Effect of Type I Collagen on Hydroxyapatite and Tricalcium Phosphate Mixtures in Rat Calvarial Bony Defects

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Abstract

To repair bone defects in the oral and maxillofacial field, bone grafts including autografts, allografts, and artificial bone are used in clinical dentistry despite several disadvantages. The purpose of this study was to evaluate new bone formation and healing in rat calvarial bone defects using hydroxyapatite (HA, $Ca_{10}[PO_4]_6[OH]_2$, Bongros®, Bio@ Co., KOREA) and tricalcium phosphate (β-TCP, $Ca_3[PO_4]_2$, Sigma-Aldrich Co., USA) mixed at various ratios. Additionally, this study evaluated the effects of type I collagen (Rat tail, BD Biosciences Co., Sweden) as a basement membrane organic matrix.

A total of twenty, 8-week-old, male Sprague-Dawley rats, weighing 250-300g, were divided equally into a control group (n=2) and nine experimental groups (n=2, each). Bilateral, standardized transosseous circular calvarial defects, 5.0 mm in diameter, were created. In each experimental group, the defect was filled with HA and TCP at a ratio of 100:0, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, and 0:100 with or without type I collagen. Rats were sacrificed 4 and 8 weeks post-operation for radiographic (standardized plain film, Kodak Co., USA), histomorphologic (H&E [Hematoxylin and Eosin], MT [Masson Trichrome]), immunohistochemical staining (for BMP-2, -4, VEGF, and vWF), and elementary analysis (Atomic absorption spectrophotometer, Perkin Elmer AAnalyst 100®).

As the HA proportion increased, denser radiopacity was seen in most groups at 4 and 8 weeks. In general radiopacity in type I collagen groups was greater than the non-collagen groups, especially in the 100% HA group at 8 weeks. No new bone formation was seen in calvarial defects in any group at 4 weeks. Bridging bone formation from the defect margin was marked at 8 weeks in most type I collagen groups. Although immunohistochemical findings with BMP-2, -4, and VEGF were not significantly different, marked vWF immunoreactivity was present. vWF staining was especially strong in endothelial cells in newly formed bone margins in the 100:0, 80:20, and 70:30 ratio type I collagen groups at 8 weeks. The calcium compositions from the elementary analysis were not statistically significant.

Many types of artificial bone have been used as bone graft materials, but most of them can only be applied as an inorganic material. This study confirmed improved bony regeneration by adding organic type I collagen to inorganic HA and TCP mixtures. Therefore, these new artificial bone graft materials, which are under strict storage and distribution systems, will be suggested to be available to clinical dentistry demands.

Key words

Bone graft, BMP, Hydroxyapatite ($Ca_{10}[PO_4]_6[OH]_2$), Tricalcium phosphate ($Ca_3[PO_4]_2$), Type I collagen, VEGF, vWF

INTRODUCTION

There is increasing interest in developing bone substitutes that can be used to restore bony defects resulting from trauma, oncologic resections, infection, or pathology. Autogenous bone grafts are the gold standard for bone defect repair. Defects with bone loss are frequently

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treated with autogenous bone grafts, either cancellous or compact, obtained from the rib, iliac crest, jaw or tibia[1-3]. This treatment is widely employed because it offers osteogenic, undifferentiated bone marrow cells, which contribute to bone regeneration. Incorporation of the bone graft, however, depends on the type of bone tissue used. Cancellous bone grafts have trabeculae that are more easily reabsorbed, preventing maintenance of graft volume. Compact bone graft volumes can be maintained for years, which is advantageous because compact bone grafts provide a scaffold for bone remodeling. In addition, it provides the host site with good mechanical resistance, which is necessary in some cases. The procedure for obtaining both compact and cancellous bone grafts involves additional surgery, which may cause postoperative complications and does not always provide sufficient bone to repair the defect[4-6]. This results in complications associated with donor site morbidity, unpredictable graft resorption, limited donor tissue, and extended surgery time.

Allogenic bone is readily available, and implants with increased osteoconductive capacity have been developed. Their use is associated with increased incidences of nonunion, fatigue fracture, and rejection[7]. The disadvantages are primarily associated with antigenicity against tissue harvested from another individual, resulting in a host immune response. Concerns have been raised regarding the possible transmission of HIV through a bone allograft[8].

