A New Anionic Bovine Tendon as Scaffold for the Repair of Bone Defects: A Morphological, Histomorphometric and Immunohistochemical Study

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ABSTRACT~~:~~

Aim: The process of bone repair is of evident importance in both the clinical and functional spheres. For this reason, the field of bioengineering has taken it as an object of study, seeking to perfect the implantation of materials that allow for adequate bone neoformation. This study investigated the process of bone repair after anionic bovine tendon grafting in rat tibias by conducting a morphological, histomorphometric and immunohistochemical ~~analysis~~study.

Methodology: The experimental model consisted of 36 rats randomly divided into two groups: a control group (CG, *n=18*), in which a surgical cavity in the tibia was filled with blood clots; and an experimental group (EG, *n=18)*, in which a surgical cavity in the tibia was filled with an anionic bovine tendon graft. In the experimental group, the major axis of the collagen fiber bundle was placed perpendicularly to the long axis of the tibia. Microscopic, morphometric and immunohistochemical evaluations were conducted at 7, 15, and 30 days postoperative.

Results: The analyzes showed an increase ~~and demonstrated increasing bone~~ in bone neoformation in the experimental group during the assessed periods. There was a significant difference between day 7 and day 30 and evident vascular proliferation was detected by the immunohistochemical analysis.

Conclusion: ~~In conclusion,~~ It can be concluded that the anionic bovine tendon collagen proved to be an adequate and biocompatible material for efficient bone regeneration, with osteogenic capabilities that allow it to be used as a scaffold for bone repair.

Key ~~W~~words: Bone regeneration; ~~B~~bone transplantation; c~~C~~ollagen; i~~I~~mmunohistochemistry; t~~T~~endons; t~~T~~issue engineering; t~~T~~issue scaffolds.

1. ~~1~~ INTRODUCTION

Bone defects cause numerous complications that are particularly relevant to the fields of rehabilitation and orthopedics. Such defects are related to pathological processes and traumatic or physiological processes, such as fractures, infections, chronic inflammatory diseases, reduced lean body mass, advanced age, immobility and the effect of glucocorticoid treatments [~~[~~1-4]~~1-4~~].

Bone regeneration in large skeletal defects is a special challenge, as it is essential for adequate bone repair [5] ~~[5]~~ and involves socioeconomic concerns regarding the correct treatment of such patients [6]~~[6]~~. Physiological bone remodeling is a coordinated process essential to bone repair and mineral homeostasis, occurring independently at several different anatomical locations [7,

8] [7, 8]. Imbalances in the quantity of removed bone in comparison to newly deposited bone lead to reduced amounts of total bone and increased risk of fractures [5][5].

Treatments for such defects require procedures such as autologous or autogenous bone grafting, or alternative metal and ceramic grafting, aimed at bone healing and repair [6][6]. Autogenous grafts have long been considered the gold standard; however, adverse effects in the donor site have been observed, leading to the development of biocompatible substitutes for this type of graft [9][9].

Efforts in this area have focused on tissue engineering and biomaterials [10] in order to study the combination of biomaterials and biological systems. The use of devices that reestablish or modify tissues or organ function leads to interactions between tissue components and biomaterials. This process is associated with the liberation of growth factors in the implantation site such as, for example, bone morphogenetic protein-2 (BMP-2), a growth factor that induces osteoblast differentiation and promotes bone regeneration [10][10].

Collagen has received special attention from the field of tissue engineering, as it is the most abundant protein in mammals, making up to 30% of the protein in the body [11][11]. It is biocompatible and biodegradable, and has low antigenicity and high resistance to traction. However, the pure form still presents limitations and its physical and chemical characteristics need to be perfected [12,13][12,13].

