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Autologous pump-perfused rat hind limb preparation for investigating muscle function and metabolism in vivo

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Abstract

Objective Oxygen delivery, underpinned by vascular tone, is the principle limiting factor in the study of skeletal muscle physiology, particularly during muscle contraction. The aim of this study was to develop an autologous perfused rat hind limb preparation for the study of skeletal muscle contractile function.

Methods Adult Wistar rats were surgically prepared using a by-pass system for pump-controlled arterial blood flow to, and venous return from the hind limb during periods of quiescence and twitch contraction of the gastrocnemius-plantaris-soleus muscle bundle.

Results During rest, hind limb perfusion pressure (102 ± 5 mmHg) was not different to systemic arterial pressure (99 ± 4 mmHg). Hind limb pressure was responsive to vasoconstrictors and vasodilators (±50 mmHg). The arterial PO2 (100 ± 3 mmHg), O2 saturation, and acid–base balance (pH: 7.42 ± 0.01) contributed to resting hind limb (a-v)O2 difference (4.8 ± 0.5 mL/100 mL) and VO2 (0.31 ± 0.03 μmol/g/min wet weight). Repetitive isometric twitch tension (1 Hz, 0.05 ms, 10 minutes) was best maintained at a flow rate of 2 mL/min (VO2 increased fivefold during muscle contraction) and efficiency of oxygen use increased from 0.27 ± 0.08–0.52 ± 0.07 N/μmol/min.

Conclusion The autologous rat hind limb provided resting vascular tone allowing maintenance of perfusion pressure at flows within the physiological range. Oxygen delivery supported repetitive twitch contractions and facilitated measurement of active metabolism.

Keywords

rat hindlimb, autologous perfusion, skeletal muscle, oxygen consumption

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Title

Autologous pump-perfused rat hindlimb preparation for investigating muscle function and metabolism *in vivo*.

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Running Title

Autologous pump-perfused rat hindlimb

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Results: During rest hindlimb perfusion pressure (102±5 mmHg) was not different to systemic arterial pressure (99±4 mmHg). Hindlimb pressure was responsive to vasoconstrictors and vasodilators (±50 mmHg). The arterial PO_2 (100±3 mmHg), O_2 saturation, and acid-base balance (pH: 7.42±0.01) contributed to resting hindlimb (a-v)O_2 difference (4.8±0.5 mL.100 mL^{-1}) and VO_2 (0.31±0.03 μmol.min^{-1}.gww^{-1}). Repetitive isometric twitch tension (1 Hz, 0.05 ms, 10 min) was best maintained at a flow rate of 2 mL.min^{-1} (VO_2 increased 5-fold during muscle contraction) and efficiency of oxygen-use increased from 0.27±0.08 to 0.52±0.07 N.μmol^{-1}.min^{-1}.

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Abbreviations

PO_2 (partial pressure of oxygen)

PaO_2 (partial pressure of arterial oxygen)

PvO_2 (partial pressure of venous oxygen)

PCO_2 (partial pressure of carbon dioxide)

$PaCO_2$ (partial pressure of arterial carbon dioxide)

$PvCO_2$ (partial pressure of venous carbon dioxide)

CaO_2 (arterial oxygen content)

$(a\text{-}v)O_2$ (arteriovenous oxygen difference)

Na^+ (sodium)

K^+ (potassium)

Cl^- (chloride)

HCO_3 (bicarbonate)

RBC (red blood cell)

Hb (haemoglobin)

P_{Tmax} (peak twitch tension)

Introduction

In laboratory perfusion systems, the importance of reproducing *in vivo* conditions, such as using the higher oxygen carrying capacity of blood during perfusion, are recognised in studies of both heart (35, 47) and liver metabolism (46). The direct comparison of blood perfusion to buffer perfusion systems in the metabolically demanding beating heart demonstrates better long term stability and maintenance of function when using whole blood (13, 35).

The *in situ* perfused rat hindlimb has become a generally accepted means of studying resting and contracting muscle metabolism. The *in situ* preparation has been progressively modified(9, 10) from that described by Ruderman and coworkers (40). The choice of hindlimb perfusate has progressed from human erythrocyte (14, 23, 37, 40) to fluorocarbon emulsions(26), to cell free perfusate (9, 10, 30, 49, 50) and more recently, bovine erythrocyte (16, 31, 38, 51). A number of factors make aspects of the *in situ* perfused rat hindlimb preparation suitable for the study of muscle metabolism. Firstly, the hindlimb consists mainly of skeletal muscle with very little adipose tissue. Secondly, normal bone, muscle, nerve and circulation relationships are minimally disrupted. Finally, control and post-perfusion muscle biopsies are readily obtainable for the measurement of intramuscular energy store, metabolites, enzyme activity and pH.

Nevertheless, some aspects of the perfusion system detract from the *in situ* model's application. It is estimated that perfusate can be re-circulated twelve to eighteen times over the duration of one experiment (38, 40). Re-circulation in large quantities can lead to gradual loss of perfusate volume to the systemic circulation

through capillary fluid shift. Moreover, during recirculation there is no clearance of metabolic waste products such as blood lactate and CO_2. One-pass perfusion systems overcome the problems of re-circulation (40), cancelling the need for repeated perfusate oxygenation and allowing for the separation of red blood cell and hindlimb metabolism. However, physiological changes in blood, such as occur in moderate to heavy exercise, are not permitted through delivered fresh perfusate. In short, both the re-circulating and the one-pass systems may compromise physiological metabolic responses to skeletal muscle stimulation.

The nature of the *in situ perfused* preparation is such that, in the absence of brainstem and baroreceptor activity, all direct neurological control of the hindlimb vasculature is lost, resulting in loss of sympathetic tone and generalised vascular dilation. As a consequence, very high perfusate flow rates are required to sustain the hindlimb perfusion pressure in the physiological range. Flows range from 6-8ml.min^{-1} during resting conditions (43, 51) to 8-20ml.min^{-1} during contractile conditions (2, 38, 43). These far exceed measures of rat hindlimb blood flow *in vivo*(15, 27, 29). Even with these high flow rates, hindlimb pressure is often less than 50mmHg (9, 31, 51). In addition, when flow rates fall below 6ml.min^{-1} in the *in situ* perfused preparation, cyanotic discolouration of the feet can occur, lactate production can be excessive and there is a high prevalence of hindlimb oedema (40) especially with cell free perfusates (2). Both high non-physiological flow rates and low pressures impact on hindlimb skeletal muscle oxygen consumption. Even with the use of bovine erythrocytes, hindlimb oxygen consumption can be regarded as low relative to delivery (2, 16, 31), suggesting overly rapid capillary transit.

In the living canine, use of the animal's own blood through controlled autologous perfusion has been demonstrated in studies of oxygen delivery and uptake in combination with skeletal muscle function (21, 24, 42). Because the animal remains alive and breathing, the canine autologous model is highly regarded for the provision of optimal tissue oxygenation (matched with physiologically relevant blood flow) and for studying *in vivo* conditions while still being able to: sample and measure metabolic and physiological responses; and control and modulate various parameters such as hindlimb flow (17-21). While the canine is ideal for technical ease of use, it is less suitable than the rat for holding costs and especially long term metabolic and dietary studies, where a wide range a relevant data is already established and quantity of high cost diet ingredients is less prohibitive. To our knowledge there has been no validation or application of perfusing the hindlimb of the living rat using the animal's own blood, known as autologous perfusion.

The aim of this study was to demonstrate that the canine hindlimb perfusion model (45) can be adapted to the smaller laboratory rat under anaesthetic and that controlled autologous perfusion can satisfy oxygen and substrate delivery, metabolic waste clearance, vascular responses, and support metabolic evaluation of contractile function. It is the premise of this study that it is possible to achieve autologous perfusion in the living rat and this method can provide optimal tissue oxygenation and perfusion to support muscle contraction for the study of rat skeletal muscle and vascular system physiology and active metabolism.

