

Research Article

IMAGE ANALYSIS IN GOMORI'S TRICHROME STAIN OF SKELETAL MUSCLES SUBJECTED TO ISCHEMIA AND REPERFUSION INJURY

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ABSTRACT: Conditions that produce ischemia and reperfusion injury include orthopedic surgeries, vascular diseases and accidents in remote places in which use of a manual tourniquet is required. Tissues under such stress suffer the consequences of evidenced by changes in their normal microscopic organization that can be reversible or irreversible according to the time and severity of lesion. An experimental model of ischemia has been designed taking into account the characteristics similar to a surgical procedure, from preparation for anesthesia up to the postsurgical follow up of each animal until it finishes the established time of reperfusion. Two muscles, soleus and extensor carpi radialis longus, dissected from Wistar rats that were underwent to short periods of ischemia and short and prolonged periods of reperfusion up to 32 days. There were no significant changes in the macroscopic weight of muscles, but significant differences were found in the area occupied by intramuscular extracellular matrix. During reperfusion, a partial recovery was observed until the last day of study. If we pretend to extrapolate these results to clinical areas, its importance focuses in the recovering of function and the following up of patients after surgical procedures as studied in the present experiment.

Key words: Ischemia, Reperfusion, Wistar rats, Soleus, Extensor carpi radialis longus.

INTRODUCTION

The technique of induced ischemia by using a pneumatic tourniquet has been widely used in animal models, especially in rat, mouse and rabbit. This procedure is quite similar to the technique applied in surgical practice and

research in order to perform histological, physiological and pharmacological analysis in oxidative and glycolytic fibers.

According to the type of investigation to carry out, a manual tourniquet with simple materials may be used (Souza *et al.*, 2009).

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However, if precise control of pressure to generate ischemia is required, it is important to select proper material to use it in the process of designing and preparation of the tourniquet in the laboratory. That must be a cuff with a correct length and diameter for the animal, which can be adapted to a source of insufflations and a manometer.

One of the main complications of orthopedic surgery with the use of a pneumatic tourniquet is the thrombo-embolism, especially if a patient is having the risk factors. The protocol before putting the tourniquet includes the ex-sanguination technique, that was performed manually by elevating the arm 90° or the leg 45° for five minutes, or by using a sterile elastic ex-sanguination tourniquet. However, some local and systemic complications were reported in both the methods (Brin *et al.*, 2015; Feldman *et al.*, 2015; Wakai *et al.*, 2001).

A practical method to ensure a complete ischemia is to apply the systolic pressure above 75-100 mm Hg (Estebe *et al.*, 2011). An essential aspect of that method is that the investigator must be sure that the occlusion covers the arterial and venous circulation to avoid edema formation due to vascular congestion. However, the skin, muscles, nerves and vessels present in the lesions show an asymmetric distribution, not only due to the induced ischemia, but also because of the mechanical pressure created under the cuff.

Ischemia and reperfusion injury are common surgical conditions still today. In the last decade, many study reports related with these aspects were published, though none of those researches presented a complete description of the procedure. To fulfill that need, the present study was conducted. The objective of this study was to establish an animal model of ischemia and reperfusion

injury, similar to a surgical procedure in clinical areas, and to describe changes in soleus and extensor carpi radialis longus in their intramuscular extracellular matrix by using Gomori's trichrome stain.

MATERIALS AND METHODS

Animals and experimental design

Forty two (42) male Wistar rats were used in this study. The animals were obtained of the vivarium of Universidad del Valle, which accomplishes requirements established in national and international guidance for the care and use of animals for experimental research. The ethics committee of Universidad del Valle authorized the procedures carried out in this work, taking into account recommendations of the manual for the appropriated used of animal models of the Faculty (Act 001-2011).

The rats were housed in the cages and were provided with food and water *ad libitum*, as well as control of temperature, humidity and light of the room during day and night.

The experimental design consisted by three phases. In phase I (the surgical phase), the rats were randomly distributed into 20 experimental groups and one control group (two animals in each group). The pneumatic tourniquets were used and as per requirement during the study period. The rules followed in operating rooms were applied during the experiment and this phase ended with performing euthanasia of animals. During Phase II, both muscles were dissected and were processed in laboratory for histochemical stain. Phase III consisted morphometric analysis and recording of results.

Phase 1 (Surgical Phase)

This phase had three sub-phases: pre-surgical, trans-surgical and post-surgical. These surgeries were classified as minor/non-invasive, according

Table 1. Procedure for Gomori's trichrome stain.

