Analysis of the Histological Effect of the Shock Waves on the Integrated Musculature of the Quadriceps of Wistar Rats

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Research article

Keywords: shock waves, neovascularization, satellite cells

Posted Date: July 29th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-46140/v1>

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Abstract

Objective: Shock waves are high-energy, high-pressure mechanical sound pulses used in medicine since 1980 with lithotripsy in urolithiasis. In addition, studies have demonstrated the osteogenic effect of shock waves indicating their use in skeletal diseases. Although several articles describe the benefits of shock waves in muscle diseases, assessments of its histological effect on the process of cell regeneration are lacking. Therefore, the objective of this study is evaluate the histological effect of extracorporeal shock wave therapy on the intact musculature of Wistar rats.

Materials and Methods: Twelve Wistar rats, with a mean age of 8 weeks, were studied for a period of 21 days. The quadriceps muscle of the right paws was submitted to 3 sessions of extracorporeal shock wave therapy at weekly intervals. The left legs were used as a control. Subsequently, the animals were euthanized and the quadriceps muscles were biopsied for histological study using histological slides stained in hematoxylin-eosin and Gomori's trichomium. Muscle fiber size, morphology and number of nuclei, existence of inflammatory infiltration, satellite cell count, and number of vessels were evaluated. Statistical calculations were performed using software R version 3.5.2 using non-parametric methods. Continuous variables relating to 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. Results were considered statistically significant when $p < 0.05$.

Result: In the musculature of the right paws, there was an increase in fibrovascular and satellite cells proliferation with a statistically significant difference ($p < 0.005$).

Conclusion: The demonstration of the angiogenic power and the proliferation of satellite cells induced by extracorporeal shock waves, it is possible to affirm that this constitutes promising therapy for several muscle injuries. The acceleration of muscle recovery in athletes stands out as a example, by minimizing the impact on the competitor's performance, reducing muscle pain and spasticity, and improving muscle strength in tendinopathies.

1. Background

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 [1–3]. The use of extracorporeal shock wave 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 [1–4]. 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 [5].

Although several articles describe the benefits of ESWT in muscle diseases, assessments of its histological effect in the process of cell regeneration are lacking [6]. 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 [7]. The main histological changes that indicate the success of this process are neovascularization and the proliferation and activation of satellite cells [7, 8].

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 quadriceps muscle of Wistar rats: in particular, angiogenesis and the proliferation of satellite cells. Therefore, the confirmation of muscular changes induced by ESWT may 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 [9].

2. Methods

2.1 Experimental Design

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

2.2 Sampling

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 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 hours, 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.

2.3 Applications of Extracorporeal Shock Waves

The researchers' blinding methods were not used during the applications of extracorporeal shock waves. The animals were anesthetized with ketamine (75 mg / kg) and xylazine (10 mg / 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.

Then, water-based contact gel was applied to the right lower limb. The quadriceps was 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 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.

2.4 Histological analysis

One week after the third shock wave application, the animals were euthanized by way of increasing intraperitoneal anesthetic, using thiopental (45 mg / 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 quadriceps 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: 1) 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; 2) 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; 3) in the 100x increase with immersion oil, the presence of blasts was investigated. In each phase, five random histological fields were evaluated.

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.

2.5 Statistical analysis

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) [10]. 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 .

3. Results

The average weight of the animals studied was 173 grams. After the three shock wave 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 1). 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. (Fig. 1, Fig. 2, Fig. 3)

Table 01
Comparison of the results obtained in the study of the right legs (test) and left legs (control). Statistical difference was admitted when $p < 0.05$. In brackets is the 95% confidence interval.

	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

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.

4. Discussion

Angiogenesis is a complex process that culminates in the formation of new vessels from pre-existing vessels [11]. It is believed that tissue neovascularization results from the release of angiogenic growth and proliferation factors, including endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor (VEGF) and nuclear cell proliferation antigen (PCNA) [1, 6, 12]. In the context of tissue injuries, vessel elongation is identified as one of the main agents promoting the release of these mediators through the influx of calcium into endothelial cells and phosphorylation of phosphatidylinositol-3-kinase (PI3K) [13, 14]. In spite of the lack of specific studies on skeletal muscle, the literature points out that the use of shock wave therapy is capable of stimulating the same pathway and stimulating the activation and proliferation of endothelial cells similar to what occurred in the injury [6, 13].

