RATS' ISCHIATIC NERVE INJURY CAUSED BY SMASHING: A VASCULARIZATION STUDY

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SUMMARY

The objective of this paper was to study acute intraneural microvascular changes in a rat's ischiatic nerve submitted to smashing with different loads. Sixty male Wistar rats were used and distributed into experimental groups according to vessels injection and smashing load. Right ischiatic nerves were isolated and submitted to smashing with loads (0.5 Kg, 1 Kg, 5 Kg, 10 kg, and 15 kg) for 10 minutes and the left ischiatic nerves served as controls. After smashing, the animals were submitted to catheter implantation on abdominal aorta and vessels injection, then 30 right and left nerves were fixed in 10% formol, dehydrated and diaphanized for longitudinal analysis of intraneural vessels and the remaining being fully removed, cut into three fragments, frozen in isopentane with dry ice and stored in a freezer at -70°C, cross-sectioned for analysis and intraneural vessels counting. Gross and microscopic analyses showed regions of endoneural and epineural hematoma on different smashing loads. The morphometrical analysis suggests that intraneural vessels injury was proportional to the smashing load, causing endoneural and epineural hematoma, which creates an unfavorable micro-environment for nervous fibers regeneration.

Keywords: *Vascularization; Smashing; Ischiatic nerve; Rats Wistar.*

INTRODUCTION

Peripheral nerves injuries show both sensorial and motor functional changes, and, if not appropriately treated, they can yield an important deficit, debilitating not only patients' quality of life, but also state systems in those cases of early retirement as a result of functional disability [1].

Peripheral nerve has a well developed microvascular system at the epineurium, perineurium and endoneurium. Its vessels are distributed along many nerve's layers, and they are interconnected through numerous anastomoses [2].

Nervous impulse transmission, as well as axonal transport, requires a continuous power supply provided by intraneural microvessels. Microvascular system has a large reserve capacity to offset local regional vessels' motion or injury. At the epineurium, vessels longitudinally oriented exhibit a characteristic pattern, that is, vessels are present in all epineurium layers, which is superficially, as well, between fascicular bundles no nerve's deep layers [3,4].

The importance of peripheral nerves vascularization is due to the fact that peripheral nerves' axons are vulnerable to ischemia because of the long distance existing between neuronal body and axon length [5].

Several studies have proposed the use of experimental models for nervous injury by smashing in order to evaluate both injury itself and functional regeneration and recovery [6,7,8], but the vascular response in an acute peripheral nervous injury by smashing remains poorly known.

Experimental peripheral nervous injuries at the ischiatic nerve may be provoked through a number of procedures, such as: smashing by compression, trans-section, stretching and freezing. Several factors such as magnitude, duration and mechanism of compressive trauma are important to determine injury degree [9].

When a nerve is submitted to external pressure, an injury can result both from pressure and from ischemia. Ischemia happens when the smashing pressure exceeds capillary perfusion pressure [3,10].

Therefore, the study of vascular changes in nervous injuries, particularly those caused by smashing, deserves investigation, since it could contribute to the improvement of treatment strategies for peripheral nervous injury.

MATERIALS AND METHODS

Male adult Wistar rats were used in the experiment, totaling 60 animals with mean weight of 250-350 grams, obtained from Central Animal Lab of Ribeirão Preto Medical College, University of São Paulo. These were set apart in appropriate cages and maintained at the Animal Lab of the Cardiovascular Physiology Laboratory of Ribeirão Preto Medical College, for at least 48 hours in the new environment for familiarization, and were fed with standard ration and water *ad libitum.*

This study was approved by the Committee on Ethics in Animal Experiments (CETEA), of Ribeirão Preto Medical College, University of São Paulo.

Study conducted at the Department of Biomechanics and Locomotive Apparatus Medicine, Ribeirão Preto Medical College, University of São Paulo.