Materials and Methods

Animals

Adult Wistar rats were obtained from the Gore Hill Animal Research Laboratory (Sydney, Australia) and housed in the University of Wollongong's Animal facility maintained at 23°C -25°C with 12 h light/dark cycle. Animals had free access to food (rat chow) and water. Experiments were conducted according to NHMRC/CSIRO/ACC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1997). A total of 37 animals were used in this series of experiments distributed separately as; Protocol 1 (n=18 resting condition with 6 rats given Evan's blue dye; n=4 hyperventilation; n=9 vascular tone). Protocol 2 (n=6 stimulated contraction).

Surgical preparation for ventilation, muscle stimulation and perfusion.

Rats were anaesthetised (sodium pentobarbital 6 mg.100 g^{-1} i.p.) with supplementary injections (2 mg.100 g^{-1} i.p.) as required during the procedure. Experiments were performed with the rat and perfusion system enclosed in a heated perspex chamber (32.2 ±0.3°C) with animal body temperature maintained at 37-38°C with the further aid of a heating lamp placed above the rat. Rectal temperature was monitored during the procedure and protocols.

The rats were prepared for artificial ventilation and blood pressure recording by cannulation of the trachea and left carotid artery. Rats were ventilated to ensure constant high arterial oxygen (rodent ventilator 7025, Ugo Basile, Italy) at a rate of 60 breaths. min^{-1} and tidal volume set according to animal body weight (1 mL.200 g^{-1}).

The rat was turned on its right side and a small incision was made in the skin, 1 cm below the iliac crest and the gluteal muscles were separated to expose the sciatic nerve trunk. A bipolar electrode (Grass Instrument Division, USA) was placed under the nerves for direct electrical stimulation of the trunk supplying the muscles of the gastrocnemius-plantaris-soleus muscle bundle. The nerve was crushed above the stimulation point to prevent retrograde conduction. Saline-soaked gauze was placed over the incision to prevent the nerve from drying.

The animal was then turned on its back and the left leg was secured at the knee and foot (rigid cable tie) to prevent movement artefact during stimulation. The gastrocnemius-plantaris-soleus muscle group tendons were tied with non-compliant silk and connected to a force transducer (FT03C, Grass Instrument Division, USA). Saline-soaked gauze was placed over the muscles to prevent them from drying.

A cannula was inserted into the right femoral artery (non-perfused leg) towards the heart and was connected to a re-sealing flexi-tube, passed through a peristaltic roller pump (Gilson Miniplus 3) and connected to a cannula that was inserted into the left femoral artery (perfused leg) towards the foot. The cannula was passed well down the femoral artery (consistently 1.5cm) to close off unwanted side branches and restrict perfusion to the muscle beds of interest. Other side branches were ligated. A T-junction inserted in the blood flow line on the perfused hindlimb side of the pump was connected to a pressure transducer (Argon CDXIII, Maxim Medical, USA) for measurement of hindlimb perfusion pressure. A Windkessel pressure dampener was inserted into the blood flow line to alleviate any large pulsatile changes in pressure.

A cannula was inserted into right jugular vein at one end and the left femoral vein of the perfused leg at the other for passive venous return to the heart and lungs for re-oxygenation. Blood could be sampled from the arterial and venous lines through in-line, thick walled, self-sealing silicone tubing located within centimetres from the arterial and venous vasculature.

When all cannulae were in place, passive re-flow was allowed from the right femoral artery of the control hindlimb to the left femoral artery of the perfused hindlimb. When all tubes had filled with the animal's own blood (< 60 s) the pump was engaged to provide a constant flow rate of 1ml.min^{-1} through the hindlimb vasculature. The perfused hindlimb was hypoxic for less than 2 min throughout surgical preparation. Prior to insertion, all cannulae (Dural Plastics Internal Diameter 0.58 mm Outside Diameter 0.96 mm) and pump tubing were fluid filled with saline containing 6% dextran (w/v) and heparin (5000 IU.100 mL^{-1}). In five rats, 0.2 mL of dye injected into the arterial line took 14.4±0.4 s to flow from the arterial catheter tip to first appearance at the venous sampling point at a perfusion rate of 1 mL.min^{-1}. The total volume of extracorporeal perfusate was measured at 2.5 mL.

Muscle contractions were initiated at optimal length for each muscle, defined as the muscle length at which the highest isometric twitch force could be generated in response to an electrical stimulus (0.05 ms duration, supramaximal voltage (6-15 V)) applied to the sciatic nerve (gastrocnemius-plantaris-soleus). Initially the muscle group was held at low tension, stimulated via the sciatic nerve and the force recorded. The length of the muscle group was then gradually increased using micrometer adjustments,

with stimulation at each length until the maximum twitch force was generated. A recovery period of 2 min was allowed between contractions while setting optimal length.

Protocol 1: Stability under resting conditions

Eighteen male Wistar rats, 12-14 weeks old and weighing 667±17 g, were used to assess and validate conditions of autologous pump-perfusion. Hindlimbs were initially perfused at 1 $ml.min^{-1}$ for 30 min derived from published rat hindlimb blood flow *in vivo* under resting and contracting conditions (15, 27, 29). The animals were then randomly allocated to one of three different groups with hindlimbs perfused for a further 120 min at either: 1 $mL.min^{-1}$ (Group 1); 1.5 $mL.min^{-1}$ (Group 2); or 2 $mL.min^{-1}$ (Group 3) (n=6 per group). Flow rates were gradually adjusted over a period of 3 min. Baseline arterial and venous blood samples were collected at the completion of the initial 30 min perfusion. Further arterial and venous blood samples (400 μL) were collected at 30 min intervals. Of each 400 μL blood sample, 240 μL was presented to the blood gas analyser for analysis of PO_2, PCO_2, Na^+, K^+, Cl^-, pH and haemoglobin. The remaining 160 μL were spun down on a bench top micro centrifuge, the plasma removed and frozen for later analysis of lactate and pyruvate. Hindlimb perfusion pressure and whole animal systemic blood pressure were recorded continually during the 120 min (200 Hz) using Labview for Windows software. At the completion of the perfusion, skeletal muscles from the perfused hindlimb were collected, freeze clamped and stored in marked aluminium foil at -80°C until further analysis of muscle glycogen.

Blood distribution. Blood distribution within the hindlimb was qualitatively investigated using six animals randomly selected from amongst the three different groups. At the completion of the perfusion trial, 1ml of Evan's Blue dye was injected into the arterial perfusion line. The flow was continued for 60 s ensuring dye appearance in the distal tubing, the muscles of the entire hindlimb were dissected and carefully separated into stained (perfused) and unstained (non-perfused) sections and weighed.

Hyperventilation. To determine the effects of ventilation, a separate group of animals (n = 4) was evaluated during two 60 min periods of constant resting perfusion at different ventilation rates. In the first period, lung ventilation and hindlimb flow were maintained at control levels and arterial and venous samples were collected every 15 min. After 60 min, ventilation rate was increased from 60 breaths.min^{-1} to 85 breaths.min^{-1} for a further 60 min while the breathing depth and hindlimb flow were kept constant. Arterial and venous blood samples (240 μL) were collected every 15 min for analysis of blood gas and haemoglobin.

Vascular tone: In nine rats at the end of 120 min autologous perfusion, a single dose of vasoactive drug (isoprenaline (10 μmol), histamine (10 μmol) or noradrenaline (16 μmol) (n=3 per group)) was delivered directly to the arterial side of the perfused hindlimb in a bolus of 0.2 mL. In a further nine rats, flow was stopped to the hindlimb (clamp) for a period of 3 min and then re-established (1 mL.min^{-1}) for a further 5 min.

Protocol 2: Stability with muscle contraction

Six male Wistar rats, 12-14 weeks old, (534±56g), were used to assess and validate conditions of hindlimb autologous pump-perfusion with repeat muscle twitch contractions over a time period of 120 min. Animals were prepared as outlined in the general methodology section including preparation of the sciatic nerve for stimulation of the gastrocnemius-plantaris-soleus muscle group. The hindlimbs of all animals were perfused initially at a flow rate of 1 $mL.min^{-1}$. Baseline arterial and venous blood samples (400 μL) were collected after 30 min of control perfusion.

