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Single Muscle Fibre Contractility Testing in Rats to Quantify Ischaemic Muscle Damage During Reperfusion Injury

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WHAT THIS PAPER ADDS

This is the first study showing reduced contractile force at a myofibrillar level as early as 48 hours following hind limb reperfusion injury on rats. Findings are promising to investigate if single muscle fibre contractility testing can also be used to detect functional outcome in the clinical setting.

Objectives: In this study, the aim was to investigate the potential for single muscle fibre contractility (SMFC) testing to detect the extent of reperfusion injury following various reperfusion periods. The hypothesis was that force generated by muscle fibres will correlate inversely with the extent of reperfusion injury.

Methods: Twenty-four Lewis rats were distributed among five groups. Group 1 served as normal muscle control. In all other groups, femoral artery flow was occluded for four hours. Muscle biopsies were obtained at 0 hour, six hours, day two, and day seven after reperfusion in Groups 2, 3, 4, and 5, respectively. Samples then underwent ultrastructural analysis (H&E stain) and SMFC testing.

Results: The maximum isometric force (mN) generated on Days two and seven after reperfusion decreased from baseline by 21% ($p < 0.05$), and 53% ($p < .001$), respectively. The specific force (kPa) followed a similar pattern with a 13% decrease at Day two ($p > 0.05$) and 31% decrease at Day 7 ($p < .001$). These results correlated inversely with the extent of quantitative injury on histology.

Conclusions: The study demonstrated an inverse relationship between single muscle fibre contractility testing and neutrophil infiltration during the reperfusion phase. Further clinical studies are needed to evaluate its potential in providing prognostic information for patient outcomes.

Keywords: Single muscle fibre contractility, Ischemia–reperfusion injury, Muscle
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INTRODUCTION

Acute limb ischaemia and compartment syndrome are life and limb threatening emergencies with an incidence of 1.5/10 000.[1] Following revascularisation, reperfusion generates oxidative damage, further cell death, and aberrant immune responses via mitochondrial reactive oxygen species.[2] Resultant remote organ injury may lead to acute renal failure, sepsis, and/or limb loss.[3] Early determination of the extent of reperfusion injury is critical in clinical practice. No method has been able to demonstrate successfully the degree of reperfusion injury and the capacity of muscles to generate force in a clinically useful timeframe.[4,5]

To address this problem, an investigation was carried out to determine whether single muscle fibre contractility (SMFC) testing has the potential to measure the extent of reperfusion injury in a tourniquet induced ischaemia model on rat hind limbs. Quantitative measurements of the muscle contractility potential force using the SMFC method were found to be valuable when assessing alterations in muscle function observed *in vivo*.[6,7] Therefore, the aim was to assess contractile force during various critical reperfusion intervals.

METHODS

All animals received humane care in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee (IACUC) approved the animal protocol (PRO00006240). Female Lewis rats ($n = 24$) weighting 250–300 g were anaesthetised with ketamine (80 mg/kg) and xylazine (10 mg/kg) and positioned supine. Body temperature was monitored and maintained between 36.5 and 37.5 °C using a heating pad connected to

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a rectal thermometer. Following induction of anaesthesia, a rubber ring (internal diameter 14.5 mm, Latex O-Rings; Miltex Instruments, York, PA, USA) was placed on the right hind limb at the level of the thigh. The effect is similar to that of inflated blood pressure cuff induced ischaemia (approximately 300 mmHg). Total vascular occlusion was further confirmed using laser flow Doppler (Periflux PF3; Perimed KB, Stockholm, Sweden). All animals were weaned and observed within temperature controlled (22−24 °C) pathogen free cages throughout the reperfusion period, with unlimited access to commercial pellets and water. Post-operatively, animals were given 0.1 mg/kg/day buprenorphine subcutaneously for analgesia for two days. The investigators who assessed the SMFC testing and the veterinary pathologist who performed histopathological examination were blinded to the animal groups.