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The animals were initially weighed for anesthetic (Thionembutal) dosage calculation purposes, and were submitted to anesthesia with a dosage of 50 mg/Kg of body weight, intraperitoneally, trichotomized, and positioned at ventral decubitus with anterior and posterior legs in full abduction and fixed to operating table. Antisepsis was performed with 20% iodinated alcohol solution.

Right and left ischiatic nerves were addressed through a skin incision on thigh lateral surface. A suture stitch (Prolene 6/0, Ethicon) was made at the epineurium in order to identify the site where smashing should be performed. Right ischiatic nerves of each experimental group were isolated and submitted to smashing forces with a device carrying different loads (0.5 Kg, 1.0 Kg, 5.0 Kg, 10.0 Kg, and 15.0 Kg) respectively, distally to suture stitch, during 10 minutes, and left ischiatic nerves were used as controls.

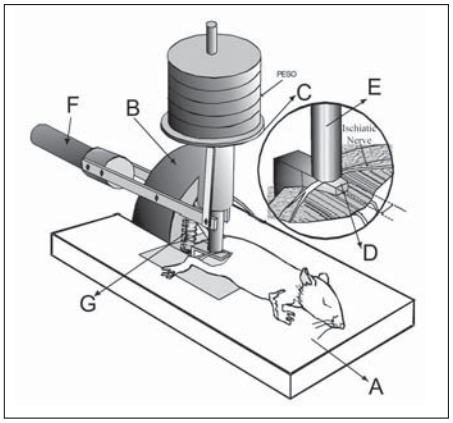

Figure 1 – *Schematic illustration of the equipment used for smashing rats' right ischiatic nerve. Smashing occurs along the whole width of the nerve, resting on 5 mm of length.*

The equipment used in the study was developed at the Bioengineering Laboratory of Ribeirão Preto Medical College, as a replacement to the assay machine used in previous similar studies and making an smashing injury on rats' ischiatic nerve easier and more reproducible.

The equipment is constituted of a platform for animal support (A) with a main structure (B) receiving the load support for measured weights (0.5 Kg, 1.0 Kg, 5.0 Kg, 10.0 Kg, and 15.0 Kg) with a telescopable axis (C), a support base for the nerve (D), pressure application axis (E), a lever intended for activating weights and positioning the nerve on the support base (F) and a spring to keep the lever in balance (G).

The smashing injury was performed in a 5-mm segment of an intermediate portion of the nerve, which remained on a support base, distally to the suture stitch previously passed through the epineurium, with different loads, during 10 minutes (Figure 1).

The sixty animals were divided into 2 experimental groups, according to the protocol of vessels injection and further subdivided into 5 groups, according to the load used for smashing.

After 10 minutes applying smashing forces with established weights, 30 animals were submitted to catheterization of the abdominal aorta with a PE-50 polyethylene tube, beneath the emergence of renal arteries. The PE-50 polyethylene tube was attached to a glass syringe with maximum volume of 20 ml and the animals were perfused with manual injection of 12 - 15 ml of a solution composed of 5% Chinese stain and jelly, dissolved in 10% formalin, heated to 37º C in double-boiler.

Immediately after perfusion was completed, right ischiatic nerves (smashed) and left ischiatic nerves (controls) were collected and sunk in 10% formalin solution during three days for tissue fixation. After three days soaked in 10% formalin dehydration of ischiatic nerves was performed in increasing concentrations of alcohol solution: 70%, 80%, 90%, 95% until absolute alcohol, remaining for 20 minutes in each solution. Then, the nerves were sunk in xylol for three days.

After three days sunk in xylol, ischiatic nerves were kept in a methyl salicylate and benzyl benzoate solution, at a ratio of 3:2 for diaphanization. The longitudinal gross analysis of the ischiatic nerves vascular meshwork was performed using a stereoscopic magnifying glass Leica M651, by transillumination with a Vilber Lourmat negatoscope and photographed with an Olympus camera attached to the magnifying glass. Photograph images were treated in a Nikon program installed in a personal computer.

