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Implants of polyanionic collagen matrix in bone defects of ovariectomized rats

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Abstract In recent years, there has been a great interest in the development of biomaterials that could be used in the repair of bone defects. Collagen matrix (CM) has the advantage that it can be modified chemically to improve its mechanical properties. The aim of the present study was to evaluate the effect of three-dimensional membranes of native or anionic (submitted to alkaline treatment for 48 or 96 h) collagen matrix on the consolidation of osteoporosis bone fractures resulting from the gonadal hormone alterations caused by ovariectomy in rats subjected to hormone replacement therapy. The animals received the implants 4 months after ovariectomy and were sacrificed 8 weeks after implantation of the membranes into 4-mm wide bone defects created in the distal third of the femur with a surgical bur. Macroscopic analysis revealed the absence of pathological alterations in the implanted areas, suggesting that the material was biocompatible. Microscopic analysis showed a lower amount of bone ingrowth in the areas receiving the native membrane compared to the bone defects filled with the anionic membranes. In ovariectomized animals receiving anionic membranes, a delay in bone regeneration was observed mainly in animals not subjected to hormone replacement therapy. We conclude that anionic membranes treated with alkaline solution for 48 and 96 h presented better results in terms of bone ingrowth.

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Introduction

Defects associated with bone loss can be repaired using autogenous grafts since these grafts prevent immunological rejection and provide cells that can immediately start the regeneration process [1]. However, autogenous grafts present disadvantages such as donor site morbidity [2], chronic pain and vascular injuries during surgery [3]. In view of these limitations, biomaterials such as ceramics, metals, silicone, polymethylmethacrylate and polyethylene have been investigated for synthetic bone graft substitutes [4]. However, the cytotoxic effects of these materials contraindicate their use. Additional problems are difficulties in filling the bone defect, the low mechanical resistance of these implants and the occurrence of corrosion when metal implants are used [5].

Polymeric membranes have been explored as an alternative in the area of biomaterials since they act as a barrier, preventing the growth of connective tissue close to the defect and permitting only bone ingrowth. Natural polymers from extracellular matrix include collagen, proteoglycans, glycosaminoglycans and elastin. These polymers play a role in the control of tissue structure and in the regulation of the cellular phenotype, simulating a native

extracellular matrix. Particularly collagen, the main organic constituent of bone tissue, has been used in the composition of biomaterials for bone reconstruction [6, 7] due to its biological properties such as biocompatibility, biodegradability and bioabsorbability; in addition, collagen presents a weak antigenic reaction and acts as a support for bone-inducing proteins [8]. Collagen influences cell differentiation, possesses sites for cell recognition [9] and stimulates cell migration and infiltration [10]. These properties are important to facilitate the process of bone regeneration [11, 12].

Among the materials synthesized from collagen, collagen matrices are particularly interesting. Artificial collagen matrices have the advantage that their properties can be altered by the chemical modification of collagen [13–15], a fact that might improve the characteristics of collagen in terms of the stimulation of osteogenesis, as well as their piezoelectric properties. An important modification in the structure of these matrices is the removal of carboxamide groups from asparagine and glutamine residues by alkaline hydrolysis and the consequent formation of carboxyl groups [16, 17]. These modifications do not alter the triple helix structure of the collagen molecule but change the self-assembled pattern of its microfibrils and its dielectric properties. In addition, the isoelectric point of the collagen is reduced to 4.6–5.0 compared to native collagen whose isoelectric point ranges from 6.7 to 7.1. Thus, these matrices are negatively charged at physiological pH [16–18]. When implanted into experimental animals, polyanionic matrices do not induce an inflammatory response and show good biocompatibility, thus indicating that the use of these substrates as bone implants might be a viable alternative [18, 19].

Despite the applicability of collagen as a bone implant, some factors, including diseases associated with bone mineral deficiency such as osteoporosis, may compromise the promising results reported in the literature. According to Frayssinet [20], the health conditions of bone are fundamental for its interaction with the implant biomaterial. Factors that increase the risk for the development of osteoporosis include a low calcium intake, smoking, alcoholism, lack of physical activity, and hormonal disorders resulting from early menopause or late menarche [21]. The risk of osteoporosis is also increased in the case of ovariectomy [22] since female gonadal hormones such as estrogen stimulate bone growth and mineralization [23]. In view of the advantages offered by artificial collagen matrices, the objective of the present study was to evaluate the process of bone ingrowth when polyanionic collagen matrix are implanted into bone defects created in the femur of rats with osteoporosis induced by ovariectomy.

