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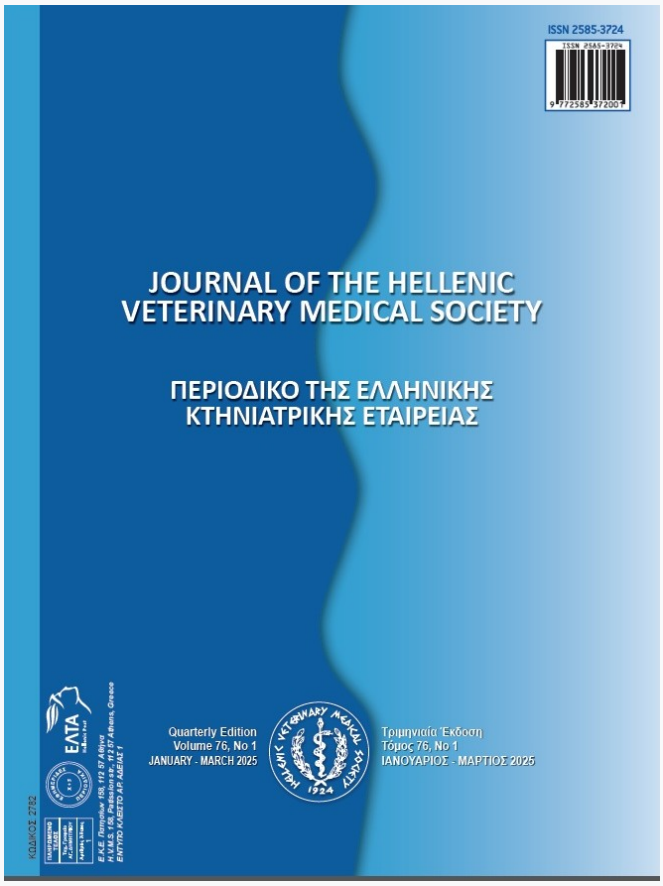

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Research article
Ερευνητικό άρθρο

Histopathological and Micro-CT Scan Evaluation of the Repair of Large Bone Lesions using Apatite Carbonate and Titanium-Containing Bioactive Glass in a Rabbit Model

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ABSTRACT: In recent years, several techniques have been used to reconstruct large bone fractures, including various scaffolds and bone reparative materials, with poor results. Therefore, this study investigates and compares the effect of new scaffolds based on apatite carbonate and bioactive glasses containing titanium on promoting the healing process of large bone lesions in animal models in laboratory conditions. After making the scaffolds, in vivo studies were done by making four circular holes in the calvarial bones of 10 adult New Zealand rabbits. Bioactive glass powders containing titanium and apatite carbonate were then randomly poured into the holes to fill them. The 3-(4, 5-Dimethylthiazol-2-yl)-2 ,5-Diphenyltetrazolium Bromide (MTT) method was used to check the survival and growth of mesenchymal stem cells. Bone defects in the study groups were also looked at using various diagnostic imaging techniques and histological analyses. The X-Ray Diffraction (XRD) analysis and Fourier-transform infrared spectroscopy (FTIR) analysis results confirmed the high purity of the fabricated Bioglass/carbonate apatite (Bg-Ca) and Bioglass/ Titanium (Bg-Ti) scaffolds. In the MTT method, the scaffolds made at a concentration of 10 mg/ml had no cytotoxicity against mesenchymal stem cells (MSCs). Also, in total, micro-CT scanning and histological findings showed a significant improvement in the healing process in rabbits treated with Bg-Ti and Bg-Ca compared to the group that received the Bg scaffold alone and the control group.

***Key words*:** Bone regeneration; carbonate apatite; bioactive glass; titanium; histopathology; rabbit

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INTRODUCTION

Bones are a key part of the body and are responsible for weight-bearing and physical support and movements (Krishnakumar, Roffi et al. 2017). Although, bone tissue has a self-healing ability, some factors avert the process of reconstruction and repair of bone defects. These factors include large bone defects, bone infection (osteomyelitis), reduced vascular blood supply to the affected site, and other unknown macromolecular factors (Lü, Bai et al. 2017). In order to overcome these obstacles, researchers use special techniques, including bone grafts, biosynthetic materials (e.g., ceramics, polymers, cement, glass, metals, etc.), bone repair compounds (e.g., glycosaminoglycan), and stem cell-based technologies (Carlini, Adamiak et al. 2016, Kouroupis, Kyrkou et al. 2016).