Synthetic biomaterials have been researched as an alternative to autogenous bone graft implants. Alternative biomaterials such as hydroxyapatite (HA) and tricalcium phosphate (TCP) have been used as substitutes for autogenous bone grafts because they are biocompatible, can be obtained in large quantities, and do not require additional surgical procedures[9-14]. Synthetic biomaterials also have an increased incidence of nonunion and fatigue fracture. We suggest that type I collagen, a major constituent of bone extracellular matrix, can be formed into native isotonic space-filling gels[15], and may provide a favorable environment for osteoconduction in bone defects. Osteoconduction is the ability of a graft to serve as a scaffold and allow for new bone ingrowth across the surface[16].

Successful bone substitutes should be biocompatible with host tissue, replaceable by, or functionally integratable into new bone[17], and osteoconductive[18-19]. Therefore, in order to evaluate the repair of rat skull bone defects treated with a hydroxyapatite, tricalcium phosphate, and collagen type I mixture, we focused on the effects of type I collagen healing of bony defects in rat skulls.

MATERIALS AND METHODS

1. Experimental design

Eight-week-old adult male rats were included in this study. The weights ranged from 250g to 300g. They were individually housed in plastic cages in a temperature controlled (24℃ to 25℃) room with a circadian light rhythm of 12 hours in 55% humidity. Twenty skeletally mature rats were randomly divided into 10 groups of two animals each. All procedures were performed using aseptic techniques. The animals were sacrificed at 4 weeks and 8 weeks post-operation using urethane.

2. Surgical protocol

Anesthesia was accomplished with 4:1 Ketamine (60mg/kg, ketamine hydrochloride, Yuhan Co., Korea) / Roumpun (3mg/kg, 2% xylazine hydrochloride, Bayer Korea Ltd., Korea).

Once adequate anesthesia was maintained, the animals were prepared and draped using povidone iodide to sterilize the area. A 3 cm incision was made along the midline of the scalp from a point midway between the base of the ears anteriorly through the full-thickness of the skin. The periosteum was resected by blunt dissection (Fig. 1-a, b). Bilateral 5 mm full thickness defects were made in the parietal bones using a dental carbide bur, under copious irrigation, with sterile saline to cool and clear any remaining debris (Fig. 1-c). Care was taken to avoid injury to the dura or midsagittal sinus.

The grafted materials were placed directly onto the dura. The volume used (0.25 cm^3) was chosen to replace the volume of bone removed, just filling the defect (Fig. 1-d). Care was taken to prevent displacement of the grafted materials into the other defect, thus protecting against cross contamination. The grafted materials contained collagen type I (3.48mg/mL, BD biosciences Co., USA) (Fig. 2-a) and a hydroxyapatite (Bongros®, Bioalpha Inc., Korea) (Fig. 2-b) and tricalcium phosphate (Sigma-Aldrich Co., USA) mixture (Fig. 2-c). The pericranium and skin were closed with resorbable Vicryl (Johnson & Johnson Co., USA) sutures (Fig. 1-e).

