**Effect of Hydroxyapatite Fiber Material with Autogenous Bone Graft on Vertical Bone Augmentation**

Junichi KIMURA¹, Makoto SHIOTA¹, Kazuhiro KON¹, Masaki FUJI¹, Hitoshi SATO² and Shouhei KASUGAI¹

¹Department of Oral Implantology and Regenerative Dental Medicine, Tokyo Medical and Dental University, Tokyo, Japan
²Yaesu Chuo Dental Clinic, Tokyo Japan

**SYNOPSIS**

**Purpose:** The aim of this study was to examine effects of hydroxyapatite fiber (HF) combining with autogenous Bone on vertical bone augmentation.

**Materials and methods:** Polytetrafluoroethylene chambers were fixed to the parietal bone on the right and left sides in thirty-six Japanese male white rabbits. HF only (30 or 60 mg) and combination of HF with autogenous bone from tibia at different ratios (30 mg bone/HF ratio: 3/1, 3/2, 1/1) were grafted into each chamber. Chambers were left empty in the negative control animals (empty group). Rabbits were sacrificed at 4 and 8 weeks. Then, total bone volume (TBV) of highly calcified bone region in the chamber was quantified with micro-CT analysis. Histomorphometric analysis was performed to evaluate the area of newly formed bone (BA). Statistical analysis was performed with the Student t-test and Tukey-Kramer’s method.

**Results:** In histology, newly formed bone conducted by HF showed a particular structure, and active bone regeneration was observed in the groups containing more HF at 8 weeks. From 4 weeks to 8 weeks BA was significantly reduced in the control, HF 30 mg and ratio 3/1 groups. At 8 weeks BA of HF 60mg was significantly higher than the one of HF30mg group.

**Conclusions:** Combination of HF with autogenous bone was effective in vertical bone augmentation although optimal ratio of the combination needs further study.

**Key words:** hydroxyapatite fiber, bone augmentation, bone graft, bone substitute, osteoclast

**INTRODUCTION**

Implant treatment is now widely accepted for the replacement of missing teeth as it provides a satisfactory outcome for patients¹,². With an increase in the demand for implant treatment, the cases with inadequate bone mass accruing. Bone augmentation could be applied for successful implant treatment in such cases. At present, autogenous bone is widely recognized as the gold standard for bone augmentation³,⁴. However, harvesting autogenous bone sometimes associates morbidity to intra
or extra oral donor sites and the volume of harvestable bone is limited. Moreover, reduction of bone volume after augmentation often impairs proper placement of implants. Johansson et al. reported that bone volume significantly decreased 6 months after autogenous bone grafting to the heavily resorbed human maxilla. Additionally, Kon et al. observed chronic bone resorption of grafted autogenous bone particles with different sizes. To maintain the newly formed bone, various synthetic bone substitutes, such as hydroxyapatite with adequate biocompatibility and osteoconductivity, have been applied in combination with autogenous bone. On the other hand, hydroxyapatite is reported to induce histological reactions which vary with its morphology. Chris et al. found that a greater number of osteoclasts was observed after bone grafting with nano-crystalline hydroxyapatite compared with TCP-HA and/or autogenous bone in rabbit experiments. Stübinger et al. performed maxillary sinus augmentation with nano-structured hydroxyapatite in humans, representing high osteoclast activity at the margin of this biomaterial. We have recently developed a nano-structured fiber formed Hydroxyapatite (HF) and demonstrated that HF is bio-degradable and that this material preserved the socket bony walls in rat experiments. Therefore, application of HF might help to maintain the volume of newly formed bone similar to other nano-structured hydroxyapatite. It is also hypothesized that HF will accelerate bone formation. The aim of this study was to examine the effect of HF application on vertical bone augmentation.

MATERIALS AND METHODS

Animals
Thirty-six Japanese male white rabbits of similar weight (3.1–3.8 kg) and size were used. The experimental protocol was approved by the Institutional Committee for Animal Care and Use of Tokyo Medical and Dental University (No.0110231B).