Materials and methods

Preparation of the artificial collagen extracellular matrices

The raw material used for the preparation of the three-dimensional collagen matrix (CM) was bovine pericardium provided by Braile Biomédica S/A (Sao José do Rio Preto, SP, Brazil). The following samples were prepared and provided by the Institute of Chemistry of Sao Carlos, University of Sao Paulo, under the supervision of Prof. Dr. Gilberto Goissis: untreated three-dimensional membranes consisting of native collagen matrix (NCM), and polyanionic collagen matrix (PCM) submitted to alkaline treatment for 48 and 96 h (PCM48 and PCM96, respectively).

Animals

Forty-eight adult female Wistar rats (Rattus norvegicus) aged 12 weeks, provided by the Multi-Institutional Animal House of the (CEMIB) State University of Campinas, were used. The animals were divided into the following groups: group 1, non-ovariectomized animals (NO); group 2, unilaterally ovariectomized animals (UO); group 3, bilaterally ovariectomized animals not submitted to hormone replacement therapy (BO); group 4, bilaterally ovariectomized animals submitted to hormone replacement therapy (BOH). Each group was divided into three subgroups of four animals each which received the NCM, PCM48 and PCM96 membrane implants, respectively.

Ovariectomy

The animals, except the non-ovariectomized, were weighed and anesthetized by intramuscular injection of ketamine (Francotar, Sespo Ind. e Com., Jacareí, SP, Brazil) and xylazine hydrochloride (2% Virbaxyl, Virbac Brasil Ind. e Com., Sao Paulo, SP, Brazil) at a proportion of 1:1 and a dose of 0.10 ml/100 g body weight. A 2-cm incision was made in the skin with a scalpel lateral to the spine to completely remove the ovary from the pelvic cavity.

Hormone replacement therapy

Estradiol hexahydrobenzoate (Benzogynestryl, Hoechst Marion Roussel) was used for hormone replacement therapy. The drug was diluted in peanut oil (All Chemistry, Sao Paulo, SP, Brazil) and the animals were injected subcutaneously with 20 mg at a mean interval of 48 h from the time of membrane implantation to the day of sacrifice after 8 weeks.

Surgical procedure for the implantation of the collagen matrix

The membranes were implanted 4 months after ovariectomy since, according to Wronski et al. [23], accelerated bone loss occurs within a period of 3–4 months after ovariectomy. After anesthesia as described above, an incision was made in the skin on the medial side of the thigh, exposing the femoral quadriceps muscle. The muscle was sectioned longitudinally in its distal third and separated anterolaterally. With the distal end of the diaphysis of the left femur exposed and the periosteum separated, a bone defect was created with a 5-mm surgical bur coupled to the pen of a mini-motor. Next, the defect was filled with the NCM or PCM. The periosteum, musculature and skin were repositioned and closed with No. 4.0 cotton suture. The animals were sacrificed 8 weeks after implantation and the femurs containing the recipient area were removed and submitted to radiological, histological and morphometric analysis.

Radiography and histological analysis

Radiographs of the left femurs were obtained with a Rigaku RU-200 apparatus with a focal point of 0.8×0.8 mm. Kodak radiographic films measuring 7.6×5.7 mm were used. After radiological analysis, the samples were submitted to routine histological processing and semi-serial 5 μm cross-sections were stained with hematoxylin and eosin.

Morphometric analysis

Using a square grid with 100 points coupled to the eyepiece of a light microscope, the volume density of newly formed bone at the site of the collagen implant was calculated for each recipient area. Newly formed bone was quantified by stereology according to the Delesse principle cited by Mandarim de Lacerda [24] using the following formula: $V_V = P_P/P_T$ (%), where V_V = volume density or relative volume, P_P = number of points over newly formed bone, and P_T = total number of points of the system.