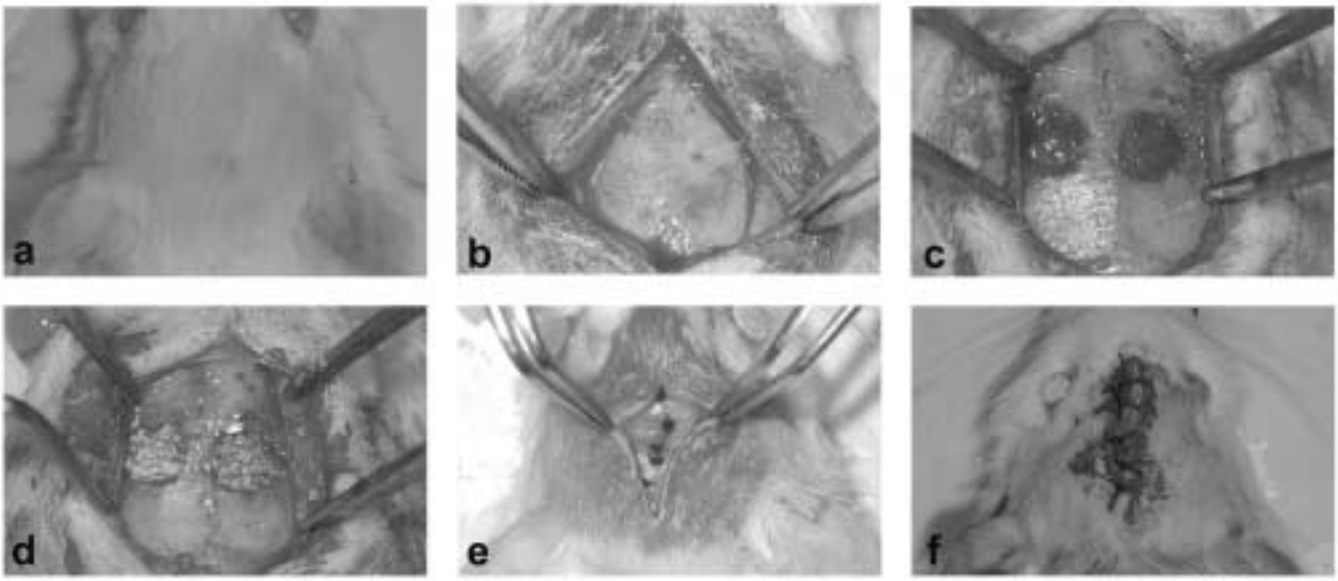

Fig. 1. Bone graft in rat calvarial bone defects using alloplastic materials.
a. Prepared rat calvarial skin
b. A 3 cm incision was made along the midline of the scalp
c. Bilateral 5 mm full thickness defects were made in both parietal bones
d. The grafted materials were placed directly onto the dura
e. The pericranium were closed
f. The skin were closed

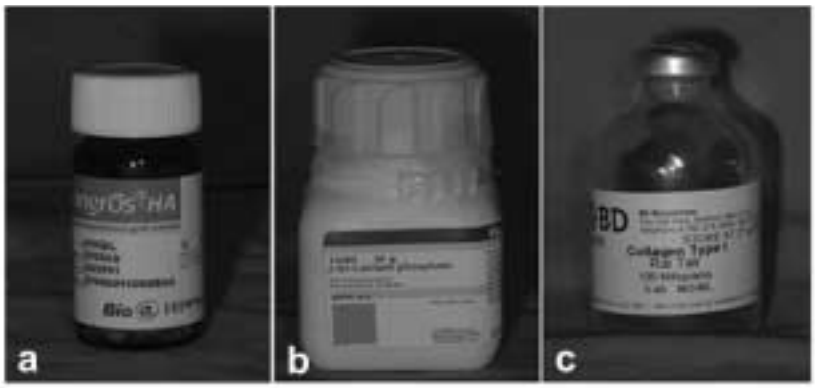

Fig. 2. Grafted bone materials used in this study.
a. Hydroxyapatite (Bongros®, Bioalpha Inc., Korea)
b. Tricalcium phosphate (Sigma-Aldrich Co., USA)
c. Collagen type I (3.48 mg/mL, BD biosciences Co., USA)

Postoperatively, animals were kept warm in individual cages until under full recovery from the anesthetic. They were then returned to the holding room with free access to water and food. For postoperative pain control, animals were given Tarasyn (2 mg/kg, Ketorolac trometha-mine, Yuhan Co., Korea) subcutaneously during 5 days. Isepacine (1.5mg/kg, isepamicin sulfate, Yuhan Co., Korea) was also used to prevent postoperative infection.

3. Test groups

Twenty rats were divided equally into 10 groups (one control and nine experimental groups). Standardized transosseous circular calvarial defects (5 mm in diameter) were made bilaterally. In each experimental group, the defect was filled with HA, TCP, and type I collagen.

3-1. Group 1: collagen versus unfilled

In group 1 (n=2), each animal had one defect filled with 0.25 cm^3 of type I collagen. The contralateral defect was left unfilled and allowed to heal spontaneously without the use of any grafting material (control defect).

3-2. Group 2: 100% HA versus 100% HA + collagen

Animals in group 2 (n=2) had one defect filled with 0.25 cm^3 HA. The contralateral defect was filled with 100% HA and collagen.

3-3. Group 3: 80% HA + 20% TCP versus 80% HA + 20% TCP + collagen

Animals in group 3 (n=2) had one defect filled with 0.25 cm^3 80% HA and 20% TCP. The contralateral defect was filled with 80% HA, 20% TCP, and collagen.

3-4. Group 4: 70% HA + 30% TCP versus 70% HA + 30% TCP + collagen

Animals in group 4 (n=2) had one defect filled with 0.25 cm^3 70% HA and 30% TCP. The contralateral defect was filled with 70% HA, 30% TCP, and collagen.