Anionic collagen is created by alkaline treatment, which gives it enhanced piezoelectric properties, which is a load change that attracts the osteoblastic action, increasing bone mineral density, favoring the deposition of minerals in the organic portion is under pressure, and this has widen therapeutic possibilities to bony tissue, a result of selective hydrolysis of carboxamide groups of asparagine and glutamine residues from carboxylic collagen [14][14]. Anionic collagen is capable of guiding attract phosphate and calcium salt deposits in accordance with its microfibrillar structure [15][15]. Glutaraldehyde can also be used as an alternative in the preparing ation of the collagenous material, emphasizing its applicability as a

biomaterial, since it functions as a stabilizer, reduces immunogenicity and increases resistance to enzymatic degradation [16-19]~~[16-19]~~. Used as scaffold systems inserted in bone defects, such biomaterials are biocompatible and can induce the formation of new bone tissue [20].~~[20].~~

The biomechanical properties of tendons characterize them as resistant and cable-like, in that they are formed by dense connective tissue composed of abundant extracellular matrices [21] ~~[21]~~**.** Fiber organization and orientation interfere in bone neoformation [20] ~~[20]~~. Thus, it is essential to study the orientation of collagenous fibers in organic tissue (in the present case, bone) in order to ensure correct morphological and functional restructuring.

There are few studies on the behavior of collagen implant tissue derived from bovine tendons [11] ~~[11]~~ and bovine collagen in the form of membranes [22]~~[22]~~, and few descriptions of such techniques are available. Therefore, the objective of the current study was to analyze the process of bone repair after anionic bovine tendon grafting in rat tibia, using morphological, histomorphometric and immunohistochemical ~~analysis~~study.

2. ~~2~~ MATERIALS AND METHODS

2.1 ~~2.1~~ Experimental ~~m~~Model

The study was approved by the ethics committee of the University of Marília (Marília, São Paulo, Brazil). Surgical defects were created in the tibias of 36 male rats (*Rattus norvegicus,* Wistar), all 60 days old and weighing an average of 245.3 grams.

The rats were randomly divided into two groups with 18 animals each: a control group (CG), in which the surgically created cavity was filled with blood clots; and an experimental group (EG), in which the medullary cavity received an anionic collagen matrix implant made from bovine calcaneous tendon. During the postoperative period, the rats were kept in individual cages and

received *ad libitum* access to food and water. Counting from the day of surgery, six animals from each group were euthanized by anesthetic overdose at 7, 15 and 30 days postoperative.

2.2 ~~2.2~~ Preparation of ~~b~~Biomaterial

This study used fresh bovine tendons (common calcaneous tendons) acquired from a commercial establishment. The material was prepared and provided by the Chemical Institute of São Carlos (University of São Paulo, São Paulo, Brazil) in accordance with the literature [16, 23-25]~~[16, 23-25]~~.

Samples were devitalized by undergoing alkaline sulfate and chlorate solution treatment for 24 hours (to remove cells). The material was neutralized and stabilized in a phosphate buffer, in accordance with collagen preparation techniques described in the literature by Bet et al. [25] ~~[25]~~, through selective hydrolysis of asparagine and glutamine amides for 24 hours. Next, the collagen was balanced with a phosphate buffer, frozen in liquid nitrogen and freeze-dried in an Edwards Modulyo freeze dryer (Thermo Electron Corporation, Waltham, USA), as described in a previous study [14]~~[14]~~.

Differential exploratory calorimetric tests, and transmission electron microscopy (TEM) and scanning ~~transmission~~ electron microscopy (SEM) analyses were also carried out, as described by Bet et al. [25] ~~[25]~~. Anionic tendons were then sterilized in ethylene oxide and hydrated during implantation with a saline solution.

2.3 ~~2.3~~ Surgical ~~p~~Procedures

The animals received general anesthesia via intramuscular injections of ketamine hydrochloride (75 mg/kg; Ceva Santé Animale, Paulínia, Brazil) associated with xylazine hydrochloride (1.5 ml/kg; Ceva Santé Animale, Paulínia, Brazil). A 20 mm longitudinal incision was made in the left hindlimb, followed by divulsion of muscle tissue surrounding the proximal tibial epiphysis and separation of the periosteum.

