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Original research

Effects of shock wave therapy on glycosaminoglycan expression during bone healing

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HIGHLIGHTS

- Glycosaminoglycans in osteogenesis after shock waves.
- ESWT stimulates chondroitin sulfate and hyaluronic acid.
- Glycosaminoglycans in matrix bone.

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ABSTRACT

Background: Several cases of delayed bone consolidation have been treated with extracorporeal shock wave therapy (ESWT) to improve bone healing and a key role of the extracellular matrix glycosaminoglycans in osteogenesis has been suggested.
Objective: In this study, we aimed to identify and quantify the amount of sulfated glycosaminoglycans (GAG) and hyaluronic acid (HA) within rat femurs following bone drilling and treatment with shock waves.
Methods: To identify and quantify the sulfated glycosaminoglycans (GAG) and hyaluronic acid (HA) within rat femurs following bone drilling and ESWT, 50 male Wistar rats were evaluated. The animals were divided into two groups, both of which were subjected to bone drilling. One of the groups was treated with ESWT. The rats were sacrificed on the 3rd, 7th, 14th, 21st, and 28th day. GAG presence was analyzed by agarose gel electrophoresis with subsequent densitometry and ELISA.
Results and discussion: The content of sulfated GAGs increased significantly from the 3rd to the 28th day ($p = 0.002$). Chondroitin sulfate was expressed more highly than the other GAGs. HA content increased significantly at the 3rd day in animals treated with ESWT compared to the control group ($p = 0.003$).
Conclusion: ESWT stimulates of sulfated glycosaminoglycans during bone healing and enhanced early expression of HA compared to the control group.

1. Methods

1.1. Bone defect model

All procedures and protocols were performed according to the guidelines of the Institutional Ethics Committee of the Federal University of São Paulo, Brazil and the Ethic's committee approved this study.

Seventy-five femurs from 50 male Wistar rats were evaluated. Rats were 3 months old and had an average weight of 300 g. Animals were housed in a propylene box (5 rats per box) subjected to

List of abbreviations: BMP, bone morphogenic protein; Cetavlon, cetyl trimethylammonium bromide; CS, chondroitin sulfate; ELISA, enzyme-linked immunosorbent assay; GAG, glycosaminoglycan; HA, hyaluronic acid; VEGF, vascular endothelial growth factor.

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12 h of light/dark cycles with food and water *ad libitum* and the temperature was controlled (20 ± 2 °C).

Animals were anesthetized by an intraperitoneal injection of ketamine and xylazine at a ratio of 2:1 using 0.2 cc/100 g per animal. Animals were restrained in the prone position, the hind legs were shaved and disinfected with 70% alcohol, and a skin incision, at the thigh, with a scalpel blade no. 15 was performed. The incisions were 3 cm in length and muscle dissection was performed in order to expose the femur. An electric drill, with a 2-mm drill, was used to create a defect in the femoral diaphysis. Drilling was performed until the cortical bone on the opposite side was reached. The wound was sutured using mononylon 4–0 sutures [9].

The animals were divided into two groups. In group I (25 animals) drilling was performed bilaterally and only the left femur (Group IA, 25 femurs) was subjected to ESWT after drilling. The right femur was no subjected to ESWT after drilling (Group IB, 25 femurs). In group II (25 animals, 25 femurs) drilling only was performed in the left femur. Following the procedure, the animals received analgesics and antibiotics for 7 days. Animals were sacrificed, with a CO_2 chamber, on days 3, 7, 14, 21, and 28 (5 animals per day).

The paws were examined daily, and no infection or clinical sign of inflammation were detected. The femurs were removed, fixed in 4% p-formaldehyde in 0.1 M phosphate buffer pH7.2 for 48 h, and decalcified in EDTA (0.7 g/L), tartrate potassium sodium (8 mg/L), hydrochloric acid (99.2 mL/L) and sodium tartrate (0.14 g/L); for 12 h. Subsequently, the fragments were soaked in absolute ethanol. Fragments of 5 mm from each femur containing the lesion area were cut, weighted and submitted to analytical procedure.

1.2. Extracorporeal shock wave therapy application

An electro-hydraulic shock wave generator EVOTRON-Vet® (SwiTech, Switzerland) with 500 pulses and an energy equivalent to 0.13 mJ/mm^2 was used. Only one ESWT session, to the left femur in Group IA, was performed after skin closure [10,11].