Over the past decade, histologists have sought to fabricate 3-D scaffolds using cells and materials such as growth factors (bone-repair stimulating materials) to find potent alternatives for autografts and other bone grafting techniques (Zhu, Cui et al. 2020). There are two types of fabricated scaffolds: natural (biological) and synthetic (artificial). Natural scaffolds include collagen, gelatin, fibrin, hyaluronic acid, polysulfated glycosaminoglycan (PSGAG), chitosan, and demineralized bone matrix (DBM) (Zeng, Liu et al. 2018, Kashirina, Yao et al. 2019). Likewise, synthetic materials include porous metals, bioactive glasses and strontium, synthetic polymers such as polylactic acid (PLA), polyglycolic acid (PGA) and polymethyl methacrylate (PMMA), and calcium phosphate-containing ceramics such as hydroxyapatite (HA), tricalcium phosphate (TCP), and calcium sulfate. In addition, factors such as bone morphogenic protein-2 (BMP-2), transforming growth factor-beta (TGF-β), and insulin-like growth factor-1 (IGF-1) stimulate the bone healing process, and their effects have been extensively researched (Ramesh, Moratti et al. 2018, Kashirina, Yao et al. 2019, Jiang, Wang et al. 2021).

HA is the crystal phase of calcium phosphate which directs the healing process and angiogenesis at the fracture site by creating a stable mechanical scaffold (Bal, Kaito et al. 2020). HA further appears to possess osteoconductive properties. According to a research HA and demineralized calf fetal growth plate (DCFGP) have shown osteogenesis capability and their accompanying use can effectively enhance the bone-repairing process. Similarly, Lett et al. (2021) have reported that the combination of HA and natural polymers can boost the osteogenesis process (Lett, Sagadevan et al. 2021).

Bioactive glass refers to a group of glass-ceramic biomaterials. The biocompatibility and bioactivity of these glasses have made them a good candidate for use as medical implants in the body to treat bone diseases and replace affected or damaged bones (El-Rashidy, Roether et al. 2017, Ege, Zheng et al. 2022). Since the early 2000s, bioactive glasses have been broadly used for biomedical applications. Research advocates that these glasses are good candidates for use as drug delivery carriers to treat various infections or diseases such as osteoporosis. Bioactive glass is also an excellent choice for the treatment of chronic wounds (van Gestel, Geurts et al. 2015, El-Rashidy, Roether et al. 2017). Although various materials have been utilized for the fabrication of bone bio-implants, a versatile organic bio-implant without any disadvantages is still lacking (Ege, Zheng et al. 2022). Therefore, this study investigates and compares the efficacy of novel carbonate apatite-based scaffolds and titanium-containing bioactive glass in enhancing the healing process of large bone lesions in rabbit femoral bone.

METHODS

Fabrication of the scaffold and investigation of its characteristics

Apatite carbonate scaffolds, bioactive glasses with titanium, synthesized by sol-gel method, were used in this study. The base formula of this scaffold is CaO-SiO2-P2O5, which, from left to right, contains 44.57% CaO, 42.35% SiO2, and 15.7% P2O5 (Bellucci, Sola et al. 2013). The pattern of scaffold decomposition was investigated at given days (i.e., days 15, 30, 45, and 60) by immersing the scaffold in the SBF solution (0.2 ml/mm^3 of the scaffold) in 50 cc Falcon tubes, followed by washing with ample deionized (DI) water per day. A scanning electron microscope (SEM) was further employed to evaluate the morphology of the scaffolds. For this, the scaffolds were first fixed in cold glutaraldehyde and then placed in the Automatic Tissue Processor for processing and preparation of microscopic slides. In the tissue processor, the samples underwent dewatering, clarification, and paraffinizing, and then were dried and coated with gold particles (Kopecká and Svobodová 2014). The surface and the inner layers of the scaffolds were examined with SEM imaging (Jalili, Naeini et al. 2022).

Cell viability test

The cytotoxicity of scaffold components against

the survival and proliferation of mesenchymal stem cells (MSCs) was assessed by the tetrazolium-based colorimetric (MTT) and colorimetric assays. Briefly, the MSCs were first cultured in Dulbecco's Modified Eagle's Medium (DMEM) enriched with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C with 5% CO2. Then, 10 mg/ml of the powders were prepared and, after sterilization with gamma rays, placed inside the insert wells containing the DMEM medium with a single layer of MSCs. Cytotoxicity and cell viability were measured using lactate dehydrogenase, 3-(4, 5-dimethylthiazol-2-yl)-2, diphenyl-, tetrazolium bromide (MTT) assay kits 24 h post-incubation according to the manufacturer's instructions (Sari, Chotimah et al. 2022).

