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The action of demineralized bovine bone matrix on bone neoformation in rats submitted to experimental alcoholism

[*Ação da matriz óssea bovina desmineralizada na neoformação óssea em ratos submetidos ao alcoolismo experimental*]

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ABSTRACT

The objective of this study was to evaluate whether demineralized bovine bone (Gen-ox®) alters bone neoformation in rats submitted to alcoholism. Forty male rats were separated into two groups of 20 rats and distributed as follows: Group E1, which received 25% ethanol and a surgical cavity filled only by a blood clot, and Group E2, which received 25% ethanol and a surgical cavity filled with Gen-ox®. The animals were euthanized at 10, 20, 40 and 60 days after surgery and necropsy was performed. The histomorphological and histometric analyses of the area of connective tissue and bone neoformation showed that the reorganization of the bone marrow and full repair of the surgical cavity in Group E1 occurred more quickly than in Group E2. It was also noted that in the final period the animals in Group E2 showed areas of connective tissue and thick bone trabeculae around the particles of the implant. It can be concluded that the use of Gen-ox® delayed the process of bone repair in alcoholic rats, although it can be used as filling material because it shows osteoconductive activity, as evidenced by bone tissue formation around the graft particles.

Keywords: rat, bone regeneration, alcoholism

RESUMO

O objetivo deste trabalho foi avaliar se a matriz óssea bovina desmineralizada (Gen-ox®) altera a neoformação óssea em ratos submetidos ao alcoolismo. Foram utilizados 40 ratos machos, separados em dois grupos de 20 animais cada, assim distribuídos: Grupo E1, que recebeu etanol a 25% e cavidade cirúrgica preenchida por coágulo sanguíneo, e Grupo E2, que recebeu etanol a 25% e cavidade cirúrgica preenchida por Gen-ox®. Os animais foram eutanasiados aos 10, 20, 40 e 60 dias após a cirurgia. Os estudos histomorfológico e histométrico da quantidade de tecido conjuntivo presente e a quantidade de tecido ósseo neoformado demostraram que a reorganização da medula óssea e a reparação total da cavidade cirúrgica no Grupo E1 ocorreram em menor espaço de tempo do que no Grupo E2. Observou-se também que, no período final do experimento, os animais do Grupo E2 apresentaram áreas de tecido conjuntivo e trabéculas ósseas espessas ao redor das partículas do material implantado. Concluiu-se que a utilização do Gen-ox® retardou o processo de reparação óssea em ratos alcoolizados, muito embora o Gen-ox® possa ser utilizado como material de preenchimento, pois demonstra atividade osteocondutiva, com a formação de tecido ósseo ao redor das partículas do enxerto.

Palavras-chave: rato, regeneração óssea, alcoolismo

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INTRODUCTION

Alcohol causes an imbalance between bone formation and resorption, acting negatively on bone repair (Maddalozzo *et al.*, 2009; Broulik *et al.*, 2010). Studies in rats have shown that moderate a consumption of ethanol (3% of caloric intake) causes a decrease in the process of bone turnover, demonstrating that even moderate levels of alcohol exert an adverse effect on bone marrow (Turner *et al.*, 2001).

Ethanol inhibits the proliferation of osteoblastic cells, leading to low bone density and increased prevalence of fractures in the alcoholic population (Garcia-Sanchez *et al.*, 1995). Acute ingestion of alcohol causes an inhibitory effect on the parathyroid and also on osteoblastic cells, contributing to the development of bone disease (Garcia-Sanchez *et al.*, 1995; Gong and Wezeman, 2004; Iwaniec *et al.*, 2008).

In animals that were exposed to different concentrations of ethanol (6%, 15% and 25%), the results showed that bone formation decreased in accordance with the increase of alcohol concentration, and it can be concluded that the 3 concentrated alcohol diets influenced bone formation in all its phases, delaying the process of bone repair (Curi *et al.*, 2008; Buchaim *et al.*, 2009).

