Alterations in the morphology of skeletal myofibres after 90 minutes of ischaemia and 3 hours of reperfusion

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Summary

Morphometric, light and electron microscopic methods were employed to determine whether skeletal myofibres were damaged by 90 minutes of tourniquet-mediated ischaemia. Open biceps muscle biopsies were obtained before 90 minutes of upper limb tourniquet ischaemia in 5 Chacma baboons. Further biopsies were obtained just before tourniquet release in 2 animals and after 3 hours' reperfusion in the remaining 3 animals. Other than a slight reduction in myofibre diameter and the anaerobic depletion of intermyofibrillar glycogen, no pathological changes were noted in skeletal myofibres after ischaemia. However, after reperfusion there was myofibre enlargement, intermyofibrillar oedema, internalisation of nuclei, myofibrillar and mitochondrial disorganisation and dissolution, and Z-band streaming. These results show that reperfusion injury affects skeletal myofibres after 90 minutes of tourniquet-mediated ischaemia.

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Limb surgery is usually performed in a bloodless field using a pneumatic tourniquet. Times of continuous tourniquet-mediated ischaemia are usually restricted to a 'safe' period of 1 - 2 hours.[1] Despite periods of ischaemia being kept well within 'safe limits', there are many reports of patients who have developed post-tourniquet tissue oedema, weakness, stiffness and persistent numbness of the limb.[2,3] These symptoms characterise a reversible condition known as the 'post-tourniquet syndrome', which is associated with significant post-operative morbidity.[4,5]

Why should this syndrome develop? The symptoms of limb swelling and stiffness are thought to occur as a consequence of the interstitial oedema associated with tissue ischaemia,[6] whereas limb weakness and numbness are reported to occur as a consequence of direct nerve compression and neural ischaemia.[7,8] Is it possible that some of the symptoms associated with the post-tourniquet syndrome are the direct result of ischaemic damage to skeletal muscle?

Most studies that describe the effect of tourniquet ischaemia on skeletal muscle base their findings on the functional, biochemical and morphological appearance of the tissue at the termination of 1 - 8 hours' ischaemia.[3,9,10] With few exceptions,[11] most investigators report no or insignificant changes in muscle function, biochemical composition or myofibre morphology after continuous periods of ischaemia up to 3 hours.[3,12] However, more recent studies suggest that any morphological damage mediated by ischaemia is exacerbated by reperfusion.[13]

Although there are reports describing the appearance of skeletal myofibres after 1 - 2 hours' ischaemia,[9,12,14] to our knowledge there are no studies that have investigated the morphology of reperfused myofibres following this 'safe' tourniquet time. Is it possible that myofibre damage occurs following 1 - 2 hours of ischaemia and subsequent reperfusion, and that this may be at least partially responsible for the weakness associated with the post-tourniquet syndrome? The purpose of this study was to determine what effect the apparently 'safe' period of 90 minutes of tourniquet time followed by 3 hours of reperfusion had on the morphology of myofibres in primate biceps muscle.

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Material and methods

Five healthy adult male Chacma baboons were studied under general anaesthesia. Induction with ketamine 100 mg was followed by intravenous administration of alloferine 0,2 mg/kg and endotracheal intubation. Anaesthesia was maintained using 70% nitrous oxide in oxygen and intermittent positive pressure ventilation with a Penlon Nuffield 200 ventilator via an ADE breathing system in its E mode. The expired partial carbon dioxide pressure $PaCO_2$ was monitored by a capnograph and was maintained between 4 kPa and 5 kPa by adjusting the fresh gas flow. During anaesthesia the animal's blood pressure, heart rate, temperature, respiratory rate and expired carbon dioxide concentration were monitored. Reversal of anaesthesia was with neostigmine 0,07 mg/kg and atropine 0,02 mg/kg. Post-anaesthetic observation was undertaken by a veterinary surgeon.

The animals were placed in the left lateral position and open muscle biopsies of approximately $1 \times 0,5$ cm were taken from the right biceps muscle. The right arms were exsanguinated using an Esmarch bandage, and a pneumatic tourniquet was applied to the upper limb proximal to the control biopsy site. The cuff was inflated to 100 mmHg above systolic pressure and the tourniquet maintained for 90 minutes. Before tourniquet deflation, muscle biopsies were obtained from the ischaemic muscle adjacent to the control site in 2 animals. The remaining 3 animals were observed under anaesthestic for a further 3 hours, after which biopsies were taken from the reperfused muscle distal to the tourniquet.

