Original Article - Comparative Study

Magnetic Field Effects on Bone Repair after Calcium Phosphate Cement Implants: Histometric and Biochemistry Evaluation

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Abstract

Objective: This work evaluated histologic and biochemically the effect of magnetic field buried in bone repair after autogenous bone graft and calcium phosphate cement implants. **Methodology:** Bone defects with 5,0 mm of diameter in the cranium of Wistar mice were used to analyse. These mice were submitted to different interventions: autogenous bone graft and calcium phosphate cement implants, both with and without magnetic stimulation. Longitudinal and transversal histometric and biochemistry analysis were made in times of 15, 30 and 60 post-operative days. **Results:** The histometric transversal analysis did not show significant differences in the bone repair between groups. Longitudinally, significant difference were found in the quantity of neoformed bone between the times 15 and 60 post-operative days in the autogenous bone graft group under magnetic stimulation. The alkaline phosphatase enzyme presented a higher activity in 30 post-operative days and the groups under magnetic stimulation presented reduced enzymatic activity in comparison to the other groups. **Conclusion:** The permanent and static magnetic field promoted significant differences in the neoformed bone in the groups autogenous bone graft.

Keywords: Calcium phosphate cement, critical size bone defect, magnetic field

Introduction

Although bone tissue has large resistance, it remains subject to biological, physical, and chemical stimuli that may persuade modifications in its structure or to cause its fracture.[1,2] Therefore, the utilization of biomaterial, such as the calcium phosphate cement,[3-5] and the stimulation of the receptor bone bed through magnetic fields[6-8] are proposals that look for reestablishing the shape and the function of the lost tissue and mainly, accelerating the bone repair.

Accounts of the use of electromagnetism, like a support in the process of bone remodelation, already appeared in the 19th century and they were intensified about 1980.[9] Since then, with the necessity of clearing the knowledge under the influence of magnetic field in the bone repair, various studies have been made employing buried magnetic field to study better the effects of these fields on bone remodelation from the stimulation of the receptor bone bed, bone graft, or implanted biomaterial.[6-8,10]

The interest to accelerate the bone repair is not only related to the researches with magnetic stimulation but also they have evaluated the rehabilitating potential of the bone replacements, such as calcium phosphate cement. This, besides the same ionic constitution to the bone tissue[11] is osteoinductor, osteoconductor, and biocompatible,[4,5] these properties help osteointegration and accelerate the bone repair.[11,12]

From the hypothesis that the calcium phosphate cement (Mimix™) is osteoconductor, absorbed and biocompatible, and the magnetic field stimulates the process of bone repair, this work evaluated histomorphometric and biochemically the bone repair after autogenous bone graft and calcium phosphate cement implant (Mimix™) under buried magnetic field.

Methodology

This study followed the rules of utilization of animals of experimentation in research projects, according to the State

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Code of Animal's Protection and Normative Resolution 04/97 of the Research Ethics Committees in Health/GPPG/HCPA and was submitted to the evaluation of the Ethics Committee in Research of the Faculty of Dentistry of the Federal University of Rio Grande do Sul, under the process number 259/08, was approved.

Sample calculation, groups of study, and experimental delineation

The sample calculation was made through Winpepi® software (Winpepi® software, Brixton Health, London, United Kingdon), Compare 2 module, version 1.62, attributing values of 5% and 80% for levels of significance and power of sample, respectively. Based on the values of standard deviation showed by Marzouk *et al*.[13] who also studied the bone repair of ostectomies in rat's calvaria, and considering a difference of 20% in the filling of the bone defect, we have the value of five animals for each experimental group. For this, 65 albino, adult, male rats were used, of 8 and 10 months age, average weight of 500 g, *Rattus norvegicus* albinos species, *Rodentia*, *Mammalia*, Wistar lineage.

