Introduction

The development of new alloplastic bone graft materials is now expected in dentistry, as well as orthopedic surgery, to improve the quality of clinical treatment (e.g., sinus lift elevation for dental implantology) [1]. Apatite is an osteo-conductive material, and large apatite plate and granules (blocks) have been employed as artificial bone skull [2] and bone-filling materials [3], respectively. These apatite devices have the large dimension of about 100 μm to several cm [2, 3]. On the other hand, nano-apatite has recently attracted the attention in bio-materials community. Apatite drastically changes physical,
3. Preparation of n-HAP/Col composites

Preparation of n-HAP/Col is briefly described, as follows. Collagen pellets (1 g) were dissolved in distilled water (28 mL) in 50 mL polystyrene conical tube at 4°C. The produced acidic sol (Fig. 1 (a)) was neutralized by 0.1N NaOH solution (6.5 mL) in disposable rectangular plastic plate (100×70×12 mm) (Fig. 1 (b)) so that collagen gel of pH = 7.5 could be obtained (Fig. 1 (c)). The n-HAP powder (1.5 g) was then manually mixed with collagen gel using plastic spatula (Fig. 1 (b) and (d)). The n-HAP collagen composite gel was then frozen at -80°C for 3 h, and freeze-dried with a freeze-drier (FD-5N, EYELA, Tokyo, Japan) for 12 h (Fig. 1 (e)). The composite sponge (sheet) stored in a holed stainless steel case was subsequently cross-linked by de-hydrothermal treatment at 140°C for 24 h in a vacuum dry oven (VO-300, AS ONE, Tokyo, Japan) (Fig. 1 (f) and (g)). Disks 6 mm in diameter and 1 mm thickness were punched out with a hole puncher. Disks stored in exclusive pouche bags were sterilized by ethylene oxide gas (Fig. 1 (h)), and kept in a vacuumed desiccator before animal tests.

As control, punched collagen sponge specimens without n-HAP (Col) were also produced.

3. Animal tests

The Ethical Committee on Animal Research of Iwate Medical University approved the protocol of this study (#22-029). Thirty-six male Wistar rats weighing 340 ± 16 g (mean± SD) were used. Groups of three rats were housed in separate cages, and provided with water ad libitum and a standard diet. Under anesthesia with a mixture of isoflurane (3 vol.%) and oxygen (0.5 L/min) gas generated by a carburter (IV-ANE, Olympus, Tokyo, Japan), the top center of the calvaria of the rats was disinfected with 10% povidone iodine, followed by administration of local anesthesia (2% lidocaine with 1:80,000 epinephrine). A flap was then raised and a 6-mm-diameter bone defect was created using a bone trephine bur. Either Col or n-HAP/Col was then implanted. The flap was repositioned and sutured with soft nylon (Sofretch 4-0, GC, Tokyo, Japan). The animals were sacrificed at 1...
day, 4 weeks, or 8 weeks after surgery. For each experimental condition consisting of two kinds of implant material and three different feeding periods, six rats were used (total 36 rats used).

4. Micro-CT
To evaluate the formation of new bone in each defect zone containing Col or n-HAP/Col in the rat calvarium, 3-dimensional (3D) images of rat skulls were obtained using a micro-CT system (eXplore Locus, GE Health Care, Wilmington, MA, USA). Each sample was scanned at 90-μm intervals at 80 kVp and 450 mA, and Vextus Factor complied storage files (VFF data) were obtained. After scanning, 3D image analysis software (MicroView Version 2.2, GE Health Care) was used to reconstruct 3D CT images of the calvaria containing defect zones (Fig. 2 (a)), and further pinpoint regions of interest (ROI; 30 x 20 x 3 mm; Fig. 2 (b)). The instrumental opac-

Fig. 1 Sequences for preparation of n-HAP/Col composite.
(a) Prepared collagen sol, (b) Materials and tools to produce n-HAP/Col composites including raw collagen pellet and n-HAP particles, (c) Collagen gel neutralized with NaOH solution, (d) n-HAP particle dropped on collagen gel, (e) Freeze-dry of mixed n-HAP/Col, (f) De-hydrothermal cross-linking, (g) Opened metal vessel which contained cross-linked n-HAP/Col sheets, (h) Punched n-HAP/Col samples after ethylene-oxide sterilization.

Fig. 2 3-dimensional (3D) micro-CT images of (a) overall rat head and (b) region of interest (ROI) of rat skull that had the same holed defect and implanted material (n-HAP/Col) at 4 weeks after operation. Notes in (b): *=Background, #=Intact bone, $=Defect implanted with material.
ity threshold value was set to 8000 to minimize the interference caused by other bony tissues (e.g., cranial base and maxilla bones). The radiographic opacity level of defect zones containing Col or n-HAP/Col was then evaluated using ImageJ software (National Institute of Health, Bethesda, MD, USA) with a circle diameter of 6 mm. Statistical analysis was conducted using Student’s t test.

