# Creation of a contusion injury in rabbit skeletal muscle using a drop-mass technique

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This study reports our experience in developing a simple, minor injury. After reviewing the literature, a 'drop-mass' method was selected where a 201 g, elongated oval-shaped weight was dropped up to 15 times through a 1 m tube onto the left *vastus lateralis* of New Zealand white rabbits. To determine the extent of injury and degree of healing, biopsies were obtained six days after injury from the healing *vastus lateralis* of each animal. The tissue was fixed in formal saline, embedded in wax, cut and stained with haematoxylin and eosin (H&E) and phosphotungstic acid haematoxylin (PTAH) and examined by light microscopy (LM). The 'optimal' injury was created after seven drops, where quite severe, mild and moderately severe trauma was caused to muscle in the juxta-bone, mid and sub-dermal regions respectively. In each region, the muscle exhibited features of healing six days after injury. The 'drop-mass' technique appears to cause a contusion within a single muscle of at least three degrees of severity. This previously unreported observation is of particular importance to other researchers wishing to investigate contusion injury in other animal models.

## Introduction

The authors investigated the effect that two physiotherapeutic modalities, deep transverse friction and compressed air massage have on myofibres and capillaries of healthy, untraumatised *vastus lateralis* muscle of rabbits (Deane, Gregory & Mars 2002; Gregory & Mars 2003) and *tibialis anterior* muscle of monkeys (Gregory & Mars 2005). In order to investigate how and/or whether these modalities influence healing, it was necessary to create a relatively minor injury that would be in a particular and reproducible stage of healing within a short period of time during the healing process. Such an injury should not cause unnecessary pain or discomfort to the study animal, be measurable at the termination of the experimental healing period, and therefore comparable with similar uninjured and untreated tissue over the same period of time.

Skeletal muscle injury produced for experimental purposes may be broadly classified as being either 'super-contraction' or 'trauma' induced. A wide range of animal species have been used, including chickens, monkeys, dogs, cats and other manageably sized mammals, especially rabbits, rats, mice and other small rodents (Tiidus 2008).

Super-contraction induced injuries are a consequence of strenuous exercise or high-force eccentric contractions. They are achieved by forcing animals to run on motorised treadmills or by electrical stimulation of muscle. Vihko, Rantamaki and Salminen (1978) forced rodents to run on a level treadmill, whereas Armstrong, Ogilvie and Schwane (1983) forced rats and Carter *et al.* (1994) mice to walk and run downhill. Downhill running was considered by Armstrong, Ogilvie and Schwane (1983) to cause injury because the antigravity (extensor) muscles would be stressed from eccentric contractions. Exercise induced injury may cause stress to the animal with concomitant endocrine and biochemical changes that may affect biochemical and or morphological parameters being measured.

To overcome this, Warren *et al.* (1994) anaesthetised rats and rabbits and electrically stimulated their dorsiflexor or plantarflexor muscles to elicit eccentric contractions. In this study, the hind foot was secured in a shoe-like structure attached to the shaft of a servomotor. Best *et al.* (1998) attempted to determine whether hyperbaric oxygen improved muscle stretch in *tibialis anterior* muscle tendon unit of rabbits. The muscle was stimulated to tetany to create an eccentric stretch injury. As electrical stimulation causes muscle to perform abnormally high-force eccentric contractions, the validity of an electrical stimulated muscle model against which 'normal' injury can be compared has been questioned (Butterfield & Hertzog 2005; Sandercock 2003).

Trauma induced injuries to muscle are almost exclusively performed on anaesthetised animals. Injuries have been induced by chemicals, cold, ischaemia, sharp trauma, crush or contusion.

Chemical injuries have been produced by injecting snake venom or local anaesthetic into muscle. Cobra venom, containing a cardiotoxin that causes skeletal muscle contraction and interferes with neuromuscular transmission, and venom of the Australian tiger snake, which contains notoxin, a myotonic phospholipase that destroys muscle tissue, have been used (Fink *et al.* 2003; Goetsch *et al.* 2003; Kirk *et al.* 2000; Ullman & Oldfors 1991; Vignaud *et al.* 2003; Zhao *et al.* 2002). Local anaesthetics, bupivacaine (Marcaine) and lidocaine have been shown to be effective in inducing fibre degeneration (Gregorevic, Lynch & Williams 2000; Shiotani *et al.* 2001). A problem with toxin or anaesthetic induced trauma is the difficulty in administering an optimal dose to provide the desired degree of injury and the unintended systemic effects when taken up in the circulation.