Statistical analysis

Linear models were fitted to each of the response variables, with the response being the variable analyzed and the factors the group to which the animals belonged (NO, UO, BO and BOH) and the type of implant (NCM, PCM48 and PCM96). The model was also fitted considering the group and type of implant interaction. Significance tests were applied to determine the effect of each factor on the response variable. In cases in which a significant difference of the factors was detected, statistical tests were applied to determine which differences were distinct from the remaining ones. To guarantee the level of significance in cases of multiple comparisons of the means, the levels of significance ($p < 0.05$) were adjusted using the Tukey–Kramer test [25]. The data were analyzed with the SAS system, version 9.1.3.

Results

Radiology analysis

Apparently, closure of the bone defect tended to be better in the NO, UO and BOH groups, especially in animals receiving the polyanionic collagen membranes. In BO animals, no marked closure of the bone defect was observed, being this closing less pronounced in the animals that received native membrane (Fig. 1).

Histological analysis

All animals receiving native collagen samples (NCM), regardless of whether they were ovariectomized or not, presented few characteristics indicative of defect closure. In addition, the bone ingrowth was located marginal to the membrane, projecting from the margins of the defect and containing cavities filled with bone marrow. In rats receiving the PCM48, the bone defect created was almost completely filled with bone ingrowth, especially in non-ovariectomized and unilaterally ovariectomized rats. The newly formed bone presented characteristics of immature (presence of round lacunae harboring osteocytes arranged in various directions) as well as mature (dense texture) bone. In rats in which the bone defect was filled with the PCM96, no apparent defect closure was observed in bilaterally ovariectomized animals and those not submitted to hormone replacement therapy. The other groups demonstrated good closure of the bone defect through bone formation from the margins. A tendency toward invasion of newly formed bone partially covering the bone defect was noted in animals receiving the PCM48 and PCM96 membranes. No fibrous tissue capsule or inflammatory process in the implant recipient area was observed in any of the groups. A thin layer of cortical bone around the implant showing characteristics of trabecular bone was noted in BO animals, in view of the presence of disorganized bone tissue in the region (Fig. 2).

Morphometric analysis

When the results obtained for the implants were first evaluated, a significant difference in the volume of newly

