Osteoconductive potential of a hydroxyapatite fiber material with magnesium: In vitro and in vivo studies

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Despite the benefits of hydroxyapatite fiber (HAf) as a synthetic bone substitute, materials capable of faster bone regeneration would be more preferable. In this study, effects of HAf with magnesium (Mg-HAf) on bone regeneration were evaluated. In vitro, levels of osteogenic genes were significantly higher in bone marrow cells cultured with Mg-HAf than in those cultured with HAf alone. Moreover, effects of HAf only (control) and 5.7 mol% Mg-HAf on the cranial bones of Japanese white rabbits were evaluated. Micro-CT imaging and histology indicated significant differences between the control and Mg-HAf groups. Significantly higher new bone volumes and percentages were observed in the Mg-HAf group than in controls at 4 and 8 weeks (p<0.05); the newly formed bone was more mature in the Mg-HAf group than in controls. These results indicated that Mg-HAf can enhance osteogenic differentiation-related gene expression and promote rapid bone formation and maturation.

Keywords: Hydroxyapatite, Magnesium, Oral implant, Osteogenesis, Fiber

INTRODUCTION

Oral implant therapy has become a common treatment approach, with satisfactory esthetic and functional outcomes1,2; several studies have reported high success and survival rates3,4. However, implant placement is often limited by advanced bone resorption after tooth loss or anatomical restrictions, such as involving the mandibular canal and maxillary sinus. In these clinical situations, ridge augmentation is frequently required in addition to implant surgery5. Bone-grafting materials include autogenous bone6, allografts7, xenografts8,9, and alloplasts10. Among these, autogenous bone remains the gold standard for augmentation procedures owing to its osteogenic, osteoinductive, and osteoconductive properties11. However, it has several disadvantages, including donor site morbidity and limited volume of harvestable bone12. Various types of synthetic bone substitutes have been developed to address these disadvantages13.

Hydroxyapatite [HA, Ca10(PO4)6(OH)2] is commonly used in the dental and orthopedic fields, owing to its excellent biocompatibility and osteoconductivity. However, HA and other bone substitutes, such as β-TCP, exhibit minimal or slow resorption in the body14,15. We had previously developed a hydroxyapatite fiber (HAf) material, made of pure HA, of 5–15-µm diameter. HAf is biodegradable and eventually replaced by newly formed bone16-18. Moreover, HAf can be easily manipulated at bone augmentation sites, owing to its cotton-like property. However, more rapid osteogenic effects at early stages are necessary to compensate for the uncontrollable absorbability of HAf.

Many studies have focused on modifying HA by doping with different elements, such as Magnesium (Mg), Zn, Sr, Ag, Mo, and Li19-22. In adult human normal calcified tissues, the proportions of Mg in the bone, enamel, and dentin are 0.72, 0.44, and 1.23 wt%, respectively23. Despite the low concentrations, Mg plays an important role in bone metabolism, particularly during the early stages of bone formation; it also strengthens bone and reduces risk of osteoporosis, similar to Ca24. Some studies have demonstrated that Mg ions enhance osteoblast differentiation in bone marrow stromal cells and bone formation25-27. Landi et al.28 prepared Mg-doped HA with different Mg/Ca molar ratios, ranging from 5.7 to 13.7, and reported that the 5.7 mol% Mg-doped HA shows superior biocompatibility based on the absence of genotoxicity, carcinogenicity, and cytotoxicity. Therefore, Mg-doped HAf is expected to have high biocompatibility and compensate for the uncontrollable absorbability of HAf. In this study, we evaluated the effect of HAf with Mg (Mg-HAf) on bone regeneration, both in vitro and in vivo.

