurement of flow in the aortic arch. Generally larger angles are used and θ must be measured by placing a marker along the axis of the vessel on the B-mode image. This itself poses difficulty if the imaging plane is not correctly aligned to the axis of the vessel. Other errors can be attributed to the processing of the reflected signal and a lack of uniformity of the ultrasound beam (Gill 1982).

4.1.4 Analysis of velocity-time waveforms

There are basically two techniques for deriving quantitative descriptions of blood flow from Doppler signals (Hoskins 1990). Measurement of volume flow is described above. The second approach is to analyse the Doppler velocity waveform.

The Doppler waveform is the graphic representation of the changes in blood flow velocity occurring during each cardiac cycle. The waveform results from the summation of forward velocities, created by contraction of the ventricles, and reverse velocities, reflected from arteriolar terminations (Taylor 1965, McDonald 1974). Each artery has a characteristic waveform shape or signature and this is determined in part by the resistance of the system (Rushmer *et al.* 1961; Zwiebel and Fruechte 1992). Downstream vascular resistance influences the waveform through altering the magnitude of the reflected velocities (Zwiebel and Fruechte 1992). If peripheral resistance is high the reflected component of the waveform is increased and this can be seen as a reduction, loss or even reversal of velocities in diastole. High resistance circuits, such as vessels supplying skin and muscle, display a transient reverse flow during diastole which is absent from vascular beds of low impedance, (Figure 4.1.1). Other factors that are important in determining waveform shape include vessel diameter and compliance of the vessel wall (Skidmore and Woodcock 1978, Skidmore and Woodcock 1980a). Thus, waveform analysis can provide information on the physiological state of the circulation.

Strandness *et al.* (1967) recognised that waveform shape was modified in patients with peripheral vascular disease and it was in this field of medicine that much of the early work with waveform analysis was conducted. In arterial occlusive disease it is a change in vessel diameter that modifies the waveform shape. Narrowing of an artery proximal to the site of measurement obstructs the inflow of blood. This slows and reduces the systolic rise in velocity producing a rounded or damped waveform (Zwiebel and Fruechte 1992).

Waveform analysis has also been applied in the field of obstetrics, where the umbilical artery waveform is used to assess placental vascular resistance. During

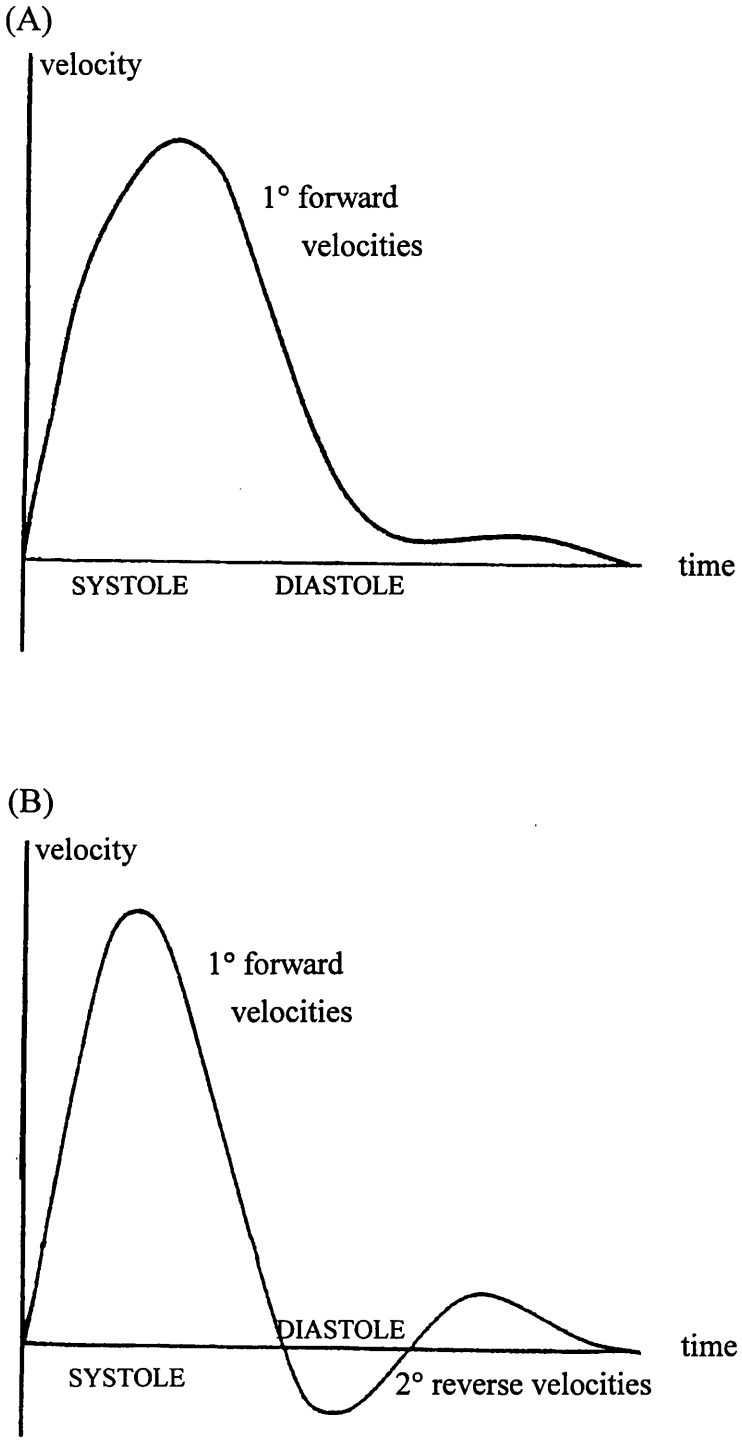


Figure 4.1.1. Illustration of an arterial velocity-time waveform. Waveform shape (A) is influenced by downstream resistance, if resistance is high the reflected component of the waveform is increased leading to reversal of velocities in diastole (B). Adapted from Johnston and Kassam (1985).

normal pregnancy there is a tendency for diastolic velocities to increase (Schulman 1986) and this can be explained by a fall in vascular resistance as the foeto-placental bed expands (Clapp 1982). Where there is foetal growth retardation this pattern is not seen. Instead there is a reduction in diastolic velocities indicating an increase in placental resistance (Erskine and Ritchie 1985; Trudinger *et al.* 1985). These changes in the umbilical artery waveform have been used to predict foetal compromise (Chambers *et al.* 1989). Other areas in which Doppler waveforms have proved useful include the prediction of renal transplant rejection (Rigsby *et al.* 1986) and the investigation of cerebral disease (Klingelhöfer *et al.* 1988).

4.1.4.1 Pulsatility index

A number of different indices, which describe the velocity waveform, have been developed to quantify the changes in flow discussed above. One of the first to be described was the Fourier pulsatility index (Fourier PI) (Gosling *et al.* 1971, Fitzgerald *et al.* 1971). The Doppler velocity-time waveform can be regarded as a steady forward flow velocity on which is superimposed a series of sinusoidal harmonics each oscillating about the forward flow (Woodcock *et al.* 1972). These harmonics reflect the elasticity of the system and so contain important physiological information. The Fourier PI is defined as the sum of the maximum oscillatory energies of the Fourier harmonics divided by the energy of the mean forward flow. Gosling and King (1974a & b) derived a simplified "peak-to-peak" pulsatility index ($PI_{p/p}$) which they defined as follows:

$$PI_{p/p} = \frac{\text{peak systolic velocity - minimum diastolic velocity}}{\text{mean velocity}}$$

The $PI_{p/p}$ has approximately the same diagnostic sensitivity as the Fourier PI and the two indices are related (Johnston *et al.* 1978):

$$PI_{p/p} = 1.9 \text{ (Fourier PI)}$$

This index has been widely used to assess vascular function. It is used in the diagnosis of peripheral arterial occlusive disease (Harris *et al.* 1974, Johnston and Taraschuk 1976, Johnston *et al.* 1983). In these circumstances a proximal stenosis reduces pulsatility index. A damping factor, calculated from the ratio of proximal to distal pulsatility indices (Gosling *et al.* 1971), has also proved useful.

One criticism of using the pulsatility index to diagnose proximal arterial stenosis is that it is also influenced by the state of the distal circulation (Barrie *et al.* 1979, Baird *et al.* 1980). This fact has been utilised in a number of different situations. In obstetrics the pulsatility index of the foetal descending aorta, external iliac or umbilical arteries has been used as a measure of placental vascular resistance

(Griffin *et al.* 1984, Tonge *et al.* 1986, Stewart *et al.* 1990). The assumption that the change in waveform pulsatility directly reflects a change in placental vascular resistance is supported by modelling studies (Adamson *et al.* 1989, Thompson and Trudinger 1990, Todros *et al.* 1992). However, direct experimental evidence is not always consistent. Generally, when vascular resistance is increased by embolisation of the placental circulation, a good correlation between resistance and waveform pulsatility can be demonstrated (Noordam *et al.* 1987, Morrow *et al.* 1989). Adamson *et al.* (1990) and Adamson and Langille (1992) confirmed this finding but were unable to show a consistent increase in pulsatility index in response to infusion of vasoconstrictive agents such as angiotensin II.

The relationship between waveform pulsatility and down stream resistance has also been investigated in other vascular beds, less complex than the foeto-placental circulation. Simplified *in vitro* models support the assertion that pulsatility index is positively correlated with peripheral resistance (Legarth and Thorup 1989b, Spencer *et al.* 1991). Fairlie and Walker (1991) have confirmed that this is the case in the brachial artery of human volunteers. Peripheral vasoconstriction, induced by immersing the subject's hand in ice-cold water, caused a significant rise in pulsatility index. The effect of vasodilation on waveform pulsatility has also been demonstrated. Legarth and Nolsoe (1990) created a postanoxic vasodilation in the hand by first occluding the blood supply with a cuff placed around the wrist. On deflation of the cuff, diastolic velocities increased and pulsatility index fell. Of more direct relevance to this study is the use of waveform indices in the investigation of Raynaud's phenomenon. In this condition, excessive digital arterial tone causes ischaemia of the fingers. Morgan *et al.* (1987) have demonstrated a reciprocal correlation between radial artery pulsatility index and digital arterial inflow, thus validating the use of radial artery pulsatility index as an indicator of vasomotor tone in the hand (Clifford *et al.* 1980).

Vascular resistance is an expression of the opposition to flow in a non-pulsatile circulation. In the arterial system vascular impedance, which describes opposition to flow in a pulsatile circulation, is perhaps more relevant (O'Rourke and Taylor 1966). Any attempt to validate the ability of pulsatility index to reflect downstream circulatory changes should include investigation of the relationship between pulsatility index and arterial impedance. Impedance reflects changes in vessel elasticity, inertia and viscosity of the blood and wave reflection (O'Rourke and Taylor 1966).

Nichols and O'Rourke (1990) explained the concept of impedance and described the different types. The most commonly used form is input impedance, which is the ratio of pulsatile pressure and pulsatile flow at a particular arterial site. It reflects not only local properties of the artery, but also the properties of all the vessels distal to that point. Input impedance is derived by transforming pressure and flow/velocity waveforms to the frequency domain using Fourier analysis (Nichols and O'Rourke 1990). This yields impedance modulus and phase. Whilst phase is expressed in radians, the units of impedance modulus vary according to the method of derivation. If impedance is determined from the flow waveform, modulus is expressed in dyne-s/cm^5, whereas if the velocity waveform is used, the units of modulus are dyne-s/cm^3. Nichols and O'Rourke (1990) prefer the latter units. Pepine *et al.* (1979) investigated input impedance in the rat and were able to show that intravenous administration of a vasodilator, such as sodium nitroprusside, reduced aortic input impedance.

Downing *et al.* (1991) and Downing *et al.* (1993) described several additional parameters of impedance:

(i) Impedance at zero frequency, Z_{pr}, is equal to peripheral vascular resistance.

(ii) Characteristic impedance, Z_o, describes the relationship between pressure and flow in the absence of wave reflection. This state does not occur naturally, however characteristic impedance approximates input impedance when the vascular bed is maximally dilated.

(iii) Reflection coefficient, R_c, which describes the degree of wave reflection at the periphery.

These workers investigated the relationship between aortic pulsatility index and arterial impedance parameters in chronically instrumented lambs. They demonstrated a good correlation between changes in pulsatility index, induced by intravenous administration of vasoactive drugs, and each of the above impedance parameters. Correlation was greatest when reflex heart rate responses were inhibited (Downing *et al.* 1993).

4.1.4.2 Other waveform indices

Numerous other indices have been described. The A/B ratio, which is simply the peak systolic velocity divided by the minimum diastolic velocity, has been used in obstetrics (Stuart *et al.* 1980, Trudinger *et al.* 1987). The resistance index (Pourcelot 1974) is related to the pulsatility index and is defined by the following equation:

$$RI = \frac{\text{peak systolic velocity - minimum diastolic velocity}}{\text{peak systolic velocity}}$$

Height-width index and path length index are measures of the damping of the waveform which occurs distal to an arterial stenosis, (Johnston and Kassam 1985). Another approach has been to define different mathematical expressions of the velocity-time waveform. The Laplace transform does so using a third order model with three poles, (Skidmore and Woodcock 1980a & b, Skidmore *et al.* 1980). The positions of these poles have been related to physiological parameters such as arterial stiffness, the degree of peripheral vasoconstriction and the proximal lumen size. Principal component analysis is a further mathematical technique that has been used to characterise the shape of the Doppler waveform (Evans *et al.* 1981, Evans 1992).

Numerous studies have compared the different methods of waveform analysis in the diagnosis of vascular disease. Campbell *et al.* (1983) and Ramsay *et al.* (1994) favoured Laplacian transformation, whilst Macpherson *et al.* (1984) concluded that principal component analysis was the most powerful method. Other authors (Johnston *et al.* 1984, Johnston and Kassam 1985, Hoskins *et al.* 1989b), found the diagnostic accuracy of the various methods to be similar and therefore preferred indices, such as the pulsatility index, for their simplicity and ease of calculation.

4.1.5 Aims of the study

The primary objective of this study was to characterise the normal femoral artery waveform in the sheep and to describe, by means of the pulsatility index, the changes induced by local infusion of vasoconstrictor and vasodilator substances. Phenylephrine was chosen as the vasoconstrictor since it is a selective agonist, that acts primarily on the α_1-adrenergic receptors on vascular smooth muscle (Hoffman and Lefkowitz 1990). It also proved to be a potent vasoconstrictor *in vitro*. Two vasodilators were chosen, sodium nitroprusside, which is an endothelium-independent vasodilator that produces relaxation through release of exogenous nitric oxide (Schultz *et al.* 1977, Feelisch and Noack 1991) and bradykinin, which is an endothelium-dependent vasodilator that causes relaxation through release of endothelial nitric oxide (Section 2.3.2.2, Marceau *et al.* 1983, Hall 1992).

The wider aim of the study was to assess the usefulness of Doppler waveform analysis, as a non-invasive tool for investigating digital vascular blood flow. It was also hoped to quantify femoral artery blood flow and thereby examine any correlation between femoral artery pulsatility index and volume flow.

4.2 Materials and methods

4.2.1 Animals

A group of 6 female greyface sheep (Border Leicester x Scottish Blackface) aged between 1 and 2 years, were purchased from Glasgow University farm. The sheep were healthy and in good condition. They were maintained indoors as a group throughout the study and were fed a diet of hay *(ad libitum)* and concentrates. Water was also available *ad libitum.*

The sheep were vaccinated annually with *Heptavac-P* (Hoechst UK Ltd., Milton Keynes, Buckinghamshire, U.K.), and were treated with a broad spectrum anthelmintic twice a year (*Valbazen* 2.5%, Pfizer Ltd., Sandwich, Kent, U.K.). Sheep were sheared each June and weighed at regular intervals over a 2 year period.

4.2.2 Drugs and drug administration

4.2.2.1 Anaesthetic agents and medications

The following drugs were used:

- ampicillin sodium (*Penbritin*, Pfizer Ltd., Sandwich, Kent, U.K.)
- flunixin meglumine (*Finadyne*, Schering-Plough Animal Health, Welwyn Garden City, Hertfordshire, U.K.)
- halothane (*Fluothane*, Mallinckrodt Veterinary Ltd., Uxbridge, Middlesex, U.K.)
- heparin sodium (Leo Laboratories Ltd., Princes Risborough, Buckinghamshire, U.K.)
- procaine penicillin (*Depocillin*, Mycofarm UK Ltd., Cambridge, U.K.)
- propofol (*Rapinovet*, Mallinckrodt Veterinary Ltd., Uxbridge, Middlesex, U.K.)
- sodium chloride 0.9% w/v (*Aqupharm* 1, Animalcare Ltd., York, U.K.)
- water for injection (Animalcare Ltd., York, U.K.)

Vials containing 500mg of ampicillin were reconstituted with 5ml of sterile water. Heparinised saline was prepared by adding 1ml of heparin, i.e. 1000 units, to 500ml of 0.9% saline, giving a concentration of 2 heparin units/ml. All other drugs were used without dilution.

Ampicillin was applied topically, procaine penicillin was injected intramuscularly and halothane was inhaled. All other drugs were given by the intravenous route.

4.2.2.2 Vasoactive agents

All vasoactive drugs were obtained from Sigma Chemical Company (Poole, Dorset, U.K.). Fresh solutions for infusion were made up in 0.9% saline on the morning of the experiment. Solutions were allowed to equilibrate to room temperature for approximately 2 hours before use. All solutions were infused intraarterially at a rate of 30ml/h.

- bradykinin

A 10^{-3}M stock solution was prepared by dissolving 10mg of bradykinin acetate in 9.43ml of saline. The solution for infusion was prepared by adding 0.3ml of stock solution to 100ml of saline in a burette giving set (Baxter Health Care, Thetford, Norfolk, U.K.). Infusion of this solution at 30ml/h delivered 1.6μg/min of bradykinin.

- phenylephrine

Solutions for infusion were prepared directly by dissolving 1.2mg, 3.6mg or 12mg of L-phenylephrine hydrochloride in 200ml of 0.9% saline. Infusion delivered 3μg, 9μg and 30μg of phenylephrine/min.

- sodium nitroprusside

Solutions for infusion were prepared by dissolving 1.2mg or 12mg of crystalline sodium nitroprusside in 200ml of 0.9% saline. Infusion at 30ml/h delivered 3μg and 30μg of sodium nitroprusside/min. Solutions were protected from light by wrapping the giving set in tin foil.

4.2.3 Ultrasonography

4.2.3.1 Ultrasound equipment

An *Apogee* Ultrasound System (Interspec Inc., Ambler, U.S.A.) with a 7.5MHz duplex probe was used for these investigations. This instrument has an advanced annular phased array for 2 dimensional sector imaging, M-Mode imaging and Doppler capability. It has the facility to adjust for misalignments of probe angle and blood flow.