After 30 min perfusion, muscle contractions were initiated by repetitive stimulation delivered to the sciatic nerve for 10 min (supramaximal voltage, 1 Hz, 0.05 ms duration). Twitch contractions of the gastrocnemius-plantaris-soleus muscle group were investigated at flow rates of 1 $ml.min^{-1}$, 1.5 $ml.min^{-1}$ and 2 $ml.min^{-1}$ in each animal and studied in random order. Flow rate at 1 $ml.min^{-1}$ was restored for 30 min recovery between stimulation bouts.

Arterial and venous blood samples were taken at the end of each 30 min rest period and then during the 10 min stimulation bout, just prior to its completion. Hindlimb perfusion pressure and twitch force production of the gastrocnemius-plantaris-soleus muscle bundle ($N.g^{-1}$ wet weight) were recorded continuously during the 10 min stimulation bouts. From the twitch force record, analyses were made of initial twitch tension, peak twitch tension (P_{Tmax}) and final twitch tension during the stimulation period (10 min). Immediately following the three stimulation protocols the hindlimb muscles were dissected free and rapidly frozen for storage. Each animal was released

from the artificial ventilator and euthanized by rapid exsanguination while still under anaesthetic. This process took 10-15 s.

Measurements

Blood pressure and twitch force. Analogue force and pressure signals were referenced to ground, amplified (Onspot Australia) and digitised. The data acquisition software, Labview for Windows (National Instruments, Austin, TX, USA) was used to collect both pressure and twitch force simultaneously during the perfusions at a sampling rate of 200 Hz.

Blood. Blood samples were drawn from the perfusion line via re-sealing silicone sections of the cannulae located proximal to the pump (arterial) and on the venous return line of the hindlimb. Two different blood gas analysers were used over the course of these experiments for measurement of arterial and venous of PO_2, PCO_2, Na^+, K^+, pH, Hb and O_2 saturation. One machine (BGElectrolytes, Instrumentation Laboratories, USA) required presentation of 240 μL for full analysis of blood, necessitating a 400 μL sample. For the majority of experiments a more efficient machine ((ABL77 Radiometer, Copenhagen) was used, requiring only 80 μL and a smaller sample size. Both analysers were routinely calibrated against control standards. The reproducibility of arterial blood-gas measures across the course of each experiment established the stability of the instruments. The blood remaining from each sample was spun down on a bench top micro centrifuge (Milipore), the plasma removed and frozen for later analysis of metabolites. Erythrocytes from the plasma collections were re-suspended in an equal volume of saline and re-injected into the venous side of the perfusion. This maintained haemoglobin levels above 12.5

g/100 mL whole blood. Procurement of the ABL77 blood gas analyser with smaller sample size (~80 μL) permitted more frequent sampling in subsequent experiments.

Oxygen consumption was calculated according to the Fick equation. Oxygen consumption ($\mu mol.g.^{-1}.min^{-1}$) = ($\underline{(a\text{-}v)O_2}$)x(hindlimb flow rate)/(weight of perfused muscle). Arterial and venous O_2 content ($mL.100\ mL^{-1}$) were calculated using oxygen content = (1.39 x Hb x RBC saturation (%) + (0.003 x PO_2), where: 1.39 = mL O_2 bound to 1 g Hb; Hb is the blood haemoglobin concentration ($g.100\ mL^{-1}$);

RBC saturation (%) is the percentage saturated of haemoglobin with O_2 calculated from PO_2, PCO_2 and pH for blood specific P_{50}; PO_2 is the partial pressure of O_2 in whole blood (mmHg); 0.003 = constant for dissolved O_2 in 100ml of plasma at 38ºC

Muscle samples. With the left limb perfused using the blood from the contralateral limb, skeletal muscle in the right limb was devoid of any blood supply. Control muscle samples (gastrocnemius, plantaris, soleus) were therefore taken from the right leg immediately prior to the right femoral artery cannulation, to be used as within-animal control tissue. At the completion of each experiment and while the blood was still flowing, left leg (perfused) muscle samples (gastrocnemius, plantaris, soleus) were rapidly extracted. All muscles were freeze-clamped and stored for later analysis. Additional muscle groups (deep posterior tibial, extensor digitorum longus, tibialis anterior) from the perfused hindlimb were collected to establish total weight of the muscle involved in the perfusion.

Biochemical Assays

Lactate and pyruvate. Stored plasma samples (200 μL aliquot) were analysed for lactate and pyruvate using Lactate and Pyruvate Kits (Sigma Diagnostics, Australia).

The method was adapted to allow samples to be analysed on the Cobas Mira (Roche Diagnostics, Australia). Absorbance was read at 340 nm wavelength and compared to prepared controls (Sigma Diagnostics, Australia).

Muscle Glycogen. Stored 100 mg sections of muscle were placed immediately into polypropylene tubes containing 2 mL potassium hydroxide (1 M) (Sigma Diagnostics, Australia) to digest the tissue. From the digested samples 100 μL duplicate aliquots were placed onto Whatman 3 mm chromotography paper (2 cm x 2 cm) along with glycogen standard (0-100 μL) (2 mg/mL) (Sigma Diagnostics, Australia). When the paper had dried, each sample (including standards) was placed into a clean tube and washed with 2 x 2 mL analytical grade ethanol (Sigma Diagnostics, Australia) to remove free glucose. The remaining glycogen was then set to the paper with 1 mL acetate (Sigma Diagnostics, Australia). Amyloglucosidase and glucose assay mixture (2 mL) (Roche Diagnostics, Australia) were added to each duplicate sample and the colourimetric reaction was stopped after 45 min when 200 μL was taken out of the tubes and placed in a micro-plate for glucosyl unit content to be read at A510 nm.

Statistical Analysis

Results are expressed as mean±SEM. All statistics were performed using Statistix for Windows (Tallahassee, USA). Data was analysed using either one or two-way ANOVA. Comparison of means was performed post-hoc using Bonferroni's Test. Alpha was set as $p<0.05$.

Results

Protocol 1

Baseline. All animals from Group 1 (638±45 g), Group 2 (638±37) and Group 3 (667±17) were included in the baseline data (N=18). There were no significant differences for lower hindlimb (Group 1: 6.25±0.34 g; Group 2: 6.60±0.55 g; Group 3: 6.04±0.35 g) or gastrocnemius-soleus-plantaris muscle weight (Group 1: 3.67±0.18 g; Group 2: 3.68±0.28 g; Group 3: 3.53±0.17 g). Mean tidal volume was 3.0±0.1 mL which at the breathing rate of 60±1 breathes.min^{-1} provided a mean ventilation rate of 182±8 mL.min^{-1}. There was no difference between mean systemic blood pressure (99±4 mmHg) and hindlimb perfusion pressure (102±5 mmHg) at rest. Table 1 represents baseline data of blood gases, electrolytes and metabolic products in all animals combined, taken following 30 min of perfusion at 1 mL.min^{-1}. The P*a*O_2 in each group was approaching 100 mmHg (Group 1: 98 ± 1mmHg; Group 2: 97 ± 1mmHg; Group 3: 98 ± 1mmHg), resulting in a mean arterial oxygen content (C*a*O_2) of 22.6±0.25 mL.100 mL^{-1} in whole blood. Compared to the arterial samples, a significant drop in venous pH, P*v*O_2 and ultimately C*v*O_2 indicated the uptake of oxygen across the resting hindlimb. The mean arterial-venous (*a-v*)O_2 difference (4.8±0.05 mL.100 mL^{-1}) was 22% of the initial C*a*O_2. The oxygen consumption for the combined groups calculated using the Fick method was 0.31±0.03 μmol.min^{-1}.g^{-1} (wet.wt). Resting hindlimb oxygen consumption was accompanied by arteriovenous differences in PCO_2, PO_2, and pH (table 1).