MATERIALS AND METHODS

Sample preparation

HAf was used as described previously16; it was made of pure HA, 5–15 µm in diameter, as determined by scanning electron microscopy. Its three-dimensional appearance resembled that of cotton (Figs. 1a, b19). Briefly, HAf was prepared as follows. First, fine apatite particles were uniformly dispersed in an aqueous solution of binder. Then, the dispersion was extruded through a plurality of spinning orifices, with heating, to evaporate
the water and allow the HA particles to bind. Finally, a fibrous HA was calcined to produce cotton-like HAf. Before the experiments, HAf was sterilized by dry-heat sterilization. In the experiments, it was used as Mg-HAf by simultaneously using HAf and a physiological saline solution containing different amounts of MgCl₂·(H₂O)₆. It was prepared as three kinds of Mg-HAf (5.7, 11.4 and 28.5 mol%) at Mg/Ca molar ratio.

**In vitro study**

1. **Cell culture**

Bone marrow cells (BMCs) were obtained from the femurs of 6-week-old male Wistar/ST rats. BMCs were cultured in α-minimum essential medium (α-MEM; Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Gibco) at 37°C in a humidified atmosphere of 5% CO₂. The culture medium was changed every 3 days and the cells were passaged at approximately 80% confluence. Cells at the 3rd passage were used in the study.

2. **Quantitative reverse-transcription PCR (RT-qPCR)**

BMCs were seeded with only HAf (control) and Mg-HAf (5.7, 11.4, and 28.5 mol%) in 24-well plates at a density of 1×10⁵ cells/well. The medium was changed to an osteogenic medium containing 0.01 μM dexamethasone, 50 μg/mL ascorbic acid, and 10 mM β-glycerophosphate. After 3, 5, and 7 days of culture, total RNA from cells, under different conditions, was isolated using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. RNA concentrations were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). The First Strand Synthesis Kit, Superscript IV VILO (Invitrogen, Carlsbad, CA, USA) was used to synthesize cDNA for the relative quantification of mRNA. RT-qPCR was performed using TaqMan® Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and the StepOnePlus System (Applied Biosystems) to determine the expression levels of selected osteogenic differentiation-related genes [alkaline phosphatase (Alp), collagen 1 alpha 1 (Col1a1), and osteocalcin (Oc)].

Preformulated TaqMan Gene Expression Assays were purchased from Applied Biosystems for the following rat genes: Alp (Rn01516028_m1), Col1a1 (Rn01463848_m1), Oc (Rn00566386_g1), and Gapdh (Rn99999916_s1). Gapdh was used as the reference gene for normalization using the ΔΔCt algorithm³⁰.

**In vivo study**

1. **Surgical procedure**

Twelve mature male Japanese white rabbits (weight: 2.8–3.2 kg) were used in this study. The protocol was approved by the Animal Care and Use Committee at Tokyo Medical and Dental University, Japan (approval number: A2018-248A). Animals were acclimated under laboratory conditions for 7 days prior to the surgery. The rabbits were initially anesthetized by an intramuscular injection of ketamine (50 mg/kg Ketalar, Sankyo, Tokyo, Japan) and thiopental sodium (25 mg/kg Rabonal, Tanabe, Tokyo, Japan) and thiopental sodium (25 mg/kg Rabonal, Tanabe, Tokyo, Japan). The surgical site was shaved and disinfected, and 1.8 mL of a local anesthetic (2% xylocaine/epinephrine 1:80,000, Dentsply Sankin, Tokyo, Japan) was injected into the surgical area. The parietal bone was selected as the augmentation model site. A 3-cm longitudinal skin incision was made sagittally between the parietal and frontal bones, subperiosteal dissection was performed, and the periosteum was raised. Hollow cylindrical polytetrafluoroethylene (PTFE) chambers with inner diameters of 5.0 mm and heights of 3.0 mm (Wilco, Kanagawa, Japan) were fixed with stainless-steel screws (FKG Dentaire, Chaux-de-Fonds, Switzerland) to the parietal bone on the right and left sides (Fig. 2a). HAf only (40 mg, control) and 5.7 mol% Mg-HAf (40 mg) were grafted into each chamber with peripheral blood (Fig. 2b). The skin flaps were sutured with 4-0 nylon. During the observation period, all rabbits were housed individually in standard cages and given water and a standard rabbit feed. Rabbits were sacrificed at 4 weeks (n=6) and 8 weeks (n=6) by a lethal dose of thiopental sodium. Following euthanasia, the entire cranial bone was removed and fixed in neutral 10% formalin for 2 weeks.
2. Micro-CT analysis
Following the fixation period, the specimens were evaluated by a micro-CT analysis (SMX-100CT, Shimadzu, Kyoto, Japan). The X-ray settings were as follows: 100 kV; 70 μA; resolution of 0.040 mm/pixel. New bone volume (NBV) and ratio of new bone formation (NB%) were calculated using analysis software (TRI/3D BON, RATOC System Engineering, Tokyo, Japan).