Experimental design

A total of 24 animals were randomly allocated to the following groups. Group 1 ($n = 4$) served as the control group, where animals were euthanised without any intervention to establish the normal microstructure and contractile forces without ischaemia and/or reperfusion time. In all other groups ($n = 5$ in each), animals had four hours of right hind limb ischaemia using rubber bands. This is the duration critical to begin irreversible changes to occur on this model allowing animals to recover from the surgery and to evaluate the effect of reperfusion injury.[8,9] In Group 2, muscle biopsies were obtained immediately after ischaemia with no reperfusion. In Groups 3, 4, and 5, muscle biopsies were obtained after six hours, two days, and seven days, respectively. Tibialis anterior (TA) muscle was used for SMFC testing (Fig. 1), and gastrocnemius (GTN) muscle was used for H&E staining.

Single muscle fibre contractility testing

The contractile properties of reperfused muscle fibres were analysed as previously described.[10] Briefly, bundles of fibres were dissected from TA muscles, placed in skinning solution for 30 minutes to permeabilise the cell membranes, then placed in storage solution and maintained at −80 °C until tested. Fibres extracted from bundles and stored at −80 °C can be tested after storage durations of up to 12 months with no apparent functional deficits.[11] On the day of fibre contractility testing, isolated bundles were thawed slowly on wet ice, and individual fibres were pulled from bundles using fine forceps. Fibres were then placed in a chamber containing relaxing solution. One end of the fibre was secured to a servomotor (Model 322C; Aurora Scientific, Aurora, ON, Canada) and the other end to a force transducer (403A; Aurora Scientific) using two ties of 10-0 monofilament nylon suture at each end. Each fibre length was adjusted to obtain a sarcomere length of 2.5 µm using a laser diffraction measurement system. The average fibre cross sectional area (CSA) was calculated assuming an elliptical cross section, with diameters obtained at five positions along the fibre from high magnification images at two different views (top and side). Maximum fibre isometric force (F_o) was elicited by immersing the fibre in a high concentration Ca^{2+} and ATP solution. For the purpose of normalizing the force produced, the specific force of fibres (sF_o) was calculated by dividing F_o by fibre CSA.

Histopathology

The harvested hind limbs were fixed in 4% paraformaldehyde for a minimum of four hours. The GTN muscle was dissected out and rinsed in Dulbeco's phosphate buffered saline for one hour. The tissue was dehydrated in 70%, 90%, and two 100% acetone rinses. The muscle was then cut transversely along the midline, at the portion of muscle with the greatest cross section, providing a representative picture of the overall injury. The samples were embedded in JB-4 glycolmethacrylate to ensure the sections were artefact free and cut in cross section at 2 µm thickness. All specimens were stained with H&E.

The fourfold divided frame counting method[12] was used to assess the extent of reperfusion injury. Each slide was divided to 15 equal fields with the same dimensions and a random number generator was used to determine which fields were scored. Six different fields were randomly chosen from each slide and split into four equal sections. One section was analysed from each field (6 sections/animal). Within each section, injured and healthy myocytes were counted by a blinded observer. The injury score was expressed as a percentage, calculated from the number of injured myocytes divided by the total number of myocytes scored within the analysed fields (percent injury = number of injured myocytes/injured and healthy myocytes score × 100).

Statistical analysis and sample size determination

All statistical analysis was performed using Prism 7 software (Prism 7, GraphPad Software, CA, USA). The data were expressed as mean ± standard deviation (SD). Based on previous experiments on SMFC testing and to have adequate power (≥.80) to detect a statistically significant difference of ≤ .05 for any time period studied, five rats were used in each ischaemia group. Changes from baseline values (Group 1) were analysed using the Mann−Whitney test. The Spearman correlation coefficient was used to quantify the correlation between SMFC and histological muscle injury score. A p value < .05 was considered significant for all statistical tests.

RESULTS

Contractile forces

Maximum isometric force (F_o), specific force (sF_o), and the average fibre cross sectional area (CSA) of permealised TA muscle fibres from all groups are shown in Fig. 2A−C. There was no statistically significant difference for either F_o or sF_o ($p = .790$ and $p = .838$, respectively) between the control group (Group 1) and the group measured after four hours of ischaemia (Group 2). F_o was initially increased 24% from baseline ($p = .077$) after six hours of reperfusion (Group 3).