The study of bone repair in the defects of 5.0 mm in the rat's cranium was made from two different interventions: autogenous bone graft, removed from the own mice's cranium with trephine drill and implant of calcium phosphate cement (Mimix™). Both interventions were evaluated with or without magnetic stimulation from buried magnets in the adjacencies of the bone defect. For the physiological control of the activity of alkaline phosphatase enzyme, five animals were used and they were not submitted to any surgical intervention (Naive group).

The magnetic field, when it is present, was conceived by two magnets of neodymium, iron and barium buried in the adjacencies of the defect. In the animals of the control groups commercially pure titanium records were used. The dimensions of magnets and titanium records were 3.0 mm of diameter by 1.0 mm of thickness. The intensity of magnetic field in the interior of the defect was calculated by a gaussmeter (Magnet-Physik FH 35, Magnet-Physik Dr. Steingroever GmbH, Germany), as also in the rats dry cranium and the 30 pairs of magnets subsequently buried in the animal's cranium. The average of intensity of the magnetic field, into the defect, was calculated in three points: Posterior, anterior, and in the central portion of the bone defect. The values found in each point of the defect were 73, 40 G, 66, 20 G, and 43, 72 G. To ensure that the magnets were buried with attractive magnetic field, and not repulsive, the north pole of all magnets was demarcated by nontoxic white nail enamel and, prior to the insertion, a test of attraction was made between the magnetic pairs.

The calcium phosphate cement (Mimix™, Walter Lorenz Surgical, Jacksonville, FL, USA) is presented commercially as a powder and a liquid that should be mixed. The powder is a material based on calcium phosphate with the addition of tetra-calcium phosphate, α-tri calcium phosphate, and dried sodium nitrate. The liquid is a solution composed of citric acid and distilled water. The powder and the liquid, when mixed (following the orientation of the manufacturers), form a material of pastose consistency that may be applied directly onto bone defects. It is manipulated from 30 to 45 s, remaining malleable from 3 to 4 min, and requires 4 to 6 min for complete endurance.

Operating technique

The animals were anesthetized with an intraperitoneal shot of ketamine hydrochloride and xylazine hydrochloride in the respective dosage of 1.0 ml/kg and 0.1 ml/kg of animal's body weight. After we made a manual trichotomy in the region of animal's cranium, followed by an antisepsis with a water solution of chlorhexidine digluconate 0.12%. A skin incision, around 1.5 cm of extension was made, allowing the tissue avulsion, incision, and periosteal ungluing. After the exposure of the bone bed and the mouse's frontal bone, a bone defect was made of 5.0 mm of diameter. Tangencing the defect from a distance of 1.0 mm, two ostectomies of 3.0 mm length by 1.0 mm width were made for burying the magnet. With a trephine drill located in the central portion of the frontal bone, the bone defect of 5.0 mm of diameter was made. The ostectomies of 3.0 mm for burying of magnets or metal records were made with multi-laminated spherical drill. After the fixation of magnets (test group), the autogenous graft or the Mimix™ implant were inserted in the bone cavity [Figure 1]. The quantity of material used was enough to fulfill the totality of bone defect. After the fulfillment of bone defect, the surgical wounds were closed, tissue reposition and skin suture in isolated points with mononylon 5-0 thread were placed. During the anesthetic recuperation, the animals were kept in contact with heated sponges and they received standard food and *ad libitum* water. The postoperating analgesia was made with paracetamol drops, 200 mg/ml, in the posology of a drop by killogram. The stability of magnets or metal dispositive, from the bone grafts and implants of calcium phosphate cement were confirmed after the death of animals through periapical radiographic incidences.

Histologic preparation

The animals were sacrificed the samples were collected and fixed in tamponed neutral formalin to 10% by 24 h. After fixation, the pieces were decalcified in a solution of formic acid 50% and sodium citrate 20% around 7 days. Subsequently, already in the plastic state, the removal of magnets (test group) and metal dispositives (control) and average longitudinal section of calvaria with the usage of a disposable knife for microtomes were made, and the section feature included the central part of two ostectomies created by the burying of magnets. Afterward, the pieces were processed according to the protocol of inclusion in paraffin and stained with hematoxylin and eosin. Three contiguous longitudinal histologic cuts were made with a thickness of 4 μm, including two orifices of ostectomies from the average longitudinal section.

