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Combined Use of Hyaluronic Acid with Nano-bioactive Glass Enhanced BiocementBased Silicate Stimulated Bone Regenerative Capacity in Tibial Bone Defects of Rabbits: In-Vivo Study

Research Article

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Abstract

Background: An ideal biomaterial for bone regeneration is a longstanding quest nowadays. Thisstudy aimed to evaluate the osteogenic potentiality of nano-bioactive glass (NanoBG) enhanced biocement based silicate with or without hyaluronic acid(HA) seeded in rabbits' tibial bone defects.
Methodology: 24 male rabbits were divided into three equal groups. All rabbit's tibia had two defects 5mm in diameter (1 defect per tibia). Group 1(control): bone defects were left untreated. Group 2: defects received nanoBG enhanced biocement based silicate cement. Group 3: defects received NanoBG cement mixed with HA. Animals in each group were divided equally for euthanization after 3 weeks and after 6 weeks. At each duration, the bone specimens were processed and examined histologically with histomorphometrically analysis of new bone area percentage.
Results: Thebone defects in group 3 showed significant improved osseous healing as compared to group 1&2 along the two durations. Upon long duration healing, the histological examination of the bone defects of group 3 showed almost filled defects with mature compact bone, however both groups 1&2 revealed less mature bone with more bone marrow spaces in-between. The morphometric analysis revealed a significant increase in the new bone area percentage in group 3 in comparison to group 1 and 2 ($P < 0.05$).
Conclusions: The present study concluded that bone defects and fractures could be treated with NanoBG and HA cement. Nano BG alone was capable of bone regeneration. Yet, the regenerative capacity of their combination was more significant.

Keywords: Bone Regeneration; Bioactive Glass; BG; NanoBioactive Glass; Calcium Silicate Cement; Hyaluronic Aacid; Tibial Bone Defect.

Introduction

Bone defects represent a serious pathological condition that can cause severe complications and affect vital components of the bone. Bone fractures' healing and union is an obstacle due to precarious blood supply that maycomplicate the treatment [1, 2]. The demand for an ideal biosynthetic material for replacement and repair of bone tissue loss has increased significantly due to the complications of autografts, allografts and xenografts. Despite the increasing number of these materials, there is no ideal bone graft substitute [3, 4]. Bone tissue engineering (BTE) is an advanced biomedical technique that is considered as an effective approach for bone regeneration and reconstruction of lost bone tissue. Currently, the paradigm of BTE depends on bone substitute materials which can promote the human body's own regenerative capacity in the repair process by stimulating expression of osteogenic genes. In this regard, the scaffold should be designed as bone tissue "regeneration" rather than mere "replacement"[5]. Synthetic materials used for bone regeneration include metal materials, inorganic non-metallic materials, organic materi-

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als, and composites, have great potential in clinical applications. Bioactive glass (BG) have been applied extensively for bone repair and regeneration as they have shown excellent bone bioactivity and in vivo-bone forming ability [6]. Nanoscale of BGshowed improvements of its bioactivity, this can be explained by the higher surface area of nanoscale BG thatpermits rapid release of ions and a higher protein adsorption. Previous researches have proven thatbone and teeth tissues mineralization were accelerated when these tissues were in contact with nanoscale particles in comparison with micron scaled particles [7]. Biocement based silicate was developed more than 20 years ago. The main advantage of silicate-based cements is the fact that Si plays an essential role in mineralization and gene activation in bone regeneration process. It was reported that silicate can be combined with Ca2+ ions, which have shown its superiority in pre-osseous and osseous tissue repair in vitro and vivo [8, 9]. Calcium silicate cements have been shown to facilitate cell attachment and integration with opposing hard tissues as well as their capability in bone regeneration. Many researchers reported that biomaterials containing CaO–SiO2 enhances mineral deposition across their surfaces and were found to bond to living bone and soft tissues through the development of a biologic hydroxyapatite layer on the surface [4]. However, the degradation of pure tricalcium silicate cement is too slow to match the rate of new bone formation, which limits its application in bone regeneration [10]. Numerous studies reportedthe efficacy of combining silicates with other materials in order to design bioactive biomaterials with better properties for tissue regeneration, especially bone tissue engineering applications [11]. Recently, hyaluronic acid (HA) act as an important natural polymer that improves and modifies the biological properties of a synthetic scaffold [12, 13]. HA was found to be capable of binding to extracellular matrix molecules and cell surface receptors. Subsequently, it helped in regulating cellular behaviour via control of the tissues' macro- and micro-environments [14]. It has been proven that HA has a great role in angiogenesis, wound healing, and tissue regeneration.HA-based scaffolds represented a source for osteoinductive elements that can subsequently promote the osteogenic effects of implanted scaffolds [12, 15]. Several previous reports on the use of nano-bioactive glass, bioactive calcium silicate cement and hyaluronic acid in bone regeneration were found. Yet, none incorporated them together as a biocomposite mixture. Therefore, this study aimed to introduce a novel composite scaffold with extrudable nanostructured bioactive glass and calcium silicate based biocement pastes using hyaluronic acid as a solvent, which may provide surprising alternatives for bone tissue regeneration.