Following two and 7 days of reperfusion, F_o was decreased from baseline values (Group 1) by 21% ($p = .077$) and 53% ($p < .001$), respectively. sF_o followed a similar pattern with significant changes observed only at two days (13% decrease, $p = .02$) and 7 days (31% decrease, $p < .001$) after reperfusion. For the CSA analysis, a statistically significant increase was observed in the six hours reperfusion group and a decrease in the seven day reperfusion group ($p = .1$ and $p < .002$, respectively). Other comparisons did not indicate statistically significant differences.

Qualitative ultrastructural analysis

All muscle samples obtained from GTN muscles demonstrated minimal ischaemic changes in Group two where muscle was subjected to four hours of ischaemia without reperfusion. In Group 3, after six hours of reperfusion, sections showed acute myofibre degeneration and necrosis without associated tissue reaction or regeneration. Lesions consisted of zones of swollen to shrunken myofibres with expansion of the interstitium with clear zones (oedema) or eosinophilic fibrillar material (fibrin), loss of cross striations, hypereosinophilia, fragmentation, and dissolution (Fig. 3E) and very few scattered neutrophils. There were clear zones of affected myofibres adjacent to unaffected myofibre bundles (Fig. 3F).

Following two days of reperfusion in Group 4, histology showed multifocal degeneration of single scattered myofibres, characterised by loss of cross striations, myofibre atrophy, cytoplasmic hypereosinophilia, and vacuolation or granularity, as well as small clusters of rounded myofibres with multiple nuclei associated with small amounts of connective tissue stroma (Fig. 3A). Adjacent myofibres appeared relatively normal and unaffected. Sections of muscle from slide F had similar clusters of myofibre bundles with degenerative changes, accompanied by minimal to mild expansion of interstitial spaces with fibrillar material

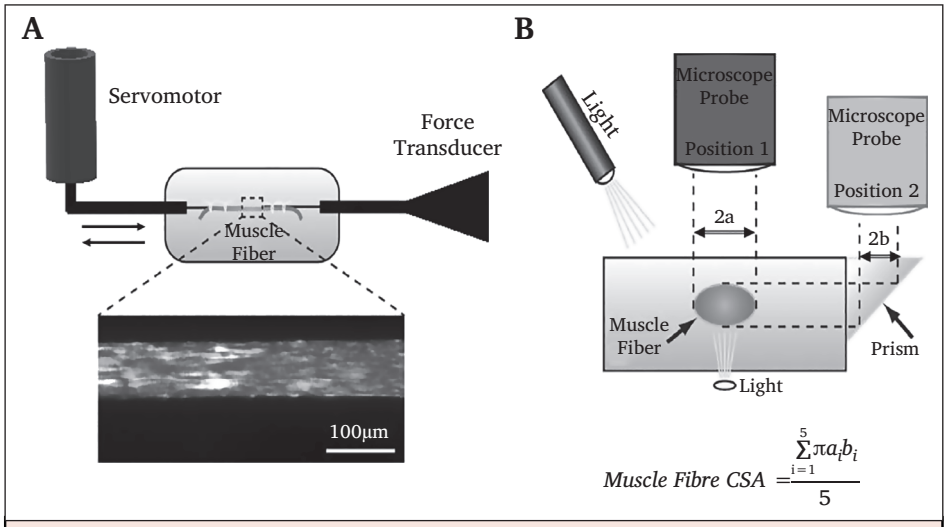

Figure 1. Muscle fibre contractility testing diagram. (A) Overview of the general orientation of the tibialis anterior muscle fibre in the testing system. (B) Five width measurements from the top and side views of the mid-portion fibre are fitted to the formula of an ellipse to calculate fibre CSA. CSA = cross sectional area. (Reproduced with permission from Transplantation.[26])