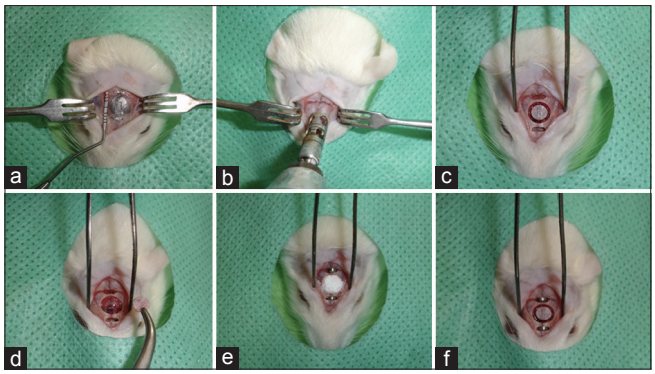

Figure 1: Image evidencing the delineation for the bone defects in the cranium. (a) Exposition of the site in the frontal bone and the area of the bone defect and the magnetic stores or metal dispositives. (b) Utilization of trephine drill for the bone defect of 5.0 mm. (c) Manufacture of bone defect bone stores of the metal dispositives and magnets. (d) Removal bone graft. (e) Fixation of the metal dispositives or magnets and implantation of the calcium phosphate's bone cement (Mimix™). (f) Fixation of the metal dispositives or magnets

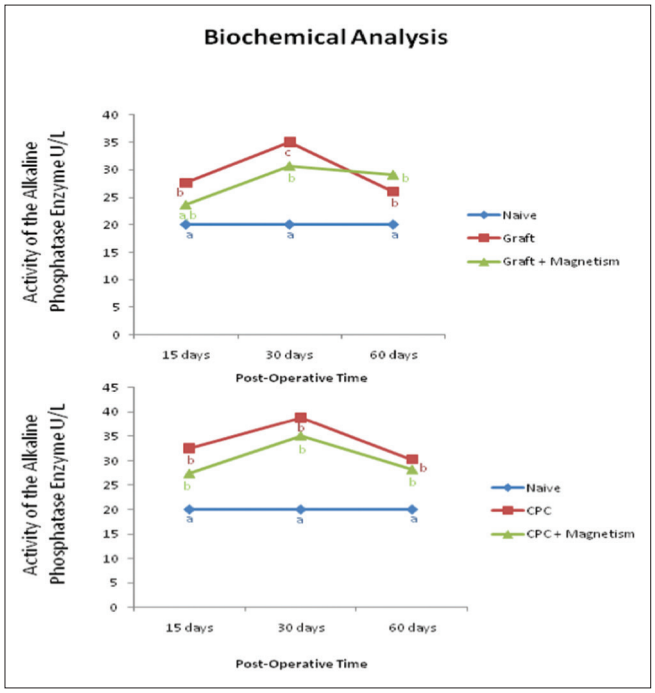

Figure 3: (A) Comparative graphic of the activity of the alkaline phosphatase enzyme between the groups Naive, autogenous bone graft (graft) and autogenous bone graft with magnetism. (B) Comparative graphic of the activity of the alkaline phosphatase enzyme between the groups Naive, implant of calcium phosphate cement and implant of calcium phosphate cement with magnetism. (a,b,c) Different small letters show a significant difference between the groups in each experimental time. ANOVA, $P < 0.05$

Histologic analysis

The images of pieces selected for the analyses were obtained through a Olympus® Video Camera (Qcolor 5 Model, Cooler, RTV), together with a binocular microscope Optical Co. CX41RF model and a Dell® Computer (Dimension 5150 model), using