A bone defect approximately 2.2 mm in diameter was created using a carbide spherical no. 6 steel drill (KG Sorensen, Cotia, Brazil) with a low-speed micromotor (KaVo Dental GmbH, Biberach, Germany), deeply affecting the medullary cavity. Throughout the procedure, the surgical site was irrigated with a sterile sodium chloride solution. In the experimental group, the major axis of the anionic collagen fiber bundle was placed perpendicularly to the long axis of the tibia (2 mm in diameter), into the defect. In the control group, the defect was maintained with no biomaterial, and filled only with blood clots. The tissues were reapproximated (including periosteum) and sutured in layers (Ethicon, Johnson & Johnson Brazil, São José dos Campos, Brazil).

2.4 ~~2.4~~ Histological P~~p~~rocessing

Following euthanasia, a portion of the defective tibia was removed, fixated in a 10% formalin solution for 24 hours, cleansed, and decalcified in a 20% ethylenediaminetetraacetic acid (EDTA) solution (Merck KGaA, Darmstadt, Germany) for 5 weeks. The solution was changed once a week, as described in the literature [20]~~[20]~~.

Next, samples underwent routine laboratory processing and were fixated in paraffin blocks. Blocks were cut into 6 µm longitudinal sections with a Leica RM 2245 microtome (Leica Microsystems, Wetzlar, Germany). The samples were then stained with hematoxylin and eosin and histomorphological analyses were performed using an Olympus BX50 optical microscope (Olympus Corporation, Tokyo, Japan) [16] ~~[16]~~.

Microscopic analyses were conducted for each group at each postoperative time. This analysis investigated the superficial surgical site, the lateral edges of residual cortical tissue, the medullary area adjacent to the superficial surgical site, and the implanted material.

2.5 ~~2.5~~ Immunohistochemistry

The immunohistochemical analysis carried out in the experimental group used two slides from each animal to detect immunoperoxidase reactions to identify primary antibodies from the following proteins: osteocalcin (OC, sc 240750), vascular endothelial growth factor (VEGF, sc 1836), alkaline phosphatase (ALP, sc 79839), tartrate-resistant acid phosphatase (TRAP, sc 30832), receptor activator of nuclear factor kB ligand (RANKL, sc 7627) and osteoprotegerin (OPG, sc 21038) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). To reveal reactions, diaminobenzidine (DAB) was used (Sigma Aldrich, St Louis, MO, USA). Images were recorded with an Olympus BX50 microscope (Olympus Corporation, Tokyo, Japan) and photographs were taken with an attached digital camera (Olympus DP 71, Tokyo, Japan) with 40x and 100x objectives.

Scores of 0 to 3, with 0 = absence of immunostaining (complete absence of immunoreactive cells), 1 = low immunostaining (staining in the extracellular matrix and in approximately 1/4 of immunoreactive cells), 2 = moderate immunostaining (staining in the extracellular matrix and in approximately 1/2 of immunoreactive cells), and 3 = high immunostaining (strong staining in the extracellular matrix and in approximately 3/4 of immunoreactive cells).

2.6 ~~2.6~~ Histomorphometric Aassessment

Quantitative analyses were performed with Image Pro-Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA) software. For morphometry, the cortical region where the tibia was perforated and the medullary region adjacent to the contralateral intact cortex were analyzed by measuring the amount of new connective and bone tissue in the region. The data were subjected to two-way analysis of variance (ANOVA) followed by Tukey's test. A significance level was established at ~~p~~*P*<~~0~~.05 for all analyses. The amount of bone tissue and connective tissue formed was measured using a light microscope with a 100-point quadrilateral grid system coupled with an ocular micrometer, according to the Delesse principle mentioned by previous studies [26]~~[26]~~.

3. ~~3~~ RESULTS

3.1 ~~3.1~~ Histomorphological ~~a~~Analysis

3.1.1 Control Group:

Fibrous tissue was present at the superficial surgical site at 7 and 15 days, but was less prevalent on day 30. Enhanced bone neoformation around the fibrotic area was observed at 7 days and was less evident at 15 and 30 days.

