Effect of BioOsteo® in combination with epidermal growth factor and ascorbic acid in a rat tibia defect

Efecto de BioOsteo® en combinación con el factor de crecimiento epidérmico y el ácido ascórbico en un defecto de la tibia de la rata

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Abstract

Background: *Treatment of bone defects derived from trauma or from removal of tumors or osteosynthesis materials can cause socioeconomic problems as a result of treatment length.* **Objective:** *The purpose of this study was to determine the effect of the implant material BioOsteo® in combinations with epidermal growth factor (EGF) and ascorbic acid (AA) on the consolidation of a non-critical size bone defect.* ***Materials and methods:*** *A unicortical non-critical bone defect was practiced in the right tibia of Wistar rats and 3 weeks later, a biomechanical property analysis was performed through a three-point bending test.* **Results:** *We found that a 1 time single-dose local application of AA + EGF + BioOsteo®, directly over the non-critical bone defect microenvironment improves its repair.*

KEY WORDS: *Bone repair. Polyurethane resin. Implants. Three-point bending test.*

Resumen

Antecedentes: *El tratamiento de los defectos óseos originados por traumatismos o por retiro de materiales de osteosíntesis o de tumores puede ocasionar problemas socioeconómicos derivados del tiempo de tratamiento.* **Objetivo:** *Determinar el efecto del material de implante BioOsteo® en combinación con factor de crecimiento epidérmico (EGF) y ácido ascórbico (AA) sobre la consolidación de un defecto óseo de tamaño no crítico.* **Material y métodos:** *Se practicó un defecto óseo unicortical no critico en la tibia derecha de ratas Wistar y 3 semanas después se realizó el análisis de las propiedades biomecánicas por medio del ensayo de flexión en tres puntos.* **Resultados:** *Encontramos que la aplicación local de una dosis única de AA + EGF + BioOsteo® directamente sobre el microambiente del defecto óseo no crítico favorece su reparación.*

PALABRAS CLAVE: *Reparación ósea. Resina de poliuretano. Implantes. Análisis biomecánico de flexión en tres puntos.*

Introduction

The loss of a bone segment, small or large, is a problem that entails various difficulties in its treatment, patients suffering from these injuries can have economic and social problems derived from the time invested in their recovery. A bone defect can be caused by fractures, trauma, removal of osteosynthesis

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material, or removal of tumors. The study of new materials, growth factors, cell cultures, and combinations among them to induce the repair of bone defects is important for orthopedic, maxillofacial, and orodental surgery[1-4].

To reconstruct bone defects, several materials that substitute bone have been studied: hydroxyapatite, tricalcium phosphate, titanium, coral, biomaterials, and combinations of substitutes with growth factors or scaffolds of different compositions with cell cultures[2,5-7]. Bone defects may be of critical size (not repaired spontaneously) or of non-critical size (repaired spontaneously), in both types of defects it is important to carry out studies to induce their complete repair, as well as to evaluate new materials that could be used as bone substitutes[6,8,9].

Animal models with both critical and non-critical bone defects are used for the study of biocompatibility, biointegration, and biodegradation of materials, as well as for testing growth factors alone and in combination with materials of different compositions[3,6,9,10]. It has been suggested that epidermal growth factor (EGF) increases the mineralization stromal cells of bone marrow[11] and has also been reported that EGF *in vitro* acts as a mitogen for fibroblasts and endothelial cells and *in vivo* induces the development of epithelial and promotes angiogenesis[12,13]. Several studies have reported that AA is essential for the synthesis of collagen and influences osteoblastic differentiation since during the repair of a fracture, it leads to the formation of the osteoid, and this leads to the mineralization of the bone matrix that eventually restores the integrity of the bone[14,15].

AA deficiency can lead to bone abnormalities[16]; it can induce spontaneous fractures, inhibit osteoblast differentiation, and promote adipocyte differentiation[17]; in addition, in a study where they supplemented postmenopausal women with Vitamin C they reported a positive role for osteoblastogenesis and they associated it positively with bone mineral density[18].

The goal of this work was to analyze the effects of the implant material BioOsteo® in combination with EGF and AA on the consolidation of a bone defect of critical size, through the biomechanical analysis of the three-point bending test. We found that after 3 weeks, the local application of a single dose of AA + BioOsteo® directly on the microenvironment of the non-critical bone defect favors the repair.