There were no significant differences between Groups 1, 2 or 3 at baseline. This included mean hindlimb perfusion pressure (figure 1), arterial PO_2 (Group 1: 102±4

mmHg; Group2: 96±4; Group 3: 103±6), venous PO_2 (Group 1: 47±2 mmHg; Group2: 49±3; Group 3: 45±4), (*a-v*)O_2 difference and oxygen consumption relative to the muscle mass perfused (figure 2A/B). There were no significant differences between groups for the arterial or venous electrolytes Na^+ or K^+ (data not shown).

Perfusion for 120 min. In Group 1 perfused at 1 mL.min^{-1} the hindlimb perfusion pressure did not alter from baseline over the 120 min, however, higher flow rates in Group 2 and Group 3 significantly increased the hindlimb perfusion pressure (figure 1) and this was sustained for the entire 120 min while the flow rate was elevated. Despite increased perfusion pressure, there was no significant change in hindlimb foot volume after 120 min perfusion with any flow rate (table 2). Muscle wet weight did increase by approximately 30% in the perfused hindlimb compare to the non-perfuse control but was not a factor of flow (and pressure per say) (table 2).

Blood haemoglobin decreased over the course of the perfusion from 16.3±0.4 g.100 mL^{-1}at baseline to 13.9±0.4 g.100 mL^{-1} after 120 min. With partial return of sampled blood by resuspended red blood cells in saline, haemoglobin was maintained at all times above 12.5 g.100 mL^{-1} with no significant difference between groups. In all groups, arterial PO_2 (Group1: 102±4 mmHg; Group 2: 96±4; Group3: 103±6) and therefore red blood cell oxygen saturation (Group1: 98.2±0.3%; Group2: 97.3±0.3; Group3: 98.0±0.4) were sustained and constant over the period of 120 min, independent of hindlimb perfusion rate. As a result, arterial oxygen carrying capacity was sustained above 18.5 mL.100 mL^{-1} whole blood throughout.

In Group 1, the venous PO_2 (120 min = 51.0±1.0 mmHg) and consequently venous red blood cell saturation (120 min = 76.1±1.6 %), together with the $(a\text{-}v)O_2$ difference (figure 2A) and oxygen consumption (figure 2B) remained constant over 120 min. At higher hindlimb flow rates the venous PO_2 of Group 2 (120 mins = 54.0±3.0 mmHg) and Group 3 (120 min = 73.0±4.0 mmHg) and red blood cell saturation after 120 min, 86.7±1.0 and 92.5±0.8 % respectively, increased with increased hindlimb flow rates. The result was a significantly lower $(a\text{-}v)O_2$ difference ($p<0.05$) (figure 2A) and significantly lower oxygen consumption ($p<0.05$) (figure 2B) compared to Group 1.

Plasma lactate levels in arterial and venous blood did not exceed 2.5 mM at any stage of the 120 min perfusion period. (Arterial lactate (mM) - Group1: 1.72±0.68; Group2: 1.63±0.41; Group3: 2.19±0.78; Venous- Group1: 2.06±0.32; Group2: 1.76±0.22; Group3: 2.24±0.60). Arterial and venous lactate/pyruvate ratios were constant and not significantly different throughout 120 min perfusion (Arterial- Group1: 12.7±0.6; Group2: 11.5±1.8; Group3: 14.7±2.0; Venous- Group1: 13.7±1.0; Group2: 11.8±2.2; Group3: 12.7±1.3). Glycogen content in the plantaris and soleus muscles decreased as a function of flow (figure 3A). The highest loss occurred with 2 $mL.min^{-1}$. This corresponded to the lowest consumption of oxygen and the highest absolute venous lactate efflux. No difference was seen in the blood concentration of Na^+ or K^+ over the 120 min of perfusion.

The staining of muscles by Evans Blue dye qualitatively demonstrated the distribution of blood through to the lower hindlimb (86±0.86% by wet weight). Muscles of the upper hindlimb had minimal staining (for example quadriceps

1.17±0.74% by wet weight), while all muscles in the lower hindlimb had a high proportion of staining, based on separated wet weight (soleus 100±0%, plantaris 83.82±7.50%, gastrocnemius 91.18±3.82%, deep tibial 83.72±6.75%, tibialis anterior 74.20±7.60% and extensor digitorum longus 83.90±7.33%).

Hyperventilation. In rats subjected to 60 min of hyperventilation, the $Pa\mathrm{O_2}$ was maintained above 100 mmHg throughout, whereas the $Pa\mathrm{CO_2}$ was significantly reduced from 31±1 mmHg to 21±1 mmHg and $Pv\mathrm{CO_2}$ from 57±1 mmHg to 38±1 mmHg. Venous $\mathrm{PO_2}$ did not fall below 50 mmHg and venous oxygen saturation remained >80%. Hindlimb oxygen consumption was significantly lower during hyperventilation compare to normal ventilation ($p<0.05$) (Normal: 0.4±0.1; Hyperventilation: 0.1±0.04 $\mu mol.g^{-1}.min^{-1}$).

Demonstration of vascular tone. The intra-arterial injection of vasodilators isoprenaline (10 μmol) or histamine (10 μmol) into the hindlimb circulation significantly decreased hindlimb perfusion pressure (baseline: 84±5 mmHg; isoprenaline: 58±6 mmHg ($p<0.001$); histamine: 67±4 mmHg ($p<0.001$)). Intra-arterial injection of the vasoconstrictor noradrenaline (16 μmol) increased hindlimb perfusion pressure significantly above baseline (138±18 mmHg ($p<0.001$ v baseline)). Figure 4 shows representative traces of hindlimb pressure from one animal following the administration of the vasodilator histamine (10 μmol) and the vasoconstrictor, noradrenaline (10 μmol). In addition, stopping the flow to the hindlimb (0 $ml.min^{-1}$) for 3 min significantly decreased hindlimb pressure in the first 2 min when flow was re-established (baseline 87±2 mmHg; Reperfusion hindlimb pressure 1^{st} min - 71±1

mmHg*, 2nd min - 76±2 mmHg*, 3rd min - 81±1 mmHg, 4th min - 81±2 mmHg, 5th min - 83±2 mmHg) (*$p<0.05$ v baseline).

Protocol 2

Baseline. Ventilation rates used in protocol 1, maintained P*a*O_2 at 91±3 mmHg, P*a*CO_2 at 29±2 mmHg, red blood cell saturation at 98.8±0.2% and C*a*O_2 at 19.9±0.9 mL.100 mL^{-1} with a mean (*a-v*)O_2 difference of 4.6±0.8 mL.100 mL^{-1} and mean oxygen consumption of 0.36±0.07 $\mu mol.g^{-1}.min^{-1}$. The mean hindlimb perfusion pressure was 103 ± 14 mmHg at 1 $mL.min^{-1}$.

Contraction. Hindlimb perfusion pressure increased in association with increased perfusion rate and O_2 consumption was a function of flow, independent of stimulation. There was no significant difference in the mean perfusion pressure during muscle contraction compared with the same flow at rest (figure 5).

Isometric twitch tension developed at the first stimulus increased with successive stimuli (staircase) to reach a maximum over 60-70 s. Developed tension for each twitch remained close to maximum then gradually declined until stimulation was stopped at 10 min. There was no significant difference in muscle peak isometric developed tension (figure 6A) at different blood flows, however there was a tendency for developed tension to be inversely related to blood flow. With a perfusion rate of 2 $mL.min^{-1}$ the developed tension at the end of 10 min of repetitive stimulation remained >80% of the peak tension, but when hindlimbs were perfused at 1 $mL.min^{-1}$ the twitch tension declined to below 60% of its peak (figure 6B).

Oxygen consumption measured after 10 min repetitive contraction was significantly increased compared to resting conditions, at all flow rates (figure 7A). The hindlimbs tended to consume slightly less oxygen during contraction if they were perfused at a higher rate, and the efficiency index ($N.\mu mol^{-1}.min^{-1}$) was significantly greater at higher flow rates (figure 7B).