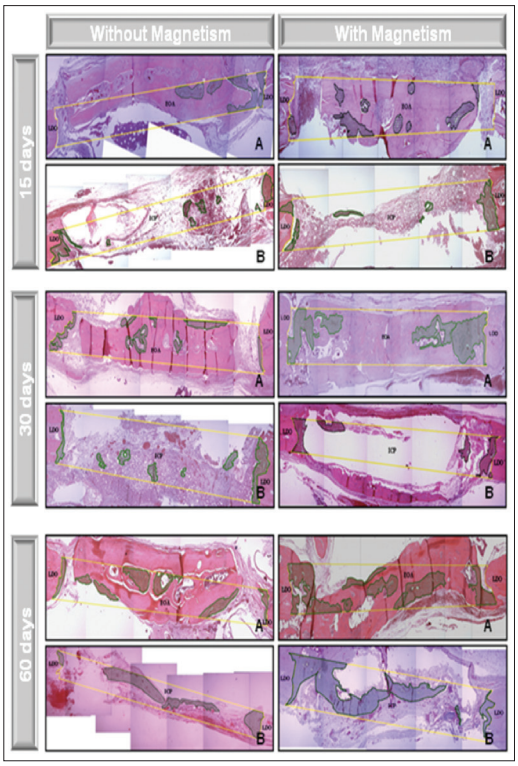

Figure 2: Image illustrating histologic cuts in the histometric analysis. Yellow lines delimit the total area of the bone defect. Green areas evidence areas of bone neoformation in the interior of the bone defect. In 30 and 60 post-operative days, in the groups with magnetism, it is observed the closure of the bone defect through the union of the bone defect limit (LDO) with the autogenous bone graft (EOA) through areas of bone neoformation. (A) Autogenous bone graft. (B) Implants of calcium phosphate cement

the Qcapture® software (version 2.81; QImaging Corporation, Inc.; 2005, Surrey, Canada), increasing by ×40, ×100 and ×200. Subsequently, to the delimitation of the total area of the bone defect, the neoformed bone tissue was evidenced using the AxioVision® software (AxionVision Imaging System, version 4.6.3, Carl-Zeiss-Stiftung, Oberkochen, Germany). The quantity of neoformed bone was expressed through percentage from the total area of the created bone defect. The image program was calibrated and manipulated by blind examiner. In a 7 days interval, the examiner made two histologic analyses of ten blades. The correlation between two histometric analyses was calculated through a coefficient of intraclass correlation, finding the value of 0.93. The choice of histologic cuts for analysis was random. All the blades were coded independently during the histologic preparation in a way that, during the histometric analysis, the examiner did not know which group the analyzed histologic cut belonged to.

For statistic means, the values of the percentage's average obtained in the cuts of each calvaria were used.

Biochemical analysis

The animals were decapitated in guillotine, 1000 µl of blood was collected through a cervical blood collection with heparin funnel. The blood collected was centrifuged for 10 min at

3000 rpm, allowing for obtaining of the blood plasma. 50 µl of this plasma was used for the dosage of alkaline phosphatase enzyme, using for laboratorial kit Labtest (Labtest Diagnóstica S/A, Lagoa Santa, MG, Brazil). The ester of phosphoric acid acts like a substratum, being hydrolyzed for the alkaline phosphatase enzyme. This reaction is interrupted by the addition of sodium hydroxide (250 mmol/l) and sodium carbonate (94 mmol/l), making the product of this reaction to have the color blue and may be read in spectrophotometer to 590 nm according to the method proposed by Roy.[14]

Statistical analysis

The analysis of data was performed using the statistical package SPSS (SPSS version 13.0 for Microsoft Windows, SPSS Company - Statistical Product and Service Solutions, Chicago, USA). The analytical unity considered was the mouse and the level of significance was established in 5%. The data of bone filling presented asymmetric distribution, being transformed using the square root. Comparisons between groups and between experimental times were made through analysis of variance of a way with a Bonferroni test such as a *post hoc*. The results were showed through averages and standard deviation of the percentual of bone neoformation after inverse transformation. For the activity of the systemic alkaline phosphatase enzyme the variance analysis of a way followed by test *post hoc* of Duncan's multiple scores were used.