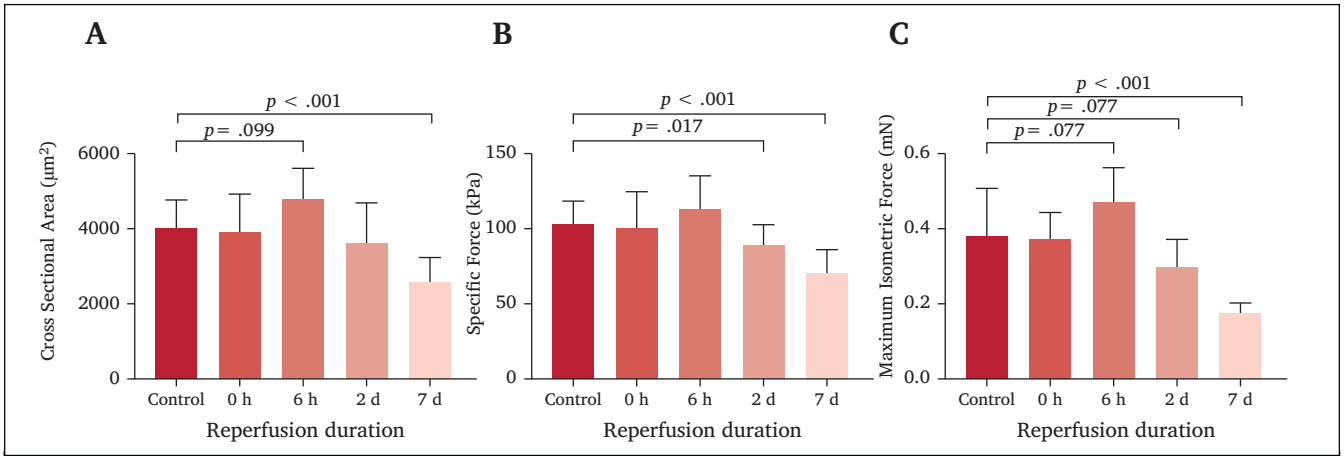

Figure 2. (A) Average fibre cross sectional area of permealised tibialis anterior (TA) muscle fibres of rats in experimental hind limb reperfusion injury model. (B) Specific force of permealised TA muscle fibres. (C) Maximum isometric force of permealised TA muscle fibres.