The mean baseline arterial plasma lactate concentration was 2.8±0.18 $mmol.L^{-1}$. At all flow rates, muscle stimulation significantly increased venous blood lactate above resting conditions ($p<0.05$). High flow rates were associated with a trend towards greater venous efflux of lactate (Group 1: 2.9±0.46 $mmol.min^{-1}$; Group 2: 3.16±0.47; Group 3: 3.65±0.41). After 90 min perfusion, including three bouts of twitch contraction, stored glycogen was markedly lower than control in all lower hindlimb muscle groups (figure 3B) reflecting the use of this stored substrate as an energy source for muscle contraction.

Discussion

Delivery of oxygen to the muscle core remains a limitation of both *in vitro* and *in situ* rat hindlimb studies (7). The present study has extended the well-documented autologous perfused canine hindlimb into the laboratory rat and demonstrated stability over more than 120 min. Autologous perfusion in the anaesthetised, breathing animal resulted in sufficient oxygen delivery to support repetitive twitch contraction bouts and resting vascular tone, demonstrated by the ability to generate both vasodilation and vasoconstriction responses in the hindlimb. The inherent tone, previously documented as autoregulation in the canine model (44), allowed perfusion pressures

to be sustained in the normal systemic pressure range at flow rates equivalent to normal *in vivo* hindlimb blood flow for the rat.

The autologous pump-controlled *in vivo* perfusion flow rates required to provide good oxygenation and perfusion pressures and sustain muscle contraction were similar to natural flow rates reported for the rat hindlimb under resting and contracting conditions *in vivo* (15, 27, 29). The autologous *in vivo* flow rates in the current investigation were always lower than those reported for *in situ* perfusions over the last 25 years (ranging from 2 to 20 $mL.min^{-1}$). The flow rates commonly used for *in situ* hindlimb perfusions are often associated with low perfusion pressure when less than $6ml.min^{-1}$ (7, 31) and perfusion pressures above 100 mmHg are rarely achieved, even with the highest rates of flow (49). Physiologically relevant hindlimb perfusion pressure demonstrates the presence of vascular tone and is an important property of the autologous perfusion preparation described in this study. Vascular tone was demonstrated by: i) perfusion pressures in the physiological range at physiological blood flows (at rest the perfusion pressure closely matched the systemic blood pressure); ii) the responsiveness to vasodilators, which cannot normally be demonstrated with *in situ* preparations without prior pharmacological vasoconstriction (49); and iii) vascular reactivity to ischaemia (dilation and decreased hindlimb perfusion pressure when hindlimb blood flow was restored following 3 min of no flow), which is synonymous with established reactive hyperaemia (32). The inherent vascular tone enables the autologous perfusion method to be used in investigations of vascular resistance (25), which have been incomplete during *in situ* hindlimb preparation displaying very low perfusion pressures. Since the modulation of oxygen delivery to metabolically active tissue is most likely mediated through local

vasodilation (11), the autologous perfused hindlimb is a more useful method to provide real vascular tone.

As a further consequence of the very high perfusion rates of *in situ* preparations, tissue oedema and hindlimb swelling provide major problems (40), especially with cell free perfusates (2). The impact of swelling may be deleterious to microvascular perfusion, perfusion pressure and oxygen consumption. In the present study, the absence of foot swelling during any flow rate (over 120 min of perfusion) indicated that the autologous perfusion has a clear advantage over published *in situ* preparations in terms of whole limb stability. When wet muscle weights were compared from the perfused and non-perfused hindlimb there was indication the experimental set up did increased muscle weights by 30%. Despite the 2ml/minute condition coupling with a significantly higher and non-physiological hindlimb pressure there was no evidence that increased muscle weights were a result of flow per say. Dry muscle weights were not recorded in the current study is a recognisable limitation that could well be considered in future work.

A further advantage of the autologous *in vivo* perfused hindlimb model is the alveolar O_2 delivery and CO_2 clearance together with enhanced arterial oxygen delivery to the muscle tissue, capable of supporting conditions of high oxygen demand, especially during muscle activity. The ability to carry oxygen via the animals own blood is obvious when compared to cell free perfusate, and still holds true compared to using perfusates with either human or bovine erythrocytes. Perfusion with aged human erythrocytes (37) was shown to be inadequate to maintain tissue oxygenation, either at rest or during stimulation. The age, fragility,

size (90 μm^3) and large volumes required for lengthy perfusion protocols, reduces viability of human erythrocytes. Bovine erythrocytes (washed and resuspended) improve the situation a little in being smaller in size (60 μm^3) and hence do not have to elongate as much in the capillary, however due to the absence of vascular tone *in vitro* or *in situ*, the system still requires significantly higher flow rates than *in vivo* to deliver adequate perfusion pressure.

Even with blood or perfusate containing red blood cells, the haematocrit can have a large influence on physiological function. In isolated hearts, an increase in haematocrit from 25% to 40% increases oxygen uptake by 60% without any increase in coronary flow (6), moreover high oxygen delivery facilitates demand-driven changes in coronary flow that could not be otherwise observed (34). In rat skeletal muscle *in situ*, oxygen uptake does not significantly increase during twitch contractions at frequencies above 1 Hz when haematocrit is maintained at 30%, however oxygen consumption does increase during tetanic isometric contractions when haematocrit is at 40% (14). In the current investigation, haematocrit was kept at or above 40% in all animals, and contraction driven O_2 consumption was demonstrated.

Animals were artificially ventilated in order to control arterial oxygen and stabilise acid base balance for the entire perfusion period, thereby minimising the potential variable influence on the oxyhaemoglobin dissociation curve and unloading of oxygen at the muscle (48). When animals were subjected to controlled hyperventilation, the $Pa O_2$ and saturation of red blood cells were maintained at 100 mmHg and 98% respectively. However, hyperventilation significantly reduced the

P*a*CO_2 and P*v*CO_2 (i.e. CO_2 was "blown off") during resting perfusion. The resulting shift to alkaline conditions impacts on the unloading of oxygen from the red blood cell at the muscle bed (39). Therefore, the low resting oxygen consumption in the present study was likely achieved through enhanced oxygen binding inducing a leftward shift in the oxyhaemoglobin dissociation curve during hyperventilation as well as lower PCO_2 as a driver of local vasodilation.

Under resting conditions and with increasing hindlimb blood flow there was an evident decline in total hindlimb oxygen consumption underpinned by reduced (*a-v*)O_2 difference and high venous PO_2. It is well documented that flow rates exceeding requirements reduce red blood cell transit time, which in turn affects oxygen diffusion and red blood cell unloading (22) and loading in even specialised arterial pathways such as the pulmonary system (36) as well as the preferential distribution of excess flow through "non-nutritive" vascular pathways. Normally during muscle activity, metabolic factors such as increased muscle temperature, decreased pH, and increased PCO_2 all act to enhance the oxygen unloading and more than counteract any increased in muscle blood flow (39). These same metabolites are responsible for local, demand driven vasodilation. Therefore another contributor to the apparent reduced oxygen consumption at higher flow rates could be the higher proportional passage of blood through the muscle in vascular shunts that are associated with low oxygen consuming tissue. The possibility of nutrient and non-nutrient vascular pathways in muscle was first suggested by (4, 5). The concept has gained increased attention again in recent years, with the majority of evidence coming from the *in situ* perfused rat hindlimb (8). If the concept holds true, then with increased flow, excess blood may transit the low oxygen consuming pathways and result in a venous admixture that dampens the

evidence of oxygen consumption in the muscle tissue. This should still be considered with simultaneously reduced transit time since increased non-nutrient blood flow does not indicate reduced total muscle blood flow.

The pattern of isometric twitch tension development, with a progressive increase in peak tension over the first ~60 s of stimulation (staircase effect), is characteristic of very low frequency stimulation (1-5 Hz) in animal and human muscle (41). The autologous perfusion, by providing good oxygen delivery, contributed to the peak twitch tension being sustained over three 10 min periods of muscle contraction. In direct contrast, contractions of skeletal muscle *in vitro* can result in very rapid fatigue (1), attributable to a generally anoxic core (3) and contractions of the *in situ* hindlimb preparation has had varing degrees of success. The autologous *in vivo* perfused preparation is also capable of sustaining more intense stimulation trains and tetanic contractions than we have reported here.