The Apogee was connected to a video recorder to enable recording of sector images and Doppler waveforms during each experiment. The data collected in this way was analysed later using the Apogee's extensive calculations package.

4.2.3.2 Measurement of ultrasound data

All Doppler signals were recorded from the femoral artery, using the following protocol. A 2 dimensional sector image of the artery was obtained by positioning the transducer in the angle between the body wall and medial surface of the hindlimb. The probe position was adjusted to give a clear longitudinal image of the vessel. Colour was then applied to highlight the blood flow within the vessel. The sample box was positioned in the centre of this flow and its height adjusted to span the diameter of the vessel (Figure 4.2.1). Angle correction was applied by aligning a pointer with the direction of flow and the system was then switched to Doppler mode. A split-screen format was used with an updated 2 dimensional sector image in the upper portion of the screen and the maximum velocity/time waveform in the lower portion (Figure 4.2.2). The sweep speed was set so that each screen displayed the waveforms produced in 4 seconds. A minimum of 2 sweeps, i.e. 8 seconds, were recorded for each reading.

Recordings were made at 1-2 minute intervals. Where possible the position of the probe was maintained between readings, with only minor adjustments being made to optimise the Doppler signal. However occasionally the probe had to be removed from position, for example when changing over infusions.

4.2.3.3 Processing of ultrasound data

Waveform analysis was conducted during video playback of the recordings. All calculations were based on the maximum spectral envelope. Calibration of the velocity and time axes was achieved by drawing lines of specified velocity or time. Four parameters were derived from the waveforms:

- maximum velocity (v_{max})
- minimum velocity (v_{min})
- mean velocity (v_{mean})
- pulsatility index (PI)

Maximum and minimum velocities were obtained by placing the cursor at the relevant point on the velocity envelope. The mean velocity, which was a time-based average, was obtained by tracing the cursor over the waveform cycle (Figure 4.2.3). The pulsatility index was calculated from these values using the simplified Fourier equation described in Section 4.1.3. For each measurement, 4 waveform cycles were analysed in this way.

Volume flow was calculated from the vessel diameter and the mean velocity. Diameter was estimated by placing cursors across the breadth of the vessel (Figure 4.2.4). Mean velocity was calculated as described under waveform analysis.

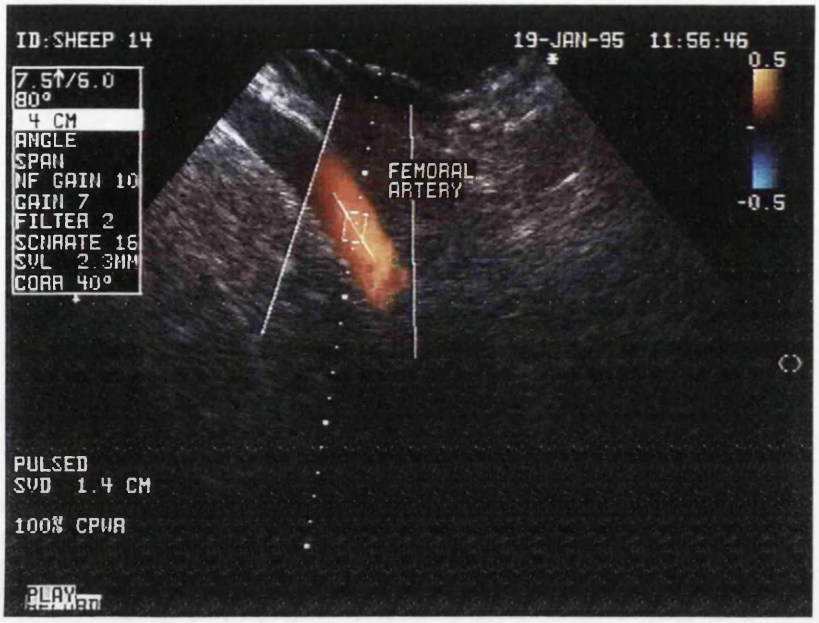


Figure 4.2.1. Two-dimensional sector image, with colour flow mapping, of the femoral artery of an anaesthetised sheep obtained using an Interspec *Apogee* ultrasound system with a 7.5MHz duplex probe.
[] sample volume
\ angle correction pointer

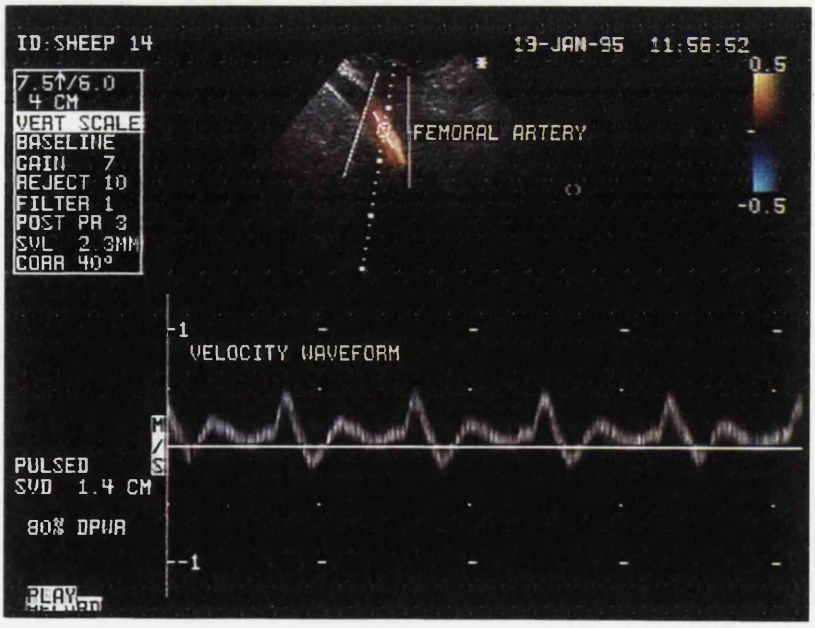


Figure 4.2.2. Split screen format showing the updated 2-D sector image of the femoral artery above the Doppler velocity-time waveform obtained using an Interspec *Apogee* ultrasound system with a 7.5MHz duplex probe.
: : 1 second interval

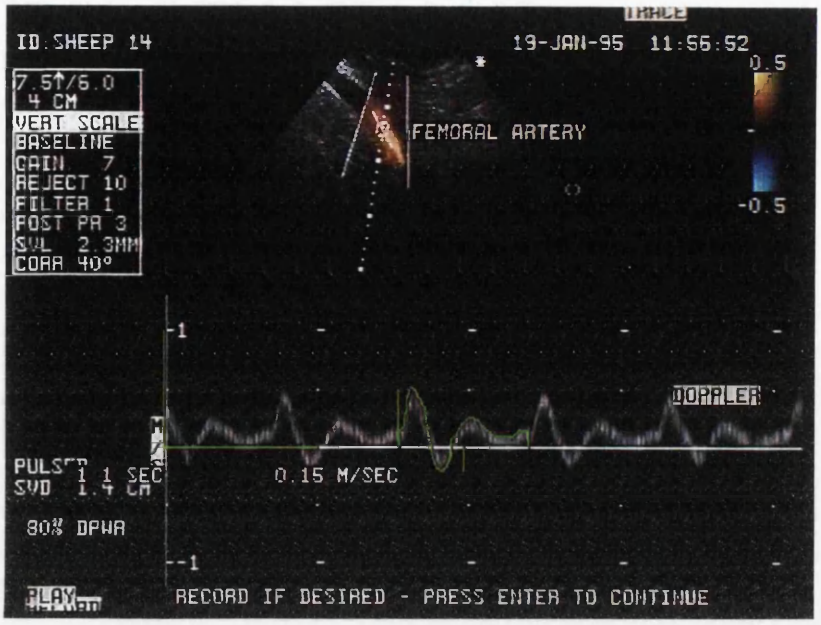


Figure 4.2.3. Doppler velocity-time waveform of the femoral artery of an anaesthetised sheep obtained using an Interspec *Apogee* ultrasound system with a 7.5MHz duplex probe. The green line traces the maximum velocity envelope in order to calculate minimum, maximum and mean velocities and thereby the peak-to-peak pulsatility index.
: : 1 second interval

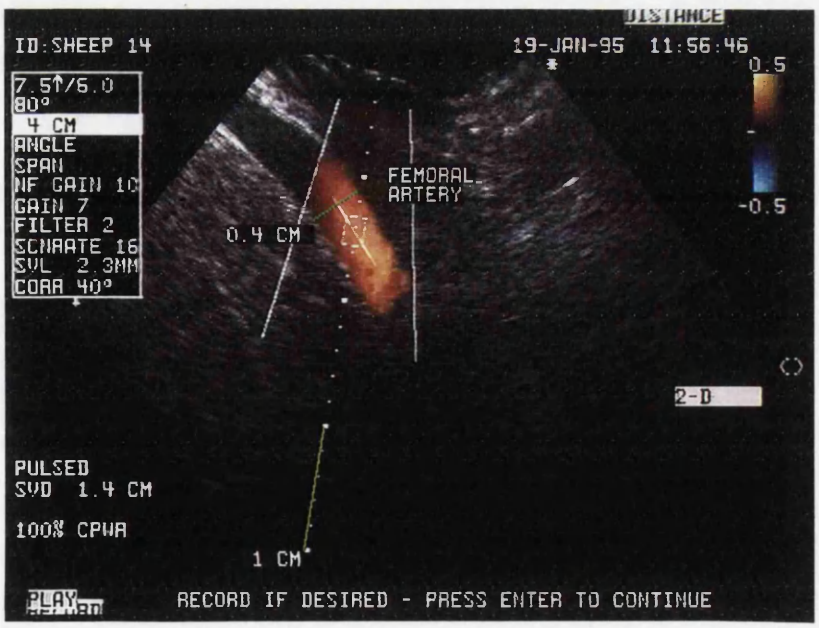


Figure 4.2.4. Two-dimensional sector image, with colour flow mapping, of the femoral artery of an anaesthetised sheep obtained using an Interspec *Apogee* ultrasound system with a 7.5MHz duplex probe. Arterial diameter is measured by plotting the distance across the artery (green line).
[] sample volume
\ angle correction pointer

4.2.4 Anaesthesia

All investigations were conducted in anaesthetised sheep with a minimum period of 7 weeks between anaesthetics. Individual procedures varied in length, each sheep being anaesthetised for between 2 and 4 hours.

4.2.4.1 Animal preparation

Each sheep was starved for 12-18 hours prior to the induction of anaesthesia. Water was not withheld. Since vasoactive drugs were administered on a "per sheep" rather than per kg basis, the sheep were not weighed prior to each anaesthetic. They were however, weighed at regular intervals to permit estimation of the dose of anaesthetic required for induction. On the morning of the procedure an 18 or 20 gauge teflon catheter, (Vygon UK Ltd., Cirencester, Gloucestershire, U.K.) was placed into a cephalic vein and secured in position using zinc oxide tape.

4.2.4.2 Induction of anaesthesia

Anaesthesia was induced with an intravenous bolus of propofol, 4mg/kg, given through the pre-placed cephalic catheter. The trachea was intubated using a cuffed red rubber endotracheal tube (Arnolds Veterinary Products, Shrewsbury, U.K.), size 11 or 12. Occasionally the initial dose of propofol was insufficient to permit successful intubation and further increments were required.

4.2.4.3 Maintenance of anaesthesia

Anaesthesia was maintained using halothane in oxygen. Initially, the vaporiser (*Fluotec III*) was set at 3%, but was reduced as anaesthesia deepened. A maintenance setting of 2% halothane was suitable for most sheep, however this was modified when the plane of anaesthesia was deemed to be too light or too deep.

The sheep were artificially ventilated using a *Flomasta Ventilator* (Medical and Industrial Equipment, Exeter, Devon, U.K.). This unit operates as a minute volume divider and provides a non-rebreathing circuit. Ventilation was adjusted by changing the total gas flow or tidal volume settings in order to maintain a partial pressure of arterial carbon dioxide of 35-45mmHg.

A sand bag was positioned under the neck to elevate the throat region so that saliva and any regurgitated material flowed out of the mouth and away from the airway thus reducing the risk of aspiration. Occasionally salivation was profuse and in these cases a suction pump was used to remove saliva as it collected.

Sterile saline (0.9%) was infused into the cephalic vein at a rate of 5ml/kg/h for the duration of the anaesthetic.

4.2.4.4 Monitoring of anaesthesia

Cardiovascular parameters were monitored using a *Minimon 7132A* (Kontron Instruments, Cumbernauld, U.K.). An ECG in lead II was displayed and from this a heart rate was derived which was recorded every 5 minutes. The *Minimon* also permitted direct measurement of arterial blood pressure. A 20 or 22 gauge teflon intravenous catheter was placed aseptically into the median artery of the forelimb or the middle auricular artery of the ear. This was then connected to the *Minimon* via a pressure monitoring kit (Medex Medical Inc., Rossendale, Lancashire, U.K.), which included a pressure transducer and flush valve. The pressure transducer was placed at the level of the sheep's heart and zero was set at atmospheric pressure. The resulting pressure trace was displayed on the monitor along with values for mean, systolic and diastolic pressure. These were recorded every 5 minutes. Heparinised saline was used to flush the catheter and maintain patency.

Respiratory rate was recorded every 5 minutes. The adequacy of ventilation was assessed subjectively by observing the movements of the chest wall and arterial blood gas analysis provided more objective information. Samples were drawn from the arterial catheter used for blood pressure monitoring approximately every 30 minutes. The majority of samples were analysed immediately using a Ciba Corning 278 Blood Gas System (Ciba Corning Diagnostics Ltd., Halstead, Essex, U.K.). Where this was not possible they were stored on ice for up to one hour prior to analysis. Values for blood pH, partial pressure of oxygen, p_aO_2, partial pressure of carbon dioxide, p_aCO_2, concentration of bicarbonate, HCO_3^- and base excess were recorded.

Rectal temperature was measured prior to sampling for blood gases permitting correction of the results to actual body temperature. Room temperature was recorded every 30 minutes to ensure there were no major fluctuations.

4.2.4.5 Recovery from anaesthesia

At the end of the procedure the sheep was first weaned off the ventilator. This was achieved by ventilating manually every 30-60 seconds until spontaneous breathing resumed. Halothane was then switched off. Once the sheep recovered sufficient consciousness to cough, the endotracheal tube, with cuff still partially inflated, was removed and the sheep was propped up in sternal recumbency.

A single intravenous dose of 75mg of flunixin meglumine was administered to reduce inflammation around the site of arterial catheterisation. A prophylactic dose of 1050mg of procaine penicillin was injected intramuscularly. The sheep remained in the operating theatre until able to stand and walk without assistance.

4.2.5 Intraarterial catheterisation for drug infusion

Anaesthetised sheep were positioned in lateral recumbency. The uppermost hindlimb was raised using a rope suspended from the ceiling. This improved access to the lower limb from which ultrasound readings were taken.

A catheter was placed into either the relatively superficial saphenous artery or the dorsal metatarsal artery of the hindlimb. Initial attempts at percutaneous catheterisation were unsuccessful and so a "cut down" technique was adopted. A short 3cm skin incision was made over the vessel which was then isolated by gentle dissection. For the initial experiments the artery was catheterised using a 22 gauge teflon catheter. However this catheter was easily displaced being only 18mm long and so a 28mm modified 2.0 French central venous catheter (Cook UK Ltd., Letchworth, Hertfordshire, U.K.), was used for the majority of studies.

Saline (0.9%) was infused into the catheterised artery at a rate of 30ml/h for a minimum of 30 minutes before any ultrasound recordings were made.

On completion of the procedure the intraarterial catheter was removed. Pressure was applied to the site for up to 5 minutes using sterile gauze swabs. Once haemostasis was achieved the wound was flushed with 500mg of ampicillin. The skin was closed using a continuous subcuticular suture in 2 metric *Vicryl* (Ethicon Ltd., Edinburgh, U.K.).

4.2.6 Experimental design

4.2.6.1 Preliminary studies

In order to show that anaesthetised sheep could maintain stable cardiovascular parameters under the conditions of the experiment, 4 sheep were anaesthetised for 4 hours. No vasoactive drugs were infused but heart rate and blood pressures were recorded every 5 minutes. All subsequent procedures were completed in less than 4 hours.

It was also necessary to demonstrate the consistency of ultrasound readings during the control infusion of saline. In 2 sheep, saline only was infused into the saphenous artery and ultrasound recordings were taken over a 1 hour period. All subsequent recordings were taken over less than 1 hour.

Estimations of femoral artery volume blood flow were made in each of the 6 sheep using the uniform isonification technique, i.e. mean velocity across the vessel was multiplied by the vessel area. This allowed the calculation of the approximate blood concentration of vasoactive drugs infused.

4.2.6.2 Intraarterial infusion of phenylephrine

All 6 sheep were used in this experiment. Baseline data were established by infusing 0.9% saline for 20 minutes and recording femoral Doppler signals every 2 minutes. Phenylephrine was then infused for a period of 10 minutes with Doppler recordings being made every minute. A single dose of 3, 9 or 30μg/min was given to each sheep. To complete the experiment 0.9% saline was infused for a further 20 minutes and waveforms were recorded every 2 minutes.

4.2.6.3 Intraarterial infusion of bradykinin

Only 1 sheep, No.134, received a bradykinin infusion. Recordings were first made during infusion of saline as for phenylephrine. Bradykinin was then infused for 6 minutes at 1.6μg/min and ultrasound recordings were made every 30 seconds. This was followed by a short infusion of saline.

4.2.6.4 Intraarterial infusion of sodium nitroprusside

All 6 sheep were used in this experiment and were given 2 doses of sodium nitroprusside on 2 different occasions. Baseline data were first established as described previously. Nitroprusside was infused for 10-15 minutes at 3 or 30μg/min. Ultrasound recordings were taken every minute.

4.2.7 Statistical analysis

Each pulsatility index was derived from analysis of 4 consecutive waveforms. Results from the preliminary saline infusions (4.3.1.2) were expressed as the mean pulsatility index ± SEM for each sheep (n = number of recordings). A coefficient of variation was also determined.

For all subsequent experiments (4.3.2), a mean pulsatility index was calculated during infusion of saline and during infusion of vasoactive drug. Results were also expressed as a percentage of baseline, where baseline was the mean pulsatility index recorded during saline infusion. Saline and drug indices were then compared by one-way analysis of variance. A p-value of less than 0.05 was taken to indicate a statistically significant difference. Unless otherwise stated, results were expressed as mean ± SEM (n = number of sheep in each group).

Other factors, such as blood pressure, heart rate, arterial carbon dioxide and halothane could also potentially modify vascular tone and thereby pulsatility index. Therefore, these parameters, before and during/after drug infusion, were also compared by one-way analysis of variance.

