Original Article

Comparing the Efficacy of Three Different Nano-scale Bone Substitutes: *In vivo* Study

Abstract

Background: Synthetic biocompatible bone substitutions have been used widely for bone tissue regeneration as they are safe and effective. The aim of this animal study is to compare the effectiveness of three different biocompatible bone substitutes, including nano-hydroxyapatite (nano-HA) nano-bioglass (nano-BG) and forstrite scaffolds. **Materials and Methods:** In this interventional and experimental study, four healthy dogs were anesthetized, and the first to fourth premolars were extracted in each quadrant. After healing, the linear incision on the crestal ridge from molar to anterior segment prepared in each quadrant and 16 defects in each dog were prepared. Nano-HA, nano-BG, and forstrite scaffold was prepared according to the size of defects and placed in the 12 defects randomly, four defects remained as a control group. The dogs were sacrificed in four time intervals (15, 30, 45, and 60 days after) and the percentage of different types of regenerated bones (lamellar and woven) and connective tissue were recorded in histological process. The data were analyzed using Mann–Whitney test ($\alpha = 0.05$). **Results:** The difference in nano-HA and nano-BG with the control group was significant in three-time intervals regarding the amount of bone formation ($P < 0.01$). After 15 days, the nano-HA showed the highest amount of woven and lamellar bone regeneration (18.37 ± 1.06 and 30.44 ± 0.54). **Conclusion:** Nano-HA and nano-BG groups showed a significant amount of bone regeneration, especially after 30 days, but paying more surveys and observation to these materials as bone substitutes seem to be needed.

Keywords: *Bone regeneration, nano-bioglass, nano-hydroxyapatite, forstrite*

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Access this article online

Website: www.advbiores.net

DOI: 10.4103/2277-9175.192627

Quick Response Code:

Introduction

Bone or massive tissue defects (caused by trauma, tumors, or degenerative disease, etc.) is still a major problem which needs sufficient bone with acceptable quality and quantity to be repaired.[1,2] As tissue engineering developed, many opportunity has been made for reconstruction bone defects. There are many patients who are just now discovering that they can use implants if their implant sites reconstructed.[3]

To achieve that goal, autogenously bone grafts have been examined for reconstructing bone defects. However, limited bone stocks, imposing additional surgical intervention, and following complications at the donor site are some of the major obstacles.[1,4] To conquer those limitations bone graft substitutes were introduced. Biocompatibility, bio-restorability, and osteogenicity are the most characteristic of an appropriate substitute.[5] Hence, allogeneic grafts was tested as alternatives such as irradiated bone from cadavers (bone mineral from bone banks) and demineralized bone matrix (bone mineral dissolved away using acids), but probably immunological cross-actions might have happened.[6]

To advance the properties, synthetic biocompatible bone substitutions have been used. they seem to be a safe and effective option in procedures such as filling with fracture defects and resolution of long bone nonunions but still some doubts remain.[5,7] They mostly consist of calcium, phosphate, ceramics, and organic materials which can provide a stable situation for supporting bone responses and reconstructions.[8] Although they fulfill the goal of repairing smaller defects, they are not appropriate for repairing extended bone defects.[9]

The examples are bioactive glass (BG) ceramics and calcium phosphate ceramics such as hydroxyapatite (HA), β tricalcium phosphate, or biphasic calcium phosphate.[10]

How to cite this article: Razavi SM, Rismanchian M, Jafari-pozve N, Nosouhian S. Comparing the Efficacy of Three Different Nano-scale Bone Substitutes: *In vivo* Study. Adv Biomed Res 2017;6:64.

Received: November, 2014. **Accepted:** August, 2015.