The medullary cavity was infiltrated by connective tissue. However, there was no bone differentiation delimitating the blood clot area, still present on day 7. At 15 days, vascular congestion was prevalent within the trabecular bone and there was rudimentary neocortical bone with primary bone tissue, which on day 30 was thicker, more organized and mature, with no well-defined periosteum. Analyses revealed the presence of mononuclear inflammatory cell infiltrates in neoformed bone at 7 days, which increased in intensity throughout subsequent days (Fig. 1).

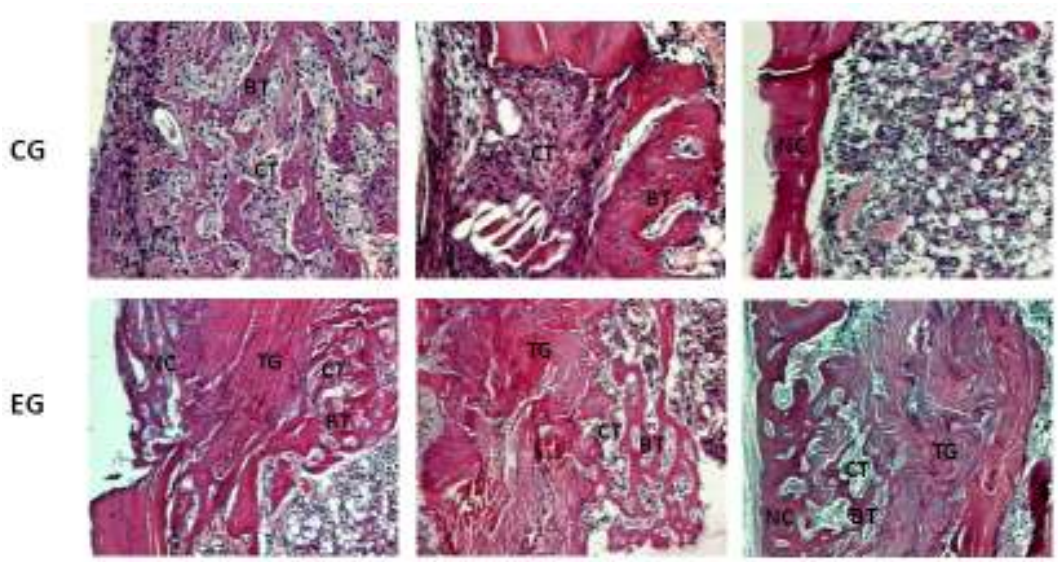
3.1.2 Experimental Group:

Superficial fibrous tissue was present during the first 15 days, notably more subtle than that in the control group. Neocortical formation was observed at 15 postoperative days and bone maturation at 30 days.

Starting on day 7, tendon fibers were multidirectional and there were fewer perpendicular fibers in relation to the long axis of the tibia when compared to the time of surgical procedure. Bone neoformation between anionic tendon fibers was observed on day 15 and was more prevalent on day 30.

Increasing bone neoformation and moderate vascular proliferation were observed in proportion to postoperative time. Osteoblast and osteoclast concentrations were found in the distal end of

the tendon. There was accentuated presence of mononuclear inflammatory infiltrates and interstitial fibrosis on all three studied days (Fig. 1).



~~FIG~~Fig. 1. Histological photomicrography stained with hematoxylin and eosin of control and experimental groups. 7 days (left), 15 days (middle) and 30 days (right): BT (bone tissue), CT (connective tissue), NC (new cortical), TG (tendon graft).