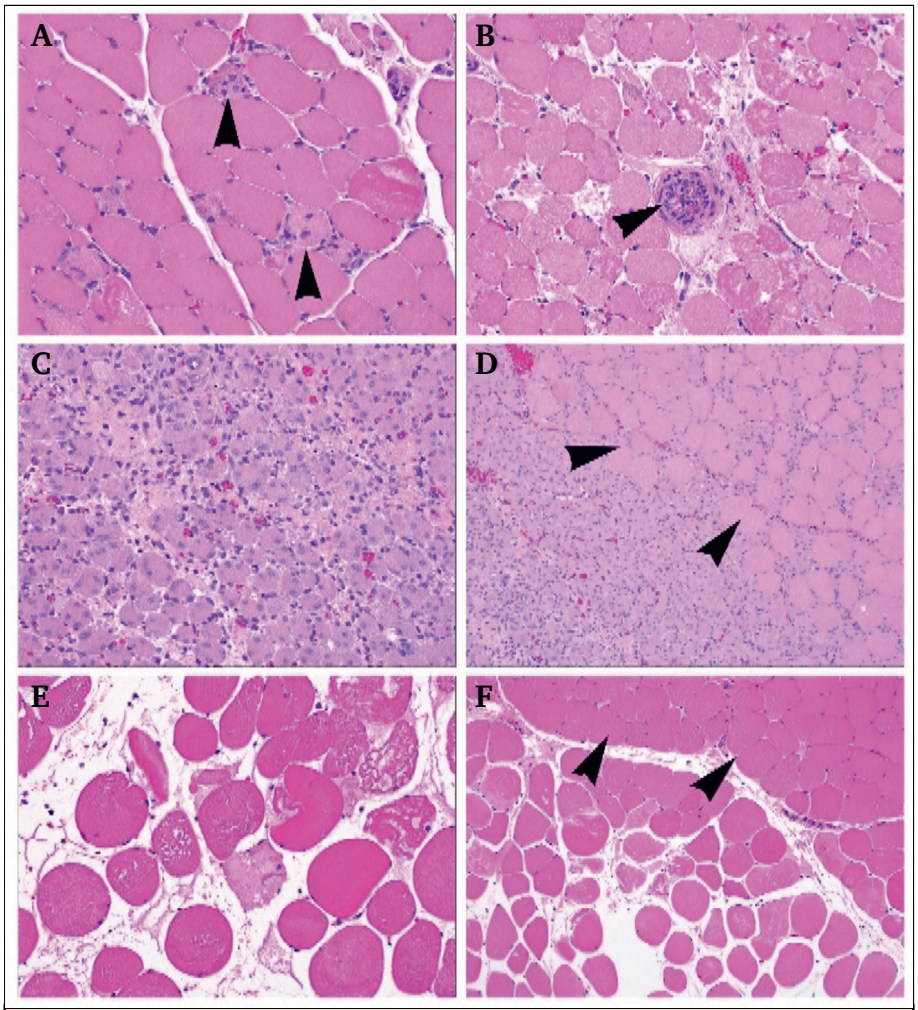

Figure 3. Representative photomicrographs from rat gastrocnemius muscle samples following six hours and two days of reperfusion. Skeletal muscle at two days reperfusion, showing focal myofibre degeneration and regeneration (A, arrowheads), interstitial oedema and mild neutrophil infiltration with hypertrophied endothelial cells in associated arterioles (B, arrowhead). Two days after reperfusion shows similar findings to seven days reperfusion; variably sized basophilic myofibres with multiple nuclei, prominent stroma, and scattered inflammatory cells (C), which merge with normal adjacent myofibres (D). Six hours after reperfusion, there is evidence of acute degeneration and necrosis of myofibres with abundant oedema, and few inflammatory cells (E), clearly delineated from adjacent normal myofibres (F, arrowheads).

and small numbers of neutrophils, and hypertrophy of endothelial cells within arterioles (Fig. 4B).

In Group 5 after seven days of reperfusion, histology showed marked ischaemic necrosis of large zones of myofibre bundles, bordered by well delineated zones of myofibre regeneration and degeneration (Fig. 4B). In zones of ischaemic necrosis, there was a loss of myofibre cross striations, loss of nuclei, fragmentation, vacuolation, and decreased myofibre tincture (Fig. 4C). Additionally, bordering areas of regeneration/degeneration were characterised by variably sized, rounded, basophilic myofibres, and prominent stromal cells containing one to multiple large round eccentric nuclei with prominent nucleoli, admixed with small amounts of reactive fibroplasia (Fig. 4D). Focally, degenerated myofibres were mineralised. A few inflammatory cells were scattered within the interstitium of zones of regeneration/degeneration, while no cells were present in zones of ischaemic necrosis.

Quantitative injury severity scores

Limbs subjected to four hours ischaemia and six hours of reperfusion (Group 3) had 26.1% of injury, vs. 50.4% and 72.9% of injury in those subjected to two days (Group 4) and 7 days of reperfusion (Group 5), respectively. Samples from no reperfusion group (Group 2) had 13.7% of injured myocytes. The injury score of each group is presented in Fig. 5.

Correlation analysis

Moderate correlations were detected between injury score and F_o ($p < .001$; $r = -0.54$ and 95% CI -0.73 to -0.27),