New bone and HAF were identified by setting radiopacities corresponding to histological sections. NBV was calculated by the radiopaque voxels observed in the chamber and were presented in mm³. NB% was evaluated based on the percentage of new bone occupying the HAF and new bone space in the chamber.

3. Histological analysis
Following micro-CT scanning, cranial bone (n=12) was dehydrated with ascending grades of ethanol and embedded in acrylic resin (Technovit 7200, Heraeus Kulzer Japan, Tokyo, Japan) to obtain non-decalcified sections. The samples were cut, using a sawing system (Exakt, Mesmer, Ost Einbeck, Germany), in the sagittal direction and ground to a thickness of about 100 μm. The sections were stained with 0.1% toluidine blue and observed under a light microscope (Biozero, Keyence of America, Itasca, IL, USA).

4. Statistical analysis
Comparison of expression data across the four groups, at the same time point, was performed using non-parametric multiple comparison test by Dunnett’s T3. Comparison of micro-CT results between 4 and 8 weeks were performed using the Mann-Whitney U-test. All statistical analyses were performed using SPSS 22.0 (SPSS, Chicago, IL, USA). Differences were considered statistically significant at p<0.05.

RESULTS

In vitro study
1. RT-qPCR
RT-qPCR was used to evaluate osteogenic differentiation-related gene (Alp, Col1a1, and Oc) expression (Fig. 3). After culturing for 3 days, 5.7 mol% Mg-HAF enhanced the expression of Alp and Oc; the levels of all osteogenic markers were significantly higher after treatment with 11.4 mol% Mg-HAF than in the control group (Fig. 3a). After 5 days, 5.7 mol% Mg-HAF significantly enhanced the expression of Alp and Col1a1 compared to the corresponding levels in the control group (Fig. 3b). However, after 7 days, Oc expression was significantly lower in the 28.5 mol% Mg-HAF group than in the rest (Fig. 3c).

In vivo study
1. Micro-CT analysis
A quantitative radiological analysis was performed by micro-CT (Fig. 4). At 4 weeks, the NBV was 22.29±2.52 mm³ in the control group and 31.13±6.73 mm³ in the Mg-HAF group (Fig. 5). At 8 weeks, the NBV was 22.39±4.72 mm³ in the control group and 32.37±7.36 mm³ in the Mg-HAF group. NBV was significantly higher at both 4 and 8 weeks in the Mg-HAF group than in the control group (p=0.015, p=0.026). At 4 weeks, NB% was 42.32±5.95% in the control group and 63.01±10.32% in the Mg-HAF group (Fig. 6). At 8 weeks, NB% was 51.43±8.17% in the control group and 69.62±15.86% in the Mg-HAF group. NB% of the Mg-HAF group was significantly higher than that of the control group at both 4 and 8 weeks (p=0.002, p=0.015).