Other methods of causing muscle trauma include freezing muscle using a metal probe cooled to dry ice temperature (Kuang *et al.* 1999; Pavlath *et al.* 1998), cutting a muscle using a scalpel blade (Zhang & Dhoot 1998), and obstructing the blood supply to the muscles of an entire limb for from 1 to 6 hours with a ligature. In this model, ischaemia does not in itself cause fibre degeneration; the injury is caused by oxidative damage and neutrophil invasion during the reperfusion phase (Huda, Solanki & Mathru 2004; Huda *et al.* 2004)**.** The direct application of force to muscle is the most common method of creating muscle injury, and can be simply achieved by crushing muscle using forceps (Fink *et al.* 2003). More complex instruments have been devised to cause contusions, an example being the spring-loaded hammer used by Jarvinen (1976a, 1976b and Hurme *et al.* (1991), which allow the impact (force) on the calf muscles of rats to be controlled by adjusting both spring and hammer mass.

A simple and easily adjusted method of producing a measurable contusion injury is by dropping weights from various heights through a guide tube onto an identifiable muscle. The degree of injury can be controlled by adjusting weight and height of drop and the number of times the weight is dropped. Many investigators have adapted this 'drop-mass' method. Crisco *et al.* (1994) dropped a 171 g weight from a height of 102 cm onto the mid-belly of the posterior surface of the *gastrocnemius* muscle of mice. Minamoto, Grazziano and Salvini (1999) injured rats by dropping a 200 g weight from a height of 37 cm to the middle belly of the *tibialis anterior* muscle. Bunn *et al.* (2004) dropped 100 g or 200 g weights from a height of 130 mm through a guide tube onto the *quadriceps* muscle of mice. To determine the effects of laser therapy on acute blunt trauma, Fisher *et al.* (2003) dropped a solid aluminium bar with a flat surface of 1.38 cm$^2$ weighing 700 g down a tubular guide through a distance of 125 mm onto the medial *gastrocnemius* muscle of rats. McBrier *et al.* (2007) and Markert *et al.* (2005) used the drop-mass technique on rats to determine the effects of therapeutic ultrasound.

No drop-mass contusion model could be found for the rabbit, the animal in which the effect of deep transverse friction to the untraumatised *vastus lateralis* muscle and its capillaries has been documented (Deane, Gregory & Mars 2002; Gregory & Mars 2003). To extend the work to traumatised muscle and other therapeutic modalities requires the development of a contusion model. The aim of this study is to report the development of a reversible, potentially measurable, contusion injury in adult rabbit *vastus lateralis* muscle.

# Methods

Based on the literature, the 'drop-mass' method was chosen to produce the injury, with a weight of 201 g dropped vertically from a height of 100 cm through a guide tube of 2 cm diameter on to the *vastus lateralis* muscle in anaesthetised New Zealand white rabbits. Muscle injury was assessed by macroscopic and light microscopic appearance of the muscle biopsy six days after injury. The number of drops required to produce a demonstrable residual injury showing features of repair was determined by serial experimentation. For each stage, the macroscopic and microscopic appearance of the tissue was examined for 'severity' of the injury before the next animal was injured. Only when the injury was deemed inadequate for further study purposes, was the next animal injured.

Eleven New Zealand, white rabbits were studied with the approval of the Ethics and Research Committees of the University of KwaZulu-Natal The animals were housed in a barrier animal facility in the animal research facilities of the University of KwaZulu-Natal and fed *ad libitum* with commercial rabbit pellets. They were maintained under the care of the staff of the facility, according to the guidelines of the Animal Ethics Sub-committee of the University of KwaZulu-Natal.