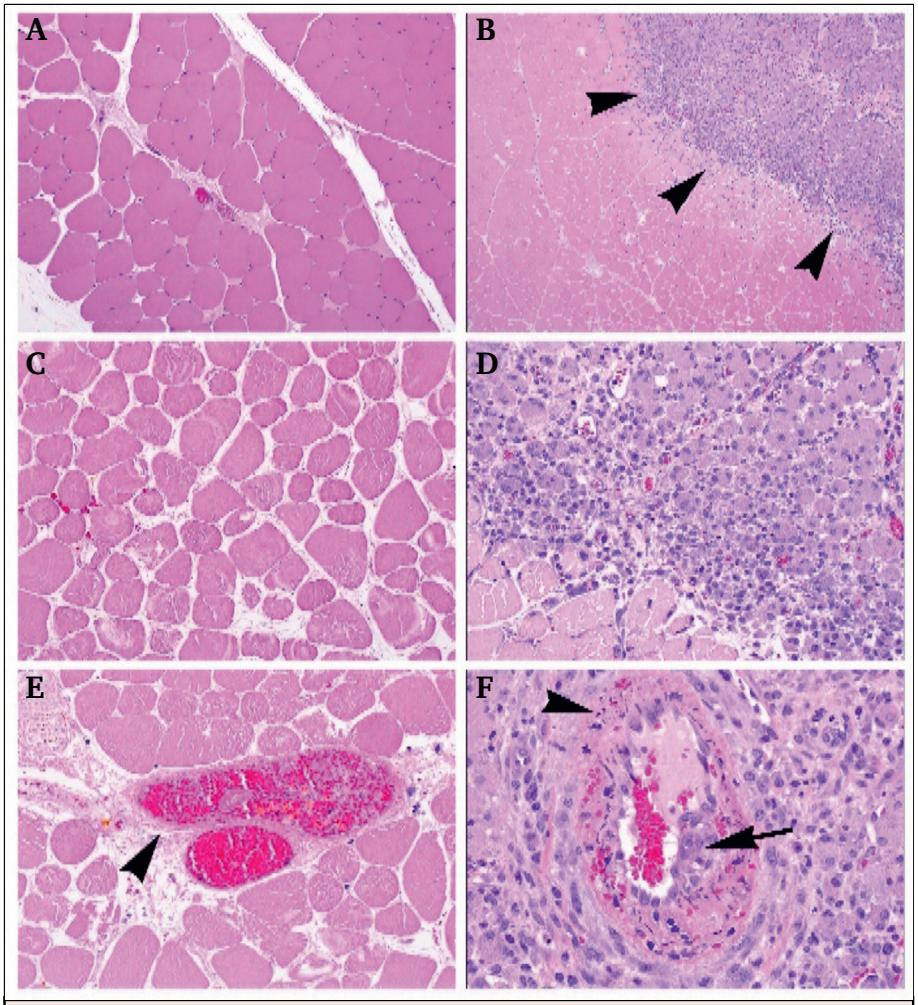

Figure 4. Representative photomicrographs from rat gastrocnemius muscle samples following 7 days of reperfusion. Sections of control skeletal muscle (A) showing normal histology. Sections of skeletal muscle 7 days after reperfusion (B–F) showing a clear line of demarcation between ischaemic necrosis and regenerative response (B, arrowheads). Necrotic zones had diffuse loss of cellular detail (C), while reparative areas showed variably sized basophilic myofibres with prominent nuclei and intervening stromal cells (D). Vascular structures in areas of ischaemia had complete loss of cellular detail (E, arrowhead), while in areas of regeneration there was disruption/remodelling of vessels (F) evidenced by cellular debris (arrowhead), vacuolated smooth muscle wall, and endothelial hypertrophy (arrow).

and between injury score and sF_o ($p < .001$; $r = -0.62$ and 95% CI −0.78 to −0.39). However, no statistically significant correlation was observed between CSA and histological injury score throughout the ischaemia–reperfusion ($p = .112$; $r = -0.23$ and 95% CI −0.50–0.07).

DISCUSSION

Single muscle fibre contractility testing is used in a wide variety of contexts.[6,10] In this study, its potential to detect the extent of reperfusion injury following various reperfusion intervals was investigated. Maximum isometric force and specific force measurements were both significantly reduced in as early as 48 h of reperfusion. These results inversely correlated with histological injury scores throughout the ischaemia reperfusion period. While this quantitative evaluation was sensitive enough to detect the injury during reperfusion, it is unclear whether this will be specific enough to reflect functional outcome in the long-term.

Several reported methods have attempted to quantify the extent of muscle injury by measuring mitochondrial enzyme activity, vessel permeability using radiolabelled albumin dye, lactate dehydrogenase activity, and neutrophil infiltration as assessed by increased tissue myeloperoxidase activity. Individually, each of these indirect measures of injury describes a specific manifestation of ischaemia reperfusion injury (IRI), but collectively, they cannot be used to describe the overall extent of ischaemia reperfusion phenomena. For this reason, spectrophotometric[13–16] and planimetric[17–19] methods are generally recommended for *in vivo* quantitative assessments by

