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Bone Augmentation Potential in Rabbit Calvaria and *Ex Vivo* Cytotoxicity of Four Bone Substituting Materials

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Abstract

Background and aims. Tricalcium Phosphate (TCP), Bovine-Derived Hydroxyapatite (BioOss™), Demineralised Freeze-dried Bone Allograft (DFDBA) and Calcium Sulphate (CaS) were compared in vitro for osteoblast cytotoxicity and in rabbit's calvaria to measure the bone histopathologic response.

Materials and methods. 34 critical size defects in the calvaria of 12 male Australian rabbits were randomly filled with the materials and 2 empty defects were used as controls. After one month, histologicalal evaluation was performed on the samples to record regenerated bone type and volume, material absorption and the amount of inflammation. Saos-2 cell line was exposed to the materials and the cell line vitality was tested with Methyl Tetrazolium Test (MTT) to determine material's osteoblast cell cytotoxicity.

Results. The type of regenerated bone did not show a significant difference between the groups ($p=1.0$) while the amount of bone inflammation was significantly different ($p=0.021$), where BioOss caused the least and DFDBA had the highest. Bone formation was also similar between the groups ($p=0.428$). DFDBA group showed the highest material absorption while TCP group had the lowest ($p=0.028$). DFDBA was associated with significantly higher Saos-2 cell line viability than TCP and Bio-Oss that were significantly less cytotoxic comparing to CaS ($p<0.0001$).

Conclusions. DFDBA group had the highest amount of material absorption and was associated with more inflammation than other materials in the rabbit calvaria. BioOss exhibited lowest amount of inflammation and TCP had the lowest amount of material absorption. Results of cytotoxicity test might be affected by different solubility constants of the test materials.

Key words: **Bone augmentation, bone substituting materials, osteoconduction, osteoinduction, rabbit calvaria.**

Introduction

For years, the regeneration of damaged or lost jaw bone tissue has been one of the major endeavors in periodontal and maxillofacial surgery.[1] Bone regeneration techniques constitute a valid surgical procedure for increasing bone quality and quantity in areas where insufficient bone volume prevents the stabilization of osteointegrated implants. Biomaterials for stimulating osseous regeneration should combine osteogenic, osteoconductive and osteoinductive properties. They should also resorb and be replaced by newly formed bone.[2] As previous studies have demonstrated, although autografts are the most suitable bone augmentation materials, their limited availability in the oral tissues and their high rate of post surgical morbidity has motivated investigators to utilize other biomaterials in bone regeneration.[3]

Allogenic biomaterials such as dematerialized freeze-dried bone allograft (DFDBA) are an alternative for autografts. There are controversies about allografts, including their osteoinductive properties.[4] There is also concern for cross-infection of pathologic factors such as prions.[5] DFDBA has been utilized previously to treat periodontal defects.[6] It has been suggested that DFDBA has osteoinductive ability.[7] It has also been shown that DFDBA is a suitable alternative for autogenous bone grafts in the treatment of bone defects.[8]

Xenogenic grafts (grafts shared between different species, such as bovine porous bone mineral or a natural coral) such as BioOss™ offer another alternative since the risk for cross-contamination can be easily minimized.[9,10] The rough topography of BioOss assists with osteoblastic anchorage, proliferation and synthesis of bone matrix on its surface.[10] BioOss has been shown to be similar to the human bone hydroxyapatite as it contains a calcium/phosphate proportion similar to bone hydroxyapatite.[11]

Synthetic calcium phosphate materials such as hydroxyapatite (HA) and tricalcium phosphate (TCP) have also been introduced and used for bone substitution; however, these groups of materials only have osteoconductive properties, which have been shown to be more rapid for HA than for TCP,[12] while the material resorption is slower for HA.[13] These materials have excellent biocompatibility; [14] they present no immunologic or infectious problems;[15] and have been suggested as an alternative to autogenous bone graft in repair of bony defects.[16]

Calcium sulphate (CaS) is one of the oldest biomaterials used in medicine, but few studies have utilized it for bone augmentation. CaS has been used for repairing the defects under sinus floor. This substitute has shown fast resorption, bone compatibility, and rapid bone remodeling.[17]

According to previous studies, a desirable characteristic of bone substituting materials is their ability to be remodeled, i.e. the biomaterial is absorbed by osteoclasts and is replaced by newly formed bone through osteoblastic activity,[18,19] and thus biomaterials are designed to have resorption speed that match bone growth rate.[20]