HA is an important component of the bone structure. Due to its biocompatibility and crystallographic structure HA is used to produce a synthetic bone substitute.[11,12] The efficacy of HA in the bone regeneration approved by studies.[13,14] Dense HA could not be degraded and remodeled in the host which was an obvious flaw for a suitable biomaterial[4,15] so nano-HA was introduced as a favorable bone substitute. Compared with micro-HA, this material is highly biocompatible and biodegradable and could be rapidly substituted by the host.[16-18]

BG is another popular bone substitute which has been emerged since 1971.[19] The capability of BG in stimulating osseointegration and forming new bone has been confirmed in different studies.[20-22] Their well surface characteristics induce the osteoblasts to generate an amorphous calcium phosphate layer which can turn to a biological hydroxycarbonate apatite and cause interfacial bonding.[19]

Gatti *et al*. examined the clinical use of BG in treating dental extraction sites before loading implants. New bone formation and biodegradation of the BG was observed.[23]

In recent years, researchers have declared that nano-bioglass (Nano-BG) has showed superiority over micro-bioglass in repairing bone defects.[24]

Using a porous scaffold with interconnected spaces provides sufficient room for cell migration and adhesion and the ingrowth of new bone tissue.[25] An ideal scaffold for bone regeneration would share loads with surrounded bone. They cannot do this when the bone defect is subjected to cyclic loads if they are brittle.[26] Forstrite (Mg_2SiO_4) is one of them. It is well biocompatible nano-composite with high bonding strength, fracture toughness and when combined with the polycaprolactone could result in a scaffold structure useful for bone tissue repair.[27-30] Tavangarian evaluated the bioactivity of forstrite, and their result revealed that Forstriete's mechanical properties make a good situation for load bearing applications in bone implant materials.[27] Based on Diba *et al*. conclusion the forsterite scaffold provides the most suitable morphology with 30% weight and steady structure.[29]

There are not enough animal studies with conclusive results regarding the efficacy of nano-particles. As forstrite is a newly invented biomaterial with nano-structural properties, and no study has compared the biological effect of forstrite with both nano-hydroxyapatite (Nano-HA) and nano-BG, this study was designed to compare the efficacy of nano-HA, nano-BG, and forstrite scaffold in repairing bone defects.

Materials and Methods

Surgical procedure

In this interventional and experimental study, four healthy male dogs aged 1–4 and weighed 32–46 kg were anesthetized initially with 10 mg/kg ketamine (ketamine HCL, Alfasan, Woerden, Holland) and 0.15 mg/kg rampone. Maintenance followed under general anesthesia by 5% halothane (Halothane, Bp, Nicholas Piramal India Limited, India) and N_2O. First to fourth premolars were extracted respectfully in each quadrant with the preservation of surrounding bone, according to Helsinki roles. Parallel periapical radiographs were taken with extension cone paralleling film holders (Rinn Co., USA) to evaluate the healing of tooth extraction sites in further 3 months.

After appropriate bone healing phase, 3.6 ml lidocain (Darou Pakhsh Pharmaceutical Mfg. Co. Tehran, Iran) was derived in the mucobuccal fold. Linear incision was prepared on the crestal ridge from molar to anterior segment and a full thickness muccoperiostal flap was elevated by a muccoperiostal elevator. Two defects with 5 mm depth and 5 mm diameter on the crestal ridge and two similar defects on the buccal surface of the ridge were surgically created using trephine #5. As a result, four defects in each quadrant and 16 defects in each dog were created overall. These 16 defects were randomly divided into four groups and filled with:

i. Nano-BG (CaNaO6PSi) (SCHOTT Vitryxx, Mainz, Germany) amorphous powder form with <250 nm (BET) particle size
ii. Nano-HA ($Ca_5(OH)(PO_4)_3$) (Sigma–Aldrich, NY, USA) amorphous powder form with <200 nm (BET) particle size, >9.4 m^2/g surface area
iii. Forstrite scaffold (Mg_2SiO_4) (New Nano, Isfahan University of Technology, Isfahan, Iran) with porosity of <83%, mean pore size of <25-45 nm
iv. Control group.

