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**ARE ANGIOGENIC STIMULI ADDITIVE? A STUDY IN THE
RAT CORRELATING STRUCTURE AND FUNCTION.**

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ABBREVIATIONS

The following abbreviations are used throughout this thesis:

ABP – Arterial blood pressure

ANOVA – Analysis of variance

C:F ratio – Capillary to fibre ratio

CD – Capillary density

EC – Endothelial cells

ECM – Extracellular matrix

EDL – Extensor digitorum longus

eNOS – Endothelial nitric oxide synthase

FI – Fatigue index

FMF – Femoral blood flow

FVC – Femoral vascular conductance

HIF – Hypoxia-inducible factors

HR – Heart rate

i.v. – Intravenous

MABP – Mean arterial blood pressure

MFA – Mean fibre area

MFBF – Mean femoral blood flow

MMP – Matrix metalloproteinase

ns – Not significant

s.c. – Subcutaneous

S.E.M. – Standard error of mean

TA – Tibialis anterior

VEGF – Vascular endothelial growth factor

ABSTRACT

The aim of this study was to elucidate whether two different types of angiogenic stimuli, known to elicit two fundamentally different types of angiogenesis in rat hindlimb skeletal muscle, produce an additive effect when applied simultaneously. When administered in drinking water, the α -1 adrenoreceptor antagonist, prazosin, elevates shear stress causing splitting angiogenesis in extensor digitorum longus (EDL) muscle; surgical extirpation of the tibialis anterior (TA) muscle, a synergist, induces sprouting angiogenesis in EDL muscle due to overload. We used an *in vivo* rat preparation to investigate how functional readouts of EDL muscle performance and hindlimb blood flow, upon indirect electrical stimulation of the common peroneal nerve, were changed, if at all, once these different types of angiogenesis had been induced. Muscle samples were analysed for structural changes and correlated with function changes, for each treatment group.

Prazosin treated animals (2 week administration at 50 mg L⁻¹) had EDL muscle that had no difference in capillary to fibre ratio (C:F ratio) to that in control animals, contrary to that found in the literature. Neither was there a change in muscle fatigue resistance for an isometric twitch contraction. Extirpated animals (allowed to recover 2 weeks post-operatively) had ipsilateral EDL muscle with an increase in C:F ratio relative to control animals, with a parallel increase in muscle fatigue resistance. Interestingly, combination treated animals (concurrently treated) had ipsilateral EDL muscle with an increase in C:F ratio relative to control animals, although with a lesser increase in C:F ratio than with extirpation alone. The muscle fatigue resistance was not significant when compared to control animals, like it was for extirpated animals. Both extirpated and combination treated animals had contralateral EDL muscles with no difference in C:F ratio or muscle fatigue resistance to that seen in control muscle.

Prazosin administered animals (either prazosin treated or combination treated animals) maintained an elevated femoral artery vascular tone to that of non-prazosin administered animals (control and extirpated animals). Irrespective of treatment, no difference in vascular tone profile was discernible during muscle contraction.

Thus, the key findings of this study were, that a supramaximal increase in capillary supply by prazosin administration, when there was no metabolic demand for such a change, did not correlate with a functional improvement in muscle fatigue resistance, although our data was confounded by the fact that this treatment did not induce an increase in C:F ratio, like that observed in the literature. Only surgical extirpation led to an increase in muscle fatigue resistance, achieved through a parallel increase in C:F ratio. Interestingly, when both stimuli were applied together, no additive increase in C:F ratio or muscle fatigue resistance was seen; moreover the structural and functional changes were less than that in animals that had undergone extirpation alone. Thus, there seems to be a feedback loop in place that prevents excessive angiogenic stimulation, a putative 'angiogenic brake', presumably to prevent signal transduction becoming pathological.

When structural and functional data across treatment groups were aligned, it was C:F ratio and not vascular tone that correlated with fatigue index, for an isometric twitch contraction. Thus it seems that for muscle fatigue resistance, local diffusion is more important than global perfusion.

INTRODUCTION

Angiogenesis: An overview

Angiogenesis, not to be confused with *de novo* vasculogenesis, is the essential process by which new blood vessels grow, expand and integrate within an existing capillary network. This process is important physiologically during development and in tissue remodelling, where changes in the vascular bed supplying a tissue are needed in order to maintain tissue performance (Risau 1997). Angiogenic signalling however is perhaps most famous when aberrant, for example in tumour progression and metastasis, contributing to disease pathology in an array of cancers and related disease conditions (Folkman 1995). Such signalling is a constant coordination of multiple pro- and anti-angiogenic growth factors that cross-talk to form an integrated signalling meshwork, many of which have been implicated in tumour angiogenesis (Carmeliet and Jain 2000) and many of which have been targeted for anti-angiogenesis therapy (O'Reilly et al. 1994; O'Reilly et al. 1997). The 'balance' of such signalling can lead to either physiological or pathological blood vessel growth (Ferrara 2001). Physiological angiogenesis is different to pathological angiogenesis in that it is controlled, non-excessive, growth that is necessary for a tissue to continue performing optimally (Ferrara 2005).

Chemical mediators of angiogenesis

Whether resulting in pathological or physiological growth, growth factor signalling cascades are key to angiogenesis (Folkman and Klagsbrun 1987). The discovery of many of the pro- and anti-mitogenic regulators owes a lot to *in vitro* cell culture (Jain et al. 1997), from which important signalling molecules have been identified, along with their dependencies on other

signalling molecules. Cell culture assays use immortal cell lines, their growth being driven by excessive chemical signalling, like in cancers which are driven by mutations in important regulatory genes (i.e. oncogenes and / or tumour suppressor genes), a variety of which are reviewed here (Blume-Jensen and Hunter 2001; Vousden and Lu 2002). Such studies have helped elucidate many of the now well characterised angiogenic signalling processes, such as that of vascular endothelial growth factor (VEGF) and its family, which is considered the key player in all angiogenic remodelling (Hicklin and Ellis 2005). However, caution must be taken when applying what is known about a particular signalling axis, known to be important in pathological angiogenesis, to physiological angiogenesis. Whilst many regulators are common, there are likely many others uniquely altered in each specific pathology, meaning each pathology is differentially regulated depending on the combination of mutations present; therefore conclusions drawn are unlikely to be universally true for physiological remodelling (Seaman et al. 2007). The response to a physiological need to induce blood vessel growth, for example during exercise training, is a much more integrative response, not one born purely out of excessive, uncontrolled growth factor signalling to maintain a growing tumour mass (Egginton 2009).

Angiogenic signalling pathways: Networks, redundancy and feedback

The angiogenic response is a plastic process that reacts to demand in a tissue in a highly specific manner, whereby many signalling components are differentially regulated at the same time, to yield a net change that will be beneficial to the tissue; this process is kept in check by feedback control mechanisms. This network system must be rigidly controlled, both spatially and temporally, because the maintenance of any one signalling component at a non-optimal level may result in a pathological process, such as the ill-effects induced by the misregulation of placental soluble fms-like tyrosine kinase 1 (sFlt1) in preeclampsia

(Maynard et al. 2003). There is much redundancy in both pro- and anti- angiogenic signalling molecules (Klagsbrun and D'Amore 1991), known from gene knockout studies and clinical trials aimed at targeting specific molecules using pharmaceutically modified antibodies. 'Anti-VEGF therapy' involves the administration of a humanised monoclonal antibody called Bevacizumab (trade name: Avastin), which targets VEGF-A and successfully inhibits blood vessel growth, in a variety of cancers. The first treatment for which Bevacizumab was FDA approved for (although many clinical trials of Bevacizumab have since highlighted its efficacy, most recently for the treatment of age-related macular degeneration (Tufail et al. 2010)) was for metastatic colorectal cancer, with the result being an extension in patient lifetime, although the cancer wasn't actually cured (Ferrara et al. 2004). However, all too often when one member of a signalling network is inhibited in one way or another, another member of the pathway is up- or down-regulated to maintain angiogenic signalling, resulting in only temporary therapeutic benefit (Bergers and Hanahan 2008; Hicklin and Ellis 2005).

Growth factor signals illicit changes in gene expression

As eluded to, growth factors act as key signals in governing blood vessel growth. There are many growth factor families, including epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), to name just a few (Coussens and Werb 2002). These factors when secreted bind receptors, known as receptor tyrosine kinases, located on the endothelial cell (EC) membrane, which transduce the signal into the cell; this initiates a molecular cascade within the cell that results in changes in gene expression (Adams and Alitalo 2007). Each growth factor has its own cognate receptor, which induces specific gene expression changes. The net result of the various signalling components is angiogenesis; individual pathways have now been elucidated, aiding our understanding of blood vessel growth.

VEGF signalling at the EC interface, is the best characterised of the angiogenic signalling components (Ferrara 2001), such that the signalling of two different VEGF receptor subtypes, VEGFR-1 (aka Flt-1 [fms-like tyrosine kinase]) (de Vries et al. 1992) and VEGFR-2 (aka Flk-1/KDR [kinase domain region]) (Terman et al. 1992), have been characterised. Both types of VEGF signalling are essential during embryonic development, in evidence by gene targeting studies that induced embryonic lethality in mice (Fong et al. 1995; Shalaby et al. 1995). Once these mice were born, postnatal development was similarly inhibited by Cre-*loxP*-mediated gene targeting, with the result being increased mortality, stunted body growth and impaired organ development (Gerber et al. 1999; Kuhn et al. 1995), although beyond 4 weeks of life, VEGF block had less detrimental effects to the animal. The presence of two subtypes of VEGF receptor is rather elegant. The two have different signal transduction profiles; VEGF-dependent tyrosine phosphorylation of Flk-1/KDR induces mitogenesis, while VEGF binding to Flt-1 does not produce a detectable response (Waltenberger et al. 1994). It was hypothesised that Flt-1 acts as a ‘decoy’ non-signalling receptor, regulating VEGF signalling via Flk-1/KDR by sequestering the ligand from its signalling receptor; this inhibition achieved by the ‘decoy’ receptor is only overcome when saturating concentrations of ligand are present (Park et al. 1994).

The knockout of VEGF was effective as signalling via the VEGF receptors on the EC membrane was inhibited; when knockout doesn't occur the net result is endothelial growth, resulting in new blood vessels. However, vital to this process is the presence of signals from other characterised ‘support’ cells surrounding the endothelium, such as pericytes (Raza, Franklin, Dudek 2010). This is achieved by reciprocal paracrine signalling between ECs and pericytes (summarised in Fig. 1). ECs release PDGF, creating a concentration gradient that causes the recruitment of pericytes local to the ECs (Gerhardt et al. 2003). The binding of

PDGF to its receptor (PDGFR) on the pericyte cell membrane causes signal transduction, resulting in stimulation of the MAPK/ Erk1/2 signalling axis, the net result being pericyte proliferation, ensuring endothelial growth is sufficiently supported and guided (Carmeliet 2003). Pericytes also secrete VEGF, which signal via the VEGFR-2 receptor on the EC membrane, initiating the PI3K/ Akt signalling axis (Gerber et al. 1998); the net result of this is EC survival, promoted by anti-apoptotic proteins Bcl-2 and A1 with pro-survival activities (Gerber, Dixit, Ferrara 1998). Other growth factors (for example, FGF and TGF) signal at the EC interface via their own specific receptors, triggering tyrosine kinase induced phosphorylation of downstream transcription factors, with the net result being the promotion of EC proliferation and their migration (Adams and Alitalo 2007). Another class of tyrosine kinases characterised in angiogenic processes are Tie1 and Tie2, which are specifically expressed on ECs (Sato et al. 1995). The Tie2 receptor was proven to be important in angiogenesis, as when it was lacking, angiogenic defects in endothelial-matrix contact were presented; similar defects were evident when its ligand Angiopoietin-1 (Ang-1) was lacking (Suri et al. 1996). Angiopoietin-2 (Ang-2), an antagonist for Ang-1 at Tie2, disrupts signalling through this receptor, hindering effective blood vessel growth (Maisonpierre et al. 1997), again highlighting the exquisite regulation present in angiogenic signalling. Cellular adhesion molecules, at the cell membrane, are also vital to achieving angiogenesis. The PECAM-1/CD31 molecule, is known to be important in mediating EC-EC contact when forming tubes that later become patent blood vessels. When these contacts were blocked by specific antibody, new blood vessels could not be induced (DeLisser et al. 1997). Other non-growth factor like regulators are also vital to angiogenic processes, for example extracellular matrix (ECM) proteases are secreted prior to EC migration, to degrade the ECM and free space to allow for the new capillary growth (van Hinsbergh and Koolwijk 2008). The panel of angiogenesis-inducing molecules are required in both a spatially and temporally specific

order to produce a viable stimulus for blood vessel growth. Without concurrent signalling, by both ECs, pericytes and other surrounding tissues, gene expression of survival, proliferation and migratory components will not maintain capillary growth through to competency (Gerhardt and Betsholtz 2003). Therefore, it is important that a coordinated suite of responses are present to regulate and support angiogenesis. Thus for angiogenesis, whether it is induced by tumour growth, tissue damage (i.e. wound healing), inflammation or by exercise, there is a lot of cross over in components activated. In tumour biology it is very daunting to attempt to develop a master-inhibitor of angiogenesis due to the sheer number of targets prone for potential manipulation. Similar scope of angiogenesis knowledge is needed in the physiological angiogenesis research field.

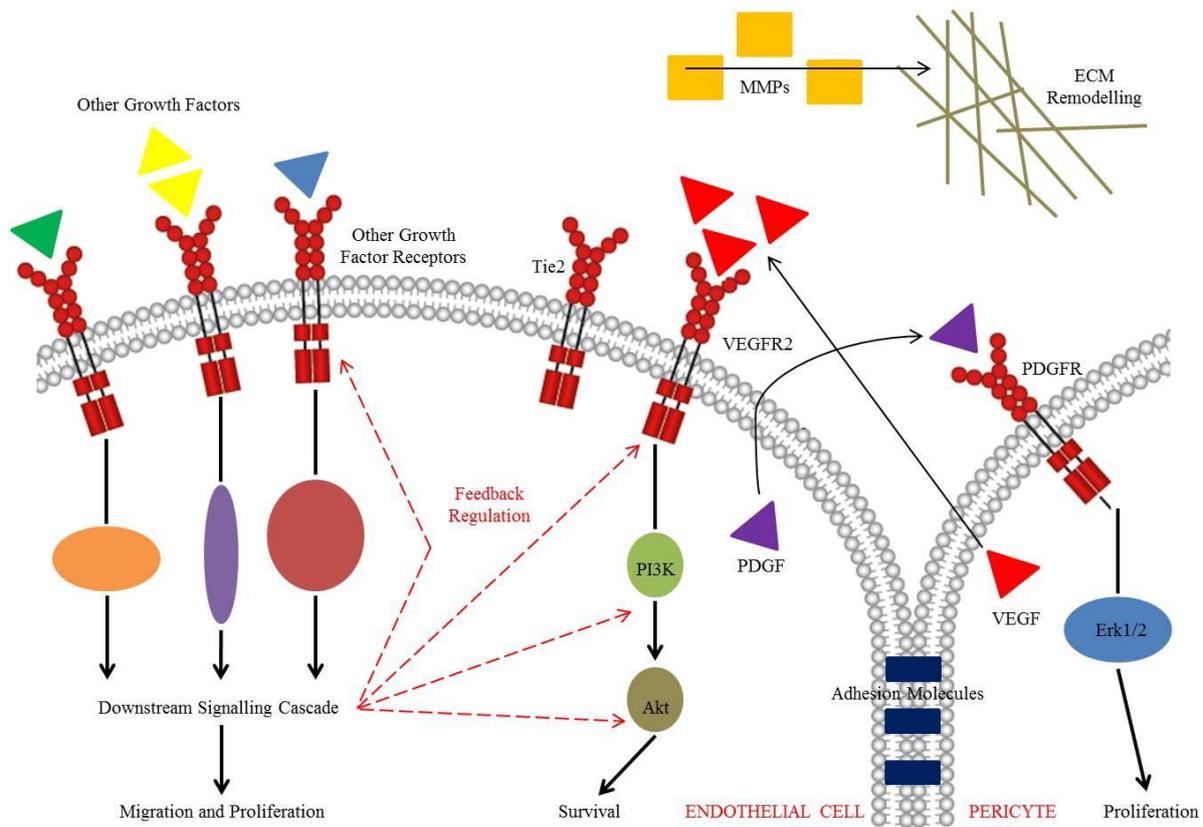


Fig 1: Schematic giving a brief overview of angiogenic signalling processes initiated at the cell membrane of an EC. Growth factor ligands bind their specific cognate receptors that span the membrane of ECs, which initiates a molecular cascade within the cell. This cascade causes modulation of multiple downstream components, often recruiting many hundred individual components, the net result being a change in gene expression, helping to support angiogenesis. For example, VEGF secreted by recruited pericytes initiates the PI3K-Akt signalling axis via VEGFR2 at the EC membrane, promoting survival. Concurrently, PDGF secreted by the EC triggers Erk1/2 signalling via PDGFR at the pericyte cell membrane, resulting in its proliferation. Other growth factor signalling families will similarly have their signal transduced across the cell membrane upon binding to their receptors on the EC, stimulating its migration and proliferation. Once MMPs have been recruited (signalled as part of the mass of gene expression changes) causing ECM remodelling, angiogenesis can then occur in the now free space. This whole physiological process is stimulated by an initial change in the local environment, for example hypoxia as a result of exercise (or indeed pathologically at the centre of a malignant tumour).

Physiological angiogenesis: Blood vessel growth in skeletal muscle

In physiological angiogenesis, particularly in the field of exercise physiology, the aim is not to inhibit but to induce blood vessel remodelling. Naturally, there are many ways to optimise exercise performance, encompassing almost every organ system. Central adaptations, stimulated by exercise, include cardiac enlargement and arteriogenesis of conduit vessels, while peripheral adaptations, include switching of skeletal muscle phenotype (improving

strength, for example in weight lifters), angiogenic remodelling of the capillary bed supplying the muscle (improving fatigue resistance, for example in cyclists and swimmers) and a combination of these adaptations, if the specific nature of the exercise training stimulates both such changes (Holloszy and Booth 1976). If the stimuli are extreme enough these can occur quite independently of one another, but most exercise co-induces multiple adaptations at the same time, albeit over different time scales. Exercise training induced changes often include co-adaptations that bring about an alignment between the vasculature and cellular respiration systems, thus enabling the support of the observed improved muscle performance. For example, in endurance exercise training (aerobic distance running but not strength training), exercise induces an increased muscle endurance response, with proportional increase in mitochondrial electron transport chain capacity (Robinson et al. 1994). Such changes within the muscle fibres occur after the increases in capillarity (Skorjanc et al. 1998), meaning muscle activity efficiency, once both adaptations have occurred, is maximised. Changes in the vasculature system supplying an exercising muscle, whether it be via bulk blood flow (gross perfusion) or the specific capillary network (local diffusion path length to the respiring tissue), optimise the delivery of nutrients to and removal of waste material from the muscle, allowing for continuing activity and the maintenance of optimal performance (Hudlicka, Brown, Egginton 1992). From here on in the only physiological adaptation of skeletal muscle of interest, is the plastic process of angiogenesis.

Mechanotransduction of the angiogenic signal

The chemical signalling milieu that induces blood vessel growth can also be manipulated upstream by important, though far less characterised mechanical influences (Hudlicka, Brown, Egginton 1992). Haemodynamic forces and physical deformation at the muscle-capillary interface are rather potent stimuli for vascular remodelling, such signalling feeding

into downstream chemical counterparts, the roles of which are better characterised. There are three examples of such factors; elevated shear stress caused by functional hyperaemia acting directly on the luminal surface of ECs, wall tension caused by blood pressure in exercising tissue, and stretch/ deformation of tissue acting on the abluminal surface of the vasculature (Egginton et al. 2001). These stimuli alter the local physical environment, i.e. the ECs, vascular smooth muscle, the ECM and other surrounding non-endothelial support cells like pericytes, which cause mechanotransduction of the signal via the host of kinases, causing an integrative angiogenic response (Egginton 2011). The investigation of mechanical, rather than chemical, influences on angiogenesis by examining the co-ordinated suite of responses observed when applying such stimuli, have led to the realisation of just how important a role such factors are in driving the process of *in vivo* capillary growth.

Exercise models of angiogenesis

Exercise models of angiogenesis are the best way to study the gross integrated response to angiogenic stimulation and are most relevant to exercise physiology. Such studies yield information about the adaptations made by certain types (e.g. treadmill running) and intensities (low to high) of exercise training, in a particular species (rats, dogs and humans are regularly used) over time, although the variation in the literature often confounds the field when attempting to make comparisons (Egginton 2009). These exercise-training studies typically assess exercise performance as well as assaying a selection of genomic transcripts and proteins (of various pro- and anti- angiogenic factors) to attempt to elucidate what underlies the exercise-induced changes, although care must be taken, as genomic and proteomic responses often do not change in parallel (Milkiewicz et al. 2006).

It has been shown that stimulation of angiogenesis may result in the release of growth factors such as VEGF, as levels of these factors increased in rat skeletal muscle during a single bout of dynamic exercise; such changes have also been shown by indirect electrical stimulation (Hang et al. 1995). This increase in VEGF after exercise decreases within a few hours to resting levels and is not sustained with further exercise bouts (Gavin and Wagner 2001). It was shown that VEGF is important both for basal capillarity and in the angiogenic response in skeletal muscle upon exercise, by elegant VEGF *Cre-loxP* skeletal muscle conditional knockout experiments (Wagner et al. 2006). The knockout decreased VEGF levels and capillarity within skeletal muscle, highlighting how essential VEGF is to angiogenic processes. In these knockout animals, the baseline capillary to fibre ratio (C:F ratio) was decreased, impairing the performance in these animals relative to controls; furthermore no increase in C:F ratio or endurance capacity could be induced upon treadmill training, unlike matched-controls who had increased C:F ratios and could run faster for longer (Olfert et al. 2009). This highlights how important VEGF and the C:F ratio is for muscle performance, both in capillary maintenance and during angiogenesis.