Materials And Methods

Ethical Statement

The study protocol was approved from the Institutional Animal Care and Use Committee (IACUC) - Cairo University. Approval number (CU/III/F/46/19).

Experimental Animals

This experiment was conducted on 24 healthy male New Zealand white rabbits weighing about 2.5 to 3.5 kg. Animals were purchased and housed in the animal house Faculty of Medicine, Cairo University. The rabbits were randomly allocated into three groups. Each group consisted of 8 rabbits. Animals were kept in separate cages and maintained under controlled temperature at 25˚C± 2˚C with 12 h light/dark cycle. They were fed pellets and fresh tap water available ad libitum with good ventilation condition throughout the experiment.

Bone defect preparation

The surgical procedure was performed under general anaesthesia upon intramuscular injection of a combination of 5mg/kg Xyaline 2%(Xyla-Ject®, Phoenix™, Pharmaceutical Inc.) and 40mg/kg Ketamine Chlorhydrate (Ketamine, Amoun pharmaceutical company) [16]. A single bone defect 5 mm in diameter was created in each tibia using a round surgical bur coupled to a low-speed hand piece usedunder constant copious irrigation withphysiological saline solution to prevent the overheating of the periphery of the bone. The bone defects were drilled until the medullary canal is reached. The defects of group 1 (control group) were left untreated (filled with blood clot), while group 2 defects were filled with nanoBG enhanced biocement based silicate mixed with distilled water. Group 3 defects were packed with nanoBG enhanced biocement based silicate mixed with HA. Postoperatively, the periosteum, muscle and fascia were then repositioned properly over the defects and sutured with resorbable #2.0 catgut and the skin was sutured with interrupted #3.0 silk sutures. Systemic antibiotic Amikacin® 1.5 mg/ kg (Amoun pharmaceutical company) was administrated as an intramuscular injection per 12 hours for 1 week [17]. Analgesic 10 mg/kg cataflam (Novartis, Egypt) was administrated to relieve postoperative pain and topical antibiotic spray; Bivatracin (Egyptian Company for Advanced Pharma, Egypt) to avoid local infection.Three weeks postoperatively,half of the animals in each group were euthanized with an intra-peritoneal overdose of Ketamine/Xylazine mixture,however, the other half after 6 weeks(18).Both tibiae were dissected free from any soft tissues; the bone specimens including the defect of each group were cut by a disc under constant irrigation to include the entire defect sites.

Histological and histomorphometry examination of H & E-stained sections

Bone specimens were fixed in 10% calcium formol solution for 48 hours and demineralized in 10% EDTA (El-Gomhouria co.) solution for 4-5 weeks. The specimens were subsequently dehydrated in ascending grades of alcohol, cleared in xylol, and then embedded in paraffin blocks. Serial 5-6 µm paraffin cross sections were cut with a microtome using diamond knife and mounted on clean glass slides, and finally stained with H and E stain. Histomorphometric analysis of the newly formed bone area percentage was obtained using Leica Owen 500 image analyser Computer system (Leica Imaging System Ltd., Cambridge, U.K. in Research unit in faculty of Oral and Dental Medicine, Cairo University). The image analyser consisted of a coloured video camera, coloured monitor, hard disc of IBM personal computer connected to the microscope and controlled by Leica Qwen 500 software.

Statistical analysis

The data obtained from the histomorphometric analysis were statistically described in the terms of mean and standard deviation (SD) values. ANOVA was used to compare different observation times within the same group. Followed by Tukey's post hoc test

to compare multiple 2-group comparisons. The significance level was set at $p < 0.05$. Statistical analysis was performed with IBM SPSS 18.0 (Statistical Package for Scientific Studies, SPSS, Inc., Chicago, IL, USA) version 22 for windows.