4.3 Results

4.3.1 Preliminary studies

4.3.1.1 Cardiovascular stability

Heart rate and blood pressure were recorded in 4 sheep anaesthetised for 4 hours. No recordings were taken for the first 20-30 minutes as time was required to set up the monitoring equipment. Heart rate was initially high (115.0 ± 9.1 beats/min) but stabilised after approximately 1 hour (Figure 4.3.1). Mean heart rate for the final 3 hours of anaesthesia was 85.0 ± 6.7 beats/min (n = 4). Arterial blood pressure was stable throughout the measurement period (Figure 4.3.2). For the final 3 hours of anaesthesia the mean arterial pressure was 88.0 ± 2.1mmHg (n = 4).

4.3.1.2 Intraarterial infusion of N-saline

Femoral artery waveforms were recorded approximately every 2 minutes for 1 hour during infusion of N-saline into the saphenous artery of the ipsilateral hindlimb. Two sheep were used. Figure 4.3.3 illustrates the change in waveform pulsatility index with time. The mean pulsatility indices for the 2 sheep were 4.27 ± 0.14 (n = 23) and 5.05 ± 0.08 (n = 31), where n equals the number of measurements taken. The coefficients of variation were respectively 16.2% and 8.4%, which gives a mean of 12.3%.

4.3.1.3 Estimation of volume blood flow

The diameter of the femoral artery measured 0.4cm on all recordings from each of the 6 sheep (Figure 4.3.4). Mean velocity of blood flow was 0.20 ± 0.02m/s (n = 6). Calculated femoral artery volume flow was therefore 148.4 ± 18.5ml/min (n = 6).

4.3.2 Intraarterial infusion of phenylephrine

A dose of 3μg/min phenylephrine was infused into the saphenous artery of 1 sheep, No. 134. Blood volume flow was assumed to be approximately 150ml/min (*vida supra*), therefore an approximate blood concentration of 10^{-7}M was achieved. This dose produced very little effect, pulsatility index increasing only slightly from a mean of 2.86 during saline infusion to 3.96 during infusion of phenylephrine (Figure 4.3.6).

A higher dose of phenylephrine, 30μg/min, was infused into the saphenous artery of sheep No. 14. This gave an estimated blood concentration of 10^{-6}M. This treatment produced such marked vasoconstriction that the Doppler signal was almost lost. Changes in the shape of the waveform are illustrated in Figure 4.3.5. The waveforms obtained during infusion of N-saline had no reverse component of flow indicating that the circulation was in a relatively vasodilated state. On infusion of

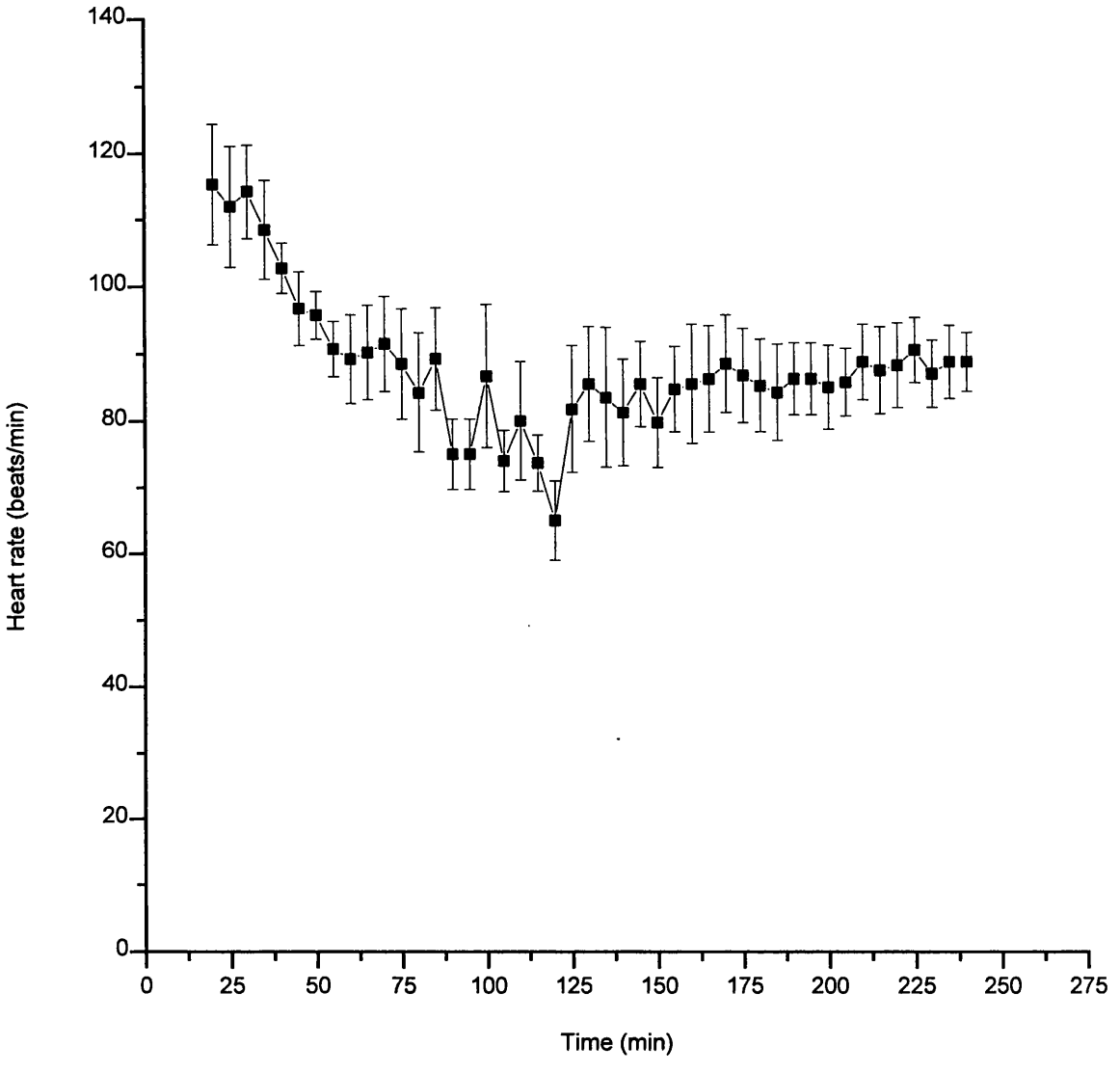


Figure 4.3.1. Heart rate recorded in anaesthetised sheep. Anaesthesia was induced with an intravenous bolus of propofol and maintained with halothane for 4 hours. Results are expressed as mean ± SEM (n=4).

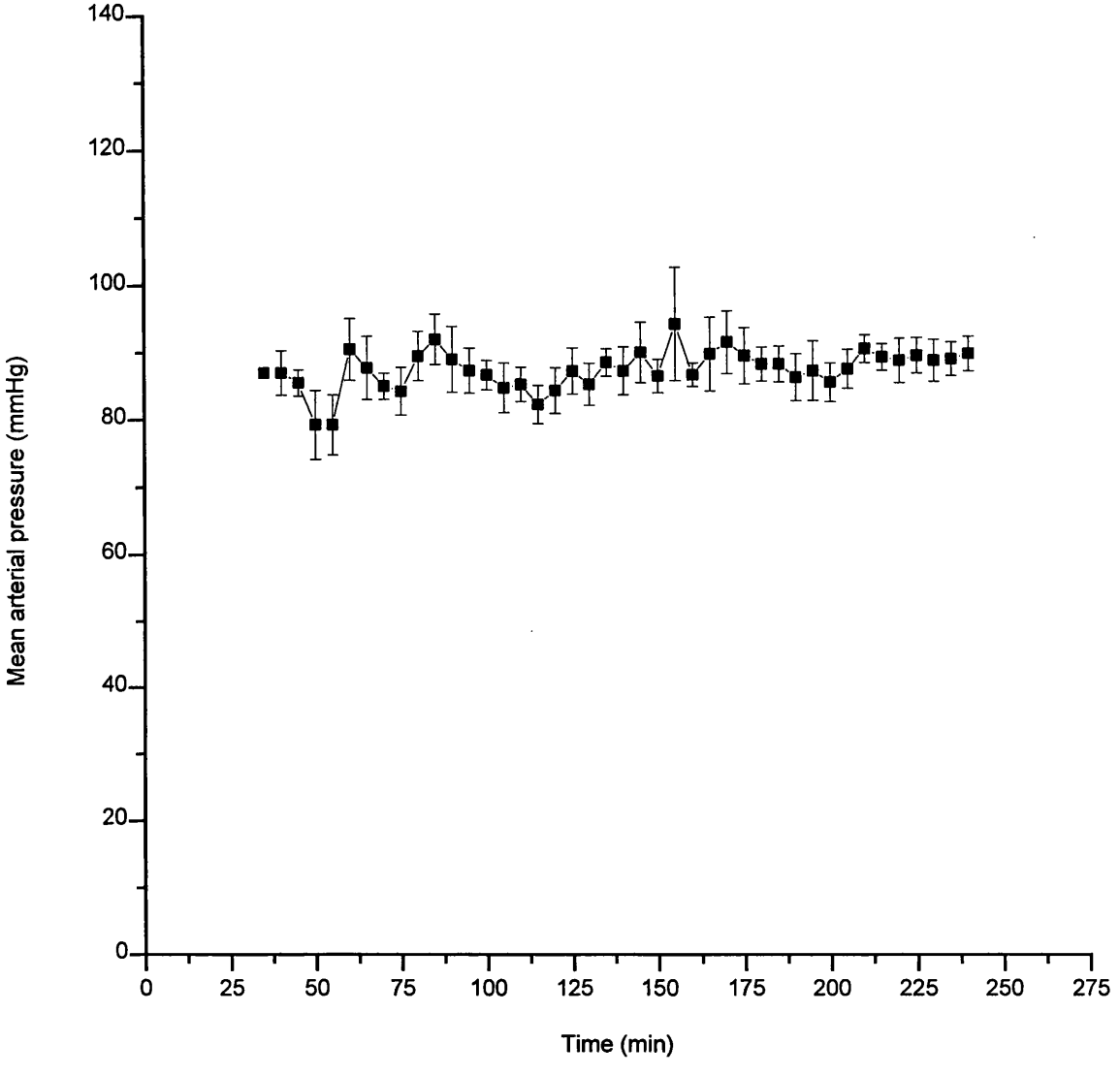


Figure 4.3.2. Mean arterial blood pressure recorded directly from a catheter in the median artery of anaesthetised sheep. Anaesthesia was induced with an intravenous bolus of propofol and maintained with halothane for 4 hours. Results are expressed as mean ± SEM (n=4).

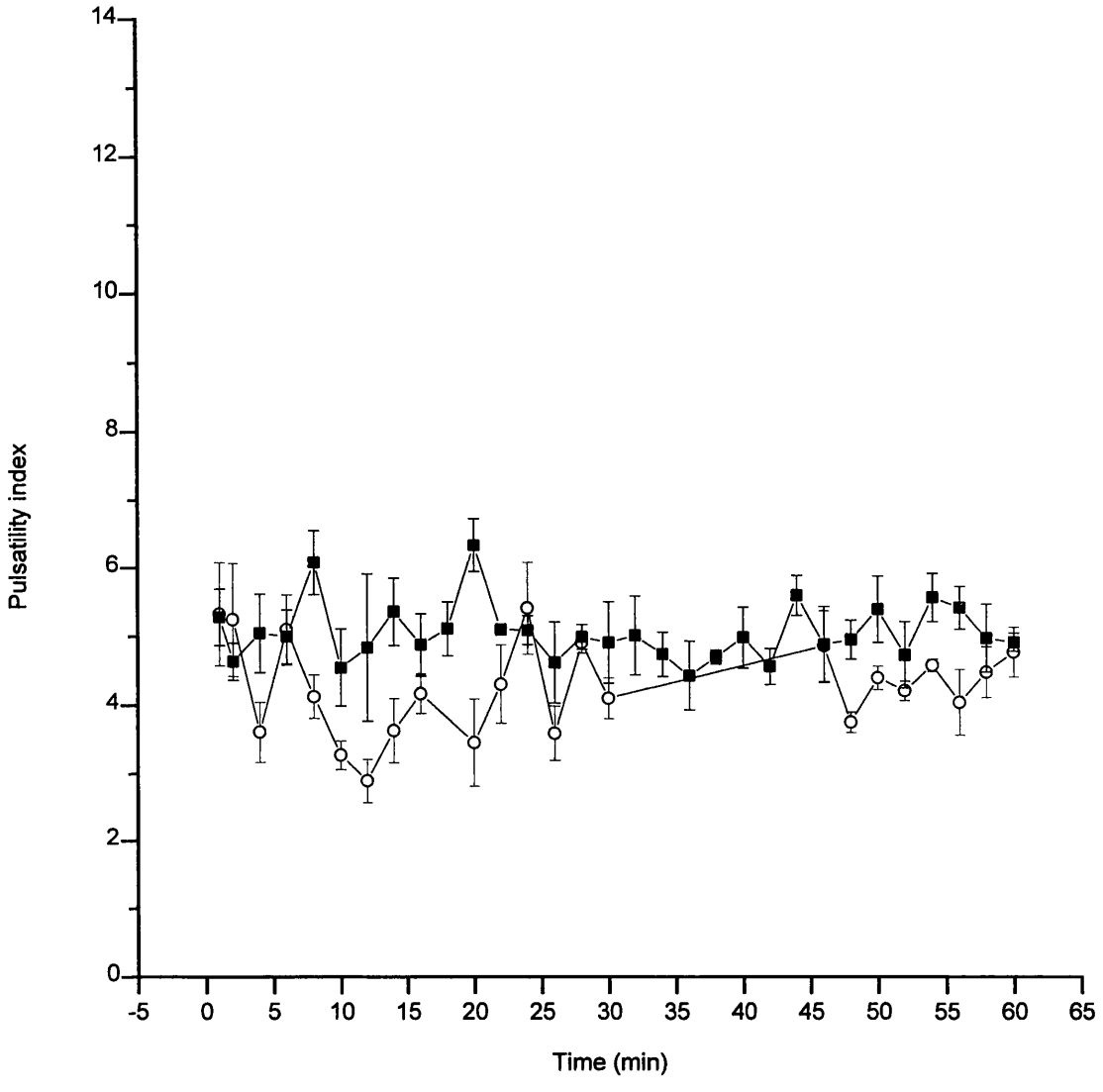


Figure 4.3.3. Femoral artery pulsatility index recorded in 2 anaesthetised sheep during infusion of N-saline into the saphenous artery of the ipsilateral hindlimb. Each result is expressed as mean of 4 recordings ± SD.

—o— Sheep 134 "Barbara"

—■— Sheep 17 "Connie"

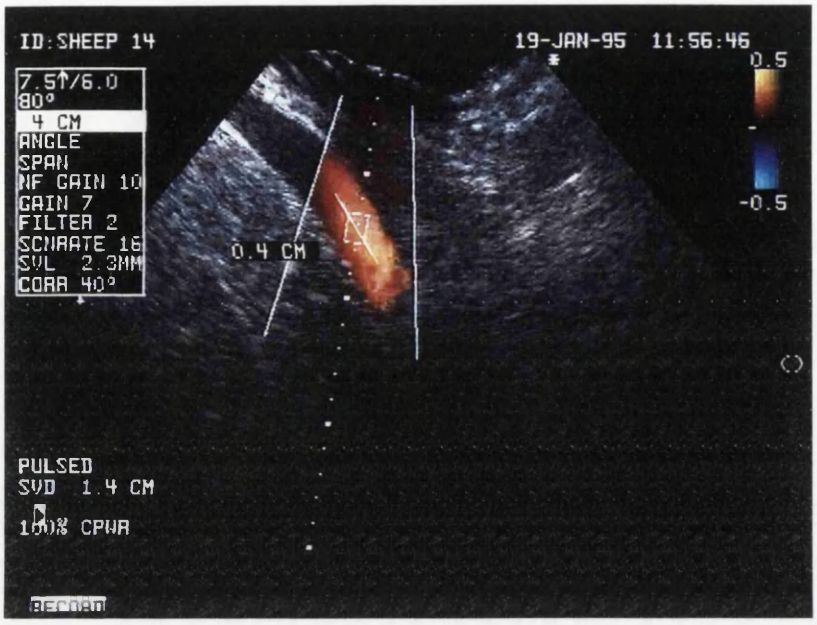
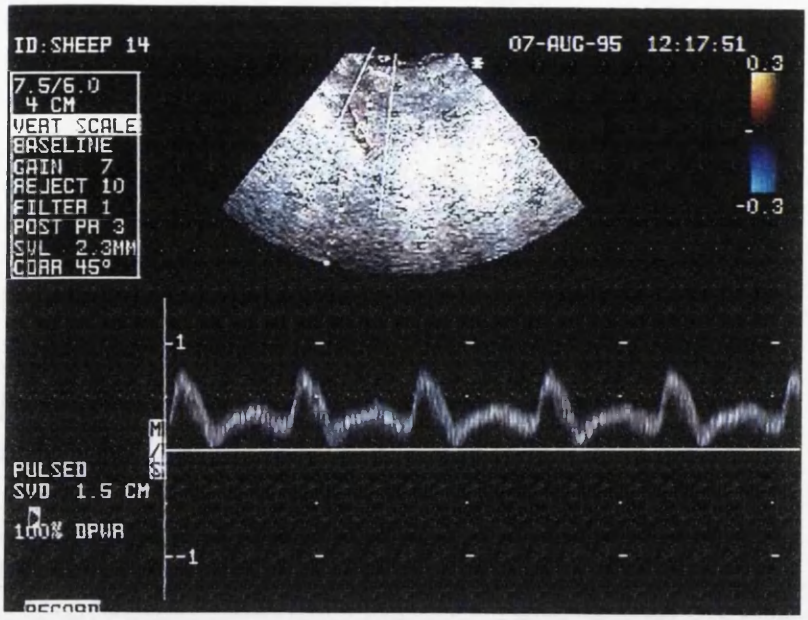


Figure 4.3.4. Two-dimensional sector image with colour-flow Doppler obtained using an Interspec *Apogee* ultrasound unit with a 7.5MHz duplex probe. Measurement of femoral artery diameter is illustrated in an anaesthetised sheep (green dotted line).