Similar studies have indicated the importance of endogenous anti-angiogenic factors, as well as the pro-angiogenic ones. It was shown that during exercise, the anti-angiogenic factor, thrombospondin-1 was also increased during exercise in rats skeletal muscle (Olfert et al. 2006), just like VEGF was (Breen et al. 1996). Upon repeated exercise bouts to train the animals, the ratio between pro- and anti-angiogenic mRNA transcripts (the balance) shifted so pro-angiogenic factors were upregulated, relative to anti-angiogenic factors which were rather downregulated, which was correlated with adaptive remodelling of capillaries (Gavin and Wagner 2001; Olfert et al. 2006). Interestingly, in the short-term, the response to exercise showed an increase in thrombospondin-1 levels; as further training occurred, the

thrombospondin-1 levels decreased, until long-term experiments where they were increased once more (Olfert et al. 2006). It seems that thrombospondin-1 is a switch that is induced with mitogens such as VEGF when exercising; they are co-expressed at a similar level initially. When angiogenesis is required, its levels are downregulated, until after training when sufficient blood vessel growth has occurred and it is upregulated once more to inhibit further angiogenic signalling. This regulation between pro- and anti-angiogenic factors, the VEGF-thrombospondin-1 axis (and others no doubt), is rather elegant and explains the exquisite control demonstrated in effective physiological angiogenesis.

Other research groups have examined vascular remodelling in human skeletal muscle, attempting to elucidate the mechanisms behind exercise-induced changes. Muscle biopsy post-exercise in one study, revealed that changes in VEGF mRNA were proportional to changes in hypoxia-inducible factor (HIF-1 α and HIF-1 β) mRNA; these changes in expression occurring in a graded manner based on the level of metabolic stress. Thus, HIF seems to be able to regulate angiogenic factors during exercise too (Gustafsson et al. 1999).

Exercise models of angiogenesis are useful when investigating the integrated angiogenic response to exercise-derived stimuli. However, as the 'exercise cycle' induces a whole host of changes, in order to attempt to clearly elucidate the mechanism of an individual mechanical activity on the integrated web of chemical components, various angiogenesis models have been developed, aiming to replicate a single individual component at a time (Egginton et al. 2001). Three of these are discussed.

Chronic electrical stimulation

Chronic electrical stimulation of animal muscles via implanted electrodes, has been used to induce exercise-like adaptations. Stimulation in the rat hindlimb, induced an increase in C:F ratio in extensor digitorum longus (EDL) muscle, within a few days, relative to control animals. “The complex pattern of vessels underwent remodelling between 2 and 7 days of stimulation, resulting in more tortuous capillaries with numerous sprouts and loops”; such changes correlated with basement membrane disruption around the endothelium. It was postulated that a combination of types of angiogenesis were occurring as a result of the stimulation regime (Hansen-Smith, Hudlicka, Egginton 1996). Further study of chronically stimulated muscle revealed that the length of stimulation duration was related to the degree of disruption of the endothelial glycocalyx layer of capillaries, a mechanism by which angiogenic stimuli are mechanotransduced within the endothelium (Brown et al. 1996). The importance of basement membrane disruption in capillary growth was confirmed by inhibition of matrix metalloproteinases (MMPs); this resulted in no new capillary growth as basement membrane proteolysis was no longer performed (Haas et al. 2000). By stimulating muscle in this manner, an integrative response was induced, just like in normal exercise; in order to elucidate the role of these individual components, simpler models were devised.

Shear stress and splitting angiogenesis

An elevated shear stress component was known to act on the endothelium, causing an observable increase in muscle C:F ratio (Zhou et al. 1998a). The muscle hyperaemia was thought to disrupt the glycocalyx meshing the endothelium and surrounding ECM together, initiating mechanotransduction (Brown et al. 1996). The luminal surface was disrupted and cytoplasmic processes observed. This was a distinct type of capillary growth known as

splitting angiogenesis, which proceeds by longitudinal splitting of the parent vessel, by filopodial extension along the centre, forming two daughter vessels (Zhou et al. 1998a). A number of key signalling components, many of which are known to be key mediators of changes in endothelial cell morphology (e.g. neuropilin, midkine and restin), have been shown to be activated in splitting angiogenesis (Williams et al. 2006c). Of the other upregulated components, some are unique to this type of angiogenesis (e.g. endothelial nitric oxide synthase (eNOS)) (Williams et al. 2006b) and others are common to all types of angiogenesis (e.g. VEGF, Flk-1, angiopoietin-2 and PECAM-1) (Williams et al. 2006a; Williams et al. 2006c). Splitting angiogenesis can be induced experimentally by the administration of the drug prazosin, an α -1 adrenoreceptor antagonist dissolved in drinking water. This produces predominantly skeletal muscle hyperaemia, with minimal systemic effects. Significant angiogenesis can be seen in rat skeletal muscle of the hindlimb within 2 weeks via this means (Zhou et al. 1998a). Induction of shear stress in *in vitro* endothelial culture has been shown to elicit graded changes in expression of genes related to endothelial migration, proportional to the grade of shear stress applied (Sheikh et al. 2003). Traditionally, one dose of prazosin (50 mg L^{-1}) (Egginton et al. 2001) has been administered *in vivo*, but theoretically, different degrees of shear stress could be investigated by changing the dose administered.

Overload and sprouting angiogenesis

The muscle stretch or overload component was known to cause changes in ECM structure, allowing for vasculature remodelling, resulting in an increase in muscle C:F ratio (Zhou et al. 1998b). This stretch of muscle causes endothelial cells to be strained; these cells mechanotransduce the signal inducing a plethora of changes in growth factor signalling, the result being the proliferation of the endothelium (Chang et al. 2003; Yamaguchi et al. 2002;

Zheng et al. 2001). The abluminal surface was disrupted and cytoplasmic processes observed. This was a distinct type of capillary growth known as sprouting angiogenesis, which originate as lamellipodial sprouts off the parent vessel (Zhou et al. 1998b). A number of key signalling components, many of which are known to be key mediators of endothelial cell migration and capillary tube formation (e.g. matrix metalloproteinase 2 (MMP-2), TIMP, SPARC and thrombospondin), have been shown to be activated in sprouting angiogenesis (Williams et al. 2006c). Of the other upregulated components, some are unique to this type of angiogenesis (e.g. MMP-2) (Rivilis et al. 2002) and others are common to all types of angiogenesis (e.g. VEGF, Flk-1, angiopoietin-2 and PECAM-1) (Williams et al. 2006a; Williams et al. 2006c). Sprouting angiogenesis can be induced experimentally in muscle by surgical extirpation of a synergist muscle; for example removal of the tibialis anterior (TA), causes overload (compensatory hypertrophy) in the EDL during a 2 week recovery period. Significant angiogenesis can be induced in rat skeletal muscle of the hindlimb within 2 weeks via this means (Zhou et al. 1998b). Different grades of muscle overload have been investigated *in vivo* based on the degree of surgical ablation performed; data suggests that the response is a graded, not threshold process (Egginton et al. 2011).

The potential of angiotherapy

Angiotherapy is the broad description given to the approach whereby blood vessel growth is the target of intervention, in a variety of normal and abnormal disease processes. By identifying, characterising and understanding the underlying physiological components of blood vessel growth and how pathology is generated by these components, researchers aim to modify the aspects of angiogenesis thought to be amenable to specific modulation, to a therapeutic end (Ferrara and Kerbel 2005). Effective angiotherapy may not be achieved via a

single intervention; thus angiotherapies may need to be concurrently applied at blood vessel differentiation, maintenance and/ or homeostasis processes (Carmeliet and Jain 2000). Angiotherapy can either be angio-inhibitory (i.e. in the control of tumour growth) or angio-stimulatory (i.e. in stimulating muscle blood vessel growth to aid exercise performance). New developments in the angiogenesis field will be of interest to those looking to develop angiotherapies, whether they be cancer researchers or exercise physiologists. Exercise physiologists may well be interested in the angiogenic changes induced by prazosin administration and by surgical extirpation, and the impact these types of angiogenesis have on muscle performance. Should any improvements in muscle performance be identified, then the work could be brought into the clinic to provide benefit to patients.

Combinational therapy?

As different mechanical stimuli are known to elicit fundamentally different types of physiological angiogenesis (Egginton 2011), it is of current interest whether two of these stimuli could be simultaneously applied to give additive capillary growth (Fig. 2). Could both types of angiogenesis induced be applied concurrently to give an optimal capillary structure within a muscle? Could such a structure, functionally be able to perform better for longer?

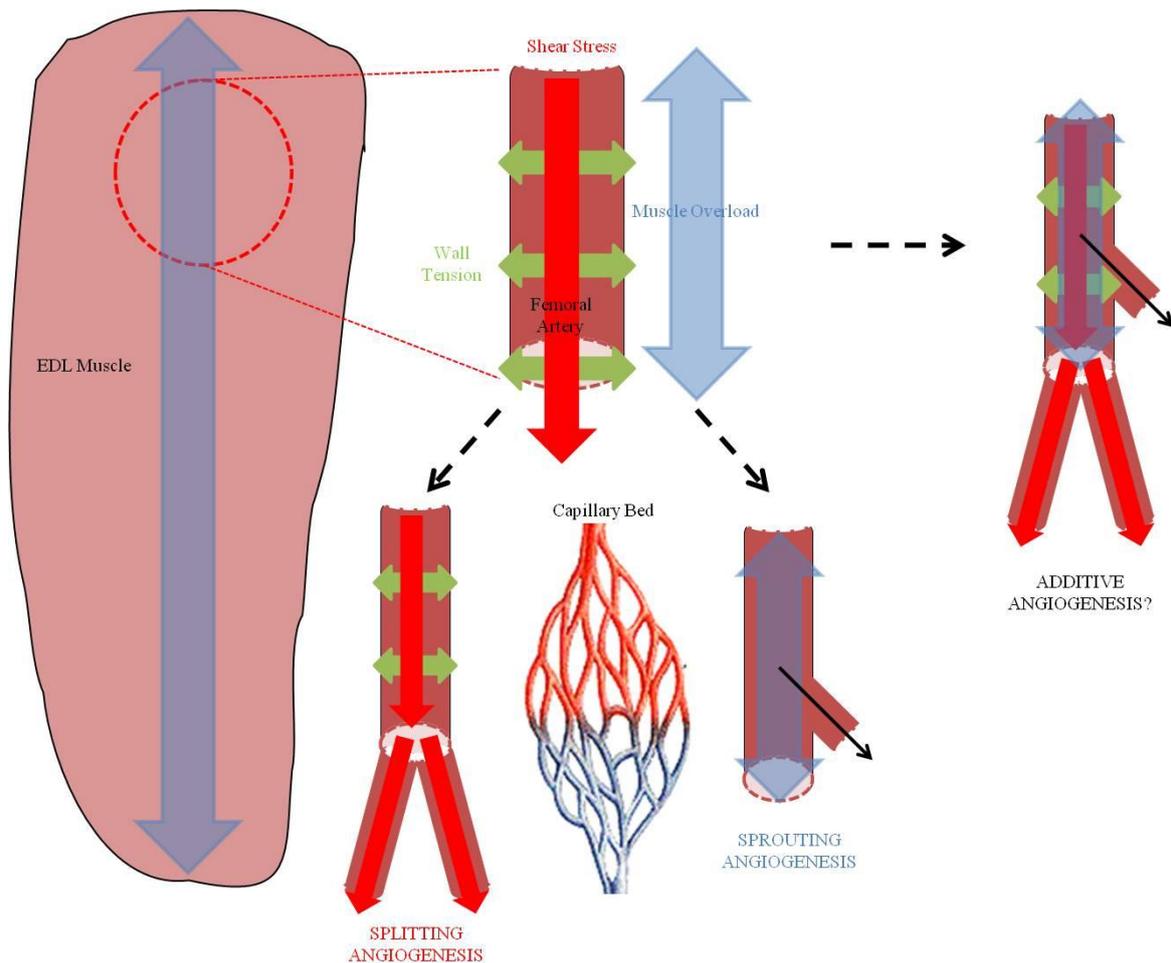


Fig 2: Schematic highlighting different mechanical stimuli that could be used to induce angiogenesis in EDL muscle. Prazosin administration, known to cause enhanced shear stress (red arrow) and wall tension (green arrow) and surgical extirpation causing muscle overload (blue arrow), induce splitting and sprouting angiogenesis, respectively. It is of interest whether additive changes in the capillary bed supplying a skeletal muscle could be induced by applying both stimuli.

Evidence has suggested that chronic electrical stimulation of muscle has resulted in both splitting and sprouting forms of angiogenic growth (Hansen-Smith, Hudlicka, Egginton 1996), raising the possibility that these distinct mechanisms may be additive. *In vivo* physiological angiogenesis has been shown to occur in a graded manner based on stimulus intensity, meaning a stronger stimuli yields a stronger response (Egginton et al. 2011). Thus, a preliminary study aimed to apply both shear stress and overload stimuli in combination, to examine whether an additive effect could be induced (Olgaard et al. 2008).

The rat EDL, a skeletal muscle of the hindlimb, is a mixed muscle of approximately equal glycolytic and oxidative fibre content, with a proportional blood flow to the rest of the hindlimb muscles (Armstrong and Laughlin 1983). Thus, it is a suitably representative candidate muscle in which to study angiogenesis and has been traditionally used for many years (Hudlicka, Brown, Egginton 1992). Indeed, it was the EDL that was studied by Olgaard et al. (2008).

Preliminary data from stained muscle sections and Western blots of key biochemical proteins assayed from EDL, indicated that an additive angiogenesis (in either capillary number or in VEGF or eNOS content) cannot be achieved when prazosin drinking water is administered for 2 weeks, alongside unilateral extirpation of the synergist TA muscle, also for 2 weeks. It is hypothesised, that during 2 weeks of simultaneous co-stimulation, that muscle overload is a maximal stimulus for angiogenesis and so the additional shear stress stimuli is essentially supramaximal, explaining why it elicits no further capillary growth (Olgaard et al. 2008).

Aims

The aim of this study was to investigate the response to these fundamentally distinct types of angiogenic stimuli (which are both induced simultaneously by exercise), applied additively in rat skeletal muscle. Our rationale, was to examine the correlation (if any) between structural and functional data, so to have statistically robust, powerful data that would hopefully reveal to what extent structural adaptation drives functional capability. For example, when subjected to the two distinct types of angiogenic remodelling stimuli to induce changes in muscle architecture (measures of C:F ratio, capillary density (CD) and mean fibre area (MFA), in a given muscle section), would different functional performance characteristics (muscle tension

and fatigue during contraction and hindlimb blood flow) be observed? Would these putative changes occur in a linear or non-linear fashion? When the different angiogenic signalling pathways applied in combination give an additive effect? Such data would be of particular interest in the physiological angiogenesis field, where it is unclear whether different angiogenic stimuli induce unique performance-based adaptations. Our approach was to use an *in vivo* rat preparation to derive the functional data. Then, once the animal was sacrificed, the muscle was sampled and structural data assessed. Structure and function relationships could then be assessed and the hypothesis proposed in Olgaard et al. (2008), investigated. Establishing whether angiogenesis is a true graded phenomenon, is an important step in designing better targeted treatments for patients.

METHOD DEVELOPMENT

To determine the functional capabilities of a skeletal muscle in the rat hind limb, an *in vivo* setup was designed in order to elucidate the whole-animal integrative response to physiological angiogenesis. The aim was to measure bilateral hindlimb blood flow (as a surrogate for EDL muscle blood flow) using perivascular flow probes positioned around the femoral arteries, upon stimulation of the common peroneal nerve to elicit EDL muscle contraction. A force transducer was used to measure developed tension and fatigue. The aim was to discover whether these variables differed in EDL muscle that had undergone different types of angiogenesis.

The original aim was to stimulate bilaterally and record tension in both hindlimbs using two force transducers. The rewiring of two Grass force transducers was attempted, however a stable signal was never obtained, preventing construction of an adequate calibration curve. Thus unilateral measurement was settled upon, making use of one alternative working force transducer. The calibration curve for this force transducer is given (Fig. 3), showing operation at the linear (i.e. most accurate) part of its dynamic range.

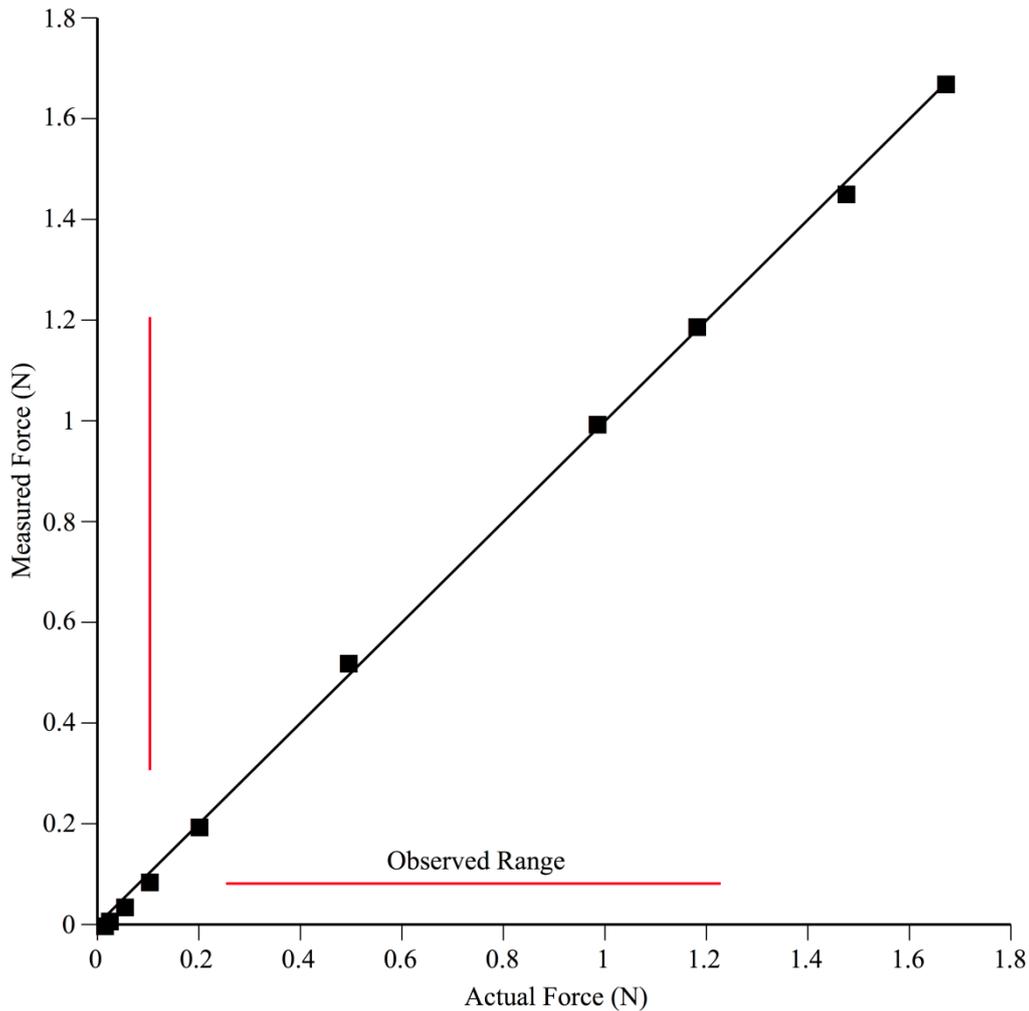


Fig 3: Calibration curve for ADI force transducer showing the accuracy of the force recorded, when a series of known weights (National Physics Laboratory Standards) were applied. The observed range during all experiments was between ~ 0.30 - 1.20 N, corresponding with the linear section of the calibration curve ($r^2 = 0.99$).

In order to optimise experimental protocols, the literature was searched to see what other groups had done, then a technique was refined to maximise sensitivity and minimise variability. The general animal setup decided upon was similar to that used by (Egginton et al. 1998; Ray and Marshall 2009a; Ray and Marshall 2009b). After practising the surgery on a number of cadavers, preparation in living animals began, to further improve surgical competency and technique, and to get used to maintaining a constant depth of Alfaxan-anaesthesia throughout the duration of the experiment. During these preliminary experiments

a final protocol was refined until robust; this was then used in all subsequent animals, across each of the treatment groups.

The setup in brief (detailed in ‘Materials and Methods’), consisted of a supine rat anaesthetised with Alfaxan via a jugular vein cannula. A tracheotomy was performed to help maintain a patent airway. The brachial artery was cannulated in order to record mean arterial blood pressure (MABP; mmHg) from which heart rate (HR; BPM) was derived. Both femoral arteries were isolated and flow probes positioned to extrapolate bilateral femoral blood flow (FBF; ml min⁻¹). From this, femoral vascular conductance (FVC; ml min⁻¹ mmHg⁻¹) was derived by dividing FBF by MABP. The ipsilateral common peroneal nerve was isolated and sectioned at the proximal end; an electrode was positioned beneath the nerve in order to provide electrical stimulation. The ipsilateral EDL muscle was isolated and sectioned at the distal end and attached to a force transducer (although for ease, both the TA and EDL muscles were assayed in early preliminary experiments) to measure tension generated upon electrical stimulation, from which fatiguability could be calculated. Data was recording in real-time on LabChart; an example of a trace is given (Fig. 4).



Fig 4: Raw trace from LabChart, for a preliminary experiment.