The established human osteosarcoma (Saos-2) cells represent a highly differentiated cell line capable of inducing bone formation and, therefore, creating a model for studying bone cell behavior,[21] that has been widely used to evaluate the effect of different biomaterials on the bone tissue *in vitro*.[22,23] Among osteoblastic cell lines, Saos-2 is considered to be a mature type of cell and like many traits of human osteoblasts, they produce high concentration of alkaline phosphatase (ALP) and they retain the mineralization ability.[24] The methyl tetrazolium test (MTT) is a simple colorimetric assay developed by Mosmann[25] as a test for cell proliferation and viability that has been adapted for the measurement of cytotoxicity. This assay involves the ability of viable cells to convert a soluble tetrazolium salt of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) into a blue formazan end product by mitochondrial dehydrogenase enzymes. The blue color reaction is used as a measure of cell viability and is analyzed by a spectrophotometer.[26] In other words, MTT assay focuses on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble tetrazolium salt into dark-blue formazan crystals that is stored in the cytoplasm of living test cells; thus the amount of formazan formed is directly proportional to the mitochondrial enzyme activity in a given cell line.[27]

The aim of the present study was to evaluate and compare the bone histopathological response to BioOss, TCP, DFDBA and CaS in the rabbit calvaria. Using the *in vitro* model, cytotoxicity of these materials was also measured.

Materials and Methods

In vivo experiment

Eight male Australian rabbits, 12 weeks old and weighing approximately 2500 ± 200 gr were used in this study. The ethics committee of Shaheed Beheshti University of Medical Sciences approved the protocol of this study. Animals were monitored for 2 weeks while they were on a standard diet mix (containing approximately 25% wheat). The cage settings

were set for 24°C, 55% humidity, and 1 atmosphere pressure and 12-hour light/dark cycle.

Graft materials

Materials used in this study were TCP (Cerasorb®, Curasan, Kleinostheim, Germany), BioOss (BioOss® Spongiosa, Geistlich Pharma, Switzerland), CaS (E. Merck AG Darmstadt, Germany) and DFDBA (Iran Tissue, Tehran, Iran). One gram of every material was prepared according to manufacturer's protocol. If not specified, materials were mixed with 1:1 ratio of sterile normal saline solution.

Anaesthesia and surgery

The animals were sedated by an intramuscular injection of Ketamine: Xylazine (35:5 mg/kg). The forehead of the rabbit was shaved and 1.8 ml lidocaine HCl containing 1:50.000 epinephrine was used as the local anesthetic agent and to control the bleeding.

A trapezoidal flap was then elevated to expose the calvaria from the forehead. Using coronal and sagital sutures as guidelines, four 6-mm-in-diameter critical-sized through-and-through defects were created adjacent to the frontal fontanel. Attention was made to prevent any meningeal membrane damages during drilling. After hemorrhage was controlled, every defect was randomly filled with one gram of the test materials (n=6 for every material). Six defects were not filled to be used as controls (Figure 1). The periosteum and the flaps were sutured back over the defects. Rabbits were then taken care of with standard diet for thirty days.

Specimen preparation

One month after initial operation, the animals were euthanized with an overdose of pentobarbital. The calvarium bone was dissected out and was placed in 10% neutral buffer formalin solution for 5 days. Fixed bone samples were dehydrated in an ascending series of alcohol rinses and in order to increase their translucency, they were placed in 50% and 100% solutions of methyl salisilate for 2 and 5 hours, respectively. The blocks were then embedded in paraffin. Transverse cross-sections with the diameter of 5-μm were made using a microtome device (Jung, Frankfort, Germany) through each hole and numbered from outside in. The section passing through the center of the lesion was used as the representative section for each lesion.

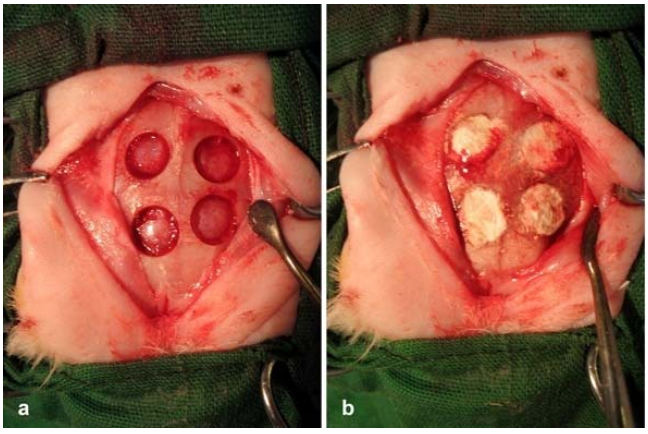

Figure 1. 6-mm-in-diameter through-and-through defects were created alongside the calvarial sutures (a). Defects were randomly filled with different test materials (b).