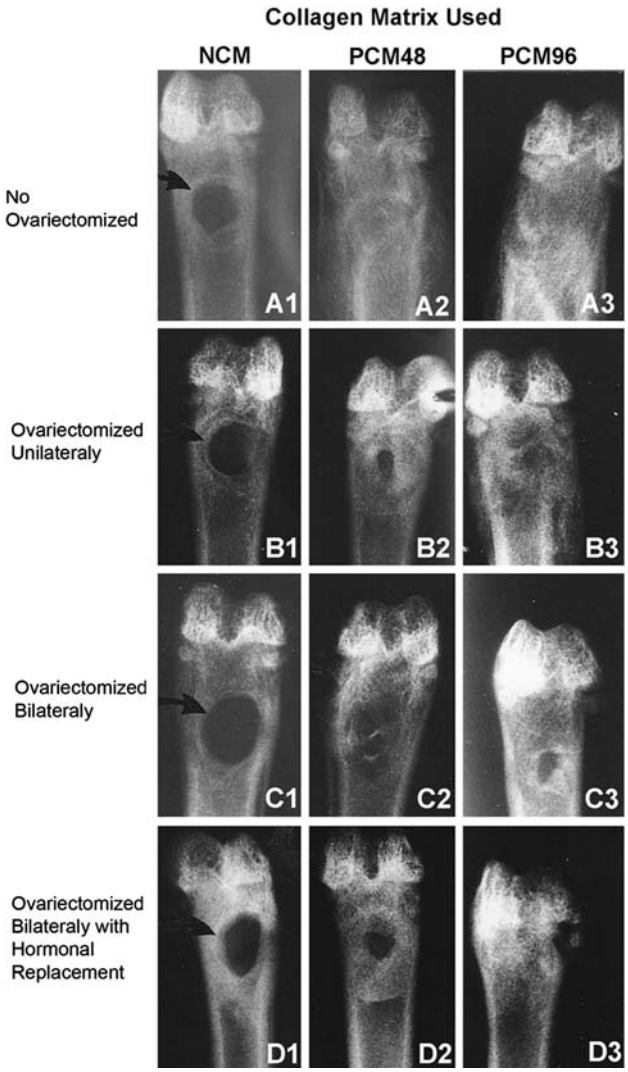

Fig. 1 Radiography analysis of animals receiving the different implants under the experimental conditions studied. (**A**) non-ovariectomized animals; (**B**) unilaterally ovariectomized animals; (**C**) bilaterally ovariectomized animals; (**D**) bilaterally ovariectomized animals submitted to hormone replacement therapy. (A1–D1), implantation of native NCM; (A2–D2) PCM48 membrane; (A3–D3) PCM96 membrane. Note the open bone defect in animals receiving the native membrane (A1–D1), mainly in bilaterally ovariectomized animals (C1). The bone defect was reduced in animals receiving the PCM48 membrane (A2–D2), except for bilaterally ovariectomized animals (C2). Apparent macroscopic closure of the bone defect was observed in animals receiving the PCM96 membrane (A3–D3), except for ovariectomized animals (C3). NCM (collagen matrix native); PCM48 (polyanionic collagen matrix submitted to alkaline treatment for 48 h); PCM96 (polyanionic collagen matrix submitted to alkaline treatment for 96 h)

formed bone was observed between animals receiving the native collagen matrix (NCM) and those receiving the polyanionic collagen membranes (PCM48 and PCM96), with the results obtained being significantly lower for the native matrix compared to the other membranes. On the other hand, no significant difference was observed between the PCM48 and PCM96 membranes. The same findings were obtained for all groups studied (NO, UO, BOH and BO).

Comparison of the groups studied showed a significantly lower volume of newly formed bone in the BO group compared to the other groups (NO, UO and BOH). The same was observed for the three types of membranes studied. The results are shown in Fig. 3.

Discussion

Studies involving laboratory animals represent an important tool for advances in the biomedical area. In this respect, laboratory animals provide a good model for the study of questions related to some human diseases, including those resulting from hormonal changes such as osteoporosis. In the literature, rats have been frequently used as an experimental model for the study of osteoporosis related to hormone alterations due to gonadal deficiency [26–34]. For this reason and because of their appropriate size for the surgical technique used for implantation of the collagen membranes in the bone defects created in the femur, rats were chosen for the present study.

In the present study, the animals were submitted to the surgical procedure for the implantation of the collagen membranes 4 months after ovariectomy. According to Wronski et al. [23], accelerated bone loss occurs within a period of 3–4 weeks after ovariectomy. The time of implantation of the membrane was standardized at 8 weeks since Rocha et al. [18] observed good bone neoformation in tibial defects filled with collagen membranes within a period of 4 weeks; however, these authors used normal animals. Since in the present study we used animals with experimentally induced osteoporosis, a period of 8 weeks was adopted to evaluate the velocity of the osteoconductive capacity of collagen in pathological bone tissue.

PCM have been widely used experimentally to fill bone defects. Among these materials, collagen membranes are particularly interesting because of their good mechanical properties. Modifications have been introduced in artificial collagen matrices to increase their mechanical resistance, as well as to reduce their degradation in vivo and to permit better integration with the recipient tissue. These alterations include the chemical modification of collagen which results in positively or negatively charged collagen matrices [12–14].

The main characteristic of modifications that change the charge of collagen is the improvement of its dielectric properties compared to native collagen. Krukowski et al. [34] showed that positively charged surfaces induced the formation of connective tissue, whereas negatively charged surfaces stimulated the formation of intramedullary bone