In order to exclude mechanical artefact, the tissue was desensitised to further mechanical trauma by immediate immersion in 0,1M cacodylate buffered Karnovsky's fixative for 10 minutes.[15] After desensitisation, the central artefact-free core of the biopsy specimen was removed, as described by Olmesdahl *et al.*[16] and bisected for light and electron microscopy.

Light microscopy and morphometry

The samples were re-immersed in fixative for a further 12 hours, dehydrated and processed for histopathological examination using conventional wax embedding techniques.[17] Sections of 5 μm were cut, mounted on slides, stained with haematoxylin and eosin and examined with a Nikon 'Optiphot' microscope. Areas containing oblique-transversely-sectioned myofibres were displayed on a computer monitor via a video

camera interfaced with the microscope. After appropriate calibration and using the 'least diameter method' of myofibre assessment,[18] a VIDS image analyser was used to determine the fibre diameters (FD) of individual myofibres in the field of view. During the course of the study, 100 measurements were made from each of the 10 specimens.

The effect of ischaemia and reperfusion was assessed by comparing the difference of the means of the skeletal muscle diameters of the biopsies of individual animals.

Electron microscopy

The samples for electron microscopy were diced into 1 mm cubes and re-immersed in fixative at 4°C for a further 50 minutes. After post-fixation/staining in 0,1M cacodylate buffered 1% osmium tetroxide, the tissue was dehydrated through increasing concentrations of ethanol before being embedded in Araldite epoxy resin.[19] One micron sections were made from the resin-embedded blocks and stained with 1% alkaline toluidine blue. Areas of interest were photographed using a Nikon microscope. Using the 1 μm sections as indicators, the blocks were orientated until myofibres could be cut in longitudinal section. Ultra-thin sections of approximately 60 nm were cut with glass knives using a Reichert 'Ultracut' ultramicrotome. The sections were mounted on uncoated copper grips and stained with 1% ethanolic uranyl acetate and Reynolds' lead citrate[20] before examination with a Zeiss EM10B electron microscope. Measurements of organelles were made from electron micrographs via a graphics tablet interfaced with a VIDS image analyser.

Results

Light microscopy/morphometry

Pre-tourniquet specimens were populated with tightly packed myofibres, the nuclei of which were positioned immediately beneath the sarcolemma at intervals along the full length of the fibre. After 1,5 hours of ischaemia, other than the presence of an occasional fibre with an internalised nucleus, no obvious abnormalities were detected. After 3 hours of reperfusion, many myofibres contained one or more internalised nuclei and some exhibited considerable intermyofibrillar oedema (Fig. 1.)

Morphometry revealed an average 14% reduction of mean fibre diameter at the end of the ischaemic period, and an average 10% increase in fibre diameter after 3 hours of reperfusion (Table I).

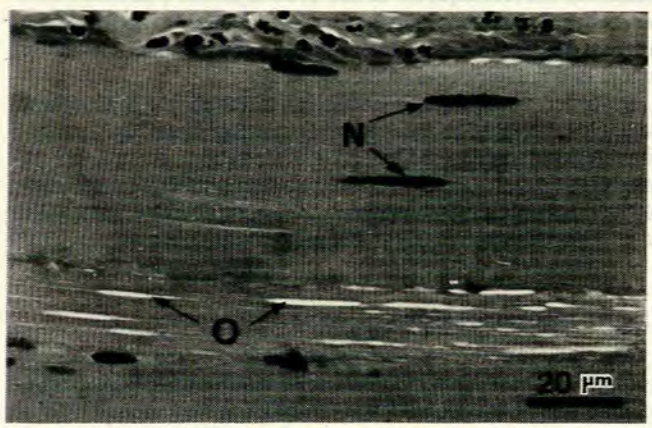

Fig. 1. Three hours' reperfusion: light micrograph showing internalised nuclei (N). Note intermyofibrillar oedema in central fibre (arrowed) (1 μm toluidine blue-stained section).

TABLE I. CHANGES IN CELL DIAMETER FOLLOWING ISCHAEMIA OR REPERFUSION

Pre-tourniquet	Ischaemic	Reperfused	*P*-value
67,4 ± 1,9	52,9 ± 1,2		< 0,00001
58,4 ± 1,7	48,9 ± 1,4		< 0,00001
52,9 ± 1,1		63,3 ± 1,5	< 0,00001
53,7 ± 1,2		56,7 ± 1,5	= 0,127
54,9 ± 1,2		60,9 ± 1,5	< 0,005

Values are expressed as means in μm and 1 standard error of the mean. Biopsies were taken before tourniquet application, at the end of the ischaemic period in 2 animals and after 3 hours of reperfusion in 3 animals.