(A)



(B)

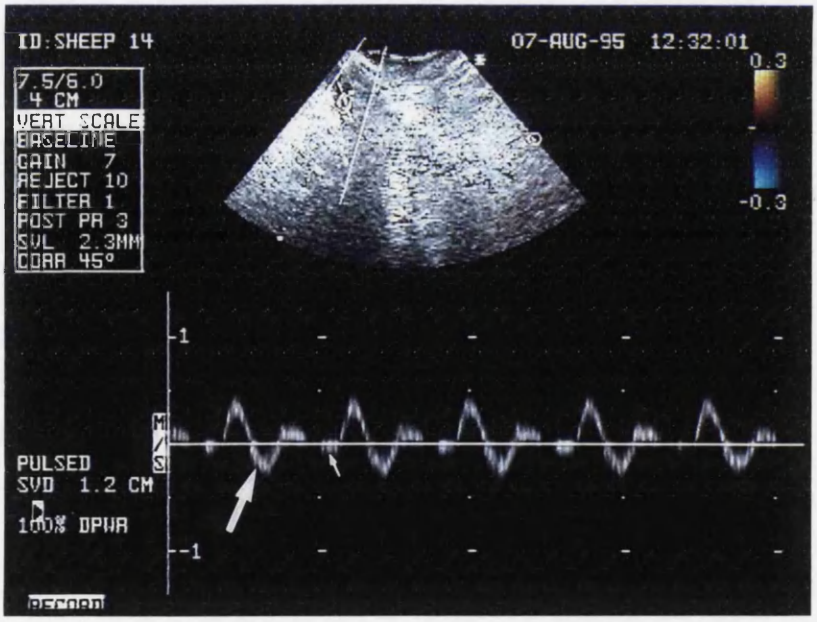


Figure 4.3.5. Doppler velocity-time waveforms recorded from the femoral artery of an anaesthetised sheep during infusion of (A) N-saline and (B) 30μg/min phenylephrine (t = 1min) into the saphenous artery of the ipsilateral hindlimb.

⇑ secondary reverse flow

⇑ quaternary reverse flow

(C)

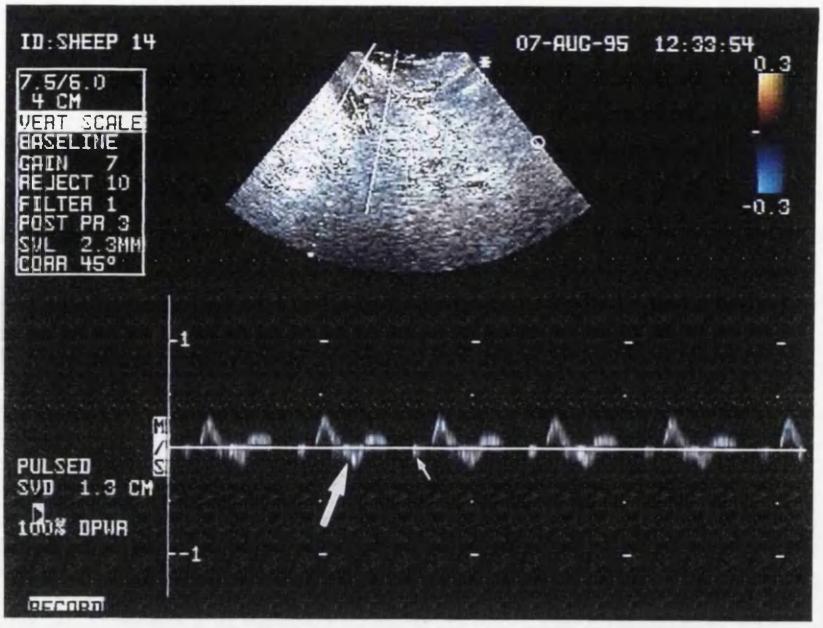


Figure 4.3.5(contd). Doppler velocity-time waveforms recorded from the femoral artery of an anaesthetised sheep during infusion of (C) 30μg/min phenylephrine (t = 3min) into the saphenous artery of the ipsilateral hindlimb.

⇑⇑ secondary reverse flow

⇑ quaternary reverse flow

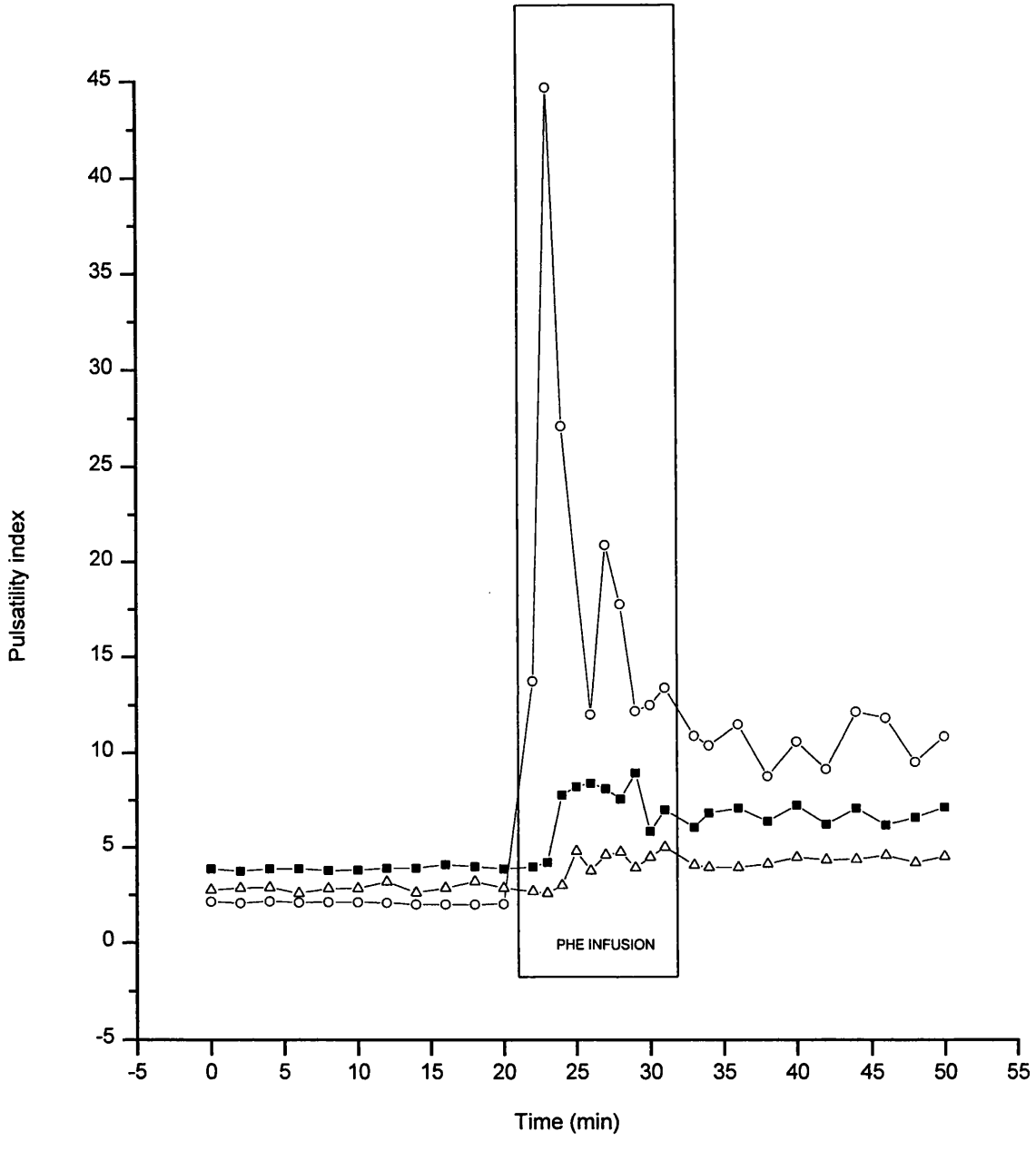


Figure 4.3.6. Mean (where appropriate), femoral artery pulsatility index recorded in anaesthetised sheep during infusion of N-saline or phenylephrine (PHE) into the saphenous artery of the ipsilateral hindlimb.

—△— 3μg/min PHE (n=1)

—■— 9μg/min PHE (n=4)

—○— 30μg/min PHE (n=1)

phenylephrine, forward velocity was reduced and secondary and quaternary reverse velocities developed. Pulsatility index increased from a mean of 2.07 during saline infusion, to a mean of 21.51 during infusion of phenylephrine (Figure 4.3.6). After 10 minutes of phenylephrine, saline was again infused and pulsatility index stabilised at 10.68.

In the light of these results, an intermediate dose of 9μg/min phenylephrine (blood concentration ≈ $3x10^{-7}$M), was infused into the saphenous artery of the remaining 4 sheep. Similar waveform changes to those occurring with the higher dose of phenylephrine were observed. A typical example is illustrated in Figure 4.3.7. There was a slight reduction in forward flow velocity, an increase in secondary reverse flow and the appearance of quaternary reverse flow. Pulsatility index increased from a baseline of 3.87 ± 0.14, to 6.99 ± 0.93 during phenylephrine infusion (n = 4) (Figure 4.3.8). One-way analysis of variance indicated that this increase was statistically significant ($p = 0.016$). Pulsatility index stabilised at 6.67 ± 0.93, on resumption of saline infusion. Slight increases in both heart rate (85.0 ± 9.9 beats/min to 87.5 ± 9.2 beats/min) and mean arterial pressure (95.7 ± 3.2mmHg to 100.0 ± 3.4mmHg) were not significant. A slight increase in arterial carbon dioxide, from 43.0 ± 2.4mmHg to 47.9 ± 3.1mmHg also failed to reach statistical significance.

4.3.3 Intraarterial infusion of bradykinin

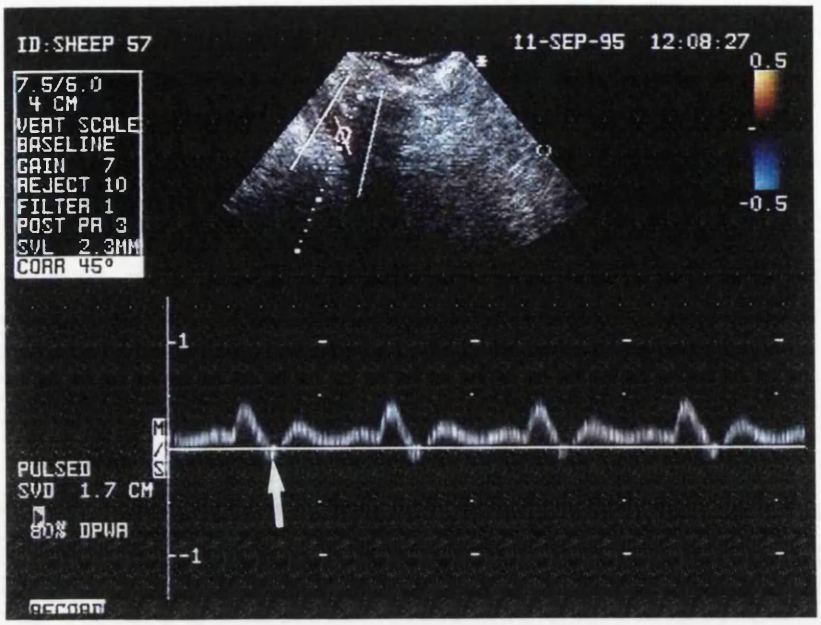
A bradykinin infusion of 1.6μg/min was infused into the saphenous artery of just 1 sheep (blood concentration ≈ 10^{-8}M). A severe local reaction developed distal to the infusion site. The skin became warm and developed erythema and pitting oedema. The infusion was therefore stopped prematurely and was not repeated. No significant change in pulsatility index was recorded (Figure 4.3.9).

4.3.4 Intraarterial infusion of sodium nitroprusside

A low dose of sodium nitroprusside, 3μg/min (blood concentration ≈ $7x10^{-8}$M), was infused into the saphenous artery of 4 sheep. In 1 of these sheep, waveform changes consistent with a degree of vasodilation were recorded (Figure 4.3.10). There was a decrease in the secondary reverse flow velocity and pulsatility index fell from a mean of 6.69 during saline infusion, to 5.16 during infusion of sodium nitroprusside. However, overall no significant changes in pulsatility index, heart rate or mean arterial pressure were recorded in this group (Figure 4.3.11).

On a separate occasion a higher dose of sodium nitroprusside, 30μg/min (blood concentration ≈ $7x10^{-7}$M), was infused into the dorsal metatarsal artery of 5 sheep. The most marked change in waveform shape was an increase in the secondary

(A)



(B)

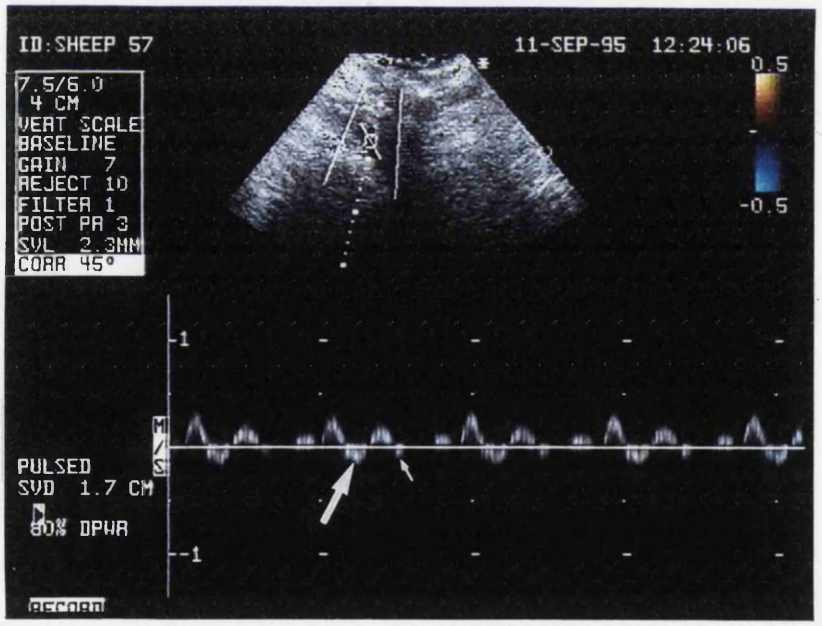


Figure 4.3.7. Doppler velocity-time waveforms recorded from the femoral artery of an anaesthetised sheep during infusion of (A) N-saline and (B) 9μg/min phenylephrine into the saphenous artery of the ipsilateral hindlimb.

⇑⇑ secondary reverse flow

⇑ quaternary reverse flow

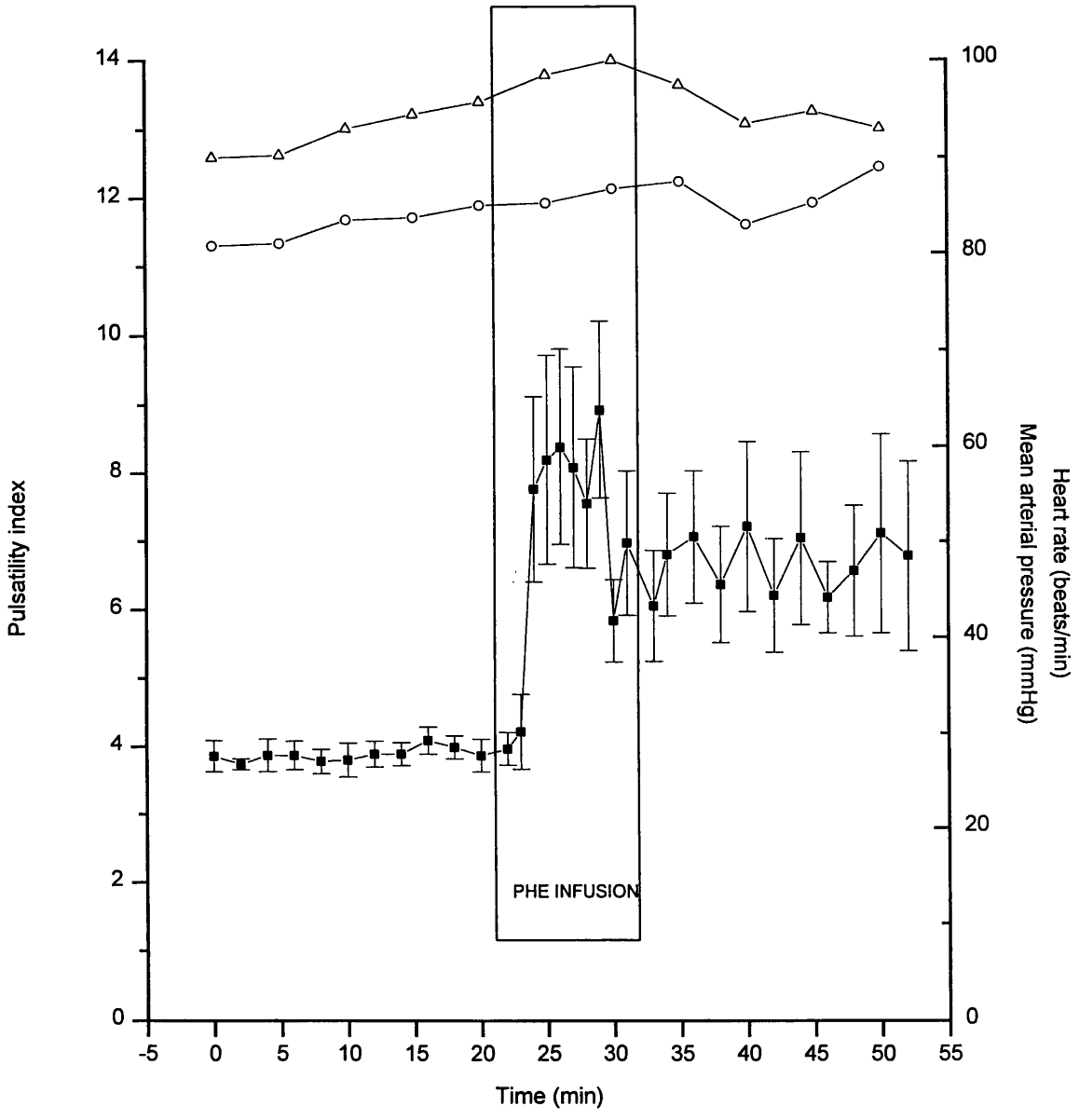


Figure 4.3.8. Femoral artery pulsatility index, heart rate and systemic mean arterial pressure recorded in anaesthetised sheep during infusion of N-saline or 9μg/min phenylephrine (PHE) into the saphenous artery of the ipsilateral hindlimb. Pulsatility index is expressed as mean ± SEM (n=4). Heart rate and mean arterial pressure are expressed as mean values (n=4).