In the present study, the cannulation and ligation technique restricted the controlled blood flow mainly to the lower leg of the rat with minimal loss into the hamstring and quadriceps muscle bundles. Consequently, stimulation of the sciatic nerve, restricted contractions to the distal muscles (14), namely the gastrocnemius-plantaris-soleus muscle bundle, representing ~70% of the perfused muscle mass, and demonstrating a good contraction to perfusion ratio. The ratio was at the higher end of the range previously reported as: 15-30% (12, 38) to: 82% (42, 43) of the perfused muscle mass contracting. At this stage there is no clear explanation for such large variations between studies, other than surgical methodology and variations in the location of the

cannula. It is appropriate for metabolic studies that a high relative proportion of the perfused muscle can contract, as achieved with this model.

Oxygen consumption rose significantly during muscle stimulation at any flow rate. Although there was no significant difference in oxygen consumption or maximum twitch tension at the different flow rates, both the final tension and the efficiency of oxygen use remained higher at the end of contraction bouts when the hindlimb was perfused at 2 $mL.min^{-1}$ than when perfused at the lower flow rates. This flow rate is in the range of observations for blood flow in the exercising hindlimb *in vivo* (27, 29). An important property of the autologous *in vivo* perfused hindlimb demonstrated by these observations is the capacity to deliver sufficient oxygen to support exercising muscle and the capacity to investigate exercise and other factors that might modulate oxygen consumption. The five-fold increase in oxygen consumption during contraction typically requires flow-rates of 14-18 $mL.min^{-1}$ when studies are conducted *in situ*, even when using red blood cell perfusates (23, 42), with further implications for the wash-out of exercise-induced metabolites. The cell-free perfusion systems are rarely used for active metabolism studies, probably because of the inablility to deliver sufficient oxygen to sustain contractions.

The model has been developed within our laboratory, specifically to investigate dietary effects on muscle metabolism (33). Hence the animals described here were of an age and size to represent rats that might have undergone many weeks of dietary intervention. The extracorporeal blood circuit represented less than 8% dilution of the animal's total circulatory volume (28). Despite the volume of blood perfused as an extra-corporal loop and the intricacy of the surgery required within the hindlimb, we

have established that the method is equally viable for rats of 400 g (33) and subsequently using younger rats less than 300 g body weight (unpublished) in which the dilution is estimated to be ~13%. We have also found it viable for investigating tetanic, and long duration (30 min) higher frequency (5 Hz) contractions.

Perspectives

The current study has developed the autologous *in vivo* perfused hindlimb system for the laboratory rat demonstrating physiologically relevant blood flow requirements and vascular responsiveness (resting tone without the prior use of vasoconstrictor drugs), improved oxygen delivery and long-term viability when compared to *in situ* rat hindlimb perfusion methods. Importantly, it can support repetitive muscle contractions, during which increased oxygen consumption can be clearly observed and metabolic responses recorded. This method expands our capacity to investigate physiological function and active metabolism in skeletal muscle in health and disease, taking advantage of the holding capacity and versatility of using the rat as a dietary model.

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Table 1: Baseline measures of blood gas, electrolyte and metabolites taken following 30 minutes perfusion (1 $mL.min^{-1}$).

Measure	***Arterial***	***Venous***
Blood Gas		
pH	7.42±0.01	7.3±0.02
PO_2 (mmHg)	100±3	47±2*
PCO_2 (mmHg)	33±1	51±2*
Hb (g/100mL)	15.4±0.2	16.4±0.2*
CO_2 (mL/100mL)	17.2±0.3	22.6±0.3*
$[HCO_3]$ (mM)	20.8±0.8	24.3±1.2*
Electrolyte		
Na^+ (mM)	133±1	137±1
K^+ (mM)	5.3±0.2	5.5±0.2
Metabolite		
Lactate (mM)	1.77±0.13	2.02±0.08
Pyruvate (mM)	0.16±0.01	0.17±0.01
Lactate / Pyruvate	11.8±1.0	12.5±0.9

*Values reported as Mean±SEM for all animals (n=18). *P<0.05 arterial versus venous samples.*

Table 2: Hindlimb swelling (foot volume - ml) and muscle weights (mg w.w) pre and post 2 hours of perfusion at flow rates 1, 1.5, or 2mL.min^{-1}.

	Hindlimb Flow Rate		
	1 mL.min^{-1}	**1.5 mL.min^{-1}**	**2 mL.min^{-1}**
Foot Volume (ml)			
Control	2.20±0.09	2.18±0.12	2.53±0.10
Pre perfused	2.21±0.09	2.23±0.14	2.51±0.08
Post perfused	2.19±0.12	2.35±0.09	2.54±0.07
Muscle weight (mg w.w)			
Control			
Soleus	177±19	220±10	145±10
Plantaris	226±83	256±8	238±20
Perfused (with % difference)			
Soleus	218±19*(23%)	277±7*(25%)	195±23*(34%)
Plantaris	330±7*(46%)	360±12*(40%)	325±11*(36%)

*Values reported as Mean±SEM for all animals (n=6 per group(foot volume) n=5 per group (muscle weights)). *P<0.05 control versus perfused wet weights within muscle.*

Figure 1: Hindlimb perfusion pressure at baseline (1ml/min) and then at three flow rates (1(●), 1.5(▼) and 2 mL.min^{-1} (■)) for the remaining 120 min perfusion. * $p<0.05$ group 3 (2 mL.min^{-1}) v group 2 (1.5 mL.min^{-1}). **$p<0.05$ group 3 (2 mL.min^{-1}) v group 1 (1 mL.min^{-1}). 2-way ANOVA. N= 6 per group. Mean ± SEM.

Figure 2 A: (Arterial – venous) oxygen difference (ml.100ml^{-1}) and **B:** Oxygen consumption (μmol.g^{-1}.min^{-1}) at baseline (1 mL.min^{-1}) and then at three flow rates (1 (●), 1.5 (▼) and 2 mL.min^{-1} (■)) for the remaining 120 min perfusion. * $p<0.05$ Group 1 (1 mL.min^{-1}) v Groups 2 (1.5mL.min^{-1}) and Group 3 (2 mLl.min^{-1}) from 30 min to 120 min. 2-way ANOVA. N = 6 per group. Mean ± SEM.

Figure 3 A: Muscle glycogen concentration (mg.g^{-1}w.w) in the soleus (Sol) and plantaris (Pl) muscles of the control (C) and perfused (P) hindlimb. **B:** Muscle glycogen concentration in the red gastrocnemius (Gas (red)), white gastrocnemius (Gas (white)), mixed gastrocnemius (Gas (mixed)), soleus and plantaris muscles of the control and perfused hindlimb. A: * $p<0.05$ 1-way ANOVA within muscles, control v perfused. B: * $p<0.05$ 1-way ANOVA between condition, control v exercise N = 6 per group. Mean ± SEM.

Figure 4: Trace example of systemic blood pressure during vasodilator histamine (C) and hindlimb perfusion pressure when A) vasoconstrictor noradrenaline (10 μM) and B) vasodilator histamine (100 μM) are administered directly to the hindlimb. Flow = 1 mL.min^{-1}.

Figure 5: Hindlimb perfusion pressure at 1, 1.5 and 2 mL.min^{-1} (from protocol 1) and during muscle stimulation at the same three experimental flow rates (1, 1.5 and 2 mL.min^{-1}). a,b,c $p<0.05$ 1-way ANOVA. N=6 per group. Mean ± SEM.6

Figure 6 A: Initial, maximum peak and final twitch tension developed (N.100g muscle tissue^{-1} w.w) in the autoperfused rat hindlimb (1 Hz, 7-12 V, 0.05 ms) with different flow rates (1, 1.5 and 2 mL/min^{-1}). **B:** Percentage of maximum peak twitch tension maintained after 10 min of muscle stimulation (1 Hz, 7-12 V, 0.05 ms) with different flow rates (1, 1.5 and 2 mL.min^{-1}). * $p<0.05$ 1-way ANOVA for 1min versus

10min independent of flow rate. a,b denotes $p<0.05$ between groups. N=6 per group. Mean ± SEM.