The trace shows a 10 Hz stimulation of the combined TA and EDL muscles, in Alfaxan-anaesthetised male Wistar rats. Contractions achieved by indirect supramaximal stimulation (0.3 ms square-wave pulse width, ≈ 10 V, 5 min [indicated by the black bar]) of the intact common peroneal nerve. The seven channels show the continuous, real-time measurement of the labelled variables. Notice the presence of a central response in HR (channel 3), the smooth decay in tension generated (channel 5) and the response in FBF and FVC to stimulation. Channels 4, 6 & 7 show hyperaemia due to femoral artery vasodilatation. The tension generated during the 5 minute stimulation period, was used to calculate a score of muscle fatiguability that could later be compared across animal treatment groups.

A number of stimulation frequencies were assayed, to replicate different intensities of muscle activity (i.e. 4, 10 & 40 Hz, akin to different intensities of exercise). A stimulation time of 5 minutes was used as previously (Egginton et al. 1998; Ray and Marshall 2009a; Ray and Marshall 2009b). Time trials indicated that there was no significant change in blood flow or tension generated beyond 5 minutes of stimulation (data not shown), and nothing was observed during preliminary experiments to indicate otherwise. Based on previous experience in the laboratory, MABP and HR data were consistent to other Alfaxan-anaesthetised

preparations (Ray and Marshall 2009a; Ray and Marshall 2009b). Importantly, electrical stimulation of the common peroneal nerve elicited no significant central response in MABP by the 'end' of the stimulation period (not significant (ns) vs. 'rest', Student's t-test, $n = 7$; Fig. 5a) regardless of stimulation frequency, but there was evidence of full body twitch, suggesting there was some systemic central response produced. This was in evidence by the significant central response in HR by the 'end' of the stimulation period ($P < 0.05$ vs. 'rest', Student's t-test, $n = 7$; Fig. 5b and Fig. 4), for 4 and 10 Hz stimulation frequencies. Such twitching may also have contributed and thus caused inaccurate tension recordings. Therefore it was decided that the main experiments would be conducted with a cut common peroneal nerve to avoid such afferent feedback. Central responses in MABP or HR were eliminated in all subsequent experiments (data not shown). Furthermore, such treatment of a nerve has been shown to give a stable preparation, for up to 5 bouts of stimulation, for the sciatic nerve (Ray and Marshall 2009a; Ray and Marshall 2009b); the cut common peroneal nerve was found to be just as stable.

To emulate different types of exercise-induced muscle activity, we compared high and low stimulation frequencies, simulating different intensities of exercise. Preliminary data showed that there was a significant increase in blood flow by the 'end' of the stimulation period, regardless of stimulation frequency ($P < 0.05$ vs. 'rest', Student's t-test, $n = 7$; Fig. 5c) which was matched by a significant increase in vascular conductance by the 'end' of the stimulation period, for every stimulation frequency except 4 Hz ($P < 0.05$ vs. 'rest', Student's t-test, $n = 7$; Fig. 5d). The greatest dynamic range in blood flow and vascular conductance was seen at 10 Hz, and was therefore used as the stimulation frequency for all subsequent experiments.

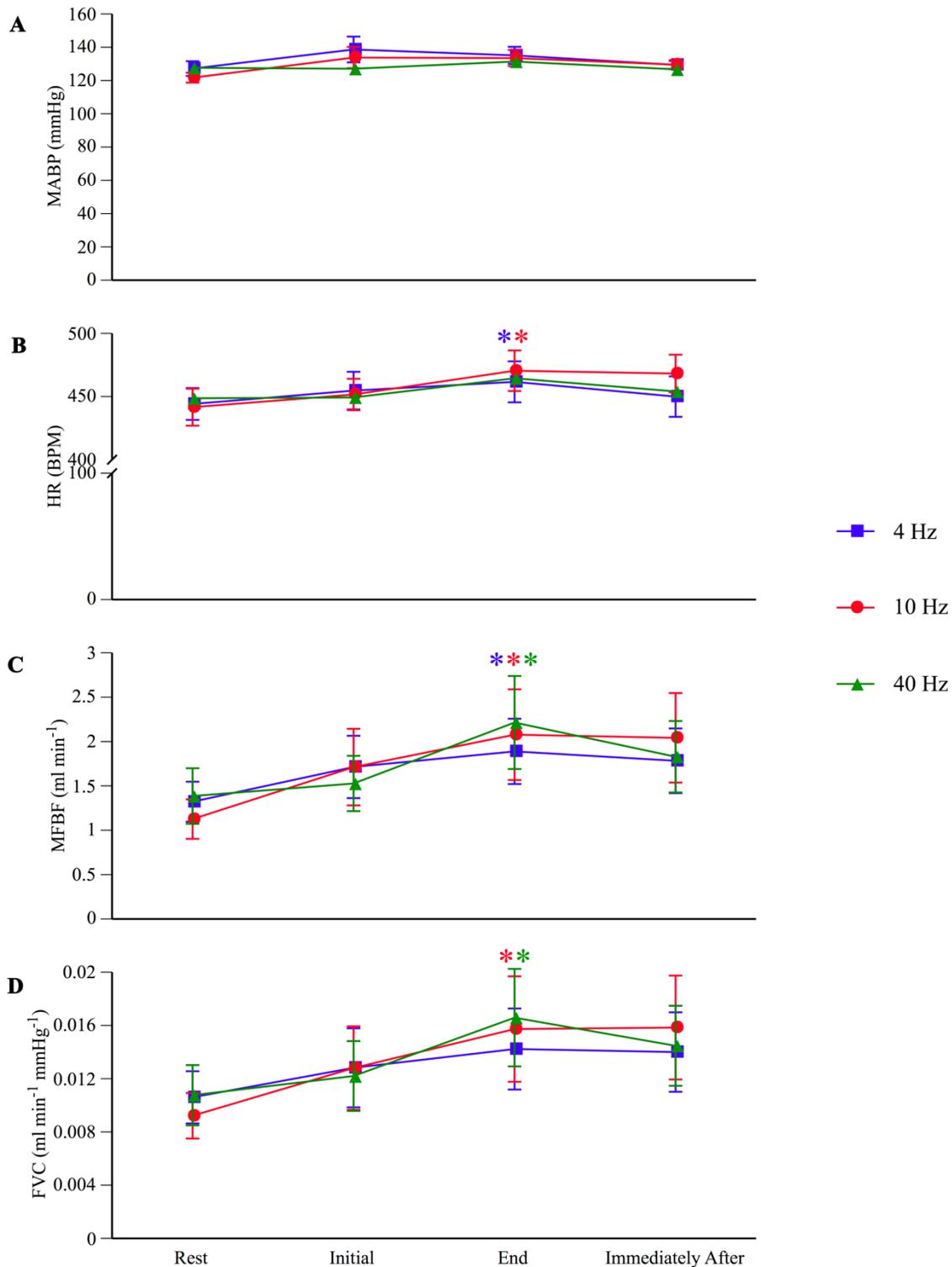


Fig 5: Integrative response to isometric muscle contraction.

A-D show mean (\pm S.E.M. error bars, $n = 7$) MABP, HR, MFBF and FVC for 4, 10 and 40 Hz stimulations of combined TA and EDL muscle, at 4 different time points, in Alfaxan-anaesthetised male Wistar rats. Contractions achieved by indirect supramaximal stimulation (0.3 ms square-wave pulse width, ≈ 10 V, 5 min) of the intact common peroneal nerve. * $P < 0.05$ vs. 'rest' (Student's t-test). There was no significant difference in MABP, HR, MFBF or FVC at any time point between different stimulation bouts.

To establish muscle tension fatigue upon stimulation, we initially tested combined TA and EDL fatiguability, which was expressed as fatigue index (FI), derived by dividing the 'end' tension by the 'initial' tension. This established the expected greater FI at high versus low stimulation frequencies, in control animals (data not shown). The intention was to assess the capabilities of animals that had undergone different angiogenic treatments, some of which would have had surgery to remove the TA. Thus, to ensure consistency between groups, we examined the influence of TA presence (because in treatment groups where animals have undergone TA removal, there was no TA present to confound what was been measured for the EDL alone). On retraction of the TA, there was no smooth decay in tension, and depending upon the force and angle of retraction, the apparent influence on EDL FI was variable (large standard error of mean (S.E.M.); data not shown). Thus, removal of the TA required some refinement, as initially nerve damage led to muscle weakness; this however was overcome upon practice. Although removal of the TA increased surgical intervention, doing so permitted comparison of blood flow and tension data, and meant that regardless of the treatment group, the setup and protocol was kept as constant as possible (assaying the EDL only, in every case), without confounding the data collected. For the EDL alone, the FI was much more consistent for a given stimulation frequency (indicated by small S.E.M.), regardless of the treatment applied. For all stimulation frequencies, there was a significant decay in tension by the 'end' of stimulation ($P < 0.01$ vs. 'initial', Student's t-test, $n = 7$; Fig. 6a). The FI was significantly different for each stimulation frequency ($P < 0.05$ vs. other stimulation frequencies, between treatments ANOVA with Scheffé Post hoc test, $n = 7$; Fig. 6b). The tension and FI for 4 and 10 Hz stimulations were both being markedly different from that of a 40 Hz stimulation (Fig. 6a and Fig. 6b). Consistent with observations of blood flow and vascular conductance, a 10 Hz stimulation frequency was chosen as the optimal

stimulation frequency to use, in assaying the differences between angiogenic treatment groups, maximising the chances of observing a significant effect.

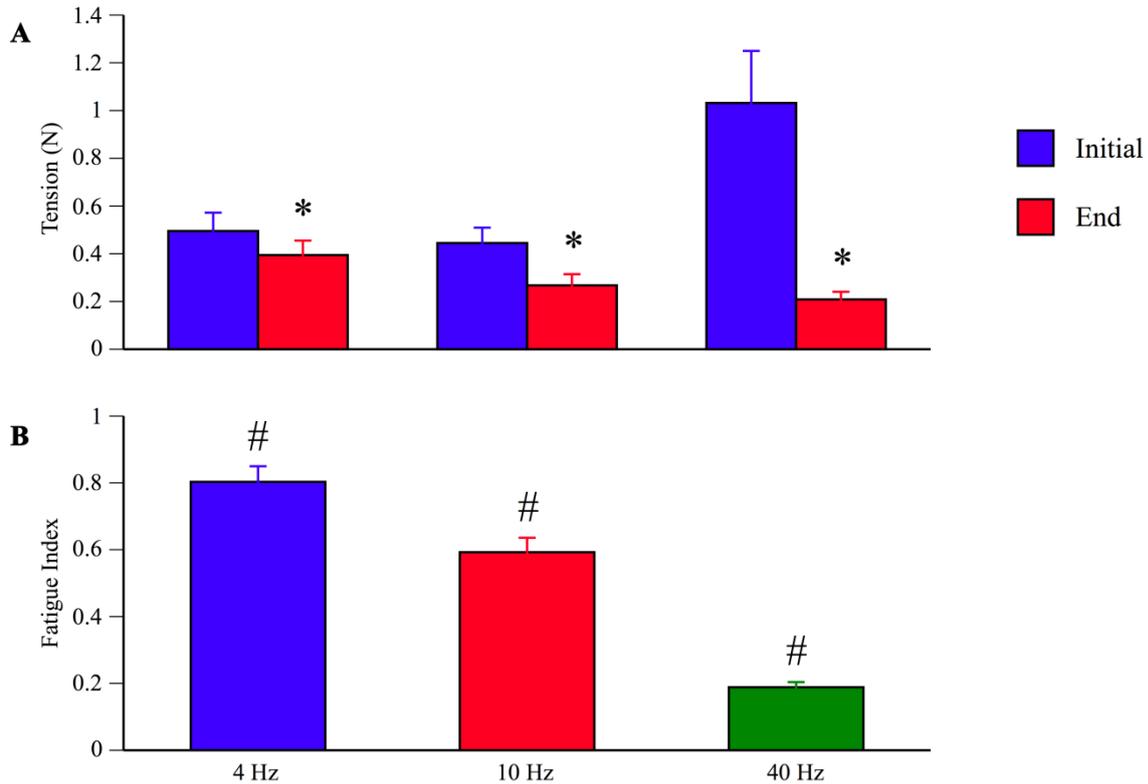


Fig 6: EDL tension and fatigue index during isometric muscle contraction.

A-B show mean (\pm S.E.M. error bars, $n = 7$) EDL tension (initial and end) and fatigue index for 4, 10 and 40 Hz stimulations of the EDL muscle, in Alfaxan-anaesthetised male Wistar rats. Contractions achieved by indirect supramaximal stimulation (0.3 ms square-wave pulse width, ≈ 10 V, 5 min) of the cut common peroneal nerve. * $P < 0.01$ vs. 'initial' (Student's t-test). # $P < 0.05$ vs. other stimulation bouts (between treatments ANOVA with Scheffé Post hoc test).

Other useful points from preliminary experiments included: deciding to fix the animal more securely using hindlimb clamps to eliminate movement upon stimulation, caring for isolated tissue (handled as carefully as possible to avoid trauma and kept bathed in physiological saline so as not to dehydrate or change pH), and deciding on an adequate time post-surgery

for baseline levels, of blood flow in particular, to return (~ 1 h). Blood flow measurement was increased from unilateral measurement to bilateral measurement at this point.

The above studies allowed us to decide on the final protocol to follow; a typical LabChart trace from such a setup is shown (Fig. 7).

- 1 - ABP (mmHg)
- 2 - MABP (mmHg)
- 3 - HR (BPM)
- 4 - Ipsi FBF (ml min⁻¹)
- 5 - Ipsi Tension (g)
- 6 - Ipsi MFBF (ml min⁻¹)
- 7 - Ipsi FVC (ml min⁻¹ mmHg⁻¹)
- 8 - Contra FBF (ml min⁻¹)
- 9 - Contra MFBF (ml min⁻¹)
- 10 - Contra FVC (ml min⁻¹ mmHg⁻¹)

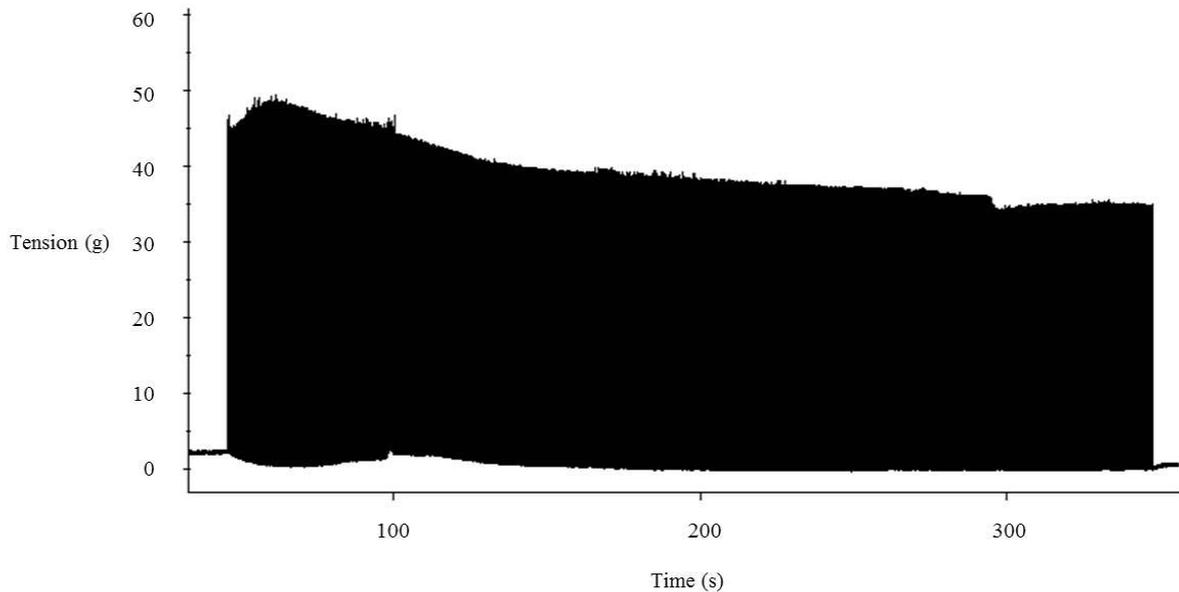
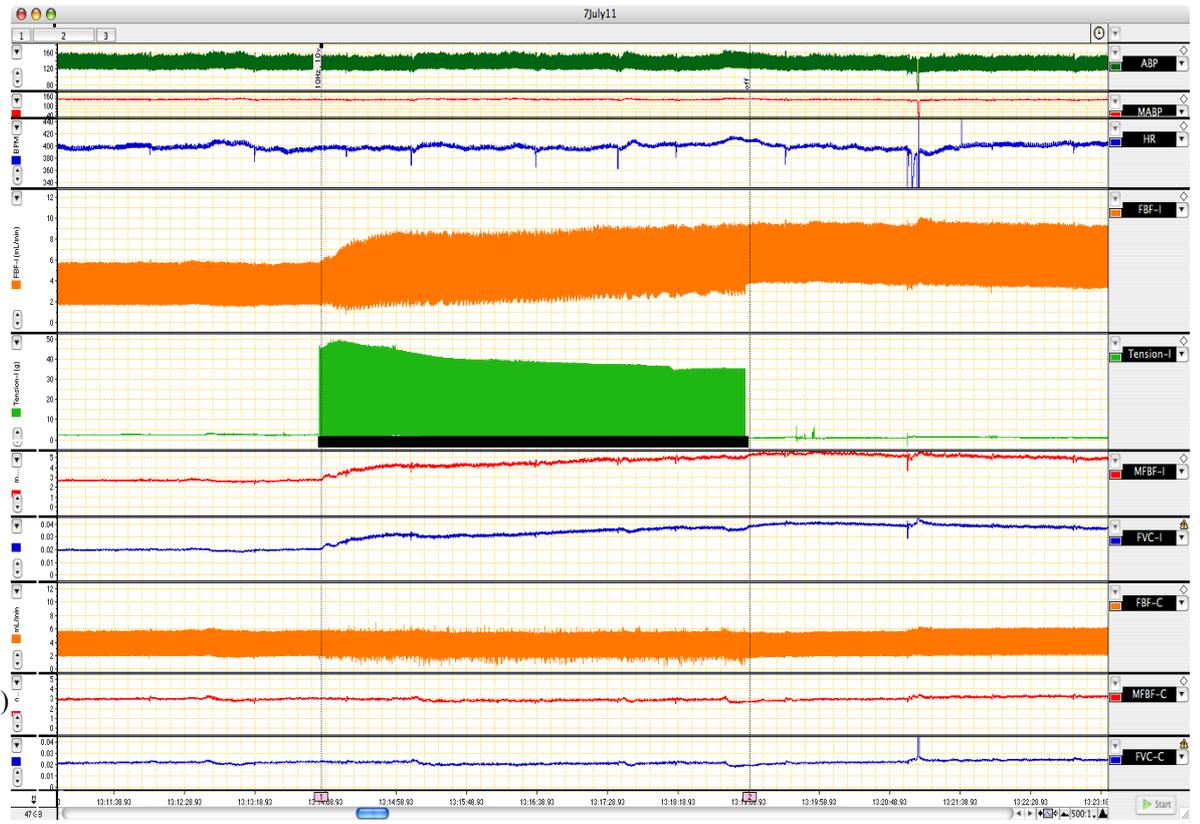


Fig 7: Raw trace from LabChart, for a final protocol experiment, with close-up of isometric tension generated.

Top panel: A trace for a 10 Hz stimulation of the EDL muscle, in Alfaxan-anaesthetised male Wistar rats. Contractions achieved by indirect supramaximal stimulation (0.3 ms square-wave pulse width, ≈ 10 V, 5 min [indicated by the black bar]) of the cut common peroneal nerve. The ten channels show the continuous, real-time measurement of the labelled variables. Notice the absence of a central response in HR (channel 3), the smooth decay in tension generated (channel 5) and the bilateral asymmetry in FBF and FVC response to stimulation. The ipsilateral channels (channels 4, 6 & 7) show hyperaemia due to femoral artery vasodilatation, while contralateral channels (channels 8, 9 & 10) show no change in blood flow or femoral artery tone. The tension generated during the 5 minute stimulation period, was used to calculate a score of muscle fatiguability that could later be compared across animal treatment groups. Bottom panel: An unaltered close-up of the tension generated for a 10 Hz stimulation.

MATERIALS AND METHODS

ANIMALS AND EXPERIMENTAL DESIGN:

Overview

Male Wistar rats were housed under Home Office regulations in the Biomedical Services Unit, University of Birmingham and were provided with standard chow and drinking water *ad libitum*. Animals were maintained at 20°C on a 12-hour light/ 12-hour dark cycle. Animals were assigned to one of four groups:

Group 1 (n = 6) – Untreated body-weight matched controls,

Group 2 (n = 6) – Administered with prazosin (50 mg L⁻¹) in drinking water for 2 weeks,

Group 3 (n = 5) – Unilateral surgical extirpation of TA, with post-operative recovery for 2 weeks,

Group 4 (n = 6) – Combinational treatment; unilateral surgical extirpation of TA, with post-operative recovery for 2 weeks & administered with prazosin (50 mg L⁻¹) in drinking water for 2 weeks (i.e. both Group 2 & 3 treatments; concurrently).

All surgical interventions were performed in accordance with UK legislation (Home Office Animals (Scientific Procedures) Act 1986). Animals were taken into final, terminal experimentation when average body weight was 327 ± 7 g (mean \pm S.E.M.). Body weights were approximately constant across each of the treatment groups, with the exception of combination treated animals, who were significantly lighter than extirpated animals ($P < 0.05$

vs. 'extirpated', between treatments ANOVA with Scheffé Post hoc test, $n = 6, 6, 5$ & 6 respectively; Fig. 8). As combination treated animals were of the same body weight as both control animals and prazosin treated animals, their body weight was not of concern. The healthy body weight across treatment groups suggests that the treatments were not having a detrimental effect on animal health, and thus animals were healthy going into terminal *in vivo* experiments.