—▪— pulsatility index

—o— heart rate

—△— mean arterial pressure

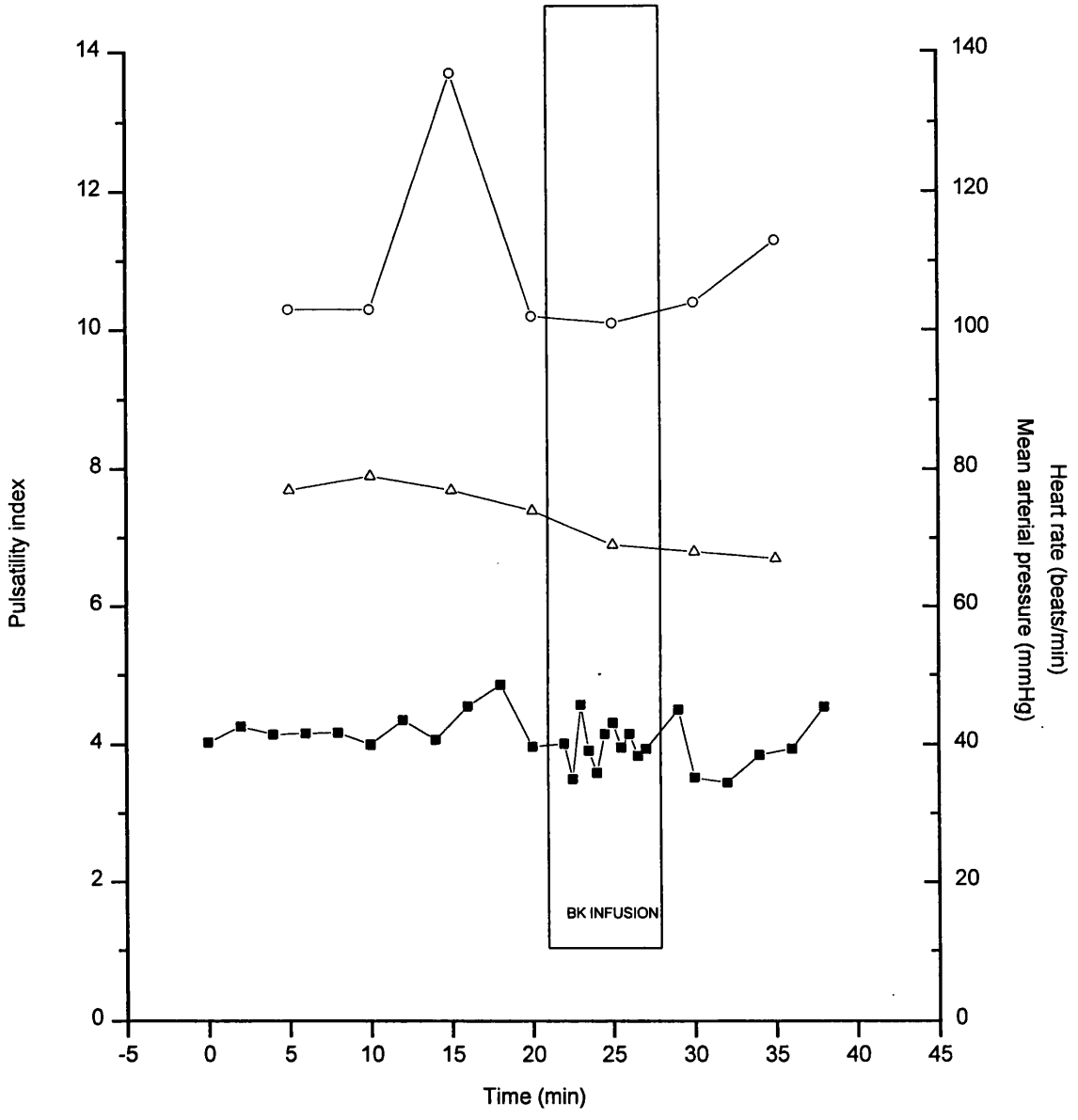


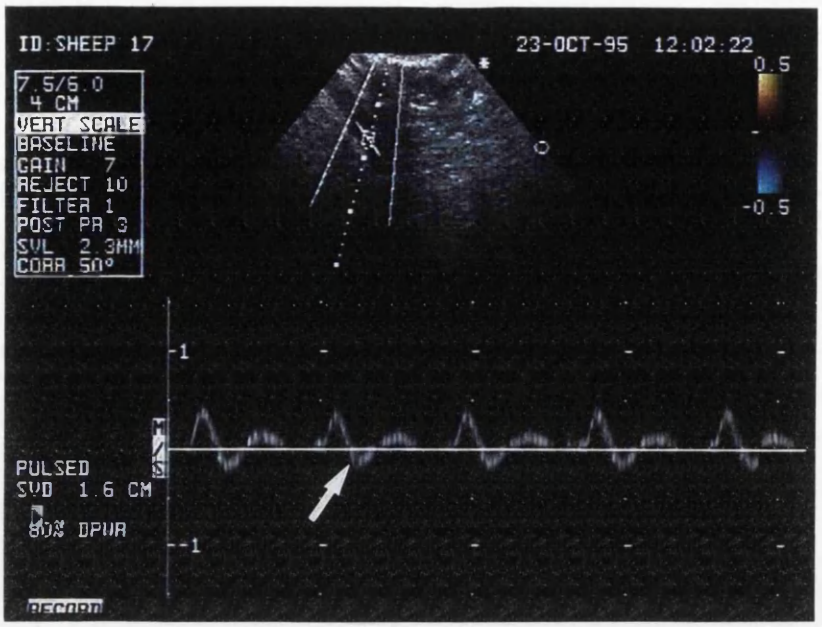
Figure 4.3.9. Femoral artery pulsatility index, heart rate and systemic mean arterial pressure recorded in 1 anaesthetised sheep during infusion of N-saline or 1.6μg/min bradykinin (BK) into the saphenous artery of the ipsilateral hindlimb.

—■— pulsatility index

—o— heart rate

—△— mean arterial pressure

(A)



(B)

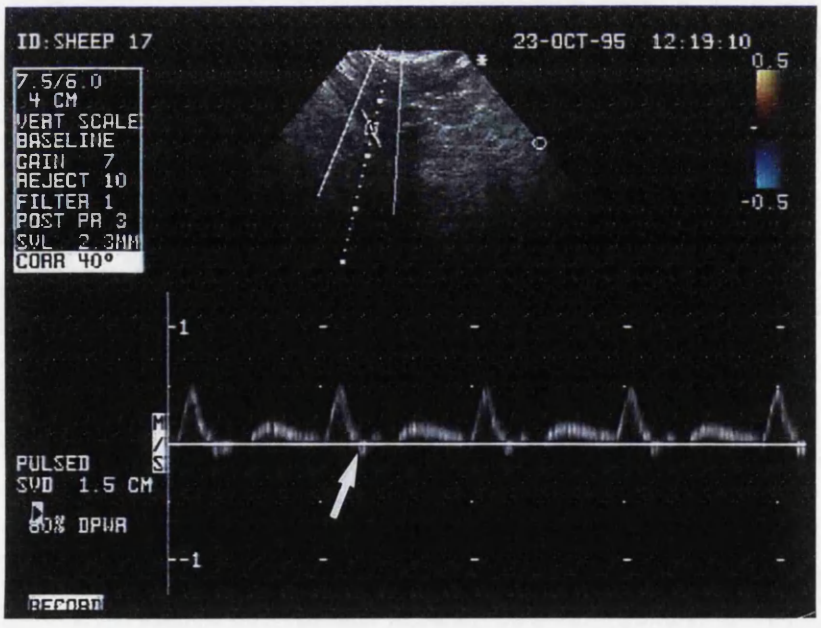


Figure 4.3.10. Doppler velocity-time waveforms recorded from the femoral artery of an anaesthetised sheep during infusion of (A) N-saline and (B) 3μg/min sodium nitroprusside into the saphenous artery of the ipsilateral hindlimb.

⇑ secondary reverse flow

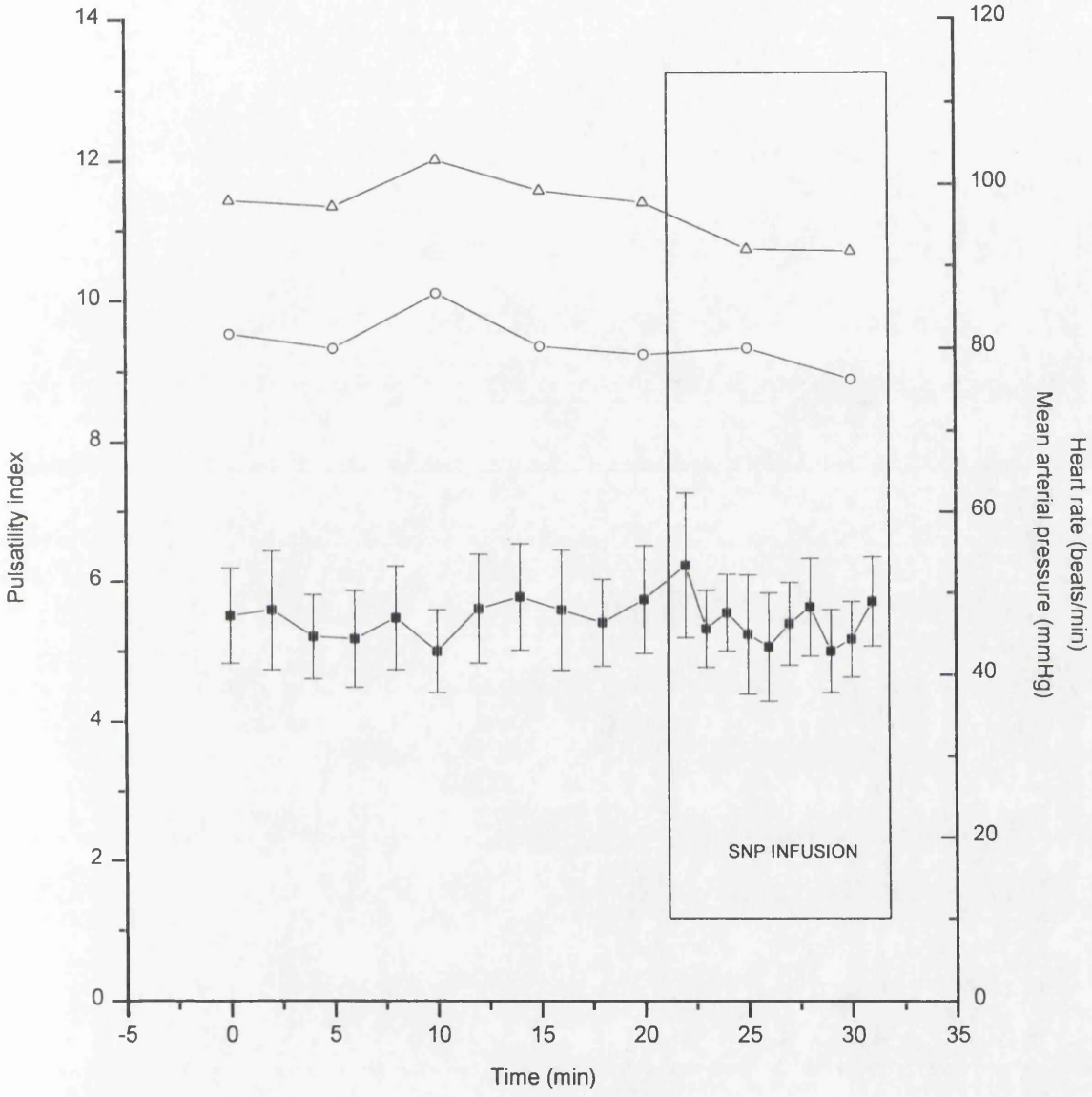


Figure 4.3.11. Femoral artery pulsatility index, heart rate and systemic mean arterial pressure recorded in anaesthetised sheep during infusion of N-saline or 3µg/min sodium nitroprusside (SNP) into the saphenous artery of the ipsilateral hindlimb. Pulsatility index is expressed as mean ± SEM (n=4). Heart rate and mean arterial pressure are expressed as mean values (n=4).

—■— pulsatility index

—○— heart rate

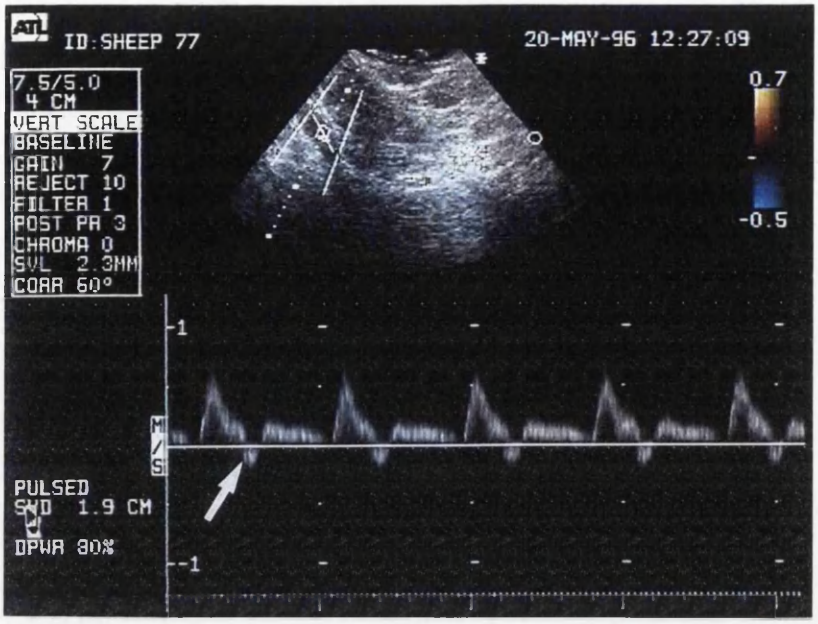
—△— mean arterial pressure

reverse flow (Figures 4.3.12 and 4.3.13). The reduction in primary forward flow velocity, seen during phenylephrine infusion, was not a feature and quaternary reverse flow was seen in only 1 sheep (Figure 4.3.13B). Pulsatility index increased from 4.86 ± 0.72 to 6.42 ± 1.18 (n = 5) (Figure 4.3.14). This increase was not statistically significant (p = 0.30). However, when pulsatility indices were expressed as a percentage of baseline, the increase, from 100.0% to 129.6 ± 13.0%, did approach significance (p = 0.052). Heart rate remained stable during infusion of the higher dose of sodium nitroprusside and although a slight fall in mean arterial pressure occurred, from 87.2 ± 4.6mmHg to 82.0 ± 5.1mmHg, this was not statistically significant. No significant change in arterial carbon dioxide was recorded, it fell only slightly from 45.4 ± 1.0mmHg to 44.3 ± 0.6mmHg.

The repeat infusion of saline, which followed infusion of both phenylephrine and bradykinin, was omitted from the nitroprusside studies since it contributed little to the results of the earlier experiments.

Changes in pulsatility index for all infusions are summarised in Table 4.3.1.

(A)



(B)

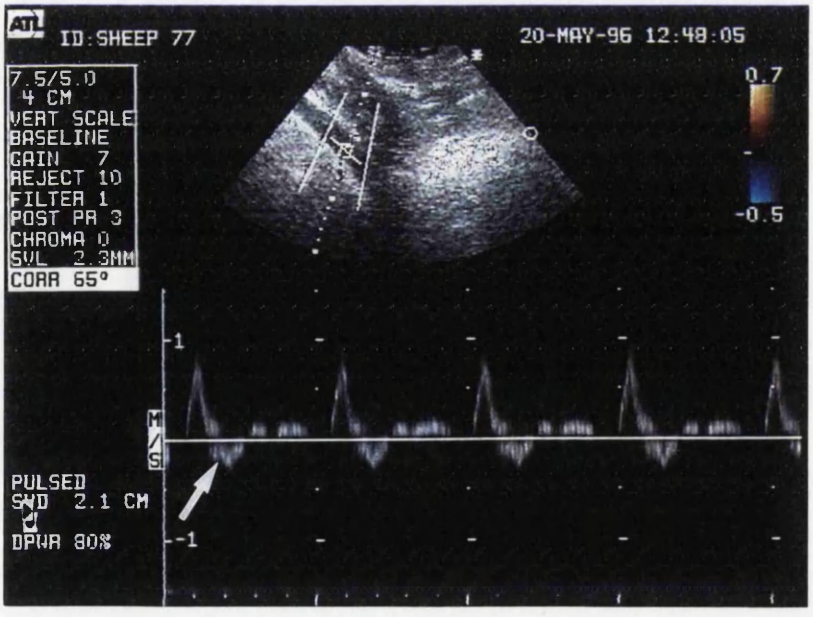
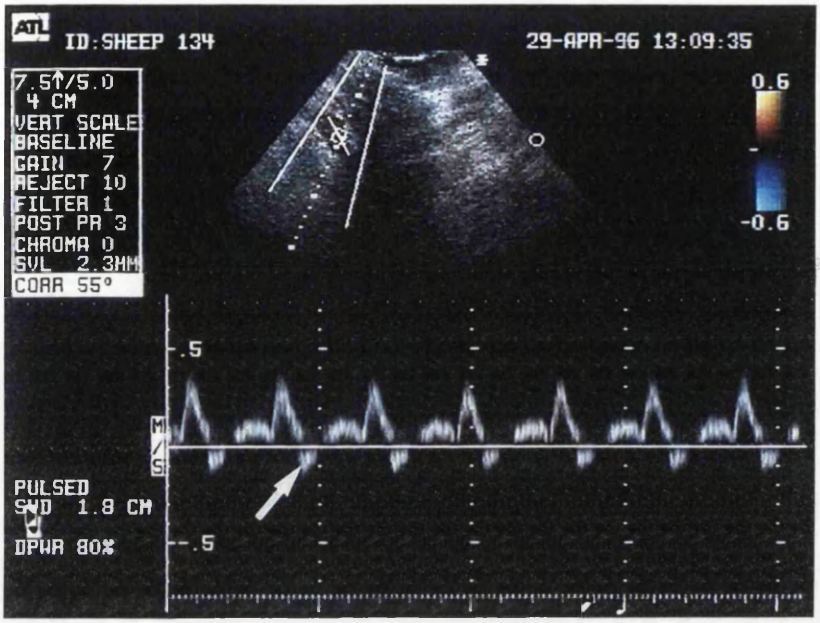


Figure 4.3.12. Doppler velocity-time waveforms recorded from the femoral artery of an anaesthetised sheep during infusion of (A) N-saline or (B) 30μg/min sodium nitroprusside into the dorsal metatarsal artery of the ipsilateral hindlimb.

⇑ secondary reverse flow

(A)



(B)

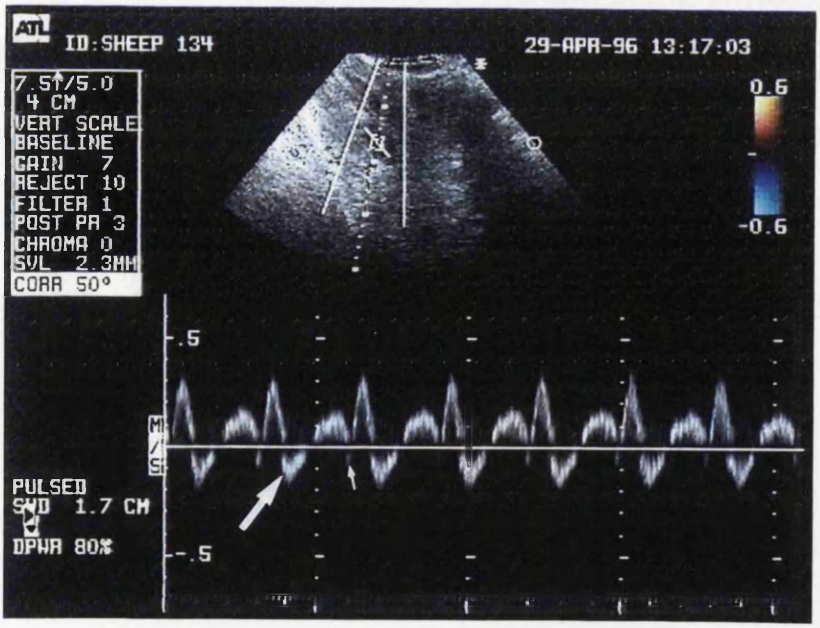


Figure 4.3.13. Doppler velocity/time waveforms recorded from the femoral artery of an anaesthetised sheep during infusion of (A) N-saline or (B) 30μg/min sodium nitroprusside into the dorsal metatarsal artery of the ipsilateral hindlimb.