Aiming at the total or partial reconstruction of lost bone mass and restoration or enhancement of biological tissues, there is growing interest in the development of biomaterials that have biological and physicochemical properties (Buchaim *et al.*, 2007).

Fibrous tissue often appears where bone should be in the repair of bone defects in patients who drink alcohol. Studies are evolving in order to examine the placement of biomaterials within cavities, such as after tooth extraction, removal of cysts or following an accident or surgical procedure (Pinheiro *et al.*, 2003).

The technique of autogenous bone grafting is widely used, but in spite of its great biological advantages, drawbacks exist, such as the need for hospitalization, interventions in other areas of the body, donor area morbidity and susceptibility to infection (Goodman, 2010).

The xenogenic graft has shown promising results. It is based on the abundance of the matrix, low cost of bovine bone and the proper techniques for mechanical and chemical preparation (Bigham *et al.*, 2008). The lyophilized inorganic bovine bone (deproteinized) follows the same process of preparation of the organic matrix; however, it does not undergo the process of decalcification, in which all the mineral components of bone are preserved and all the organic parts are eliminated (BMPs, collagen, proteins), i.e. the inorganic part is preserved (Sanada *et al.*, 2003; Gerbi *et al.*, 2005; Marin *et al.*, 2007).

The role of the bone induction carrier factors can potentially be played by a bovine bone marrow (macro or micrographic, deproteinized), as has been demonstrated in clinical studies (Yukna *et al.*, 1998). Besides providing a support structure and osteoconductive properties, it can also provide a high calcium and phosphorus content, essential for new bone tissue formation (Damien *et al.*, 1995; Sciadini *et al.*, 1997).

The objective of this study was to evaluate whether demineralized bovine bone (Gen-ox®) alters bone formation in rats submitted to alcoholism.

MATERIAL AND METHODS

This study was approved by the Ethics Committee in Animal Experimentation from Universidade Estadual Paulista in Araçatuba, SP, protocol 62/04. Forty adult (sixty days old) male rats were used (*Rattus norvegicus*), weighing on average of 285 grams.

The animals were separated into two groups of 20 rats each: Group E1, which received 25% ethanol and the surgical cavity filled only by a blood clot; Group E2, which received 25% ethanol and the surgical cavity filled with demineralized bovine cortical bone.

Both groups received a liquid diet *ad libitum* and were initially gradually adapted to ethanol to achieve the maximum level, 8% in the first week, 16% in the second week and finally 25% in the third week. The animals then continued to receive diet beverages in the final concentration (25%) for a period of 90 days. After this period, animals underwent experimental surgery and

remained intoxicated until the period corresponding to the eutanasia of each group.

For surgery, the rats underwent general anesthesia via intramuscular injection of tiletamine hydrochloride (125.0 mg) combined with zolazepam hydrochloride (125.0 mg) at a dose of 50.0mg/kg IM (Zoletil 50, Virbac).

After shaving the ventral region of the left pelvic limb, a linear incision of 20 mm was made extending in the craniocaudal direction. A dental bur (n^{o} 6) mounted on a low-speed handpiece was used to prepare a cavity of approximately 3mm in diameter and depth in the tibia, reaching the bone marrow. The cavity irrigation was performed with a solution of 0.9% sodium chloride.

In all animals in Group E1 the cavity was filled with a blood clot, and in all animals in Group E2 the cavity was filled with inorganic demineralized bovine cortical bone (Gen-ox®, Genius, Baumer S.A.) associated with saline. The tissues were repositioned and sutures made. Using excessive injections of the anesthetic previously mentioned, five rats from each group were euthanized on days 10, 20, 40 and 60 days from the day of surgery.

The tibias were collected, dissected and fixed in 10% buffered formalin for 24 hours, washed in water for 12 hours and then decalcified in a solution of sodium citrate and formic acid in equal parts for 45 days. Thereafter, the sections went through the usual laboratory routine for paraffin. Blocks with a longitudinal thickness of six micrometres were obtainded, resulting in semi-serial sections that were stained by Masson's trichrome for histomorphological and histometric study. The slices were examined under a microscope (Olympus BX50) and photographs taken with a digital camera attached (Olympus DP 71).