Surgical procedures
Animals were anesthetized preoperatively with an intramuscular injection of ketamine (50 mg/kg Ketalar, Sankyo, Tokyo, Japan) and thiopental sodium (25 mg/kg Rabonal, Tanabe, Tokyo, Japan). The surgical area was shaved and disinfected. Local anesthetic (18 ml; 2% xylocaine/epinephrine 1: 80,000, Dentsply Sankin, Tokyo, Japan) was injected into the surgical sites. Autogenous bone was harvested from the tibia with bone forceps and bone particles, approximately 1-2mm³, were standardized with sieves.

Skin incision and subperiosteal dissection were carried out sagittally between the parietal and the frontal bone, and the periosteum was raised. Polytetrafluoroethylene chambers (hollow cylinders with 5.0-mm inner diameter and 3.0-mm height; having an outer brim) were fixed with stainless-steel screws (FKG Dentaire, Chaux-de-Fonds, Switzerland) to the parietal bone on the right and left sides. HF only (30 or 60 mg) and HF combined with harvested bone (bone 30 mg/HF ratio: 3/1, 3/2, 1/1) were grafted into each chamber with peripheral blood. HF was kindly provided by Mr. S. Fujii (NRC, Saitama, Japan) (Fujii S., United State Patent No. 4659617). The material used in this study was exactly similar to the one in our previous study. The diameter of HF is 5-15 μm and HF consisted of hydroxyapatite, which was confirmed with X-ray diffraction. Chambers were left empty in the negative control animals (empty). The skin flaps were sutured with 4-0 silk. During the observation period, all rabbits were given water and a standard rabbit feed ad libitum. Rabbits
were sacrificed at 4 weeks (n=18) and 8 weeks (n=18) with a lethal dose of thiopental sodium. The cranial bone was removed and fixed for 14 days in neutral 10% formalin.

**Micro-computed tomography (micro-CT) analysis**  
After fixation, total bone volume (TBV) of highly calcified bone region in the chamber was quantified with micro-CT (SMX-90CT, Shimadzu, Kyoto, Japan) configured in accordance with the manufacturer's instructions. In this configuration, newly formed bone was not detected in the control group. TBV was described as mm$^3$.

**Histological processing**  
After fixation, to obtain non-decalcified sections, all samples (n=72) were dehydrated in ascending grades of ethanol and then embedded in polyester resin (Rigolac-70F, Rigolac-2004, Nisshin EM Co., Tokyo, Japan). These sections were cut (Exakt, Mesmer, Germany) in the sagittal direction and ground to a thickness of approximately 20 μm. The sections were finally stained with 0.1% toluidine blue. All samples were then observed under a light microscope.

**Histomorphometric measurement**  
Histomorphometric analysis was performed to evaluate the area of newly formed bone (total bone area: BA) with ImageJ (National Institutes of Health, MD, USA). BA was described as a percentage of bone area versus whole area inside the chamber.

**Statistical analysis**  
To evaluate the difference between TBV and BA at 4 and 8 weeks, all data were statistically compared with the Student $t$-test. Comparison among HF 30 mg, 60 mg, control groups and Bone/HF ratio 3/1, 3/2, 1/1 groups at identical time points was analyzed with the Tukey-Kramer's method. P-values < 0.05 were considered statistically significant. All statistical analyses were performed with commercial software (Statcel2, OMS Co., Saitama, Japan).

**RESULTS**

**Histological Results**

1. **Control group**
   1) **4 weeks**  
The height of newly formed bone was approximately one-third of the chamber height (Fig. 1a). Newly formed bone was slightly stained at some parts, and on the densely stained bone surface, osteoblast-like cells were aligned. Most of the newly formed bone contained thin trabeculae including large bone lacunae, though lamellar structure was observed at some parts close to the host bone. Some adipose tissue and blood vessels were observed between the trabeculae (Fig. 1b).

   2) **8 weeks**  
The height of newly formed bone decreased compared with 4 weeks especially at the center portion (Fig. 2a). Bone trabeculae were relatively thick and comprised most of the lamellar bone. The area connected to the host bone increased. Some parts of the newly formed bone surface were slightly stained and multinuclear and mononuclear cells were observed at the stained site. The inter-space between the trabeculae was mainly filled with adipose tissue and some blood vessels (Fig. 2b).