All the mentioned biomaterials were purchased from their exclusive company at least 1-year before the expiration date. The above nano-HA and nano-BG powders were mixed with distilled water according to the manufacturers' instructions of each material; the blocks of forstrite scaffold prepared according to the size of defects and rinsed with distilled water too. Finally, all of those materials were placed separately in different defects. Therefore, 16 defects in each dog were filled randomly by nano-HA, nano-BG and forstrite scaffold and four defects were left as a control group. Then, all the defects were covered by a nonresorbable membrane (PTFE Whatman, Kent, UK).

The dogs were sacrificed at four-time intervals (15, 30, 45, and 60 days, each dog at each time point). Fifteen days after the surgery, a lethal injection of 40 ml pentobarbital sodium at 100 mg/ml in 290 g/1000 ml spiritus fortis, 100 mg/kg was given to one of the dogs. All 16 samples were extracted using trephine #8 with sufficient amount of surrounding bone. These procedures were also carried out in 30, 45, and 60 days after the surgery.

Histological analysis

Extracted specimens were kept in glutaraldehyde solution for 6 h. Longitudinal ground sections were prepared using Microtome (Accutom-50, Stuers, Copenhagen, Denmark). The samples were stained by Hematoxylin and Eosin stain (H and E) and mounted on the histological lams. All stained specimens were investigated under an optical microscope (×40) (Ziess, Germany), and percentage of different types of regenerated bones (lamellar and woven), and connective tissue were recorded. The cross sections of specimens were surveyed by Adobe Photoshop 7.0 (SanJose, CA, USA) and the amount of the regenerated bone was re-evaluated to confirm data. Data analysis were performed by SPSS software version 15 (IBMCorporation, NY, USA) and Mann–Whitney test ($\alpha = 0.05$).

Ethics

This study was approved by the Animal Department of Torabinejad Dental Research Center and Local Ethical Committee of the Isfahan University of Medical Science.

Results

Using the Mann–Whitney test showed that the difference in nano-HA and nano-BG with the control group was significant in three-time intervals regarding the amount of bone formation ($P < 0.01$). Table 1 represents the hard tissue responses to the tested biomaterials at different intervals.

After 15 days, both nano-HA and nano-BG besides forsterite scaffold showed the significant difference with the control group in amount of woven bone production; also the results of nano-BG and forstrite scaffold had significant difference with nano-HA too ($P < 0.05$). The amount of lamellar was noticeable in forstrite and nano-HA with the control group ($P < 0.05$). The nano-HA showed the highest amount of woven and lamellar bone regeneration (18.37 ± 1.06 and 30.44 ± 0.54) [Table 1]. The mean percentage of connective tissue was the highest in control group (63.03 ± 0.83) and the difference between other groups was not significant ($P > 0.05$) except forstrite and control group with nano-HA ($P < 0.05$).

Table 1: The hard tissue regenerative response to different tested biomaterials at different intervals

Intervals	Groups	Mean±SD of woven bone	Mean±SD of lamellar bone	Mean±SD of connective tissue
15	Nano-HA	18.37±1.06	30.44±0.54	51.81±0.36
	Nano-BG	16.53±1.07	26.84±6.27	56.66±6.37
	Forstrite scaffold	15.87±0.83	25.22±3.84	58.97±4.18
	Control	15.37±0.50	21.59±0.66	63.03±0.83
30	Nano-HA	19.87±3.22	29.53±1.41	50.59±2.65
	Nano-BG	23.09±2.12	28.53±1.16	48.37±1.18
	Forstrite scaffold	18.28±2.35	26.56±6.68	54.78±8.63
	Control	16.50±0.71	22.37±3.44	61.12±3.81
45	Nano-HA	16.69±1.08	27.94±2.02	55.44±2.64
	Nano-BG	16.00±2.79	23.44±3.32	60.57±3.82
	Forstrite scaffold	16.37±1.24	25.22±3.28	58.37±2.61
	Control	16.81±1.64	25.81±2.75	57.37±1.47
60	Nano-HA	15.84±1.49	22.44±1.60	60.72±2.93
	Nano-BG	19.66±5.98	29.62±2.60	50.72±8.00
	Forstrite scaffold	14.53±1.31	26.66±4.25	58.19±5.66
	Control	18.78±2.48	25.44±5.27	57.03±5.70