⇑ secondary reverse flow

⇑ quaternary reverse flow

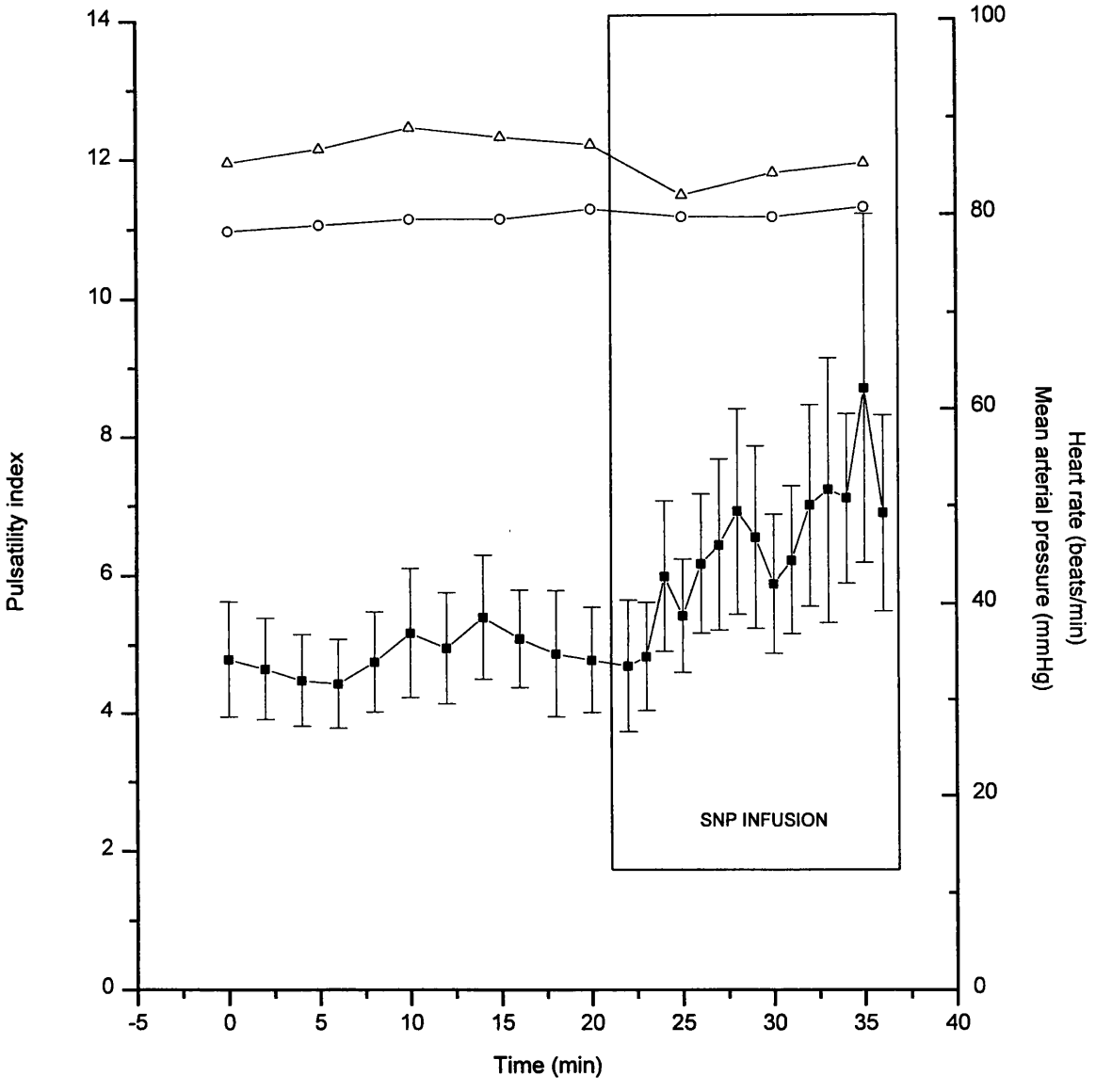


Figure 4.3.14. Femoral artery pulsatility index, heart rate and systemic mean arterial pressure recorded in anaesthetised sheep during infusion of N-saline or 30μg/min sodium nitroprusside (SNP) into the dorsal metatarsal artery of the ipsilateral hindlimb. Pulsatility index is expressed as mean ± SEM (n=5). Heart rate and mean arterial pressure are expressed as mean values (n=5).

—■— pulsatility index

—○— heart rate

—△— mean arterial pressure

(A)

		MEAN PI ± SEM		ANOVA
INFUSION	n	N-saline	Treatment	p
PHE 3μg/min	1	2.86	3.96	NA
PHE 30μg/min	1	2.07	21.51	NA
PHE 9μg/min	4	3.87 ± 0.14	6.99 ± 0.93	0.016**
BK 1.59μg/min	1	4.23	3.99	NA
SNP 3μg/min	4	5.46 ± 0.70	5.43 ± 0.63	0.972
SNP 30μg/min	5	4.86 ± 0.72	6.42 ± 1.18	0.292

(B)

		MEAN PI (%) ± SEM		ANOVA
INFUSION	n	N-saline	Treatment	p
PHE 3μg/min	1	100.0	138.5	NA
PHE 30μg/min	1	100.0	1039.0	NA
PHE 9μg/min	4	100.0	179.0 ± 17.9	0.005**
BK 1.59μg/min	1	100.0	94.4	NA
SNP 3μg/min	4	100.0	101.1 ± 8.1	0.893
SNP 30μg/min	5	100.0	129.6 ± 13.0	0.052*

Table 4.3.1. Mean pulsatility indices (A) of the femoral artery recorded in anaesthetised sheep during infusion of N-saline followed by phenylephrine (PHE), bradykinin (BK) or sodium nitroprusside (SNP). Pulsatility indices (PI) were also expressed as a percentage of baseline, i.e. as a percentage of PI during infusion of N-saline (B). Drugs were infused into the saphenous or dorsal metatarsal artery of the ipsilateral hindlimb. Saline and treatment groups were compared by one-way analysis of variance (ANOVA).

Where appropriate, results are expressed as a mean value ± SEM and n indicates the number of sheep used.

** indicates that treatment produced a statistically significant effect, i.e. $p < 0.05$

* indicates that treatment effect approached significance, i.e. $p \approx 0.05$

4.4 Discussion

The main aim of this work was to investigate the effects of vasoconstrictor and vasodilator agents on the femoral artery waveform and to characterise these effects in terms of pulsatility index. Originally the intention was to measure pulsatility index in conjunction with volume blood flow and investigate any correlation between the 2 parameters. However, it became clear on measuring femoral artery diameter that distance could only be estimated to the nearest mm. This introduced an error of ± 10% in measuring the diameter of a 4mm artery. Flow measurements are also dependent upon the probe-to-vessel angle (Gill 1985) and whilst this angle can be estimated it remains a source of error. In the light of these inaccuracies, attempts to quantify blood flow were abandoned and the peak-to-peak pulsatility index ($PI_{p/p}$) was chosen as the primary measurement parameter.

$$PI_{p/p} = \frac{\textit{peak systolic velocity - minimum diastolic velocity}}{\textit{mean velocity}}$$

This index has the advantage of being independent of the probe-to-vessel angle (θ), since inaccuracies attributable to θ contribute to both the numerator and denominator they therefore cancel out. This is particularly valuable where studies are carried out *in vivo* using a hand-held ultrasound probe.

Whilst the pulsatility index is independent of probe-to-vessel angle, it is subject to a number of other errors. Indices may be derived from the mean or maximum velocity waveforms, the latter was used in this study. If Doppler shifts of venous origin are erroneously measured within the sample volume they reduce the mean velocity but not the maximum (Gosling & King 1974b, Reneman 1981). Low frequency artefacts induced by probe or vessel wall movements, which are not excluded by the filter, will also reduce the spectral average. It is therefore preferable to derive indices from the maximum velocity waveform (Thompson *et al.* 1986a), as was the case in this study. Another problem which may occur with pulsed Doppler units is velocity/range ambiguity or aliasing (Woodcock 1992). Pulsed Doppler flowmeters have a single transducer, which both transmits and receives the signal. Thus each pulse must travel to the target and then back again before the next pulse is emitted. This is not always possible if high velocities are being measured at long range. If shift frequency exceeds half the pulse repetition frequency, aliasing occurs. Fortunately, this produces a characteristic alteration in the waveform, the peak of the wave appears at the bottom of the reverse flow channel (Figure 4.4.1). Aliasing was not a problem in this study.

The repeatability of the pulsatility index has been studied. Reuwer *et al.* (1984) investigated the influence of a number of factors, including the observer/operator

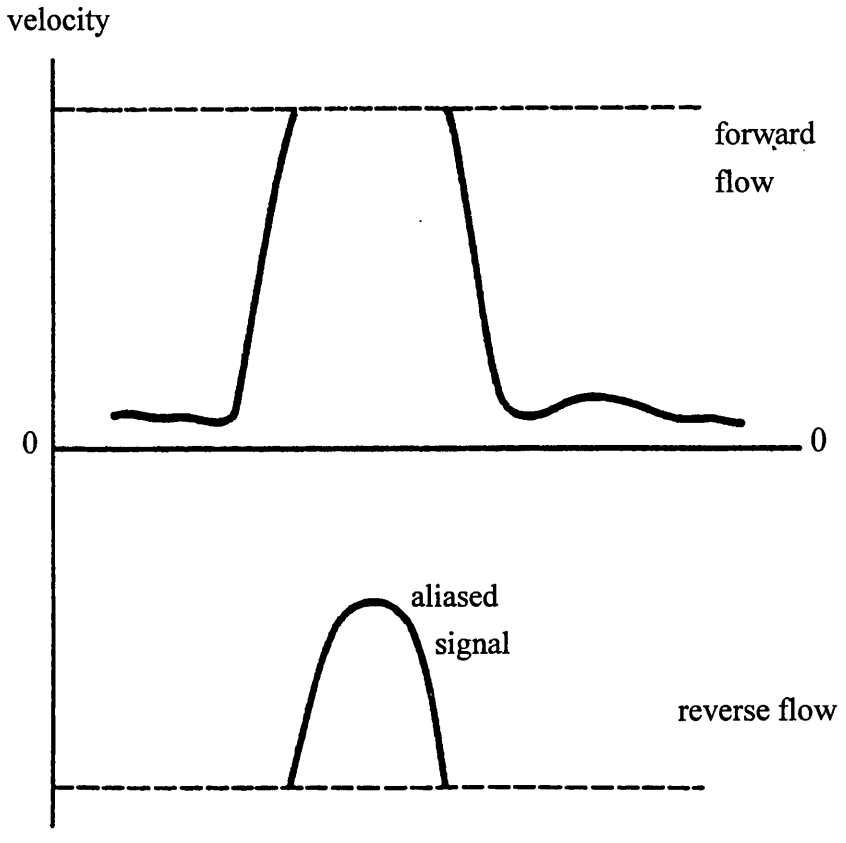


Figure 4.4.1. Illustration of the alteration in the velocity-time waveform seen when aliasing occurs. The aliased portion of the spectrum appears at the bottom of the reverse flow channel. Adapted from Woodcock (1992).

and the time of day, on the reproducibility of umbilical artery pulsatility index. Neither factor exerted a significant effect and so the authors concluded that umbilical artery pulsatility index is an intra- and inter-observer reproducible variable. Others have suggested that Doppler waveforms are influenced by the experience of the operator (Bernstein and Fronek 1982). This has been most clearly demonstrated in association with a more complex form of waveform analysis, Laplacian transformation (Campbell *et al.* 1984). These workers recorded a decrease in the coefficient of variation of femoral artery Laplacian coefficients from 16.9 to 12.5%, as the operator gained experience. This may also account for the decline in variation noted in the study reported here. In preliminary studies, a mean coefficient of variation of 12.3% was calculated, based on ≥ 23 recordings made in each of 2 sheep, during intraarterial saline infusion. However, the overall coefficient of variation recorded during all subsequent saline infusions, i.e. ≥ 10 recordings from each of 6 sheep on 2-3 occasions, was 8.5%. This degree of variation is consistent with that deemed acceptable by other workers in this field (Qamar *et al.* 1986, Rasmussen 1987, Voet *et al.* 1996).

In this study, intraarterial infusion of 9μg/min phenylephrine induced a significant increase in femoral artery pulsatility index. Phenylephrine is a vasoconstrictive drug that acts primarily by stimulating α_1-adrenergic receptors on vascular smooth muscle (Hoffman and Lefkowitz 1990), thereby increasing vascular resistance. These results, therefore, support the assertion that pulsatility index increases in response to increasing vascular resistance. This is consistent with the findings of Fairlie & Walker (1991) in man and Downing *et al.* (1993) in sheep. These workers recorded increases in pulsatility index in response to cold- and phenylephrine-induced vasoconstriction, respectively.

The effect of vasodilation on pulsatility index has also been investigated. Legarth & Nolsoe (1990) demonstrated a fall in brachial artery pulsatility index during post-anoxic vasodilation in people. Whilst Downing *et al.* (1991, 1993) recorded a fall in aortic pulsatility index in new-born lambs treated with intravenous hydralazine. Therefore, a fall in pulsatility index in response to infusion of sodium nitroprusside might have been predicted in sheep. This was recorded in only 1 of 4 sheep given the lower dose of 3μg/min. Paradoxically, the higher dose of 30μg/min nitroprusside tended to increase pulsatility index, although initial analysis of the data did not show this increase to be statistically significant. The baseline pulsatility index, for each of the 5 sheep receiving the higher dose, was very variable, ranging from 2.20 to 6.17. In order to standardise the starting point, all indices were expressed as a percentage of baseline. On analysis of these data, the increase in pulsatility index in response to 30μg/min sodium nitroprusside approached statistical significance, i.e. $p = 0.052$.

An explanation for this trend is not immediately apparent. Sodium nitroprusside is a potent endothelium-independent vasodilator, which relaxes vascular smooth muscle through release of nitric oxide (Schultz *et al.* 1977, Feelisch and Noack 1991). Tissue bath studies have confirmed that it is a vasodilator in sheep arteries (Section 2.3.1.4) and so vasoconstriction is unlikely to be the cause of the increased pulsatility index. This view is supported by qualitative assessment of the waveforms obtained during infusion. Nitroprusside did not reduce the forward component of flow, in contrast to phenylephrine (Figure 4.3.5). In fact forward velocities were enhanced in many cases (Figure 4.3.12). The most striking feature of these waveforms was the decrease in diastolic velocity, i.e. the increase in the reverse component of flow, which suggests an increase in reflection from the periphery. This decrease in diastolic velocities could account for the increase in pulsatility index. Pulsatility index is derived from the maximum minus the minimum velocity divided by the mean, therefore enhanced reverse flow reduces mean velocity, i.e. the denominator, and tends to increase the numerator, hence pulsatility index increases.

A number of studies, investigating the effect of vasodilators on pulsatility index, have been conducted in sheep. Downing *et al.* (1991, 1993) demonstrated a fall in aortic pulsatility index in response to intravenous hydralazine, which they correlated with a fall in aortic input impedance parameters, including peripheral vascular resistance. Other authors have recorded the opposite effect. Adamson and Langille (1992) measured flow pulsatility, i.e. pulsatility index derived from the flow waveform rather than the velocity waveform, in the thoracic aorta and umbilical artery of foetal sheep. They recorded no significant changes in aortic flow pulsatility index in response to infusion of sodium nitroprusside, however, unexpectedly, they found that umbilical artery pulsatility index increased. In a similar study, Rybakowski *et al.* (1995) were able to demonstrate an increase in pulsatility index of the abdominal aorta on administration of nitroprusside to foetal sheep. They used the velocity waveform to derive pulsatility index and found similar changes to those recorded in this study, i.e. a tendency for systolic velocity to increase and diastolic velocity to decrease. Adamson and Langille (1992) suggested that this anomaly may be explained if flow pulsatility index (PI) is determined not only by downstream vascular resistance but also by impedance and pressure. They proposed that these parameters are related as follows:

$$\textit{Flow PI} \approx \frac{\textit{pulse pressure}}{\textit{mean pressure}} \times \frac{\textit{vascular resistance}}{\textit{fundamental impedance}}$$

where *fundamental impedance* = vascular impedance at cardiac rate

The authors recorded a decrease in impedance on infusing nitroprusside that was greater than the decrease in resistance and suggested that this increase in the resistance/impedance ratio was responsible for the increase in pulsatility index. However they also recorded a fall in mean pressure which would also tend to increase pulsatility index. Rybakowski *et al.* (1995) proposed an additional theory that the ratio of downstream resistance to upstream resistance is an important determinant of pulsatility index. They suggested that nitroprusside may significantly reduce upstream resistance, therefore encouraging the flow of blood back to the heart and causing diastolic velocities to fall.

If these assessments are correct, there is no reason why they might not equally apply to human studies, however this does not appear to be the case. There are numerous studies conducted in people which consistently demonstrate a fall in arterial pulsatility index in response to administration of vasodilators (Belfort *et al.* 1993, Belfort *et al.* 1994, Miyazaki 1995, Voet *et al.* 1996). Of particular relevance, are those human studies that have examined the pulsatility index of an artery supplying the distal limb. Decreases in brachial or radial artery pulsatility index have been recorded following administration of the vasodilators prostaglandin E_1 (Clifford *et al.* 1980) and nifedipine (Lewis *et al.* 1987, Morgan *et al.* 1987).

This study in sheep, demonstrated an increase in pulsatility index in response to a vasodilator, a result that is in contrast to the findings of equivalent studies in people. The suggestion by Rybakowski *et al.* (1995), that a fall in upstream resistance may be responsible, cannot be applied in this case. Nitroprusside was administered locally and was unlikely to exert a significant systemic effect since arterial blood pressure did not change. Perhaps these differing patterns of flow are a consequence of the dissimilarity between the human and ungulate distal limb. In ungulates the hoof forms a semi-rigid shell which encloses the foot. If blood flow to the foot is increased by vasodilation the capacity to expand and accommodate the increased flow is limited and pressure within the hoof will rise. This rise in pressure may contribute to the increase in wave reflection and thereby pulsatility index. Within the distal phalanx, the capacity of the distal phalangeal artery to vasodilate will similarly be limited by the dimensions of the ungular canal and this may also contribute to an increased pressure. This is not the first time that an increased pulsatility index has been related to changes in pressure. Van Vugt *et al.* (1988) demonstrated an increase in umbilical artery pulsatility index on occluding the umbilical vein. This occurred despite a slight fall in placental vascular resistance and was attributed to an increase in venous pressure.