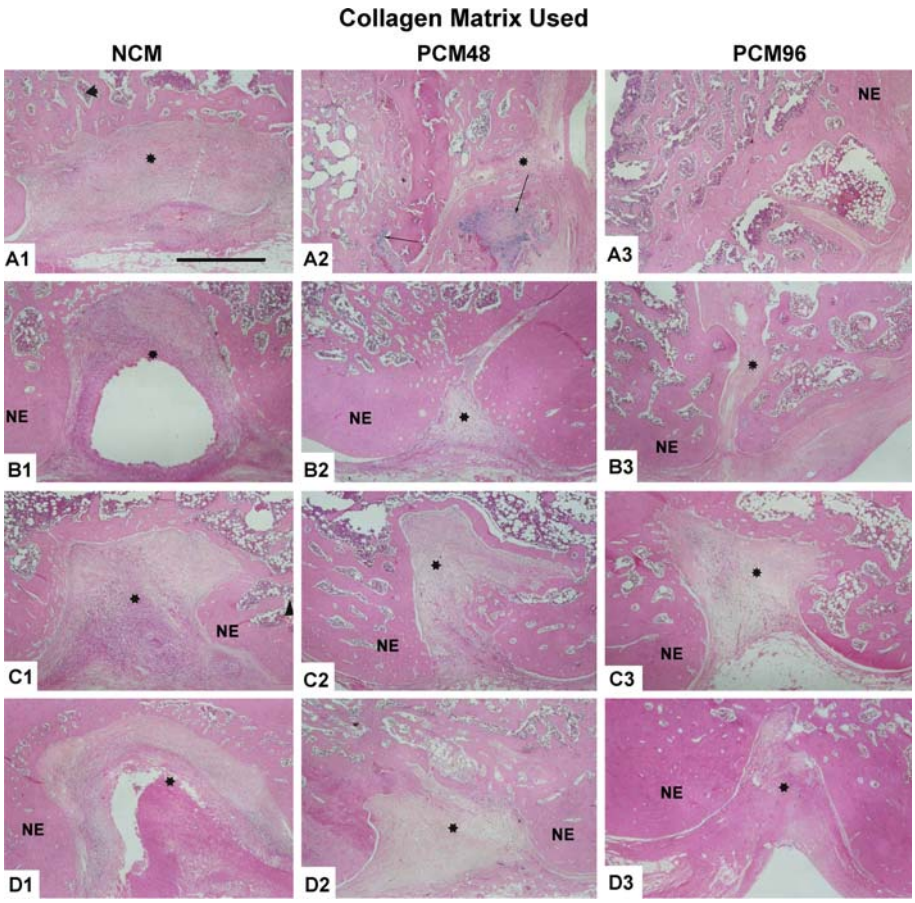

Fig. 2 Histological analysis of animals receiving the different implants under the experimental conditions studied. (**A**) non-ovariectomized animals; (**B**) unilaterally ovariectomized animals; (**C**) bilaterally ovariectomized animals; (**D**) bilaterally ovariectomized animals submitted to hormone replacement therapy. (A1–D1) implantation of NCM; (A2–D2) PCM48 membrane; (A3–D3) PCM96 membrane. Lack of closure of the bone defect and the presence of newly formed bone (NE) around the implant (*) were noted in animals receiving the NCM (A1–D1), with bone thickness being apparently lower in bilaterally ovariectomized animals (C1). Note the formation of new bone (NE) from the margins of the bone defect in the direction of the implant (*) in animals receiving the PCM48 (A2–D2), which was more significant in non-ovariectomized (A2) and unilaterally ovariectomized (B2) animals. Results similar to those obtained for the (A2–D2) group were observed for animals receiving the PCM96 (A3–D3). Scale bar = 40 μm for all pictures. NCM (collagen matrix native); PCM48 (polyanionic collagen matrix submitted to alkaline treatment for 48 h); PCM96 (polyanionic collagen matrix submitted to alkaline treatment for 96 h)