The quantitative analysis was performed on the computer using the Image Pro-Plus 6.0 software. For morphometry, the cortical region where the tibia was broken and the medullary region adjacent to the contralateral intact cortex was analyzed. In this region, the amount of connective tissue and amount of new bone was measured. The data collected were subjected to the statistical two-way analisys of variance test followed by the Tukey test. For all analyses $P<0.05$ was considered statistically significant.

RESULTS

The histological analysis will be described according to the experimental periods:

Ten days after surgery, in Group E1 rats, connective tissue and newly formed blood vessels were abundant in the shallower regions. In group E2 animals, the implanted material occupied much of the surgical cavity with neoformed connective tissue in its vicinity. In the surgical area superficial bone formation was present.

Twenty days after surgery, in animals from Group E1, bone healing occurred in the part of the surgical cavity with areas of tissue still present (Fig. 1A). In animals from Group E2, early bone formation occurred around the particles of the implant and cortical reorganization of disrupted bone (Fig. 1B).

Forty days after surgery, in rats from Group E1 the surgical cavity was repaired with a reorganization of the bone marrow, and there was incomplete new formation of the cortical bone. In anomals from Group E2, in most specimens the implanted material had connective tissue rich in collagen fibers surrounding them, with partial bone repair to the site.

Sixty days after surgery, in rats from Group E1 reorganization occurred in the bone marrow, as well as total cortical repair of the area ruptured at surgery (Fig. 2A). In animals from Group E2, there was no total bone repair of the surgical cavity, leaving areas of connective tissue. Thick bone trabeculae are observed around the particles of the implanted material (Fig. 2B).

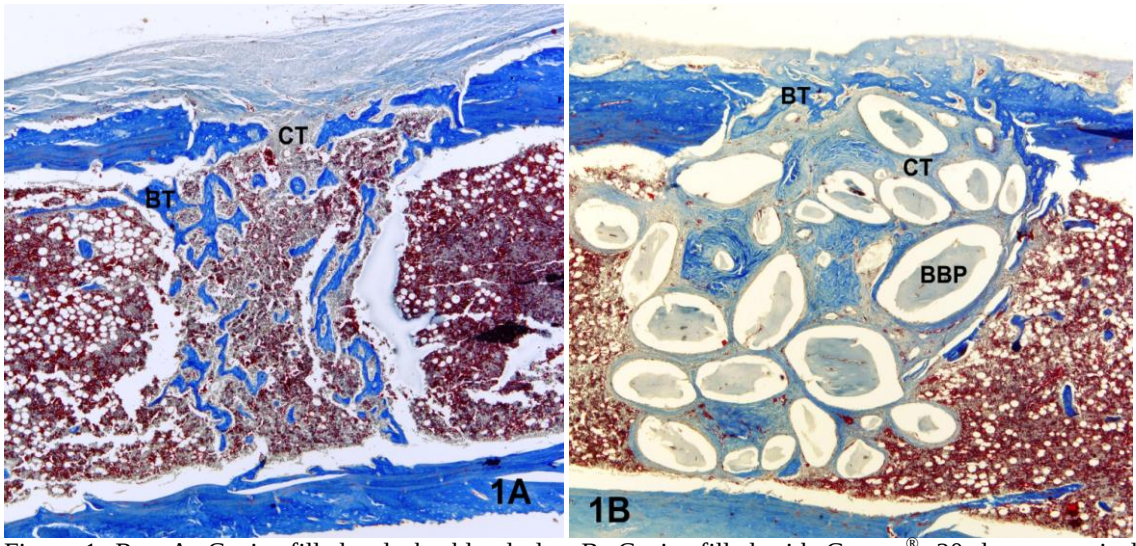

Figure 1. Rat. A: Cavity filled only by blood clot. B: Cavity filled with Gen-ox®, 20 days - surgical cavity, 25 X. Bone Tissue (BT), Connective Tissue (CT) and Bovine Bone Particles (BBP).