The day before the procedure, the lower limbs of the animals were shaved and hair further removed with commercially available hair remover (No Hair® Adcock Ingram Healthcare [Pty] Ltd.) to facilitate observation of any inflammatory reaction and facilitate treatment. Before injury, all animals were weighed and anaesthetised with an intramuscular injection of the combination of 30 mg/kg bw ketamine (Anaket-V, Bayer [Pty] Ltd. Isando South Africa) and 4 mg/kg bw xylazine (Xylavet 2%, Intervet SA (Pty) Ltd. Isando South Africa) followed by subcutaneous injection of 5 mg/kg bw morphine sulphate (Morphine sulphate Fresenius PF 10 mg/mL Bodene [Pty] Ltd. Port Elizabeth South Africa). The rabbit was placed in lateral recumbency and the left hind leg was supported manually with the hip flexed 135° and the knee flexed at 90°. The mid belly of the *vastus lateralis* muscle was identified by palpation and the skin marked with indelible ink. A guide tube, 100 cm in length and 2 cm diameter, was held vertically and centred over the mark (Figure 1) and an elongated oval weight of 201 g (Figure 2) dropped on the muscle. The point of impact was calculated to be approximately 4 mm$^2$.

### Creation of injury

**Part 1:** Rabbits 1–5 were subjected to 1, 2, 5, 10 and 15 drops respectively. The appearance of the skin was noted
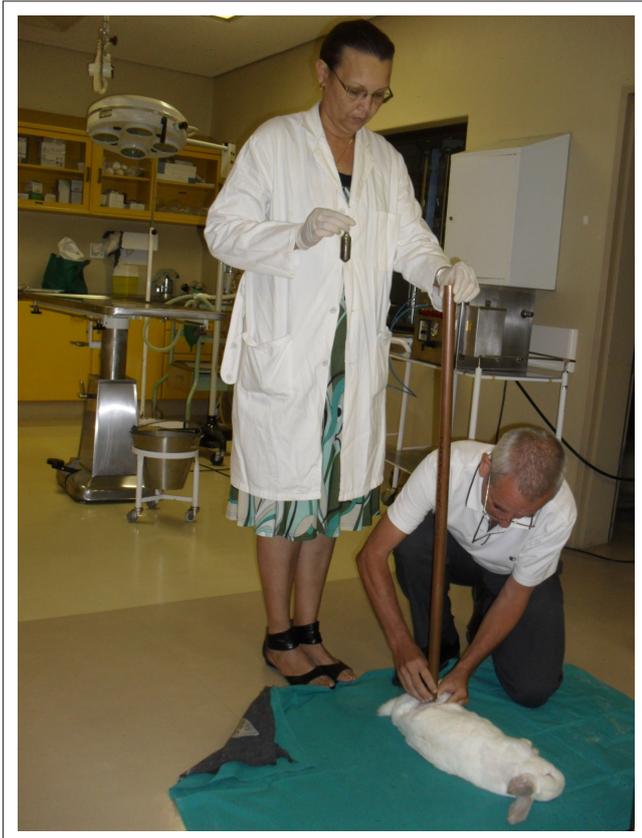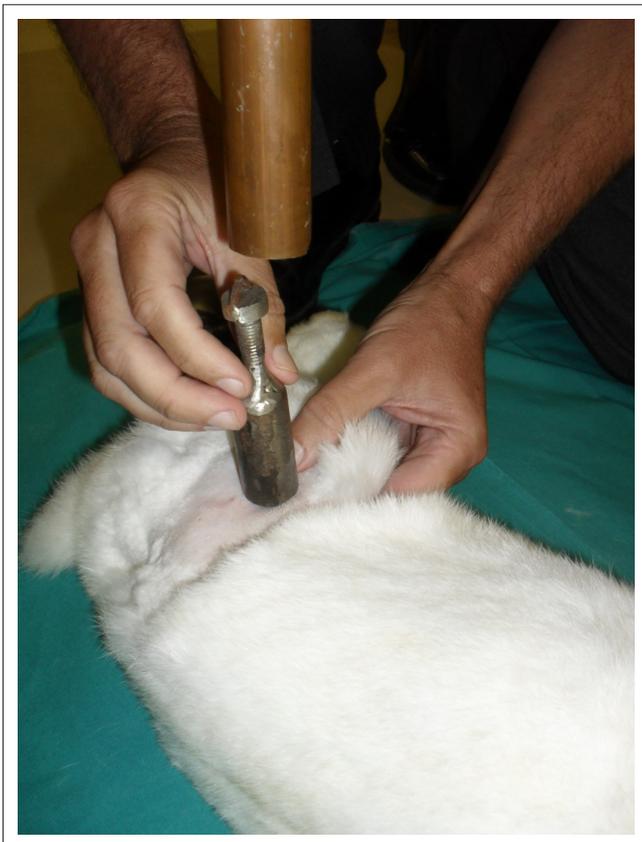
**FIGURE 1:** A 100 cm guide tube held vertically and carefully centred over the marked *vastus lateralis* muscle.