The other 30 animals, after 10 minutes of smashing, were submitted to catheterization of abdominal aorta, as previously described and perfused with manual injection of a solution composed of 5% Chinese stain and jelly, dissolved in saline solution, heated at 37º C in double-boiler.

Once perfusion was completed, the animals were kept in freezer at -20º C for one hour. Then, nerves were dissected and thoroughly removed, cut into three fragments at the following regions: proximal (above smashing site), medial (smashing site), and distal (below smashing site), identified and immediately frozen with isopentane in dry ice, and stacked with Tissue-Tek. They were stored separately in cryotubes, in a freezer at -70º C and then cross-sectioned (semi-seried cuts of 20 mμ) in cryotome[19]. The different solutions used for vessels injection are due to the kind of analysis on collected material.

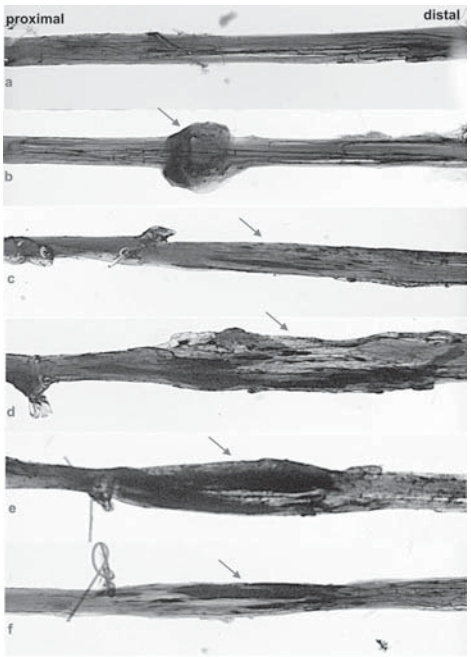

Figure 2 – *Photographs of a control rat's ischiatic nerve (a) showing its longitudinal vascular meshwork, and smashed with 0.5 Kg (b), 1.0 Kg (c), 5.0 Kg (d), 10 Kg (e) and 15 Kg (f), evidencing hematoma at medial region (arrow). Magnification: 40x.*

For microscopic analysis and endoneural capillary count, three cross-sections were chosen by means of KS 300 software, and the perfused vessels count in those three selected images of each region for obtaining the average number of filled vessels on the smashed side and on control in the different experimental groups was performed by means of KS Lite software.

Data concerning to the count were submitted to statistical analysis and performed by means of non-parametric variance analysis technique (ANOVA) for the model of repeated measurements in two factors [12].

Differences were considered as significant when $P<0.05$.

RESULTS

A gross evaluation of control ischiatic nerves' vascular meshwork under trans-

illumination magnifying glass showed that the nerve has a well defined vascular pattern. It includes a meshwork of superficial and spaced longitudinal vessels with cross-penetrating branches, which are responsible for supplying blood flow to endoneural vascular meshwork (Figure 2a).

The gross view of smashed ischiatic nerves was characterized by the presence of a disorganized vascular meshwork with the presence of hematoma and vessels expansion at the smashing site and beneath the injury, which increases with heavier smashing loads (Figures 2b, 2c, 2d, 2e, and 2f).

The microscopic view of smashed ischiatic nerves' vessels and related controls was taken at regions above (proximal) and below (distal) smashing site and exactly at smashing site (medial).

In contralateral control group, cross-sections of left ischiatic nerve of all animals with different loads showed a normal vascular pattern (Figure 3a).

In the group smashed with 0.5 Kg, an intact epineurium with filled vessels was observed at medial region (smashing site), as well as a subtle digression of injected solution, indicating the presence of an endoneural hematoma with normal regions of filled vessels, apparently smaller than controls (Figure 3b).