Solution	Reactive	Amount	Procedures	Stain
Harris's Hematoxylin				Kept in the solution for 5 minutes. Washed 3 times with distilled water.
Trichrome	Fast green	0.3 gr	All the chemicals were mixed and shaken before use	Kept 20 minutes in trichrome solution. Then immersed twice in glacial acetic acid 2%.
	Chromotrope 2R	0.6 gr		
	Phosphotungstic acid	0.3 gr		Dehydrated in alcohol 95% twice for 2 minutes each. Then in 100% alcohol twice for 2 minutes each. Placed in xylene twice quickly.
	Glacial acetic acid	1 ml		
	Distilled water	100 ml		
			Results: Connective tissue: Green Muscle fibers: Red	

to the veterinary guidelines for anesthetic risk, based on the Physical Status Classification System of the American Society of Anesthesiologists (Muir 2007). In pre-surgical sub-phase, there was a one-control group, which showed normal characteristics of muscles (Fig. 2). There were a total of 10 experimental groups of ischemia of one hour and reperfusion of zero hour, one hour, 16 hours, 24 hours, 48 hours, 96 hours (4 days), 192 hours (8 days), 384 (hours), 576 hours (24 days) and 768 hours (32 days). In the same way, there were 10

experimental groups of three hours of ischemia and the same periods of reperfusion. Two animals were kept to each group, following the concept of reduction, according to the guiding principles for ethical use of animals in testing (Franco and Olsson 2014).

The anesthesia was performed first as an induction of isoflurane 100% (ISOFLUORANO USP® - Baxter) through inhalation keeping the rat inside a glass chamber. Then, to maintain the anesthetic condition, injection of pento-barbital

Table 2. Acquisition parameters of images.

Parameter	10x
<i>Exposure to light</i>	Automatic
<i>Manual adjustment of light</i>	Low
<i>Bright</i>	50%
<i>Saturation</i>	1.1
<i>Gamma</i>	0.7

(Penthal® - INVET 64.8 mg/ml) was given intraperitoneally. No premedication and no avoidance of food and water were performed before surgery.

The trans-surgical phase began with the use of the pneumatic tourniquet (Fig. 1). This step was designed as a part of the present research. The measurements to construct the tourniquet were 14 cm X 4 cm. It was covered with a cloth of Kodraand. A center was made of a synthetic material. A belt of adhesive material was added in order to fix the dispositive to the limb of the rat. Besides, the center was connected with two hoses of 9 cm length. One of them was in contact with a conventional manometer, and the other one with a source of insufflations and a valve for the control of the amount of air and pressure to create ischemia (Estebe *et al.*, 2011).

Two tourniquets were used in the total system. One was put around the right upper limb to induce ischemia during one hour or three hours in the extensor carpi radialis longus, and the other tourniquet was used to put it above the left hind limb to induce an ischemic condition of one hour or three hours in the soleus muscle (Fig. 1). These tourniquets were kept contra-laterally in order to avoid a vascular decompensation (Mendoza 2002).

Phase II (Processing of samples)

This phase began with dissection of the muscles, soleus and extensor carpi radialis

longus. The dissection was performed within 15 minutes to as it is required to perform enzymatic histochemistry techniques afterwards (Dubowitz *et al.*, 1973). Weight of each muscle was measured in an electronic scale.

Each sample obtained after dissection was added with Tissue Tek (O.C.T.), then put in isopentane (2-metylbutane 99.5%) for one minute and afterwards in liquid nitrogen for 3-4 minutes. These samples were finally stored at -70°C.

Each section of muscle was cut by using a freezing microtome at a temperature of -20°C before study. Each cut was placed in ionized slides for adequate adherence. Each slide contained six to eight cuts. The quality and correct orientation of tissues were observed by hematoxylin and eosin stain. After that, Gomori's trichrome stain was performed (Table 1). Gomori's trichrome stain is a conventional histochemical technique useful to observe the intramuscular extracellular matrix visible in a green contrast. The muscle fibers appeared red or more intense. The abnormal vesicles in the cytoplasm stain reddish (Sanoudou *et al.*, 2006).

Normal characteristics of skeletal striated muscle tissue (Fig. 2) included hexagonal fibers with peripheral nuclei, surrounded by a loose connective tissue (endomysium). Muscle fascicles surrounded by a dense connective tissue formed perimysium, that lead to association of groups to form the muscle, which was surrounded by a dense connective tissue, epimysium.