Materials and methods

Implant material

The implant was prepared as indicated by the BioOsteo® team (Biomecânica, São Paulo, Brazil): one part of prepolymer, one part of Ca(HCO3)2 and 0.85 part of polyol (polyurethane resin Ricinus communis).

AA

A solution of 5 µg/5 µL of AA (Biocheika) was prepared.

EGF

A 100 ng/5 µL solution of murine EGF (GIBCO-BRL) was prepared.

Animals

A total of 24 Wistar rats (males) with a body weight (bw) of 300-340 g (g) were placed in boxes with three rats each, with dark-light cycles of 12:12, they were fed with a commercial balanced diet and purified water *ad libitum*. The animals were randomly divided into four groups of six rats each and were organized as follows: G-1, control rats not treated with age and weight matched to the experimental ones, G-2, rats with defect implant material and application of 100 ng of murine EGF (GIBCO-BRL) in a single dose on the day of surgery, G-3, rats with defect implant material and application of 5 µg of AA (Biochemika) in a single dose on the day of surgery, and G-4, rats with defect implant material and application of 5 µg of AA followed by 100 ng of murine EGF in a single dose on the day of surgery. All the rats were allowed to recover for 3 weeks. The handling and maintenance of the animals were carried out with strict adherence to the Official Mexican Standard NOM-062-ZOO-1999: technical specifications for the production care and use of laboratory animals. The smallest possible number of rats was used to carry out this study.

Non-critical bone defect

The non-critical bone defect was practiced in the right tibia of all the experimental animals in the following way: general anesthesia was induced with

sodium pentobarbital (50 mg/kg, i.p.). The right hind limb was shaved and washed with Dermodine (antiseptic and germicidal solution of Iodine-povidone 8 g/100 mL; DEGASA), a 1 cm incision was made through the skin directly on the tibial crest, and special care was taken of not damaging the underlying bone or the adjacent muscle. The superficial fascia was separated from the skin and retracted to expose the tibia. A 1-mm diameter unicortical defect was made in the center of the region of interest by an electric drill (Mini-drill Pros Kit Model PK-500) with a ball-shaped tungsten dental drill for bone surgery; the area was cleaned and the skin was sutured with simple discontinuous stitches with surgical silk 000 (Atramat, Mexico) mounted on a round needle. The animals were monitored every 3rd day, verifying their general health status and the member subjected to particular experimentation, and the PC of each of the animals was recorded at the end of the experiment. At the end of the experiment, all the animals were sacrificed in a CO_2 chamber.

Biomechanical test

The tibias were resected, stripped of soft tissues, and prepared for the destructive three-point bending tests[8,19,20] in a universal testing machine (Instron 4502, Instron Inc., Canton, MA) and a load cell of 1 kN. The right tibia (Fig. 1A) was placed in two separated rods at a distance of 14 mm and a preload of 3.6 ± 0.1 N was applied. The tests were performed at a speed of 2.5 mm/min. The right tibia was placed in the middle of the bars, on the traction side of the bone, taking care that the tibia was aligned in the bars of the device at the center of the supports and the load was applied on the opposite side until its rupture. To the left tibia (Fig. 1B), the load was applied to the same level of the right tibia following the same procedure. All tests were performed without exceeding the first 30 min after euthanasia.

The load-displacement data were recorded through the interface of the universal test machine and captured in a conventional computer. The load-displacement graphs were made with the data obtained during the test, and from these data, we calculated the rigidity, the resistance, the energy at maximum load, and the maximum energy (Fig. 2) through the Origin 8 program (OriginLab, MA, USA). For each group, the measurements were normalized with the tibia control of each animal.