Results

From 65 animals, two were excluded. One, due to the postoperating infection and another, due to the magnetic dislocation, respectively, belonged to the groups of the autogenous bone graft without magnets of 30 postoperative days and autogenous bone graft with magnetism with 15 postoperative days.

Histologic analysis

The analysis of histologic cuts evidenced complete closure of the bone defect in only two animals. These belonged to the groups of the autogenous bone graft with magnets of 30 and 60 postoperating days [Figure 2]. In the groups without magnetic stimulation with autogenous bone graft and in the groups with implants of calcium phosphate cement, with or without magnetic stimulation, there was no complete closure of the defect.

The histometric analysis [Table 1] did not evidence significant differences in the quantity of neoformed bone tissue from a transversal histometric analysis (comparison between the different groups in the same experimental time). On the other hand, the longitudinal histometry (comparison between the same groups in different postoperating times) evidenced, significantly, more bone formation in the group submitted to the autogenous bone graft under magnetic stimulation.

Still, according to Table 1, the comparison between the quantity of neoformed bone tissue in the group with autogenous bone graft and implant of calcium phosphate cement did not evidence significant differences, as much in the groups with magnetism as in the groups without magnets, according to the transversal and longitudinal histometric analysis.

The histologic analysis did not evidence inflammatory reactions, such as reaction to the foreign body to the implanted biomaterial.

Biochemical analysis

The biochemical analysis evidenced significant statistical differences in the activity of alkaline phosphatase between the Naive group and the 30 days group with autogenous bone graft, autogenous bone graft with magnetism and 60 postoperating days in the group with autogenous bone graft with and without magnets. In 15 days, only the autogenous bone graft group without magnetism showed a significant difference in relation to Naive, while the group of the autogenous bone graft with magnetism showed enzymatic activity statistically equal to the Naive group and also equal to the autogenous bone graft group with magnetism of 15 days. Still, in 30 postoperating days, there were different significant statistics between the autogenous bone graft group with or without magnets, being the group that was under influence of the magnetic field showed an enzymatic activity statistically smaller than the autogenous bone graft without magnets [Figure 3A].

The comparison between the alkaline phosphatase activity of the Naive group with the group that received an implant of the calcium phosphate cement with magnetic stimulation did not show statistically significant difference in the time of 15 postoperating days. In the time of 15 days without magnetic stimulation, 30 and 60 days, with or without magnetism, there were statistical differences, according to Figure 3B.

Table 1: Transversal and longitudinal histometric analysis of the percentage of bone neoformation in the groups with autogenous bone graft and implants of calcium phosphate cement, both with and without magnetic stimulation in the periods of 15, 30 and 60 postoperating days (average + standard deviation)

	15 days		30 days		60 days	
	Average±SD	*	Average±SD	*	Average±SD	*
Graft + Magnet**	8,15±2,32[a]	A	14,84±4,10[ab]	A	19,95±7,00[b]	A
Graft**	11,23±3,92[a]	A	12,09±1,76[a]	A	14,64±6,36[a]	A
Mimix™ + Magnet**	9,94±3,85[a]	A	10,91±1,56a	A	16,50±15,38[a]	A
Mimix™**	7,23±2,53[a]	A	8,28±4,30[a]	A	10,73±3,12[a]	A

*Different capital letters show significant difference between the groups in each experimental time (Bonferroni test, $P < 0.05$), **Different small letters show significant difference between the experimental times inside each group (Bonferroni test, $P < 0.05$)

Discussion

The oral and maxillofacial surgery field has studied several biomaterials[15-17] and developed alternatives[6-8,18,19] that may accelerate the bone repair or substitute this tissue when lost.