Phase III (Image analysis)

The acquisition of image was made in 10x magnification, by using a microscope Leica DM750 adapted to a digital camera DFC295



Fig. 1. Surgical phase, induced ischemia of one and three hours. A. Two pneumatic tourniquets were put to the animal, one in the right upper limb and the other in the left hind limb. B. The position of the tourniquet in the upper right limb for ischemia in the extensor carpi radialis longus. C. The pressure was up to 250 mm Hg. D. The position of the tourniquet in left hind limb. The periods of reperfusion were of zero hour, one hour, 16 hours, 24 hours, 2 days, 4 days, 8 days, 16 days, 24 days and 32 days.

and to a software LASV3.8. The parameters of contrast, exposure to light and others, were standardized (Alfaro *et al.*, 2003).

The image analysis was performed by the software Image Pro Plus version 7.0 Media Cybernetics.

Previous to image analysis is required image processing consisted define regions of interest and techniques to measure those regions (Young *et al.*, 1998). The method to establish regions of interest and no interest to be recognized by the

software, is the conversion of images to gray scale and applying masks of colors to improve visual determination for measurements, in this case for connective tissue (Fig. 3 and Fig. 4).

Distribution of connective tissue and muscle fibers during reperfusion were studied. Though conventional and enzyme histochemistry made visible important aspects of tissue, image analysis measurements are also important. Conversion to gray scale is based on color channels of red, green and blue (RGB), which

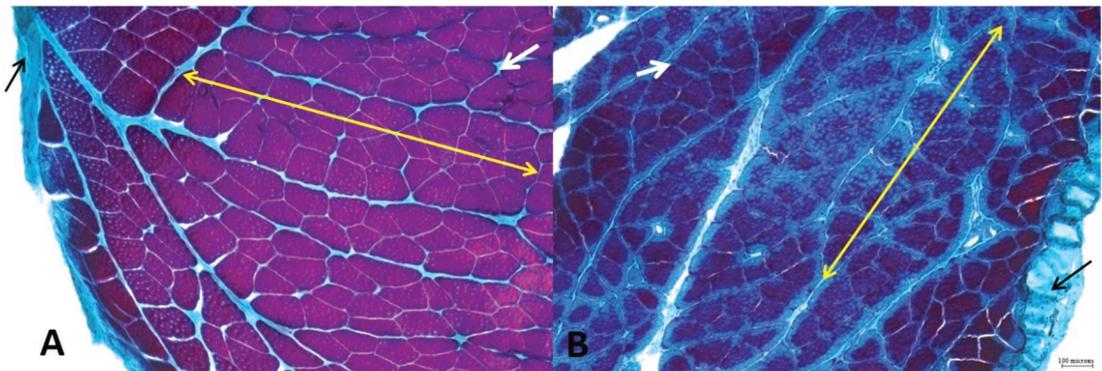


Fig. 2. A. Extensor carpi radialis longus muscle. Control group. B. Soleus. Control group. Both images evidence muscle fascicles (yellow double arrow) surrounded by perimysium. Note the epimysium externally (black arrow) and the endomysium internally (white arrow). Magnification 10x. Gomori's trichrome stain. Scale bar: 100ums

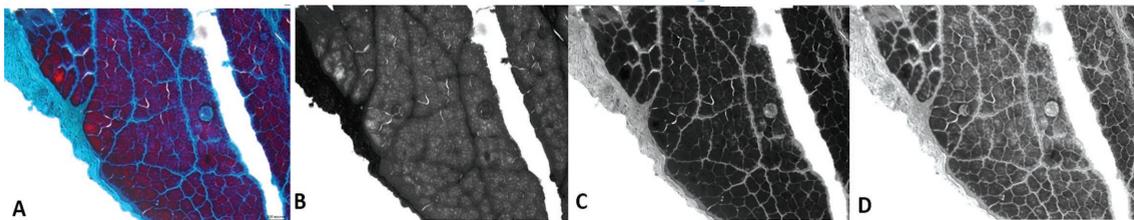


Fig. 3. Digital image processing – Color channel. A. Extensor carpi radialis longus with visible epimysium (external), perimysium (surrounding muscle fascicles) and endomysium (surrounding fibers), seen in green contrast. Gomori's trichrome stain. B, C and D. Gray scale image in the Red, Green and Blue channels of color. Gomori's trichrome stain. Magnification 10x. Scale Bar: 100 ums.

permits digital application of color to each pixel, according to the intensity of each region in the range of 0 (white) to 255 (black).

In this study, the green channel was selected to observe characteristics of skeletal striated muscle tissue in a better way. Fig. 3 and Fig. 4 indicate steps of Image Processing.

