COMPARATIVE STUDY OF BONE NEOFORMATION USING AUTOLOGOUS GRAFTING AND THREE REPLACEMENTS: BONE DEFECTS IN RATS

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ABSTRACT

Objective: Compare the percentage of bone neoformation promoted by autologous bone grafting and three kinds of replacement materials with different characteristics in rats' femoral holes. Methods: Two holes measuring 5.4 x 2.7mm, were produced on each femur (right and left) of 14 isogenic Wistar rats. Each of the four defects produced was filled by autologous bone or by one of three tested materials – hydroxyapatite (HA), Genphos® (HA+ ß-TCP) and *GenMix*® (a combined bovine bone graft). In the end of the 6-week (n = 6) and 12-week (n = 8) periods, the animals were sacrificed. The sections (stained with Picro-Sirius) were assessed by optical microscopy and specific software. Results: The groups with autologous bone were shown to be significantly superior to the others at both assessed times, showing a mean bone formation rate ± SD of 90.6 ± 10.8% in six weeks, and 98 ± 9.2% in 12 weeks ($p > 0.0001$ for both assessed times). In six weeks, the results for the other groups were the following: Genphos®, 46 ± 7.1%; HA, 43.1 ± 8.4%; and *GenMix*®, 57.3 ± 4.5%. In 12 weeks: Genphos®, 47.8 ± 11.1%; HA, 39.9 ± 5.4%; GenMix®, 59.7 ± 4.8%, significant ($p = 0.007$). Conclusions: In both assessed times, the three bone replacement materials tested in the study showed to be inferior to autologous bone graft for bone neoformation percentage.

Keywords – *Bone regeneration; Bone transplantation; Durapatite; Calcium phosphates; Transplantation, heterotopic; Transplantation, autologous; Biocompatible materials; Rats, Wistar*

INTRODUCTION

There are numerous accidents or diseases in which there is loss of bone tissue that needs to be replaced (in the U.S. alone, there are an estimated about 500,000 bone graft procedures per year)[1]. To the present day, the treatment of choice to recover from this type of injury is the autogenous bone graft (gold standard), an established and very effective surgical procedure that is far from perfect. Among its disadvantages that could be cited are its difficulty of acceptance by patients (because it is necessary to remove bone from another area), the volume and shape of the limited donor sites, the defect generated during graft extraction, and postoperative state of the donor area, which usually presents more complications than the recipient area[1-6]. Because of these limitations, there is a constant search for a grafting material that can replace autogenous bone, with the same quality of treatment, but with fewer drawbacks. It seems that an ideal substitute has yet to be created[6-8].

A bone substitute with ideal characteristics should have physicochemical properties similar to bone to facilitate bone regeneration, should combine osteoinductive and osteoconductive properties, and should be biocompatible and resorbable, to be completely replaced by bone[8,9].

The objective of this study was to compare the percentage of new bone formation after grafting between the current gold standard (autogenous bone graft) and three bone substitutes with different characteristics to

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see if any of these would be able to replace the standard without significant loss of quality.

METHODS

We used 14 young adult male isogenic Wistar (Rattus norvegicus) rats of the Kyoto strain between two and four months old, weighing between 220 and 300 grams, from the Microsurgery and Medical Skills Laboratory at PUC/RS, where all surgical procedures were performed. The specimens were randomly assigned into two experimental groups which received the same treatment but were sacrificed and analyzed at different times, at six weeks (n = 6) and 12 weeks (n = 8).

In each treated specimen, anesthesia was administered intraperitoneally with a solution consisting of 0.2 ml of chlorpromazine hydrochloride (5 mg/ml) + 0.8 ml of ketamine (50 mg/ml), at a dose of 0.3 ml of solution/100 grams of rat body weight (which is equivalent to 0.3 mg chlorpromazine + 12 mg of ketamine/100 g of rat body weight). Trichotomy was performed in the area of surgical access over both femurs (left and right). Maintenance doses of anesthetic were prepared to be administered in the course of surgery as needed (half the initial dose).