In the group smashed with 1.0 Kg, an intact epineurium with vessels was also observed at medial region, but with a higher digression of injected solution, indicating a bigger endoneural hematoma. Some filled vessels were still present at the endoneurium (Figure 3c).

In the group smashed with 5.0 Kg, we observed that, at medial region, the size of hematoma was even bigger, also involving epineural vessels. Rare filled endoneural vessels (Figure 3d).

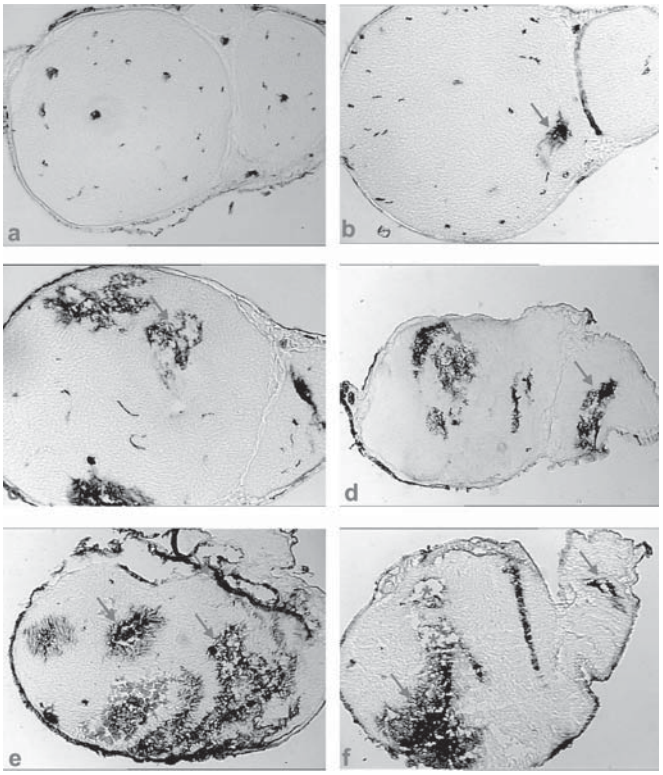

Figure 3 – *Cross-sectional photomicrographs at medial region of a control rat's ischiatic nerve (a) and smashed with 0.5 Kg (b), 1.0 Kg (c), 5.0 Kg (d), 10.0 Kg (e) and 15.0 Kg (f). Please, note the presence of filled intraneural vessels (capillaries) (a) and the presence of endoneural hematoma (arrow), epineural hematoma (arrow head) and tissue destruction (asterisks). Magnification: 100 X.*

Group (G)	Load (Kg)	Region		
		Proximal	Medial	Distal
Smashed	0.5	36.0±18.0 *	15.0±11.5 #	33.0±11.0.*
	1.0	29.0±15.0 *	13.5± 7.0 #	37.0± 6.0 *
	5.0	41.5± 4.5 *	7.5± 1.5	41.5± 7.0 *
	10.0	35.5± 7.0 *	8.0± 3.5	39.0± 5.0 *
	15.0	42.0±10.5 *	3.5± 9.5	39.0± 8.5 *
Control	0.5	20.0±18.6	30.0±10.5 Δ	29.5± 7.5
	1.0	24.0±13.5	25.0±13.0 Δ	24.0±10.5
	5.0	38.0±14.5	39.0±13.5 Δ	39.0±11.0
	10.0	34.5±13.0	32.5±10.5 Δ	30.5± 6.0
	15.0	32.0±12.5	36.0±10.5 Δ	36.5± 7.0

(P<0.05) (L0.5 = L1.0) vs (L5.0 = L10.0 = L15.0) in smashed group at medial region
Δ (P<0.05) for Smashed vs Control in all loads at medial region
* (P<0.05) for (Prox= Distal) vs Medial in all loads of smashed group

Table 1 – *Median and total semi-amplitude of the number of perfused vessels, according to group (G), load (Kg) and region (proximal, medial, and distal).*

Group (G)	Load (Kg)				
	0.5	1.0	5.0	10.0	15.0
Smashed	30.0±9.5	27.0±8.0	29.0±3.5	27.0±4.5	28.0±5.0 *
Control	25.5±9.0	29.0±8.0	26.5±11.0	31.5±8.5	34.5±6.0 *

* (P<0.05) for Smashed vs Control with 15 Kg load.