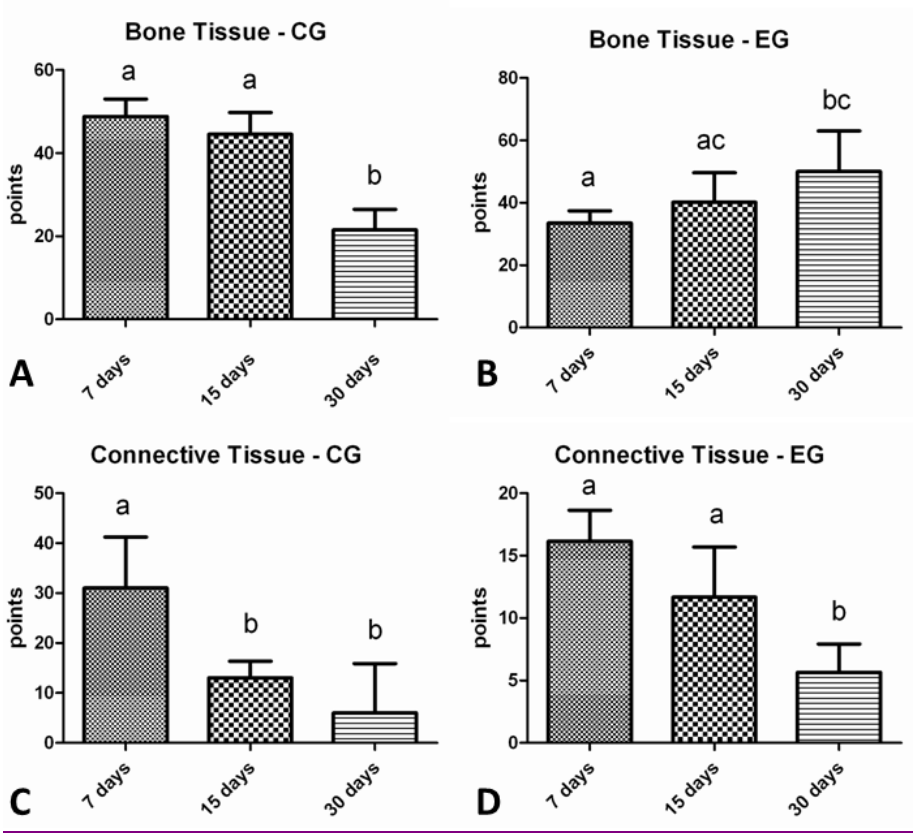
3.2 ~~3.2~~ Histomorphometric ~~a~~Analysis

3.2.1 Control Group:

Measurement of neoformed bone tissue revealed significant differences between day 30 and the other assessed periods. Regarding connective tissue, a significant difference was observed between day 7 and the other investigated periods (Fig. 2).

3.2.2 Experimental Group:

Measurement of neoformed bone tissue revealed a significant difference between day 7 and day 30. A significant difference was observed regarding connective tissue at 30 days in comparison to the other assessed periods (Fig. 2).

