Skeletal Muscle Ischaemia-reperfusion Injury: Further Characterisation of a Rodent Model


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Background: postischaemic damage in skeletal muscle may be reflected in changes to microvascular blood flow, vascular permeability, and subsequent tissue viability. Previous preclinical studies have not addressed all these parameters, and have not used periods of ischaemia and reperfusion relevant to the clinical setting. This study aimed to develop an animal model hindlimb ischaemia-reperfusion to simulate acute lower limb ischaemia.

Methods: a rodent model of hindlimb tourniquet-induced ischaemia-reperfusion was employed. Gastrocnemius muscle blood flow (GMBF; radio-labelled microspheres), oedema (GMO; using a wet:dry ratio method) and viability (GMV; histochemistry and computerised planimetry) were quantified.

Results: 6 h ischaemia per se resulted in neither muscle oedema nor loss of viability, but these changes were apparent following 4 h reperfusion. Early reperfusion at 10 min demonstrated low reflow, with GMBF improving at 120 min before declining sharply at 240 min.

Conclusion: prolonged hindlimb ischaemia followed by reperfusion in this rodent model caused significant reductions in gastrocnemius muscle blood flow, associated with muscle oedema and necrosis. These three parameters have not been previously reported together in the same model. This reproducible model could be used in the evaluation of potential therapeutic intervention strategies aimed at ameliorating skeletal muscle reperfusion injury.

Key Words: Rodent; Ischaemia; Muscle; Reperfusion injury; Animal model.

Introduction

Tissue damage occurring during revascularisation following a prolonged period of critical ischaemia is termed reperfusion injury. Following surgery for acute limb ischaemia a major amputation may be required in up to 15% of cases despite a technically successful operation; this may be the result of skeletal muscle reperfusion injury (SMRI). Considerable evidence now exists which implicates reperfusion injury as a cause of significant tissue damage following organ revascularisation with its deleterious effects being both local and systemic. The pathophysiology underlying this inflammatory process is complex, and current work has been directed at elucidating various aspects of these mechanisms of cell injury, such as neutrophil-endothelial cell interaction, and by inflammatory mediator blockade.

A rodent model of acute hindlimb ischaemia and reperfusion has been developed in this laboratory in which we have previously quantified gastrocnemius muscle microcirculatory blood flow. In the present study, this model was further characterised in a series of experiments in which gastrocnemius muscle oedema and viability were assessed in conjunction with muscle perfusion.

Materials and Methods

All the experiments were conducted under a Home Office project licence.

The experimental protocol used in this study has been described previously. Briefly, anaesthetised male Sprague-Dawley rats weighing approximately 500 g were ventilated via a tracheostomy. The left external jugular vein, and left ventricle (via the right carotid artery) were cannulated. The method of Rosenthal was employed to induce limb ischaemia, in which a rubber band tourniquet was applied proximally in the thigh to the intact lower limb to produce total ischaemia. In each experiment, muscle blood flow, oedema and viability were assessed. The contralateral limb (without tourniquet application) was used in each
case as the control limb. Ten normal (10 h general anaesthesia (GA), no ischaemic insult to either hindlimb), 10 control (6 h ischaemia followed by 4 h reperfusion) and 10 ischaemic (10 h of ischaemia, no reperfusion) animals were studied.

Postischaemic gastrocnemius muscle blood flow (GMBF) was measured by injecting three separate radiolabelled microspheres ($^{46}$Sc, $^{85}$Sr, $^{141}$Ce) at 10, 120 and 240 min reperfusion respectively via the carotid artery into the left ventricle. Following release of the tourniquet (and at matched time points in the normal and ischaemic groups), GMBF was measured at 10, 120 and 240 min of reperfusion.

A perfusion Index (PI) was calculated as the ratio of radioactive emission from the study limb to that from the contralateral control limb in each animal (the left to right limb ratio in the normal and nonischaemic groups). A ratio of 1 would thus indicate that the gastrocnemius muscle blood flow was identical in each limb.

Muscle oedema was quantified by the wet:dry weight ratio method and an oedema index thus calculated in a similar manner. Muscle viability was assessed by histochemical staining with nitroblue tetrazolium (Sigma Chemicals Co, U.K.) and quantified as a percentage by means of computerised planimetry (Summagraphics Digit version 2 software, Summagraphics, Fairfield, Connecticut, U.S.A.). At the conclusion of each experiment the animals were given a lethal intravenous dose of pentobarbitone (200 mg).