In vivo studies

This study was independently reviewed and approved by Shiraz University's ethics committee (ethical code: IR.SHU.13972334). The experiments were conducted on 10 New Zealand white male rabbits (average weight: 2 to 2.5 kg; age: 1 to 5 months) to evaluate the efficacy of apatite carbonate and titanium-containing bioactive glass in repairing bone lesions. Premedication was performed via the intramuscular (IM) injection of acepromazine maleate (1 mg/kg of body weight, Alfasan,Woerden, Netherlands), while anesthesia was performed via the IM injection of 10% ketamine hydrochloride (100 mg/kg of body weight, Alfasan,Woerden, Netherlands) and 2% xylazine hydrochloride (10 mg/kg of body weight, Alfasan,Woerden, Netherlands). After anesthesia, 10 mm of calvaria bone was removed circularly using a trephine (model Strong 204 micromotor handpiece; SAESHIN; China), and the bio-implants were placed in the bone defect. The animals were then kept in the recovery room post-surgery and received postoperatively buprenorphine (Buprenex®, Indivior Inc. North Chesterfield, VA, USA, 0.05 mg/kg) and enrofloxacin (Baytril® (2.5%), ElancoUK, Hampshire, United Kingdom, 10 mg/kg) for 3 days. (Jalili, Naeini et al. 2022). The rabbits were split into four groups based on the type of bone defect treatment, which include the ontrol group, bioimplant containing bioglass group, bioimplant containing bioglass and titanium group and bioimplant containing bioglass and carbonate apatite group (Table 1).

Micro-CT scan

Micro-CT images were taken on day 60 post-treatment for therapeutic evaluations. For this, calvaria bone samples were evaluated by a micro-CT scanner (model SCANCO, Switzerland, µCT35 scanner; 70 kV, 114 µA for 800 ms). In this assay, the bone volume fraction (BV/TV), bone mineral density (BMD), cortical bone thickness (Ct.Th), and trabecular bone thickness (Tb.Th) values were evaluated and compared in different groups.

Histopathology

Calvaria bone tissue samples were taken on weeks 4 and 8 after treatment with the powders and immediately fixed in a 10% formalin container for 48 hours. The samples were then decalcified with 10% EDTA buffer at a pH of 7.4 for 30 days. Paraffin plaques were subsequently prepared from the samples after dehydrating in an alcoholic series. Ultimately, a 5-µm section of the samples was cut using an A35 Feather disposable microtome blade (Feather, Tokyo, Japan) in order to prepare pathology slides from the cut samples. The tissue sections were then stained with hematoxylin and eosin, and Masson's trichrome, and the prepared slides were covered with coverslips. The slides were placed under an Olympus microscope (Olympus, Tokyo, Japan) at 400x and 1000x magnifications for histological examination.

Statistical analysis

All the quantum data were reported as mean ± standard deviation (SD). The statistical differences in

Table 1. Groups of rabbits based on the type of bone defect treatment

Group	Description	The number of rabbits (30 day trial)	The number of rabbits (60 day trial)
Control group	Defect without treatment	1	1
Treated group	Bioimplant containing bioglass	1	1
	Bioimplant containing bioglass and titanium	1	1
	Bioimplant containing bioglass and Carbonate apatite	1	1

measured data among different groups within a given period were measured using one-way ANOVA and Tukey post hoc tests. The results with a p-value of less than 0.05 were considered to be statistically significant. Statistical tests were performed in GraphPad Prism software, Version 6.0.

RESULTS

Characteristics of Bg, Bg-Ca, and Bg-Ti composite powders

SEM images revealed that Bg-Ca and Bg-Ti composites are spherical particles that have been aggregated as massive assemblies. SEM images further revealed the angular and jagged morphology of Bg powders. After grinding, the Bg powders were imaged and their arrangements were investigated. Fig. 1 demonstrates the SEM images of Bg, Bg-Ca, and Bg-Ti composites.

XRD analysis of Bg-Ca and Bg-Ti composites

The FTIR and XRD patterns of Bg-Ca and Bg-Ti composites are shown in Fig. 2. Analysis of the XRD patterns revealed the consistency of the results for Bg-Ca particles with those reported in the ICDD database. However, from 20 to 40 degrees, the characteristic diffraction peaks for Bg-Ca particles were subdued by the amorphous peak of Bg-Ti by nearly 2 θ.

