

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

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29 **ABSTRACT**

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

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

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

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

47

48 **Keywords:** Bone regeneration; bone transplantation; collagen; immunohistochemistry; tendons;
49 tissue engineering; tissue scaffolds.

50

51 1. INTRODUCTION

52

53 Bone defects cause numerous complications that are particularly relevant to the fields of
54 rehabilitation and orthopedics. Such defects are related to pathological processes and traumatic
55 or physiological processes, such as fractures, infections, chronic inflammatory diseases,
56 reduced lean body mass, advanced age, immobility and the effect of glucocorticoid treatments
57 [1-4].

58 Bone regeneration in large skeletal defects is a special challenge, as it is essential for adequate
59 bone repair [5] and involves socioeconomic concerns regarding the correct treatment of such
60 patients [6]. Physiological bone remodeling is a coordinated process essential to bone repair
61 and mineral homeostasis, occurring independently at several different anatomical locations [7,
62 8]. Imbalances in the quantity of removed bone in comparison to newly deposited bone lead to
63 reduced amounts of total bone and increased risk of fractures [5].

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

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

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

80 Anionic collagen is created by alkaline treatment, which gives it enhanced piezoelectric
81 properties, which is a load change that attracts the osteoblastic action, increasing bone mineral
82 density, favoring the deposition of minerals in the organic portion is under pressure, and this
83 has widen therapeutic possibilities to bony tissue, a result of selective hydrolysis of
84 carboxamide groups of asparagine and glutamine residues from carboxylic collagen [14].

85 Anionic collagen is capable of attract phosphate and calcium salt deposits in accordance with its
86 microfibrillar structure [15]. Glutaraldehyde can be used in the preparation of the collagenous
87 material, emphasizing its applicability as a biomaterial, since it functions as a stabilizer, reduces
88 immunogenicity and increases resistance to enzymatic degradation [16-19]. Used as scaffold
89 systems inserted in bone defects, such biomaterials are biocompatible and can induce the
90 formation of new bone tissue [20].

91 The biomechanical properties of tendons characterize them as resistant and cable-like, in that
92 they are formed by dense connective tissue composed of abundant extracellular matrices [21].
93 Fiber organization and orientation interfere in bone neoformation [20]. Thus, it is essential to

94 study the orientation of collagenous fibers in organic tissue (in the present case, bone) in order
95 to ensure correct morphological and functional restructuring.

96 There are few studies on the behavior of collagen implant tissue derived from bovine tendons
97 [11] and bovine collagen in the form of membranes [22], and few descriptions of such
98 techniques are available. Therefore, the objective of the current study was to analyze the
99 process of bone repair after anionic bovine tendon grafting in rat tibia, using morphological,
100 histomorphometric and immunohistochemical study.

101

102 **2. MATERIALS AND METHODS**

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104 **2.1 Experimental Model**

105

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

109 The rats were randomly divided into two groups with 18 animals each: a control group (CG), in
110 which the surgically created cavity was filled with blood clots; and an experimental group (EG),
111 in which the medullary cavity received an anionic collagen matrix implant made from bovine
112 calcaneous tendon. During the postoperative period, the rats were kept in individual cages and
113 received *ad libitum* access to food and water. Counting from the day of surgery, six animals
114 from each group were euthanized by anesthetic overdose at 7, 15 and 30 days postoperative.

115

116 **2.2 Preparation of Biomaterial**

117

118 This study used fresh bovine tendons (common calcaneous tendons) acquired from a
119 commercial establishment. The material was prepared and provided by the Chemical Institute of
120 São Carlos (University of São Paulo, São Paulo, Brazil) in accordance with the literature [16,
121 23-25].

122 Samples were devitalized by undergoing alkaline sulfate and chlorate solution treatment for 24
123 hours (to remove cells). The material was neutralized and stabilized in a phosphate buffer, in

124 accordance with collagen preparation techniques described in the literature by Bet et al. [25],
125 through selective hydrolysis of asparagine and glutamine amides for 24 hours. Next, the
126 collagen was balanced with a phosphate buffer, frozen in liquid nitrogen and freeze-dried in an
127 Edwards Modulyo freeze dryer (Thermo Electron Corporation, Waltham, USA), as described in
128 a previous study [14].

129 Differential exploratory calorimetric tests, and transmission electron microscopy (TEM) and
130 scanning electron microscopy (SEM) analyses were also carried out, as described by Bet et al.
131 [25]. Anionic tendons were then sterilized in ethylene oxide and hydrated during implantation
132 with a saline solution.

