INTRODUCTION

With the development of minimally invasive osteosynthesis and surgery, bone regeneration procedures have become basic and frequent. The selection of implantation materials has always been performed with autologous or allogeneic bone\(^1,2\). However, autologous bone transplantation is limited by the complicated method of obtaining autogenous bone, addressing additional wound complications in the removal area, and the quality and post-implantation effects of the implanted bone\(^3,4\). Allogeneic bone transplantation also has drawbacks linked to ethical issues and the possibility of disease transmission by xenotransplantation\(^5,6\).

In the human body, hydroxyapatite (HA) \([\text{Ca}_{10} (\text{PO}_4)_{6} (\text{OH})_2]\) constitutes 60–70% of bones and 98% of teeth and is the toughest component of human hard tissues\(^7-9\). Nowadays, synthetic HA has repeatedly proven its good biocompatibility, space maintenance, and bone conduction potential\(^10-13\) because its surface supports the adhesion, growth, and differentiation of osteoblasts (OBL). Hydroxyapatite (HA) \([\text{Ca}_{10} (\text{PO}_4)_{6} (\text{OH})_2]\) has a high degree of chemical similarity with the mineral composition of animal bone. Hydroxyapatite fiber scaffold (HAF) is a biological material with a highly interconnected porous structure. We aimed to study the physical and biological characteristics of HAF and compare the osteogenic effects of HAF, natural osteogenic materials (NOM), and carbonate apatite (CO\(_3\)Ap-DP) in the parietal defects of a rabbit’s skull. X-ray analysis and histological assessment showed that HAF followed a trend of early initial osteogenesis and bone trabecular structure formation, especially at the cortical bone portion. Compared to the other two materials, HAF was more absorptive. Results indicated that HAF had the same osteoconductive and new bone formation properties as NOM and CO\(_3\)Ap-DP. These findings will provide options for future material development and novel protocols for use in surgeries, ultimately leading to better patient outcomes.

Keywords: Hydroxyapatite, Scaffold, Cranial model, Porous geometry, Bone regeneration

MATERIALS AND METHODS

Synthesis of the hydroxyapatite fiber scaffold

This research sought to improve the HAF manufacturing
method that has been used in previous studies\textsuperscript{21}. Mixed a 3% deflocculant (Serna D-305, Chukyo Yushi, Aichi, Japan) with an aqueous solution of HA (1 nm to 10 μm; median particle size=2 μm) (Tomita Pharmaceutical, Tokushima, Japan) and added pullulan (trisaccharide, Hayashibara, Okayama, Japan) to prepare a neutral spinning raw material. We then added this raw material to a spinning machine (RMX-200: Remedio, Tokyo, Japan) to produce a nonwoven HA fabric. We placed the fabric in a high-temperature roaster and gradually heated it to 1,200°C to remove the binder and harden the HA fibers. We impregnated the hardened fibers with the above-mentioned HA aqueous solution, dried it, and subjected it to secondary roasting at 1,200°C. After cooling, we obtained the final HAF to use in our experiment. We used commercially available, natural osteogenic materials (NOM) (Bio-oss\textsuperscript{®}, S-size Geistlich Pharma, Wolhusen, Switzerland) and carbonate apatite (CO\textsubscript{3} Ap-DP) (L-size Cytranz\textsuperscript{®} Granules, GC, Tokyo, Japan) —without further modification.

**Microstructure and compositional analysis**

For X-ray diffraction (XRD) analysis, we first ground all three samples to powder. We recorded the powder-X-ray diffraction spectrum at room temperature using a fold-back X-ray device (Smart Lab -SP/IUA, Rigaku, Tokyo, Japan) at 40 kV and 30 mA (radiation source: Cu Ka=1.5406 Å) as the radiation source. We recorded the diffraction pattern in the 2θ range from 10°–40° and its corresponding value.

**Observation of surface features**

Before observing the surface characteristics, we sprayed the sample surface with a Pt-Pd alloy using an ion-sputtering instrument (E102, Hitachi, Tokyo, Japan). We then analyzed the surface topography using a field-emission scanning electron microscope (FE-SEM; JSM-7900F. Hitachi).