Whilst the Rosenthal tourniquet method of producing limb ischaemia results in some amount of local muscle injury at the site of application, it has been shown not to affect limb reperfusion on removing the tourniquet. Any metabolic products released into the venous effluent from local muscle injury would enter the systemic circulation and thus affect microvascular perfusion in both limbs. Accurate positioning of the tourniquet is important. An elastic band was wound tightly (10 times) round a no. 15 cork borer and a thin metal lever. The lever was positioned to project just beyond the end of the borer, with the elastic band as close to the end of the borer as possible. The left paw of the rodent is passed through the centre of the borer and the elastic band levered onto the thigh above the gastrocnemius muscle. This method reliably produces complete limb ischaemia. If placed too low the gastrocnemius muscle would continue to receive blood from its nutrient artery whilst too proximal a position would result in failure to reperfuse due to direct injury to the femoral artery. A satisfactory position can be confirmed by observing the immediate development of limb pallor and poor or absent capillary return.

Muscle oedema was quantified by measuring the wet to dry weight ratio of the gastrocnemius muscles to calculate an oedema index. Following careful excision, both gastrocnemius muscles were weighed immediately to determine the wet weight and then dried at 80°C in a convection oven till constant weight was achieved (dry weight). The measurements were all made on the same balance and the weights recorded to 4 decimal places. An index of muscle oedema was calculated as the ratio between the ischaemic-reperfused study limb and the contralateral control limb in each animal. A theoretical argument against this method is based on the fact that the muscle blood content may affect the measurement. Thus when muscle blood flow is high the wet:dry weight ratio may correspondingly increase which could be wrongly interpreted as an increase in muscle oedema. However, the contribution of muscle blood volume to overall wet muscle weight has been shown to be negligible in a similar rat model, and hence the observed differences in the wet:dry weight ratios will reflect the degree of tissue oedema.

Muscle viability was assessed using the histochemical stain nitroblue tetrazolium. Each gastrocnemius muscle was sectioned in the coronal plane through midbelly and both halves incubated in a solution of the stain (50 mg per 100 ml, dissolved in 200 mMol tris HCl, pH 7.4) at 37°C for 20 min. Viable muscle stains deep blue whereas non-viable muscle remains pale and unstained. Following incubation with the stain, the outline of each half of the muscle and the stained and unstained areas on its surface were carefully traced in duplicate on to transparent acetate sheets. Percentage muscle viability was then calculated by means of computerised planimetry.

This method was initially used for mapping areas of myocardial infarction. More recently, other groups have demonstrated the validity of this method for detecting skeletal muscle necrosis.

The results of the experiments conducted involved the comparison of two independent groups of animals. The maximum number of animals per group was ten and hence data from each group was not normally distributed. Since the requirements for the use of parametric tests were not fulfilled, the non-parametric Mann–Whitney U-test was used for statistical analysis of the data.

**Results**

**Gastrocnemius muscle blood flow (GMBF)**

In normal animals, the PI remained constant throughout the duration of the experiment. These results dem-
onstrate that neither the duration of the experiment nor the general anaesthetic influenced muscle blood flow in this group of animals.

In the control group of animals in which one hind-limb was subjected to 6 h of ischaemia followed by up to 4 h of reperfusion, the median PI (interquartile range in brackets) after 10, 120 and 240 min of reperfusion was as follows: 0.08 (0.01–0.13), 0.29 (0.09–0.59), 0.05 (0.01–0.14). These results are in stark contrast to those of the normal group above (PI of 1.05, 0.97, 1.01 at 10 min, 120 min, and 240 min respectively; p<0.01 at all time points), and demonstrate the effect of 6 h of ischaemia upon post-reperfusion muscle blood flow. At 10 min of revascularisation, a period of low reflow was observed. This was followed by a period of partial improvement in flow at 120 min when muscle perfusion increased to approximately 30% of the normal value. However, following 240 min of revascularisation, muscle blood flow became almost negligible and not significantly different from that of the ischaemic group (see below); thus representing reperfusion injury of the gastrocnemius muscle.