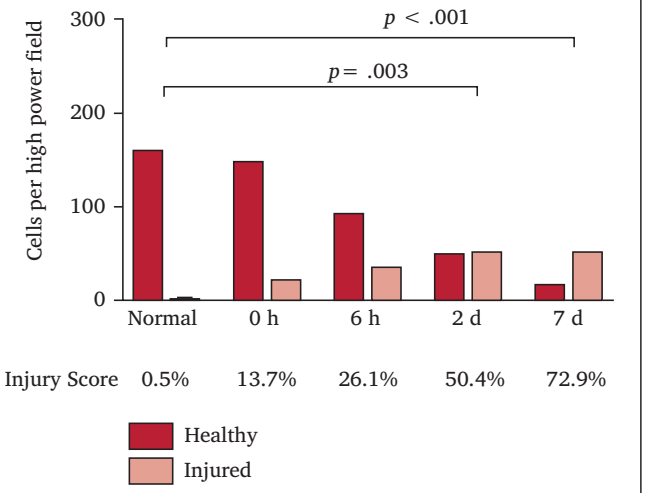

Figure 5. Relative number of injured myocytes in comparison to total number of myocytes per high power field (Injury Score) in different time points during rat hind limb reperfusion injury. Healthy represents the group of mycocytes without injury on per high power field. Injured represents the number of myocytes with structural changes on per high power field. Control group without ischemia (normal) shows very few myocytes with injury. Y axis shows the average number of cells per high power field including healthy and injured. X axis shows reperfusion time following four hours of ischaemia.

tetrazolium reduction as an indicator of mitochondrial integrity.[18] Although both methods were reported to correlate well with the extent of muscle injury,[20] they are not suitable for bedside diagnostic purposes due to long preparation times and/or large sample requirements. Muscle injury severity score on H&E stained samples offers a percentage of fibres with structural changes that cannot predict long-term functional deficit or force generation. This study provides novel and useful information regarding the effects of reperfusion injury on the generation of force at the myofibrillar level as early as 48 hours. This time frame may appear late in case of critical limb ischaemia. However, in a haemodynamically stable patient following revascularisation, the effects of reperfusion injury on the limb and muscle circulation are awaited, which is often no less than 48 hours. This time is needed to allow muscle cells to recover from the injury and activate their internal repair or replacement mechanisms including apoptosis, necrosis or regeneration. SMFC testing does not predict the outcome of muscle cells and currently there are no methods of evaluation to predict the ultimate outcome. It only provides information about the force generation of those cells following critical ischaemia. Compared with H&E, this remains a further step towards obtaining quantitative information regarding the strength of an individual fibre as early as 48 hours. It is unclear at this point if this numeric value will eventually reflect the function of the extremity.

At the early reperfusion phase, a temporary increase in Fo and sFo was observed after six hours ($p > .05$). At this point, there is no a mechanism to explain this observed phenomenon. One plausible explanation is that reperfusion injury causes increased membrane permeability, osmotic overload and cytosolic calcium accumulation, which result in the opening of the mitochondrial permeability transition pore and myocyte hypercontracture.[21] Also, reperfusion injury induced, increased membrane permeability may cause cellular swelling in the overall fibre volume. This swelling increases the distance between actin and myosin filaments resulting in reduced calcium sensitivity of the myofilament system. In this study, there is only histology demonstrating swollen myofibres with the expansion of the interstitium (Fig. 3E). Further studies are required to explain the mechanism of temporary force increase in SMFC test.

Since reperfusion of a severely injured ischaemic extremity may increase complication rates, rapid diagnosis and quantitative determination of the extent of reperfusion are of utmost clinical importance. Direct observation of muscle histopathology has been widely used in both animal and clinical studies to provide direct evidence of damage on muscle fibers.[22] Although the oxidative capacity (mitochondrial density) of fast fibres varies considerably among different species and depends on fibre type specific antioxidant level,[23] rodent GTN and TA muscles are both comprised highly of “fast” fibres.[24] Therefore the inverse correlation observed between reduced fibre force generation on TA and increased muscle injury score in GTN muscles probably represented the overall status of the limb.[25] It is unclear however how these structural changes affect the overall force generation and function of the extremity. The current study offers a potential method for quantifying force generation in this context.