SD: Standard deviation, Nano-HA: Nano-hydroxyapatite, Nano-BG: Nano-bioglass

After 30 days of the experiment, both nano-BG and nano-HA revealed a significant difference in the amount of connective tissue, woven and lamellar bone regeneration with the control group ($P < 0.05$). Another significance is between forstrite scaffold and nano-BG ($P < 0.05$). The nano-BG showed the highest rate of woven bone (23.09 ± 2.12) and nano-HA showed the highest rate of lamellar bone regeneration (29.53 ± 1.41). The percentage of connective tissue was the highest in the control group (61.12 ± 3.81) and the lowest in nano-BG (48.37 ± 1.18) [Table 1].

After 45 days, there was a significant lamellar bone difference between nano-HA and nano-BG groups ($P < 0.05$). The amount of lamellar bone production was the highest in nano-HA (27.94 ± 2.02) but the amount of connective tissue was the highest in nano-BG (60.57 ± 3.82) [Table 1]. The nano-HA reflected a significant difference with nano-BG and forstrite ($P < 0.05$), but the significance between nano-BG and control group should not be overlooked ($P < 0.05$).

After 60 days, the difference in woven bone production was significant among forstrite and nano-HA with the control group ($P < 0.05$) however they (forstrite and nano-HA) had discrepancies with each other ($P < 0.05$). Another significance was among forstrite and nano-BG with the nano-HA group in lamellar bone regeneration ($P < 0.05$). The amount of woven and lamellar bone was the highest in nano-BG (19.66 ± 5.93 and 29.62 ± 2.60). Also, the rate of connective tissue was the highest in nano-HA (60.72 ± 8.00) [Table 1].

It should be noted that the mean amount of woven regenerated bone reached to its highest rate in nano-BG group after 30 days. Furthermore, the highest rate of lamellar bone regeneration was after 60 days by seeding nano-BG. Furthermore, Figures 1 and 2 show the microscopic appearance of regenerated woven and lamellar bones in four groups after 15 and 45 days [Figures 1 and 2].

Discussion

Tissue engineering techniques currently have offered great fortune to meet the clinical need for bone substitutes. Besides that, there has been growing interest in using three-dimensional scaffolds for supporting bone regeneration.[6,31-33]

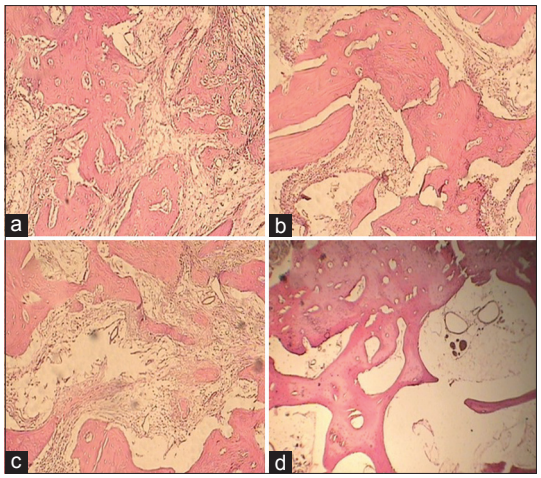

Figure 1: Histopathology of the samples after 15 days. (a) nano-bioglass, (b) nano-hydroxyapatite, (c) forstrite, (d) control

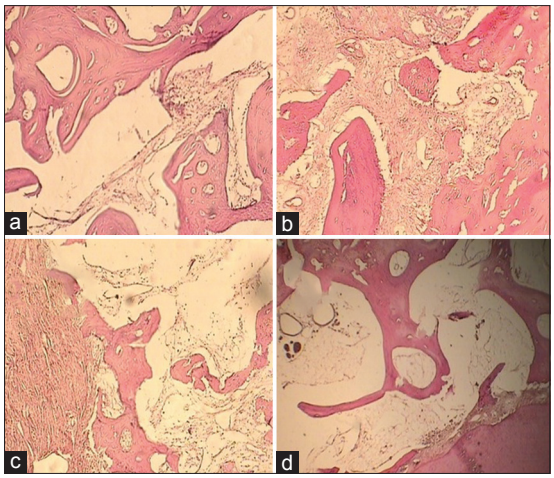

Figure 2: Histopathology of the samples after 45 days. (a) nano-bioglass, (b) nano-hydroxyapatite, (c) forstrite, (d) control