Materials

Tetraethyl orthosilicate (TEOS), triethyl phosphate ethanol (TEP), nitric acid (65%) used as a catalyst, calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$), ammonia (NHOH), and silver nitrate ($AgNO_3$) were used to prepare the silver bioactive glass and calcium silicate cement by sol-gel method. The silver bioactive glass system formula reached was $60SiO_2$:35CaO:4P2O5:1 Ag_2O_3(19). The novel biocement was prepared by mixing 80% of calcium silicate cement to 20% of silver bioactive glass [20]. Either high molecular weight hyaluronic acid (1750 kDa) (Sigma-Aldrich) or distilled water was used to prepare the cement paste which was subsequently moulded into the critical sized bone defect [21].

Results And Discussion

Transmission electron microscope (TEM) analysis of silver nanoBGbased silicate biocement and silver nanoBGbased silicate biocement /HA:

TEM analysis of novel sliver nanoBG/calcium silicate biocement-showed heterogeneous shape of the nanoparticles with formation of crystalline dark and amorphous transparent nanoparticles. The average particle size of nanoparticles of the clumped distributions was between 9.46 and 18.36 nm. (fig. 1A) While the TEM images of novel biocement mixed with HA showed a uniform distribution with large hydrated cloudy clusters encapsulating many nanoparticles ofdifferent morphology. The average nanoparticles size ranged from 12.09 to 15.31 nm in diameter. (fig. 1B).

Histological (H & E stain) results

Three weeks postoperatively, group 1 showed almost open bone defect with some granulation tissue in the middle of the defect and few newly formed bone trabeculae at the edges enclosing large bone marrow cavities in-between. (fig. 3 A&B) Thin and interconnected neobone trabeculae were formed around the graft material in group 2 with wide bone marrow cavities in-between. (fig. 3 C&D) Group 3 revealed newly formed bone trabeculae filling the defect with thick trabeculation and appearance of primary osteons having wide haversian canals as well as scattered areas of woven bone. Bone defect showed a highly vascularized periosteum coverage. The interface between newly formed bone and old pre-existing bone was about to be sealedwith scalloped border. (fig. 3 E&F) Six weeks postoperatively, group 1 defects revealed newly formed interconnecting bone trabeculae filling almost all the defect as compared to the same group at 3 weeks postoperatively. Dispersed areas of woven bone with different degrees of basophilia were detected. (fig.4 A&B) Group 2 showed bone defect almost filled with newly formed lamellar bone with thick trabeculation enclosing smaller bone marrow spaces.Indistinguishable interface was observed between old bone and newly formed bone with significant difference in the orientation of the lamellae between old and new. (fig.4 C&D) Group 3 demonstrated completely restored defect with densely packed compact bone tissue that could not be distinguished from the old bone with completely sealed interface. Dense compact bone compromised lamellae assumed in concentric arrangement around a haversian canal, forming a typical osteon. (fig.4E&F).

Figure 1. TEM image of sliver nanoBG/calcium silicate cement nanopowder. (A) TEM image of sliver nanoBG/calcium silicate cement nanopowder mixed with HA. (B) (x100 nm).

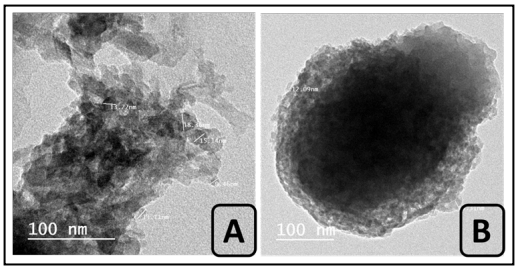
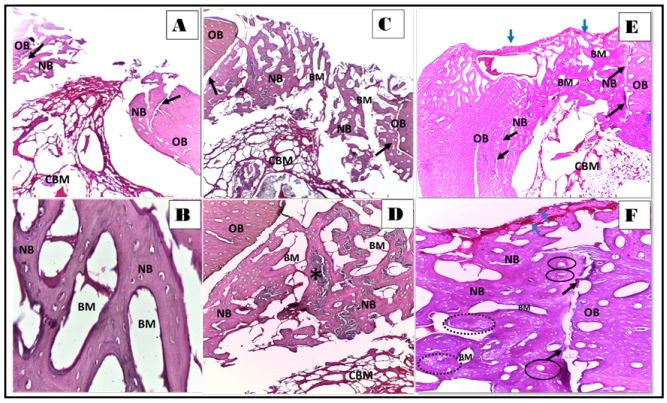


Figure 2. Photomicrographs of H& E-stained sections of bone defects (3 weeks). (A &B) represent group 1 or control group. (C&D) represent group 2 or nanoBG group. (E&F) represent group 3 or nanoBG and HA group. OB: old bone, NB: new bone, BM: bone marrow, CBM: central bone marrow, black arrows: interface between old pre-existing and new bone, blue arrows: periosteum, black asterisk: graft material remnants, black circles: primary osteons, dashed black circle: woven bone. (A,C&E x40, D&F x100, B x400).