Statistical analysis

The intra class correlation coefficient was established first. One of the investigator (author) and two experts (different to authors), performed two measurements of 14 randomly selected images to evaluate concordance and

accuracy of data. The obtained intra class correlation coefficient was 0.99, p - value 0.000. Then, three measurements for area of connective tissue were performed to 123 images.

For comparisons between one and three hours of ischemia during reperfusion, Mann Whitney test was applied. Periods that showed significant differences in the median were analyzed by multiple comparisons test, as non-parametric Bonferroni post-ANOVA. Kruskal – Wallis test was applied for comparisons among groups of reperfusion (Gómez *et al.*, 2003). SPSS version 22 was the statistical software for this analysis.

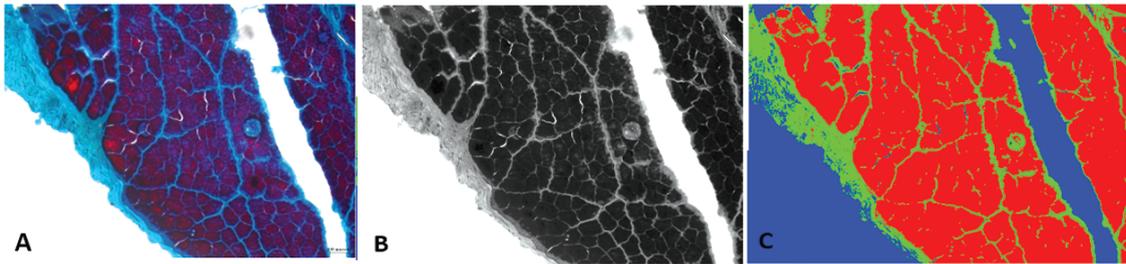


Fig. 4. Digital image processing – Segmentation by regions. A. Extensor carpi radialis longus in Gomori's trichrome stain. B. Image evidenced in green channel of gray scale. C. Regions of interest are in red for muscle fibers and in green for connective tissue. The regions of no interest are in blue. Magnification 10x. Scale Bar: 100 ums.