A longitudinal incision of approximately 4 cm was made parallel and anterior to the axis of each femur, one at a time. The anterior area of each femur was exposed with the delicate dissection of muscle and periosteum. With the aid of a BLM 600 Plus electric motor against a 1 x 1 dental angle, two 5.4 mm cavities were made using a 2.7 mm trephine drill in two adjacent cavities, 2.7 mm x 2 = 5.4 mm. To prepare these cavities, a constant torque was set to 45N, speed was set at 45,000rpm, and copious irrigation (70% – adjustment of the electric motor) with saline for viability of bone regeneration.

After drilling, the bone fragments were carefully removed (Figure 1) and stored in sterile dappen dishes. Later, these will be used as autogenous bone to be grafted (after being particulated with dental minialveolotomy) in one of the cavities. Each of the remaining cavities was filled with one of the bone substitute materials to be tested: hydroxyapatite (HA) (Bionnovation, Inc., Brazil), (HA + ß-TCP) – Genphos® (Baumer, Inc., Brazil) and (a bovine composite bone graft) – *GenMix*® (Baumer, Inc., Brazil). The distribution of the position of each material in the cavities of the femurs underwent clockwise rotation for the standardization of grafted areas with each material.

The periosteal, plane, and skin sutures were performed with mononylon 4-0 (*Ethilon*®, Johnson & Johnson, Brazil). The rats were kept in individual cages after surgery.

For the sacrification of animals at the determined times, the previously mentioned dose of anesthesia was administered; later, an 100 mg/kg intracardiac overdose of pentobarbital sodium was applied to the previously anesthetized rat.

After the euthanasia of the animals, there was a delicate removal of the right and left femurs (Figure 2), which were immediately placed in formalin 10% for three days. The femurs were then sectioned with the help of a dental carborundum disk, the same electric motor used in other surgeries, and the 1 x 1 straight piece. About 0.5 to 1 mm of healthy bone was sectioned beyond the grafted area on both sides of the treated area.

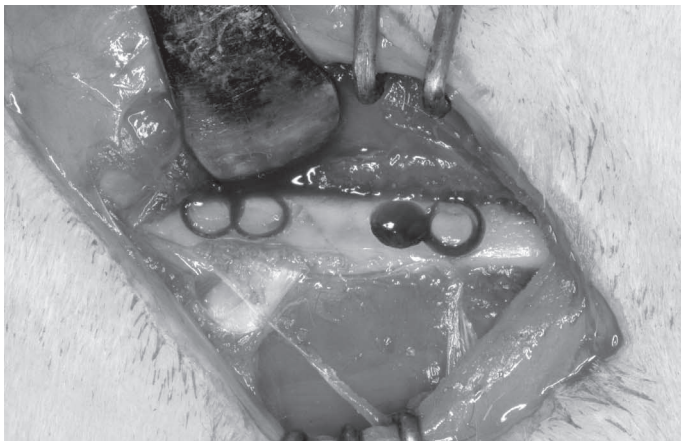

Figure 1 – Photograph showing the perforations made and the beginning of bone removal, which will be used as a source for autogenous grafting

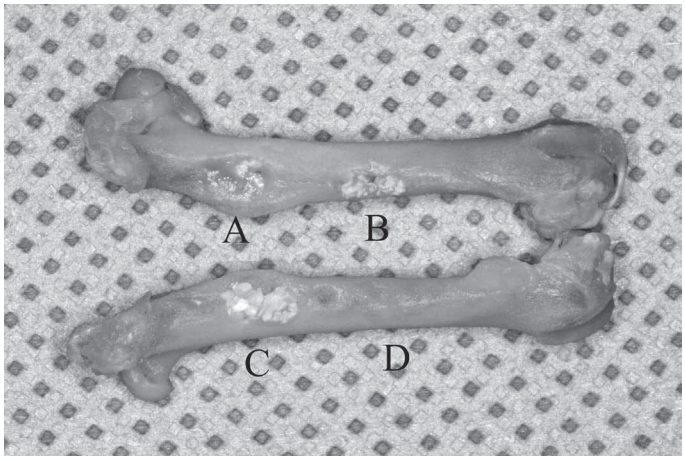

Figure 2 – Photograph showing the appearance of the four groups tested after 12 weeks: A) *Genmix*®, B) *Genphos*®, C) HA (Bionnovation), D) Autologous. Note the difficulty of locating the area of the autogenous graft (D)

All treatments were separated according to the type of graft performed; slow decalcification was performed with formic acid at a 30% concentration in an oven at 37°C, changed every three days for a period of two weeks. After complete decalcification, specimens were embedded in paraffin for microtome cutting and production of histological slides.