Note: Shaven area over *vastus lateralis* muscle.
**FIGURE 2:** A 201 g elongated oval weight used to cause injury.

immediately after injury and before biopsy. Six days after injury, the skin was incised longitudinally over the mid-thigh, the fascia opened and the macroscopic appearance of the muscle noted. A biopsy approximately 8 mm by 7 mm by 3 mm was taken from the mid portion of the *vastus lateralis* of the injured left limb of each animal. Following biopsy, the animal's skin was sutured and the animal monitored for any post-operative discomfort during and after recovery from the anaesthetic. The animals were mobile immediately after recovery from the anaesthetic, both after the injury and within 12 h of biopsy. Two weeks after surgery, the wounds had healed completely with no apparent ill effects demonstrated by any animal. The wounds were regularly cleaned with 2% hibitane solution prior to suture removal.

All biopsies were immediately immersed in 10% formal saline for 24 h and dehydrated through increasing concentrations of ethanol prior to clearing with xylene and embedding in paraffin wax. Sections 4 μm in thickness were cut and stained with haematoxylin and eosin (H&E) to show general cellular features, and phosphotungstic acid haematoxylin (PTAH) to show damaged or necrotic myofibres. The sections were examined using a Nikon Compound Microscope (Nikon Eclipse 8i) and images of the tissue were captured at ×10, ×20, & ×40 magnification and stored in jpeg format.

Each biopsy was assessed microscopically for residual injury and phase of healing and compared with the biopsy from the healthy untraumatised right limb of each animal.

**Part 2**: Based on observation of a superficial and deep injury to muscle after 10 and 15 drops, the protocol was amended. Ten drops were applied to 1 limb and 2 drops to the other in rabbit 6 and 5 drops and 7 drops to the left and right limbs of rabbit 7. As before, the macroscopic appearance of both the surface injury site and the underlying muscle was recorded and biopsies performed after six days. Smaller biopsies (approximately 3 mm by 5 mm by 3 mm) were taken from deep, juxta-bone and superficial, subdermal positions within the *vastus lateralis* muscle from each limb and assessed as before. Having established the optimal number of 'drops' to be 7, the left limbs of rabbits 8–11 were injured with 7 drops and superficial and deep biopsies taken after six days from each injured left limb and control right limb.

## Results

**Part 1:** There was no evidence of trauma either macroscopically or microscopically in biopsies from the mid portion of the *vastus lateralis* muscle after 1, 2 or 5 drops. There was a light reddening of the skin immediately following the 10 and 15 drops, but six days after injury there was no macroscopic evidence of trauma to the skin. Control muscle from each right limb was morphologically normal (Figure 3). Only after 15 drops to rabbit 5 left limb (R5L) was there any microscopic evidence of damage to the muscle obtained from the central portion of the injured *vastus lateralis*. Here, occasional abnormal, possibly necrotic myofibres or myofibres in the

process of regeneration were seen in some regions. However, after 10 and 15 drops, it was noticed prior to biopsy that there was a darker discolouration with bruising of the muscle at the impact zone and haemolysed muscle close to the bone. It appeared that the mid-region of the muscle had been largely cushioned from the effect of the impact and was not the best region to examine. The protocol was changed to include superficial and deep biopsies of the injured muscle.