5. Histological observations

The post-operative bone formation in the cranial defect zone was examined with non-calcified tissue histology using Villanueva stain and sequential fluorescent double staining technique. The rats implanted with n-HAP/Col and fed for 8 weeks received an intraperitoneal injection of tetracycline (TC) (2 mg/100-g animal weight) dissolved in phosphate-buffered saline (PBS(-)) (40 mL) at 5 weeks after surgery, followed by calcein (CL) (1 mg/100-g animal weight) in PBS(-) (40 mL) at 7 weeks and 7 weeks + 5 days (2 days before sacrifice) after surgery. After comfort sacrifice, tissue blocks of rat calvaria containing defect zones (~5 × 10 × 20 mm) were cut using a diamond band saw, and processed for non-decalcified histology using poly-methylmethacrylate resin. After fixation for about 1 week in 70% ethanol at 4°C, the sample was dehydrated in a graded series of ethanol (1 day at each concentration) and then placed in pure acetone for 24 h. The sample was then stained with Villanueva solution (222-01445, Wako, Osaka, Japan). Finally, the samples were impregnated with methylmethacrylate for 4 days and chemically polymerized for 10 days. The resulting non-decalcified block (~15 × 15 × 20 mm) was cut sagittally using a circular diamond cutter (MC-201 Microcutter, Maruto, Tokyo Japan). Sections were mounted on plastic slides, ground to a thickness of 20 μm using a precision lapping machine (ML-110N, Maruto, Tokyo, Japan), and then ground by hand. Histological evaluations were conducted using fluorescence microscopy (All-in-one BZ-9000, Keyence, Osaka, Japan).

Results

1. Micro-CT analyses

Fig. 3 Top indicates 3-dimensional (3D) micro-CT horizontal images of ROI of cranial defects filled with n-HAP/Col of rats fed for 1 day, 4 weeks, or 8 weeks (2 cases: (a) and (b)) after surgery. Bottom: 2-dimensional micro-CT sagittal sections of ROI of cranial defects filled with n-HAP/Col of rats fed for 1 day, 4 weeks, or 8 weeks (2 cases: (a) and (b)) after surgery.
4 weeks, or 8 weeks (2 cases: (a) and (b)) after surgery. Fig. 3 Bottom shows two-dimensional (2D) micro-CT sagittal sections of ROI of cranial defects filled with n-HAP/Col of rats fed for 1 day, 4 weeks, or 8 weeks (2 cases: (a) and (b)) after surgery. At 1 day, n-HAP particles in n-HAP/Col provided slight opacity in both the horizontal image and sagittal section. Opacity levels in two directions at 4 weeks became quite larger than those at 1 day, indicative of new bone formation by n-HAP/Col at 4 weeks after implantation. At 8 weeks, however, opacity levels tended to decrease, compared with those at 4 weeks. Opaque regions at 8 weeks became segregated, indicative of elimination of n-HAP particles and partial loss of newly formed bone.

Numerical data from 3-dimensional (3D) horizontal micro-CT images of ROI of cranial defects filled with implant materials (Col or n-HAP/Col) are shown in Table 1 (n = 6). With Col, opacity increased from 1 day to 4 weeks (p < 0.05) and from 1 day to 8 weeks (p < 0.05), although opacity was similar between at 4 weeks and at 8 week with the value of around 105. Fig. 4 indicates those of cranial defects filled with n-HAP/Col of rats fed for 1 day, 4 weeks, and 8

| Table 1 | Micro-CT opacity of ROI of rat cranial defects filled with Col or n-HAP/Col after feeding for 1 day, 4 weeks, or 8 weeks, expressed as mean values with standard deviations (in parenthesis) of six measurements. Those of intact bone and background were also presented. |
| Raw data | Col | n-HAP/Col | Intact bone | background |
| Feeding period | 1 day | 4 weeks | 8 weeks | 1 day | 4 weeks | 8 weeks |
| Col | 69.7 (6.4) | 102.9 (1.9) | 108.7 (17.6) | 102.9 (6.5) | 123.4 (25.3) | 232.2 (10.8) |
| n-HAP/Col | 109.0 (8.8) | 148.6 (6.5) | 123.4 (25.3) | 148.6 (6.5) | 123.4 (25.3) | 51.1 (12.5) |

(b) Statistics

Note: NS=statistically not significant (p>0.05); *=P<0.05; **=P<0.01

| Col | n-HAP/Col |
| Feeding period | 1 day | 4 weeks | 8 weeks | 1 day | 4 weeks | 8 weeks |
| Col | 1 day | ** | ** | ** | ** | ** |
| | 4 weeks | NS | NS | ** | NS |
| | 8 weeks | NS | NS | NS |
| n-HAP/Col | 1 day | NS | NS | NS |
| | 4 weeks | NS | NS |

All six samples were different at** from intact bone.
Col 1 day and other five samples were different from background at * and at **, respectively.