All these studies have been made to supply feasible alternatives for not only autogenous bone graft, that, although is considered the gold standard in maxillofacial bone reconstructions, has some limitations, like higher postoperating morbidity and limited available bone quantity.[20]

Therefore, the better way to study the interaction between the biomaterials and methodologies proposed for the stimulation of reparative processes is through the utilization of animal models,[6-8,21,22] mice are the first choice preferred as they are easy to handle, require less space for accommodation, and make the experimentation cost-effective.[23]

Ostectomies in animals' models, more specifically in the cranial calvarium of mice, allows for many biomaterials and ways of stimulation of bone repair to be tested with the purpose of reconstructing lost structures and accelerate the reparative processes of the bone tissue. Among these alternatives are the calcium phosphate cement[24-27] and the usage of magnetic fields.[6-8]

The studies involving biomaterials based on calcium phosphate cement show large diversity of these biomaterials. These differences are mainly related to the physical-chemical structure and the commercial presentation. However, the different ways and presentations of the cements based on calcium phosphate makes it an appropriate bone replacement, what may be noticed in this work, once the histometric analysis did not evidence significant statistical differences in relation to the quantity of neoformed bone tissue between the implants of calcium phosphate cement (Mimix™) and the autogenous bone graft, that it is the gold standard in bone graft.[28]

The prerequisites for biomaterials for bone replacement should be ease of manipulation, resistance to fractures, adhesion and cellular proliferation and the capacity of cell's induction with osteoprogenitor phenotype.[15,29,30] The calcium phosphate cement (Mimix™) fulfilled these prerequirements, keeping its shape along the study, allowing the colonization and cellular proliferation, according to what is noticed through the bone neoformation by means of phenomenon of osteoconduction.

The histologic analysis did not evidence inflammatory reactions of the kind of reaction to a foreign body in any of the studied groups, evidencing the biocompatibility of the biomaterial, that is widely evidenced in the literature.[4,5,31,32] The standard of bone formation, however, showed a difference in relation to osteogenesis. While the neoformed bone in the groups with bone grafts extends on the board of the defect in direction to the graft, in the groups with Mimix™, the bone neoformation occurred mainly from the board of the defect in a direction to the center, through osteoconduction.

The bone neoformation that occurred in the biomaterial, statistically equalled to what occurred in the group with autogenous bone graft, may be explained as the chemical structure of the calcium phosphate cement is constituted by ions of calcium and phosphorus, the same ions that form the bone tissue. This promoted a positive interaction of this biomaterial with host bone tissue.[11] Still, the presence of micro- and macro-pores in its constitution makes this biomaterial an ideal location for the processes of colonization and cellular proliferation. While the macro-pores are related to the adhesion and cellular proliferation, the micro-pores become a net of nutritional canaliculus that allow the passage of body liquids to feed the cells that are colonizing the biomaterial.[33-35]

With the understanding of the cellular activity involved in the process of bone repair, in the 19th century, electromagnetism was used as supporting therapeutic modality in the process of bone remodeling.[9] From then, the search for ways to accelerate the process of bone repair intensified and researches studied the usage of magnetism, developing methodologies that evaluated the electromagnetic stimulation in areas of fractures,[9,36,37] they studied the influence of magnetic fields generated for permanent magnets in the repair of wounds in skin[38,39] and they studied the burying of permanent magnets and implanted metal dispositives in rat's femurs.[7,8]

All these described methodologies, although they used different forms of promoting the magnetic stimulation under the surgical bed, have a common point that is the utilization of magnetic fields with variable flow in the area of study. This study used natural magnets, with permanent and constant flow. These magnets, composed by a league of neodymium, iron, and barium were introduced under pressure in bone without causing micro-movements during the study period. This fact, unlike the other studies, determines the incidence of a field with static and permanent magnetic flow in the bone defect.