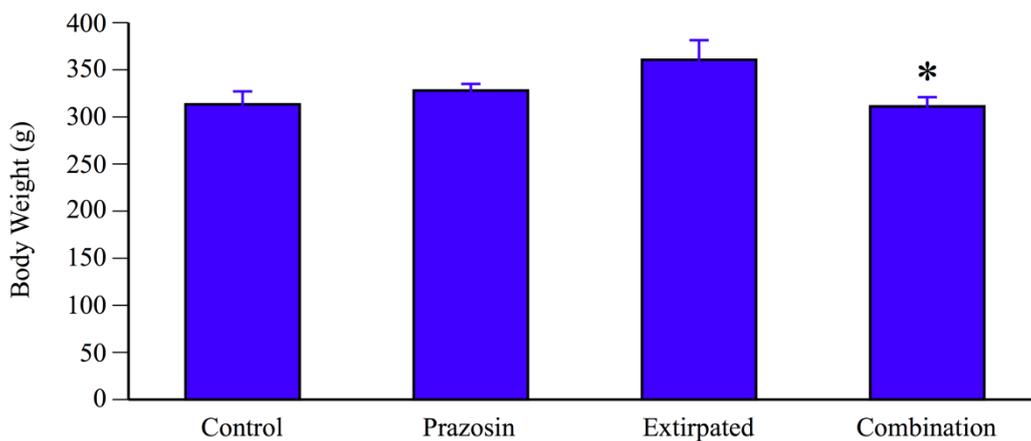


Fig 8: Animal body weights going into terminal *in vivo* experiments.

Mean body weight (\pm S.E.M. error bars) for each of the treatment groups ($n = 6, 6, 5$ and 6 respectively) going into terminal *in vivo* experiments. * $P < 0.05$ vs. 'extirpated' (between groups ANOVA with Scheffé Post hoc test).

Advance preparation

Group 2 animals were administered with 50 mg L^{-1} prazosin hydrochloride (Fluka Analytical, 81515, Sigma-Aldrich) gradually dissolved overnight in distilled water, with a pinch of sucrose (Sigma-Aldrich) to mask the bitter taste and maintain consumption. This was provided *ad libitum* as drinking water for 2 weeks. Previous studies have shown that this regime induces elevated shear stress and shear-induced angiogenesis (Egginton et al. 2001; Hudlicka, Brown, Egginton 1992; Zhou et al. 1998a).

Group 3 animals underwent unilateral surgical extirpation of the ipsilateral (right) TA (see ‘Surgical preparation for terminal experiments’ below), under Isoflurane inhalation anaesthesia (3.5% in Oxygen; Merial). Animals were treated perioperatively with systemic analgesic (2.5 ml kg⁻¹ buprenorphine, subcutaneous (s.c.), Temgesic, National Veterinary Services) and topical antibiotic (Duplocillin LA, National Veterinary Services). Animals were carefully monitored for signs of pain or discomfort during the 2 week recovery period (locomotion recovers within days – hanging on cage bars – not having a detrimental effect on behaviour (Hudlicka, Brown, Egginton 1992)). Previous studies have demonstrated that this regime causes overload of the EDL and overload-induced angiogenesis (Egginton et al. 2001; Hudlicka, Brown, Egginton 1992; Zhou et al. 1998b).

Group 4 animals were treated with a combination of the treatments in Groups 2 & 3. Surgical extirpation was performed and during the 2 week recovery period from surgery, prazosin was also administered in drinking water (started one day post-surgery to avoid excessive bleeding).

EXPERIMENTAL PROTOCOL:

Surgical preparation for terminal experiments

Anaesthesia was induced with the inhalation anaesthetic Isoflurane (3.5% in O₂), using an anaesthetic trolley (013406, General Anaesthetic Services; Harvard Apparatus Veterinary Fluosorber) and was judged to be at an adequate surgical level when the pedal withdrawal reflex was absent. The animal was fixed supine on a homeothermic heating blanket

positioned on a board, its forelimbs taped in place using micropore tape. Upon cannulation of the right jugular vein (Portex Translucent PVC Tubing, 0.63 mm ID, 1.40 mm OD), anaesthesia was maintained at a surgical level with continuous infusion of the steroid anaesthetic Alfaxan (9 mg ml^{-1} Alfaxalone diluted with physiological saline (0.9%) to 4 mg ml^{-1} , infused at $12\text{-}20 \text{ mg kg}^{-1} \text{ h}^{-1}$ intravenous (i.v.); Vétoquinol, UK) using a Perfusor Device (Perfusor Secura FT, B.Braun (50 ml)); experiments were performed thereafter under this anaesthetic. Animals were allowed to breathe spontaneously via a tracheal cannula (stainless steel), helping to maintain airway patency. The left brachial artery was cannulated (Portex Fine Bore Polythene Tubing, 0.40 mm ID, 0.80 mm OD), to monitor systemic arterial blood pressure via a blood pressure transducer (MLT844, ADInstruments); the cannula was filled with heparinised (Heparin (mucous), LEO) saline (in 0.9% NaCl (Sigma-Aldrich)) to prevent blood clotting. A rectal probe was applied to the animal, which works by feedback with the underlying homeothermic heating blanket (Harvard Apparatus) to maintain animal body temperature at 37°C , as body temperature homeostasis is poorly maintained under anaesthesia. Bilaterally, a single incision through the skin and blunt dissection through the underlying connective tissue was made to allow isolation of the femoral artery from surrounding tissue, particularly below the inguinal ligament and around the circumflex branch. Surgical suture was inserted through the overlying muscle and skin was used to pull this tissue away from the incision, using retractors, for ease of access. The ipsilateral (right) common peroneal nerve was located in a ‘key-hole surgery’ manner, freed from the connective tissue by blunt dissection. A length of 6-0 surgical suture was tied around the proximal end of the nerve and the nerve cut on the proximal side of this tie; this region was kept hydrated by the application of physiological saline (0.9% NaCl) to tissue paper placed over the incision. Lastly, a single incision using a scalpel blade was made along the length of the ipsilateral (right) hindlimb, through the skin and underlying tissue, revealing the hindlimb

muscle. Blunt dissection was used to isolate the TA (unless already surgically removed 2 weeks before; groups 3 & 4), and a scalpel to cut the tendon around the ankle joint. Blunt dissection was again employed to free the TA from the connective tissue between the TA and EDL, and holding the tendon tightly with forceps, a single cut of the scalpel blade used to remove the TA at its proximal end, along the plane of the neurovascular bundle. When performed effectively little bleeding occurred. The EDL was isolated in a similar manner and a double throw of 4-0 length of surgical suture looped around the tendon knotted tightly in place; the other end of the suture was used to attach the muscle to the force transducer. The EDL was also kept bathed in physiological saline, to avoid dehydration.

Positioning the animal for experimental protocol

Once surgery was completed, both hindlimbs were secured in place using paw clamps, taking care not to pull the hindlimbs too far out of place, thus restricting blood flow. Surgical suture was looped around the incisors and taped tightly to keep the animal as straight as possible; the tail base was similarly taped to keep the animal stretched out. The blood pressure transducer was calibrated with known pressure using a sphygmomanometer and the brachial artery cannula connected, avoiding the introduction of air bubbles. Two perivascular flow probes (0.7V; Transonic Systems Inc.), pre-calibrated with known flow and connected to a flow meter (T206, small animal blood flow meter; Transonic Systems Inc.), were placed around the femoral arteries for continuous measurement of FBF as an index of hindlimb blood flow. The suture tied around the EDL tendon was attached to a pre-calibrated, isometric force transducer (MLT1030/A, 1030/465, ADInstruments) to record tension developed in response to stimulation. Finally, using the suture tied around the sectioned common peroneal nerve, the nerve was positioned (without stretching it) so that a bipolar

silver electrode, connected to a Stimulator Unit (Grass Instruments, S8 stimulator) could be positioned beneath, to allow stimulation of the muscle.

The blood pressure transducer was connected to a QUADBridge Bridge Amplifier (ML110, ADInstruments), the force transducer to a Bridge Pod (ML301, ADInstruments) and the flow probes, were all connected to the PowerLab hardware (4/30, ML118, ADInstruments) linked to an Apple Power PC G5 computer. Data was collected via LabChart software (v5.5, ADInstruments), to give a continuous real-time record of each of the variables. A ten channel settings file was designed to do so, including raw ABP, MABP, HR from the pulsatile ABP trace, raw ipsilateral and contralateral FBF traces, mean derivations of each (MFBF), ipsilateral and contralateral FVC (derived online by dividing MFBF by MABP) and ipsilateral EDL tension.

Once all inputs were in place, the outputs on screen could be monitored. Using the tension trace, the optimal muscle length and stimulation voltage could be determined. By lengthening and shortening the muscle by moving the force transducer with a vernier clamp (making sure to keep it in plane) and stimulating the nerve, the length that produced maximal EDL twitch could be established. This was achieved by using a supramaximal stimulation voltage (around 10 V). Once the preparation was set ready for stimulation, the preparation was allowed to rest and stabilise, for at least 1 h, before stimulation began. The depth of anaesthesia was checked regularly via either the corneal reflex, the palpebral reflex or by pinching the ear or tail (the pedal reflex could not now be used effectively as the hindlimbs were secured in clamps) and the general health of the animal was indicated by the cardiovascular variables monitored.

The overall completed setup is summarised in Fig. 9 and Fig. 10.

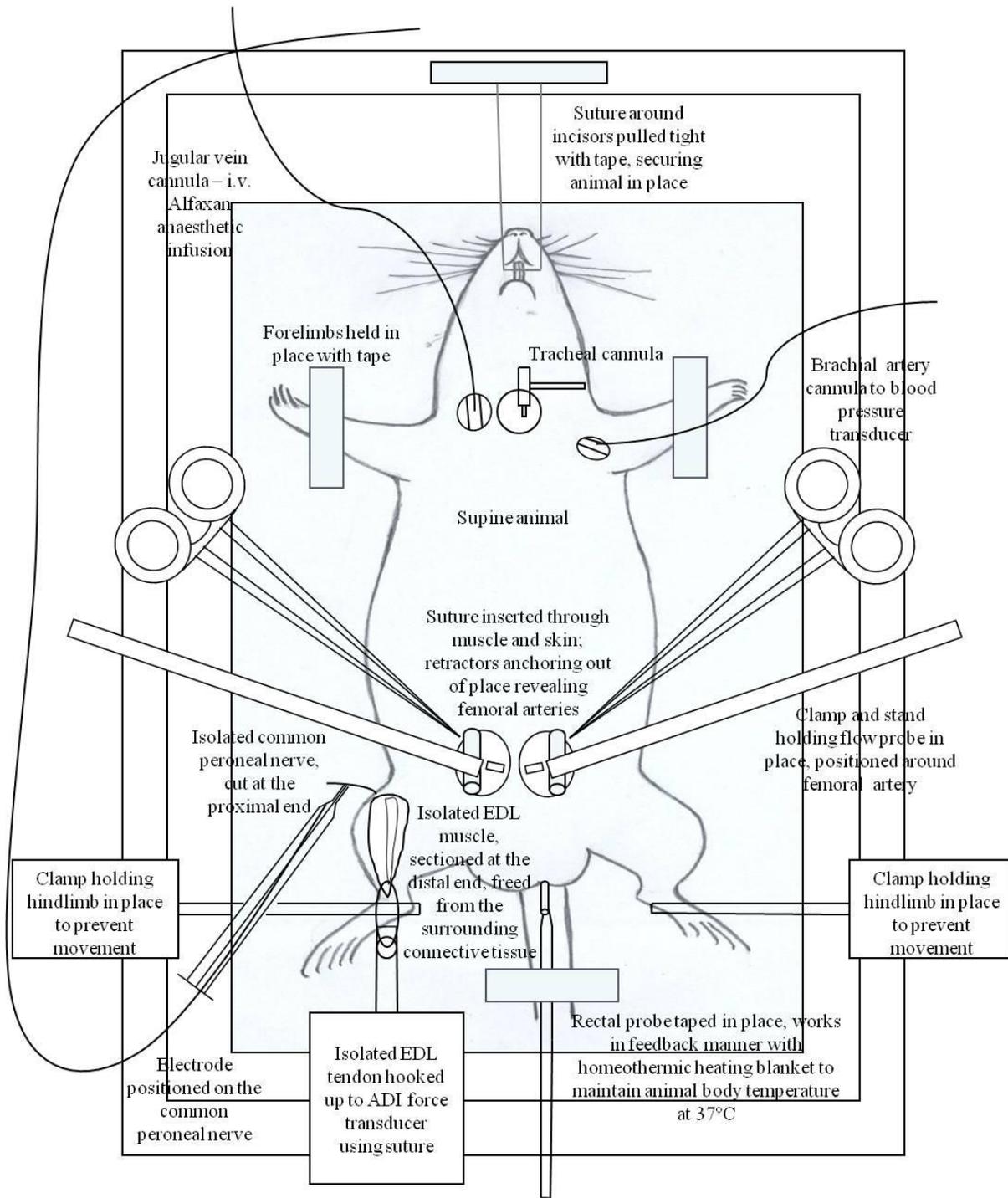


Fig 9: Schematic showing experimental setup.

The supine animal was secured in place by suture, tape and clamps. Anaesthesia was maintained continuously via a venous cannula. MABP was measured and HR derived via an arterial cannula. Bilateral MFBF was measured via perivascular flow probes and FVC calculated by MFBF/MABP. The isolated common peroneal nerve, cut at the proximal end, was stimulated electrically by an electrode, eliciting EDL muscle contraction. The EDL muscle was sectioned at the distal end and tied tightly to a force transmitter, to which it transmitted the muscle tension. The animal's body temperature was maintained at 37°C using feedback control between the rectal temperature probe and the homeostatic heating blanket on which the animal was positioned.

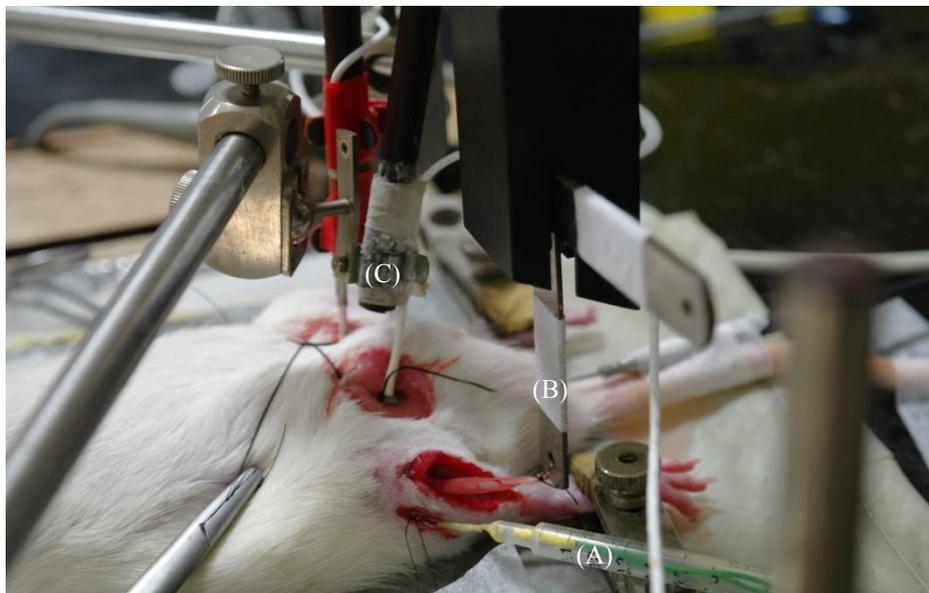
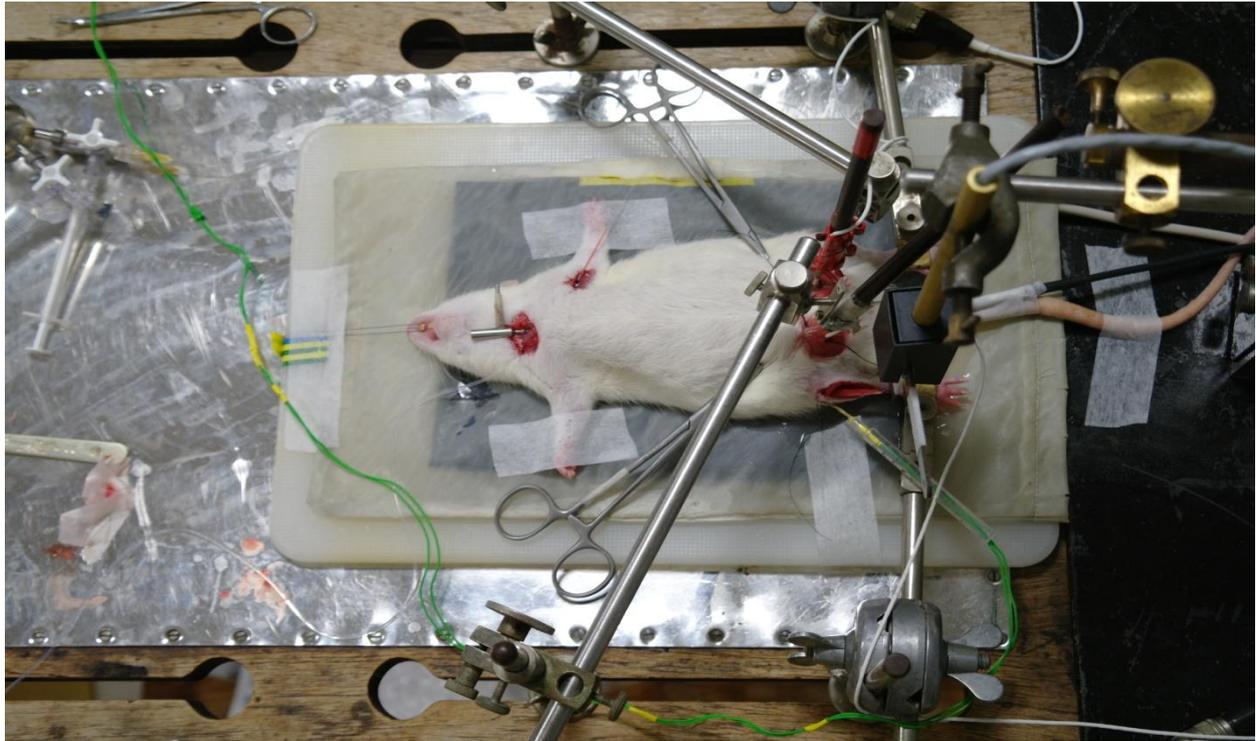


Fig 10: Photographs showing the overall setup.

Top panel, shows an overview of the completed setup. Bottom panel, shows a close up of the isolated common peroneal nerve (cut at the proximal end) with electrode positioned in place (A) and the EDL muscle (sectioned at the distal end) attached to the force transducer (B), in the natural plane of the muscle, and set at the determined optimal length; the flow probe positioned around the femoral artery is also shown (C). When the preparation was given sufficient time to recover and for baseline values to be restored, stimulation would commence.

In vivo measurement of hindlimb blood flow, muscle tension and fatigue

Once adequate time had been given for the preparation to obtain resting baseline levels, the stimulation began. Keeping the muscle moist, but ensuring the nerve was dry, contractions were evoked from rest. Indirect stimulation was applied (0.3 ms square-wave pulse width and supramaximal voltage, usually ~10 V) for a 5 min period, at a stimulation frequency of 10 Hz. This frequency was shown to elicit maximal MFBF in preliminary experiments. Due to the earlier setting of the muscle, isometric twitch contractions were stimulated with a supramaximal voltage at this frequency, when the muscle was of optimal length, with no indication of a change in muscle length. A rest period was allowed at the end of the stimulation to allow baseline values to return.

Blood gas analysis

When the blood gas analyser was available (n = 6), arterial blood samples were taken from the left brachial artery cannula. Typically 120 µl was sampled using a capillary collection tube. Samples were analysed (IL-682, GEM Premier 4000, Instrumentation Laboratory) for blood gases and electrolytes. Blood was typically sampled at rest, and 30 s after muscle stimulation.

Study caveat

Once the 10 Hz stimulation was complete in each animal (including a 1 h rest period for baselines to be restored) but before the muscle was sampled and the experiment terminated, an additional 40 Hz stimulation was performed, in exactly the same way as that for a 10 Hz stimulation. This was done purely out of interest, to see whether any

potential changes revealed by a 10 Hz stimulation were also evident for a 40 Hz stimulation, or whether adaptations were intensity specific.

Sampling of muscle

Once the stimulation protocol was completed, the EDL was removed from both hindlimbs and weighed. A piece from the middle belly of the muscle was removed using a fine, sharp razor blade (Wilkinson Sword), mounted on a cork section in OCT Embedding Matrix (Raymond-Lamb) and rapidly frozen in isopentane pre-cooled in liquid nitrogen. The top and tail of the muscle were placed in Eppendorf tubes and frozen in liquid nitrogen. Samples were stored at -80°C for later histological studies.

Experiment termination

At the end of the experiment, all animals were terminated by overdose of the sodium pentobarbital anaesthetic, Euthatal (Merial); death was confirmed by cervical dislocation.

TISSUE HISTOLOGY – SECTIONING, STAINING AND COUNTING:

Muscle sections (10 μm) were cut from frozen muscle samples, stored at -80°C , using a clinical cryostat (Clinicut) at -25°C . Cut sections were applied to Polylysine microscope slides (631-0107, VWR International) and checked for quality using an Olympus BH-2 Light Microscope. Muscle sections were allowed to dry at room temperature ($\sim 25^{\circ}\text{C}$), ringed with a wax pen (Vector Laboratories, UK) and fixed in 4% paraformaldehyde (TAAB Laboratories) for 1 min. Once washed with phosphate buffered saline (PBS) (Sigma-Aldrich), muscle sections were incubated with Lectin (Fluorescein conjugated *Griffonia (Bandeiraea) simplicifolia* Lectin, FL-1101, Vector Laboratories, UK) for 1 h at room temperature to mark ECs, washed again with PBS, stained with DAPI (Vectashield Mounting Medium with DAPI, H-1200, Vector Laboratories, UK) to identify nuclei, covered with cover glass and immediately imaged to prevent fluorescence quenching.