Electron microscopy

Pre-tourniquet

All myofibres were longitudinally orientated and no supercontraction artefact was observed in any specimen. The myofibres were relaxed at the time of fixation with sarcomere lengths ranging from 1,7 μm to 2,2 μm. Nuclei, approximately 2,6 μm in diameter and from 10 μm to 13 μm in length, were situated beneath the sarcolemma (Fig. 2). There was generally a layer of chromatin just beneath the nucleolemma with aggregates of chromatin dispersed throughout the nucleoplasm. The double membraned nucleolemma had a regular intermembrane space of 20-25 nm. The mitochondria in the juxtanuclear regions were elongated, being approximately 0,4 μm in diameter and up to ± 1,7 μm in length. The christae were clearly visible and the mitochondriosol was quite electron dense. The mitochondria in the intermyofibrillar spaces were generally rounded (possibly as a consequence of plane of sectioning) being from 0,13 μm to 0,4 μm in diameter. Large quantities of glycogen were present in the inter-myofibrillar spaces and within myofibres, especially in the A-band region proximal to Z-bands. The sarcoplasmic reticulum was not swollen and T tubes were from 34 nm to 38 nm in diameter. Occasional small fat droplets were present both in the juxtanuclear and inter-myofibrillar spaces.

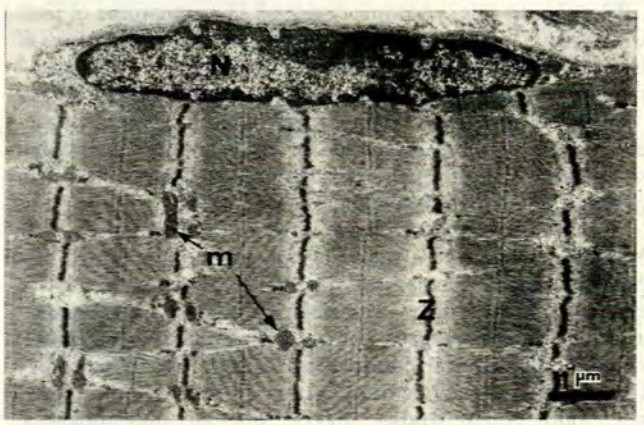

Fig. 2. Pre-tourniquet: electron micrograph of a normal skeletal myofibre (N = nucleus; Z = Z-bands; m = mitochondria).

1,5 hours of ischaemia

In both cases, myofibres were longitudinally sectioned. There was a substantial reduction in the quantity of intermyofibrillar and myofibrillar glycogen (Fig. 3) and an apparent increase in the length of some intermyofibrillar mitochondria (2,2 μm). The mitochondriosol of occasional mitochondria had pale

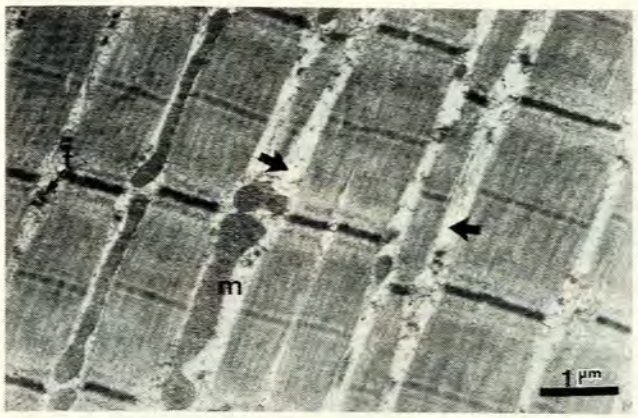

Fig. 3. One and a half hours' tourniquet ischaemia: electron micrograph showing depletion of intermyofibrillar glycogen (arrowed). Note elongated mitochondria (t = t tubes).

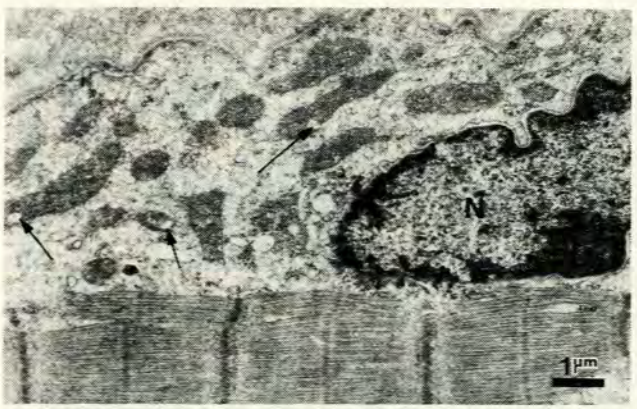

Fig. 4. One and a half hours' tourniquet ischaemia: electron micrograph showing pale structureless areas in some mitochondria (arrowed).