After discarding the first millimeter of each region tested, 10 histological slides were prepared with two to five 50μm cuts of the femur each, repeating the procedure for every 1 mm of the region analyzed.

After 24 hours in an oven at 60°C, the slides were placed in a solution of 1% picrosirius for an hour, then washed in running water for 20 minutes. When dried, the slides were examined and cataloged to choose the areas to be photographed and analyzed.

All groups were examined with standard light microscopy at 5x magnification. When capturing images with the aid of a Cool Snap Pro camera, a magnification similar to a 10x objective lens is produced. Thus, the resulting images are magnified 50x.

For the analysis, at least three different areas were stipulated with a minimum of 1 mm of space between them. In the region adjacent to the lesion, an area on the same slide with similar length and width as each treated area was selected as a 100% bone neoformation control.

The captured images were analyzed with Image Pro Plus, version 4.5.1, used by the Department of Pathology, Hospital São Lucas, PUC/RS.

With help of this program, the area corresponding to the bone in each captured image was selected (Figures 3, 4, 5, and 6), and a specific "mask" was applied. This area, when measured with the program described above, generates a count of the number of matching pixels. The percentage of new bone formation in the treated area was calculated by comparing the bone in this (treated) area to the bone present in the adjacent untreated (control) area, which was set as 100% of neoformation (ideal).

Data are presented as means and standard deviations. To compare new bone formation between the groups, we used analysis of variance (ANOVA) followed by Tukey post hoc test. The comparison of the times was performed by Student's t-test. The level of significance was set at $\alpha = 0.05$. Data were analyzed with SPSS.

The research protocol of the experiment was submitted to the Research Ethics Committee of the Pontifícia Universidade Católica do Rio Grande do Sul, with no changes or modifications made to the proposed procedures.

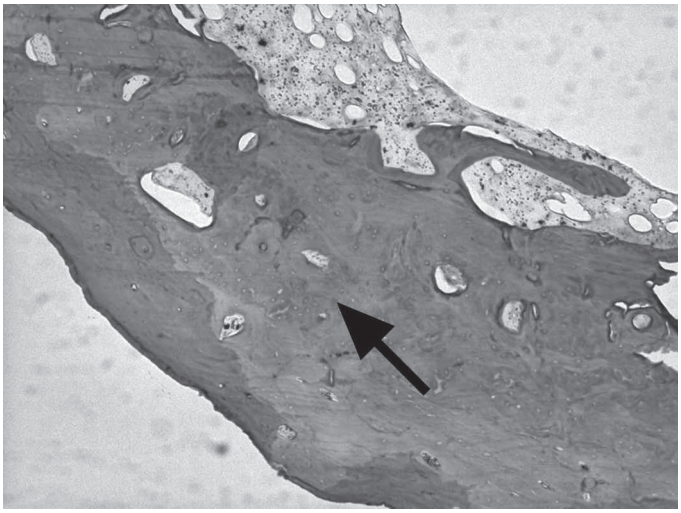

Figure 3 – Image showing the characteristics of an area treated with autogenous bone after 12 weeks, stained with picrosirius, at 50x magnification. The arrow shows the normal neoformed bone

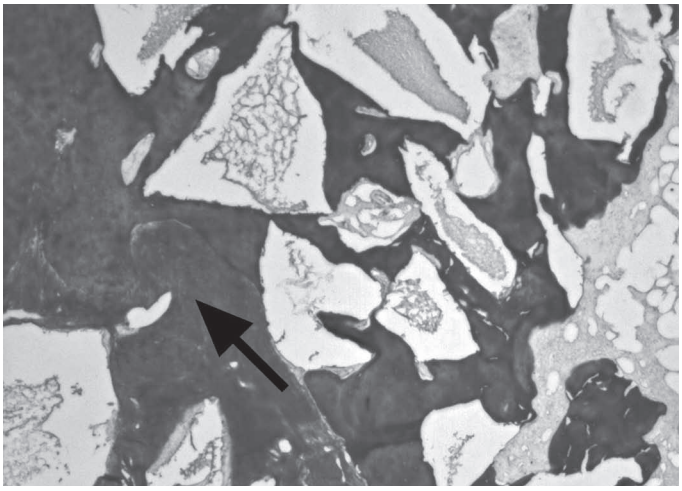

Figure 4 – Image showing the characteristics of an area treated with Genphos® after 12 weeks, stained with picrosirius, at 50x magnification. The arrow shows the normal neoformed bone