Table 2 – *Median and total semi-amplitude of the average number of perfused vessels according to group (G) and load (Kg).*

In the group smashed with 10.0 Kg, at medial region, we could similarly observe the extensive hematoma involving epineural vessels, indicating epineural and endoneural hematoma, with subtle tissue destruction (Figure 3e).

In the group smashed with 15. Kg, at medial region, apparently, the hematoma seemed to be smaller, but nerve's tissue destruction was observed, which probably caused the injected solution to be lost. No intact endoneural vessel was found (Figure 3f).

The morphometrical analysis of perfused vessels was performed at proximal, medial, and distal regions to smashing site of right and left ischiatic nerves in all animals of each group. The number of vessels was compared between smashed and contralateral control groups and among smashing loads at proximal, medial, and distal regions to injury (Tables 1 and 2) (Figure 4).

When we compare loads at medial region, we notice a statistic difference between them in the smashed group, that is, it was seen that in that medial region (smashing site) a significant reduction of the number of vessels occurred with heavier loads, which means that, from 5 Kg load on, results were statistically similar and lower when compared to 0.5 and 1.0 Kg loads (Figure 5).

In brief, in the smashed group (all loads), the number of perfused vessels has shown to be significantly lower at medial region (P<0.05). Also, in this situation, the control group showed a higher response rate (P<0,05) than the smashed one (Table 1).

By comparing the summation of the 3 regions of smashed and control groups with different loads, we notice that only with the 15.0 Kg load a statistically significant difference was found between smashed and control groups (Table 2).

Therefore, vascular response was statistically significant when we consider the region where smashing was performed, which probably explains the mechanical injury caused by stress forces applied to the nerve submitted to smashing with different loads presenting a lower number of filled vessels at medial region when compared to proximal and distal regions of the smashed group.

DISCUSSION

In this study, a method of vascular perfusion was used for evaluating acute vascular injury, without bias from fixators, which may distort vessels anatomy and size. The solution composed of 5% Chinese stain and jelly and saline solution was perfused through the abdominal aorta artery in order to assure the whole delineation of vascularization at the posterior region of rats while those where kept under anesthesia until euthanasia. Most of previous studies addressed vascularization during degeneration and regeneration process in an ischiatic nerve injury [13,14,15, 16].

Although the Sunderland's second-degree injury (axonotmesis) is the most commonly used model in peripheral nerves researches, there is a lack of standardization of the method to produce a nervous injury. In fact, many surgical instruments [7,19], as well as compression devices[3] with different smashing times are presented in literature. Those devices are dependent, particularly, on the pressure manually applied to tweezers or to pull threads, and are difficult to control and standardize.

The rat's ischiatic nerve was selected in our study on intraneural vessels because of its similarity to human peripheral nerves. This is the longest in body, easily accessible, with a number of fascicles and dense blood flow.

Peripheral nerves may be submitted to smashing injuries in a variety of circumstances, including car accidents, labor accidents, fractures, dislocations. Functional deficiencies after a smashing injury are not only related to smashing impact, but include important components, such as limb ischemia.

The lack of standardization of stress application may be a significant source of results variation among published experimental studies. If interest is towards the study of vascularization after smashing injury on rats' ischiatic nerves, it is essential to measure and standardize smashing forces.

In our study, we introduced a device especially developed to produce a smashing injury on rats' ischiatic nerves with a standardized system of measured weights from 0.5 to 15 Kg. It enables the direct application of smashing forces on a peripheral nerve, with no bias from soft tissues, and facilitates its use in rats.