2. **HF alone group**
   1) **4 weeks**  
The height of newly formed bone reached almost half of the chamber height, and HF and mononuclear cells occupied the upper region of the chamber (Fig. 3a, b). The newly formed bone appeared as a net-like structure containing some HF inside; however, no lamellar-structured bone was observed.
Fig. 1 Histological images of control group at 4 weeks. Low magnification (a) and high magnification (b). Newly formed bone (NB), adipose tissue (AD) and blood vessels (BV).

Fig. 2 Histological images of control group at 8 weeks. Low magnification (a) and high magnification (b). Adipose tissue (AD), blood vessels (BV), newly formed bone (NB), host bone (HB) and osteoclast-like cell (arrow).

Fig. 3 Histological images of HF 30 mg group (a) and HF 60 mg group (b, c) at 4 weeks. Low magnification (a, b) and high magnification (c). Newly formed bone (NB) and osteoclast-like cell (arrow).
The outline of newly formed bone was not as smooth as natural bone, and showed “moth-eaten-like appearance”. HF and mononuclear cells were mostly observed between the trabeculae. Large number of osteoclast-like cells and osteoblast-like cells were observed in contact to HF and newly formed bone (Fig. 3c).

2) 8 weeks
The bone height was reduced by almost half compared to 4 weeks in both groups, while the newly formed bone still showing “moth-eaten-like appearance” was interconnected and it is widely connected to the host bone compared to 4 weeks in the HF 60 mg group (Fig. 4a, b). The newly formed bone contained HF, and some part of the newly formed bone was mostly composed of HF (Fig. 4c), while lamellar-structured bone was still not observed. Relatively large bone lacunae compared with 4 weeks and control groups were observed. Osteoclast-like and osteoblast-like cells were still observed on the surface of newly formed bone and HF (Fig. 4c).

3. Autogenous bone + HF group (Bone/HF ratio 3/1, 3/2, 1/1 groups)
1) 4 weeks
The bone height exceeded the top of the chamber in the ratio 3/2 and 1/1 groups, and almost the same height as the chamber peak in the ratio 3/1 group (Fig. 5a, b, c). The histological images of each composition appeared almost identical. The net-like-structured newly formed bone was slightly stained and connected to grafted and host bone. Lamellar structure in newly formed bone was observed especially at the site close to grafted and host bone; Grafted bone was resorbed at many sites. As in the newly formed bone observed in HF alone group, the surface of newly formed bone was slightly stained and showed “moth-eaten-like appearance”, and a large number of osteoclast-like and osteoblast-like cells were observed on HF, newly formed bone, grafted bone and host bone (Fig. 5e). The inter-space between host, grafted and newly formed bones, was filled with HF and mononuclear cells. Blood vessels and adipose tissue were observed in the inter-space only in the ratio 3/1 group: minimum HF combined with autogenous bone (Fig. 5d).

2) 8 weeks
The bone height was comparable in ratio 3/2 and 1/1 groups to that at 4 weeks, however, it was reduced to 75% in the ratio 3/1 group (Fig. 6a, b, c). Trabeculae were relatively thick and tightly connecting to the grafted and host bone. Lamellar bone was observed at the same site as in 4 weeks. In the ratio 1/1 and 3/2 groups, the moth-eaten-like appearance was still observed, and newly formed bone appeared to extend along HF and incorporated HF. However, residual HF which was not incorporated into newly formed bone was also observed in the inter-space. Many osteoclast-like and osteoblast-like cells were still observed on the surface of HF, newly formed, grafted and host bone (Fig. 6e). The finding in the ratio 3/1 group is different from the other two groups. Lamellar-structured trabeculae did not show “moth-eaten-like appearance”, and adipose tissue and blood vessels were observed in the inter-space as seen in the control group (Fig. 6d).