Fig. 4 CT opacity of ROI of cranial defects filled with n-HAP/Col of rats fed for 1 day, 4 weeks, and 8 weeks after surgery. Note: At 8 weeks, CT opacity values had large SD while the minimum and maximum values were 88 and 166, respectively. Squares indicate raw data values at 8 weeks.
Fig. 5  (a) Images of rat cranial defect filled with n-HAP/Col after feeding for 8 weeks (Case 1 of maximum bone-regenerated one), double-stained with TC (5 weeks after surgery) and CL (7 weeks and 7 weeks + 5 days). Top: Original Villanueva-stained image, second: TC fluoro image (yellow), third: CL fluoro image (green), bottom: Overlay TC+CL. Bar = 2 mm. (b) The red-encircled section in the upper image was magnified below in lower images. Top left: Villanueva-stained image, top right: TC fluoro image (yellow), bottom left: CL fluoro image (green), bottom right: Overlay TC+CL fluoro image. Lower bar = 200 μ m. Note: Active new bone formation activity was recognized.

Fig. 6  (a) Images of rat cranial defect filled with n-HAP/Col after feeding for 8 weeks (Case 2 of minimum bone-regenerated one), double-stained with TC (5 weeks after surgery) and CL (7 weeks and 7 weeks + 5 days). Top: Original Villanueva-stained image, second: TC fluoro image (yellow), third: CL fluoro image (green), bottom: Overlay TC+CL. Bar = 2 mm. (b) The red-encircled section in the upper image was magnified below in lower images. Top left: Villanueva-stained image, top right: TC fluoro image (yellow), bottom left: CL fluoro image (green), bottom right: Overlay TC+CL fluoro image. Lower bar = 200 μ m. Note: New bone formation activity was sluggish.
weeks after surgery. It was confirmed that CT opacity of the defects filled with n-HAP/Col at 4 weeks was significantly larger than those at 1 day, implying good osteo-conduction by n-HAP/Col at 4 weeks. At 8 weeks, CT opacity values were not statistically different from those at 1 day and 8 weeks; and fluctuated with large standard deviations (SD) while the minimum and maximum values were 88 and 166, respectively. In another words, implantation of n-HAP/Col in rat cranial defect resulted in two extreme contradicting cases of the minimum opacity (i.e. poor osteo-conduction) and the maximum opacity (i.e. excellent osteo-conduction).

All defects implanted by Col or n-HAP/Col fed for three periods (1 day, 4 weeks or 8 weeks) had larger and smaller opacity than background and intact bone, respectively (Fig. 2 (b), Table 1).

2. Histological observations

Fig. 5 (a) shows images of rat cranial defect filled with n-HAP/Col after feeding for 8 weeks (Case 1 of maximum bone-regenerated one). The top was Villanueva-stained image, the second was TC fluoro image (yellow), the third was CL fluoro image (green) and the bottom was overlay TC+CL. Active dynamic bone formation was identified in cranial defect zone, especially at the left-hand, while that was relatively sluggish at the right-hand. The red-encircled area in the upper image of Fig. 5 (b) was magnified below in lower four images. In lower four images, the top left was x2.5-magnified Villanueva-stained image, the top right was TC fluoro image (yellow), the bottom left was CL fluoro image (green) and the bottom right was overlay TC+CL fluoro image. In Villanueva stain, older bone appeared yellow to brown, while newer bone did white; osteoid (i.e., non-calcified bone) appeared purple, and cell nuclei did blue. Bones had characteristic spots arising from osteocytes. Collagenous tissues appeared as tangled threads. All these elements were found in the magnified Villanueva stain. Double-stained fluoro images identified active dynamic new bone formation in the red-encircled zone. No n-HAP/Col remain was observed in this actively formed new bone area.

Fig. 6 (a) shows images of rat cranial defect filled with n-HAP/Col after feeding for 8 weeks (Case 2 of minimum bone-regenerated one). The top was Villanueva-stained image, the second was TC fluoro image (yellow), the third was CL fluoro image (green) and the bottom was overlay TC+CL. New bone formation activity was sluggish in the cranial defect zone. The red-encircled area in the upper image of Fig. 6 (b) was magnified below in lower four images. In lower four images, the top left was x2.5-magnified Villanueva-stained image, the top right was TC fluoro image (yellow), the bottom left was CL fluoro image (green) and the bottom right was overlay TC+CL fluoro image. Although single bone island was found in the defect zone, other spaces were largely occupied by non-osseous (soft) connective tissues. No n-HAP/Col remain was found. Dynamic bone formation activity around new bone was identified just in the red-encircled region.