133

134 **2.3 Surgical Procedures**

135

136 The animals received general anesthesia via intramuscular injections of ketamine hydrochloride
137 (75 mg/kg; Ceva Santé Animale, Paulínia, Brazil) associated with xylazine hydrochloride (1.5
138 ml/kg; Ceva Santé Animale, Paulínia, Brazil). A 20 mm longitudinal incision was made in the left
139 hindlimb, followed by divulsion of muscle tissue surrounding the proximal tibial epiphysis and
140 separation of the periosteum.

141 A bone defect approximately 2.2 mm in diameter was created using a carbide spherical no. 6
142 steel drill (KG Sorensen, Cotia, Brazil) with a low-speed micromotor (KaVo Dental GmbH,
143 Biberach, Germany), deeply affecting the medullary cavity. Throughout the procedure, the
144 surgical site was irrigated with a sterile sodium chloride solution. **In the experimental group, the
145 major axis of the anionic collagen fiber bundle was placed perpendicularly to the long axis of the
146 tibia (2 mm in diameter), into the defect [20].** In the control group, the defect was maintained
147 with no biomaterial, and filled only with blood clots. The tissues were reapproximated (including
148 periosteum) and sutured in layers (Ethicon, Johnson & Johnson Brazil, São José dos Campos,
149 Brazil).

150

151 **2.4 Histological Processing**

152

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

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

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

165

166 **2.5 Immunohistochemistry**

167

168 The immunohistochemical analysis carried out in the experimental group used two slides from
169 each animal to detect immunoperoxidase reactions to identify primary antibodies from the
170 following proteins: osteocalcin (OC, sc 240750), vascular endothelial growth factor (VEGF, sc
171 1836), alkaline phosphatase (ALP, sc 79839), tartrate-resistant acid phosphatase (TRAP, sc
172 30832), receptor activator of nuclear factor κB ligand (RANKL, sc 7627) and osteoprotegerin
173 (OPG, sc 21038) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). To reveal reactions,
174 diaminobenzidine (DAB) was used (Sigma Aldrich, St Louis, MO, USA). Images were recorded
175 with an Olympus BX50 microscope (Olympus Corporation, Tokyo, Japan) and photographs
176 were taken with an attached digital camera (Olympus DP 71, Tokyo, Japan) with 40x and 100x
177 objectives.

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

183

184 **2.6 Histomorphometric Assessment**

185

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

194

195 **3. RESULTS**

196

197 **3.1 Histomorphological Analysis**

198

199 **3.1.1 Control Group:**

200

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

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

211

212 **3.1.2 Experimental Group:**

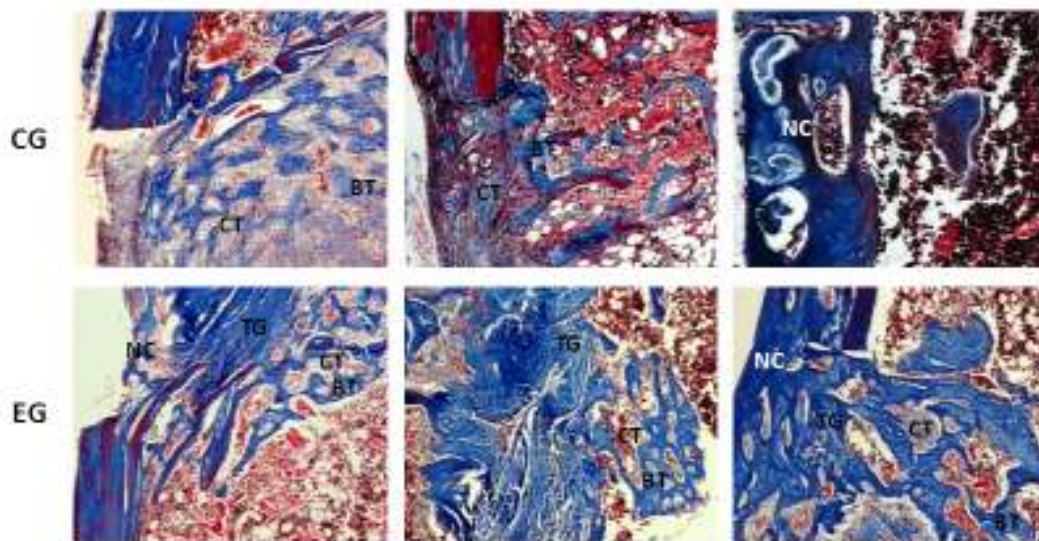
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214 Superficial fibrous tissue was present during the first 15 days, notably more subtle than that in
215 the control group. Neocortical formation was observed at 15 postoperative days and bone
216 maturation at 30 days.