Histomorphometrical analysis
The area of newly formed and grafted bone (bone area: BA (\%)) is shown in Figs. 7 and 8. There were significant differences between 4 weeks and 8 weeks in control, HF 30 mg and in the ratio 3/1 groups (P<0.05). At 8 weeks, there was a significant difference of BA between the HF 30 mg and HF 60 mg groups (P<0.05).
Fig. 4 Histological images of HF 30 mg group (a) and HF 60 mg group (b, c) at 8 weeks. Low magnification (a, b) and high magnification (c). Newly-formed bone (NB), blood vessels (BV) and osteoclast-like cell (arrow).

Fig. 5 Histological images of ratio 3/1 group (a, d), ratio 3/2 group (b, e) and ratio 1/1 group (c) at 4 weeks. Low magnification (a, b, c) and high magnification (d, e). Newly formed bone (NB), grafted bone (GB), blood vessels (BV), adipose tissue (AD), osteoblast-like cell (OB) and osteoclast-like cell (arrow).
Fig. 6 Histological images of ratio 3/1 group (a, d), ratio 3/2 group (b, e) and ratio 1/1 group (c) at 8 weeks. Low magnification (a, b, c) and high magnification (d, e). Newly formed bone (NB), grafted bone (GB), host bone (HB), blood vessels (BV), adipose tissue (AD), osteoblast-like cell (OB) and osteoclast-like cells (arrow).

Fig. 7 Area of newly formed and grafted bone. HF was combined with harvested bone at different ratios. Bone 30 mg/HF ratio: 3/1, 3/2, 1/1. The area was measured histomorphometrically. * Significantly different, P < 0.05.

Fig. 8 Area of newly formed bone. Thirty or 60 mg of HF was applied whereas nothing was applied in the control group. The area was measured histomorphometrically. * Significantly different, P < 0.05.
Micro-CT analysis
The total bone volume (TBV: mm³) in HF 30 mg, 60 mg, Bone/HF ratio 3/1, 3/2 and 1/1 groups are presented in Figs. 9 and 10. There was a significant difference between 4 weeks and 8 weeks in the HF 30 mg and ratio 3/1 groups (P<0.05). At 8 weeks, there was a significant difference between the HF 30 mg and HF 60 mg groups (P<0.05). In the control group newly formed bone was detected with micro-CT although it was histologically detected.

DISCUSSION
Autogenous bone is widely recognized as a gold standard of grafting material for bone augmentation3, 4. However, harvesting autogenous bone sometimes associates morbidity to intra or extra oral donor sites. Moreover, bone augmentation using autogenous bone grafts sometimes does not produce adequate bone mass as expected because of chronological reduction of bone volume. Johansson et al. reported that bone volume decreased to 49.5% six months after autogenous bone grafting to the heavily resorbed human maxilla5. Additionally, Steven et al. reported that 5 months after sinus augmentation by autogenous bone or β-TCP, the bone height was significantly reduced in both groups6. In an animal study, Kon et al. reported that vertical bone augmentation on rabbit parietal bone using autogenous bone of large particle size (1–2 mm in diameter) maintained bone height, while bone volume was more reduced at 8 weeks than at 4 weeks7. In the present study, similarly, significant reduction of bone volume including newly formed bone and/or grafted materials with time occurred in the control, HF 30 mg and ratio 3/1 groups. These reductions of grafted bone would negatively affect the expected outcome in both function and aesthetics. The present study was designed to examine effect of HF material on vertical bone augmentation focusing on volume preservation.

In this study, newly formed bone tightly bridged the grafted and host bone and occupied the inter-space between trabeculae in the ratio 3/2 and 1/1 groups. The structure of newly formed bone in more HF groups (HF 60 mg, ratio 3/2 and 1/1 groups) was different from those in control and less HF groups (HF 30 mg and ratio 3/1 groups). In more HF groups, newly formed bone appeared to extend along HF incorporated, and its outline showed "moth-eaten-like appearance". It is likely that this rough outline of newly formed bone was created by particular resorption by osteoclast-like cells. Unfortunately, we could not count osteo-
clast-like cell number in this study because of the thick un-demineralized ground sections. Osteoclast-like cells were observed even at 8 weeks in the greater HF groups, but fewer in the less HF groups and almost none in the control group. Chris et al. reported that a larger number of osteoclasts was observed by bone grafting with nano-crystalline hydroxyapatite compared with TCP-HA and/or cancellous bone grafts in a rabbit femur\textsuperscript{12}. In a clinical study, Stübinger et al. performed maxillary sinus augmentation with nano-structured hydroxyapatite, and the histological analysis of the bone biopsies revealed high osteoclast activity at the margin of the biomaterial which was well integrated into the newly formed bone\textsuperscript{13}. These results indicated that nano- or fiber-structured apatite affects the number of osteoclast-like cells. In an \textit{in vitro} study, Lemaire et al. and Bonfoh et al. proved that during bone remodeling, osteoclasts and osteoblasts interact with each other\textsuperscript{15,16}. It is likely that continuous presence of osteoclast-like cells closely associated with acceleration of bone remodeling.