3-5. Group 5: 60% HA + 40% TCP versus 60% HA + 40% TCP + collagen

Animals in group 5 (n=2) had one defect filled with 0.25 cm^3 60% HA and 40% TCP. The contralateral defect was filled with 60% HA, 40% TCP, and collagen.

3-6. Group 6: 50% HA + 50% TCP versus 50% HA + 50% TCP + collagen

Animals in group 6 (n=2) had one defect filled with 0.25 cm^3 50% HA, and 50% TCP. The contralateral defect was filled with 50% HA, 50% TCP, and collagen.

3-7. Group 7: 40% HA + 60% TCP versus 40% HA + 60% TCP + collagen

Animals in group 7 (n=2) had one defect filled with 0.25 cm^3 40% HA and 60% TCP. The contralateral defect was filled with 40% HA, 60% TCP, and collagen.

3-8. Group 8: 30% HA + 70% TCP versus 30% HA + 70% TCP + collagen

Animals in group 8 (n=2) had one defect filled with 0.25 cm^3 30% HA and 70% TCP. The contralateral defect was filled with 30% HA, 70% TCP, and collagen.

3-9. Group 9: 20% HA + 80%TCP versus 20% HA + 80% TCP + collagen

Animals in group 9 (n=2) had one defect filled with 0.25 cm^3 20% HA and 80% TCP. The contralateral defect was filled with 20% HA, 80% TCP, and collagen.

3-10. Group 10: 100% TCP versus 100% TCP + collagen

Animals in group 10 (n=2) had one defect filled with 0.25cm^3 100% TCP. The contralateral defect was filled with 100% TCP and collagen.

4. Clinical and radiographic evaluation

All animals were analyzed qualitatively once they were sacrificed with Urethane (U2500, minimum 99%, Sigma-Aldrich Co., USA). The cranial vault and attached pericranium was carefully removed from each animal, visually examined for gross inflammation on both the cranial and dural sides, and photographed (Fig. 3, 4). The calvarial bones were radiographed (standardized plain film, Kodak Co., USA) (Fig. 5) and analyzed for radiopacity to examine new bone formation and test material remineralization.

5. Histological and immunohistochemical evaluation

Immediately after visual inspection and radiography, the specimen was immediately fixed in 10% neutral buffered formalin solution at 4℃ for a week. Specimens were prepared, dehydrated in a graded ethyl alcohol series (70%, 80%, 90%, 95% and 100%), infiltrated, and embedded with methyl methacylate (Technovit 7200 VLC®, Kulzer Co., Germany). They were then affixed onto acrylic slides, sectioned along the coronal surface of rat skull, and ground to 3 um thickness from the middle of the defect (the greatest diameter across the defect). Histomorphological analysis was carried out and stained (Hematoxylin-Eosin, Masson trichrome stain, and immunohistochemical staining for vWF, VEGF, BMP-2, and BMP-4) to compare cellular morphologies and bone formation.

Immunohistochemical staining was carried out by the indirect PAP method. Briefly, after rehydration in PBS (pH 7.4), the microsections were incubated in 0.6% hydrogen peroxide for 30 minutes to inactivate endogenous peroxidases and incubated with normal swine serum (1:50) in PBS to block nonspecific background. Slides were incubated in mouse polyclonal angiogenin (1:100, Dako Co., Denmark) or rabbit polyclonal VEGF (1:100, Pharmingen Co., USA) primary antibody for 1 hour. The horseradish peroxidase-conjugated secondary antibody was then applied for 1 hour. Finally, all sections were incubated with 3,3'-diaminobenzidine tetrahydrochloride chromogen (DAB, Dako Co., Denmark) substrate for up to 10 minutes.

6. Elementary analysis

The calcium content of the specimens was determined by elementary analysis (Perkin Elmer AAnalyst 100®, Atomic absorption spectrophotometer). In a 1,000 ml volumetric flask, 1.249 g anhydrous calcium carbonate ($CaCO_3$) was added to 50 ml deionized water and dissolved by adding 10 mL concentrated hydrochloric acid (HCL) dropwise. The solution was diluted to 1 L with deionized water. The resulting was a 500 mg/L standard solution.

13.37 g lanthanum chloride ($LaCl_3.7H_2O$) was dissolved in deionized water to create a 0.5% lanthanum solution. Each standard, control, and sample was spiked with a 9:10 ratio of 0.5% lanthanum solution. A Ca-Mg hollow cathode lamp was used in the Atomic Absorption Spectrometer using the Default Conditions using an oxidizing (lean, blue) air-acetylene flame. The machine was calibrated with standards that bracket the sample concentration. The correlation coefficient was greater than or equal to 0.990. The calibration curve was checked for drift, accuracy, and precision with standards and controls after every 20 samples.