Cell viability and cytotoxicity assays

The effects of Bg, Bg-Ti, and Bg-Ca powders on

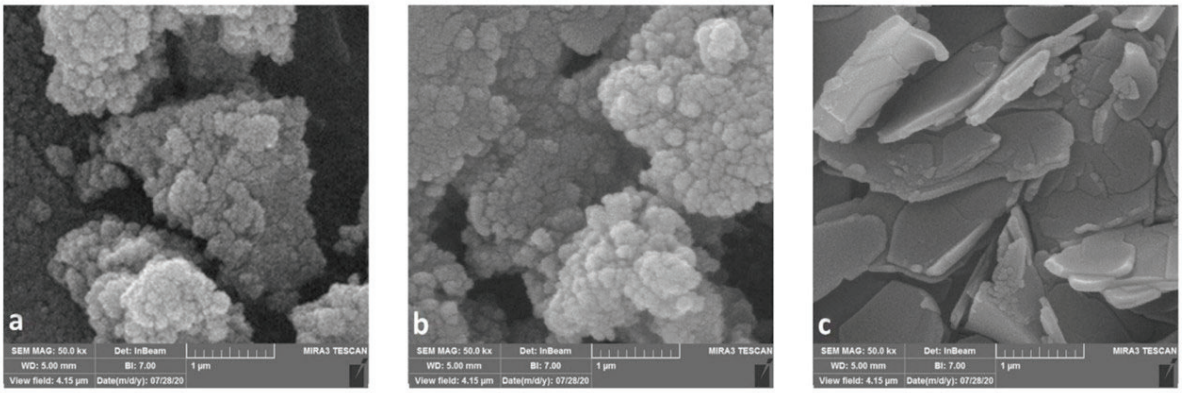

Fig. 1. Morphology and laboratory analysis of Bg, Bg-Ca and Bg-Ti composite by electron microscopy, a: Bg, b: Bg-Ca, and c: Bg-Ti

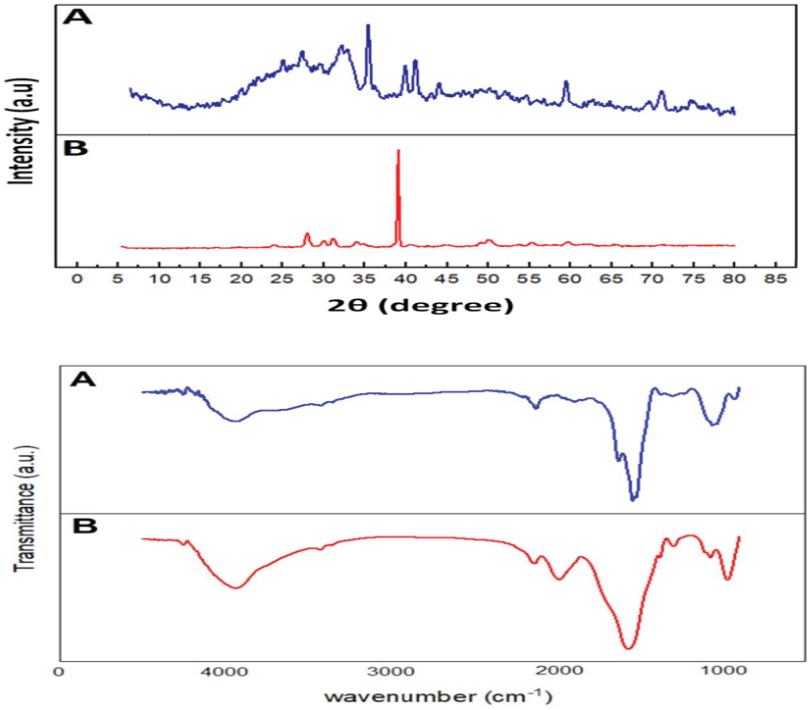

Fig. 2. Determining the characteristics of the fabricated scaffold by XRD and FTIR analysis. A: Bg-Ca composite, B: Bg-Ti

the survival and proliferation of human bone marrow-derived MSCs were investigated and measured using MTT assays. According to Tables 2 to 4, there is no significant cytotoxicity observed on days 1, 3, and 7 at a concentration of 10 mg/ml (P=0.95). However, the cytotoxicity of Bg-Ti powder was higher than that of Bg-Ca powder.

Micro-CT scan results

The micro-CT scan results were used to evaluate the bone tissues which are newly constructed in the 8th week after implantation of substitute materials. Results were reported as BV/TV values for all critical defects of the calvaria bone. The BV/TV ratio was investigated for Bg, Bg-Ca, Bg-Ti, and control (NC) groups. The difference in the BV/TV ratio in the defect area was significant among Bg-Ti, Bg-Ca, and NC groups. The BV/TV ratio was also significantly higher in Bg-treated rabbits than in rabbits in the NC group (P=0.021) (Fig. 3).