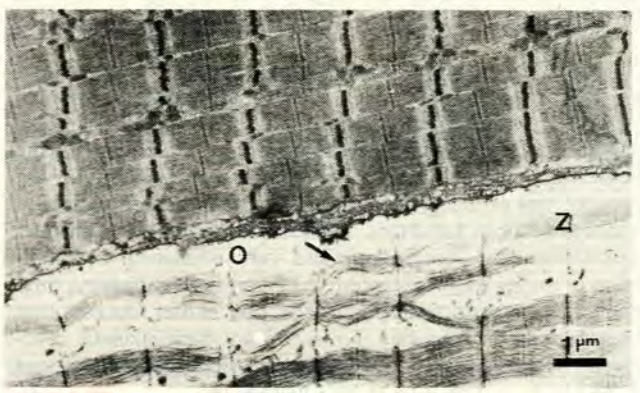

Fig. 5. Three hours' reperfusion: electron micrograph showing part of a normal and oedematous fibre (O). Note the disassociation of myofilaments from thinned Z-bands in the oedematous fibre (arrowed).

structureless areas that were suggestive of early mitochondrial oedema (Fig. 4). T tubes were a little dilated, being from 41 nm to 50 nm in diameter.

3 hours of reperfusion

Intermyofibrillar oedema was a common phenomenon in all reperfused specimens. A qualitative electron microscope and light microscopic assessment of myofibres in ultra-thin and 1 µm toluidine blue-stained sections suggested that approximately 20% of myofibres were oedematous. The Z bands in oedematous fibres were often thin with myofilaments becoming unravelled from electron 'pale' Z-band cores (Fig. 5). Internalised nuclei were more common in these than in ischaemic specimens. In oedematous fibres, internalised nuclei were often necrotic and had a 'beaded' appearance (Fig. 6), whereas those in non-oedematous fibres were morphologically normal (Fig. 7). Many myofibres exhibited Z-band streaming. Generally, 1 - 3 sarcomeres were involved (Fig. 8). There were, however, occasional myofibres in which Z-band streaming was more severe (Fig. 9). In some myofibres, myofilaments appeared to have become unravelled from 'moth-eaten' Z-bands to form a pool of disoriented filamentous material (Fig. 10). Severely swollen mitochondria were present in many myofibres from all reperfused specimens (Fig. 11). Many had become degenerate and were probably responsible for the increased number of myelin figures in the intermyofibrillar spaces (Fig. 12). The glycogen had returned to pre-tourniquet levels in non-oedematous fibres and was dispersed in the swollen intermyofibrillar spaces of oedematous fibres. The diameters of T tubes were unexpectedly normal in both swollen and non-swollen fibres.

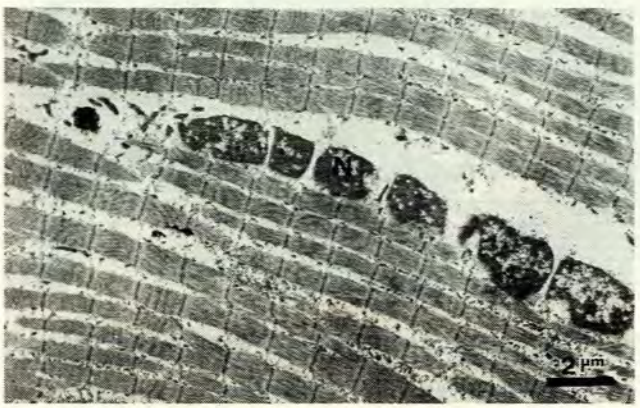

Fig. 6. Three hours' reperfusion: electron micrograph showing internalised 'beaded' nuclei in an oedematous fibre.