Figure 7 A: Resting oxygen consumption ($\mu mol.g^{-1}.min^{-1}$) at 1, 1.5 and 2 $mL.min^{-1}$ (from protocol 1) and during stimulation at the same three flow rates (1, 1.5 and 2 $mL.min^{-1}$). a,b $p<0.05$ within rest *$p<0.05$ between rest and exercise. 1-way ANOVA. N=6 per group. **B:** Efficiency index ($N.\mu mol^{-1}.min^{-1}$) in the autoperfused rat hindlimb after 10 min of muscle stimulation (1 Hz, 7-12 V, 0.05 ms) with different flow rates (1, 1.5 and 2 $mL.min^{-1}$). a,b,c $p<0.05$. N=6 per group. Mean ± SEM.

Figure 1

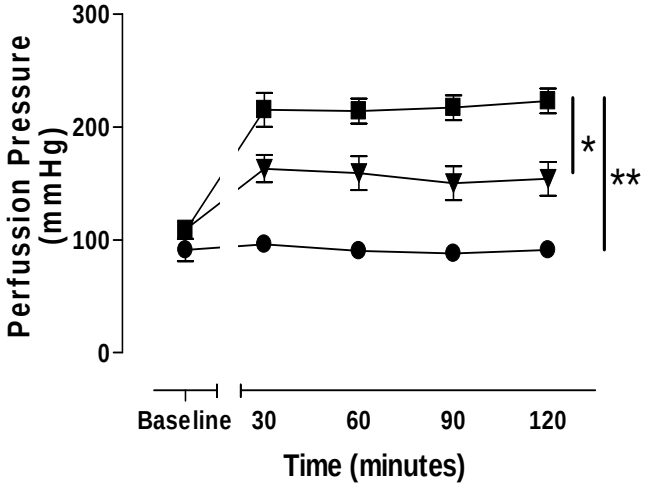


Figure 2

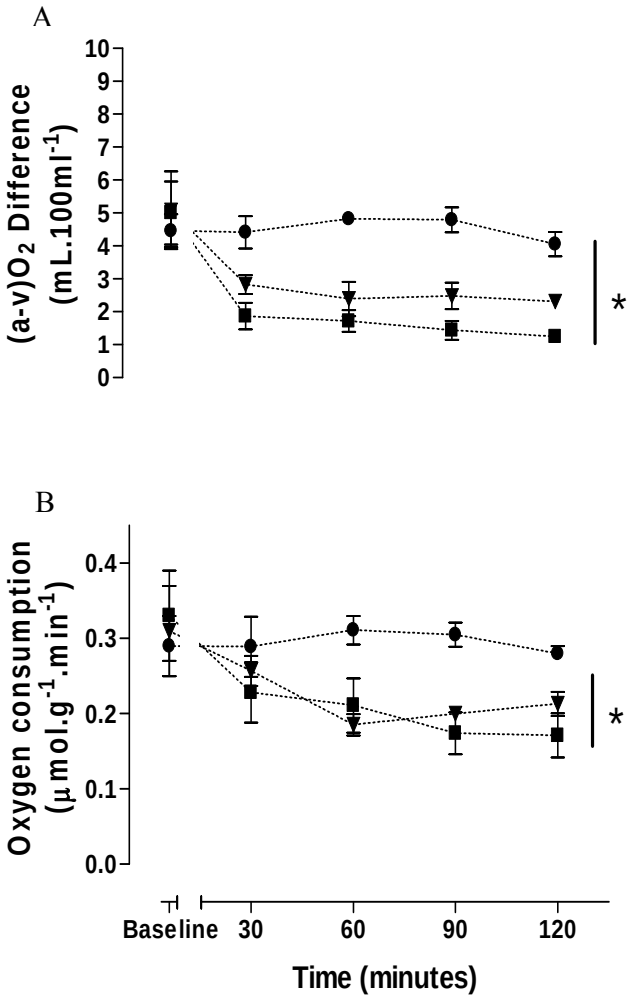


Figure 3

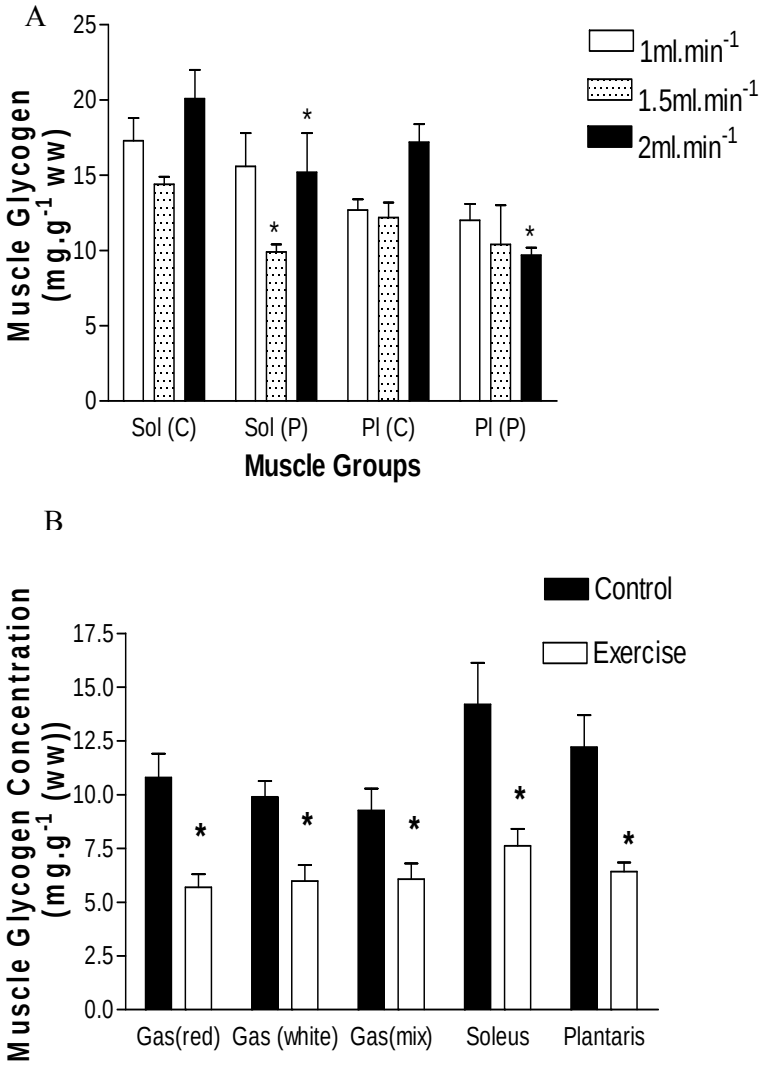

A
Muscle Glycogen (mg.g-1 ww)
25
20
15
10
5
0
1ml.min-1
1.5ml.min-1
2ml.min-1
Sol (C)
Sol (P)
Pl (C)
Pl (P)
Muscle Groups
B
Muscle Glycogen Concentration (mg.g-1 (ww))
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
Control
Exercise
Gas(red)
Gas (white)
Gas(mix)
Soleus
Plantaris