This study has several limitations. Single muscle fibre contractility testing requires expertise, advanced training, and specialised equipment. Despite those challenges, quantitative assessments can be performed within one day. The myofibrillar unit tested during the study is a small fraction of the entire muscle likely to be representing the entire compartment in this ischaemia model. Compartment syndrome due to crush injuries will require further testing. Only the contractile properties of TA muscle following four hours of ischaemia were examined. For technical reasons histology and SMFC samples were obtained from different muscles (TA and GTN, respectively). Although TA and GTN muscles are both fast twitch muscles that are susceptible to ischaemic injury, further studies using other methods of evaluation are required to confirm whether the same pathology was observed on other muscle compartments. This study had a small sample size with one ischaemia time. Additional studies with larger sample sizes, additional immunohistochemistry and

cell staining analysis (such as CD31) at various ischaemia reperfusion intervals may be required before conclusions can be reached regarding clinical application. Finally, the function of the extremity as a whole could not be mentioned in this model. The only available method of evaluation, walking track analysis, often conducted six to 12 weeks following injury measuring toe spread and contracture development, is validated primarily for peripheral nerve injuries, not post-ischaemic muscle injury. Further clinical studies are needed to reliably assess its potential to reflect extremity function.

The primary function of skeletal muscle is to generate a contractile force. A complex sequence of events plays a critical role throughout that process. Following IRI, the intensity of inflammatory response disrupts muscle viability. Therefore, diagnosis and quantitative determination of the extent of reperfusion injury are of utmost clinical importance. Since SMFC testing shows reduced force generation as early as 48 hours after reperfusion, the assessment of fibre contractility produces reliable information and could be used as a novel method to detect the extent of reperfusion injury as early as 48 hours. However, this study is only the first step for opening new questions to be answered in future studies, including the effect of increasing ischaemic times on the single muscle fibre contractility testing and its correlation with the function of the extremity.

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CONFLICTS OF INTEREST

None.

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COUP D'OEIL

"Type II Endoleak" and Popliteal Artery Aneurysm Rupture after Surgical Repair

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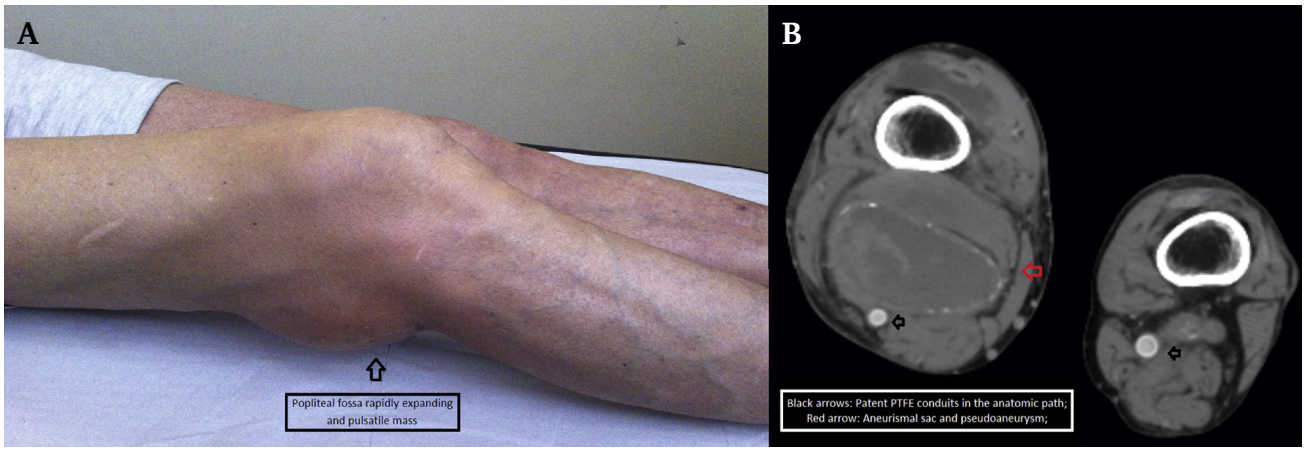

A 79 year old man underwent popliteal artery aneurysm (PAA) exclusion and bypass by a medial approach. Seven years later he was admitted with a rapidly expanding mass in the right popliteal fossa with no symptoms/signs of limb ischaemia (panel A). Computed tomography angiography revealed a contained PAA rupture with pseudo-aneurysm development and a patent (prosthetic) conduit (panel B). Via a posterior approach, back flowing collaterals were underrun and the haematoma was drained. Post-operatively, the patient recovered uneventfully. Continued perfusion of the PAA sac through collaterals resulted in its continued pressurisation (similar to type II endoleak after endovascular aneurysm repair) and rupture with massive acute pseudo-aneurysm expansion, as recognised in this scenario.

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