Discussion

Three-dimensional (3D) micro-CT analyses have been widely employed to assess new bone formation of bony defect zones implanted with various bio-materials [17]. Addition in the CT opacity suggests the increased rate of new calcified-bone formation. It became evident from three-dimensional (3D) micro-CT experiments conducted (Figs. 3 and 4) that n-HAP/Col induced quick osteo-conduction at 4 weeks after operation in rat cranial defect zone. We speculate that at 4 weeks implantation, most n-HAP/Col was eliminated and the increase in CT opacity might be attributed to new bone formation. On the other hand, it became evident that the rate of new bone formation at 8 weeks after operation tended to decrease with respect to those at 4 weeks, and had large data variation ranging from 88 to 166 (Fig. 4). The reason for the latter phenomenon is considered in the next paragraph. As for Col, CT opacity values at 4 and 8 weeks were similar, while both were greater than that at 1 day (Table 1). This increment, however, might reflect invasion of connective soft tissues at 4 and 8 weeks, which replaced first-formed blood clots at 1 day.

Histological observations by non-de-calcified tissue histology method also demonstrated that rat cranial defect zones implanted with n-HAP/Col for 8 weeks brought about both better and worse osteo-conduction (Figs. 5 and 6, respectively). During animal feeding, we noticed that rats scratched repeatedly the cranial defect areas, especially over 4
weeks after operation, possibly due to irritating cutaneous sensation that produced a desire to scratch [18]. It was speculated that scratching the defect zone by hand and leg caused hemorrhage in the defect zone, leading to loss of once acquired new bone and disruption of new bone formation by concomitant inflammation [19]. Because such scratch action was animal individual dependent, CT opacity values of rat cranial defect zones at 8 weeks after operation might have large data fluctuations (Fig. 4). If scratch action could be prevented, it was postulated that n-HAP/Col might be continuously osteo-conductive even over 4 weeks after operation, always achieving the maximum CT opacity level of around 166 (Fig. 4).

We have already reported that n-HAP/Col was basically safe and bio-compatible in the subcutaneous soft tissues in the back of mice [15]. It was found that no severe adverse tissue reactions (e.g. necrosis of surrounding tissues) were observed, and n-HAP/Col was gradually digested through conventional inflammation and healing process with time from 1 to 2 weeks. We thought that Col was first-digested by enzyme (collagenase), while n-HAP particles in n-HAP/Col were phagocytized by cells (i.e. neutrophil and macrophage) in encircling granulation tissues. It was also found that n-HAP had a minor angiogenetic capability [12, 15].

In osseous defects like cranial bone defect, it was probable that similar soft tissue reaction might first occur against n-HAP/Col. In addition; other osseous tissue reactions might take place from neighboring bone and osteo-periosteum with time. It could be considered that n-HAP with vast surface areas was dissolved chemically by the body fluid, releasing small amounts of calcium ions and phosphate ions, which might contribute to new bone formation [20] as well as vascular activities. Such ions might activate cell signaling pathway associated with new bone formation [21], and might be chemotactic to vascular endothelial cells and neural cells.

Referring to other studies, which applied nano-apatite for bone re-generation, growth factors (e.g. bone morphogenetic protein (BMP)) [22] and/or stem cells (e.g. mesenchymal stem cells (MSC)) [23] were added to the nano-apatite-containing composite scaffolds. Very positive results for osteo-conduction and osteo-induction were reported in these composite constructs [22, 23]. We are also looking forward to using growth factors and MSC in n-HAP/Col for bone re-generation at cranial defect zones in future studies.

**Conclusion**

We self-prepared nano-hydroxyapatite/collagen composite (n-HAP/Col); and evaluated its usefulness as bone-substitute material by the animal test model using rat cranial critical-size bone defects. The following results were obtained.

(1) In rat critical-size cranial defects, n-HAP/Col caused quick osteo-conduction at 4 weeks after operation. At 8 weeks after implantation, however, the rate of new bone formation slightly declined, partly due to rat scratching action and resultant hemorrhage/inflammation.

(2) For longer implantation beyond 4 weeks, it was recommended to prevent rat-scratching action and to add growth factors and stem cells to the cranial defect zone so that n-HAP/Col could be better utilized as a new noble bone substitute material.

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**Disclosure**

The authors report no conflicts of interest in this work.

**References**


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