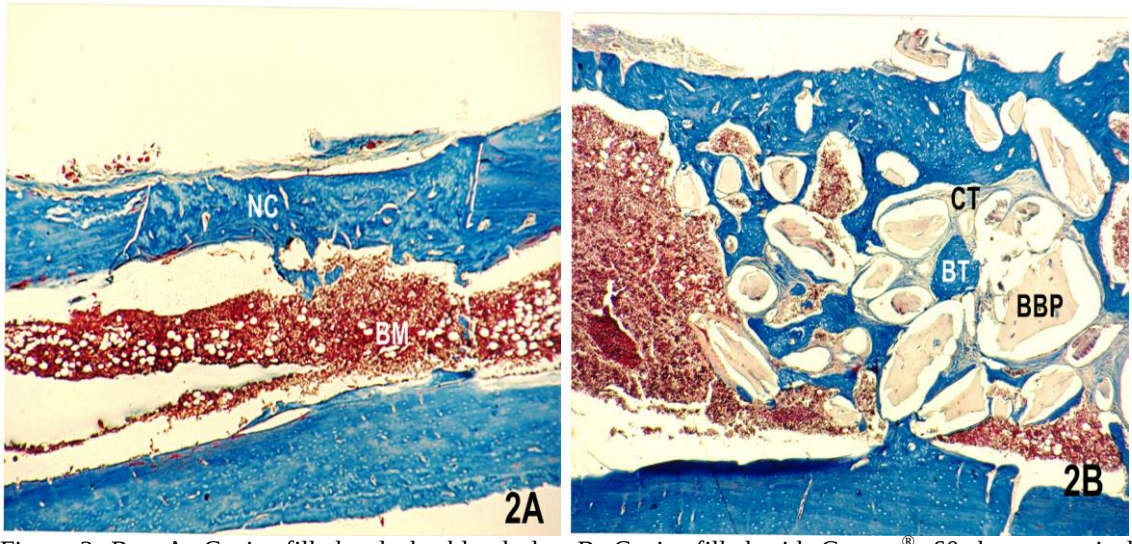

Figure 2. Rat. A: Cavity filled only by blood clot. B: Cavity filled with Gen-ox®, 60 days - surgical cavity, 25 X. Newly Cortical (NC), Bone Marrow (BM), Bone Tissue (BT), Connective Tissue (CT) and Bovine Bone Particles (BBP).

The histometric analysis will be described in a sequence of measurement areas.

For area of bone tissue, in group E1, there were no significant differences between periods. In group E2, there were no significant differences between the periods of 10 days and 20 days and between the periods of 20 days and 40 days. All other periods compared were statistically different. In comparing the two groups in each period, there were significant differences only at 10 days (Table 1).

Regarding the connective tissue area, in group E1 there were significant differences between all periods. In Group E2 there were significant differences only between the 10 day and 60 day periods. When comparing the two groups in each period, there were no significant differences at 10 days (Table 2).

Table 1. Bone tissue area (mm^2) comparing groups E1 (filled only by blood clot) and E2 (filled with Gen-ox®) in different periods

Periods	Groups	
	E1	E2
10	0.39±0.06Aa	0.21±0.04Ba
20	0.38±0.05Aa	0.29±0.06Aab
40	0.41±0.04Aa	0.39±0.06Ab
60	0.47±0.05Aa	0.53±0.06Ac

Different letters indicate significant differences by two-way analisys of variance followed by the Tukey test (P<0,05). Capital letters indicate comparison between the experimental groups in the same period (line). Lower case letters indicate comparison in the same group in different periods (column).

Table 2. Connective tissue area (mm^2) comparing groups E1 (filled only by blood clot) and E2 (filled with Gen-ox®) in different periods

Periods	Groups	
	E1	E2
10	0.58±0.07Aa	0.54±0.07Aa
20	0.22±0.04Ab	0.48±0.07Bab
40	0.12±0.02Ac	0.44±0.03Bab
60	0±0Ad	0.38±0.04Bb

Different letters indicate significant differences by two-way analisys of variance followed by the Tukey test (P<0,05). Capital letters indicate comparison between the experimental groups in the same period (line). Lower case letters indicate comparison in the same group in different periods (column).