Histological analysis

The representative section for each defect was stained with hematoxilline and eosine. The specimens were observed with an optical microscope, linked through a digital camera to a personal computer equipped with an image capturing software. To avoid possible bias, coded samples were used in this study. All of the samples were evaluated by one examiner to determine the regenerated bone type (lamellar vs. Woven), the amount of bone regeneration (the percentage of bone trabeculae comparing to the total observed area), the amount of inflammation (mild, moderate or severe) and the presence of inflammatory cells (macrophages, lymphocytes, monocytes, foreign body giant cells and plasma cells).

Statistical analysis

SPSS 16.0 statistical software (SPSS, Inc, Chicago, IL, USA) was used for data analysis. ANOVA with post hoc Scheffe test was used for parametric data and Kruskal-Wallis statistical test was used for non-parametric data. A p-value < 0.05 was considered statistically significant.

In vitro experiment

Material preparation

One-mm of every material was ground down to fine particles with average diameter of 10 μm after initial preparation according to manufacturer's protocol. The obtained powder was then mixed with 5 ml of RPMI media 1640 (Gibco® cell culture systems, Invitrogen Ltd, Paisley, UK). The mixtures were then placed in a 37°C incubator with humidified 5% CO_2-95% air for a period of 72 hours.

Preparation of the cells

The Saos-2 osteoblast-like cells (Institute of Pasteur, Tehran, Iran) were grown in RPMI medium with 10% FBS, 100 μg/ml Penicillin, 10 μg/ml Strepto-

mycin and 2 mMol Glutamine, according to International Standard for Biological Testing of Medical Devices (ISBTMD). The cells were grown in 24-well plates at 37°C incubator with humidified 5% CO_2-95% air atmosphere for a period of 72 hours. 1×10^5 cells were seeded in each well.

After 72 hours, 1 ml of the RPMI solution containing the extract of the materials was added to each well (10 wells for each material) and the wells were placed in the incubator for 24 hours. One milliliter of cold sterile distilled water and complete medium culture (CMC) were used as positive and negative controls, respectively.

24 hours later, the solution within each well was replaced with 1 ml of CMC medium and the plates and the vitality of the cells was tested with MTT.

The MTT test

In order to perform the MTT test, 100 µl of MTT (5mg/ml in PBS) was added to each well. The plates were then incubated for 3 hours at 37°C. Then the supernatant was carefully removed and 120 µl acidic isopropanol (containing 0.04 N HCl) was added to each well. After gentle shaking for dissolving the formazan crystals, 100 µl of the blue solution was transferred to a 96-well plate and the optical density was read in the microplate-reader, using a test wavelength of 540 nm.[26]

Samples were omitted from the study due to improper responses to tests. ANOVA with post hoc Scheffe test was used for data analysis.

Results

In vivo experiment

All the animals survived the follow-up period and no complications due to the surgical procedure were noted. In all the defects filled with the test materials, bone formation was observed subjectively, while the control defects showed no sign of bone formation.

Histological evaluation

The results of the histological evaluations are demonstrated in Tables 1 & 2. The type of regenerated bone in the defect area did not show any significant difference between groups, according to the Fisher's exact test (p= 1.0). The amount of the inflammation was considerably different between the groups according to the Kruskal-Wallis test (p=0.021). BioOss (Figure 2a) had the least amount of inflammation while DFDBA (Figure 2e) was associated with the highest amount of inflammation (Table 1).

Homogeneity of variance of groups for bone regeneration and material absorption was confirmed using Leven's test (p=0.236 and 0.062, respectively). Normal distribution of data was confirmed with Kolmogorov-Smirnov test for bone regeneration and material absorption (p=0.582 and 0.261, respectively). According to the ANOVA test, there was no significant difference between groups in terms of bone regeneration (p=0.428) while there was a significant difference in the amount of material absorption between groups (p=0.028). Post hoc Scheffe test indicated that TCP had significantly less material absorption and DFDBA had the highest amount of material absorption (Table 2).