RESULTS

1. Clinical and Radiographic Evaluation

A clinical evaluation of group 1 at 4 and 8 weeks revealed a thin, fibrous scar covering the entire defect area, and remaining bony defects. The collagen filled defect was relatively more intact than its unfilled counterpart. Radiographs of group 1 examined at 4 and 8 weeks appeared clearly radiolucent. Only a small amount of increasing radiopacity was seen, and only in the defect margin (Fig. 3, 4).

Radiographs analyzed at 4 and 8 weeks showed denser radiopacity when compared with non-collagen filled counterparts. At 4 weeks, all other groups showed a denser radiopacity than group 1. Group 10 shows radiolucency because of the TCP radiolucency. Radiopacity increased with the HA ratio. At 8 weeks, radiopacity did not increase dramatically, but the defect margin showed increased radiopacity (Fig. 5).

2. Histologic Evaluation

During the observation period, specimens were examined using a light microscope at × 40, × 100, and × 200 magnification (Fig. 6, 7, 8, 9). Calvarial defects in group 1 were primarily bridged by fibrous connective tissue. Some appositional new bone growth was observed on the defect periphery adjacent to the lamellar calvarial bone.

Histologic observation revealed that defect filled with collagen, HA, and TCP was able to induce new bone formation throughout the defects, bridging the defects by 4 weeks. At 8 weeks, bony healing was not complete, though some new bone formation had occurred. Defects in groups treated with collagen showed near-total repair by immature bone. New bone formed within the defects extended subperiosteally away from the defects in many cases.

Immunohistochemical findings with BMP-2, -4, and VEGF were not compared significantly, but marked vWF antibodies were found especially in endothelical cells of new formed bony margins especially in 100:0, 80:20, and 70:30 ratio type I collagen mixed group at 8 weeks (Fig. 10).

3. Elementary analysis

In 8 weeks, mean calcium content of group 2, 4, 6, and 10 was 1.38, 1.259, 0.770, and 1.22 ppm, each. But, these calcium content determined by elementary analysis was not significant statistically (Fig. 11).

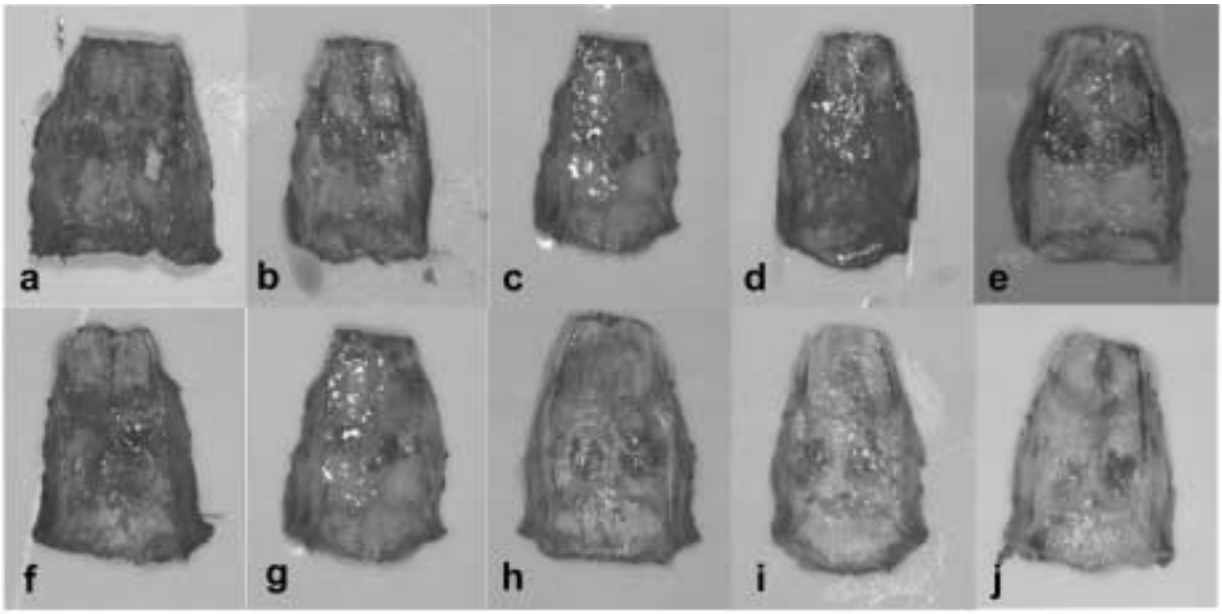

Fig. 3. Cranial vault appearance in 4 weeks.