Histromorphometric and statistical analysis

The histomorphometric analysis of the bone area percentage between groups during both time intervals showed the highest bone area percent in group 3 which revealed a statistically significant increase in the mean of bone area percent relative to group 1 and 2. Moreover, bone area percentage mean value significantly increased by time in all groups.(fig. 4, table 1 and 2).

Discussion

Beforeevaluating biomaterials in human, a perfect bone substitute ought to be tried in vitroand in vivo, to be certain beyond any doubt that it works viably and securely. Therefore, establishingan appropriate animal model is an essential step when assessing the mechanical property and biocompatibility of bone tissue biomaterials [22]. Silica based BG has been exclusively applied for bone repair and regeneration as they showed excellent bone bioactivity and in vivo bone forming ability. In this study, BG composite was the material of choice with replacing the sodium component with silver.Silver ions were found to be perfect in enhancing the antibacterial and osteogenic activities [23]. Numerous literatures indicates that HA acts primarily to promote healing at fracture site by stimulating callus formation. Furthermore, HA of a specific molecular weight when used in vitro, was reported to significantly increase alkaline phosphatase activity and stimulate osteoblastic cell proliferation and differentiation [24].

Through the current study nanoBG cement alone and upon addition to HA promoted bone regeneration in critical-sized tibial bone defects along short and long-time intervals however, his-

Figure 3. Photomicrographs of H& E-stained sections of bone defects (6 weeks). (A &B) represent group 1 or control group. (C&D) represent group 2 or nanoBG group. (E&F) represent group 3 or nanoBG and HA group. OB: old bone, NB: new bone, BM: bone marrow, CBM: central bone marrow, CB: compact bone, black arrows and dashed rectangle: interface between old pre-existing and new bone, green arrows: different orientation of bone lamellae, black circles: typical osteons, dashed black circle: woven bone. (A,C&E x40, D&F x100, B x400).

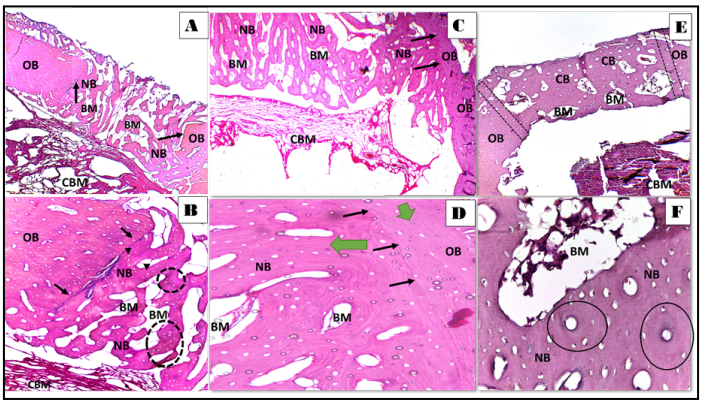


Figure 4. Column chart showing bone area % mean value of with 95% confidence interval error bars in all groups for 3 and 6 weeks postoperatively.

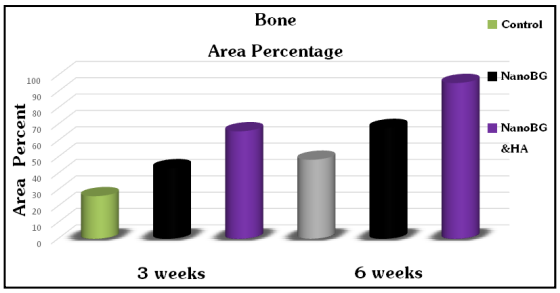


Table 1. Bone area percentage between groups.

Duration	Group	Mean	Standard deviation	Std. Error Mean	95% Confidence Interval for Mean		Min	Max
					Lower bound	Upper bound		
3 weeks	Group 1	26.13^{D}	3.35	1.18	23.53	28.73	20.50	29.80
	Group 2	43.221^{C}	2.589	0.915	40.621	45.821	39.110	46.340
	Group 3	65.79^{B}	4.65	1.64	63.20	68.39	59.51	72.00
6 weeks	Group 1	48.40^{C}	4.17	1.47	45.80	51.00	40.70	53.21
	Group 2	67.84^{B}	4.31	1.52	65.24	70.44	60.80	72.89
	Group 3	95.317^{A}	2.039	0.721	92.718	97.917	92.990	98.780

ANOVA test: Significant means with different superscript letters are significantly different.