Table 1. Bone type and amount of inflammation in different groups compared using Kruskal-Wallis test

Index	Category	TCP (n=6)	BioOss (n=6)	CaS (n=6)	DFDBA (n=6)	p value
Bone Type	**Woven**	5(80%)	5(80%)	5(80%)	5(80%)	1.0
	Lamellar	1(20%)	1(20%)	1(20%)	1(20%)	
Inflammation	**Mild**	4(66.7)	6(100%)	2(33.3%)	1(16.7%)	0.021
	Moderate	2(33.3%)	0(0%)	2(33.3%)	3(50%)	
	Severe	0(0%)	0(0%)	2(33.30%)	2(33.3%)	

Table 2. Mean and standard deviation of bone regeneration and material absorption was compared using ANOVA test

Index	Material	N	Mean	Std. Deviation	Minimum	Maximum	p-value
Bone regeneration (%)	**TCP**	6	19.66	8.98	5.00	30.00	0.428
	BioOss	6	28.33	16.93	5.00	50.00	
	DFDBA	6	15.83	9.70	5.00	30.00	
	CaS	6	29.16	24.57	5.00	75.00	
Material Absorption (%)	**TCP**	6	28.66	13.73	10.00	50.00	0.028
	BioOss	6	40.83	10.68	25.00	50.00	
	DFDBA	6	61.66	11.25	50.00	75.00	
	CaS	6	46.66	27.86	10.00	90.00	

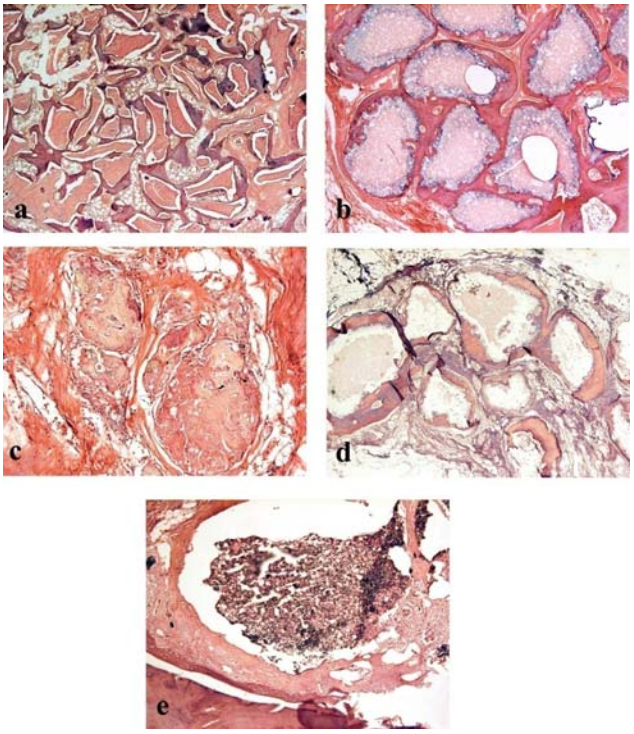

Figure 2. Test groups after 30 days. (a) BioOss; new bone formation can be seen around BioOss particles; (b) TCP; Compact bone formation can be seen around the particles; (c) CaS; woven bone formation can be seen.(d) DFDBA; bone formation and inflammation is present within the defect (e) Control; no bone formation can be seen.

In vitro Experiment

Saos-2 cell viability after incubation with test material extracts for 24 hours was measured using the MTT viability test. Original optical density values of the test cultures were initially multiplied by 10^3. Anova with post hoc Scheffe test was used to measure the differences between groups (Table 3).

The DFDBA group showed the highest biocompatibility with Saos-2 osteosarcoma cells ($p<0.0001$) and the CaS group the least ($p<0.002$), while the TCP and BioOss groups did not had a significant difference regarding this matter ($p=1.0$), although their biocompatibility with Saos-2 cells was significantly less than DFDBA and more than Cas.

Discussion

According to previous studies a 4-week period of implantation is sufficient for observing bone formation in several animal models, like rabbits, at the defect side that experimental biomaterials are grafted.[28-30]

Other authors claim that biomaterials with slow *in vivo* resorption can interfere with bone growth instead of enhancing it; however, we could not observe this effect in our study because the control samples always had much less bone augmentation than the test groups.[31-34]

The results of this study demonstrate that BioOss is the most promising biomaterial for clinical situations in which bone formation is desired. Successful bone regeneration through biomaterials must meet certain biologic principles, i.e., biomaterial properties, wound stabilization and sufficient space making. Tamura et al[19] showed that porous β-TCP block is a promising biomaterial for clinical situations requiring bone formation.