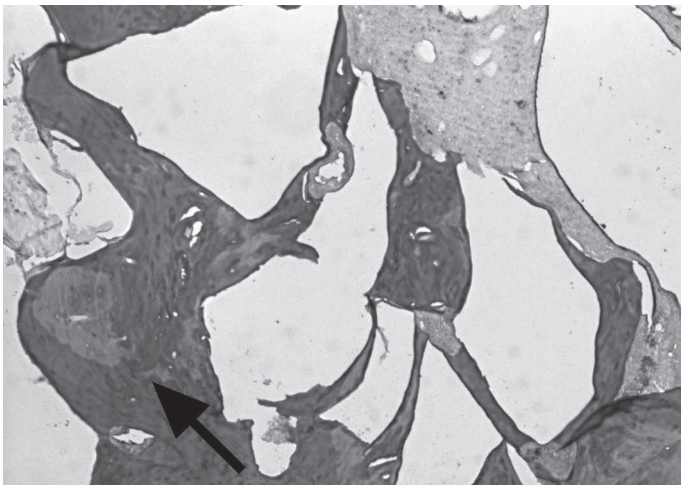

Figure 5 – Image showing the characteristics of an area treated with hydroxyapatite (HA) after 12 weeks, stained with picrosirius, at 50x magnification. The arrow shows the normal neoformed bone

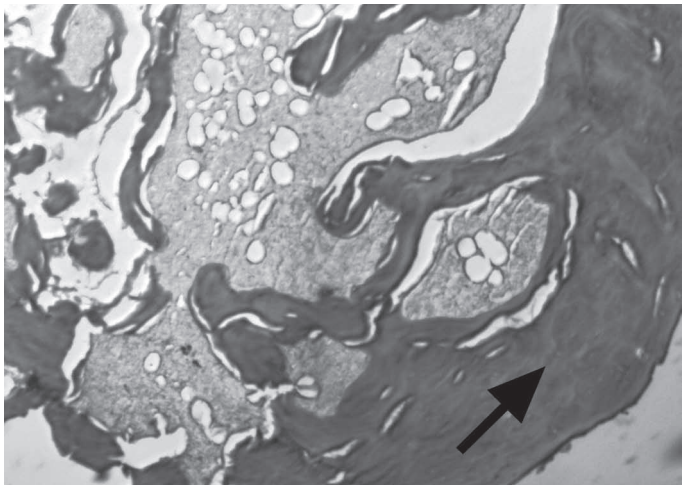

Figure 6 – Image showing the characteristics of an area treated with *Genmix*® bone after 12 weeks, stained with picrosirius, at 50x magnification. The arrow shows the normal neoformed bone

RESULTS

None of the rats used died after the procedure or during the experiment. Despite all the animals appearing healthy – none showed restrictions in their movement – dissection of the fractured femurs revealed three fractures in the six week group and five fractures in the 12 week group (Table 1), probably caused by the large size of the cavities made and the fact that the lesions were performed in both femurs.

The remaining losses were due to failures in decalcification or in preparation of the slides, thus having no means by which to be analyzed, and were therefore excluded.

The mean values of bone neoformation and their respective standard deviations are shown in Table 2 and Figure 7.

DISCUSSION

There are currently numerous bone substitutes on the market (HA, ß-TCP, bioglass) with many different features to try to replace autogenous bone. The companies that make them use strong marketing to try to convince the consumer that this or that produces better results and that, as if it were obvious, their product is of a superior quality to those of other companies.

Good studies on many of these materials are still scarce and inconclusive. Normally, only two or three different materials are compared and rare are the experiments that compare several groups of substitutes with the current gold standard, the autogenous bone graft.