**Animal experiments**

In this study, we used 8 Japanese white rabbits weighing 3.0–3.3 kg as animal models. The protocol was approved by the Institutional Animal Care and Use Committee of the Tokyo Medical and Dental University (Approval No. A2019-315A). Throughout the experimental period, we fed all the animals a standard laboratory diet and housed them in a facility approved by the Tokyo Medical and Dental University. The food and water consumption of the rabbits were checked in every day and ensured that their pain and discomfort were minimal. The animal protocol conformed to the NIH guidelines stated in the Principle of Laboratory Animals Care (NIH publication no.86-23; revised in 1985). The rabbits were anesthetized with an intramuscular injection of ketamine hydrochloride (50 mg/kg Ketalar, Sankyo, Tokyo, Japan) and thiopental sodium (25 mg/kg Rabonal, Tanabe, Tokyo, Japan). Selected the skull parietal bone as experimental model site. The surgical site was shaved and disinfected, and 2 mL of a local anesthetic (2% xylocaine/epinephrine 1:80,000, Dentsply Sankin, Tokyo, Japan) was injected into the surgical area. A 3 cm longitudinal skin incision was made on the parietal, dissection the peristeum to expose the bone surface. A 5 mm-diameter drill was used to remove bone fragments in four areas under continuous cold saline irrigation to prepare the bone-defect model. CO\textsubscript{3}Ap-DP, NOM, and HAF were placed in the areas with the bone-defect and prepared the similarly control group. Covered the bone surface with a Bio-Gide membrane (Bio-Oss\textsuperscript{®}, Geistlich Pharma) (Fig. 1). The skin flaps were sutured with 4-0 nylon. The animals were sacrificed after 4 or 8 weeks (for each group, n=4) and the implantation sites with surrounding bone tissue was excised and stored them in 10% formalin for further evaluation.

**Microfocus X-ray computed tomography (CT)**

We took CT images of the specimen area using a microscope (SMX-100CT, Shimadzu, Kyoto, Japan) with 10 μm resolution (voltage=100 kV; current=30 μA; scan slice thickness=0.022 mm). We used the scan data to reconstruct the micro-CT image. We constructed the volume of regenerated bone according to the bone tissue and bone regenerative filler in the calculated defect area and considered the total defect volume as the total space present inside the round bone defect. We calculated the internal volume of the defect according to different tissue parts and transmission shadows, and finally obtained the new bone volume/total volume (BV/TV).

**Histological analysis**

The bone samples were fixed in a formalin solution for 7 days. Following this, some samples were decalcified in an neutral deliming solution (EDT-X, Falma, Tokyo, Japan) for 5 days, performed gradient dehydration in 60%–100% alcohol, and embedded them in paraffin. After dewaxing the sections, we used a microtome to cut 5 μm-thick sections and stained the samples with hematoxylin-eosin according to distinguish between the various tissue types. We subjected the other samples to gradient dehydration from 60% to absolute ethanol and embedded them in methyl methacrylate. We sliced this with a tungsten steel blade. Subsequently, we stained all

![Fig. 1](image-url)  
(A) A 5 mm-diameter bone defect was prepared on the cranial bone of the rabbit.  
(B) The defects in the experimental groups were filled with bone-forming materials.
the sections with Goldner’s trichrome staining method. We observed all the slices using an optical microscope (BZ-X700, KEYENCE, Tokyo, Japan). We used the ImageJ program to measure new bone areas and the material surface base in the Goldner-stained section.

Statistical analysis
We performed statistical analysis using the SPSS statistics software (version 22, IBM, Armonk, NY, USA). All data are presented as mean±standard deviations. We evaluated the between-group differences using a one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. *p<0.05* was considered statistically significant.

RESULTS

Material property analysis
1. XRD analysis
According to Fig. 2A, by comparing the XRD waveforms of HAF and standard HA, we were able to match all the peaks and prove that both materials were composed of HA. Figure 2B summarizes the XRD patterns of HAF (Fig. 2B-1), NOM particles (Fig. 2B-3), and CO3Ap-DP (Fig. 2B-2). All the samples showed apatite waveforms, which suggested that these three materials did not contain substances of other crystal-phase components. The waveform of HAF was sharper, showing a higher crystallinity and higher density than the other two materials.

