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# Angiogenic effect of the application of radial shock waves on the integral musculature of the Wistar rat quadriceps - a new perspective for muscle physiology

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To the Editor,

Shock waves consist of high-energy, high-pressure mechanical sound pulses that are applied in a short time to a specific part of the body (1, 2). The use of extracorporeal shockwave therapy (ESWT) in medicine began in 1980 with lithotripsy in urolithiasis (1). Later studies demonstrated the benefits of ESWT in bone ailments such as delayed fracture healing and pseudoarthrosis (1). The osteogenic effect of ESWT is well defined in medical literature due to the fact that the activation of the integrin-cytoskeleton-nucleus protein system acts in the conversion of the mechanical to biological stimulus and promotes the release of growth factors (3).

Although several articles describe the benefits of ESWT in muscle diseases, assessments of its histological effect in the process of cell regeneration are lacking (3). It is known that in the recovery of skeletal muscle there is a period of degradation and inflammation, followed by an extensive repair phase and, finally, a period of maturation and redevelopment (4). The main histological changes that indicate the success of this process are neovascularization and the proliferation and activation of satellite cells (4, 5).

Taking these aspects into consideration, the objective of this study was to verify whether the stimulus produced by electrohydraulic shock waves is capable of inducing histological changes compatible with the cell recovery process in the quadricep muscle of Wistar rats: in particular, angiogenesis and the proliferation of satellite cells. The confirmation of muscular changes induced by ESWT may therefore permit its recommendation in improving the muscular conditions of professional and amateur athletes, the rehabilitation of patients undergoing osteoarticular procedures by reducing the effects of disuse, and in neurological injuries with intact musculature, such as spasticity (6).

## MATERIALS AND METHODS

This is a prospective experimental study to evaluate the histological effects of shockwave therapy on the whole quadricep muscles of Wistar rats. The study was approved by the Ethics Committee on Animal Use (CEUA - FAME Barbacena) with protocol number 01/2017, and followed current regulations on animal research.

The sample size was estimated using a 5% confidence interval and 80% power. The variable used to estimate sample size was the existence of blasts. Assuming that no control sample has blasts and that 50% of the test samples do, it would be necessary to have a sample of 8 animals

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in Fischer's exact test. A sample consisting of 12 animals (12 right test members and 12 left control members) was chosen so that, even if an evaluation of 1/3 of the samples was not feasible, the minimum sample size was respected.

Twelve male healthy Wistar rats, provided by the Biology Reproduction Center of the Federal University of Juiz de Fora, Minas Gerais, were evaluated in the vivarium of the Faculty of Medicine of Barbacena. The average age of the animals was 8 weeks. The animals were kept under stable temperature conditions in a ventilated unit at 22°C in controlled light/dark cycles of 12 h, with open access to water and *ad libitum* food. The right legs of the 12 animals were used as test legs and the 12 left legs were used as control legs. There were no exclusions of animals during the study. As all animals were tested (right paws) it was not necessary to use methods of separating the animals between the test group and the control group.

The researchers' blinding methods were not used during the applications of extracorporeal shock waves. The animals were anesthetized with ketamine (75mg/kg) and xylazine (10mg/kg) in order to produce a dissociative effect with the cerebral cortex and a state of deep analgesia without a loss of protective reflexes. Both drugs were administered intraperitoneally. Water-based contact gel was then applied to the right lower limb. The quadriceps were then subjected to a stimulus of 2000 pulses at 4.0 bar of air output pressure and a frequency of 20 Hz through the SwissDolorclastSmart® 20 device with the Evo Blue® air gun. After the procedure, the rats were placed in collective cages for 3 animals. The second application was performed with the same criterion after 7 days, and the third after 14 days.

The protocol used followed the recommendations of the manufacturer of the Swiss Dolorclast Smart® device in use for muscle tissues and in articles by different authors for use in skeletal muscle (4). The general data of the animals' health, behavior, and gait were evaluated daily and changes were noted. Prior to the start of the shock wave therapy sessions and after the end of the anesthesia effect, the animals were observed with an emphasis on changes in the test paws. One week after the third shock wave application, the animals were euthanized by way of increasing intraperitoneal anesthetic, using thiopental (45mg / kg).

After the loss of foot and corneal reflexes, trichotomy of the right and left lower limbs was performed and, with the aid of a scalpel and Metzenbaum scissors, part of the quadricep muscle was removed. The conservation of biological material was carried out in a previously designated plastic container containing 10% formaldehyde solution. Animal carcasses were then disposed of in accordance with current regulations for biological waste.

The histological blades were made by a qualified professional in a specialized laboratory. In order to standardize the depth of the cut of the muscles to be studied, 5 microtomy cuts were made in each paraffin block, which were disregarded. The following microtomy cut was used to make the histological blade to be studied: for each paw evaluated, two histological blades were made, one stained in hematoxylin and eosin and the other stained by Gomori's trichrome.