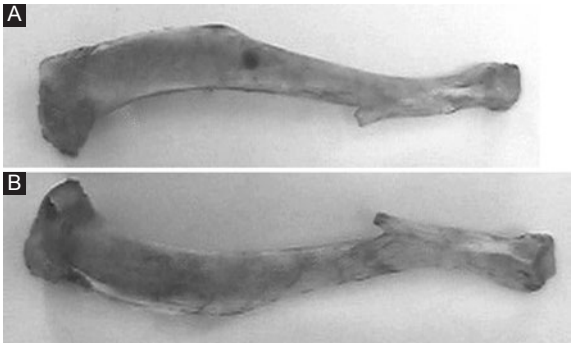

Figure 1. *Resected tibias.* **(A)** *The non-critical defect is shown in the right tibia and* **(B)** *the left tibia is shown intact control.*

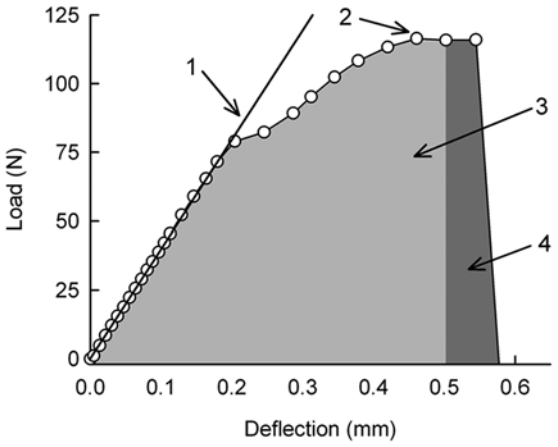

Figure 2. *Measurement of biomechanical parameters. Stiffness is the slope of the load-displacement curve in its initial linear portion (1). The resistance is the maximum load recorded during the test and corresponds to the highest point of the graph (2). The energy at maximum load is the energy that is required for the tibia to reach its point of greatest strength and is determined by calculating the area under the curve (3). The maximum energy is the energy that is absorbed during the test to take the specimen to the fault and is taken as the total area under the curve (sum of the areas 3 + 4).*

Statistical analysis

Based on the size of the sample and assuming non-normality of the data, it was decided to perform the statistical analysis with non-parametric tests[8,21]. The Kruskal–Wallis test is used to compare three or more groups, so it was used initially to know if there were differences between the groups (G-1, G-2, G-3, and G-4). However, if there were differences, the test does not indicate which ones. Therefore, in case of finding significant differences ($p < 0.05$), the Mann–Whitney U-test was then used, comparing the groups to each other (e.g., G-1 vs. G-2, G-1 vs. G-3, etc.). The level of significance was 0.05 and the analysis was performed with the software SPSS 9.0 (SPSS Inc., Chicago IL, USA).

Table 1. Results of the Kruskal–Wallis test.

Energy at maximum load (N-mm)	Maximum energy (N-mm)	Strength (N)	Stiffness (N/mm)
0.013*	0.013*	0.006*	0.075

*Statistically significant difference $p<0.05$.

Table 2. Results of the Mann–Whitney U-test.

Groups	Energy at maximum load (N-mm)	Maximum energy (N-mm)	Strength (N)	Stiffness (N/mm)
G1 versus G2	0.015*	0.009*	0.002*	0.65
G1 versus G3	0.041*	0.394	0.041*	0.004*
G1 versus G4	0.004*	0.041*	0.065	0.240

*Statistically significant difference $p<0.05$.

Results

During the course of the experiment, the rats showed good general health, they used the experimental limb and did not present infections. The animals were fed and drank *ad libitum*, showing an increase in weight according to their age and strain.

The body weight of the rats at the beginning of the experiment was between 300 and 340 g and after 3 weeks before sacrifice the weight was of 400-452 g, having a weight gain between 100 and 112 g, reflecting similar growth for their age and race.

During the analysis of the biomechanical parameters between groups with the Kruskal–Wallis test, we found significant differences for three of the biomechanical parameters studied, except for stiffness (Table 1). The results of the Mann–Whitney U-test (Table 2) between the G-1 versus G-2 groups showed no significant differences for any of the biomechanical parameters measured, except for stiffness. For groups G-1 versus G-3, they revealed significant differences of three biomechanical parameters except for maximum energy. For groups G-1 versus G-4, they revealed significant differences for energy at maximum load and for maximum energy, but not for strength and stiffness.

Discussion

Progress in the treatment of bone defect repair is an important issue for orthopedic surgery, maxillofacial surgery, and orodental surgery since reducing the time spent on treatment reduces the costs and both of the patient and of the institutions of Health. BioOsteo® can be used as an implant material in orthopedic surgery, its biocompatibility and biointegration have been demonstrated *in vivo* and *in vitro* as some authors have reported[22,23]. In addition, regarding osseointegration, BioOsteo® has been reported that there are resorption and substitution of this polymer by bone tissue[23]. Here, we present biomechanical evidence that the BioOsteo® implant material in combination with EGF, AA, or both improves the repair of a non-critical bone defect. Furthermore, our results were obtained in a model of biomechanical analysis of three-point bending test[8] that can be used to follow the consolidation process.