DISCUSSION

The diet (alcoholic) drinks used in this study (25%) are in agreement with previous studies, where the experimental groups were subjected to a gradual adaptation, rendering these animals chronic alcoholics (Curi *et al.*, 2008; Buchaim *et al.*, 2009; Broulik *et al.*, 2010). Alcohol causes growth retardation, a decrease in animal weight and weight gain inversely proportional to the dosage of alcohol (Gong and Wezeman, 2004).

In previous studies, where a group treated with water (control Group) was also evaluated in the same periods of this paper and the surgical cavity was filled with clot, it was observed that the cavity was completely repaired by new bone in a period of 40 days (Buchaim *et al.*, 2009).

When using a graft material, the response that is expected is that the defect is repaired with higher speed and quality. But the effects of alcohol impair the potential benefits of biomaterials, which are widely used for this purpose (Iwaniec *et al.*, 2008). The desired effect in this experiment is not obtained since the total repair of the defect occurred in a shorter period of time only in the cavity filled by blood clot.

In groups E1 and E2, within 60 days the specimens achieved total cortical bone repair in the tibia which was broken in the making of the surgical cavity. Histological observation showed that the newly formed cortical thickness was less than the original (Broulik *et al.*, 2010).

The quantification of the bone tissue area of group E2 showed no significant differences when comparing the periods of 10 days and 20 days and between 20 days and 40 days. All other periods compared were statistically different. It is important to report that all biomaterial placed in a cavity generates an initial inflammatory response in the receptor tissue, unlike the hollow with the absence of implanted material populated only by a blood clot (Buchaim *et al.*, 2007; Marin *et al.*, 2007). This can be seen in the empty cavities 40 days after surgery where the cavity has repaired.When comparing groups E1 and E2 in area measurement of bone tissue, there were significant differences only at 10 days.

In the morphometry of the connective tissue area of group E1, there were significant differences between all periods. This is mainly caused by the speed of the repair process of the surgical cavity without implant material, as discussed earlier, mainly in rats (Pinheiro *et al.*, 2003).

In Group E2, there were significant differences only between the periods of 10 days and 60 days. The repair of the cavity is slower and at the final period of 60 days there is still a relative amount of connective tissue without bone differentiation (Gerbi *et al.*, 2005).

In comparing the two groups in each period, there were no significant differences only at 10 days. Despite the surgical cavity, at 60 days in Group E2 there is further indication of tissue areas, and new bone formation around the particles of Gen-ox® demonstrates satisfactory repair of the defect with xenogenic graft material (Bigham *et al.*, 2008).

The satisfactory repair of the Gen-ox® surgical cavity was found in all animals of group E2 within 60 days. Besides the presence of their particles in the cavity, it showed well-organized trabecular bone surrounding the material and also the presence of blood vessels (Sanada *et al.*, 2003; Marin *et al.*, 2007). The resorption of deproteinized bovine bone is slow when compared with frozen human demineralized bone powder (Yukna *et al.*, 1998; Sanada *et al.*, 2003).

The osteoconductive material is one that directs cell proliferation and may be encompassed by new bone, becoming part of the new tissue. Gen-ox® showed these characteristics, being a graft material for possible use in medical and dental care, not with the expectation of accelerating the formation of new bone tissue, but to create conditions for new formation, especially in patients who continuously ingest alcohol, which reduces the activity of osteoblasts (Damien *et al.*, 1995; Gerbi *et al.*, 2005).

CONCLUSIONS

Based on these results it can be concluded that the use of Gen-ox® delayed the process of bone formation in rats experimentally exposed to alcohol on a regular basis. Although not the objective of the work, it was noted that the Gen-ox® can be used as a filling material because it shows osteoconductive activity, with the formation of bone tissue around the graft particles.

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