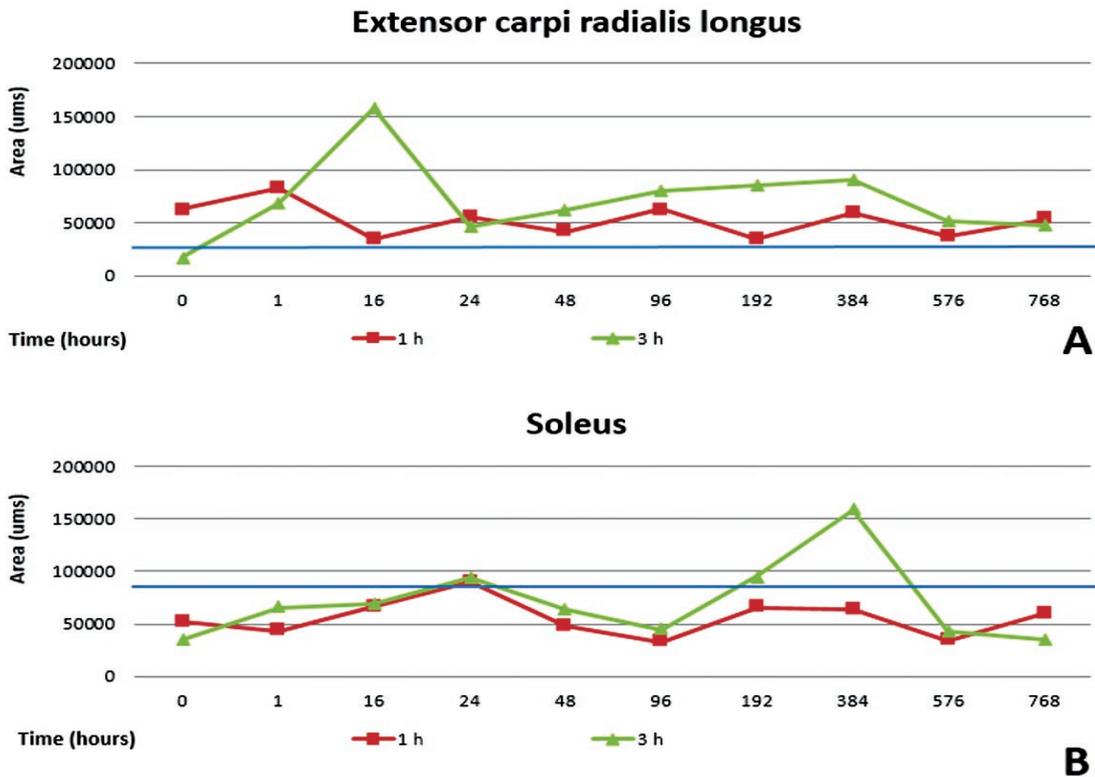


Fig. 5. Comparison of the median in the intramuscular extracellular matrix during periods of reperfusion following one hour and three hours of ischemia. The blue line indicates control group measurements. X-axis: Time; Y: Area. A. Extensor carpi radialis longus. The tendency is increase of the area. There are differences between zero hours and 32 days (768 hours) of reperfusion, with more variation of values in three hours. B. Soleus. The tendency in this case is decrease of the area. In prolonged periods of reperfusion, complete recovery of the area was not observed.

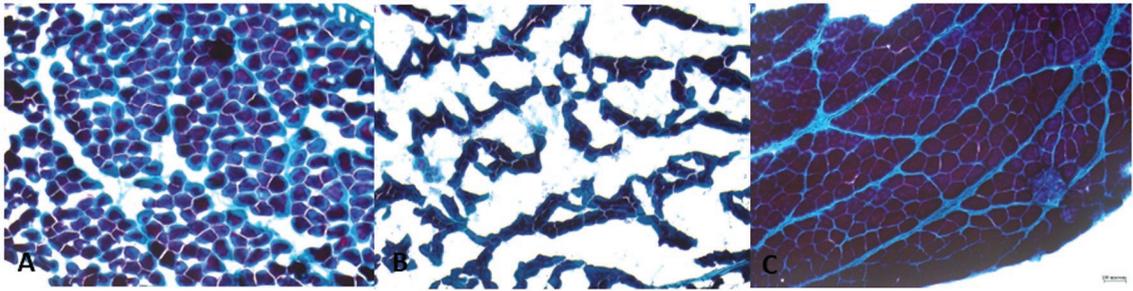


Fig. 6. Extensor carpi radialis longus. **A.** Muscle that underwent to ischemia of one-hour, and reperfusion of one hour. There were increase of the spaces occupied by connective tissue. **B.** Ischemia of three hours and reperfusion of 16 hours, muscle fibers presence a smaller size than in the normal tissue; there were no muscle fascicles and there was cellular infiltration in areas among fibers. **C.** That muscle corresponds to ischemia of three hours and reperfusion of 768 hours (32 days). The muscle recovered its normal characteristics partially similar to the control. Gomori's trichrome stain. Magnification 10x. Scale Bar: 100 ums.

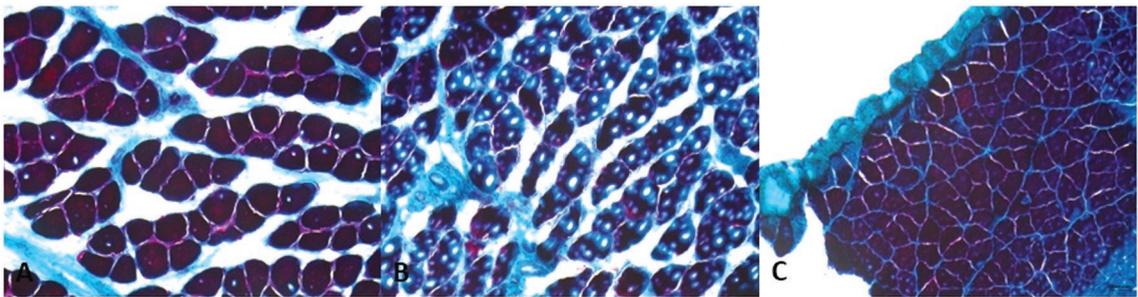


Fig. 7. Soleus. **A.** Muscle that underwent to ischemia of one-hour and reperfusion of 24 hours. Muscle fibers appear with a bigger size than the normal tissue. **B.** That muscle corresponds to ischemia of three-hours and reperfusion of 24 hours. No muscle fascicle and fiber appeared bigger and less hexagonal than in the normal tissue. Vesicles in the cytoplasm of fibers are present. **C.** That tissue corresponds to ischemia of three-hours and reperfusion of 576 hours (24 days). The muscle had recovered its normal characteristics partially similar to the control. Gomori's trichrome stain. Magnification 10x. Scale Bar: 100 ums.