By using this smashing device and rats as models, we observed the influence of smashing forces on rat's ischiatic nerve vascularization. A time period of 10 minutes was chosen for smashing duration. Considering this, we used a standardized nervous smashing injury, in terms of load (pressure) as well as the time of smashing, in order to avoid additional variables.

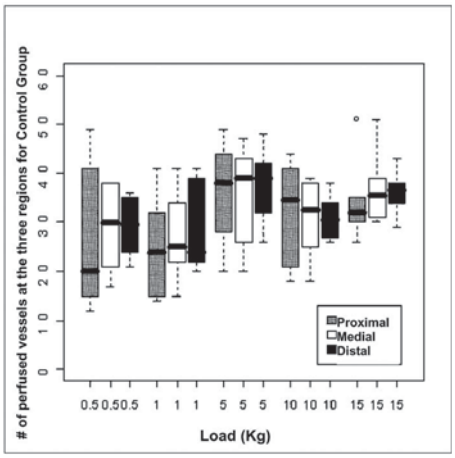

Figure 4 – *Number of perfused vessels in control group with different loads at proximal, medial, and distal regions.*

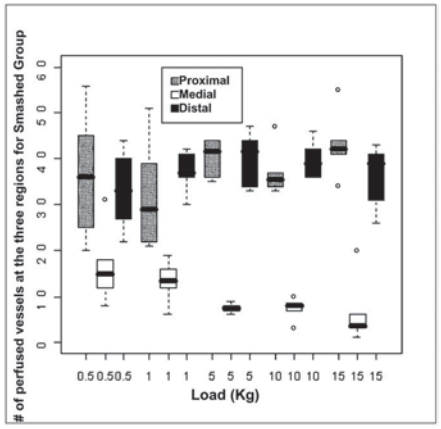

Figure 5 – *Number of perfused vessels in smashed group with different loads at proximal, medial, and distal regions.*

It is a known fact that after a smashing injury, morphological and functional changes become worse due to a smashing-related ischemia. With a sustained pressure, the reduced oxygen levels can increase endothelium permeability of endoneural capillaries, causing edema enlargement and the creation of the endoneural compartment syndrome. Furthermore, ischemia may be an additional factor, interfering on nervous regeneration after an injury [17].

Many authors who used models similar to ours, with load of 100g, 500g and 15000g during 10 minutes, achieved results where functional recovery was shown after smashing and that this was directly related to injury severity, that is, was proportional to the smashing load, and even with heavy loads (15000 g), motor function returned to normal levels within up to 60 days [8].

Some researchers show an increased vascular space as well as an increased endoneural capillary permeability after frogs' ischiatic nerve smashing, drawing a conclusion that the injury changes perineural and endoneural vascular permeability eliciting the onset of edema[15,17] and endoneural edema may seriously affect several functions of nervous fibers, even with differences in animals, methods and smashing and collection times [6].

Our results provide an analysis of vascular changes after smashing injury with different loads. A strong correlation was found between the amount of strength (load) and vascular changes. After strengths of 0.5 Kg and 1.0 Kg were applied, changes were detected, such as subtle hematoma, but with endoneural capillaries still preserved and filled. In contrast, loads of 5.0 Kg, 10.0 Kg, and 15 Kg resulted in more extensive changes, with endoneural and epineural hematoma with tissue destruction, in addition to the non-preservation of endoneural vessels.

The number of endoneural capillaries has increased after 2, 4, 6, and 8 weeks after smashing, transections and ischemic injuries, but not after permanent axotomy. These results suggested that post-trauma distal endoneural neovascularization is dependent on two variables: the degree of regeneration and the degree of ischemia [17].