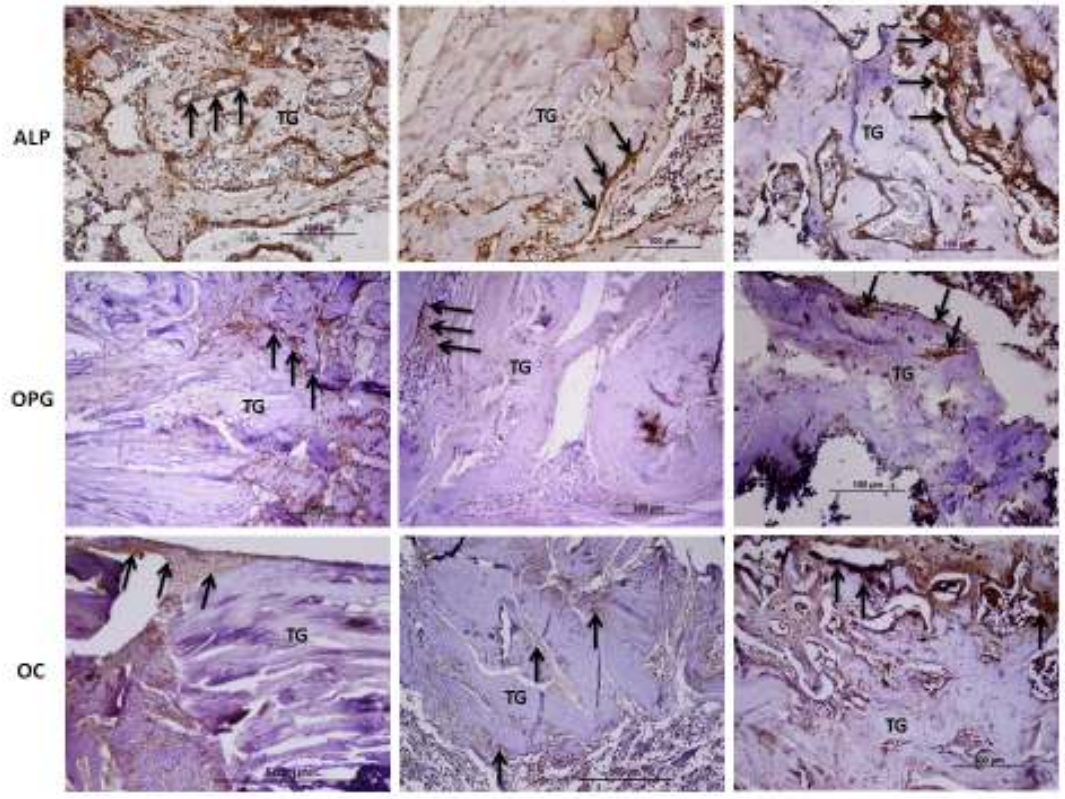


~~FIG~~Fig. 2. Histomorphometry of the amount of newly formed bone and connective tissue~~,~~ in ~~both control~~CG (control group) and EG (experimental group~~s~~)~~,~~ at 7, 15 and 30 days postoperative. Different lowercase letters indicate significant differences among the groups by means of ANOVA, followed by Tukey's test (~~p~~*P*<~~0~~.05).

3.3 ~~3.3~~ Immunohistochemical ~~a~~Analysis

Immunohistochemical samples for each protein marker used in this study are illustrated in Fig. 3 and Fig. 4. Involved in the osteoclastogenesis regulating mechanism, OPG and RANKL proteins showed similar levels during all periods; OPG presented peak intensity (score 3) at the

biomaterial-tissue interface at 7 days, and RANKL at 15 days (score 3). Our findings indicated that TRAP, a specific protein marker expressing bone reabsorption, was more intensely prevalent at 15 and 30 days (score 3). Osteocalcin, a protein from the synthetized extracellular matrix secreted during osteoblast differentiation and primarily expressed in the final phase of bone formation, was present during all assessed periods, reaching peak intensity at 30 days (score 3). In addition to OC, ALP is also used as a marker for osteoblasts with a role in bone matrix mineralization, and it was detected during all of the studied periods in expressive quantities (score 3). The VEGF protein, a factor expressed by osteoblasts and intimately connected with angiogenic processes, was present in all periods, with peak intensity at 15 and 30 days postoperative (score 3).



~~FIG~~Fig. 3. Immunolabeled proteins (arrows) used for assessing tendon graft (TG). ALP, alkaline phosphatase; OPG, osteoprotegerin; OC, osteocalcin~~; RANK, receptor activator of nuclear factor kB; TRAP, tartrate-resistant acid phosphatase; VEGF, vascular endothelial growth factor.~~. 7 days (left), 15 days (middle) and 30 days (right).