1.3. Extraction and quantification of sulfated glycosaminoglycans and hyaluronic acid

Demineralized bone fragments (5 mm) were pulverized using liquid nitrogen. Samples were incubated with 1 mg mL^{-1} papain (Calbiochem, Darmstadt, Germany) in 0.08 M phosphate buffer-cysteine pH 6.5, containing 0.02 M EDTA for 18 h at 60 °C.

Afterwards, peptides and nucleic acid fragments were removed by precipitation with 10% trichloroacetic acid (TCA) at 4 °C. After centrifugation (10 min, 3500*g*, 4 °C), the supernatant containing GAGs were precipitated by adding two volumes of methanol for 18 h at 4 °C. The precipitate was collected by centrifugation (10 min, 3500*g*, 4 °C), dried, suspended in 40 μL of distilled water and analyzed for sulfated GAGs and HA contents. Recovery of GAGs extracted from the bones through that method was around 95% [26]. Sulfated GAGs were identified and quantified by agarose gel electrophoresis in 0.05 M 1,3-diaminopropane acetate buffer, pH 9.0. An aliquot of 5 μL of each sample was submitted to electrophoresis for 1 h at 100 V; GAGs were precipitated in the gel with 0.1% Cetavlon (cetyl trimethylammonium bromide) for 2 h at room temperature. The gel was dried and stained with a 0.1% solution of toluidine blue in acetic acid–ethanol–water (0.1:5:4.9, vol/vol). Quantification was carried out by densitometry at 530 nm of the toluidine-blue-stained electrophoretic slide. The extinction coefficients of the GAGs were calculated using standards of chondroitin sulfate (CS), dermatan sulfate and heparan sulfate. Identification of the sulfated GAGs was based on the migration of the compounds compared with those of standards. The identities of GAGs present in the samples were further confirmed by treatment with specific enzymes. Samples of the GAGs isolated from the bones were degraded with chondroitinase AC and chondroitinase ABC (Sigma) in 0.05 M Tris–HCl buffer pH 7.0 at 37 °C. The electrophoresis was performed in triplicate. Results of the absolute amounts of GAGs were expressed per weight of decalcified femur (μg g^{-1}) [12].

HA was quantified by fluorometric noncompetitive enzyme-linked immunosorbent assay (ELISA)-like assay that can detect 2–500 g/L of HA. The ELISA plates had a fixed probe and 100 μl/well of standard HA solutions, at various concentrations (0–500 g/L), were added. The samples were diluted (1:100) in a buffer Tris–HCl 0.05 M, with 1% bovine serum albumine and added to the ELISA plates in triplicate. The plates were incubated at 4 °C for 12 h, followed by three washes with Tris–HCl 0.05 M.

Next, 100 μl of the probe (1 mg/ml) diluted (1:10.000) in the assay buffer was added. The plate was incubated for 2 h on a shaker and washed nine times with a wash buffer. Streptavidin labeled with europium diluted 1:10.000 in assay buffer was added to each well (100 μl; Sigma, Germany). Streptavidin has an affinity for the biotin-conjugated probe. The plate was incubated 30 min on a shaker and washed nine times with Tris–HCl 0.05 M. To release the europium bound to streptavidin (Sigma), an enhancement solution (280 μl/well) was added. The plates were agitated for 5 min and the europium-free plate fluorescence was read on a fluorimeter. The results were expressed in ng/ml [13,14].

1.4. Statistical analyses

Mean values between groups were compared using an Two-way ANOVA [15], followed by Tukey's multiple comparison. A p-value of ≤0.05 was considered to be statistically significant.

2. Results and discussion

2.1. Characterization and quantification of sulfated glycosaminoglycans (Groups IA, IB and II)

The electrophoretic behavior in agarose gel of the glycosaminoglycans extracted from femurs of each experimental condition showed the presence of a single band migrating as chondroitin sulfate.

ESWT leads to an increase in the amounts of CS compared with the non treated femur.