tissue. This fact indicates that polyanionic collagen might be useful in implants aimed at bone regeneration. Alkaline treatment for 72, 48, 36 and 24 h results in an increase of 113 ± 15, 87 ± 17, 66 ± 12 and 46 ± 12 carboxyl radicals in amino acid residues, respectively [17], decreasing the isoelectric point of collagen to 4.6–5.0 compared to native collagen (6.7–7.1). Thus, these matrices are negatively charged at physiological pH [17], a fact altering the association pattern of the microfibrils, with the microfibrils forming a matrix different from that of native collagen [15–17]. An excessive carboxyl group content alters the self-aggregation pattern of the collagen molecules in such a way as to inhibit the formation of microfibrils, thus producing an amorphous matrix. This is observed for matrices submitted to alkaline treatment for 72 h or longer [16, 17]. In addition, it has been demonstrated that the preparation of polyanionic collagen matrices results in a "contamination" with 3.2 ± 1.0% elastin, a value lower than that observed for native collagen membranes whose elastin content is about 4.8 ± 0.8% [17]. From a biological point of view, the addition of a negative charge is desired to a certain extent since cells generally show a preference for a negatively charged substrate during cell adhesion [18, 35, 36]. However, in the case of some cell types an increase in negative charge seems to inhibit or even block adhesion [37].

Anionic collagen matrices have been chemically characterized in previous studies [16]. Matrices treated for 24 h presented an increase in the number of carboxyl groups per collagen molecule of 46 ± 12, whereas matrices treated for 48 h presented an increase of 87 ± 17 [17]. However, the

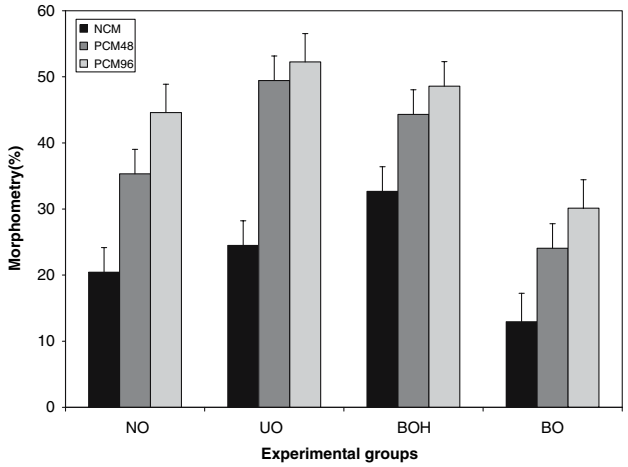

Fig. 3 Average quantity of newly formed bone determined by morphometric analysis. A significant difference in the volume of newly formed bone was observed between animals receiving the native collagen matrix (NCM) and those receiving the polyanionic collagen membranes (PCM48 and PCM96), with the results obtained being significantly lower for the native matrix compared to the other membranes. On the other hand, no significant difference was observed between the PCM48 and PCM96 membranes. The same findings were obtained for all groups studied. Comparison of the groups studied showed a significantly lower volume of newly formed bone in the ovariectomized bilateraly group compared to the other groups (no ovariectomized, ovariectomized unilateraly and ovariectomized bilaterally with hormonal replacement). NCM (collagen matrix native); PCM48 (polyanionic collagen matrix submitted to alkaline treatment for 48 h); PCM96 (polyanionic collagen matrix submitted to alkaline treatment for 96 h); NO (non-ovariectomized animals); UO (unilaterally ovariectomized animals); BO (bilaterally ovariectomized animals not submitted to hormone replacement therapy); BOH (bilaterally ovariectomized animals submitted to hormone replacement therapy)

triple helix structure of collagen was preserved in all membranes, irrespective of the increase in the number of carboxyl groups [38]. In addition, absorption of water by the matrices and their hydrophilic potential were shown to increase linearly with the increasing carboxyl groups [38]. According to Bet et al. [37], these characteristics have a much larger impact on the behavior of cell adhesion to these matrices than their surface morphology. Morphological studies of these matrices have demonstrated that the dense aspect of the native matrix is transformed into a sponge-like structure with pores of different shapes and diameters after chemical treatment, with this structure being important for osteogenesis [18].

Studies have shown that polyanionic collagen developed as a support for bone tissue reconstruction was able to induce the deposition of calcium phosphate salts in vitro. It was thus suggested that dielectric changes in the collagen matrix may have induced a process of collagen mineralization in vitro similar to the stage of bone mineralization [39]. When bovine osteoblast cells were cultured on these polyanionic collagen matrices, the matrices promoted the production of alkaline phosphatase, followed by mineralization of the substrates and in vitro osteoblast differentiation [40]. In vivo, when implanted into experimental animals these polyanionic matrices induced no inflammatory response and presented very good biocompatibility, a fact also observed in the present experiment. These matrices have also been tested in bone tissue engineering studies regarding their ability to promote bone regeneration [18, 19], yielding satisfactory results in terms of some aspects [19]. In vitro studies have shown that polyanionic collagen membranes present no sign of toxicity and are more receptive to cell adhesion than native collagen [38].