Histological analysis was performed in the pathology laboratory at the College of Medicine of Barbacena, Minas Gerais and in the angiogenesis sector of the Institute of Biological Sciences at the Federal University of Minas Gerais. The review was carried out at the TECSA Laboratory - Technology in Animal Health, where the reading was made by two distinct pathologists in a blind system with diagnostic agreement on all slides.

The microscopic reading of the histological blades was divided into: i) in the 10x increase, the existence of gross changes suggestive of malignant transformation was evaluated, as well as the general appearance of the muscle fibers; ii) in the 40x increase, the size and width of muscle fibers, the morphology and number of nuclei, the existence of inflammatory infiltrate, the number of satellite cells, and blood vessels were measured; iii) in the 100x increase with immersion oil, the presence of blasts was investigated. In each phase, five random histological fields were evaluated.

*Statistical analysis*

The data obtained were transcribed into the database and an Excel spreadsheet was made with the data, identifying the animals in regard to the results of the test and control paws. All statistical calculations were performed using the software R-3.5.2, using non-parametric methods, continuous variables regarding the legs of the same animal were compared using the Wilcoxon paired test (signed-rank). Discrete variables were compared using Fisher's exact test with mid-p correction. Statistical significance was considered when $p < 0.05$. Boxplot charts were used to illustrate the distribution of data between different subgroups. Continuous variables were described as median (interquartile range) and binary variables as absolute number (%). The results were calculated considering the 95% confidence interval.

## RESULTS

The average weight of the animals studied was 173 grams. After the three shockwave therapy sessions, the right quadriceps presented a hematoma with a pattern similar to all animals studied. It is worth noting that during the reapplications, there was no initial hematoma in any animal. During the 21 days of evaluation, beginning with the first application, no changes in the animals' behavior, gait, and general condition were observed.

Histological analysis was performed, comparing the test and control paw of the same animal. Vascular proliferation, an increase in the number of fibers and satellite cells in the test paws was shown, with a statistically significant difference ($p < 0.05$, Table I). Right paws showed an increase of 40% in the number of satellite cells and 66% in the number of vessels when compared to the left paw of the same animal. (Figs. 1-3). There was no statistical difference in the number of blasts, interstitial edema, and in the integrity of the muscle fibers. Additionally, both groups studied showed no signs of malignant tissue transformation.



**Table I.** *Comparison of the results obtained in the study of the right legs (test) and left legs (control).*

| | Control | Test | P-value |
|---|---|---|---|
| Nuclei per fiber | 3 (3 - 3.25) | 4 (3.75 - 5) | **0.0206** |
| Fiber cores | 3 ( 2 - 4 ) | 5 ( 4.75 - 6.25) | **0.0105** |
| Blasts | 0 (0%) | 3 (25%) | 0.1087 |
| Vessels | 2 (2 - 3.25) | 6 (5 - 6.25) | **0.0024** |

*Statistical difference was admitted when p <0.05. In brackets is the 95% confidence interval.*

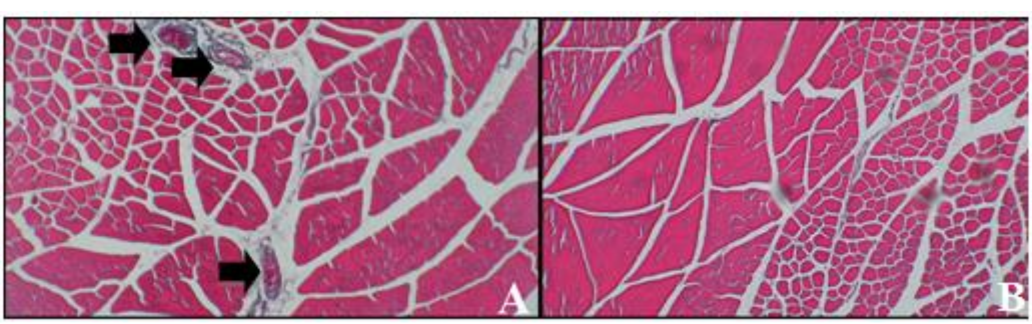


**Fig. 1.** *Histological slides stained in hematoxylin and eosin comparing the findings of the right (**A**) and left (**B**) paws of animal 1. The presence of vessels in A and absence in B is evident.*