In this study, TBV and BA in the HF 60 mg, ratio 1/1 and 3/2 groups were maintained during the experimental period, whereas significant reduction was observed in the HF 30 mg and ratio 3/1 groups. As seen in histology in the ratio 1/1 and 3/2 groups at 8 weeks, trabeculae were relatively thick and tightly connected to the grafted and host bone and appeared mature at some parts close to grafted and host bone, whereas active bone remodeling was still observed at the well-stained part. Similar structure of newly formed bone and active bone remodeling were observed in the HF 60 mg group. Meanwhile, newly formed bone in the ratio 3/1 group mostly did not connect to grafted and host bone, some adipose tissue was observed in the inter-space, and few signs of bone remodeling were observed at 8 weeks. It has been reported that the number of adipose tissue is in inverse proportion to that of osteoblasts and bone volume\textsuperscript{17}. Thus, presence of fat cells was one of the indices used to identify stable and mature bone, and at stable phase of bone formation, resorption of newly formed bone becomes normal again. On the other hands, in the HF 60 mg, ratio 1/1 and 3/2 HF groups, active bone regeneration was continued even at 8 weeks. As a result, bone volume was maintained. It is assumed that the long-term continuous active bone regeneration could maintain bone volume.

At 8 weeks TBV and BA of HF 60mg group were larger than the ones of HF30mg group. The reduction of TBV and BA from 4 weeks to 8 weeks was significant in ratio 3/1 group (less HF group) whereas this reduction was prevented in ratio 3/2 and 1/1 group (more HF group). On the other hand, there was no significant difference among the ratio 3/2 and 1/1 group at the ideal time points. These results indicate that the ratio of combination affects regeneration and bone remodeling and bone volume at different time points. Thus, adequate ratio of autogenous bone and HF should be further studied, which has not been clarified in the present study.

HF is cotton-like and easy to handle and we can apply this material to any surgical site without difficulties, which is a great advantage of this material. We have previously reported that HF works as biodegradable osseoconductive material in socket healing model\textsuperscript{14}. In the present study, effect of HF on vertical augmentation was evaluated and we observed that HF also worked as osseoconductive material in vertical augmentation. Notably, the space for augmentation was maintained with a chamber, compensating the mechanical weakness of HF in vertical augmenta-
tion. In clinical situation, combination of HF with autogenous bone or other bone substitutes would be clinically practical to keep the shape for augmentation.

In the present study although we did not have an experimental group, in which only autogenous bone was applied, we demonstrated that combination of HF with autogenous bone worked effective in vertical bone augmentation. Combination of autogenous bone with HF can reduce the amount of autogenous bone required for bone augmentation. Reducing autogenous bone amount is not a perfect solution. However, it can also reduce the patient mobility if this material is clinically used in the future.

In conclusion, combination of HF with autogenous bone was effective in vertical bone augmentation although optimal ratio of the combination needs further study.

REFERENCES


Corresponding author:
Junichi KIMURA, D.D.S., Ph.D.
Department of Oral Implantology and
Regenerative Dental Medicine,
Tokyo Medical and Dental University,
1-5-45, Yushima Bunkyo-ku,
Tokyo113-8549, Japan
Tel: +81-3-5803-5774
Fax:+81-3-5803-5774
E-mail:kimura.irm@tmd.ac.jp