Sections were imaged using a Zeiss Photomicroscope III and computer with AxioVision4 software, at x20 magnification. Four sampling frames were obtained from each section, spread equally across the section; a calibrated scale bar was applied to each image (Fig. 11).

Capillary and fibre counts were performed on each sampling frame offline, using an unbiased counting method consisting of inclusion and exclusion lines, explained in (Egginton 1990). Such counts were performed using ImageJ (v1.440) (Fig. 11).

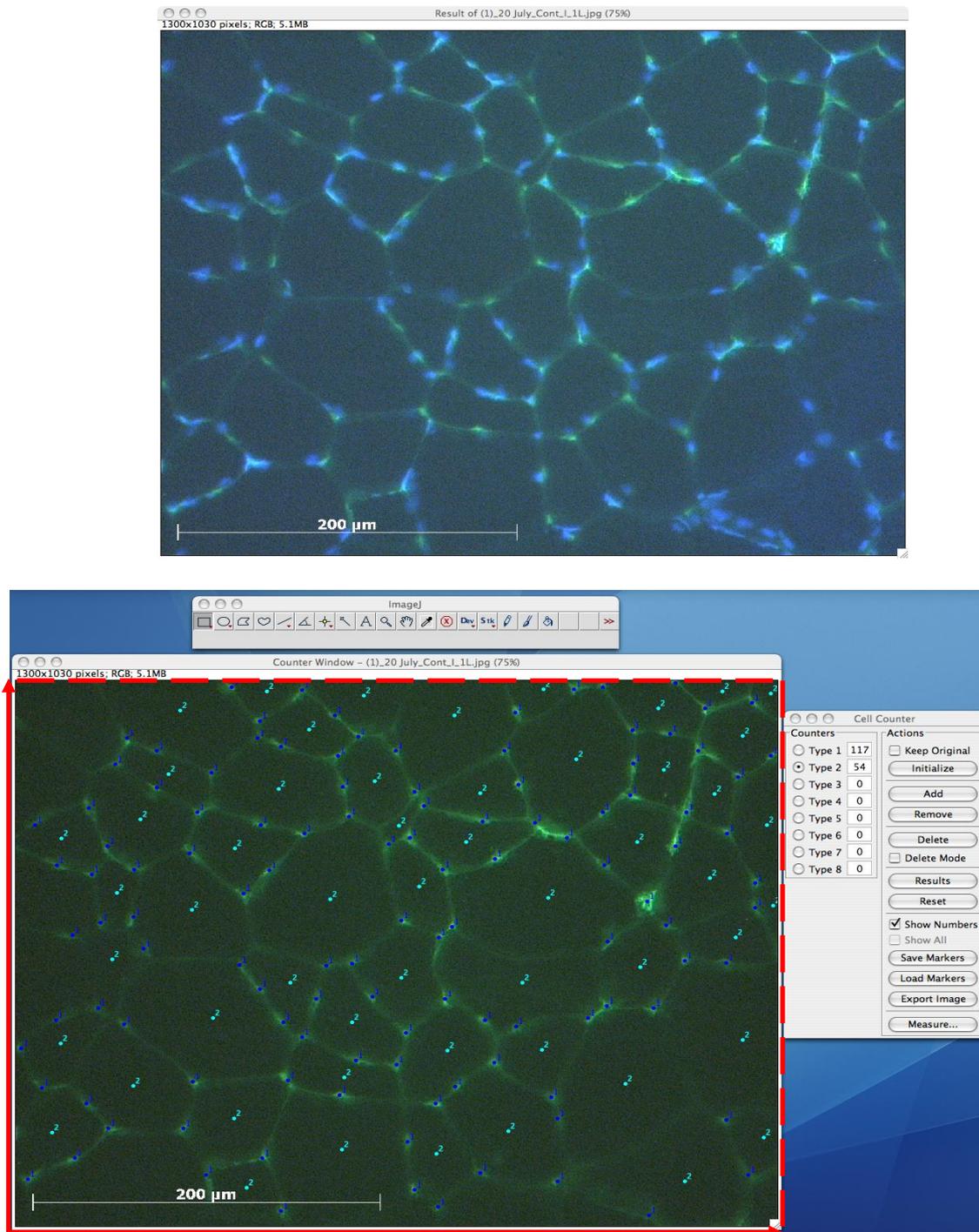


Fig 11: Performing capillary and muscle fibre counts on a stained muscle section using ImageJ.

The top panel shows an example image of a sampling frame across a muscle section. Lectin (green) stained endothelium and DAPI (blue) stained nuclei images have been overlaid. A 200 µm, pre-calibrated scale bar has been applied to the image to aid the calculation of capillary densities and mean fibre areas. The bottom panel illustrates the unbiased process by which capillary and muscle fibres counts were performed on a muscle section using ImageJ. The cell counter function was used to mark individual components, counter 1 for capillaries and counter 2 for muscle fibres. The top and right adjacent sides of the counting frame (indicated by the dashed red lines) were the inclusion lines, so components touching these lines were included. The bottom and left adjacent sides of the counting frame (indicated by the solid red lines) were the exclusion lines, so component touching these lines were excluded. This ensures that only components that lie entirely within the designated sampling frame are counted, giving the best unbiased estimate.

DATA ANALYSIS:

All subsequent analysis was performed offline.

Analysis set 1 – Gross response

The DataPad function of LabChart was used to analyse the gross response to muscle stimulation. Mean MABP, mean HR, ipsilateral & contralateral MFBF, mean ipsilateral & contralateral FVC and (cyclic maximum – cyclic minimum) ipsilateral tension for 30 s time periods (baseline pre-stimulation, initial 30 s, end 30 s of stimulation, immediate 30 s post-stimulation and baseline post-stimulation, up to 1 h later) were acquired. Fatiguability during the 5 min stimulation was expressed as FI by the ratio of end developed cyclic maximum tension to initial developed cyclic maximum tension (shown in Fig. 12).

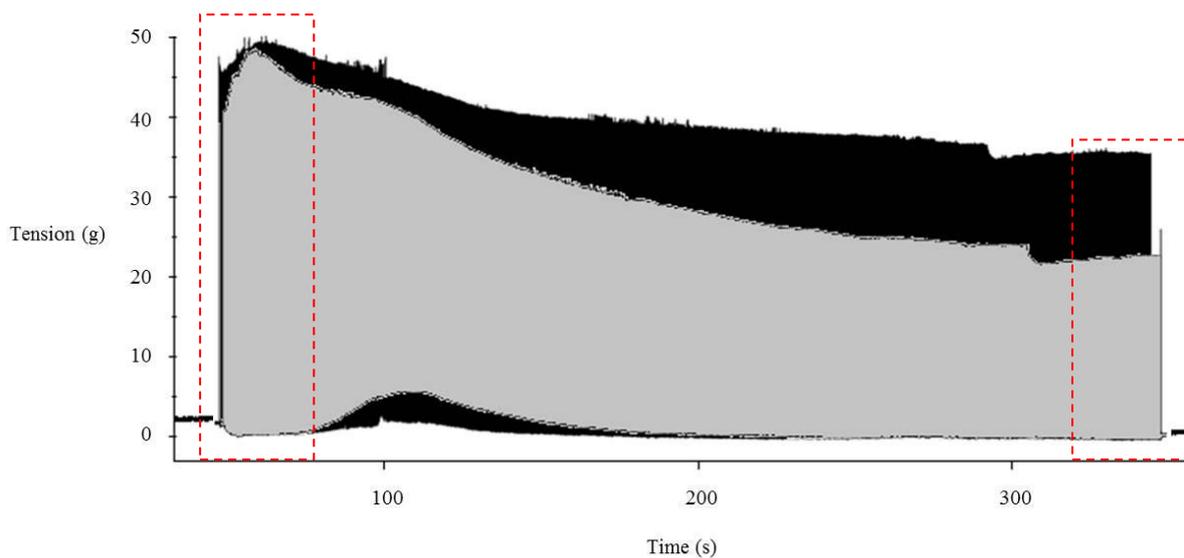


Fig 12: Calculating FI from tension traces.

A diagram showing two overlaid, unmanipulated tension traces (for EDL muscle of prazosin treated animal in grey and EDL muscle of combination treated animal in black) from LabChart. The isometric tension generated by supramaximal 10 Hz stimulation, decays over the 5 minute stimulation period. FI is calculated by expressing the mean cyclic maximum tension between the initial 30 s and end 30 s of stimulation (indicated by red dashed boxes) as a ratio [end/ initial]. In this instance there appears to be a difference in FI between these two differently treated groups of animals, suggesting a treatment effect.

Analysis set 2 – Twitch characteristics

The DataPad function of LabChart was used in conjunction with the PeakParameters plugin. The rise time and fall time (defined as the time taken to rise from 25% to 75% of the twitch peak from baseline and the time taken to fall from 75% to 25% of the twitch peak to baseline, when baseline is set by the user; shown in Fig. 13) for five successive twitches at the absolute peak of a contraction and five equal-amplitude successive twitches in the final 10 s of a contraction, were acquired. For each twitch, the ‘baseline’ was set as the initial 20 ms of baseline before an individual twitch was evoked. Each of the five successive twitches were averaged, before comparison was made between treatment groups.

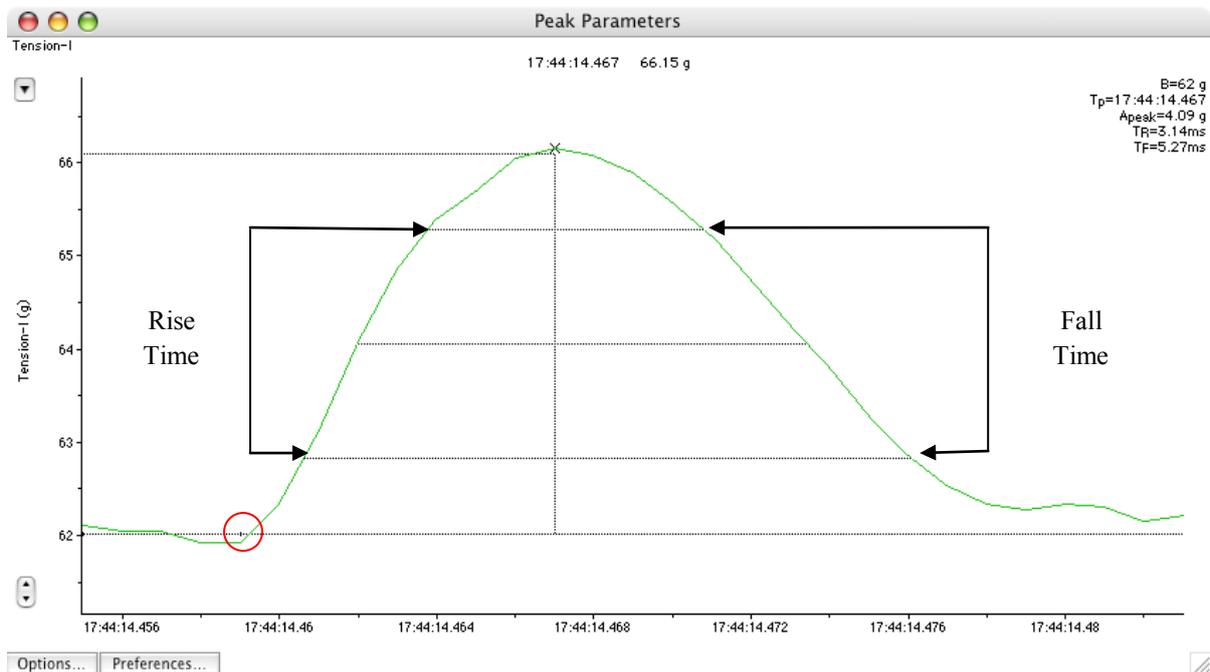


Fig 13: The peak parameters window used to derive twitch rise and fall times.

For a 10 Hz stimulation, the initial 20 ms of the window was set as the baseline, indicated to the left of the black dot set in the red circle. This point was set as the baseline relative to the absolute twitch peak (automatically detected). The lines indicated by arrows at the first and third quartile were the limits between which the rise and fall times were calculated.

Analysis set 3 – Half relaxation tension and time

For each muscle tension profile generated by stimulation, the ‘absolute peak’ tension and ‘end’ tension were recorded and the specific half relaxation tension (N) was calculated (i.e. $0.5 \times [\text{‘absolute peak’ tension} - \text{‘end’ tension}]$). The time into the stimulation period at which this decay in tension was achieved was recorded to give the specific half relaxation time (s).

Analysis set 4 – Muscle histology

Capillary and fibre counts, once performed on each sampling frame (four across each muscle section), were averaged for one muscle section for the EDL (ipsilateral **and** contralateral muscles for extirpated animals and combination treated animals only) of every animal. Such counts were expressed as C:F ratio (by simple division) and CD (μm^{-2}) and MFA (μm^2) by using the scale bar to calibrate pixels to section area. Data was then compared between muscles (both between ipsilateral muscles only and between ipsilateral and contraclateral muscles where the two muscles within the same animal had been differentially treated) in each treatment group.

Where appropriate, data were normalised and expressed per unit muscle weight. All graphs were produced using DeltaGraph (v5.7.5, SPSS Inc., Red Rock Software).

STATISTICAL ANALYSIS:

All data are presented as means \pm S.E.M. error bars, with numbers of animals (n) given in parentheses.

Differences between treatment groups (i.e. for functional data [MABP, HR, MFBF, FVC, tension and calculated FI, rise and fall times & half relaxation tension and time] at a specific time point for ipsilateral muscles; for structural data [muscle weights, C:F ratio, CD & MFA] of ipsilateral muscles) were determined by between treatments ANOVA with Scheffé post hoc test where appropriate. When a pair-wise comparison was desired (i.e. for functional data [tension 'initially' and 'at end'] for a particular muscle contraction; for structural data [muscle weights, C:F ratio, CD & MFA] between ipsilateral and contralateral muscles within the same treatment group), data were compared using Student's t-test. Differences within treatment groups across a time course (i.e. for functional data [MABP, HR, MFBF & FVC profile in time] within ipsilateral muscles) were determined by within treatments ANOVA for repeated measures, with Scheffé *post hoc* test where appropriate. All statistical analysis was performed using Aabel3 (20/20 Data Vision, Gigawiz). Statistical significance was set at $P < 0.05$.

RESULTS

The data that makes up the figures in this chapter are from completely different animals to that in the Method Development chapter, using the refined final protocol described in the Materials and Methods chapter. The ‘Study caveat’ data is described here but the figures related to the ‘Study caveat’ are included in the Appendix.

Did the angiogenic stimuli induce angiogenesis?

Two week prazosin administration is known to induce splitting angiogenesis in EDL muscle, resulting in an increase in C:F ratio (Zhou et al. 1998a). Unfortunately, there was not an increase in C:F ratio observed in our prazosin treated animals (ns, between groups ANOVA with Scheffé Post hoc test, $n = 6, 6, 5$ & 6 respectively; Fig. 21a), suggesting it is unlikely that any splitting angiogenesis was successfully induced. However our prazosin treated animals had elevated femoral artery blood flow relative to both control animals and extirpated animals ($P < 0.05$ vs. ‘control’ and ‘extirpated’, between groups ANOVA with Scheffé Post hoc test, $n = 6, 6, 5$ & 6 respectively; Fig. 14a), suggesting there was perhaps a partial effect on the endothelium.

Two week recovery from surgical extirpation of the TA muscle is known to induce sprouting angiogenesis in EDL muscle, resulting in compensatory muscle hypertrophy in the ipsilateral muscle relative to the contralateral muscle (by around 20%), with an approximately parallel increase in C:F ratio (Egginton et al. 1998; Zhou et al. 1998b). Fortunately, this was the case in our extirpated animals ($P < 0.05$ vs. ‘contralateral’, Student’s t-test, $n = 6$; Table. 1) and ($P < 0.05$ vs. ‘contralateral’, Student’s t-test, $n = 6$; Fig. 21a), suggesting that sprouting

angiogenesis was successfully induced. Compensatory muscle hypertrophy was induced in the ipsilateral muscles of both extirpated animals and combination treated animals, relative to their contralateral muscles by approximately 22% and 34% respectively. Interestingly the ipsilateral muscle of the combination treated animals experienced a higher degree of hypertrophy than the ipsilateral muscle of the extirpated animals ($P < 0.05$ vs. ‘extirpated % change’, Student’s t-test, $n = 6$; Table. 1).

Table 1: EDL muscle weights across the treatment groups.

Tabulated individual animal EDL muscle weights for each of the four treatment groups ($n = 6$), including both ipsilateral and contralateral muscle weights for extirpated animals and combination treated animals. Mean data and S.E.M. have been calculated. * $P < 0.05$ vs. ‘contralateral’ (Student’s t-test). ^ $P < 0.05$ vs. ‘extirpated % change’ (Student’s t-test).

Rat #	Control	Prazosin	Extirpated			Combination		
			Ipsi	Contra	% Change	Ipsi	Contra	% Change
1	141.4	155.6	203.4	161.7	25.8	174.9	141.1	24.0
2	133.3	161.9	255.0	210.9	20.9	236.8	167.5	41.4
3	133.2	173.2	217.4	175.9	23.6	191.8	155.7	23.2
4	156.3	129.3	219.5	153.1	43.4	187.2	117.3	59.6
5	176.7	173.3	162.0	148.9	8.8	147.8	113.3	30.5
6	172.1	164.8	175.1	158.9	10.2	162.0	127.3	27.3
Mean	152.2	159.7	205.4	168.2	22.1	183.4	137.0	34.3
			*			*		^
S.E.M.	7.8	6.7	13.7	9.3	5.1	12.6	8.8	5.7

As one stimuli was apparently induced and the other was not, it is brought into question whether two angiogenic stimuli were actually been applied in combination treated animals.

Effect of stimulation on blood flow

During the 10 Hz stimulation, there was no discernible central response in MABP or HR regardless of treatment group (ns, within groups ANOVA for repeated measures, with Scheffé Post hoc test, $n = 6, 6, 5 \text{ \& } 6$ respectively; data not shown), showing both the efficiency of motor nerve transection and that the localised responses to altered blood flow had little effect on systemic cardiovascular variables (thus, there was little difference whether muscle perfusion was measured as blood flow or vascular conductance). The functional hyperaemia associated with muscle activity was significant by the 'end' of the 5 minute stimulation for all treatment groups ($P < 0.05$ vs. 'rest', within subjects ANOVA for repeated measures, with Scheffé Post hoc test, $n = 6, 6, 5 \text{ \& } 6$ respectively; Fig. 14a). Relative blood flow was significantly increased in prazosin treated animals at 'rest', 'initially' and at the 'end' of contractions ($P < 0.05$ vs. 'control' and 'extirpated', between groups ANOVA with Scheffé Post hoc test, $n = 6, 6, 5 \text{ \& } 6$ respectively; Fig. 14), but no different to that in combination treated animals. Contralateral blood flow was unaffected, showing no evidence for a bilateral steal effect, during ipsilateral stimulation (ns, within groups ANOVA for repeated measures, with Scheffé Post hoc test, $n = 6, 6, 5 \text{ \& } 6$ respectively; data not shown). The functional hyperaemia profile observed as stimulation progressed remained in proportion across each of these treatment groups, i.e. no shift in the gradient at which blood flow changed across the treatment groups. Values returning to baseline levels at 'rest', around 1 h later.

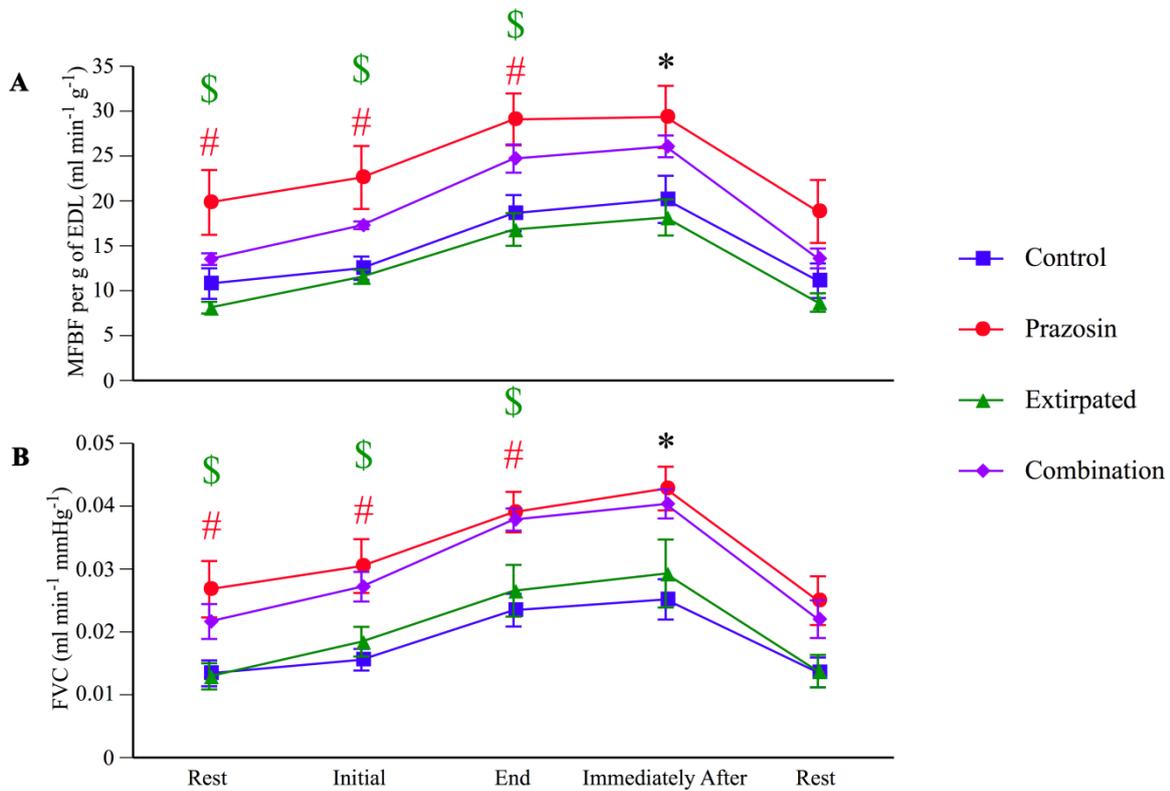


Fig 14: Vascular response during 10 Hz isometric contraction of EDL muscle.