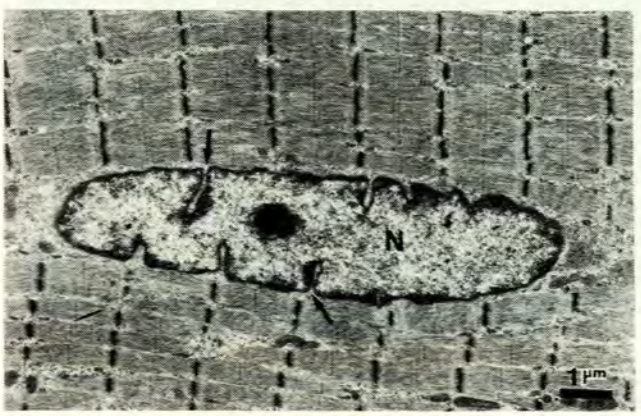

Fig. 7. Three hours' reperfusion: electron micrograph of an internalised nucleus in a non-oedematous fibre. Note the deeper than normal invaginations of the nucleolemma (arrowed).

Discussion

Striated myofibres may go into a state of supercontraction when mechanically damaged.[21,22] The biopsy procedure causes

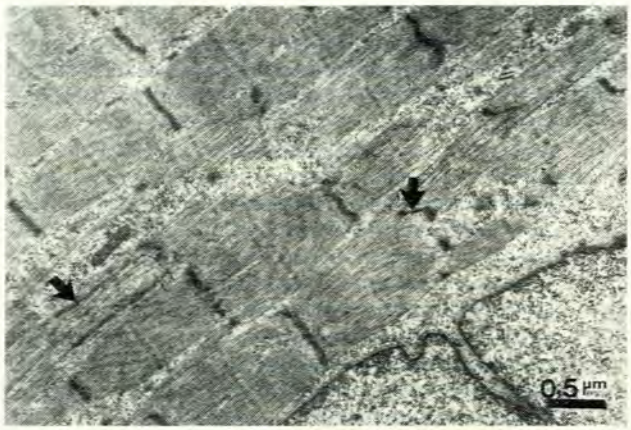

Fig. 8. Three hours' reperfusion: electron micrograph showing mild Z-band streaming in a non-oedematous fibre (arrowed).

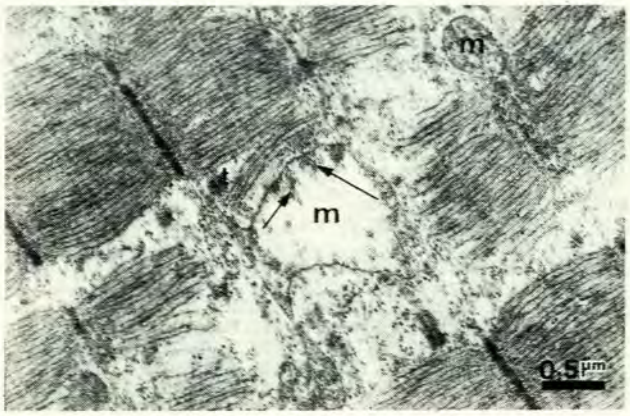

Fig. 11. Three hours' reperfusion: electron micrograph showing severely swollen mitochondria (cristae arrowed).

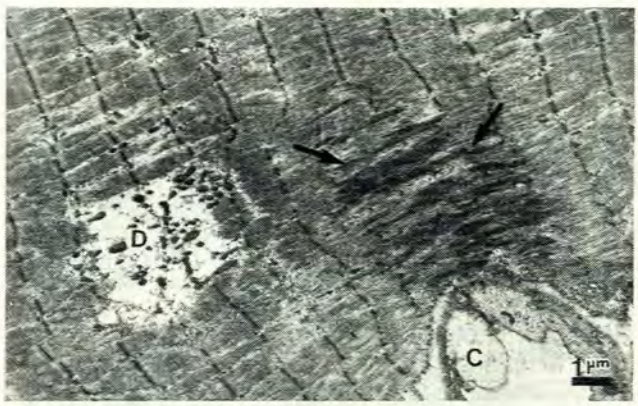

Fig. 9. Three hours' reperfusion: electron micrograph showing more severe Z-band streaming (arrowed). **Note the area of myofibrillar dissolution (D) (C = capillary).**

Fig. 12. Three hours' reperfusion: electron micrograph showing myelin figure (My) in intermyofibrillar space.