Table 2. Cell survival assay after exposure to BG, BG-Ti and BG-Ca powders on days one, three and seven

Cell Viability (%)				Group	Day
BG -Ca	BG -Ti	BG	NC		
95	93	92	100	1	1d
92	90	97	100	2	
96	91	95	100	3	
94.33	91.33	94.66	100	Ave	
2.08	1.52	2.51	0	STD	
90	85	89	100	1	3d
86	81	87	100	2	
91	87	93	100	3	
89	84.33	89.66	100	Ave	
2.64	3.05	3.055	0	STD	
87	86	87	100	1	7d
91	83	92	100	2	
93	90	89	100	3	
90.33	86.33	89.33	100	Ave	
3.05	3.51	2.51	0	STD	

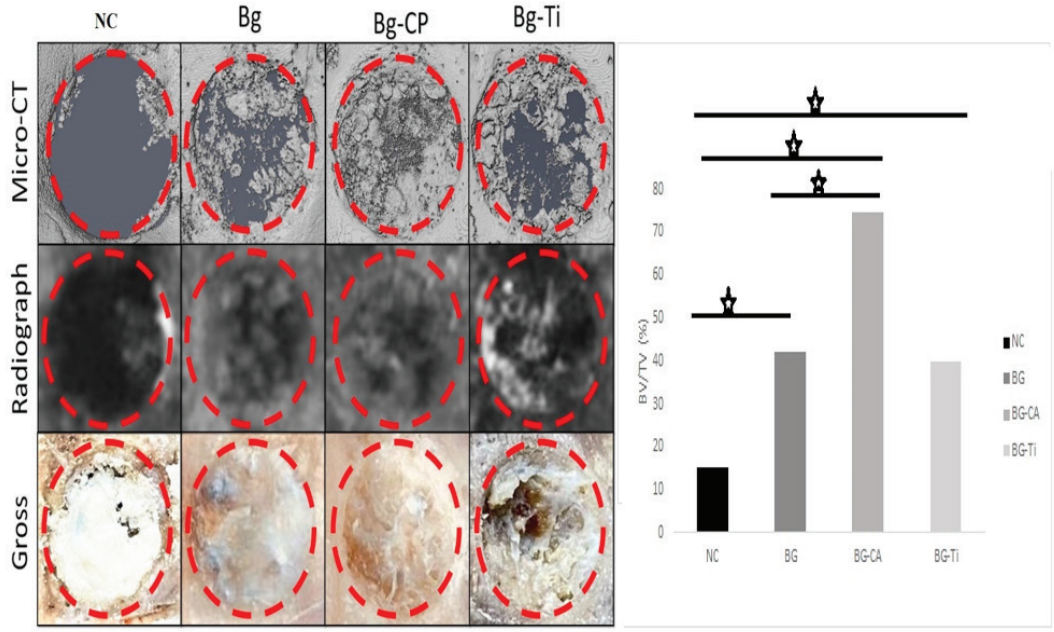

Fig. 3. Gross images, radiology and micro-CT scans of untreated (NC) and treated bone defects eight weeks after treatment and analysis of related results. * indicates a statistically significant difference between the experimental and control groups (P≥0.05).

Histological findings

Bone defects were histologically assessed in weeks 4 and 8 post-surgery (Fig. 4, Fig. 5). The histological images of the bone defect revealed a higher rate of osteogenesis in rabbits receiving Bg-Ti and Bg-Ca treatments than in rabbits receiving Bg alone and those in the NC group. At week 4 after implantation, the bone defect area in the NC group was observed to be filled with loose connective tissue (LACT), which contained loosely organized collagen fibers, fibroblasts, and abundant blood vasculature. Regarding the bone defects of this group, LACTs were found to transform into fibrous connective tissue (FCT) after 8 weeks. After 4 weeks, it was determined that the defect in the calvarial bone was filled with FCT tissues in rabbits receiving Bg and Bg-Ti treatments. It was observed that the remaining implanted materials were still located in the defect area. After 4 weeks, the defects treated with Bg-Ti and Bg-Ca scaffolds were the only defects exhibiting osteogenesis of varying degrees within this period, with the greatest rate of the reconstruction of bone tissues observed in the Bg-Ca group. Furthermore, at week 8 post-treatment, woven bone formation in the Bg-Ti group was much higher than in the Bg group.

The histomorphometric results of reconstructed bone tissues, including bone growth and fibrotic tissues in the 4th and 8th weeks, are depicted in a graph. According to the results, the greatest rate of bone