Table 2. Bone area percentage for each group at different observation times.

Group	Duration	Mean	Standard deviation	Std. Error Mean	95% Confidence Interval for Mean		T-Value	Adjusted P-Value
					Lower bound	Upper bound		
Group 1	3 weeks	26.13	3.35	1.18				
	6 weeks	48.4	4.17	1.47	16.83	27.71	12.22	0.000*
Group 2	3 weeks	43.221	2.589	0.915				
	6 weeks	67.84	4.31	1.52	19.18	30.06	13.51	0.000*
Group 3	3 weeks	65.79	4.65	1.64				
	6 weeks	95.317	2.039	0.721	24.09	34.96	16.2	0.000*

Tukey's post hoc test:Pairwise comparison of bone area % between different time intervals within the same group.(*Significance level P≤0.05)

tological and histomorphometric examinations revealed superior results in HA groups at both time intervals.

The histological results in the nanoBG group at both time intervals showed better bone regeneration than control group. They showed interconnected bone trabeculae filling almost all the defect perimeters which appeared thicker with smaller bone marrow cavities 6 weeks postoperatively. Moreover, the bone area percentage was significantly higher in nanoBG group. BG showed unique properties in bone tissue regeneration by formation of carbonated hydroxyapatite layer (HCA) when exposed to biological fluid. This layer is responsible for the strong bonding between bioactive glasses and human bone [11]. In coincidence with our findings, Abirman et al., 2002; concluded that after 6 weeks of BG implantation in tibial bone defects in rabbits the periosteal and the endosteal regions were completely closed [25]. As well asPinto et al., 2013; reported that tibial bone defects implanted with biosilicate ceramics showed highly organized newly formed bone filling the whole defect after 45 days postoperatively [26]. Another study demonstrated that the quantitative woven bone volume was significantly higher in BG group than in control group after 20 days of implanting BG in tibial bone defects of rats [27].

NanoBG with HA group showed superior histological results than the other 2 groups throughout the whole experiment. Newly formed bone was observed filling the defect with thick trabeculation and intimate bonding with the defect pre-existing old bone, however this bone was more organized and uniform in form of dense compact bone enclosing typical haversian systems after 6 weeks. Superior bone regenerative results seen in nanoBG and HA group could be assumed to the characteristic role of HA in cell adhesion, chemotaxis, differentiation, and proliferation, signalled through several macromolecules and especially during wound healing and tissue regeneration [28, 29]. Similarly, Shamma et al., 2017; confirmed that addition of HA into bone graft around dental implants placed in sockets of extracted mandibular third premolar of dogs after 6 weeks showed entirely filled mature well-formed bone with obvious complete osseointegration with the native bone [30].

On contrary, Ahmed et al., 2020; revealed that HA implanted in combination with biphasic calcium phosphate cement in femoral bone defects of rats didn't give superior bone regeneration in comparison with the cement alone 4 and 10 weeks postoperatively. They explained their findings by assuming that the low molecular weight (less than 1000 kDa) of the HA used in their study was the reason [31]. The HA ability to enhance the osteogenic and osteoinductive properties of bone graft materials was dependent on its dose and molecular weight. It was found that HA of higher molecular weight (more than 1000 kDa) promoted mesenchymal stem cells (MSCs) proliferation and differentiation [28]. This may confirm the osseous regenerative potentiality of HA used in our present study which had high molecular weight (1750 kDa).

Parallel to our results, Elkarargy, 2013; demonstrated that combining HA to synthetic bone graft increased the new vital bone formation bone area percentage upon implantation in sockets of extracted lower lateral incisors in rabbits when compared with bone graft alone and empty control group after 4weeks and 8weeks postoperatively [32]. Moreover, Shirakata et al., 2021;concluded that adding HA either alone or combined with collagen matrixin 5 mm intrabony defects on the walls of mandibular premolars in dogs enhanced the periodontal wound regeneration [33].

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Conclusion

From our study, we can conclude that the combined use of HA and nanoBG enhanced silicate biocement for osteogenic regeneration of osseous defects is a potential treatment alternative for accelerated healing than using these biomaterials alone.This conclusion is a new breakthrough in the field of bone graft materials since BG overcomes the limitations associated with other synthetic and natural bone grafts and make it promising bone substitute material in critical bone defects in clinical applications.

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