Animals in the ischaemic group had the tourniquet left in situ throughout the duration of the experiment. The median PI (interquartile range in brackets) at matched time points, i.e. at 6 h, 10 min, 8 h and 10 h were 0.02 (0.00–0.04), 0.02 (0.01–0.04) and 0.04 (0.00–0.06) respectively. Against the controls, at 6 h 10 min p<0.05, and at 8 h p<0.01. At 10 h p=ns with respect to controls representing true reperfusion injury. These results demonstrate that muscle blood flow was virtually absent when the tourniquet was in situ with almost none of the microspheres entering the limb. Small amounts of radioactive emission were detected in these ischaemic limbs which could have been due to random background activity. Also, it is likely that a small amount of blood could still reach the gastrocnemius muscle via nutrient vessels within the femur and tibia which would obviously not be influenced by the tourniquet. Nevertheless, it is clear from these results that application of a tourniquet reliably produces an acutely ischaemic limb.

Gastrocnemius muscle oedema

These results are shown in Figure 2. Neither 10 h of general anaesthesia nor ischaemia alone for 6 h caused any muscle swelling. However, when 6 h of ischaemia was followed by 4 h of reperfusion, significant limb oedema ensued (normal and ischaemic groups vs controls p<0.01). The oedema indices (median values with interquartile ranges in brackets) of the normal, ischaemic and control groups were 1.00 (0.98–1.01), 1.01 (0.99–1.03) and 1.23 (1.09–1.37) respectively.

Gastrocnemius muscle viability

These results are shown in Figure 3. The duration of general anaesthesia per se had no detrimental effect on muscle viability, which was also the case in animals subjected to 6 h of ischaemia with no reperfusion. All muscles in these two groups had 100% viability. In contrast, rats subjected to 4 h of reperfusion following the 6 h of ischaemia showed marked evidence of muscle necrosis. The median muscle viability in this control group was 53% (interquartile range 33–61) indicating that significant muscle necrosis occurred during postischaemic reperfusion (p<0.01 vs normal and ischaemic groups).
which was associated with marked muscle necrosis (viability of 52.9% vs 100% in the normal and ischaemic groups). Thus the postischaemic decrease in perfusion was accompanied by significant muscle oedema and necrosis.

Confirming the hypothesis that reperfusion per se is injurious to tissues, the literature to date supports the view that reperfusion injury manifests as two pathological processes occurring principally within the muscle microcirculation and which together produce tissue loss and organ dysfunction. These processes have been termed “no reflow” and the “reflow paradox”.

No reflow was originally described by Ames et al. in a model of impaired microvascular perfusion following prolonged cerebral ischaemia. Other workers have since described this phenomenon in a variety of organs including the kidney and myocardium. In skeletal muscle the no reflow phenomenon was originally noted at 15 min following a 2.5 h period of ischaemia, with restoration of perfusion at 2 h. Longer periods of reperfusion were not used in this particular study.

Tissue perfusion following a period of ischaemia has been shown to be heterogeneous with areas of increased and decreased flow. It has also been shown that the degree of no reflow experienced by the tissues is directly dependent upon the duration of the preceding ischaemia, but that if the ischaemic period was short, there then followed a phase of reactive hyperaemia with no reperfusion injury demonstrable. This feature has been confirmed in our own laboratory. Other groups have described similar rodent models of reperfusion injury with shorter periods of ischaemia ranging from 2.5–4 h. In this setting the beneficial effects of various potentially therapeutic compounds in ameliorating SMRI may be spurious.

The “reflow paradox” or “oxygen paradox” was described by Menger et al. and denotes the detrimental events occurring in the tissues upon revascularisation and hence reoxygenation. Neutrophil activation with upregulation of leucocyte surface integrins (such as CD11b/CD18) and endothelial intercellular adhesion molecules (ICAM) causes further inflammatory cell accumulation within the reperfused tissue and therefore promotes the further detrimental generation of chemoattractants and reactive oxygen species, thus increasing the injurious process despite the restoration of perfusion. Hallmarks of this process are increased microvascular permeability and tissue oedema, hence the parameters measured in this model.

This study has clearly shown that reperfusion injury
Rodent Model of Ischaemia-reperfusion Injury

is associated with decreased microcirculatory blood flow, increased oedema and muscle necrosis, highlighting the importance of examining all these aspects in the same model in order to obtain a clear understanding of the deleterious sequelae of reperfusion. Previous studies examining only some of the above parameters and utilising shorter periods of ischaemia and reperfusion may not have given a true reflection of SMRI, particularly when our results have confirmed that the duration of ischaemia is a key determinant for the occurrence of reperfusion-related tissue injury. In our continuing efforts to ameliorate reperfusion injury, preclinical models such as the one described in this study could be used to evaluate potential therapeutic strategies.

References


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