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

221 Increasing bone neoformation and moderate vascular proliferation were observed in proportion
222 to postoperative time. Osteoblast and osteoclast concentrations were found in the distal end of
223 the tendon. There was accentuated presence of mononuclear inflammatory infiltrates and
224 interstitial fibrosis on all three studied days (Fig. 1).

225



226

227

228 **Fig. 1.** Histological photomicrography stained with Masson's trichrome of control and
229 experimental groups. 7 days (left), 15 days (middle) and 30 days (right); BT (bone tissue), CT
230 (connective tissue), NC (new cortical), TG (tendon graft).

231

232 3.2 Histomorphometric Analysis

233

234 **3.2.1 Control Group:**

235

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

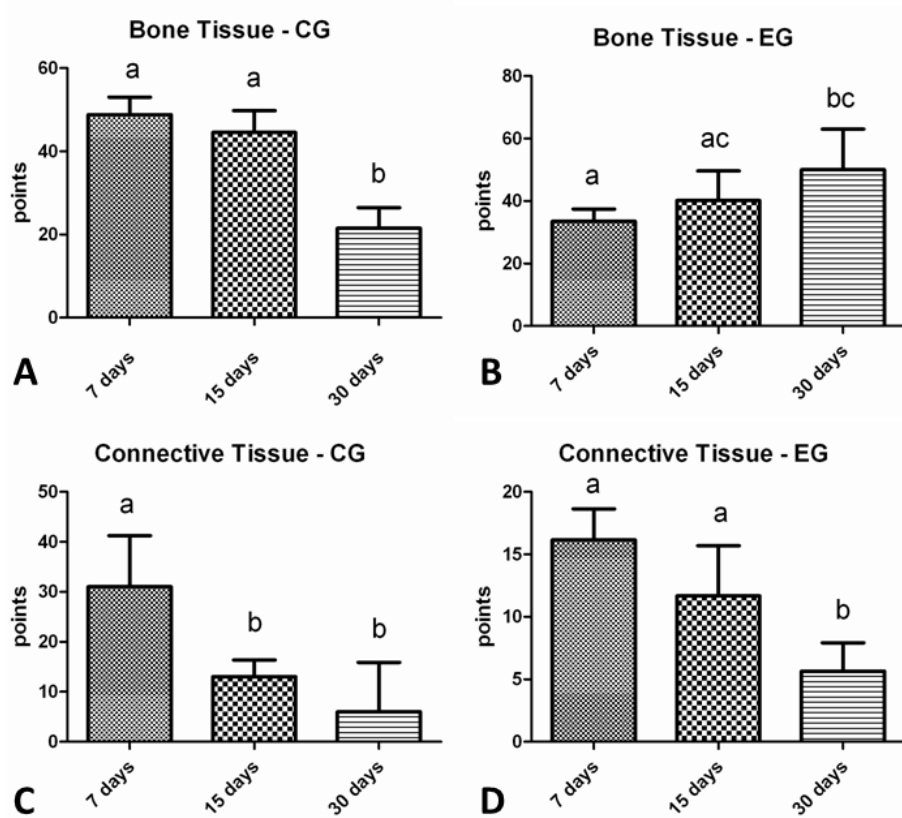
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240 **3.2.2 Experimental Group:**

241

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

245



246

247

248 **Fig. 2.** Histomorphometry of the amount of newly formed bone and connective tissue in CG

249 (control group) and EG (experimental group) at 7, 15 and 30 days postoperative. Different

250 lowercase letters indicate significant differences among the periods by means of ANOVA,
251 followed by Tukey's test ($P<.05$).