GAG content (ug/g tissue) in Group IA (ESWT group) and Group

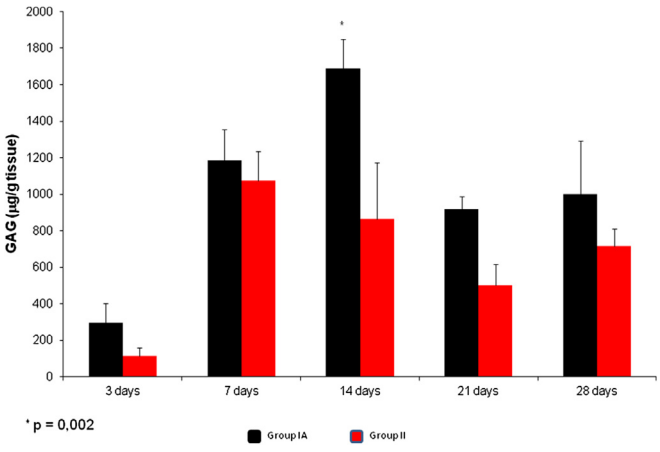

Fig. 1. GAGS Group IA and Group II.

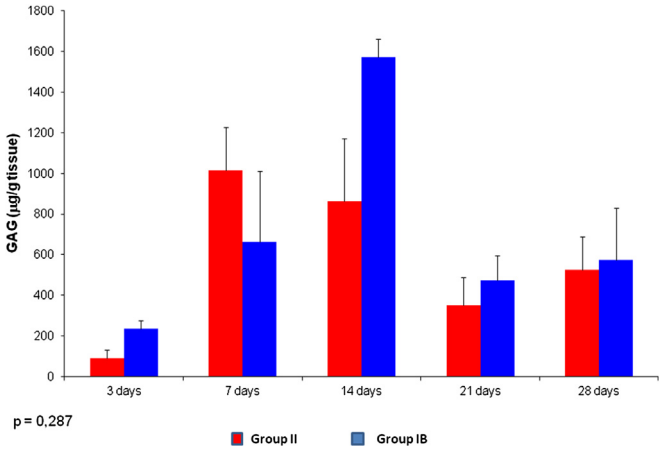

Fig. 2. GAGS Group II and Group IB.

IB (contralateral to ESWT) progressively increased on 3rd, 14th day and 28th day (Fig. 1). Group II (control) GAG content increased on 3th, 7th day, and 28th day. This difference was most pronounced between the groups (IA and II) at day 14 ($p = 0.002$). The difference in GAG content between groups IB and Group II was not significant ($p = 0.287$ (Fig. 2).

2.2. Hyaluronic acid quantification

The results revealed a significant HA expression difference ($p = 0.003$) at day 3 between Group IA and Group II. No differences were observed at subsequent time points (Fig. 3).

Previous studies [1–16] indicate that animals subjected to ESWT undergo neoangiogenesis and display increased expression of angiogenic markers, endothelial nitric oxide synthase, VEGF, and proliferating cell nuclear antigen. ESWT has been shown to induce membrane hyperpolarization and activation of Ras, a factor that operates in the initial phases of osteogenesis. ESWT also has a dose-dependent effect on bone mass and strength [11–17].

The present study aimed to better understand the role of ESWT on sulfated GAGs and HA during bone healing. Bone-related GAGs, including CS, heparan sulfate, and HA have become a focus of interest because of their involvement in bone formation. In our study, GAG content displayed a homogeneous behavior pattern in all three groups.

Specifically, we observed an initial increase in GAG content that reached a peak maximum, followed by a fall and subsequent rise to a plateau below the previous peak.

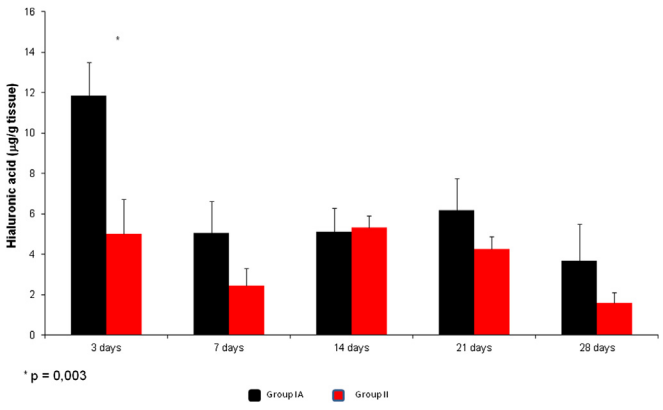

Fig. 3. Hialuronic acid in the Groups.