**Part 2:** With 15 drops resulting in severe juxta-bone trauma, the number of drops was reduced. After 10 drop**s**, macroscopically the tissue was haemolysed near the bone. Microscopy revealed that the muscle in this region had been severely damaged. Necrotic myofibres had been removed by numerous phagic cells and repair was underway with many myotubes and small myofibres being present in cellular areas. The epimysium was severely thickened in these regions and contained numerous fibroblasts, some of which appeared to be migrating into the muscle body. Whilst less extensive than in the deep biopsy, muscle morphology in the superficial region was similar. After 2 drops, all sectors of the muscle, superficial and deep, appeared normal. After 5 drops there was evidence of trauma in the juxta-bone position similar to that seen in the superficial biopsy of rabbits that received 10 drops, but little or no evidence of injury in superficial regions.

Following 7 drops, macroscopically the muscle was bruised near the bone. Microscopically, there was significant residual injury and evidence of repair in this region, similar to but less extensive than that described above after 10 drops. The epimysium was significantly thickened with cells migrating into the muscle body. In the immediate sub-epimysial region, there was an absence of myofibres with myotubes and small myofibres developing within a disorganised cellular matrix (Figure 4). Peripheral to the cellular area were darkly staining, large, rounded myofibres that were probably injured myofibres in the process of regeneration (Figure 4). The biopsy from the superficial region showed a lesser injury with a much thinner disorganised cellular region surrounded by swollen or regenerating myofibres beneath a less thickened epimysium. Myofibres in muscle bundles far from the epimysium, near the mid-regions of the muscle, in both the superficial and deep biopsies appeared generally normal. There were, however, occasional fibres showing evidence of myofibril disorganisation suggesting that even this central portion of muscle had experienced minor injury (Figure 5). The findings in the 4 further animals injured with 7 drops were similar and consistent with those described above.

## Ethical considerations

This study was approved by the Ethics and Research Committees of the University of KwaZulu-Natal on a yearly basis to accommodate any changes that might have occurred in the process of research project using animals from 2009. References: 022/09/Animal; 018/10/Animal; 022/11/Animal; 010/12/Animal; and 069/13/Animal.
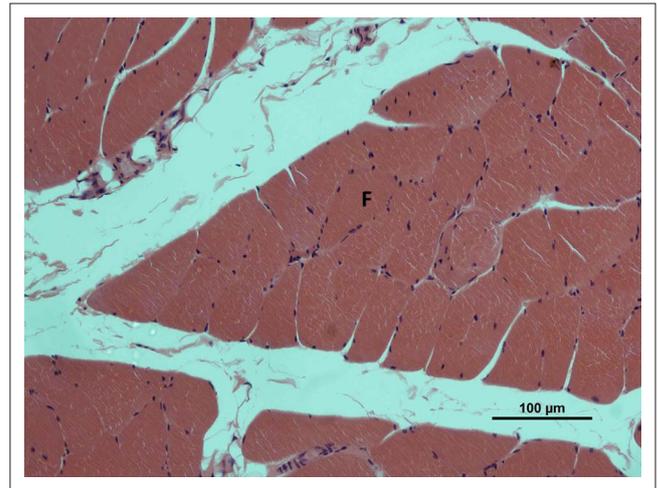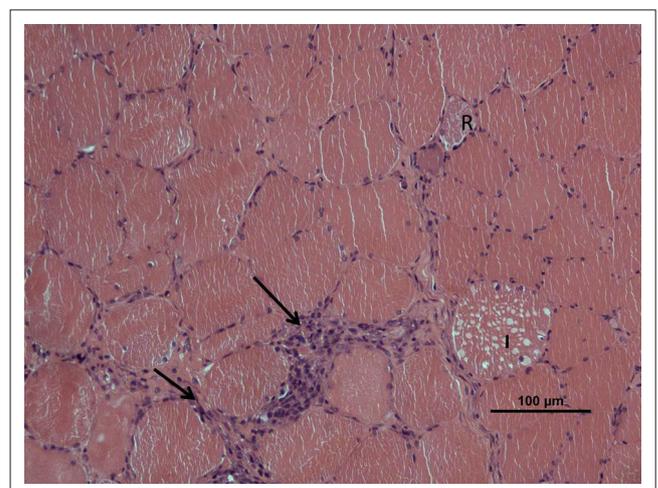


F, myofibres.