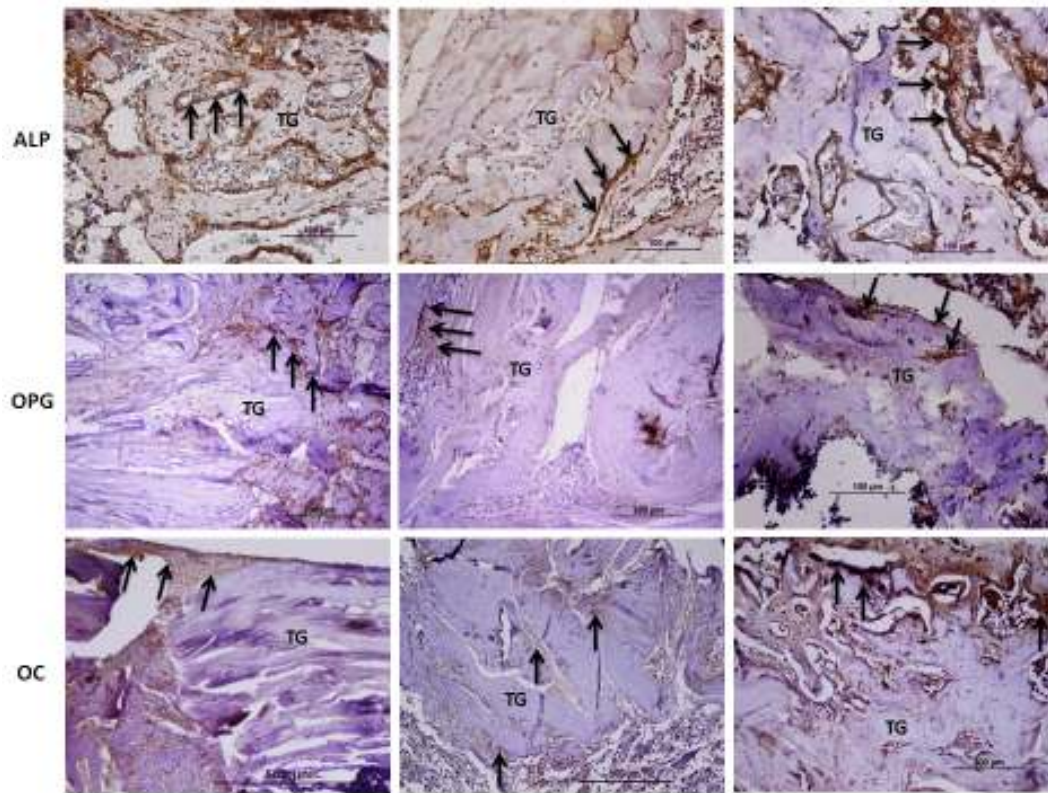
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253 **3.3 Immunohistochemical Analysis**

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255 Immunohistochemical samples for each protein marker used in this study are illustrated in Fig. 3
256 and Fig. 4. Involved in the osteoclastogenesis regulating mechanism, OPG and RANKL proteins
257 showed similar levels during all periods; OPG presented peak intensity (score 3) at the
258 biomaterial-tissue interface at 7 days, and RANKL at 15 days (score 3). Our findings indicated
259 that TRAP, a specific protein marker expressing bone reabsorption, was more intensely
260 prevalent at 15 and 30 days (score 3). Osteocalcin, a protein from the synthesized extracellular
261 matrix secreted during osteoblast differentiation and primarily expressed in the final phase of
262 bone formation, was present during all assessed periods, reaching peak intensity at 30 days
263 (score 3). In addition to OC, ALP is also used as a marker for osteoblasts with a role in bone
264 matrix mineralization, and it was detected during all of the studied periods in expressive
265 quantities (score 3). The VEGF protein, a factor expressed by osteoblasts and intimately
266 connected with angiogenic processes, was present in all periods, with peak intensity at 15 and
267 30 days postoperative (score 3).

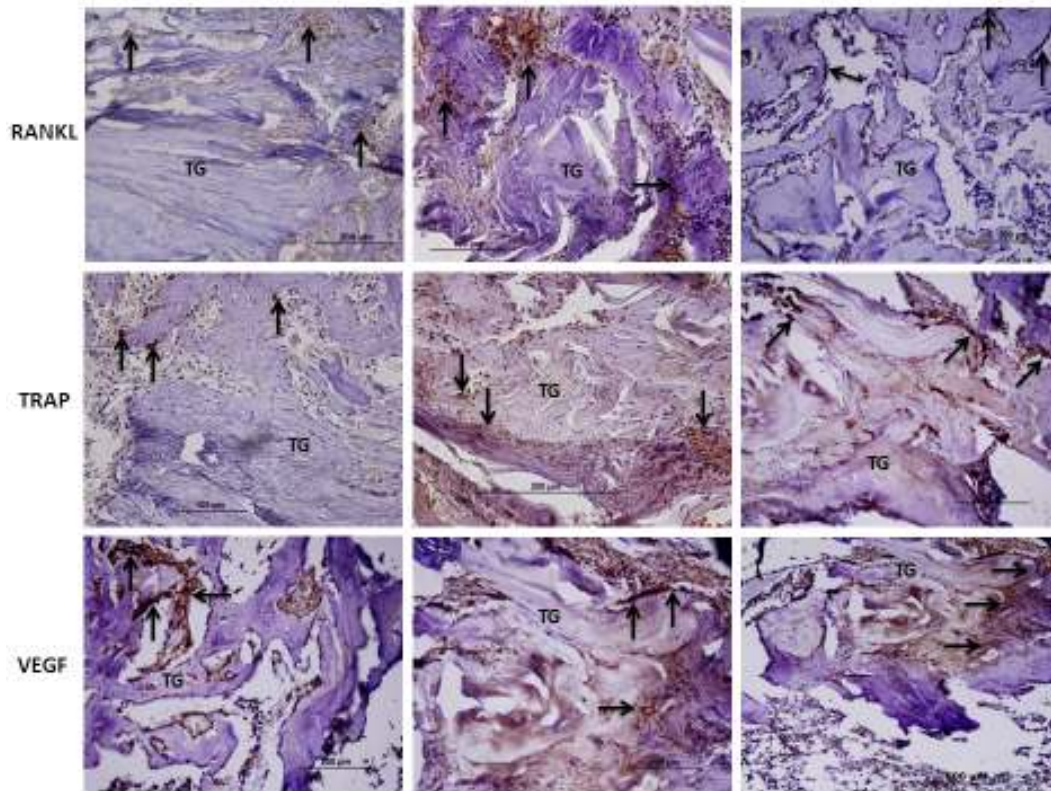
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269