Other researchers investigated vascular response by means of morphometric analyses after 1, 2, 3, 6, and 9 weeks of smashing injuries with haemostatic tweezers in three sequential periods of 10 seconds. The response was constituted of two phases: the first one, up to one week, vessels increased in size. At the second phase, up to six weeks after smashing, the number of vessels and

their densities increased. They observed that the complex process of degeneration and regeneration is assisted by a vasogenic response with two phases that are related to tissue's functional and metabolic need [16].

The morphometric analysis of ischiatic nerve vessels in our study showed a different behavior in the three studied regions, verifying a lower number of vessels only at the smashing region, where vessels have probably been submitted to mechanical injury related to smashing stress forces.

Several experimental studies have shown that the combined effect of mechanical injury and ischemia is more severe than the mechanical effect or ischemia alone [3,10].

Regarding the mechanisms promoting compressive injuries, many researchers suggest that those occur as a result of mechanical stress, ischemia or both.

In this study, we correlated the mechanical effect and the ischemic effect, showing that the mechanical effect may be more important with lighter loads, once the vessels were preserved in these groups. The ischemic effect gets stronger as loads increase, since we showed endoneural capillaries destruction with loads of 10 and 15 Kg.

Another important point we noticed is that from 5 Kg load, results were statistically similar and lower with loads of 0.5 and 1.0 Kg and that, therefore, it indicates that there is no need to use loads above 5.0 Kg, because this is enough to cause injury to intraneural vessels, particularly endoneural capillaries.

Researchers have investigated this issue using compression models with fixed load (150 mmHg), applied during periods ranging from 10 minutes to 4 hours, or during a fixed time of 2 hours with different loads (0, 20, 50, 150, and 300 mmHg). The results showed that structural changes after acute compression are much more a result of cellular events related to mechanical forces than to those induced by ischemia [19].

This is due, according to some authors, to the fact that acute compression is performed in a very short interval to produce ischemic changes to the nerve. Furthermore, the pathological changes found by the author were consistent to with mechanical injury and differed from changes produced in experimental models of ischemia. Differences were found in distribution, length of time, and kind of affected fibers, and, thus, structural changes in compressive acute injuries would be related to compression stress forces, more than to ischemia. Nevertheless, those arguments do not fully rule out the possibility of an ischemic component to contribute to a stronger severity of an injury after a period of days or weeks [10].

According to some researchers, when tissues are submitted to load or pressure, they distort, and pressure gradients are formed, redistributing compressed tissues towards low-pressure areas [4].

Microscopic analysis of ischiatic nerve's vessels showed regions of endoneural and epineural hematoma with the different loads used, indicating that smashing forces used in our research were enough to injure intraneural vessels, particularly endoneural capillaries, but critical values of pressure and duration regarding nervous injury are unknown. Hematoma is the result of an increased vascular permeability by direct injury to capillary walls caused by smashing forces.

In a study addressing the endoneural microenvironment in a smashing injury with haemostatic tweezers for 30 seconds (acute injury) or 3 hours (extended injury) contradict the concept that a microvascular injury is a major component of peripheral nerve smashing and conclude that the acute compression of the nerve does not injure *vasa nervorum*[19]. Nervous fibers injuries in acute compression or smashing (brief) seem to result more frequently from mechanical effects [20].

In the present study, we clearly showed that an important vascular compromise exists in smashing injuries, particularly in groups with heavy loads. Vessels destruction should lead to ischemia, making regeneration processes slower. The formation of an endoneural hematoma will certainly create an unfavorable microenvironment for nervous fibers regeneration, which were also injured in this model.

CONCLUSIONS

Smashing causes direct injury of endoneural vessels, with endoneural hematoma formation of which severity is directly proportional to smashing load (strength). The use of light loads (0.5 and 1.0 Kg) preserves some endoneural capillaries' integrity, with this preservation being similarly jeopardized with loads equal or above 5.0 Kg. Intraneural vessels injury and the endoneural hematoma formation are factors that may damage nervous regeneration, since they interfere with nervous function.

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