In addition to sodium nitroprusside, an endothelium-dependent vasodilator, bradykinin, was studied. This agent was chosen in preference to acetylcholine,

which produced less consistent relaxation of ovine digital arteries *in vitro* (Section 2.3.1.3). Intraarterial infusion of 1.6μg/min bradykinin produced local erythema and oedema, but failed to alter the femoral artery waveform shape or pulsatility index. This lack of change in the velocity waveform was consistent with the response to low dose sodium nitroprusside, but provided no evidence of the secondary contractile phase observed in ovine digital arteries *in vitro* (Section 2.3.1.2). This implies that bradykinin-induced contraction is less prominent *in vivo*, although it is possible that the local inflammatory response may have masked the effects of bradykinin on vascular tone. However, this agent was administered to just 1 sheep and further studies, perhaps using lower doses of bradykinin are needed to clarify the vascular effects of this agent *in vivo*.

Vascular tone can be modified by factors other than the vasoactive drugs infused and it was necessary to minimise or at least stabilise these influences during the study. Heart rate variability was one such factor. Intuitively, an inverse relationship between heart rate and pulsatility index might be predicted. Slowing of the heart increases path length, allowing velocity to fall to a lower level, thus decreasing the mean velocity and causing pulsatility index to rise. Opinions vary as to whether this relationship requires consideration when interpreting clinical data. A study examining the umbilical artery waveforms in sheep, showed that slowing of the foetal heart induced an increase in pulsatility index independent of any change in placental vascular resistance (van Huisseling *et al.* 1989). Legarth and Thorup (1989a) also suggested that waveform indices are correlated to pulse rate. However, other authors have reached a different conclusion. Thompson *et al.* (1986a) were unable to demonstrate a correlation between path length and pulsatility index, whilst Irion and Clark (1990) concluded that this correlation was only significant at heart rates in excess of 170 beats/min.

A normalisation formula, which corrects for changes in path length, has been described by Hoskins *et al.* (1989a).

$$\textit{normalised pulsatility index} = \frac{(PI \times 7.48) - (5.19 \times DP)}{(7.48 + DP)}$$

where PI = pulsatility index

DP = path length (seconds) - 0.46

These authors went on to compare the ability of normalised and unnormalised waveform indices to detect antenatal foetal compromise and concluded that normalisation offered no advantage, in fact they preferred the simplicity of the unmodified indices (Hoskins *et al.* 1989b).

Preliminary ultrasound examinations were conducted in conscious sheep, restrained in dorsal recumbency in a purpose-made cradle. Those sheep that struggled showed rapid fluctuations in heart rate and rates in excess of 160 beats/min were recorded. Such fluctuations were undesirable for the reasons outlined above and so it was considered appropriate to conduct the study in anaesthetised sheep. This ensured a more stable cardiovascular system and in addition permitted incision of the skin to facilitate arterial catheterisation. Normalisation of pulsatility index was not attempted, instead every effort was made to keep heart rate stable during infusion of drugs, through maintenance of an even plane of anaesthesia. This was achieved in all but one sheep, whose heart rate rose dramatically during infusion of N-saline (Figure 4.3.9). This was the sheep which received a bradykinin infusion and so no conclusions regarding changes in pulsatility index were drawn from these results..

Whilst general anaesthesia was advantageous, it could itself influence vascular tone. The majority of anaesthetic agents reduce cardiac output and in many cases, systemic vascular resistance (Prys-Roberts 1989). The induction agent, propofol causes a reduction in peripheral vascular resistance within the first few minutes of bolus administration but beyond this changes are minimal (Dundee 1989). Thus, waveform analysis was not performed within the first hour of anaesthesia to minimise the effects of the induction dose of propofol. Halothane was used to maintain anaesthesia and this agent produces hypotension, predominantly through myocardial depression (Forrest 1989), although a degree of vasodilation occurs as a result of reduced sympathetic tone. Halothane may also alter vascular tone directly by depressing endothelium-dependent vasodilation (Muldoon *et al.* 1988, Greenblatt *et al.* 1992). It was therefore important to ensure a constant concentration of halothane during the measurement period. As an anaesthetic vapour analyser, to monitor the end tidal concentration of halothane, was not available, a semiclosed non-rebreathing anaesthetic circuit was employed. Such systems ensure that the inspired concentration of halothane is close to that set by the vaporiser (Hartsfield 1987). During each anaesthetic the target vaporiser setting was 2% halothane. Whilst this was not always achieved, i.e. some sheep required more halothane than others, in all but 1 sheep, a constant level of halothane was maintained throughout the measurement period. This sheep, which was infused with high dose sodium nitroprusside, appeared to lighten during saline infusion and so halothane was increased to 2.5%. A slight fall in pulsatility index was observed at this time but this did not detract from the increase in pulsatility index that occurred on infusion of nitroprusside.

Another factor which may alter vascular tone is the partial pressure of carbon dioxide (p_aCO_2). An increase in p_aCO_2, i.e. hypercapnia, triggers a local arteriolar dilation, for example in active or reactive hyperaemia (Vander *et al.* 1985). Most

anaesthetics depress respiratory function therefore hypercapnia is a potential complication of general anaesthesia. This is especially so in ruminants (Trim 1987), therefore the sheep were ventilated artificially and arterial blood gas analysis was performed regularly. Arterial p_aCO_2 values before and during/after drug infusion were recorded but did not differ significantly.

Arteries are sensitive to trauma and it is likely that catheterisation of the saphenous and dorsal metatarsal arteries would itself induce a degree of vasoconstriction and so modify the femoral artery waveform. Arterial catheterisation was always performed before the start of waveform recording, thus any effect on pulsatility index would apply equally during infusion of saline and vasoactive drugs. Catheterisation proved to be quite difficult, even though pulses were easily palpable. Cannulation of the hindlimb arteries required incision of the skin and superficial dissection. Inevitably, a degree of fibrosis occurred around the artery and so they were rarely catheterised more than once.

No adverse reactions were recorded in response to infusion of either phenylephrine or sodium nitroprusside. Doses were selected after examination of experimental, and where appropriate, therapeutic protocols. The therapeutic dose of phenylephrine, used in hypotensive dogs to increase blood pressure, is 1-3μg/kg/min, by continuous intravenous infusion. Experimentally, Downing *et al.* (1993) administered intravenous boluses of 10μg/kg to newborn lambs and recorded significant changes in both heart rate and mean arterial pressure. For this study, a localised response with minimal systemic side-effects was required, so a lower dose, given intraarterially was preferred. Doses of 0.2-2.4μg/min were infused into the brachial artery of human volunteers in a study to determine if hyperinsulinaemia modulates vascular responses to phenylephrine (Sakai *et al.* 1993). No changes in blood pressure or heart rate were recorded at this dose rate. Therefore a phenylephrine infusion of 3μg/min seemed a reasonable starting point for this study. The therapeutic dose of sodium nitroprusside, used in dogs with heart failure to reduce afterload, is 1-10μg/kg/min by continuous intravenous infusion (Dhupa and Shaffran 1995). Intraarterial administration of sodium nitroprusside has been reported in rabbits, at doses of 0.1-100μg/kg/min (Ward and Angus 1993), whilst García *et al.* (1992) administered intracoronary boluses of 10-300μg to anaesthetised goats. Doses at the lower limit of these ranges were chosen for this study , i.e. 3μg and 30μg/min.

Bradykinin, the second vasodilator used, has numerous pro-inflammatory actions, including a tendency to increase vascular permeability (Marceau *et al.* 1983, Hall 1992) and these effects may well account for the local erythema and oedema that developed during intraarterial infusion of this agent. Doses of up to 3μg/min have

been infused into the brachial artery of human volunteers without adverse effect (Cockcroft *et al.* 1994), therefore the dose of 1.6μg/min used in this study would seem reasonable. However, lower intraarterial doses have been suggested by other authors, for example, up to 0.1μg/min in man (O'Kane *et al.* 1994) and up to 0.3μg boluses in dogs (White *et al.* 1993). The route of administration may also be significant in determining the effect on vascular permeability. For example, histamine have been shown to increase capillary permeability when administered by the intraarterial route (Robinson *et al.* 1976) but not following intravenous infusion (Robinson and Scott 1981).

The primary objective of this study was to characterise the normal femoral artery waveform in the sheep and to describe, by means of the pulsatility index, the changes induced by local infusion of vasoconstrictor and vasodilator substances. Since both classes of drug appeared to increase pulsatility index, this parameter cannot be used in isolation to distinguish vasodilation and vasoconstriction of the digital vascular bed in ungulates. However this finding, which is in contrast to similar studies in man, is itself of interest and worthy of further investigation. Vascular impedance appears to be a crucial determinant of pulsatile blood flow and its measurement (Section 4.1.) during administration of vasoactive drugs may provide an explanation for these unexpected changes in the velocity waveform. Studies comparing pulsatility index and impedance in non-ungulate species would also be of value to ascertain that this effect is limited to hoofed animals. Another approach would be to quantify volume blood flow in conjunction with pulsatility index, as had been intended originally. Whilst this was outwith the capability of the ultrasound unit used in this study, more modern systems, with sufficient resolution to accurately quantify blood flow in vessels of this size, are now available. This approach could determine if the increased reverse velocities observed during infusion of nitroprusside exert a significant effect on volume flow, i.e. are there circumstances in which vasodilation can reduce net volume flow to the digit? It is clear that further studies are needed to elucidate fully the impact of the hoof on digital blood flow.

5. General Discussion

The ultimate aim of this study was to increase our knowledge of the factors that regulate digital blood flow in sheep, with a view to understanding the pathogenesis of laminitis. Laminitis, a disease of ungulates, has a complex aetiology and there are conflicting views as to how the condition develops. This project sought to investigate the relationship between one theory of pathogenesis, i.e. disturbed digital blood flow and endotoxin, a potential aetiological factor, through examination of the responses of the isolated ovine digital artery to vasoactive agents. To complement these *in vitro* studies, the response to infusion of similar agents was evaluated using Doppler ultrasound in the live animal. This approach allowed assessment of the influence of the hoof on the pattern of limb blood flow.

These studies were conducted in sheep and it is not clear how easily the results can be extrapolated to other ungulate species, such as cattle and horses, in which laminitis occurs more frequently. There are anatomical differences between the ruminant and equine digit, in both the form of the hoof and the laminar interdigitation, which lacks secondary dermal and epidermal laminae in the ruminant (Schummer *et al.* 1981b). However, it is unclear if this influences the development of disease. Physiological differences may also occur, although the results of this study suggest that the regulation of vascular tone is similar in both the sheep and the horse. It seems likely that the sheep is a good model for laminitis, although further work is needed to clarify the effects of endotoxin on the vasculature in different ungulate species before this aspect of pathophysiology can be compared.

The *in vitro* work demonstrated the importance of endothelium-derived mediators, such as nitric oxide, in the modulation of ovine digital artery tone. This finding was consistent with similar studies of equine digital arteries and veins (Elliott *et al.* 1994, Baxter 1995, Cogswell *et al.* 1995). Clearly, disturbances in the production of such a key mediator could alter digital vascular tone and contribute to the pathogenesis of laminitis.

Endotoxin, a factor predisposing to laminitis in both horses and cattle (Blanchard *et al.* 1987, Maclean 1965), has been shown to modify nitric oxide production in bovine aortic endothelial cells (Myers *et al.* 1992). If this were to occur in digital vessels, the resultant changes in vascular tone could contribute to the pathogenesis of laminitis. However in this study, incubation with endotoxin did not alter the vascular responses of the ovine digital artery and this is in agreement with previous studies using equine digital vessels (Baxter *et al.* 1989, Bailey and Elliott 1996).

Baxter *et al.* (1989) accounted for this by suggesting that the effects of endotoxin are mediated by cytokines, generation of which may be inadequate in isolated vessels. Whilst this is not a limiting factor in isolated rat aorta (McKenna 1990), it is possible that species differences in cytokine generation occur. In a subsequent study, Baxter (1995) demonstrated that the vascular responses of digital arteries removed from horses treated with endotoxin *in vivo*, were altered. Arteries from endotoxin-treated horses exhibited enhanced contraction to noradrenaline and impaired endothelium-dependent relaxations when compared to saline-treated controls. Similar changes were demonstrated following incubation of equine digital arteries with tumour necrosis factor (TNF-α, Baxter 1994). The author suggested that these changes were a consequence of endotoxin-mediated damage to the endothelium, which in turn reduced constitutive nitric oxide production. Furthermore, it was proposed that the resultant increase in vascular tone could be responsible for the development of digital hypoperfusion in laminitis. There is evidence to support this view. Endotoxin-mediated endothelial cell injury has been demonstrated by histopathological studies (Templeton *et al.* 1985, Mishra and Sack 1987) and both endotoxin and cytokines have been shown to reduce nitric oxide synthesis in cultured bovine endothelial cells (Myers *et al.* 1992, Zhang *et al.* 1997). Infusion of endotoxin caused a reduction in digital blood flow in the isolated perfused digit (Hunt *et al.* 1990) and in the conscious horse (Ingle-Fehr and Baxter 1996). In addition, a recent study by Hinckley *et al.* (1996a) showed that transdermal administration of glyceryl trinitrate, a nitric oxide donor, enhanced recovery from laminitis, a conclusion based on subjective assessments of the degree of lameness and depression in treated and untreated laminitic ponies. The authors presented systemic blood pressure data to support this assertion, however the small number of untreated controls and lack of statistical analysis prevented meaningful comparison of the 2 groups.

In considering the effects of endotoxin and cytokines on nitric oxide production expression of inducible nitric oxide synthase (NOS) should not be neglected. In other species, particularly the rat, endotoxaemia is characterised by a reduced response to vasoconstrictors (Wakabayashi *et al.* 1987). Since this vascular hyporeactivity can be reversed by inhibitors of guanylate cyclase or NOS, it has been attributed to increased nitric oxide production following expression of inducible NOS (Beasley 1990, French *et al.* 1991). This process has been documented in isolated rings of bovine pulmonary artery (Dekimpe *et al.* 1994b), and cultured bovine endothelial cells (Lamas *et al.* 1991), following more prolonged exposure to cytokines. In this study, the sensitivity of ovine digital arteries to phenylephrine was reduced by incubation with cytokines, in contrast to the findings of Baxter in equine digital arteries (1994, 1995). This may reflect increased nitric

oxide production as a result of NOS induction, although further studies are needed to confirm this. There is also some evidence to suggest that a reduction in vascular responsiveness occurs in the course of laminitis (Baxter *et al.* 1989) and it is feasible that this could be a consequence of increased nitric oxide synthesis. Expression of inducible NOS has been shown to occur in equine chondrocytes exposed to endotoxin or IL-1β (Frean *et al.* 1997), but has not yet been demonstrated in equine vessels.

Thus, endotoxin and cytokines can have opposing effects on nitric oxide synthesis and thereby vascular tone, depending on the balance between inhibition of constitutive NOS and expression of inducible NOS. In the studies by Baxter (1994, 1995) digital arteries were exposed to TNF-α or endotoxin for relatively short periods of time, 10 minutes and 1-2 hours respectively. Such short incubation periods may not have allowed sufficient time for NOS induction. Beasley (1990) reported that exposure to endotoxin for 2-3 hours was required to induce NOS in rat aorta, whilst studies of bovine endothelial cells suggest that longer incubation, more than 8 hours, may be required in this species (Lamas *et al.* 1995). Thus, the studies by Baxter (1994, 1995) may only have assessed the more immediate effects of endotoxin or cytokines on the vasculature. In the carbohydrate overload model of laminitis in horses, lameness develops approximately 12 hours after the first peak in plasma endotoxin (Sprouse *et al.* 1987), implying that a delayed effect may be involved. A similar time interval, 8-12 hours, elapsed between endotoxin treatment and the development of vascular hyporeactivity in sheep (Nelson *et al.* 1991). However this comparison assumes that lameness marks the start of the disease process. It has been suggested that laminitis is a form of reperfusion injury (Hood *et al.* 1993) and that lameness marks the reperfusion stage, the earlier ischaemic phase going unnoticed clinically. This is not however supported by scintigraphic studies that show laminar perfusion to be reduced at the onset of lameness (Galey *et al.* 1990).

The *in vitro* studies examined just one aspect of digital blood flow, i.e. the effect of vasoactive mediators on digital artery tone. The second part of this project sought to evaluate the impact of such changes in tone on digital blood flow in the live animal, thereby allowing for the influence of the hoof. The velocity waveform of an artery changes in response to alterations in downstream resistance or impedance (Zwiebel and Fruechte 1992) and such changes can be quantified by calculation of the pulsatility index (Legarth and Thorup 1989b, Downing *et al.* 1993). In this study, the femoral artery velocity waveform was examined and the response to infusion of vasoactive agents was described in terms of the pulsatility index.

The response to local infusion of phenylephrine was predictable, downstream resistance was increased, reflected velocities were increased and pulsatility index rose. However, the response to high dose sodium nitroprusside infusion was unexpected. There was a tendency for pulsatility index to rise, resulting mainly from an increase in reflected velocities. Whilst this result was not statistically significant at the 5% level ($p = 0.052$), its biological significance cannot be discounted. The implication of this finding is that the blood flow response to intense vasodilation is modified in the intact animal, possibly by the hoof, which forms a semi-rigid compartment enclosing the distal limb. It is feasible that increasing blood flow into this compartment might increase the pressure within, thereby leading to an increase in wave reflection, i.e. an increase in reverse velocities.

What is the significance of this for laminitis? Capillary and tissue hydrostatic pressures within the digit have been measured in experimentally-induced laminitis and shown to be markedly elevated (Allen *et al.* 1990, Eaton *et al.* 1995). It has been hypothesised that in laminitis these high pressures exceed the closing pressure of capillaries leading to capillary shut down and ischaemia. Is it possible that such pressure increases could be the consequence of increased blood flow? Clinical findings of a warm hoof and bounding digital pulse imply that blood flow is increased in laminitis (Colles and Jeffcotte 1977) and this is supported by studies of isolated perfused digits from laminitic ponies (Robinson *et al.* 1976). Increased blood flow was also a finding in the study by Eaton *et al.* (1995) and the authors conceded that it may have contributed to the increase in capillary pressure. The *in vitro* study by Baxter *et al.* (1989) demonstrated a reduction in vascular responsiveness in arteries from horses with early laminitis and this decrease in vascular tone is consistent with an increase in blood flow to the digit. Furthermore, the vascular hyporeactivity was more pronounced in arteries than veins, leading to a relative increase in postcapillary resistance and a tendency for blood to be retained within the digit, exacerbating the rise in capillary pressure.