**FIGURE 3**: Haematoxylin and eosin-stained section showing normal cross-sectioned myofibres in normal rabbit skeletal muscle.
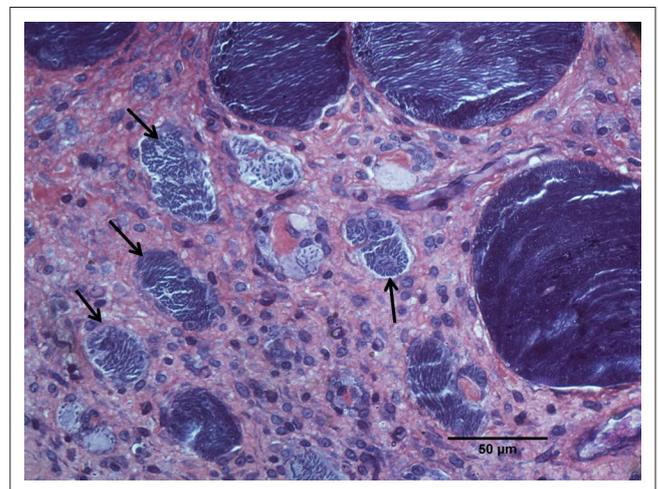


Note: Rounded injured myofibres at the periphery of the cellular area.

**FIGURE 4:** Phosphotungstic acid haematoxylin-stained section from biopsy showing small regenerating myofibres in a cellular matrix.



Arrows, cellular infiltration.

**FIGURE 5**: Haematoxylin and eosin-stained section from subdermal biopsy showing an injured myofibre (I) and possible regenerating (R) fibre within a bundle of apparently normal myofibres.

# Discussion

A reproducible injury to the *vastus lateralis* muscle in the New Zealand white rabbit was developed and described using a drop-mass technique. The objective was to produce an injury that could equate to a treatable contusion injury in man. The contusion, therefore, had to provide residual evidence of trauma or significant evidence of healing at the termination of our experimental period which was six days after injury. The residual degree of muscle pathology or phase of healing, by inference, informed the authors of the 'severity' of the contusion at the time of injury. Seven drops of a weight of 201 g from a height of 100 cm achieved this.

An unexpected finding was that there appear to be at least three zones of injury in this model. The expectation was that the injury would be maximal at the point of impact, reducing with distance from the point of impact. This was not the case. The most severe injury occurred adjacent to the bone, where the muscle tissue is seemingly crushed against the denser bone and capillaries are damaged, leading to local ischaemia. The superficial tissue at the point of impact is less severely injured whilst the central area shows the least injury. That there is not a continuum of injury may be explained by the elasticity of muscle and the fact that muscle is a functional gel. The impact can be likened to a set of shock waves set up at the point of impact. The force is localised at that point, but dissipates as the waves spread in the gel. When the force wave reaches the denser bone it cannot be transmitted and is dissipated in the muscle tissue adjacent to the bone, causing the most severe injury.

A contusion injury, therefore, causes complex, position-dependent processes of muscle regeneration and repair through the thickness of the muscle. These observations should be taken into consideration when creating an experimental injury and when treating a contusion injury with a physiotherapeutic modality.

The epimysium appears to play a role in the injury repair process. Epimysial thickness varied considerably in superficial and deep biopsies, with both being very much thicker than in control specimens. In deep biopsies, it was markedly thickened and contained numerous fibroblasts that appeared to be migrating into the muscle body. The role of the epimysium in muscle repair is not well documented and requires further investigation.

# Conclusion

A model of reproducible contusion injury showing signs of repair and regeneration six days after injury was developed using a drop-mass method in the rabbit. At least three zones of injury of differing severity were identified. The morphological differences in degree of damage and extent of healing in superficial and deep biopsies may enable the creation of a morphological index that could be used to semi-quantify the degree of repair. This would enable the efficacy of therapeutic regimens to be assessed and compared. Further work on the development of a scoring system is required before treatment modalities are assessed.

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