In another comparative study between BioOss and a novel β-TCP for bone formation in rabbit calvaria,[2] it was concluded that the novel cement was resorbable and generated more bone tissue than BioOss. Taylor et al[34] considere BioOss a non-resorbable biomaterial as this substitute needs several years (3-6 years) of implantation before showing some slow resorption through osteoclasts activity. Although in our study the amount of material resorption was different, the capability of bone formation did not show any significant differences between the bone substitutes. This indicates that material resorption could not substantially affect bone formation capacity.

DFDBA in our study showed the highest amount of inflammation; however, the amount of bone formation was adequate in comparison with the other materials.

The question of whether results from *in vitro* ex-

Table 3. Mean optical density readings of the MTT assay as a measure for Saos-2 cell viability after incubation with test material extracts for 24 hours was compared

Test Material	Repeats	Mean	Standard Deviation	95% Confidence Interval for Mean	
				Lower Bound	Upper Bound
Negative control	10	380.70	33.38	356.81	404.58
DFDBA	9	334.55	32.86	309.29	359.81
BioOss	9	195.44	19.31	180.59	210.29
TCP	7	191.85	5.78	186.50	197.20
CAS	9	148.00	13.38	137.70	158.29
Positive control	10	113.50	22.05	97.72	129.27

periments can be applied to the clinical situation remains to be investigated. There is evidence that *in vitro* methods adequately measure cytotoxicity and, therefore, could reasonably be used as a screening tool to evaluate biocompatibility of test materials.[35] The results of such *in vitro* cytotoxicity tests may not highly correlate with the *in vivo* data. However, it is safe to say that if a test material consistently induces a strong cytotoxic reaction in cell culture tests, it is also very likely to exert toxicity in living tissue. A reduction in the number of animal tests and resulting expenses might be additional benefits of such a screening approach. [35]

In this study, we used material extracts instead of the materials themselves. The reason behind this decision was made as it has been reported that when the model cells are challenged with the extracts, the behavior is very close to that recorded with the corresponding solid samples, except that the cells are allowed to function and to grow better in contact with the extracts, comparing to the solids.[25]

Cytotoxicity assays are the initial screening tests in assessing biocompatibility of a material. Such assays may not correlate highly with *in vivo* data.[36] Preparation of the test materials to be studied has crucial importance in these studies and is a subject of debate.[37,38] Materials obtained from the dissolution in the culture medium have commonly been tested. This limits the investigations to those substances that are water-soluble, thereby eliminating non-water-soluble substances from the test. Solubilizing test materials using Tween-80 or alcohol that has been suggested by some authors, carries the risk of altering the test material in an unpredictable manner.[39] It has been suggested that caution is also necessary in selecting toxicity parameters, because materials consisting mostly of hydrophilic components are likely to create changes in the intracellular enzyme activities involved in energy production and consumption at lower concentrations than those at which they will influence membrane permeability. The converse may be true for lipophilic substances, as they will disrupt the integrity of the lipid bilayer of a cell membrane, probably at lower concentrations than they will modify the activity of a mitochondrial enzyme. Thus, comparison of the results achieved from experiments using different designs should be done with great care, because the results may reflect the procedure used.[40]

The results of this study showed that CaS had the highest cytotoxicity and the DFDBA the lowest. As noted, along with the cytotoxicity of the materials that can alter the viability of Saos-2 cells, the solubility of the materials can impact the final results. The solubility constant of the materials tested in this study differ and this can have an impact on the final results of the study. How much this factor influences the final results needs to be further investigated. These conclusions are in accordance with a study performed by Ignatius et al[40] that suggest the toxicity of the materials depends on their solubility.

In summary, the viability of Saos-2 cells in contact with extracts of four osteoconducting materials was mostly affected by CaS followed by TCP, BioOss, and DFDBA that showed the least cytotoxicity. Due to limitations of this *in vitro* study and the effect of different solubility constant of the materials on the final results, further *in vitro* studies should be performed for complete evaluation of the cytotoxicity and biocompatibility of these materials.

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