This is the classic pattern of GAG content during bone healing described in previous studies [18,19]. We also observed that animals treated with ESWT (Groups IA and IB) displayed a prolonged increase in GAG content, which reached a peak on the 14th day. Animals in Group II, that did not receive ESWT, reached the GAG peak earlier, at the 7th day. This change in behavior pattern of GAG content during bone healing in the animals subjected to ESWT suggest that shock waves can prolong anabolism of GAGs and may have systemic effects caused by the prolonged anabolism, as the contralateral femurs (Group IB) not subjected directly to ESWT also displayed the prolonged GAG increment.

As previous studies [20,21,28,29], our results showed that CS was the predominant sulfated glycosaminoglycan present in the injured femurs. ESWT increases CS expression (14th day). Studies showed that on the bone mature (21st day) occurs a decrease in the amounts of CS which parallels the decrease in the SLRP biglycan and decorin [30] who are expressed in extracellular bone matrix and seem to play a role in bone cell differentiation and proliferative activity. It has been proposed that SLRPs modulate local storage and ⁄or availability of different growth factors [30]. Merli et al. (2012) using bone treated with laser showed the presence of decorin and biglycan at the initial stages after bone injury. Previous studies show the influence of CS and SLRPs on fibrogenesis collagen control [27,30–32].

HA is a unique among the GAGs in that no contain any sulfate and for that it quantified was done by ELISA-like assay who is able to detect low concentrations of this GAG [33]. An increase in HA expression in the femurs subjected to ESWT 3–4 days following the therapy was observed. Recent studies [22,23] indicate that HA has an impact on early bone tissue healing processes by acting on initial signaling mechanisms.

The increased HA early expression resulting from ESWT might indicate a beneficial effect. Expression of HA, an indispensable component of ECM. Interestingly, the amount of HA decreases as the bone matures. HA is related to cell migration in healing process and tissue repair [34,35] and appears to be associated with bone remodeling by controlling osteoclasts, osteoblasts and osteocytes behavior [36].

ESWT has also been shown to promote increased bone mineral density, increased bony callus, and higher calcium content [11]. These increases could be related to a shock wave-induced increase in the content of sulfated GAGs and HA during the bone mineralization process. Interestingly, ESWT has been shown to initiate other bone-related processes including the stimulation of neovascularization by inducing precursors of neoangiogenesis [16], stimulation of BMP-2 [24], and stimulation of osteoblasts [25]. This indicates that ESWT interact with the many factors involved in the complex process of bone regeneration. In this study, we show that the pattern of sulfated GAG and HA expression is altered during the process of bone healing and after stimulation with ESWT. An increase in the content of GAGs within bone tissue and a prolonged period of anabolism resulting from the ESWT could be factors that promote bone formation to create more resistant sites of new bone growth with higher callus content.

In the present work, it was possible to demonstrate that ESWT in perforated rat femurs stimulated expression of sulfated glycosaminoglycans during bone healing and enhanced early expression of HA compared to the control group. Study limitations include the model used to evaluate bone healing by a bone perforation that resembles a stable fracture and may not represent the same response for unstable fractures. Although ESWT has been the focus of intensified studies, the literature lacks an optimal protocol regarding each animal and bone evaluated, so the application methodology presented here may still not be the optimal.

3. Conclusions

Extracorporeal shock wave therapy stimulated regeneration and bone healing by increasing the concentration and prolonging the anabolism period of sulfated glycosaminoglycans in addition to early enhanced expression of hyaluronic acid.

Ethical approval

Comite de Ética em Pesquisa da Universidade Federal de São Paulo sob numero 0048/09.

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Author contribution

Paulo Roberto Dias dos Santos – study design ,data collections ,writing.
Joao Paulo Freire Moura – data collections.
Valquiria Pereira de Medeiros – data analysis.
Carlos Eduardo da Silveira Franciozi – writing.
Flavio Faloppa – study design.
Helena Bonciani Nader – study design.

Conflicts of interest

I haven't conflicts of interest.

Guarantor

Paulo Roberto Dias dos Santos.

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