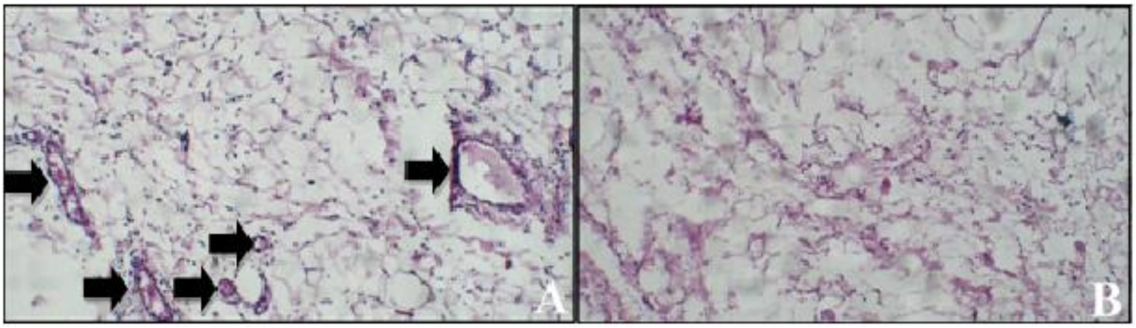


**Fig. 2.** *Histological slides stained by Gomori trichomium showing the connective tissue of the right (****A****) and left (****B****) paws of animal 1 with a clear difference in the count and dimensions of the vessels, corroborating the assessment of neovascularization in the test paw (A).*

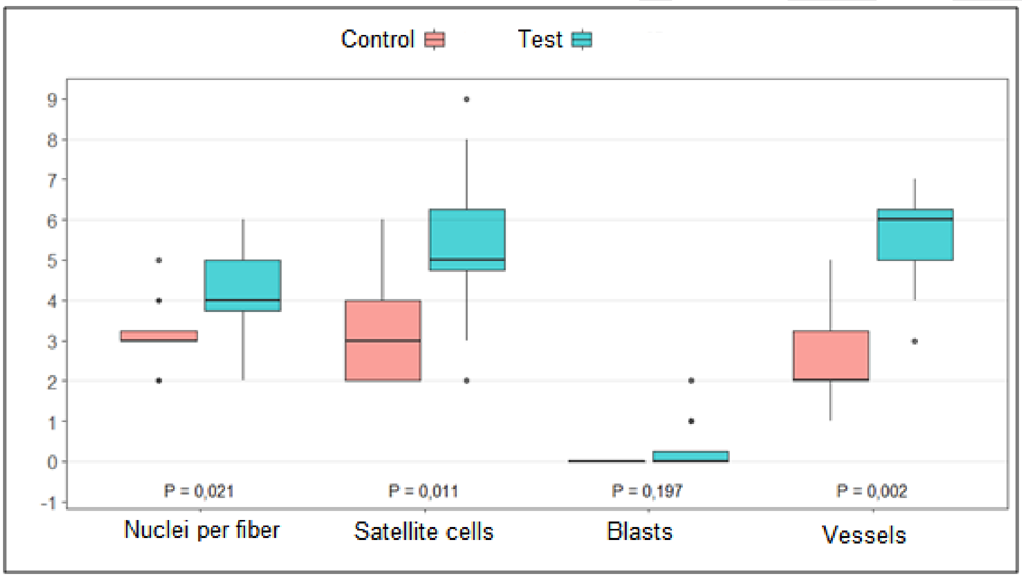


**Fig. 3.** *Boxplot comparing the variables studied in test paws and control paws.*

## DISCUSSION

The regeneration process of muscle injuries is a complex process that includes the formation of new vessels and proliferation of satellite cells, which represent the muscle reserve (1, 5, 7). In spite of the lack of specific studies on skeletal muscle, the literature points out that the use of radial ESWT is capable of stimulating the proliferation of endothelial cells and satellite cells similar to that which occurred in the injury (8). The capacity of radial ESWT accelerates the signaling pathways of muscle regeneration can be especially important among athletes, seeing that injuries are commonly associated with pain and functional loss that impact the competitor's performance (5, 8). In healthy muscles, the increase of vascularization can improve the tissue oxygenation (4, 8). The therapy could therefore help to reduce muscle fatigue and improve the performance of professional athletes (4).

Other applications of neovascularization induced by radial ESWT are the improvement of muscle pain, hyperreflexia, and strength recovery after injury. In painful muscle syndromes and in the hyperreflexia after stroke, the neoangiogenic effect of ESWT can reduce muscle stiffness and tension (10, 11). In patients with tendinopathies, the demonstration of satellite cell proliferation after the use of radial ESWT can aid in the recovery of muscle strength (12).

In tissue injuries, neovascularization and the proliferation of satellite cells are the main processes that promote tissue regeneration. In the present study, the evaluation of the effect of radial shock waves on the skeletal muscle of Wistar rats demonstrated the capacity of the method to stimulate the proliferation of vessels and satellite cells, which can be applied to assist in the restoration of muscle integrity in several muscular pathologies. Thus, there were no other factors that could stimulate the proliferation of vessels and satellite cells, with this effect being attributed only to radial shock waves.

It is worth noting that the results may present some bias since, only one model of radial equipment was tested, and future studies are necessary for a better understanding of the effect on integral musculature. Additionally, the histological evaluation technique must be complemented with electron microscopy and VEGF protein research in order to deepen the knowledge of the effects of ESWT on skeletal muscle.

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