2. Histological analysis
At 4 weeks, in the chamber of both groups, three vertical layers were observed (Figs. 7, 8). The bottom layer was mainly composed of newly formed bone that continued directly to the host parietal bone. It included many bone lacunae and incorporated HAF. In the intermediate layer, deeply stained amorphous component appeared...
Fig. 3 Osteogenic gene expression levels in BMCs cultured on each sample type. Expression of osteogenic genes was measured by RT-qPCR at the following time points: (a) day 3; (b) day 5; (c) day 7. \(n=6\); *\(p<0.05\). Expression levels of all osteogenic markers were significantly higher in the test groups (5.7 mol\% and 11.4 mol\%) than in the control group on day 3. \(\text{Alp} \) and \(\text{Col1a1} \) levels were significantly higher in test groups (5.7 and 11.4 mol\%) than in the control group on day 5. \(\text{Oc} \) levels in 28.5 mol\% group were significantly lower than those in the control group on day 7.

Fig. 4 Image of the graft site obtained by micro-computed tomography (micro-CT).

Fig. 5 NBV at 4 and 8 weeks post-operation, as determined by micro-CT. There were significant differences among the groups at both 4 and 8 weeks. Values are presented as means±standard deviation \(n=6\); *\(p<0.05\) according to the Mann-Whitney \(U\)-test.

Fig. 6 Ratios of new bone formation at 4 and 8 weeks post-operation, as determined by micro-CT. There were significant differences across the groups at both 4 and 8 weeks. Values are presented as means±standard deviation \(n=6\); *\(p<0.05\), **\(p<0.01\) based on Mann-Whitney \(U\)-test.

With densely packed HAf. In this layer, only few cell components were observed. In the uppermost layer, only HAf, without any cell component, was observed, and no connective tissue invagination from the top of the chamber was seen. At 4 weeks, the newly formed bone was found at approximately one-third of the total height of the chamber in the control group (Fig. 7) and at almost half of the chamber height in the Mg-HAf group (Fig. 8).

At 8 weeks, both groups showed nearly the same tissue structure as at 4 weeks (Figs. 9, 10). The newly formed bone reached more than half of the chamber in the control group (Fig. 9) and reached about three-quarters of the chamber in the Mg-HAf group (Fig. 10). HAf was still found in newly formed bone at the bottom.
of the chambers. In some Mg-HAf groups, fat cells were detected around the host bone. Unlike at 4 weeks, the uppermost layer was either slightly or not at all observed in both groups.

Fig. 7  Histological images of the control group at 4 weeks (a, b).
The height of newly formed bone reached approximately one-third of the chamber height. (a) Magnification 4×; (b) Magnification 50×; NB, Newly formed bone.

Fig. 8  Histological images of the Mg-HAf group at 4 weeks (a, b).
Newly formed bone reached almost half of the chamber height. (a) Magnification 4×; (b) Magnification 50×; NB, Newly formed bone.

Fig. 9  Histological images of the control group at 8 weeks (a, b).
A large amount of new bone formed from the host bone. (a) Magnification 4×; (b) Magnification 50×; NB, Newly formed bone.
DISCUSSION

Autogenous bone is considered the most useful material for augmentation procedures related to implant treatment, owing to its osteogenic, osteoinductive, and osteoconductive properties. However, it has various limitations, including increased operative time, limited availability, and significant morbidity. Therefore, there has been a growing demand for synthetic bone substitutes to address these shortcomings. HA has been widely studied in bone tissue engineering and has also been used as a synthetic bone substitute owing to its high biocompatibility and osteoconductivity. HAf, previously developed and improved by us, is resorbable and eventually replaced by new bone, while HA and β-TCP are very slowly resorbed, if at all, in the body. The shape of a bone substitute is considered an important determinant of its effects. Fibrous scaffolds have been reported to absorb more proteins compared to solid scaffolds and exhibit enhanced cell attachment, hence suggesting the fibrous scaffolding of HAf to be superior for tissue engineering. Various effects of HAf on bone formation have been demonstrated using the rabbit cranium chamber model. Kimura et al. indicated that HAf, combined with autogenous bone, is highly effective for vertical bone augmentation.