270 **Fig. 3.** Immunolabeled proteins (arrows) used for assessing tendon graft (TG). ALP, alkaline
 271 phosphatase; OPG, osteoprotegerin; OC, osteocalcin. 7 days (left), 15 days (middle) and 30
 272 days (right).

273



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275

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

279

280 **4. DISCUSSION**

281

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

287 Studies investigating how to repair bone defects have found that scaffolds are frequently
 288 needed to induce the growth of new bone tissue [14]. Researchers have been searching for
 289 new, enhanced and increasingly more biocompatible materials to minimize complications in
 290 bone repair. Biomaterials can present granulomatous inflammation on a chronic basis, which is

291 intimately connected with the healing process of bone implantation [16,27-29]. Anionic collagen
292 displays high biocompatible and biodegradable potential, in addition to low antigenicity and low
293 levels of inflammatory reactions, thus enhancing bone neoformation [13,30].

294 After the native tendon is hydrolyzed, fibers are modified by opening the pores, favoring bone
295 cell migration and growth in the matrix, especially due to the generated anionic charge in
296 addition to the presence of growth factors. Hydrolysis is also responsible for removing cells that
297 can cause dystrophic calcification, intense local inflammatory reactions and foreign body
298 reactions from the matrix [14,20,25,31-33]. The anionic tendon has low levels of inflammatory
299 response which can be associated with improvements in new bone formation, allowing the
300 restoration of osseous defects may occur within a shorter period such as, for example, within 15
301 days in this study, according Rocha et. al (2002) that reported osteoblast deposition in its own
302 matrix along the formed scaffold and collagen removed since the remodeling, demonstrating the
303 enhanced performance of anionic collagen when compared to materials that must be
304 reabsorbed in order to allow for bone regeneration.

305 Morphometric analysis aimed to verify bone and tissue neoformation [20,34,35]. The amount of
306 neoformed bone tissue in the control group was significant at day 30. The experimental group
307 presented significant amounts at day 7 in comparison to day 30. This finding was in accordance
308 with that of a previous study [20] that showed that new bone was formed starting on day 7 and
309 increased with the time after bone implantation. This study also showed that formation of new
310 bone over collagenous tendon fibers was evident from day 15 onwards. The results of the
311 present study were also in accordance with those of Pan et al. [36], who observed the presence
312 of endochondral bone neoformation in all experimental groups, and Uchida et al. [34], who
313 found altered properties of bone composition, such as increased bone matrix formation, mineral
314 concentration, cortical thickness and volume of trabecular bone.

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

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

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

336 The occurrence of osteoclastogenesis is demonstrated by the presence of RANKL and OPG
337 [40,49,50], which in the present study were present at similar levels during all three assessed
338 periods; OPG presented peak intensity at the biomaterial-tissue interface at 7 days, and RANKL
339 at 15 days. The present results were in agreement with previous research [16,44,45] regarding
340 bone regeneration, narrowing the gap between neoformation of the vascular bed and
341 osteogenesis.

342

343 **5. CONCLUSION**

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

347

348 **COMPETING INTERESTS**

349 Authors have declared that no competing interests exist.

350

351 **AUTHORS' CONTRIBUTIONS:**

352

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

358

359 **ETHICAL APPROVAL**

360

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

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