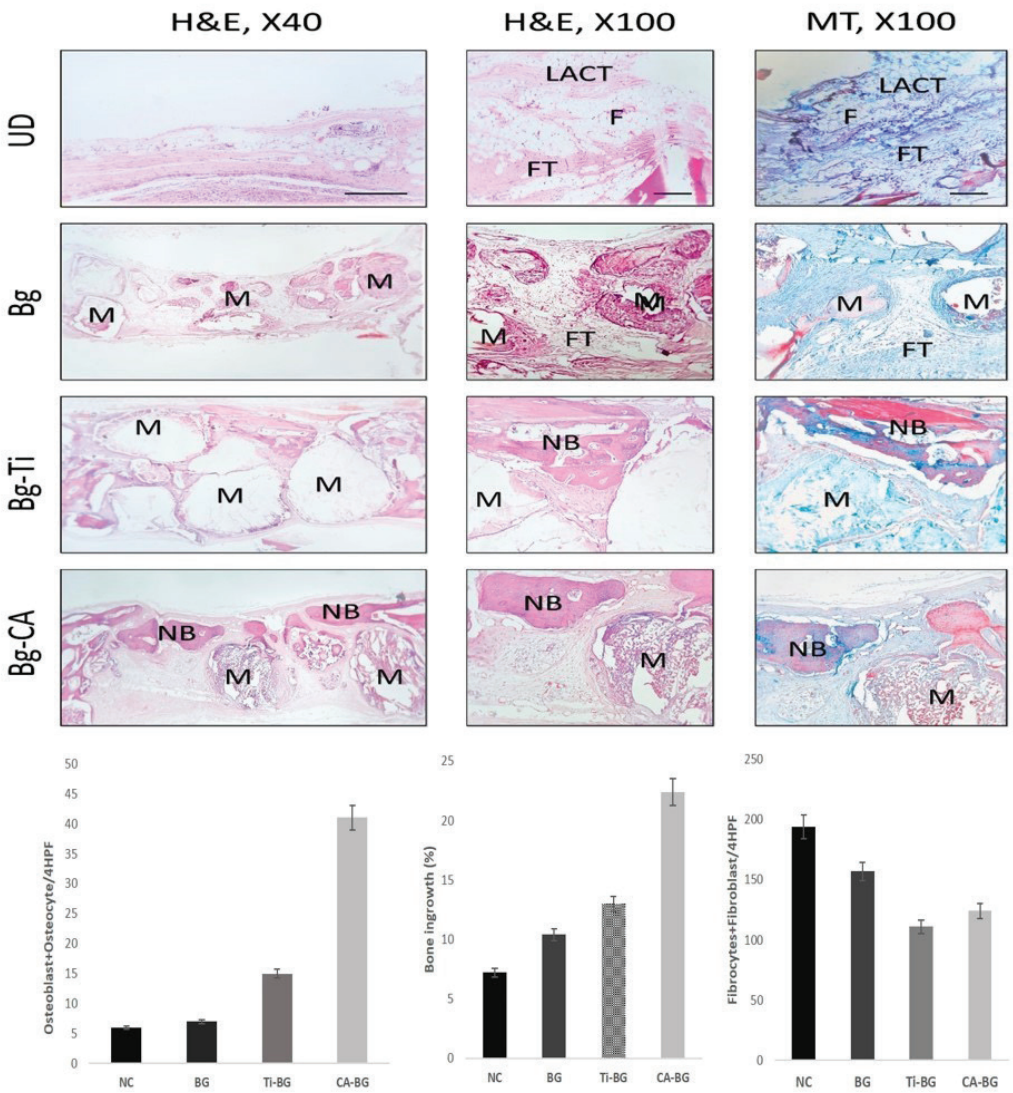

Fig. 4. Microscopic images of calvarial bone defects 4 weeks after surgery. Remnants of the implanted material were evident in the treated defect area, and new bone formation had begun in the defects treated with Bg-Ti and Bg-Ca. LACT: areolar loose connective tissue. FT: fibrous connective tissue; NB: new bone formation. M: Residue of planted material. F: fat, H&E and MT stained sections, magnification: x40: 500μm, x100: 150μm.