Ion doping is a trusted method to enhance the biodegradability, biocompatibility, and biomechanics of bio-ceramics, such as HA. Mg-doped materials have the potential to stimulate osteoblast proliferation, enhance osteoblast differentiation in bone marrow stromal cells and promote bone formation. Moreover, Mg-substituted HA-coated dental implants induce enhanced osteoblast differentiation in vitro and exhibit improved osseointegration in vivo. Zreiqat et al. reported that Mg stimulates new bone formation and may increase osteoblast cell adhesion and stability by interactions with integrins. Rude et al. indicated that Mg depletion results in decreased osteoblasts, impaired bone growth, and increased osteoclastic resorption in the trabecular bone. In the present study, higher levels of osteogenic differentiation-related gene expression were observed in the Mg-HAf group, especially in the 5.7 mol% Mg-HAf group, than in control groups at the early stage (Fig. 3). This result was consistent with those from past studies. However, our data showed that a high concentration (28.5 mol%) of Mg-HAf inhibited osteogenic differentiation-related gene expression, thereby suggesting cytotoxicity at higher concentrations of Mg ions. Yoshizawa et al. reported that cell proliferation is reduced at high concentrations of MgSO4 in vivo. Other metal ions (Na, Cr, Mo, Al, Ta, Co, Ni, Fe, Cu, Mn, and V) could also significantly reduce osteoblast function in a concentration-dependent manner. Therefore, new bone formation is related to ion concentration, and 5.7 mol% was optimal in this study.

Fujii et al. demonstrated that HAf graft density affects new bone formation in a vertical bone augmentation model of rabbit parietal bone. In their study, an intermediate HAf density (40 mg of HAf in the chamber with 5-mm inner diameter and 3-mm height) showed the greatest NBV among all groups. Accordingly, in the present in vivo study, 40 mg and 5.7 mol% Mg-HAf were adopted.

A micro-CT analysis showed that NBV and NB% of the Mg-HAf group were significantly higher than those of the control group at 4 and 8 weeks after surgery (Fig. 5). This result strongly indicated that Mg promotes new bone formation. Histological observations supported the micro-CT results. At 4 and 8 weeks, the groups showed nearly identical tissue structures, although the Mg-HAf group showed more newly formed bone than the control group (Figs. 7–10). Accordingly, Mg was able to stimulate faster bone formation and bone maturation. Some in-vivo studies have suggested that Mg released from Mg-doped implant surfaces can enhance bone formation and osseointegration at the early stage of healing, which is highly desirable for the immediate/early loading of the implant. Mg also stimulates
vascularization on the bone, which is closely related to bone remodeling\cite{galli1995long}. Galli et al.\cite{galli1995long} indicated that more rapid angiogenesis around Mg-releasing implants is associated with notably increased VEGFA, a vascular growth factor in endothelial cells, suggesting that the release of Mg\cite{momo2005fiber} stimulates faster bone formation at the early stages. Such osteogenic features of Mg are thought to positively affect bone formation when using Mg-HAf.

The present study had some limitations. First, in this study, Mg was not incorporated into HAf, but Mg was used together with HAf as an aqueous solution. Landi et al.\cite{landi2005fiber} reported Mg-doped HA which is releasing Mg. However, in this study, we investigated the effect of Mg which was all administered at the beginning and HAf which was moisted by Mg aqueous solution. Mg-incorporated HAf may have a prolonged effect on new bone formation owing to sustained release. Slower Mg release is expected to exhibit a continuous effect. Second, in vivo study results were obtained using rats while in vitro study results were from rabbits; therefore, the current results may not be directly generalizable to humans, owing to the differences between animal and human models. Further studies would be required to resolve these limitations.

CONCLUSIONS

In an in vitro study, we showed that Mg-HAf has the potential to enhance osteogenic differentiation-related gene expression at the early stages of bone formation. Furthermore, an in vivo study using rabbits demonstrated that this material was effective for vertical bone augmentation. Under our experimental conditions, 5.7 mol\% Mg-HAf was the optimal dose required for bone formation. Taken together, our results suggested that Mg-HAf scaffolds are promising bone substitutes for bone augmentation procedures during dental implant treatment.

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