In addition to autogenous bone, we tested a ß-TCP + HA (*Genphos*®) and a resorbable HA (Bionnovation)

Table 1 – Distribution of animals per group, losses, and treatment areas analyzed

Group	Treated areas	Number and causes of losses	Analyzed treatment areas
6 semanas	24 (6 x 4)	3 femur fractures; 2 decalcification and slide preparation problems.	4 Autogenous 5 Genphos® 4 HA 4 GenMix® 17 TOTAL
12 semanas	32 (8 x 4)	5 femur fractures; 4 decalcification and slide preparation problems.	5 Autogenous 5 Genphos® 5 HA 5 GenMix® 20 TOTAL

Table 2 – Variation in mean ± SD for the rate of bone neoformation in the two periods analyzed

Time	Type of graft	n	Mean	Standard deviation
6 weeks	Autogenous	4	90.5	10.8
	Genphos®	5	46.0	7.1
	Resorbable HA	4	43.1	8.4
	GenMix®	4	57.3	4.5
	Total	17		
12 weeks	Autogenous	5	98.0	9.1
	Genphos®	5	47.8	11.1
	Resorbable HA	5	39.9	5.4
	GenMix®	5	59.7	4.8
	Total	20		

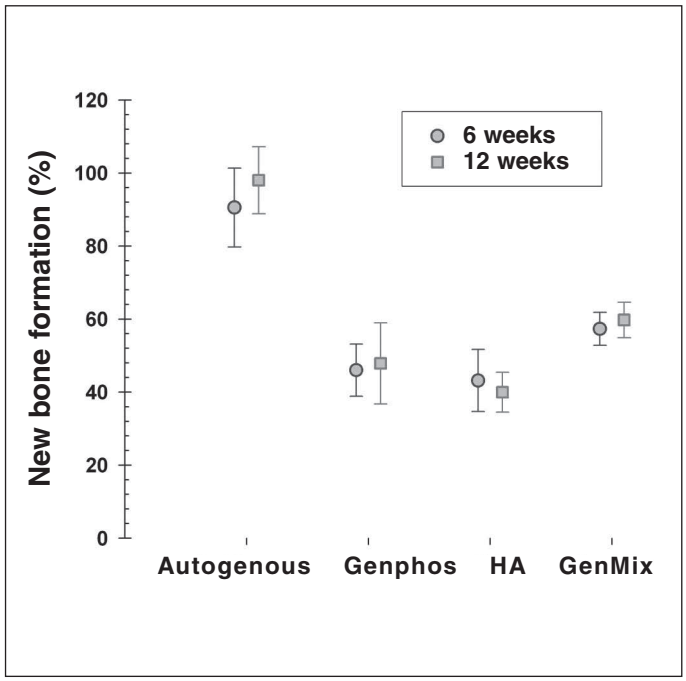

Figure 7 – Graph of means and standard deviations representing the values of new bone formation between the study groups

– two alloplastic grafts that would have only osteoconductive characteristics – and *GenMix*®, an example of a xenograft, which could theoretically have osteoconductive characteristics, because of its inorganic matrix, and osteoinductive characteristics, because of its organic matrix, which would, on the other hand, theoretically decrease its carrying capacity[9].

The allografts derived from synthetic HA (and its variations) are highly biocompatible and appear to have biological responses similar to those of bone[9]. The results in the literature depend on various formulations and are still inconclusive as to the quantity and quality of neoformed bone[6-8,10-14]. By changing its composition (such as the addition of ß-TCP) and its method of production, the speed of resorption of HA, which naturally occurs very slowly, could be modified[8,9].

Xenografts have the great advantage of having the greatest similarity with natural bone – when analyzing its inorganic component (varying in crystallinity and morphology according to the method of preparation) [15] – and could, in theory, retain osteoinductive features in their inorganic component[6,9], which would supposedly have greater immunogenic potential[3]. With the association of the two components, *GenMix*® would either combine the benefits of the two or would have its osteoinductive capacity and/or support affected by this mixture[6,10,13,16].

Human and rat osteoclasts show a similar pattern of resorption. Thus, the animal model used in this study would be valid for the study of the resorption of bone substitutes[17].