A-B show mean (\pm S.E.M. error bars) MFBF and FVC for control, prazosin, extirpated and combination animals ($n = 6, 6, 5$ and 6 respectively) upon stimulation of the EDL muscle, at 5 different time points, in Alfaxan-anaesthetised male Wistar rats. Contractions achieved by indirect supramaximal stimulation (0.3 ms square-wave pulse width, ≈ 10 V, 5 min) of the cut common peroneal nerve. * $P < 0.05$ vs. 'rest' (within groups ANOVA with Scheffé Post hoc test). # $P < 0.05$ vs. 'control' and \$ $P < 0.05$ vs. 'prazosin' (between groups ANOVA with Scheffé Post hoc test).

Developed muscle tension and fatigue index

When the gross response to muscle contraction was assessed, the muscle tension and FI derived were consistent, indicating a stable preparation and a robust sampling regime. Furthermore the decay profile was as expected for the EDL, stimulated at this frequency. For all treatment groups there was a significant decay in tension by the 'end' of the stimulation period, ($P < 0.05$ vs. 'initial', Student's t-test, $n = 6, 6, 5$ & 6 respectively; Fig. 15a). There was no treatment effect between the groups on 'initial' tension generated (ns, between groups ANOVA with Scheffé Post hoc test, $n = 6, 6, 5$ & 6 respectively; Fig. 15a). There was however a tendency for extirpated animals and combination treated animals to have an increased 'end' tension generated, (although ns, between groups ANOVA with Scheffé Post hoc test, $n = 6, 6, 5$ & 6 respectively; Fig. 15a). This did however lead to a significantly increased FI for extirpated animals ($P < 0.05$ vs. 'control' and 'prazosin', between groups ANOVA with Scheffé Post hoc test, $n = 6, 6, 5$ & 6 respectively; Fig. 15b). This indicates that the muscle in extirpated animals had a significantly decreased fatiguability, relative to the muscle in both control animals and prazosin treated animals, though no such change was seen for the muscle in combination treated animals. Specific tension, allowing for significant variation in muscle mass among groups, suggested a slight weakening of the EDL following extirpation, with or without prazosin administration (Fig. 16).

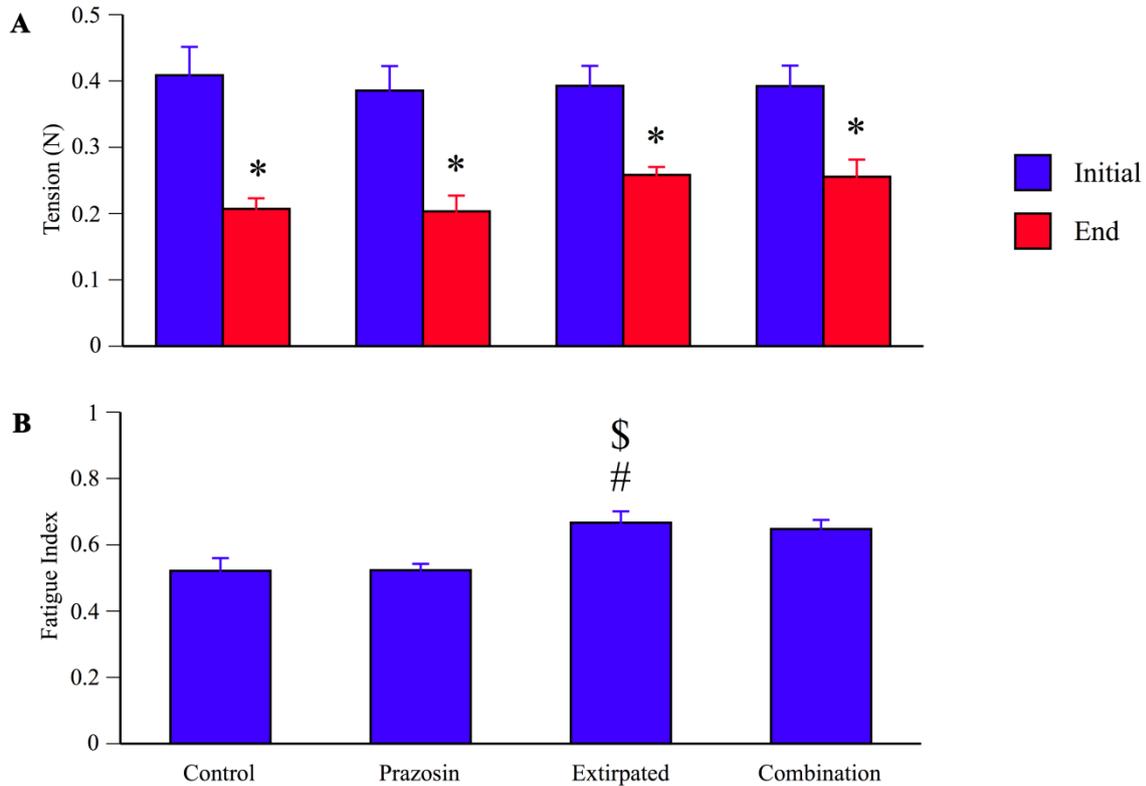


Fig 15: EDL tension and fatigue index during 10 Hz isometric muscle contraction.

A-B show initial and end mean (\pm S.E.M. error bars) EDL tension and overall fatigue index for control, prazosin, extirpated and combination animals ($n = 6, 6, 5$ and 6 respectively) upon stimulation of the EDL muscle, in Alfaxan-anaesthetised male Wistar rats. Contractions achieved by indirect supramaximal stimulation (0.3 ms square-wave pulse width, ≈ 10 V, 5 min) of the cut common peroneal nerve. * $P < 0.05$ vs. 'initial' (Student's t -test). # $P < 0.05$ vs. 'control' and \$ $P < 0.05$ vs. 'prazosin' (between groups ANOVA with Scheffé Post hoc test).

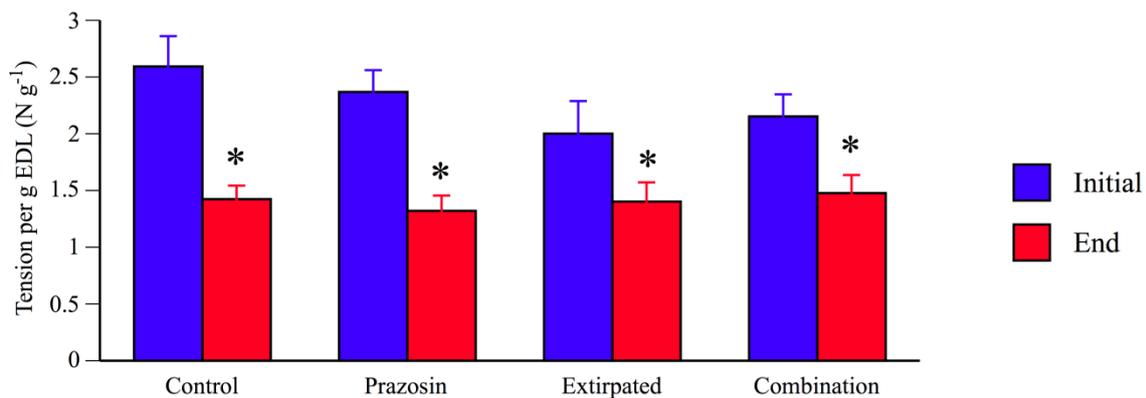


Fig 16: Specific EDL tension during 10 Hz isometric twitch contraction.

Graph shows the initial and end mean (\pm S.E.M. error bars) EDL tension, normalised for EDL muscle weight to allow for variation between groups, for control, prazosin, extirpated and combination animals ($n = 6, 6, 5$ and 6 respectively) upon stimulation of the EDL muscle, in Alfaxan-anaesthetised male Wistar rats. Contractions achieved by indirect supramaximal stimulation (0.3 ms square-wave pulse width, ≈ 10 V, 5 min) of the cut common peroneal nerve. * $P < 0.05$ vs. 'initial' (Student's t -test).

Investigation of muscle characteristics

Analysis of tension decay for a 10 Hz stimulation, consistent with the derived FI, indicated that the developed half relaxation muscle tension was not changed between treatment groups (ns, between groups ANOVA with Scheffé Post hoc test, $n = 6, 6, 5 \text{ \& } 6$ respectively; Fig. 17a), however the time taken to reach the half relaxation muscle tension (the half relaxation time) was significantly increased in extirpated animals ($P < 0.05$ vs. 'control' and 'prazosin', between groups ANOVA with Scheffé Post hoc test, $n = 6, 6, 5 \text{ \& } 6$ respectively; Fig. 17b) but not in combination treated animals ($P < 0.05$ vs. 'extirpated', between groups ANOVA with Scheffé Post hoc test, $n = 6, 6, 5 \text{ \& } 6$ respectively; Fig. 17b). This suggests that the muscle tension in extirpated animals decays to approximately an equal half relaxation tension to that in the other treatment groups, but takes a significantly longer period of time to reach that tension (the half relaxation time). This decrease in fatiguability is lost in the muscle of combination treated animals, like it is for FI. At 10 Hz, the twitch rise time and fall times were unaffected by treatment, either 'initially' or at the 'end' of stimulation (ns, between groups ANOVA with Scheffé Post hoc test, $n = 6, 6, 5 \text{ \& } 6$ respectively; Fig. 18).

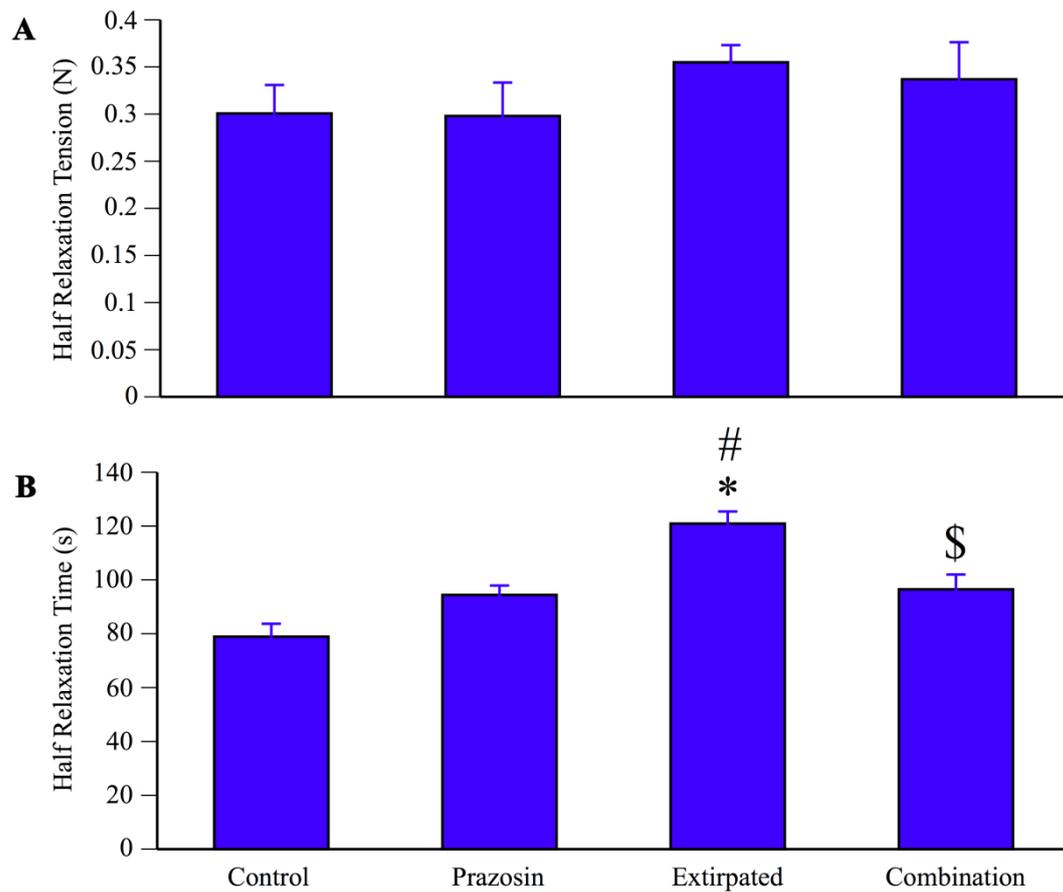


Fig 17: Half relaxation properties of EDL muscle undergoing a 10 Hz isometric twitch contraction.

A-B show mean (\pm S.E.M. error bars) EDL muscle half relaxation tension and half relaxation time for control, prazosin, extirpated and combination animals ($n = 6, 6, 5$ and 6 respectively) upon stimulation of the EDL muscle, in Alfaxan-anaesthetised male Wistar rats. Contractions achieved by indirect supramaximal stimulation (0.3 ms square-wave pulse width, ≈ 10 V, 5 min) of the cut common peroneal nerve. * $P < 0.05$ vs. 'control', # $P < 0.05$ vs. 'prazosin' and \$ $P < 0.05$ vs. 'extirpated' (between groups ANOVA with Scheffé Post hoc test).

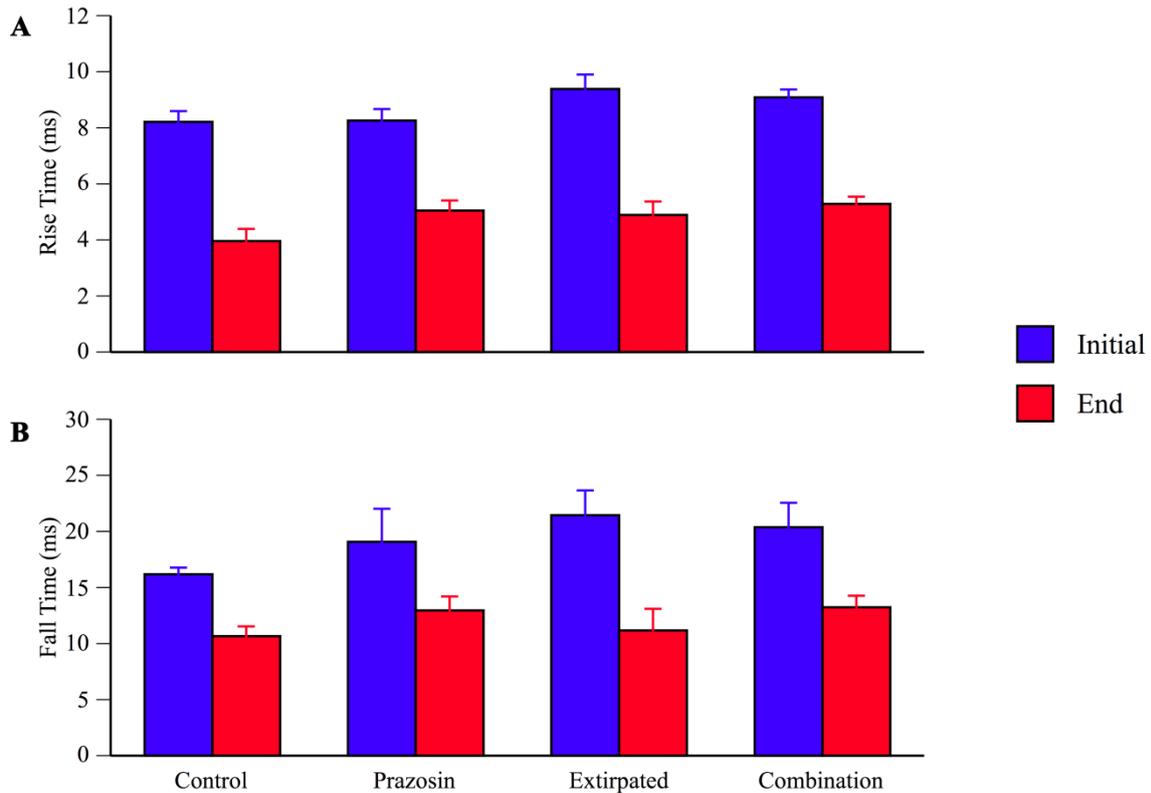


Fig 18: Muscle twitch rise time and fall times, for EDL muscle undergoing 10 Hz isometric twitch contraction.

A-B show mean (\pm S.E.M. error bars) individual EDL muscle twitch rise time and fall times, initially and at the end of the stimulation period, for control, prazosin, extirpated and combination animals ($n = 6, 6, 5$ and 6 respectively) upon stimulation of the EDL muscle, in Alfaxan-anaesthetised male Wistar rats. Contractions achieved by indirect supramaximal stimulation (0.3 ms square-wave pulse width, ≈ 10 V, 5 min) of the cut common peroneal nerve. There was no significant difference in either rise time or fall times between group during the initial or end period of the bout of contraction.

Blood chemistry

No change was seen in any blood gas component post-stimulation (ns, within subjects ANOVA for repeated measures, with Scheffé Post hoc test, $n = 6, 6, 5$ & 6 respectively; data not shown), suggesting muscle stimulation produced a largely local, not systemic response.

Study caveat

(For figures see Appendix).

The additional 40 Hz stimulation, when analysed, yielded similar data to that of a 10 Hz stimulation, at least for blood flow data. The functional hyperaemia associated with muscle activity was again significant by the 'end' of the 5 minute stimulation for all treatment groups ($P < 0.05$ vs. 'rest', within subjects ANOVA for repeated measures, with Scheffé Post hoc test, $n = 6, 6, 5$ & 6 respectively; Fig. A1a). Again, relative blood flow was significantly increased in prazosin treated animals at 'rest', 'initially' and at the 'end' of contractions ($P < 0.05$ vs. 'control' and 'extirpated', between groups ANOVA with Scheffé Post hoc test, $n = 6, 6, 5$ & 6 respectively; Fig. A1), but no different to that in combination treated animals. As seen for a 10 Hz stimulation, for all treatment groups there was a significant decay in tension by the 'end' of the stimulation period, ($P < 0.01$ vs. 'initial', Student's t-test, $n = 6, 6, 5$ & 6 respectively; Fig. A2a). However, converse to a 10 Hz stimulation, there was no treatment effect between the groups for neither 'initial' or 'end' tension generated, meaning there was no significant change in FI (all ns, between groups ANOVA with Scheffé Post hoc test, $n = 6, 6, 5$ & 6 respectively; Fig. A2a&b). Specific tension, again suggested a tendency for slight weakening of the EDL following extirpation, with or without prazosin administration (Fig. A3). The developed half relaxation muscle tension and time were not changed between treatment groups (ns, between groups ANOVA with Scheffé Post hoc test, $n = 6, 6, 5$ & 6 respectively; Fig. A4a&b), highlighting another difference to that of the 10 Hz stimulation. The twitch rise time and fall times were again unaffected by treatment, either 'initially' or at the 'end' of stimulation (ns, between groups ANOVA with Scheffé Post hoc test, $n = 6, 6, 5$ & 6 respectively; Fig. A5).

Muscle composition: capillaries and fibres

Example photomicrographs of stained muscle sections for each of the treatment groups are included (Fig. 19) to give a visual representation of the scope of histological changes in muscle, from which counts were performed.

Analysis of muscle section counts revealed a tendency for an increase in C:F ratio in muscle from prazosin treated animals, relative to control animals (though ns, between groups ANOVA with Scheffé Post hoc test, $n = 6$; Fig. 20a); however a significant increase in C:F ratio was seen in muscle from extirpated animals ($P < 0.05$ vs. 'control' and 'prazosin', between groups ANOVA with Scheffé Post hoc test, $n = 6$; Fig. 20a) and to a lesser extent in combination treated muscle ($P < 0.05$ vs. 'control', between groups ANOVA with Scheffé Post hoc test, $n = 6$; Fig. 20a). For both of these treatments, the increases in C:F ratio in ipsilateral muscles were significant relative to the contralateral muscles ($P < 0.05$ vs. 'contralateral', Student's t-test, $n = 6$; Fig. 20a) in the same animals (at levels indistinguishable from controls). CD data suggested a tendency for a decrease in muscles from prazosin treated animals relative to control animals, and an increase in ipsilateral muscles from extirpated animals, relative to control animals, however this wasn't the case (ns, between groups ANOVA with Scheffé Post hoc test, $n = 6$; Fig. 20b). The only significant change for CD was highlighted between the ipsilateral and contralateral muscles of extirpated animals ($P < 0.05$, Student's t-test, $n = 6$; Fig. 20b). MFA's remained approximately constant regardless of treatment (ns, between groups ANOVA with Scheffé Post hoc test, $n = 6$; Fig. 20c), although contralateral muscles from combination treated animals had a tendency to have a decreased MFA, relative to all other treatment groups (Fig. 20c).