Antagonistic effects have been reported with respect to the action of EGF on bone tissue. Some studies report that EGF contributes to bone synthesis[24], while other studies have reported that EGF contributes to bone resorption[25]. This antagonistic effect of EGF could explain that under our experimental conditions we did not observe a favorable effect on the process of repair of a non-critical bone defect during the application of EGF in a single dose (G2) since none of the parameters changed.

There are reports that EGF acts in the early phase of repair where it promotes and regulates the proliferation of osteoblasts[26,27]. It is also mentioned that in the late phase of repair EGF is involved in bone differentiation and mineralization and in inhibiting the formation of mineralization nodules. This would explain that the EGF recovered the energy at maximum load of the bone defect when applied in combination with AA (G3) but not for the other parameters analyzed. The reported antagonistic effect of EGF could explain the contradictory results reported in the literature, as well as those found in this study; however, these results may be due to the different microenvironments generated either only with BioOsteo® + EGF or BioOsteo® + EGF and AA.

It is known that AA stimulates the synthesis of collagen and proteoglycans, as well as organic components

of the extracellular matrix in various tissues such as teeth and capillary endothelium; in this context, the production of collagen is a complex process of protein synthesis, as well as its post-translational modifications. This leads us to think that AA applied locally and in a single dose stimulates the production of collagen and, consequently, the mineralization of the bone matrix[28,29], which ultimately leads to the recovery of the biomechanical properties of bone. It was a surprise to find that the BioOsteo® + AA in single dose (G3) only recovered energy at maximum load under our study conditions, although we know that when there are variations of AA in the diet, the formation of bone matrix is affected. In young populations, the lack of Vitamin C changes the formation of the bone matrix and the resorption of cartilage, which leads to bone fragility and fracture of the growth plate[29,30].

It has been reported that there is a sodium-dependent carrier for AA in the plasma membrane of osteoblasts[31], which indicates a nutrient function for the synthesis of this population of cells. In addition, AA stimulates the action of alkaline phosphatase and induces osteoblastic differentiation of stromal cells *in vitro*[28,30], these reports agree with what we observed here since we found that the group with BioOsteo®, EGF + AA (G4) is the one that recovered the resistance and rigidity, which suggests that the process of bone repair of the defect is more advanced than in the other groups analyzed.

Previous studies report that during the repair of the fracture, AA does not favor repair when compared to the control group[31]. At the molecular level, AA increases the production of collagen, which is essential for the mineralization process during the repair of the fracture[30,32]. In addition to participating in the synthesis of collagen, AA can inhibit the differentiation of osteoclasts that participate during bone remodeling[33,34]. It is also reported that AA has a dual effect on bone tissue as it accelerates bone remodeling and affects the formation and death of osteoclasts[34] since osteoclastogenesis is promoted by AA in cultures that contain both osteoclasts and osteoblasts. The antagonistic effects of both EGF and AA can explain the results obtained in this work since we observed different effects when applying EGF and AA individually and in combination of BioOsteo®, EGF, and AA.

There are different strategies to induce bone repair, which focus on some of the various processes that are carried out during such repair, for example, some studies induce vascularization to increase bone mass and thus stimulate repair; another strategy is to inhibit cells that degrade bone (osteoclasts) and/or stimulate bone-forming cells (osteoblasts), the application of growth factors and compounds that participate during bone repair, as well as materials or biomaterials that stimulate such repair, increasing the possibilities of cell migration to replace these biomaterials with bone tissue.

In this search, various strategies have been tried, from the simplest to the most complex; here, we used a simple strategy with a relatively inexpensive cost that can be an option in the treatment of bone defects to accelerate its repair, with a single dose applied locally directly on the microenvironment of a non-critical bone defect. In addition, this strategy could be used to study the repair of critical bone defects as well as fractures.

Conclusion

We found that a 1 time single-dose local application of AA + EGF + BioOsteo®, directly over the non-critical bone defect microenvironment improves its repair; this effect would significantly contribute to decrease the socioeconomic problems during integral patient treatment.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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