In the present experiment, morphometric analysis showed a better capacity of bone neoformation at sites filled with the polyanionic collagen treated for 48 and 96 h in alkaline medium (PCM48 and PCM96) compared to areas implanted with native collagen, suggesting that the higher the porosity of the material, the improve its capacity to permit cell migration. Similar results have been reported by Rocha et al. [18] and Rosa et al. [19] who implanted polyanionic collagen membrane treated for 24, 36 and 48 h in alkaline medium into the rat tibia. Bone formation was observed for all matrices, especially that treated for 48 h. These data support the thesis that polyanionic collagen are a promising biomaterial for the regeneration of bone defects because of their biocompatibility and osteoconductivity.

Saadeh [40] implanted collagen type I into rat mandibular defects and observed no closure of the defect after 6 weeks, although collagen is considered to be a promising material for the reconstruction of bone defects.

Although the results reported above are highly promising, they have been obtained experimentally in young or adult healthy animals. In humans, most prostheses or substrates used for the stimulation of bone regeneration are implanted in elderly patients or patients with diverse bone diseases. According to Albrektsson et al. [41], a healthy bone condition is essential for the interaction with biomaterial implants, whereas poor bone health can alter the expected results. Thus, the data obtained in studies using healthy animals cannot be directly extrapolated to humans. In this respect, Camilli et al. [42] investigated the effect of alcohol on the osteointegration of hydroxyapatite implanted into the femur of rats and concluded that there is a delay in bone ingrowth in the implanted areas due to the abnormal conditions of bone tissue. César-Neto et al. [43] studied the healing around titanium implants used to fill bone defects created in the tibia of rats submitted to nicotine administration. The authors observed a negative effect on the degree of bone-to-implant contact due to the presence of fibrous tissue. Similar results have been reported by Nociti et al. [44] and by Zechner et al. [45].

Studying the bone–implant interaction in cases of osteoporosis induced by hormonal alterations, some investigators performed ovariectomy in rats and then implanted titanium into the bone cavities created in these animals. The results of these studies showed a poor interaction between the implanted material and bone tissue, as well as a low amount of newly formed bone, and were attributed to the osteoporosis-like conditions as a result of gonadal deficiency induced by ovariectomy [28, 46–53]. Similar results have also been reported by Jung et al. [26], Pan et al. [52], DeBenedittis et al. [48], Fini et al. [50] and Hayashi et al. [51] who, however, used hydroxyapatite as implant material.

The present results showed a significantly smaller volume of bone ingrowth in the implanted areas of BO animals, irrespective of the type of collagen membrane used. Statistical analysis revealed a mean difference of 11.0694, 19.6875 and 19.4792 between the BO and the NO, UO and BOH groups, respectively, with this difference being significant ($p < 0.05$). This analysis confirms the effects of estrogen deficiency on bone metabolism in response to the implant used. No significant difference was observed between the NO, UO and BOH groups. Thus, bone neoformation close to the collagen membrane can be expected even in ovariectomized animals, but it is slower and the bone volume is smaller. In addition, we noted the presence of trabecular bone in the implanted areas of these animals. Similar results have been reported by Cho et al. [31] who studied the bone-dental implant interface in ovariectomized rats. The authors concluded that osteoporosis-like conditions induced by hormone deficiency may compromise the mechanical stability of the implant in view of the results demonstrating a reduction of the cortical layer and an increase of trabecular bone in the recipient area.

We conclude that bone neoformation can occur in bone defects even under pathological conditions of the bone resulting from gonadal deficiency, especially when the defect is filled with polyanionic membranes like those used in the present study. Thus, further studies are necessary since this aspect becomes relevant in some situations, as the time of the experiment and new approaches to evaluate the parametes biomechanical as regards force of traction and compression of the bone.

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