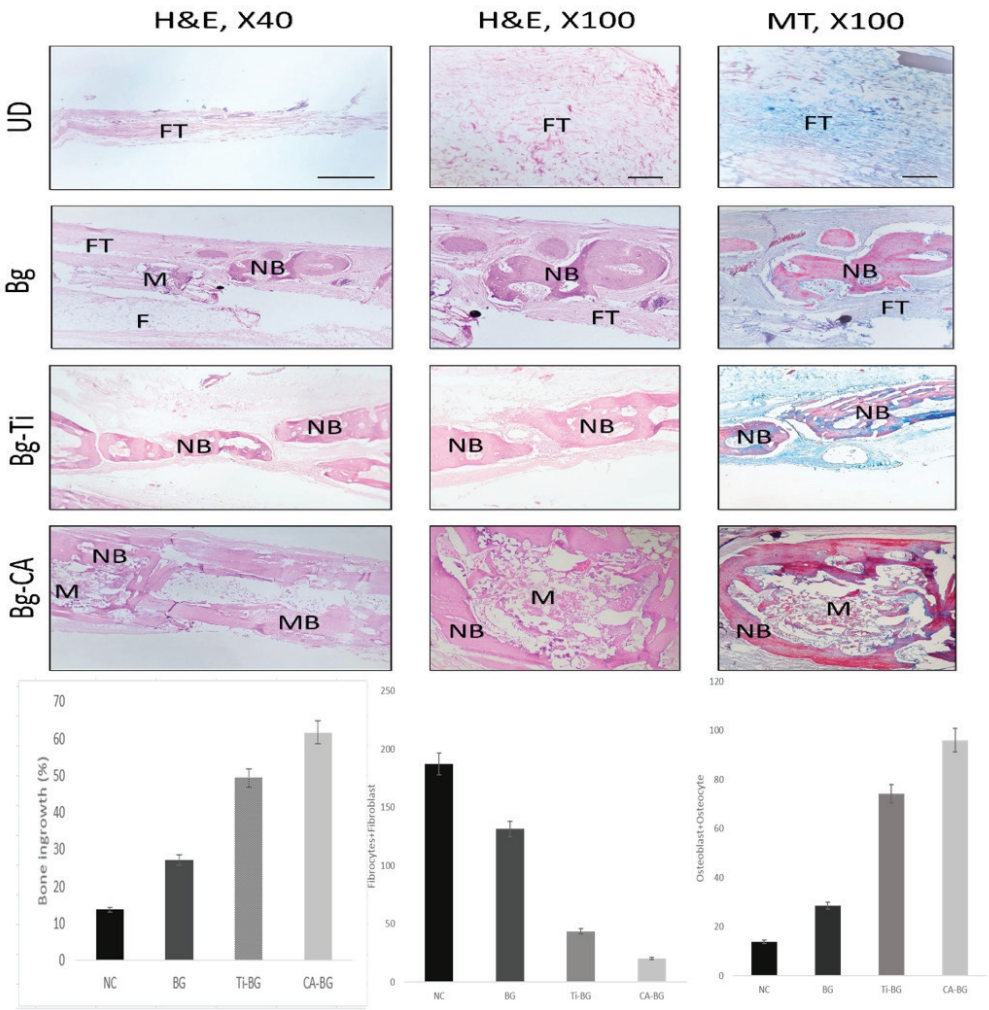

Fig. 5. Microscopic images of calvarial bone defects 8 weeks after surgery. Remnants of the implanted material were evident in the treated defect area, and new bone formation had begun in the defects treated with Bg-Ti and Bg-Ca. LACT: areolar loose connective tissue. FT: fibrous connective tissue; NB: new bone formation. M: Residue of planted material. F: fat, H&E and MT stained sections, magnification: x40: 500μm, x100: 150μm

growth and the number of osteoblasts/osteocytes are found in the Bg-Ca group, followed by the Bg-Ti group in the 8th week. Contrarily, the number of fibrocytes and fibroblasts and the density of collagen fibers in the NC group were significantly higher than in the other groups (P≥0.02).

DISCUSSION

Bones can grow, deform, and self-heal after a fracture, but the repair of large bone defects is still a big challenge for orthopedic surgeons and researchers. There is a growing demand for the reconstruction of bone, concerning the multitude of clinical bone conditions (e.g., bone infections, bone tumors, bone loss from wounds and injuries, etc.) (Currey 2013, Ansari 2019). Grafting techniques and materials employed to cover the defects for restoring the shape and function of the lost bone are typically restricted due to issues such as graft rejection, restrictions with a bone donation, prolonged surgery duration, infections, pain, and eventually likely mortality (Ansari 2019, Battafarano, Rossi et al. 2021). There are many researches within the past decades ,such as research by Hu and Olsen 2016 and Zeng et al. 2018, that have broadly explored potent materials as substitutes for bone tissue repair (Hu and Olsen 2016, Zeng, Liu et al. 2018). Many synthetic materials have been developed as bone substitutes and bone material substitutes (de Melo Pereira and Habibovic 2018).

During the past decades, bioactive glass has been employed by scientists as a bone reconstruction

agent. Due to their biocompatibility and bioactivity, these glasses are widely used as medical implants in living organisms to treat Osteoporosis disease and replace affected or damaged bones (Bellucci, Cannillo et al. 2018, Fernandes, Gaddam et al. 2018). The first use of bioactive glass in animals was reported in 1986 from Amsterdam (Netherlands), where bioactive glass cubes were implanted in the tibia of Indian pigs. The SEM images revealed the better growth of bone cells and blood vasculature in the implant area, implying the biocompatibility of the implants (Schneible 2020). However, due to the breakability and poor mechanical properties of bioactive glasses compared to natural bone, researchers have significantly modified these materials by adding various pollutants, structural and surface modifications, modifying synthesis methods, altering the ratio of constituent compounds, additives, etc. to enhance the function of these materials. Accordingly, this study investigated the synergistic effect of apatite carbonate and titanium-containing bioactive glass and evaluated the performance of these materials as a new composition and scaffold in bone reconstruction (Fernandes, Gaddam et al. 2018, Schneible 2020).