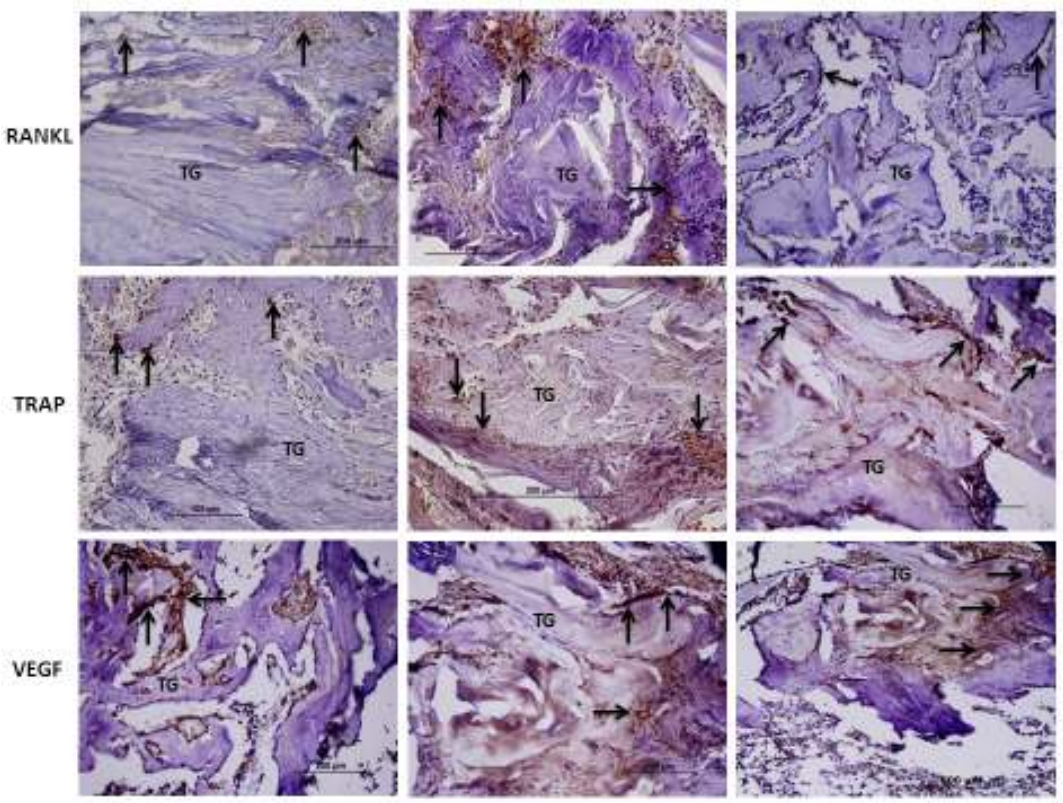


Fig. 4. Immunolabeled proteins (arrows) used for assessing tendon graft (TG). RANKL, receptor activator of nuclear factor kB ligand; TRAP, tartrate-resistant acid phosphatase; VEGF, vascular endothelial growth factor. 7 days (left), 15 days (middle) and 30 days (right).

4 DISCUSSION

The present study aimed to analyze bone neoformation following new anionic bovine tendon grafting in rat tibias, by means of morphological, histomorphometric and immunohistochemical analyses. Anionic bovine tendon was shown to be an adequate and biocompatible material for efficient bone regeneration, with osteogenic capabilities that allow it to be used as a scaffold for bone repair.

Studies investigating how to repair bone defects have found that scaffolds are frequently needed to induce the growth of new bone tissue [14]~~[14]~~. Researchers have been searching for new, enhanced and increasingly more biocompatible materials to minimize complications in bone repair.

Biomaterials can present granulomatous inflammation on a chronic basis, which is intimately connected with the healing process of bone implantation [16,27-29]~~[16, 27-29]~~. Anionic collagen displays high biocompatible and biodegradable potential, in addition to low antigenicity and low levels of inflammatory reactions, thus enhancing bone neoformation [13,30] ~~[13, 30].~~

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After the native tendon is hydrolyzed, fibers are modified by opening the pores, favoring bone cell migration and growth in the matrix, especially due to the generated anionic charge in addition to the presence of growth factors ~~[14, 31, 32]~~. Hydrolysis is also responsible for removing cells that can cause dystrophic calcification, intense local inflammatory reactions and foreign body reactions from the matrix [14,20,25,31-33]~~[14, 20, 25, 33]~~.