Another important factor in the regeneration process of muscle injuries is the proliferation of satellite cells, which represent the muscle reserve and are activated in the presence of an aggressive factor [8, 15]. As seen in the literature, the mechanical stimulus of ESWT is capable of stimulating biological signals that are noted for promoting the activation of satellite cells, similar to what occurs in muscle damage [8].

4.2 Clinical Implications

Muscle regenerative capacity is especially important among athletes, seeing that injuries are commonly associated with pain and functional loss that impact the competitor's performance [8]. Therefore, the use of ESWT can accelerate the signaling pathways for neovascularization and proliferation of satellite cells, establishing muscle regeneration and earlier clinical recovery [8, 13]. In this study, the evaluation of the effect of ESWT on skeletal muscle was performed on the intact quadriceps of Wistar rats. Thus, there were no other factors that could stimulate the proliferation of vessels and satellite cells, with this effect being attributed only to shock waves.

Despite being controversial, the literature still presents the possibility of using ESWT on patients with healthy muscles [7]. Considering the increase in muscle vascularization, as observed in this study, an increase in tissue oxygenation can be acknowledged [7, 13]. So, the therapy could help reduce muscle fatigue and improve the performance of professional athletes [7].

Other applications of neovascularization induced by shock waves are the improvement of muscle pain, hyperreflexia, and strength recovery after injury. In painful muscle syndromes, an extremely prevalent condition in the general population, the neoangiogenic effect of ESWT can benefit the patient by reducing muscle stiffness and tension, in addition to interfering with the signaling mechanism of substance P [16]. The reduction of stiffness is also beneficial in pathologies that present hyperreflexia, with ESWT commonly used to improve the motor performance of patients after stroke [17]. In patients with tendinopathies, the demonstration of satellite cell proliferation after the use of ESWT can aid in the recovery of muscle strength [18].

4.3 Limitations

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

5. Conclusion

In tissue injuries, neovascularization and the proliferation of satellite cells are the main processes that promote tissue regeneration. In this study, the evaluation of the effect of 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. The acceleration of muscle recovery in athletes stands out as an example, by minimizing the impact on the competitor's performance, reducing muscle pain and spasticity, and improving muscle strength in tendinopathies.

Abbreviations

ESWT

extracorporeal shock wave therapy

eNOS

endothelial nitric oxide synthase

VEGF

vascular endothelial growth factor

PCNA

nuclear cell proliferation antigen

Declarations

6.1 Ethics approval and consent to participate

The study was approved by the Ethics Committee on Animal Use (CEUA - FAME Barbacena/Brazil) with protocol number 01/2017 and followed current regulations on animal research. As this is an animal study, consent was not obtained from the participants.

6.2 Consent for publication

this is an animal study, consent was not obtained from the participants.

6.3 Availability of data and material

All data generated or analysed during this study are included in this published article [and its supplementary information files].

6.4 Competing interests

The authors declare that they have no conflict of interest.

6.5 Funding

No funding was obtained for this study.

6.6 Authors' contributions

All authors contributed to the study conception and design. Material preparation and analysis were performed by RSA, RIF, IGGFM and MAPA. The first draft of the manuscript was written by RSA and RIF. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

6.7 Acknowledgements

The authors thank research the statistician and veterinarian Márcio Heitor Stelmo, for ongoing advice regarding study design and statistical analysis. They also thank the Faculty of Medicine of Barbacena, which provided the laboratory and the vivarium for the study. The authors are grateful for helpful support from School of Medicine of the Federal University of Minas Gerais, Brazil.

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Figures

Figure 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.

Figure 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).

Figure 3

Boxplot comparing the variables studied in test paws and control paws.

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