This study investigated the efficacy of titanium-containing bioactive glass in combination with carbonate apatite in the reconstruction of calvaria bone. The biocompatibility of Bg-Ca and Bg-Ti composites and their ability to stimulate osteogenesis were evaluated through various analyses *in vitro* and *in vivo*. SEM images revealed that the Bg-Ca and Bg-Ti composites are visible as spherical particles that have aggregated into bulky assemblies. Bg-Ca and Bg-Ti composites were further examined *in vitro* using XRD diffraction patterns and FTIR evaluations. The analysis of XRD patterns showed that the peaks of Bg-Ca and Bg-Ti occur from 35 to 40 °C, results that comes in agreement with previous results reported in the ICDD database. Investigation of the FTIR spectra for Bg-Ca and Bg-Ti composites revealed that the produced spectra are related to both scaffolds and exhibited no impurities in the synthesized compounds.

Further *in vitro* investigations have shown that Bg-Ca and Bg-Ti have good biocompatibility, with minimal cytotoxicity against human bone marrow MSCs, indicating the possibility of using concentrations of up to 10 mg/ml of Bg-Ca and Bg-Ti powders in animal models. Shamsi et al. (2018) reported that S455 bioactive glass had no cytotoxicity against the growth and differentiation of MSCs (Shamsi, Salimi et al. 2018). Similarly, Mirjalili et al. (2018) reported no cytotoxicity of the fluorapatite-bioactive glass composite against Vero cells (Mirjalili, Manafi et al. 2020).These studies, in line with this study, imply the non-cytotoxicity of bioactive glass and other elements against eukaryotic cells, suggesting the potency of these materials which could have clinical use in humans and animals.

The use of HA scaffold and bioactive glass for bone regeneration has been the topic of interest in many studies such as Al-Bakhsh et al. 2019, Abulyazied et al. 2021. (Al-Bakhsh, Shafiei et al. 2019, Abulyazied, Alturki et al. 2021). However, recent studies have sought to enhance bone reconstruction by combining these materials with several bone regenerators. Towards to many researches concurrent use of HA and DCFGP (Oryan, Monazzah et al. 2015), Nano HA with bone marrow (Yadegari, Bigham et al. 2020), HA with natural polymers (Radulescu, Neacsu et al. 2022), HA with bone marrow and platelet-rich plasma (Yun, Yoo et al. 2012), and HA with Royal gel promoted bone regeneration and induced bone reconstruction at the bone defect site of laboratory animals (Bigham-Sadegh, Torkestani et al. 2020), indicating the synergistic effect of two or more reconstructive compounds. The research conducted by Smith et al. (2021) also advocates the capability of titanium-containing bioactive glass in bone regeneration and the repair of bone lesions (Smith, ElKashty et al. 2021). Alaa Emad Eldeeb et al. (2022) used titanium-containing bioactive glass NPs (BGT5; 1 wt%) to repair bone defects in rats and reported that the fabricated scaffold is more potent (by several folds) than bioactive glass NPs alone in repairing the bone defects (Eldeeb, Salah et al. 2022). Titanium also has some applications in dentistry. Mistry et al. (2011) coated titanium alloy with bioactive glass and HA and evaluated various clinical and radiological parameters after implanting the prosthesis in the patients. It was found that HA and bioactive glass (as coating materials) are non-toxic and biocompatible, and the implants coated with titanium alloy can successfully achieve bone integration and support final restorations (Mistry, Kundu et al. 2011). In this study, the combination of titanium and bioactive glass has shown no cytotoxicity against eukaryotic cells. Considering its marked effect on the regeneration of the rabbit calvarias bone, the titanium-containing bioactive glass could be a good candidate for bone regeneration in other animals in the future. However, the clinical use of these materials requires further research into clinical trials.

CONCLUSION

In this study, the Bg-Ca and Bg-Ti composites were fabricated using the sol-gel method. XRD and FTIR analyses were employed to assess the right formulation of composites and confirm the presence of no impurities in the fabricated composites. The MTT assay revealed that the synthesized scaffolds exhibit no cytotoxicity at a concentration of 10 mg/ml on eukaryotic cells after 7 days of implantation. The biocompatibility and osteogenic capacity of HA with titanium-containing bioactive glass were further investigated. It was found that the Bg-Ca and Bg-Ti composites possess a stronger capacity for the regeneration of rabbit calvaria bone than the NC group.

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