2. Analysis of structure and surface morphology
The shapes of the three materials in SEM were markedly
different (Fig. 3). The particle diameter of NOM was 0.5–1 mm, and that of CO3Ap-DP was 0.4–0.8 mm. HAF measured about 3 mm and was trimmed according to the usage. HAF (A) was composed of fibers with a diameter of 10–15 μm, and there were a large number of irregular holes between the fibers (D), each having a diameter of 5–200 μm. The NOM particles (B) had a porous structure, and each particle had an average of 2–4 micropores, with pore sizes ranging from 100–250 μm, and many tiny scale-like structures on its surface (E). However, CO3Ap-DP (C) had spherical particles and small irregular holes were seen on the smooth surface (F).

In vivo sample analysis

1. Clinical and radiological analysis of the defect area

We clinically observed all the experimental samples. After all the defects were filled with bone-forming materials, the surgical sites healed well. No graft material infection or exposure occurred during our observation. In Fig. 4, sagittal and coronal X-ray sections revealed the bone defects and implantation areas of each sample at 4th and 8th week. In the 8th week HAF group (Fig. 4A), the implanted site had a higher density than in all the other groups. Also, the material range of the CO3Ap-DP group (Fig. 4C) was reduced and did not differ much from the material areas of the NOM group (Fig. 4B). In the control group (Fig. 4D), there was significant natural bone formation in the defect area and on its edge.

During the 4 and 8 weeks periods, we performed micro-CT measurements on the implanted area. The BV/TV of each defect-implanted area was significantly different from that of the control group (Fig. 5A). Fourth week after surgery, the BV/TV values of the HAF, NOM, and CO3Ap-DP groups were 42.02±2.49%, 34.90±2.80%, and 30.73±2.68%, respectively. In 4th week, all the experimental groups had significantly higher BV/TV values than the control group (13.15±1.25%). Eight weeks after surgery, the BV/TV values of the HAF, NOM, and CO3Ap-DP groups were 54.69±4.68%, 44.22±3.12%, and 42.67±3.53%, respectively. In this period too, all the groups showed higher values than the control group (23.40±2.19%). In 4th week, there was a significant difference between NOM and HAF (p<0.05).

In both the 4th and 8th week groups, the percentage of remaining implantation material particles was used as an indicator to summarize the resorption rate of the HAF, NOM, and CO3Ap-DP groups (Fig. 5B). Between 4th to 8th week, remaining percentage of graft material was 54.90±4.31%, 91.54±6.05%, and 65.03±5.82% in the HAF, NOM, and CO3Ap-DP groups respectively. There were statistically significant differences between the HAF and NOM groups, and the CO3Ap-DP and NOM groups (p<0.001) respectively.

2. Histological assessment

In the Villanueva Goldner stain-histological observation,

![Fig. 4](image-url) Micro-CT cross-sectional images of the HAF group (A), NOM group (B), CO3Ap-DP group (C), and control group (D) at 4th and 8th week.

![Fig. 5](image-url) (A) Post-operative status at 4 and 8 weeks using micro-CT to determine ratios of new bone formation. It was found that the amount of bone formation in the implant area was significantly different from the control group, but there was no significant difference between the implanted material groups. Values are presented as mean±standard deviation (n=4); * p<0.05 *** p<0.001 **** p<0.0001 (B) The range of new bone formation in the entire defect area was analyzed using the ImageJ software at 4 and 8 weeks after implantation of the osteogenic material. The experimental group had obvious histological differences as compared to the control group. (C) The reduction ratio of the implant face in the 4th and 8th week sections was calculated using the Image J software. There were significant differences between the NOM and HAF groups (p<0.001).
we found that in 4th week, the HAF group (Fig. 6A) showed a large amount of calcified bone formation at the upper part and edge of the transplantation area, and there was early formation of trabecular bone structure. However, some calcified tissue was scattered at the center and bottom of the transplantation area. Both the NOM (Fig. 6B) and CO3Ap-DP (Fig. 6E) groups had a thin layer of new bone on the implantation material particles. In the control group (Fig. 6F), only a small amount of bone was formed around the HAF-group area bordering the Original bone (OB). In 8th week, the HAF group (Fig. 7A) showed trabecular bone tissue on the edge of the implantation materials with a further-calcified upper portion, and the proportion of calcified tissue scattered in the center and the bottom areas also increased significantly. The new bone in the NOM (Fig. 7B) and CO3Ap-DP (Fig. 7E) groups grew around the surface of the implantation materials, but the early bone structure was not as obvious as in the HAF group. In the control group (Fig. 7F), a large amount of osteoid tissue was absorbed and a small amount of calcified bone was seen in the central area of the defect.