Forstrite is a nano-crystal bioceramic made of talc, alumina, and magnesium carbonate with not only lots of macropores but also plentiful micropores (on the scale of 1–10 μm) on the macroporous walls.[29]

Zamet, Froum, and Nandi studies approved the positive effects of bioglass on bone formation.[14,34,35]

Maybe it is needed to mention that in the initial stage of bone reconstruction, the matrix proteins can be attracted by nano-bone granules so a vascular rich protein matrix would be formed. In consequences, the ossteogenesis happens on this matrix, and the final bone is regenerated. As nano-scale structures provide a larger surface area, the amount of attracted proteins would be much more. This can be an explanation why the use of nano-materials can accelerate the bone regeneration. On the other hand, these larger surface areas pretend as biological materials.[36] Hence, the mechanical reliability and osseoconductivity could be improved, and the osteogenic differentiation of stem cells would be induced.[37,38] For instance, Huang *et al*. claimed in their results that the size (micro/nano) of HA is an important factor in stimulation of the osteogenic differentiation.[39]

Nano-hydroxyapatite

Based on present results, application of nano-HA provided promising results after 15, 30, and 45 days of reconstruction of bone defects which was in accordance to some studies. Götz *et al*. concluded that nano-porous HA materials show osteoconductive capacity and have the capability to integrate with the host bone.[40] In Schwarz study, different cases with intrabony defects around dental implants were investigated. They applied nano-crystalline hydroxyapatite and bovine-derived xenograft in combination with a collagen membrane (BDX + BG). The final result was repair of all bone defects repaired and reduction of pocket depth.[41] This result can be explained by osseocunductivity and biocompatibility of nano-HA bone material, and is in agreement with the Welch *et al*.,[42] den Boer *et al*.,[43] and Kruse *et al*.[44] studies. Also, the present study revealed that the use of nano-HA showed better result than nano-BG, which is somehow in accordance with Rodenas–Roshina J examination. They conclude that presence of bioactive particles is not necessary to reach the higher amount of bone regeneration.[31]

Nano-bioglass

Based on current results, the induced regenerated bone tissue by nano-BG was at second place after 15, 30, and 60 days. The bioactivity of BG is initiated exactly after mixing with saline or blood, and silicon oxide bonds break down. As a result, the silicic acid can be aggregated on the surface of particles and form a negatively charged gel. As time lasts, calcium hydroxide would be formed on these surfaces to form a new apatite layer which initiates the bioactivity of BG.[45] These can be a good reason why the formation of new bone revealed a significant difference in nano-BG compared to the control group. However, the means of regenerated bone tissue was not promising after 45 days which might be due to inappropriate implantation of the biomaterial. Azenha *et al*. concluded that new bone formation around the materials is influenced by their location within the bone.[46]

Forstrite scaffold

The published *in vivo* research about the bioactivity of forstrite is not extensive as it has been newly invented. Based on obtained results, the highest amount of regenerated bone tissue was only observed after 45 days, and at the other three time intervals it was at the third place of biomaterials ranking. That might be due to its degenerative properties and apatite formation which are not remarkable. In an *in vitro* study, Ni and Chang compared degenerative properties and apatite formation of Mg_2SiO_4, the major composition of forstrite, with two other biomaterials. Their result confirmed that the its mentioned properties were poor.[47]

Conclusion

With the limitation of this study, the nano-HA group is the first choice which showed a significant amount of bone regeneration, especially in the first 30 days. The second recommended choice is nano-BG but paying more surveys to these materials as bone substitutes are needed.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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