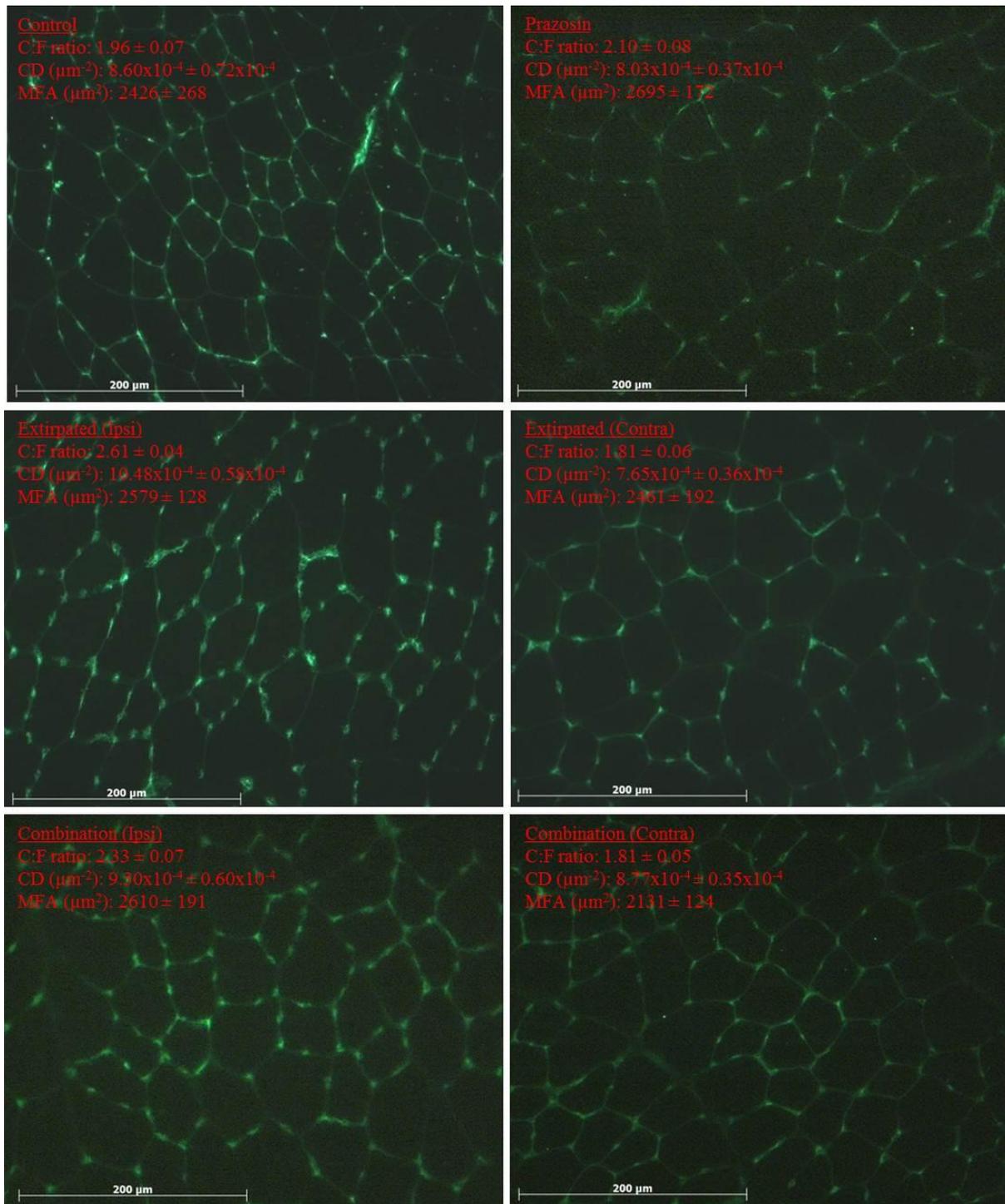


Fig 19: EDL muscle photomicrographs.

Photomicrographs show typical EDL muscle sections (at x20 magnification), stained for Lectin (Fluorescein conjugated *Griffonia (Bandeiraea) simplicifolia* Lectin), which were used to generate histological count data (C:F ratio, CD and MFA). For extirpated and combination animals, both the ipsilateral and contralateral EDL muscles were sampled and analysed, to show relative change. Four such photomicrographs across the section of each EDL muscle were averaged for each animal, before being compared to other animals in other treatment groups. Mean (\pm S.E.M.) C:F ratio, CD and MFA values for each treatment group ($n = 6$) are indicated.

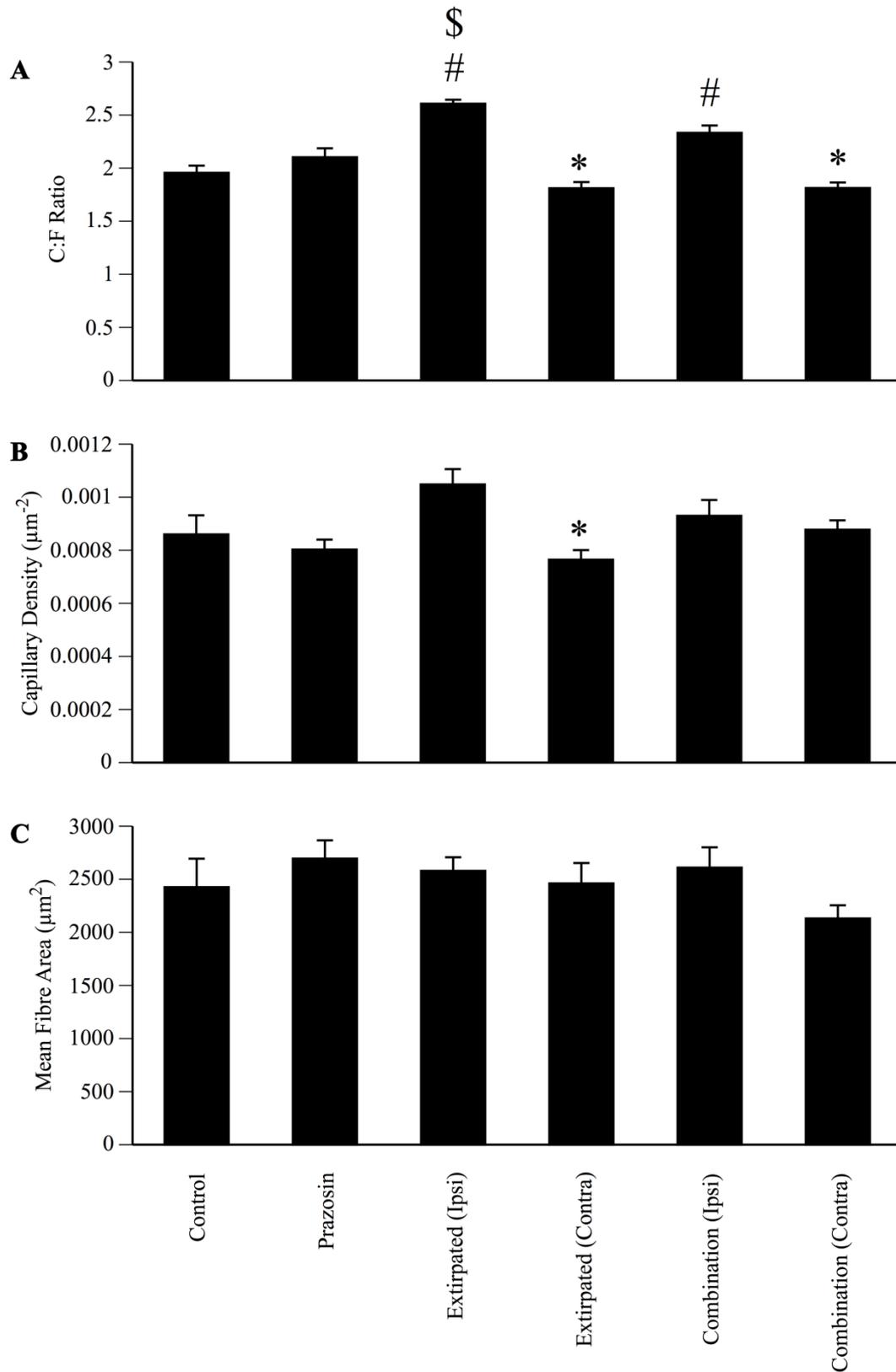


Fig 20: Histological counts.

A-C show mean (\pm S.E.M. error bars) C:F ratio, CD and MFA counts performed from stained EDL muscle sections sampled from control, prazosin treated, extirpated (ipsilateral and contralateral muscles) and combination treated (ipsilateral and contralateral muscles) animals ($n = 6$ for each). * $P < 0.05$ vs. 'ipsilateral' (Student's t-test). # $P < 0.05$ vs. 'control' and \$ $P < 0.05$ vs. 'prazosin' (between groups ANOVA with Scheffé Post hoc test). There was no significant difference in MFA between groups.

DISCUSSION

The final setup and protocol implemented allowed us to collect quality data, in replicate, from which we were able to draw appropriate comparisons.

Comparisons with the literature

This study aimed to build on the work of others (Egginton et al. 2011), that showed certain physiological angiogenic stimuli caused angiogenesis in a graded, not threshold manner. Further, preliminary work by Olgaard et al. (2008) had indicated that when two fundamentally different stimuli, that were shown to evoke two different types of angiogenesis, were applied concurrently, no additive effect in C:F ratio was produced. Unfortunately, our data is confounded by the fact that the administration of prazosin didn't seem to induce an increase in C:F ratio, like it does in the literature (Zhou et al. 1998a). Surgical extirpation however, did induce the expected increase in C:F ratio (Zhou et al. 1998b).

The functional hyperaemia responses observed during EDL muscle contraction were comparable to that seen in similar studies (Ray and Marshall 2009a; Ray and Marshall 2009b), although slightly different to muscle blood flow data obtained using radioactive microspheres (Hargreaves, Egginton, Hudlicka 1990). The tension generated by EDL muscles, across the treatment groups, was also comparable to that achieved by others (Egginton et al. 1998; Fulgenzi et al. 1998; Ray and Marshall 2009a; Ray and Marshall 2009b).

Surgical extirpation of the TA, induced compensatory hypertrophy of the EDL after 2 weeks, to the same degree compared to contralateral muscles ($\approx 20\%$), to that seen in the literature (Egginton et al. 1998). A parallel increase in C:F ratio, relative to the contralateral muscle was also evident (Zhou et al. 1998b). Our study used a 10 Hz contraction to assay EDL performance as it was the stimulation frequency that produced the maximum blood flow; this was different to the 4 Hz used in the literature (chosen for the same reason) (Hawker and Egginton 1999). There was however, no significant difference in blood flow or tension generated, between the two frequencies in our preliminary experiments and so general comparisons could be made. Blood flow data was not different to that seen in controls, like that in the literature (Egginton et al. 1998). Similarly, the generated tension here, was no different to that in controls, agreeing with some literature (Egginton et al. 1998), but not others (Frischknecht and Vrbova 1991), although the length of treatment in our study was far less, so this was not surprising. The FI for a 4 Hz contraction did not differ from that in contralateral muscles after 2 weeks (Egginton et al. 1998). For a 10 Hz contraction however there was a significant improvement in the fatigue resistance relative to that seen in controls; it took 8 weeks of treatment for such significance to be seen at 4 Hz (Egginton et al. 1998). Furthermore here, the increased C:F ratio (in parallel with the increase in muscle weight) was aligned with the improved FI. It seems that the compensatory hypertrophy is responsible for the improvement in fatigue resistance. Although not assayed for, this was likely achieved through a shift in fibre phenotype, from type IIb glycolytic fibres towards type I oxidative fibres (Degens et al. 1992; Yamaguchi et al. 1996). Finally, it is evident that in muscle that has undergone compensatory hypertrophy (of the whole muscle – muscle hypertrophy), that the increased muscle mass is accounted for by fibre hyperplasia (Antonio and Gonyea 1993) and not because of fibre hypertrophy (James 1973), as indicated by the constant MFA across treatment groups.

Prazosin administration did not induce an increase in C:F ratio in EDL relative to controls, like that seen in the literature (Fulgenzi et al. 1998; Zhou et al. 1998a). Our study used a 10 Hz contraction to assay EDL performance as it was the stimulation frequency that produced the maximum blood flow; this was different to the 4 Hz used in the literature (Fulgenzi et al. 1998). This was a study investigating fatigue resistance in ischaemic muscle (for combined TA and EDL) and the benefits in muscle performance of prazosin administration (hence having a similar setup to our own and why data [untreated controls vs. prazosin treated; ischaemic data ignored] can be compared). Blood flow data was elevated in prazosin treated animals to that seen in controls, like that in the literature (Fulgenzi et al. 1998; Milkiewicz et al. 2001). The generated tension and calculated FI in our study was no different to that in controls, agreeing with what seen for combined TA and EDL in the ischaemic muscle study (Fulgenzi et al. 1998) and for EDL alone in a different study (Ziada et al. 1984). Finally, there was no change in MFA or fibre phenotype, relative to controls, which is as expected (Hudlicka, Brown, Egginton 1992).

Data from combination treated animals was interesting as both the C:F ratio and FI (which are proportional) for their muscles had intermediate values to that seen for prazosin treated animals and extirpated animals (although these were not significant relative to controls, like extirpated animals were). This is intriguing as even though it seems that prazosin administration alone didn't increase C:F ratio, there is a decrease in the C:F ratio and FI from that seen in animals treated with extirpation only, in the combination treated animals. Thus it seems that prazosin and/ or a downstream component of the splitting angiogenesis pathway is interacting with the sprouting angiogenesis pathway, preventing any additive effects and furthermore, reducing the normal effects induced by extirpation alone. It seems prazosin is having an effect on blood flow, but not the desired effect on capillary growth, that normally

follows the systemic hyperaemia! A further confounder is the huge compensatory hypertrophy seen in these animals (significantly larger than for extirpated animals!); for these animals there wasn't a parallel relationship evident between muscle mass, C:F ratio and FI, like there was in extirpated animals.

'Additive angiogenesis'; not exactly additive!

Although our data has been confounded by the reality that one of our stimuli has not induced capillary growth, it could be suggested, like in Olgaard et al. (2008), that additive angiogenesis of the two stimuli used in this study, cannot be seen in any of the structural or functional readouts we assayed. In Olgaard et al. (2008), the C:F ratio for ipsilateral muscle in extirpated animals and combination treated animals was constant, whereas this study has shown that the ipsilateral muscle C:F ratio in combination treated animals is in fact less than that in extirpated animals. This is certainly not an additive effect. Thus, it seems that for physiological angiogenesis, a given stimuli may well induce blood vessel growth in a graded manner (Egginton et al. 2011), but different stimuli cannot be applied concurrently to yield an additive effect. This may be because the net angiogenic stimuli present in these animals is 'excessive' and supra-physiological. Thus, feedback regulation may be keeping angiogenic signalling under control, like an 'angiogenic brake'. This putative component of angiogenic signalling is likely to be an endogenous angiogenesis inhibitor, similar in nature to (or actually) thrombospondin-1 (Olfert and Birot 2011).

Aligning structure and function

Interestingly, when the range of C:F ratio's across the treatment groups were correlated by means of a regression plot, with the range of FI's for the 10 Hz stimulation, a trend emerges ($r^2 = 0.89$; Fig. 21a). For example, in the muscle of extirpated animals, there was a 33% increase in C:F ratio compared to control muscle, correlating with a 28% increase in FI. This was not the case when FI was compared with the maximal ipsilateral vascular conductance, for a 10 Hz stimulation; no such correlation was discernible ($r^2 = 0.0046$; Fig. 21b). These plots suggest that the fatiguability of the EDL is proportional to the C:F ratio of the muscle, rather than the net blood flow to the muscle; ergo the local capillary supply (local diffusive path length between capillary and muscle fibre) is more important than global perfusion (bulk flow to and from the capillary network), in determining fatigue resistance. This is in line with current thinking (Egginton and Gaffney 2010).

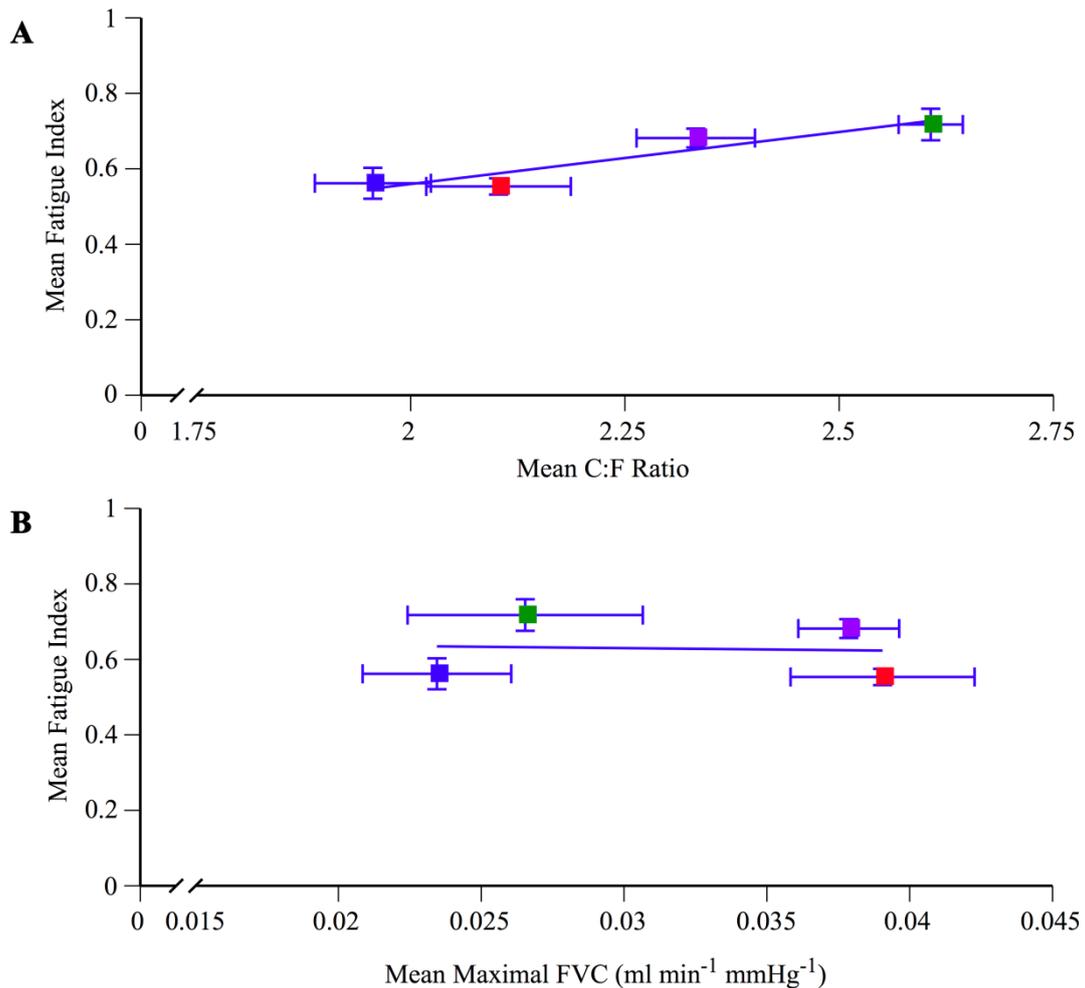


Fig 21: Alignment of structural and functional variables.

A-B are regression plots (S.E.M. xy error bars) correlating mean C:F ratio (A) and mean maximal FVC (B) with mean FI for a 10 Hz stimulation, across all treatment groups ($n = 6$; defined by a coloured point: control, prazosin, extirpated and combination animals). A: $r^2 = 0.89$; B: $r^2 = 0.0046$.

Study caveat

The additional 40 Hz stimulation yielded data with no treatment specific effects. This may be because of insufficient rest time (typically 1 h) after the 10 Hz stimulation for baseline levels to be restored, before the next stimulation was initiated. Two hours is known to be sufficient time for glycogen repletion of fibres (Hudlicka et al. 1994);

perhaps because a full two hours wasn't given for recovery, glycogen depleted fibres were more fatiguable, accounting for the lack of significance observed.

Critique and improvements

Should this *in vivo* setup and protocol be used again for future experiments, a number of improvements could be made. For example, it would be better if a specially designed board/frame, as used by the group before (Hudlicka et al. 1994), was used to completely eradicate animal movement upon stimulation of the nerve. This would ensure the most accurate tension was recorded by the force transducer. Alternatively, the animal could be securely fixed by drilling pins into the femur and securing said pins rigidly in place (Egginton and Hudlicka 1999). Methodological alterations would improve the quality and reliability of data obtained. Upon hindsight it is clear that bathing the muscle with room temperature saline, to avoid dehydration, wasn't enough; 37°C saline would have been better to maintain muscle surface temperature as close to that of body temperature as possible. Mitochondrial enzymes, active during cellular respiration, work optimally at body temperature, so any deviation from the optimal temperature will yield sub-optimal data. Another significant alteration to the method that should be made is the use of a 'water diary'. By recording the volume of water consumed by each animal, the exact prazosin dose required could be administered to each animal. This would have ensured sufficient dosing of those animals consuming prazosin, which may have resulted in a significant increase in C:F ratio in muscle of those animals, as seen in the literature!

Future directions

Apart from perhaps repeating the study once prazosin administration was refined to obtain conclusive data, future direction should focus on obtaining genomic and proteomic readouts of key angiogenic signalling components (by qPCR and Western blotting respectively), from the frozen muscle sampled. Such data may indicate why the combinational therapy was less effective than either of the individual treatments and to what extent each is driving angiogenesis. The makeup of the putative 'angiogenic brake' would also be an interesting avenue to explore, a candidate being thrombospondin-1 (Olfert and Birot 2011), if it is indeed this that is inhibiting excessive signaling. It would be instructive to perform such a study, as it would complement the structural and functional data derived here by probing the extent of interaction among the angiogenic signalling pathways.