RESULTS AND DISCUSSION

The normal weight for extensor carpi radialis longus was 0.187 gm and that of soleus was 0.220 gm. The mean value for extensor carpi radialis longus during reperfusion after one-hour of ischemia increased to $0.198 \text{ gr} \pm 0.039$ (p value 0.07) but after three-hours of ischemia this value decreased to 0.181 ± 0.037 (p value 0.045). On the other hand, the mean value for

soleus during reperfusion after one-hour of ischemia changed to $0.190 \text{ gr} \pm 0.023$, and after three hours of ischemia increased to $0.206 \text{ gr} \pm 0.041$, with no significance differences for this muscle (p value > 0.05).

The normal area of intramuscular connective tissue in 10x magnification was 27900 ums for extensor carpi radialis longus, and 86552 ums for soleus.

For extensor carpi radialis longus during reperfusion after one-hour of ischemia, normal area was increased without significant differences ($\bar{\chi}$ =54675, p value > 0.05). In the same way, during reperfusion after three-hours of ischemia the area was increased, and these changes were significant ($\bar{\chi}$ = 64899, p value 0.02) (figure 5A). At day 32, the measured area was 53407 ums after one-hour and 56186 ums after-three hours.

For soleus muscle, during reperfusion after one-hour of ischemia, the area of connective tissue decreased and the changes were not significant ($\bar{\chi}$ =56001, p value > 0.05). On the other hand, during reperfusion after three hours of ischemia, changes in the area of connective tissue were significantly decreased ($\bar{\chi}$ =64743, p value 0.04). At day 32, the measured area was 49357 ums after one hour, and 38537 ums after three-hours (Figure 5B).

Comparison of results between one and three hours of ischemia evidenced no significant differences in areas occupied by intramuscular extracellular matrix (p value > 0.05). Fig. 6 and Fig. 7 showed changes in determined periods of reperfusion.

For both muscles, lesions were reversible. The extensor carpi radialis longus deals well with injury during reperfusion after three hours of ischemia though is very susceptible during early stages of reperfusion. Soleus muscle evidenced recovering more immediately than the extensor carpi radialis longus. At day 32, both muscles had an increasing in areas of connective tissue associated to changes in areas occupied by muscle fibers, probably by edema or atrophy. One of the aspects that lead to reperfusion injury is the non-reflux syndrome, in which ischemia is produced by an obstruction of capillary circulation accompanied by

oxidative stress and cellular infiltration (Da Silveira and Bonneti 2004; Vignaud *et al.*, 2010). So, many authors have been agreed that short periods of ischemia can induce significant changes in histological characteristics of muscles, which can be exacerbated during reperfusion. In the same way, not all fibers of skeletal muscles respond similarly to injury and the association of myocytes with the connective tissue has influence in the mechanisms of adaptation evidenced during reperfusion (Carmo-Araújo *et al.*, 2007; Mars and Gregory 1991; Rácz *et al.*, 1997; Walters *et al.*, 2008; Woitaske and McCarter 1998).

The pneumatic tourniquet applied in the present study was having similarity with the tourniquets used in orthopedic surgeries. Some authors defend the occlusion arterial technique because the technique is more effective to produce ischemia directly (Ghaly and Marsh 2010; Itoh and Kudoh 2011). However, this technique is quite invasive, and produces risk factors to the animal during post-surgery. Some other workers used a manual tourniquet without a control of the applied pressure (Carmo-Araújo *et al.*, 2007), with the inconvenience that is not possible to ensure a complete interruption of capillary blood flow.

In 2008, Walters *et al.* used a pneumatic tourniquet which pressure was above 230 mmHg. By using a Doppler evaluation technique, they determined that at this pressure with ± 20 mmHg, there was a total obstruction including microcirculation (Walters *et al.*, 2008). For this reason, in the present study, the pressure applied to experimental groups was over 250 mm Hg. The main advantage was the diminishing of risks of infection and hypothermia so that this model can be used in a non-invasive way.

The model of ischemia-reperfusion presented in this study by using Wistar rats and a pneumatic tourniquet, required a thorough planning since the first phase in order to avoid unnecessary deaths during reperfusion and the use of more animals, by applying the principles of Reduction and Refinement of the 3R's (Franco and Olsson 2014).

CONCLUSION

During post-surgery, the following up of patients is an important aspect. In such cases, some people do not recover the complete function or movement of the muscle in the same proportion as previously. From the present study, it may be assumed that same type of characteristic changes happens in connective tissue during partial recovering of muscles in humans after surgeries that acquired ischemia for a variable period.

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