We analyzed the image using the ImageJ program (Fig. 5C). The results showed that the new bone area at 4th week in the HAF, NOM, and CO3Ap-DP groups was 5.44±0.42, 5.41±0.32, and 4.04±0.35 mm², respectively. At 4th week, HAF, NOM and CO3Ap-DP groups were significantly different from the control group (1.46±0.12 mm²) respectively, however there was no statistical difference between the three groups. The new bone area statistically differed between the HAF (7.1±0.63 mm²) and CO3Ap-DP groups (5.26±0.51 mm²) (p<0.05) at 8th week, however, there was no statistically significant difference between the HAF and NOM groups (6.12±0.53 mm²) at 8th week. There were statistically significant differences between the HAF and control groups (2.05±0.16 mm²) (p<0.0001) at 8th week.

In the HE-stained decalcified sample section (Fig. 8), we observed a large number of osteocytes (OC) in the holes and on the surface of the bone structure around the bone graft material (BM). The three BM groups were covered with a new layer of new bone (NB) tissue, and the surface of the bone tissue in contact with the three groups of BMs showed OBLs. The space between the BM
and NB was filled with osteogenic fibrous tissue. In the 8th week HAF group (Fig. 8E), the formation of bone fibrous tissue with OBL was visible in the material.

**DISCUSSION**

The ideal substitute material in bone regeneration should be biocompatible, osteoconductive, and bioactive (on degrading, it should fuse with the implantation site)\(^2\). Our study evaluated the bone-regeneration ability of HAF through its physical and biological properties.

In our XRD peak chart, HAF had a high crystallinity, as compared to NOM and CO\(_3\)Ap-DP. Nano-level HA is closer in structure to the bone tissue and has a larger electrochemical effect and ratio of surface area to volume, which is comparable to that of coarse HA crystals. With better biological activity and degradability\(^2\),\(^3\), it is a biocompatible and bioactive material that facilitates the adhesion, proliferation, and diffusion of different cells\(^2\). After the HA material is implanted, its surface begins to absorb various growth factors and other proteins that promote healing\(^2\). It is reported that, compared with solid scaffolds, fibrous scaffolds absorb more protein and exhibit enhanced cell attachment, indicating that the fibrous scaffolds of HAF are superior for tissue engineering\(^7\).

While preparing the HAF scaffold, micro- or nano-HA particles can be added to enhance the properties and biological response of the support fibers on the surface of the scaffold\(^2\). Moreover, adding a dissipative cellulose binder after sintering can form micropores on the surface of the scaffold, which increases the surface area, thereby promoting surface adsorption and biological reactions. According to SEM, the HAF scaffold has a highly interconnected porous 3D structure. Its size, density, and interconnected structure contribute to its osteoconductivity, vascular infiltration, and material degradation\(^2\),\(^3\). In HAF, pores of various diameters are interconnected, which forms voids that allow interstitial fluid to pass through the matrix, regulate osteogenesis, and enhance the bone regeneration ability of the bone graft\(^2\),\(^3\). Thus, it acts as a bioabsorbable scaffold with certain mechanical properties and biocompatibility.