At all times analyzed, the area treated with autogenous bone was even difficult to locate because of its excellent regeneration (Figure 2); in all other materials the presence of the inorganic portion of their granules is quite noticeable, and there is none that has been completely reabsorbed. These findings can be confirmed by the histological results (Figures 3, 4, 5, and 6), and may also be seen on x-ray images (Figure 8) taken after dissection of the femurs.

There were no areas with complete graft failure, and the materials served to maintain the relative volume of the treated area. The question to be explored should really be about the amount of normal bone present in these areas and the quality of bone resulting from this treatment. In autogenous bone, on the other hand, there was even a thickening in some areas in relation to the control image, which explains why some values are above 100% in the statistics.

In conclusion, at both times analyzed (six and 12 weeks) and the model tested, autogenous bone graft showed a mean percentage of new bone formation far superior to that of the tested bone substitutes. Among the replacements, the only statistically significant differences were found between the groups with HA and *GenMix*® at 12 weeks, with p = 0.007 (Table 3).

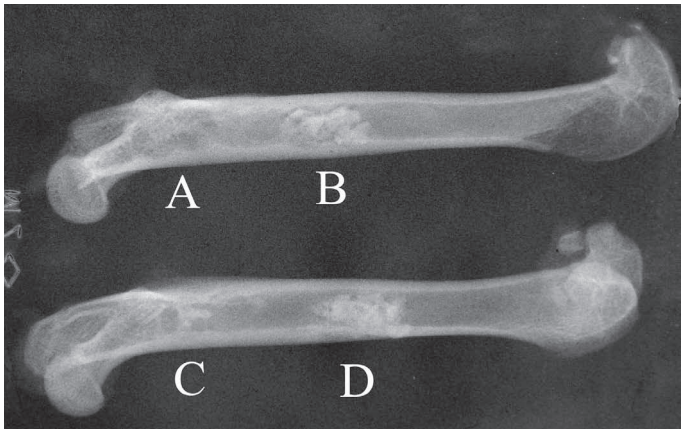

Figure 8 – X-ray showing radiological findings after 12 weeks: A) Autogenous, B) HA (Bionnovation), C) *GenMix*®, D) Genphos®

Tabela 3 – Statistics

Dependent variable: mperc Tukey HSD

Time	(I) group	(J) group	Mean difference (I-J)	Standard deviation	Sig.	95% confidence interval	
						Maximum value	Minimum value
6	1	2	44.54885*	5.37275	.000	28.7792	60.3185
		3	47.37370*	5.66338	.000	30.7511	63.9963
		4	33.22033*	5.66338	.000	16.5977	49.8430
	2	1	–44.54885*	5.37275	.000	–60.3185	–28.7792
		3	2.82485	5.37275	.951	–12.9448	18.5945
		4	–11.32852	5.37275	.201	–27.0981	4.4411
	3	1	–47.37370*	5.66338	.000	–63.9963	–30.7511
		2	–2.82485	5.37275	.951	–18.5945	12.9448
		4	–14.15337	5.66338	.107	–30.7760	2.4693
	4	1	–33.22033*	5.66338	.000	–49.8430	–16.5977
		2	11.32852	5.37275	.201	–4.4411	27.0981
		3	14.15337	5.66338	.107	–2.4693	30.7760
12	1	2	50.13669*	5.10926	.000	35.5190	64.7544
		3	58.07482*	5.10926	.000	43.4571	72.6925
		4	38.27371*	5.10926	.000	23.6560	52.8914
	2	1	–50.13669*	5.10926	.000	–64.7544	–35.5190
		3	7.93813	5.10926	.431	–6.6796	22.5558
		4	–11.86298	5.10926	.134	–26.4807	2.7547
	3	1	–58.07482*	5.10926	.000	–72.6925	–43.4571
		2	–7.93813	5.10926	.431	–22.5558	6.6796
		4	–19.80111*	5.10926	.007	–34.4188	–5.1834
	4	1	–38.27371*	5.10926	.000	–52.8914	–23.6560
		2	11.86298	5.10926	.134	–2.7547	26.4807
		3	19.80111*	5.10926	.007	5.1834	34.4188

*. The mean difference is significant at .05.

CONCLUSIONS

At both times analyzed, autogenous bone graft showed a mean percentage of new bone formation far superior to that of the tested bone substitutes.