Figure 4

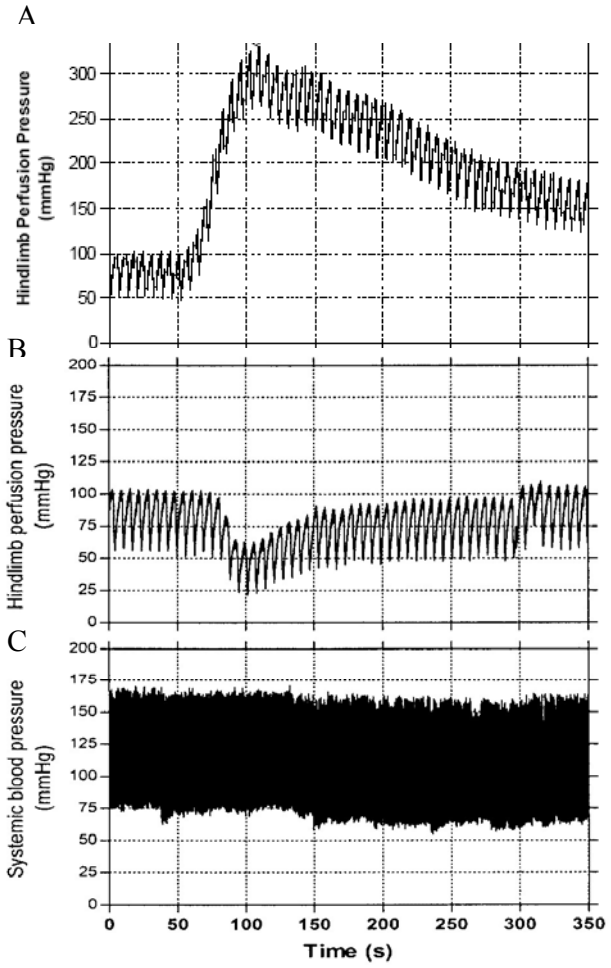

A
Hindlimb Perfusion Pressure (mmHg)
300
250
200
150
100
50
0
B
Hindlimb perfusion pressure (mmHg)
200
175
150
125
100
75
50
25
0
C
Systemic blood pressure (mmHg)
200
175
150
125
100
75
50
25
0
0
50
100
150
200
250
300
350
Time (s)

Figure 5

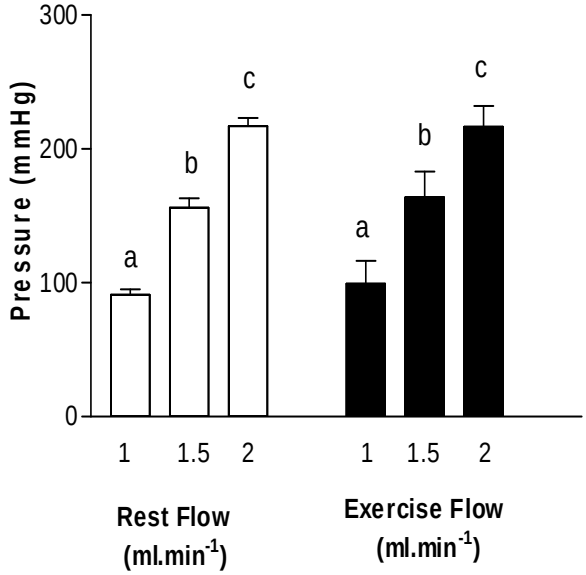
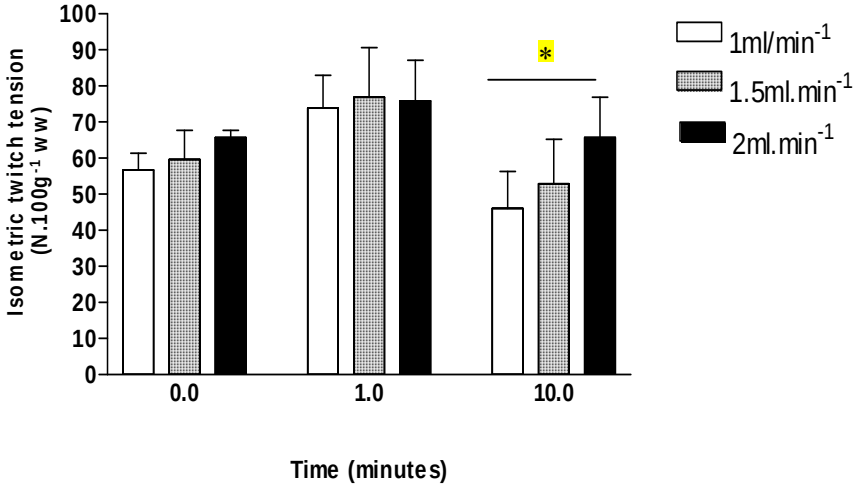

300
200
100
0
Pressure (mmHg)
a
b
c
a
b
c
1
1.5
2
1
1.5
2
Rest Flow
(ml.min-1)
Exercise Flow
(ml.min-1)

Figure 6

A


Isometric twitch tension (N.100g-1 ww)
100
90
80
70
60
50
40
30
20
10
0
*
0.0
1.0
10.0
Time (minutes)
1ml/min-1
1.5ml.min-1
2ml.min-1

B

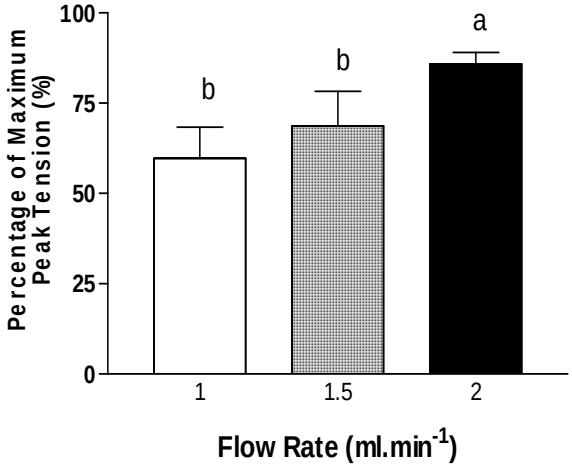
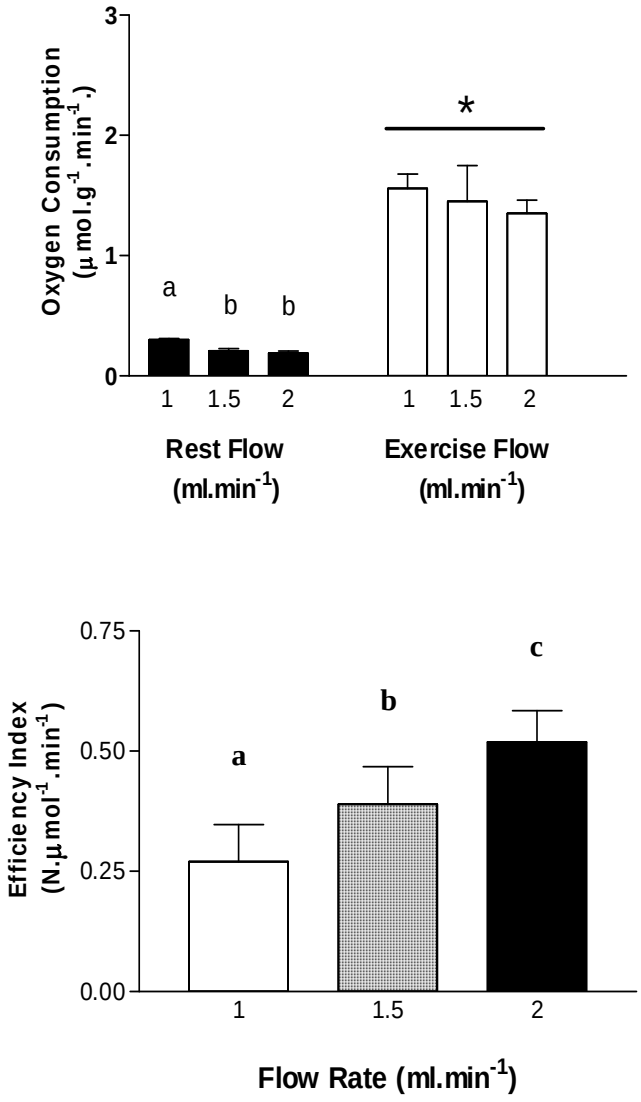

Percentage of Maximum Peak Tension (%)
100
75
50
25
0
b
b
a
1
1.5
2
Flow Rate (ml.min-1)

Figure 7


Oxygen Consumption (µmol.g-1.min-1.)
3
2
1
0
*
a
b
b
1
1.5
2
1
1.5
2
Rest Flow (ml.min-1)
Exercise Flow (ml.min-1)
Efficiency Index (N.µmol-1.min-1)
0.75
0.50
0.25
0.00
a
b
c
1
1.5
2
Flow Rate (ml.min-1)