However, if vasodilation does not improve digital perfusion, how can we account for the beneficial effects of vasodilators such as prazosin and glyceryl trinitrate in equine laminitis (Galey *et al.* 1990, Hinckley *et al.* 1996a)? Laminitic horses are hypertensive (Garner *et al.* 1975a) and some benefit may ensue from a reduction in arterial blood pressure, which will in turn lead to a reduction in capillary hydrostatic pressure. However, improved digital perfusion following transdermal treatment with glyceryl trinitrate has also been demonstrated, by near infrared spectroscopy (NIRS), in clinically normal horses and ponies (Hinckley *et al.* 1996b). It is possible that vasodilation only increases blood flow to the digit if blood pressure is

maintained, as in this study, and only if blood flow is increased will the pressure within the digit rise. In the study by Hinckley *et al.* (1996b), blood pressure was not measured therefore its influence cannot be assessed. Clearly, further studies are needed to clarify the effect of local vasodilation on both net blood flow to the digit and perfusion within.

These studies have identified a number of areas for future research. The sensitivity of the ovine digital artery to phenylephrine was depressed after prolonged *in vitro* incubation with a mixture of cytokines, interferon-γ, interleukin-1β and tumour necrosis factor-α. It is not clear if this response was limited by unsuitable experimental conditions and some improvements could be made. Incubation in an oxygen enriched environment for a more prolonged period, up to 24 hours, may be beneficial. The mechanism of this response also requires further investigation. If it is the result of increased nitric oxide synthesis following induction of NOS, then this may have implications for laminitis. If inducible NOS is expressed in digital vessels from laminitic animals, horses and cattle, then vascular tone may be reduced, not increased as others have suggested (Baxter 1994,1995). This study has also shown that the hoof modifies the blood flow response to intense local vasodilation, possibly through increasing pressure within the digit. However, it is not clear if the changes in waveform shape observed during infusion of high dose nitroprusside reflect alterations in digital perfusion or net blood flow. Further studies are needed to investigate this possibility. With the development of techniques such as NIRS and laser Doppler, accurate assessment of perfusion within the digit is now possible *in vivo*. Ultrasound technology has also progressed and measurement of volume flow in small arteries is feasible. Combining these more precise methods with techniques such as waveform analysis that examine the pattern of limb blood flow as a whole may reveal not only the nature of the blood flow changes that occur in laminitis, but also how they arise.

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7. Appendix: Pilot studies

7.1 Aims

The ultrasound study described in Chapter 4 was conceived as a result of earlier work conducted in conscious sheep. This work had a number of aims. Firstly, it was necessary to answer a couple of questions relating to the methodology, namely which ultrasound transducer to use and which artery to study. In addition, it was important to practice the technique of scanning arteries. Whilst this is not a difficult procedure, a degree of operator experience is considered optimal if repeatable arterial waveforms are to be obtained. Finally, the feasibility of using arterial waveform analysis to describe drug-induced alterations in blood flow was examined by looking at the effects of temperature on waveform shape.

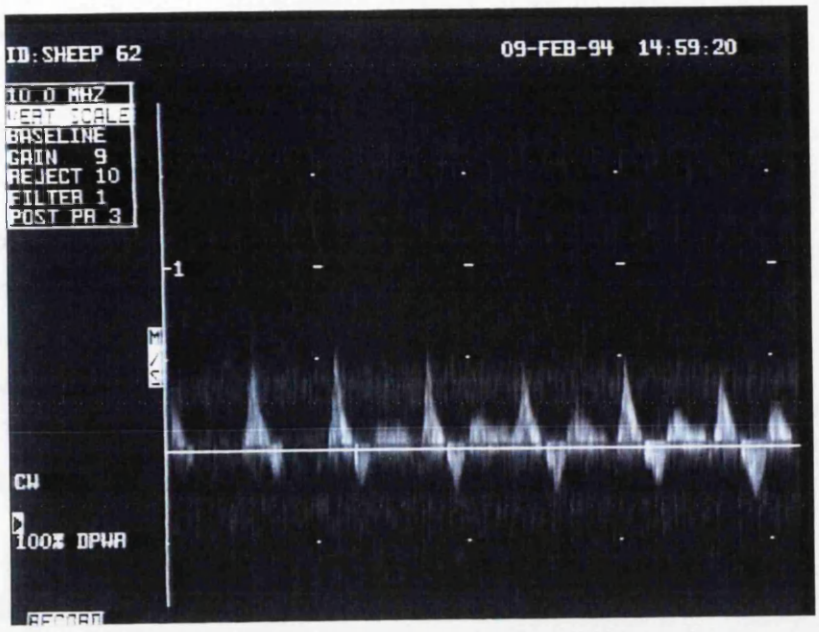
7.2 Choice of transducer

Two transducers were available for use in this study, a 10MHz continuous wave probe and a 7.5MHz pulsed wave duplex probe. Both were used initially to examine different ovine arteries.

Arterial waveforms of reasonable quality could be obtained from the outset using the 10MHz transducer. This probe being smaller could be positioned more easily. However, continuous wave transducers provide no information on the position of a reflector within the isonated tissue and all reflectors in the path of the ultrasound beam can potentially contribute to the returning signal. Therefore, continuous wave velocity-waveforms tended to have increased background noise, which obscured finer details such as quaternary reverse flow (Figure 7.1A). Furthermore, this transducer had no imaging capability and without visualisation of the artery the angle θ could not be estimated. Although in theory waveform indices, such as pulsatility index, are independent of this angle, the lack of vessel imaging was considered a disadvantage nonetheless.

The 7.5MHz pulsed wave duplex transducer had the converse qualities. Being bulkier it was more difficult to position at a suitable angle, however with practice good waveforms could be obtained. The imaging capability allowed the sample volume to be positioned in the centre of the visualised artery. This conferred two advantages, firstly the returning signal could be range-gated, i.e. only signals originating from a specific depth contributed to the final waveform. This resulted in

(A)



(B)

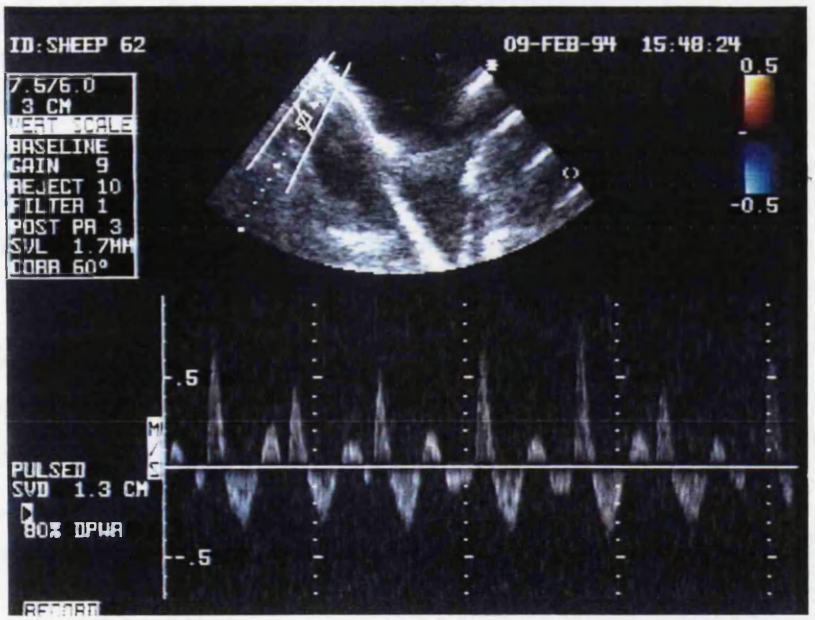


Figure 7.1. Doppler velocity-time waveforms recorded from the plantar proper digital artery of conscious sheep. Waveforms were obtained using (A) a 10MHz continuous wave transducer and (B) a 7.5MHz pulsed wave duplex transducer.

the production of a cleaner waveform (Figure 7.1B). Secondly, the angle θ could be estimated permitting more accurate calculation of velocity. In the light of these advantages, the pulsed wave duplex transducer was chosen for use in this study.

7.3 Choice of artery

In the forelimb, the median artery was examined where it coursed over the medial aspect of the carpus. Using the 10MHz transducer, waveforms could be obtained consistently but were poorly defined (Figure 7.2A). Use of the 7.5MHz probe did not improve definition, possibly as a result of positioning difficulties. As the median artery ran parallel to the skin surface, it was difficult to achieve an angle of approach of less than 90° and since the cosine of 90° is zero the signals were weak (Figure 7.2B).

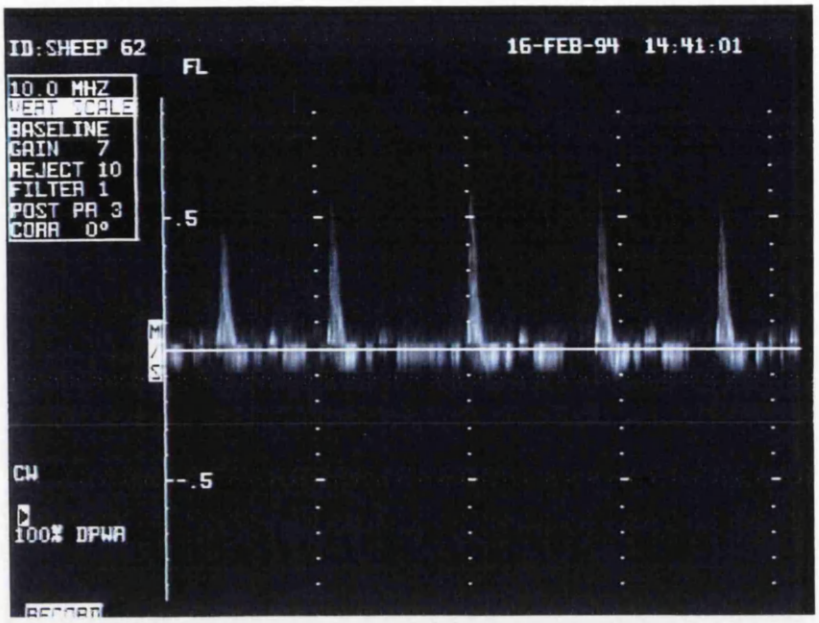
In the hindlimb, the femoral artery was the first vessel to be examined. This artery being large was relatively easy to image and velocity waveforms were well defined (Figure 7.3A). As the initial aim of the study was to assess digital blood flow, a more distal artery was considered preferable. Therefore the plantar proper digital artery was the next vessel to be examined. This artery proved difficult to image and considerable practice was required to obtain good quality velocity waveforms. However, the shape of the waveform was quite characteristic, being more pulsatile than that of the femoral artery (Figure 7.1B). Despite its advantages, a major drawback became apparent during subsequent studies of this vessel. Vasoconstriction produced by cooling the foot obliterated the Doppler signal. For this reason, the more proximal but more repeatable femoral artery waveform was chosen for study.

7.4 Effect of warming or cooling on femoral artery velocity waveform

The aim of this experiment was to assess the influence that temperature-dependent changes in blood flow had on waveform shape and thereby pulsatility index. This approach, being non-invasive, could be employed in conscious sheep.

A number of different methods were adopted to warm or cool the foot, including warm and cold water or ice packs and warm compresses. The former method could be more carefully controlled and was therefore chosen for use in the following experiment. Sheep were restrained in dorsal recumbency in a specially designed cradle. Ultrasound recordings (4 velocity-time waveforms) were made before and

(A)



(B)

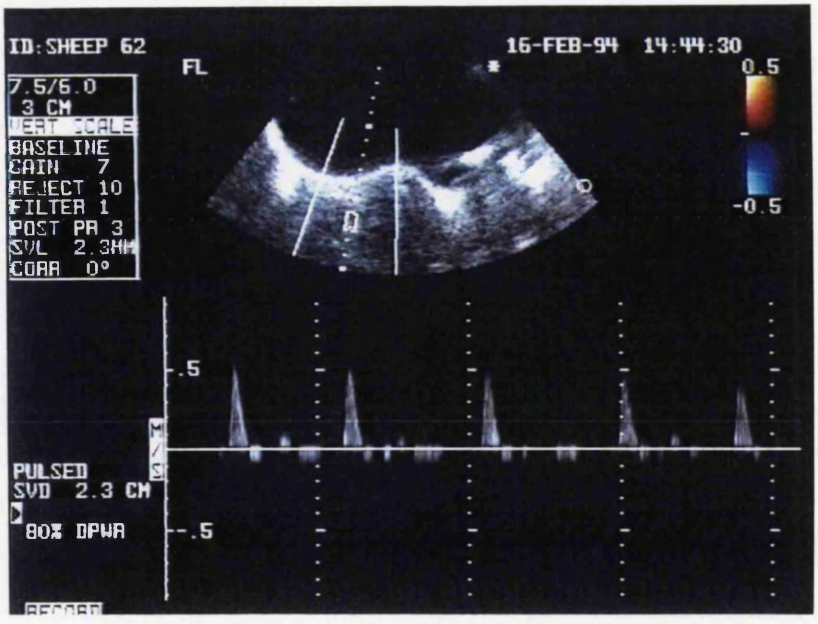
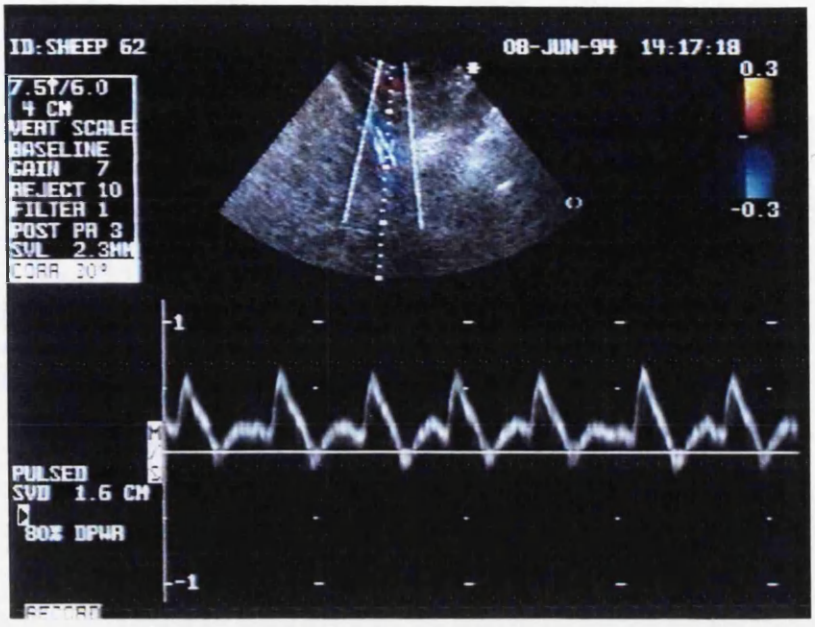


Figure 7.2. Doppler velocity-time waveforms recorded from the median artery of conscious sheep. Waveforms were obtained using (A) a 10MHz continuous wave transducer and (B) a 7.5MHz pulsed wave duplex transducer.

(A)



(B)

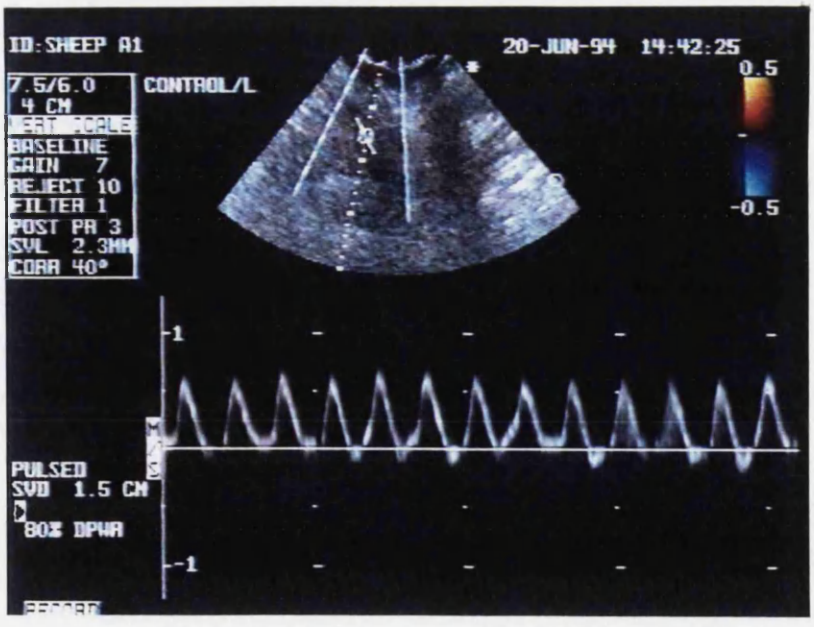


Figure 7.3. Doppler velocity-time waveforms (A & B) recorded from the femoral artery of conscious sheep. Sheep A1 from which waveform (B) was recorded, struggled during the measurement period and consequently had a higher heart rate.

after immersing the distal limb in warm (40°C) or cold (10°C) water for 10 minutes. A mean value for pulsatility index was calculated from each set of 4 waveforms. These measurements were repeated in 6 sheep and each "warm" or "cold" PI value was compared to the appropriate control by one-way analysis of variance.

The results are summarised in Table 7.1. Overall, immersion in cold water increased mean pulsatility index slightly from 3.91 ± 0.53 to 4.09 ± 0.61, whilst immersion in warm water increased pulsatility index from 3.33 ± 0.46 to 4.07 ± 0.67. However, these changes were not statistically significant.

A number of problems were encountered during this experiment, which made interpretation of the results difficult. The efficiency of the warming or cooling achieved was questionable. Several of the sheep resisted handling and kicked quite vigorously during the procedure, especially when the distal limb was dipped into the water. This may have contributed to the lack of effect by both reducing the period of immersion and delaying the recording of waveforms. Furthermore, 2 of the sheep became very agitated and had heart rates in excess of 160 beats/min. When heart rate is this high, the path length is reduced leading to loss of the tertiary and even secondary phases of the velocity waveform (Figure 7.3B). Under these circumstances, the information that can be derived from waveform shape is severely limited.

The main conclusion drawn from this experiment was the necessity of having a stable heart rate and an immobilised patient. As a result, subsequent studies were conducted in anaesthetised sheep.

	PULSATILITY INDEX			
SHEEP NO.	Control (Right limb)	Cold	Control (Left limb)	Warm
24	3.89	4.77	4.19	5.61
62	5.27	6.29	4.86	4.22
A1	2.68	3.27	2.52	2.81
A2	2.57	2.03	1.92	1.93
A3	3.39	3.38	2.66	3.60
12	5.65	4.78	3.84	6.25

Figure 7.1. Mean pulsatility indices recorded from the femoral artery of conscious sheep, before and after warming or cooling of the distal limb.