REFERENCES

1. Bostrom MP, Seigerman DA. The clinical use of allografts, demineralized bone matrices, synthetic bone graft substitutes and osteoinductive growth factors: a survey study. HSS J. 2005;1(1):9-18.
2. Burg KJ, Porter S, Kellam JF. Biomaterial developments for bone tissue engineering. Biomaterials.2000;21(23):2347-59.
3. Lane JM, Yasko AW, Tomin E, Cole BJ, Waller S, Browne M, *et al*. Bone marrow and recombinant human bone morphogenetic protein-2 in osseous repair. Clin Orthop Relat Res.1999;(361):216-27.
4. Pollock R, Alcelik I, Bhatia C, Chuter G, Lingutla K, Budithi C, *et al*. Donor site morbidity following iliac crest bone harvesting for cervical fusion: a comparison between minimally invasive and open techniques. Eur Spine J. 2008;17(6):845-52.
5. Öztürk A, Yetkin H, Memis L, Cila E, Bolukbasi S, Gemalmaz C. Demineralized bone matrix and hydroxyapatite/tri-calcium phosphate mixture for bone healing in rats. Int Orthop. 2006;30(3):147-52.
6. Soost F, Koch S, Stoll C, Amthauer H, Grosse-Siestrup C, Zorn P. Validation of bone conversion in osteoconductive and osteoinductive bone substitutes. Cell Tissue Bank. 2001;2(2):77-86.
7. Fariña NM, Guzon FM, Pena ML, Cantalapiedra AG. In vivo behaviour of two different biphasic ceramic implanted in mandibular bone of dogs. J Mater Sci Mater Med. 2008;19(4):1565-73.
8. Spector M. Basic principles of tissue engineering. In: Lynch SE, Genco RJ, Marx RE, editors. Tissue engineering: applications in maxillofacial surgery and periodontics. Chicago: Quintessence Pub. Co.; 1999. p.3-16.
9. Giannoudis PV, Dinopoulos H, Tsiridis E. Bone substitutes: an update. Injury. 2005;36(Suppl 3):S20-7.
10. Rodrigues CV, Serricella P, Linhares AB, Guerdes RM, Borojevic R, Rossi MA, *et al*. Characterization of a bovine collagen-hydroxyapatite composite scaffold for bone tissue engineering. Biomaterials. 2003;24(27):4987-97.
11. Nair MB, Suresh Babu S, Varma HK, John A. A triphasic ceramic-coated porous hydroxyapatite for tissue engineering application. Acta Biomater. 2008;4(1):173-81.
12. Allegrini S Jr., Yoshimoto M, Salles MB, Konig B Jr. The effects of bovine BMP associated to HA in maxillary sinus lifting in rabbits. Ann Anat. 2003;185(4):343-9.
13. Busenlechner D, Tangl S, Mair B, Fugger G, Gruber R, Redl H, *et al*. Simultaneous in vivo comparison of bone substitutes in a guided bone regeneration model. Biomaterials. 2008;29(22):3195-200.
14. Ye F, Lu X, Lu B, Wang J, Shi Y, Zhang L, *et al*. A long-term evaluation of osteoinductive HA/beta-TCP ceramics in vivo: 4.5 years study in pigs. J Mater Sci Mater Med. 2007;18(11):2173-8.
15. Accorsi-Mendonca T, Conz MB, Barros TC, de Sena LA, Soares Gde A, Granjeiro JM. Physicochemical characterization of two deproteinized bovine xenografts. Braz Oral Res. 2008;22(1):5-10.
16. Kasten P, Luginbuhl R, van Griensven M, Barkhausen T, Krettek C, Bohner M, *et al*. Comparison of human bone marrow stromal cells seeded on calcium-deficient hydroxyapatite, beta-tricalcium phosphate and demineralized bone matrix. Biomaterials. 2003;24(15):2593-603.
17. Monchau F, Lefevre A, Descamps M, Belquin-myrdycz A, Laffargue P, Hildebrand HF. In vitro studies of human and rat osteoclast activity on hydroxyapatite, beta-tricalcium phosphate, calcium carbonate. Biomol Eng. 2002;19(2-6):143-52.