The utilization of a static and permanent magnetic field, from the transversal histometric analysis, made in this work, did not evidence significant statistical differences in relation to the percentage of neoformed bone between the groups of autogenous bone graft and implants of calcium phosphate cement, in the groups with and without magnets. However, the longitudinal histometric analysis evidenced significant differences in the quantity of neoformed bone tissue between the times of 15 and 60 postoperative days under magnetic stimulation, what corroborate the finds of the descriptive histology of other studies which evidence a more acceleration in the groups under magnetic stimulation.[7,8] The comparison of these results, however, should be made cautiously, once the methodologies used are different in relation to the bone sites and the shape of magnetic stimulation.[7,8,10,37] The studied bone sites are different for bigger and smaller concentration of medullary bone tissue. The cavity created in the femur, unlike created in the cranial calvarium, offers a huge extension of medullary bone.

Some works studied the molecular component involved in the bone repair under the magnetic stimulation. The increase in the concentration of growth factors in the site where the

magnetic fields are acting, apparently affects magnetic flow to cause alterations in the cellular membrane, modulating the transmembrane signalization and accelerating the bone tissue growth, justifying, therefore, the more intense bone formation in sites under magnetic stimulation.[18,40]

The influence of magnetic field under mitochondrial activity did not establish relation to the magnetic field[41] and other works evidenced the reduction of vascularization and reduction of the quantity of calcium's ion in the interior of the cell when the animals were exposed, in a chronic way, to magnetic fields.[42]

With this variability of results, we questioned the difference between cellular stimulus generated static fields and magnetic fields with variable flow. Therefore, it is hypothesized that the cells with osteoprogenitor lineage could be stimulated to produce bone tissue when submitted to variable magnetic fields and, then, the results of this work did not evidence differences between the groups with and without magnets from the transversal histometric histologic analysis. To such hypothesis may be noticed the realization of other studies suggested, using the same methodology for evaluation of bone neoformation, however with comparative analysis of bone repair under variable magnetic stimulation and under static magnetic stimulation. It is suggested, yet, the realization of histologic cuts, not only sagitals, like those made here but also coronary and axial cuts, that would allow a three-dimensional evaluation of the area of the bone defect.

The complete understanding of the influence of magnetic field under bone repair did not study evidence just in histologic parameters, but it seems to influence the concentration of growth factors,[18,40] the disposition of calcium ions during the ossification[6] and it seems to influence the plasmatic membrane.[40] Therefore, membrane's mediator, such as alkaline phosphatase enzyme, consequently could be influenced by the magnetic field. This enzyme is deeply related to the bone metabolism and the process of matrix secretion made by osteoblasts,[43] being for that, considered a peripheral mark of the bone metabolism.[14]

The results of this study evidenced an activation of this enzyme when compared to the results of normal values of physiologic activity. The exception of the experimental period of 15 postoperating days with autogenous bone graft under magnetic stimulation, the other groups evidenced statistical differences in relation to the physiologic activity.

In 30 postoperating days, the enzymatic activity showed the tendency of a peak. However, in this experimental time, the comparison between the groups of the autogenous bone graft with and without magnets showed significant statistical differences, evidencing a bigger enzymatic activity in the group of the autogenous bone graft without magnetic stimulation.

This enzyme has co-factors (substances that may be necessary for its function), ions zinc and magnesium, and its active site has a league based on manganese.[44] These metal ions related to its activity, speculating, therefore, if the activity of the reduced alkaline phosphatase in the groups under the influence of magnetism could affect the action in the magnetic field under these ions. However, some studies suggest that the action of magnetic field seems to be related also to factors with involvement in molecular level, like growth factors and, even, enzymes like osteocalcin and osteopontin.[18,40]

Conclusion

Histomorphometric analysis showed that the bone repair accelerated in the groups submitted to the autogenous bone graft under magnetic stimulation. Biochemically, the measure of systemic activity of the alkaline phosphatase enzyme was more expressive in the postoperating time of 30 days. Based on the results of this study we suggest clinical use of a magnetic field, a creation of magnetized covering screws in implantology or the creation of magnetized masks for use in postoperative orthognathic surgery.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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