The anionic tendon has low levels of inflammatory response which can be associated with improvements in new bone formation, allowing the restoration of osseous defects may occur within a shorter period such as, for example, within 15 days in this study, according Rocha et. al (2002) that reported osteoblast deposition in its own matrix along the formed scaffold and collagen removed since the remodeling, demonstrating the enhanced performance of anionic collagen when compared to materials that must be reabsorbed in order to allow for bone regeneration.

Morphometric analysis aimed to verify bone and tissue neoformation [20,34,35] ~~[20, 34, 35]~~. The amount of neoformed bone tissue in the control group was significant at day 30. The experimental group presented significant amounts at day 7 in comparison to day 30. This finding was in accordance with that of a previous study [20] ~~[20]~~ that showed that new bone was formed starting on day 7 and increased with the time after bone implantation. This study also showed that formation of new bone over collagenous tendon fibers was evident from day 15 onwards. The results of the present study were also in accordance with those of Pan et al. [36]~~[36]~~, who observed the presence of endochondral bone neoformation in all experimental

groups, and Uchida et al. ~~[34]~~[34], who found altered properties of bone composition, such as increased bone matrix formation, mineral concentration, cortical thickness and volume of trabecular bone.

Several studies have used immunohistochemistry to analyze bone neoformation [37-42]~~[37-42]~~. Osteoblasts express ALP, which plays a very important role in the mineralization of the bone matrix ~~[42, 43]~~. In the present study, ALP was present during all of the analyzed periods, thus demonstrating constant bone mineralization, as was the case in other studies that demonstrated high mineralization levels at the end of experiments [42,43]~~[42]~~.

Vascular endothelial growth factor indicates that vascularization in the receptor bed is occurring at a constant rate [40]~~[40]~~; it was observed in the present study as a marker in all the analyzed periods, reaching peak intensity at 15 and 30 days. Another study ~~[40]~~[40] found that VEGF reached its highest level at 10 days, but decreased after day 20 until reaching a statistically significant difference after 60 days in the experimental group. As explained by Carano and Filvaroff [44]~~[44]~~ and Hankenson et al. ~~[45]~~[45], angiogenesis is essential to bone regeneration, in that it provides cells, oxygen, nutrients and growth factors to the implantation site. Corroborating the findings of the present study, Miguel et al. [16]~~[16]~~ diverged from other authors and did not find any evidence of vascular formation at the points surrounding mineralization nuclei between matrix fibers.

The use of TRAP expressed by osteoclasts provides the rate of bone remodeling [41,46-48]~~[41, 46-48]~~. In the present findings, this marker was more prevalent in the experimental group on days 15 and 30. Pedrosa et al. [40]~~[40]~~ demonstrated similar TRAP curves in their control and experimental groups, with a maximum peak at 10 days. Furthermore, they noted that a constant level of receptor bed vascularization throughout the experiment showed that graft remodeling was occurring at a proportional rate.

The occurrence of osteoclastogenesis is demonstrated by the presence of RANKL and OPG [40,49,50]~~[40, 49, 50]~~, which in the present study were present at similar levels during all three

assessed periods; OPG presented peak intensity at the biomaterial-tissue interface at 7 days, and RANKL at 15 days. The present results were in agreement with previous research [16,44,45]~~[16, 44, 45]~~ regarding bone regeneration, narrowing the gap between neoformation of the vascular bed and osteogenesis.

5. CONCLUSION

In conclusion,

~~**5 Conclusions**~~

the ~~The~~ findings of this study showed that anionic bovine tendon is an adequate and biocompatible material for efficient bone regeneration, with osteogenic capabilities that allow it to be used as a scaffold for bone repair.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS:

This work was carried out in collaboration between all authors. Authors RLB, JCA, DVB, ACR, JSR, DDR and GG participated in concept and design, data collection and data analysis/interpretation. RO participated performing immunohistochemical analysis. MPOR, ALS and GMRJr participated in manuscript creation involving critical writing and revising of the content. All authors read and approved the final version of this manuscript.

ETHICAL APPROVAL

The study was approved by the ethics committee of the University of Marília (Marília, São Paulo, Brazil).

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