The inorganic bovine bone particles present in NOM are slender and have large pores, and they cannot maintain their geometric structure after the particles are processed\(^1\). CO\(_3\)Ap-DP is solid and approximately spherical, without a 3D spatial structure. This proves that HAF has a better geometric structure. However, because HAF has a rich 3D spatial structure, it is sensitive to high mechanical pressure. Therefore, as compared to NOM or CO\(_3\)Ap-DP, HAF may be more suitable for implantation in areas that are not susceptible to high mechanical pressure, such as for retention of tooth extraction sites or implantation of induced bone fillers after maxillary sinus elevation.

According to our micro-CT analysis, the amount of bone formation in the HAF group at 4 weeks was 28.87% higher than that of the control group. Compared to the control group, the NOM and CO\(_3\)Ap-DP groups have a slightly higher value. We inferred that the 3D structure of HAF and coordination between its pores may be the reason for its superior performance during early osteogenesis.

In non-decalcified Goldner-stained tissue sections, some mineralized bone formation was observed. Because the blood supply in the edge area was better than that in the center and bottom, sufficient blood circulation accelerated the reconstruction and promoted the new bone in the edge and top area. The HAF group showed a better degree of mineralization in the cortical bone. Even in the early 4 weeks of healing, trabecular bone was formed. This was not obvious in the two other groups. The area of new bone in the HAF group was also
higher than that of the NOM and CO3Ap-DP groups. HAF material has a certain amount of absorption during the experiment. In the experimental groups of NOM and CO3Ap-DP, the bone formation near the edge area was also better than that in the center and bottom area. On the other hand, the degradation of HAF provides more space for subsequent osteogenesis. In the experimental groups of NOM and CO3Ap-DP, the osteogenic materials occupy a large area of the bone defect area, the newly formed bone only occurred on the periphery of the osteogenic material and less likely to occur in between the material.

In the HE-stained sections, we found OBLs on the surface of the bone tissue and around the grafting material in all three groups, which suggested that all the materials possess bone conduction properties. During the growth process, new bone formation and resorption took place simultaneously. We observed osteogenic fibrous tissue in the 8th week HAF group. To the best of our knowledge, none of the materials had cytotoxic effects on the surrounding bone and soft tissues.

In the 4th and 8th week HAF groups, X-ray clearly showed a large reduction in the surface of the base. In the non-decalcified tissue slices, the base reduced by 45.1% more after 4 weeks than after 8 weeks. This explained the replacement of new bone; however, this change only occurred in 8.46% of the NOM specimens. Although previous experiments have proved that direct absorption of HA is difficult under normal circumstances, the production process of HAF overcomes this limitation by using raw material with nano-sized HA and a 3D fiber structure. The bio-absorption of HA is usually mediated by osteoclasts and phagocytosed by macrophages, and our HE slices also showed a large number of multinucleated macrophages and osteoclasts around the HAF scaffold. Reportedly, the absorption time of NOM in the body is 2–3 years.

Both NOM and CO3Ap-DP are commonly used to clinically induce bone regeneration. However, in this study, NOM showed no significant absorption after 8 weeks. The absorption rate of CO3Ap-DP was higher than that of NOM; however, HAF showed significant new bone formation and a significantly higher resorption rate than the other two materials. Although the tendency of early osteogenesis is good, HAF material replacement occurs prematurely in the early stage, and the long-term healing effect requires further study.

This experiment was the first time to use HAF, an osteogenic material in animal experiments. The main purpose of the experiment was to observe the performance of the three osteogenic materials in the formation of new bone in the early stage of osteogenesis. However, it was found that HAF has a higher biodegradability after 8 weeks. Further studies are necessary for the better understanding of HAF biodegradation and endogenous bone replacement.

CONCLUSION

HAF is a highly interconnected porous material composed of HA. Its 3D structure promotes osteoblast colonization and vascular regeneration. In our experiment, as compared to the control group, the HAF scaffold group histologically showed excellent osteoconductivity, and the X-ray analysis and tissue sections showed significant new bone formation. Within 8 weeks, the HAF scaffold was gradually replaced by new bone tissue and it showed a significantly higher resorption rate than NOM or CO3Ap-DP. This finding indicated that the HAF scaffold material could be used as an osteogenic substitute due to its superior osteogenic effect and biodegradability. Further studies with larger sample sizes are necessary to better understand the long-term effects of HAF on the regeneration of bone defects.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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