Conclusion

The key finding of this study was that muscle overload, but not elevated shear stress (possibly due to insufficient prazosin consumption by the animals), resulted in an increase in EDL C:F ratio, which was correlated with its increased fatigue resistance for a 10 Hz stimulation. Application of both of the two stimuli yielded a lesser response in both C:F ratio and fatigue resistance, than for muscle overload alone. This suggests, at this 'level' of overload (degree of surgical ablation)/ shear stress (dose of prazosin), that an additive effect is not possible, perhaps due to modulation by endogenous angiogenesis inhibitors (Fig. 22). Translation of physiological angiogenesis research into the clinic should aim at establishing minimal-effort exercise regimes that generate maximal health gains.

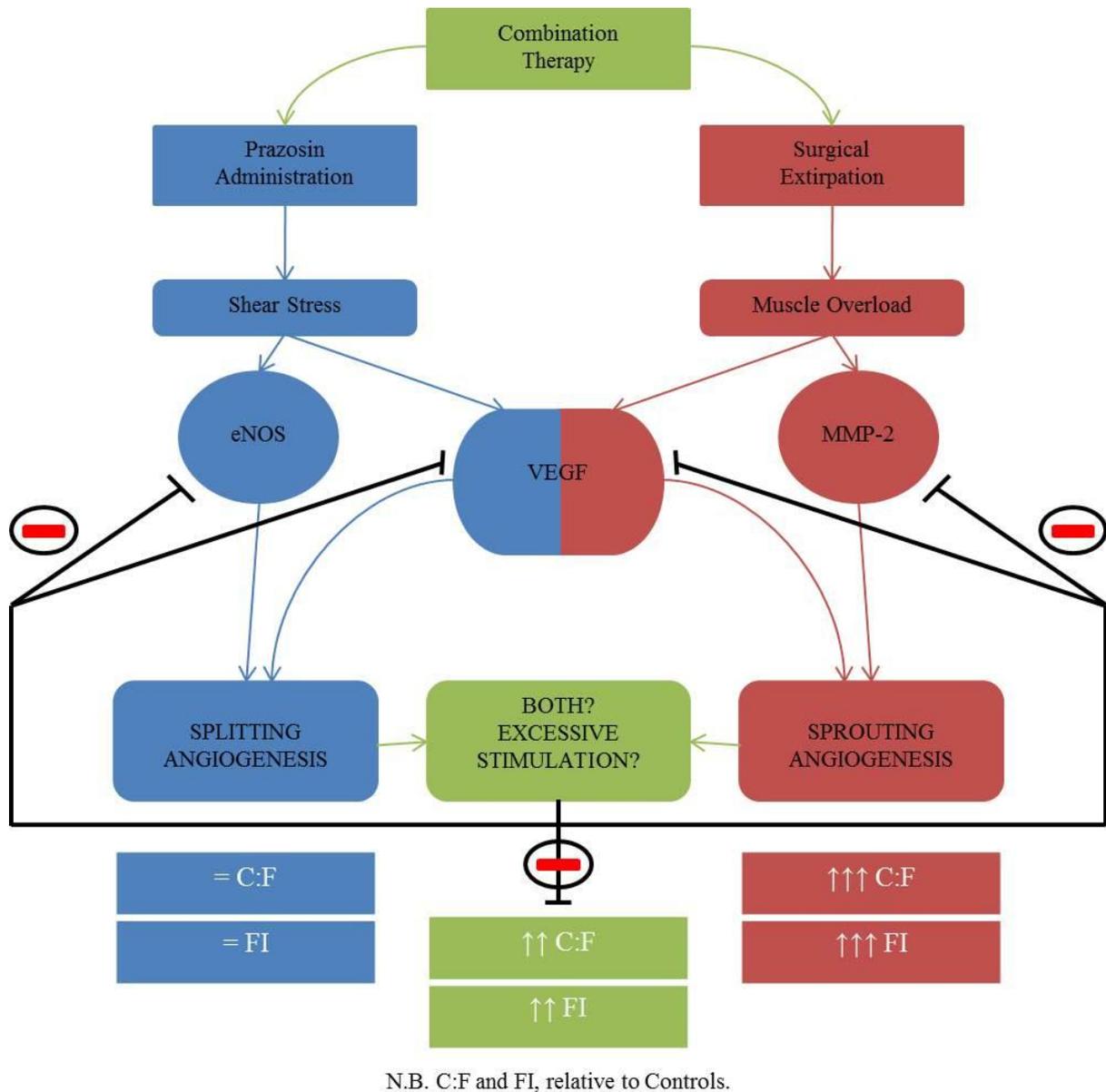


Fig 22: A much simplified schematic summarising the key points of this study.

The mechanical stimuli explored in this study induce two different types of angiogenesis, via similar and different chemical components of the angiogenesis signalling network. In this case, prazosin administration did not yield a significant increase in EDL C:F ratio, relative to controls, unlike surgical extirpation which caused a significant increase in EDL C:F ratio. Combination treatment resulted in EDL C:F ratio between the two treatments individually, though not significant relative to controls. EDL C:F ratio correlated with improved fatigue resistance in ipsilateral muscle from extirpated animals and combination treated animals compared to controls, although no change in fatigue resistance was evident in muscle from prazosin treated animals. It is of interest, to what extent each angiogenic signalling pathway contributes to the change seen in combination treated animal muscle structure and function and the identity and molecular activity (hypothesised by inhibitory signs) of the putative feedback regulator that orchestrates the ‘angiogenic brake’.

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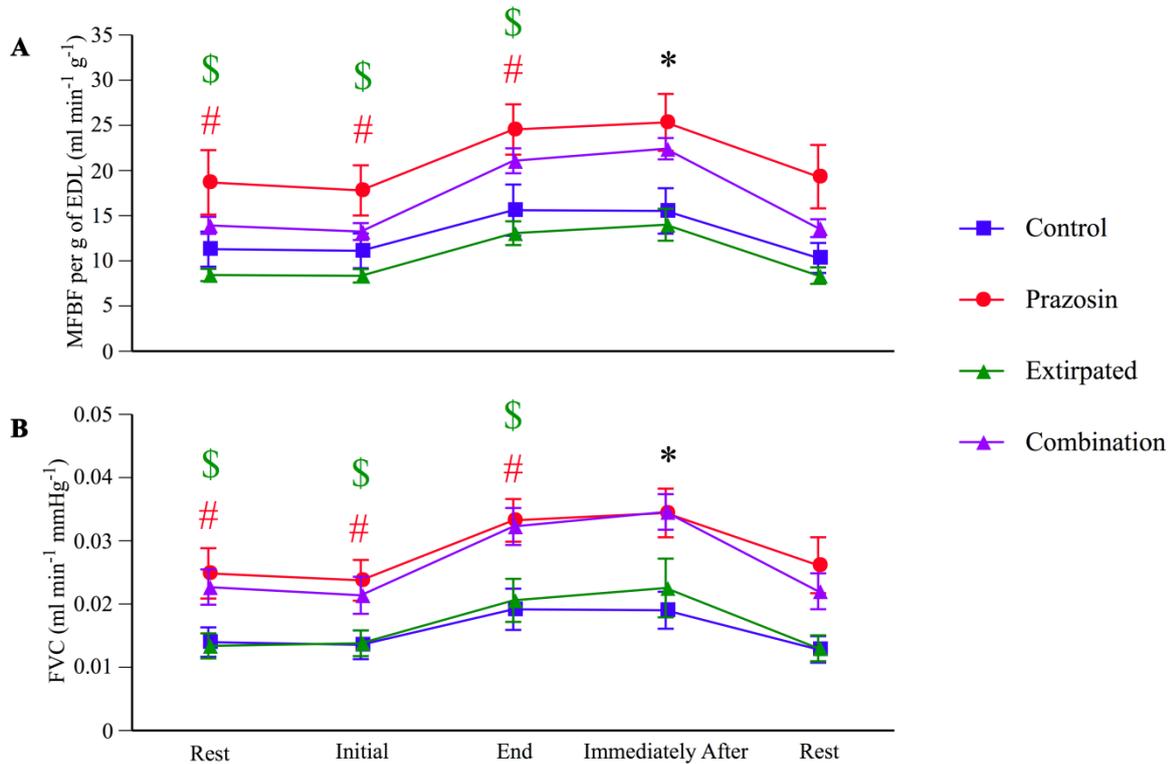
APPENDIX

Fig A1: Vascular response during 40 Hz isometric contraction of EDL muscle.

A-B show mean (\pm S.E.M. error bars) MFBF and FVC for control, prazosin, extirpated and combination animals ($n = 6, 6, 5$ and 6 respectively) upon stimulation of the EDL muscle, at 5 different time points, in Alfaxan-anaesthetised male Wistar rats. Contractions achieved by indirect supramaximal stimulation (0.3 ms square-wave pulse width, ≈ 10 V, 5 min) of the cut common peroneal nerve. * $P < 0.05$ vs. 'rest' (within groups ANOVA with Scheffé Post hoc test). # $P < 0.05$ vs. 'control' and \$ $P < 0.05$ vs. 'prazosin' (between groups ANOVA with Scheffé Post hoc test).

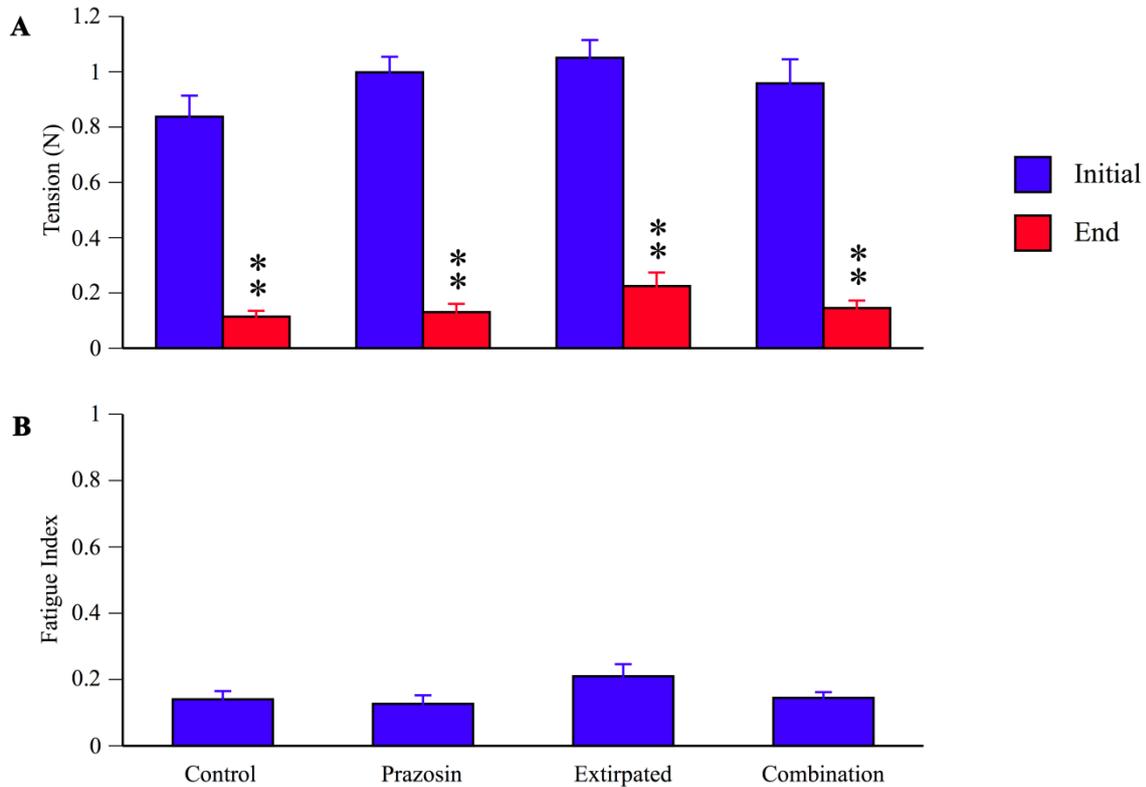


Fig A2: EDL tension and fatigue index during 40 Hz isometric muscle contraction.

A-B show initial and end mean (\pm S.E.M. error bars) EDL tension and overall fatigue index for control, prazosin, extirpated and combination animals ($n = 6, 6, 5$ and 6 respectively) upon stimulation of the EDL muscle, in Alfaxan-anaesthetised male Wistar rats. Contractions achieved by indirect supramaximal stimulation (0.3 ms square-wave pulse width, ≈ 10 V, 5 min) of the cut common peroneal nerve. ** $P < 0.01$ vs. 'initial' (Student's t-test). There was no significant difference in fatigue index between groups.

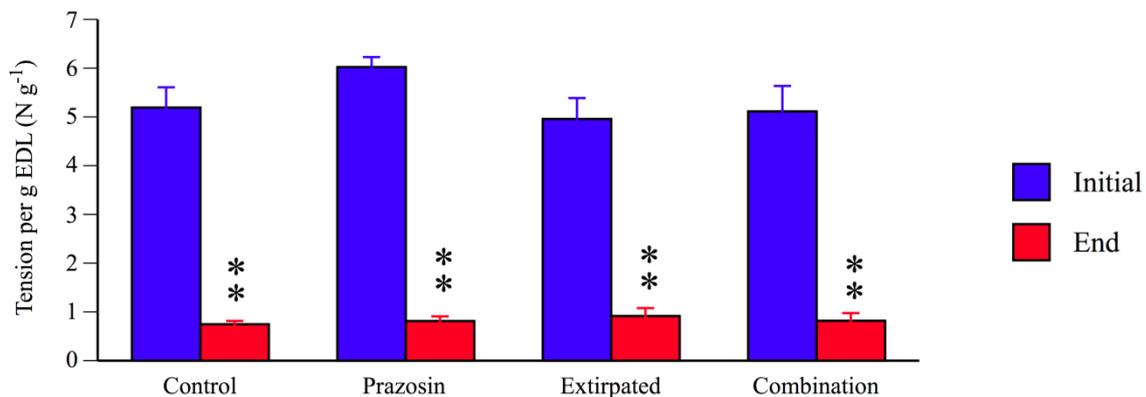


Fig A3: Specific EDL tension during 40 Hz isometric twitch contraction.

Graph shows the initial and end mean (\pm S.E.M. error bars) EDL tension, normalised for EDL muscle weight to allow for variation between groups, for control, prazosin, extirpated and combination animals ($n = 6, 6, 5$ and 6 respectively) upon stimulation of the EDL muscle, in Alfaxan-anaesthetised male Wistar rats. Contractions achieved by indirect supramaximal stimulation (0.3 ms square-wave pulse width, ≈ 10 V, 5 min) of the cut common peroneal nerve. ** $P < 0.01$ vs. 'initial' (Student's t-test).

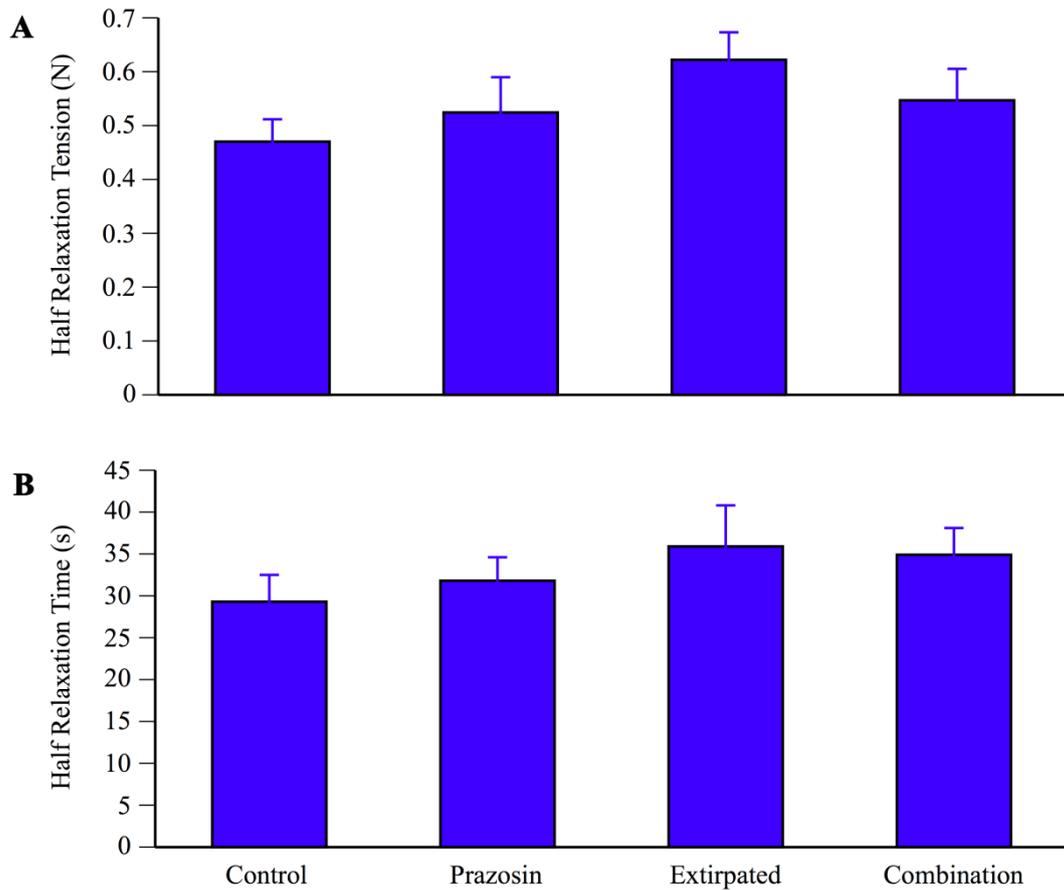


Fig A4: Half relaxation properties of EDL muscle undergoing a 40 Hz isometric twitch contraction.

A-B show mean (\pm S.E.M. error bars) EDL muscle half relaxation tension and half relaxation time for control, prazosin, extirpated and combination animals ($n = 6, 6, 5$ and 6 respectively) upon stimulation of the EDL muscle, in Alfaxan-anaesthetised male Wistar rats. Contractions achieved by indirect supramaximal stimulation (0.3 ms square-wave pulse width, ≈ 10 V, 5 min) of the cut common peroneal nerve. There was no significant difference in EDL muscle half relaxation tension or half relaxation time between groups.

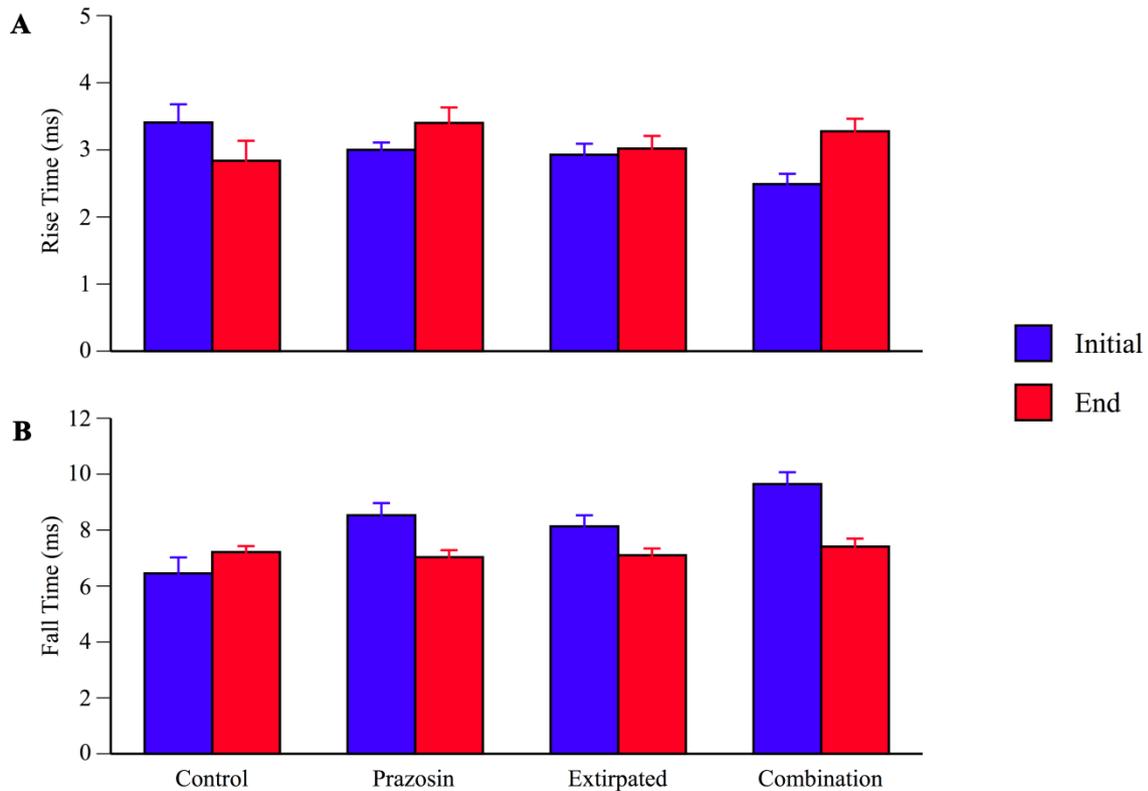


Fig A5: Muscle twitch rise time and fall times, for EDL muscle undergoing 40 Hz isometric twitch contraction.

A-B show mean (\pm S.E.M. error bars) individual EDL muscle twitch rise time and fall times, initially and at the end of the stimulation period, for control, prazosin, extirpated and combination animals ($n = 6, 6, 5$ and 6 respectively) upon stimulation of the EDL muscle, in Alfaxan-anaesthetised male Wistar rats. Contractions achieved by indirect supramaximal stimulation (0.3 ms square-wave pulse width, ≈ 10 V, 5 min) of the cut common peroneal nerve. There was no significant difference in either rise time or fall times between group during the initial or end period of the bout of contraction.