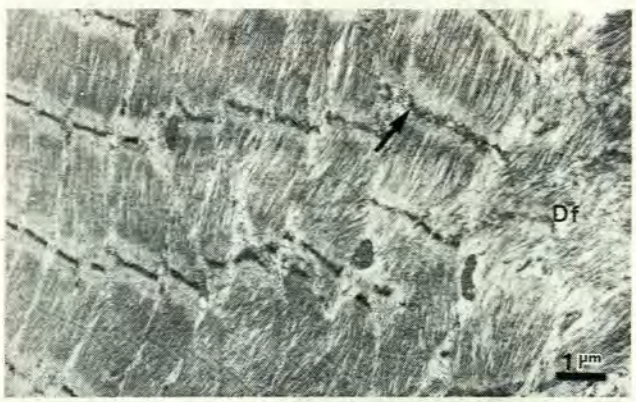

Fig. 10. Three hours' reperfusion: electron micrograph showing disorientation of myofilaments (Df). Note 'moth-eaten' Z-bands (arrowed).

supercontraction of sarcomeres adjacent to the cut surface and subsequent dicing of the tissue for electron microscopy damages the tissue still further.[16] Mechanical artefact may mimic changes caused by disease.[23] In this study, in order to ameliorate mechanical artefact, the method of Olmesdahl[16] was used. It is important to note that the results discussed below were derived from artefact-free tissue.

The most obvious alteration in myofibre morphology following 90 minutes of tourniquet ischaemia was the substantial decrease in the amount of intermyofibrillar glycogen. This may be a natural consequence of anaerobic metabolism during the ischaemic period, and the consumption of intermyofibrillar glycogen was probably responsible for the measured reduction in mean myofibre diameters. The minor fine structural changes in mitochondrial morphology and very occasional internalisation of nuclei was not suggestive of significant ultrastructural injury. These results support reports by earlier workers that short periods of ischaemia, *per se*, do not harm skeletal myofibres.[12] However, after 3 hours' reperfusion, many alterations to the fine structure of myofibres were noted.

Based on their morphological appearance, reperfused myofibres could be categorised into two types: those that exhibited intermyofibrillar oedema and those that did not. The internalisation of nuclei, swelling and degeneration of mitochondria and presence of myelin figures was common to both types of fibres, whereas nucleolemmal swelling and beading of internalised nuclei was only observed in oedematous fibres. The myofilaments of oedematous fibres often appeared to be unravelling from thin electron-pale Z bands into the intermyofibrillar spaces. Although Z bands often had a 'moth-eaten' appearance, Z-band streaming was not observed in oedematous fibres. Conversely, Z-band streaming was a common phenomenon in non-oedematous fibres. Although rare, myofibrillar dissolution also occurred in these fibres.

The cause and consequence of Z-band streaming and internalisation of nuclei are still a matter for debate. Whereas myofibrillar dissolution, myofilament disorganisation and mitochondrial swelling and lysis are probably degenerative phenomena,[24,25] Z-band streaming and internalisation of nuclei may be either pathological or regenerative features.[26,27] Irrespective of whether the phenomena are degenerative or regenerative, their presence in myofibres 3 hours after reperfusion indicates that 90 minutes of ischaemia causes significant submicroscopic injury, which only manifests after reperfusion. The mitochondrial and myofibrillar alterations described above may reduce myofibre contractility and be a cause of post-tourniquet limb weakness.

The significant increase in mean fibre diameter after reperfusion was evidence of myofibre oedema. The higher resolution light microscope preparations, together with the electron microscopic data, revealed that in each specimen approximately 20% of myofibres were oedematous. Mammalian skeletal muscle has been categorised into three primary types: oxidative (type 1); oxidative glycolytic (type 2a); and glycolytic (type 2b).[18] The consistency of incidence of myofibre oedema in each specimen suggested that such oedema was limited to one fibre type. This premise was supported by the morphometric data. After 90 minutes' ischaemia the coefficient of variation of fibre diameter was similar to that of the pre-tourniquet data showing that the myofibres became uniformly smaller. After reperfusion, however, the coefficient of variation was very much higher than in pre-tourniquet specimens, suggesting a disproportionate increase in the size of a specific group of myofibres. The fibres most likely to exhibit post-ischaemic injury are those that rely predominantly on oxidation to provide energy for metabolic processes — namely, the type 1 fibre. Although it appears that post-ischaemic oedema was limited to type 1 myofibres, this was not proven, and further investigations employing histochemical methods to discriminate between the three fibre types are being undertaken in order to identify the type of oedematous fibres.

In conclusion, although few morphological alterations were detected in myofibres following 90 minutes of ischaemia, significant pathomorphological changes were present in myofibres after 3 hours of reperfusion. This suggests that the present 'safe' period of 1 - 2 hours' tourniquet time may not be entirely without risk, and that short-term tourniquet-induced ischaemia and subsequent reperfusion may damage myofibres and contribute to the morbidity of the post-tourniquet syndrome.

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