a. Collagen versus unfilled
b. 100% HA versus 100% HA + Collagen
c. 80% HA +20% TCP versus 80% HA +20% TCP + Collagen
d. 70% HA +30% TCP versus 70% HA +30% TCP + Collagen
e. 60% HA +40% TCP versus 60% HA +40% TCP + Collagen
f. 50% HA +50% TCP versus 50% HA +50% TCP + Collagen
g. 40% HA +60% TCP versus 40% HA +60% TCP + Collagen
h. 30% HA +70% TCP versus 30% HA +70% TCP + Collagen
i. 20% HA +80% TCP versus 20% HA +80% TCP + Collagen
j. 100% TCP versus 100% TCP + Collagen

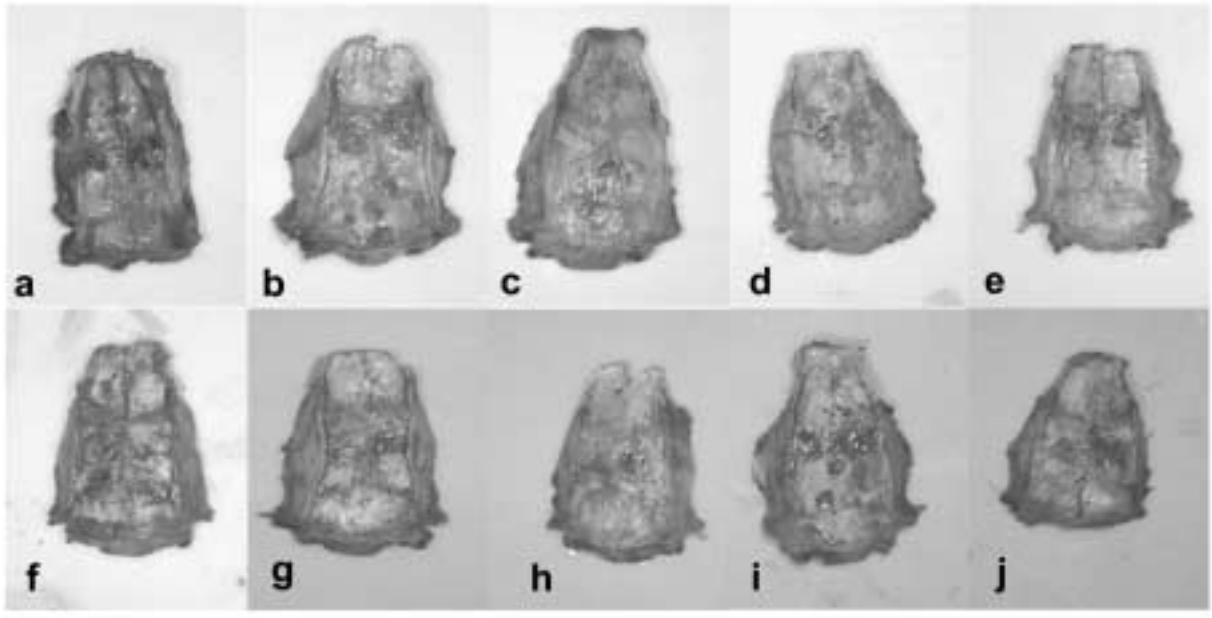

Fig. 4. Cranial vault appearance in 8 weeks.

a. Collagen versus unfilled
b. 100% HA versus 100% HA + Collagen
c. 80% HA +20% TCP versus 80% HA +20% TCP + Collagen
d. 70% HA +30% TCP versus 70% HA +30% TCP + Collagen
e. 60% HA +40% TCP versus 60% HA +40% TCP + Collagen
f. 50% HA +50% TCP versus 50% HA +50% TCP + Collagen
g. 40% HA +60% TCP versus 40% HA +60% TCP + Collagen
h. 30% HA +70% TCP versus 30% HA +70% TCP + Collagen
i. 20% HA +80% TCP versus 20% HA +80% TCP + Collagen
j. 100% TCP versus 100% TCP + Collagen

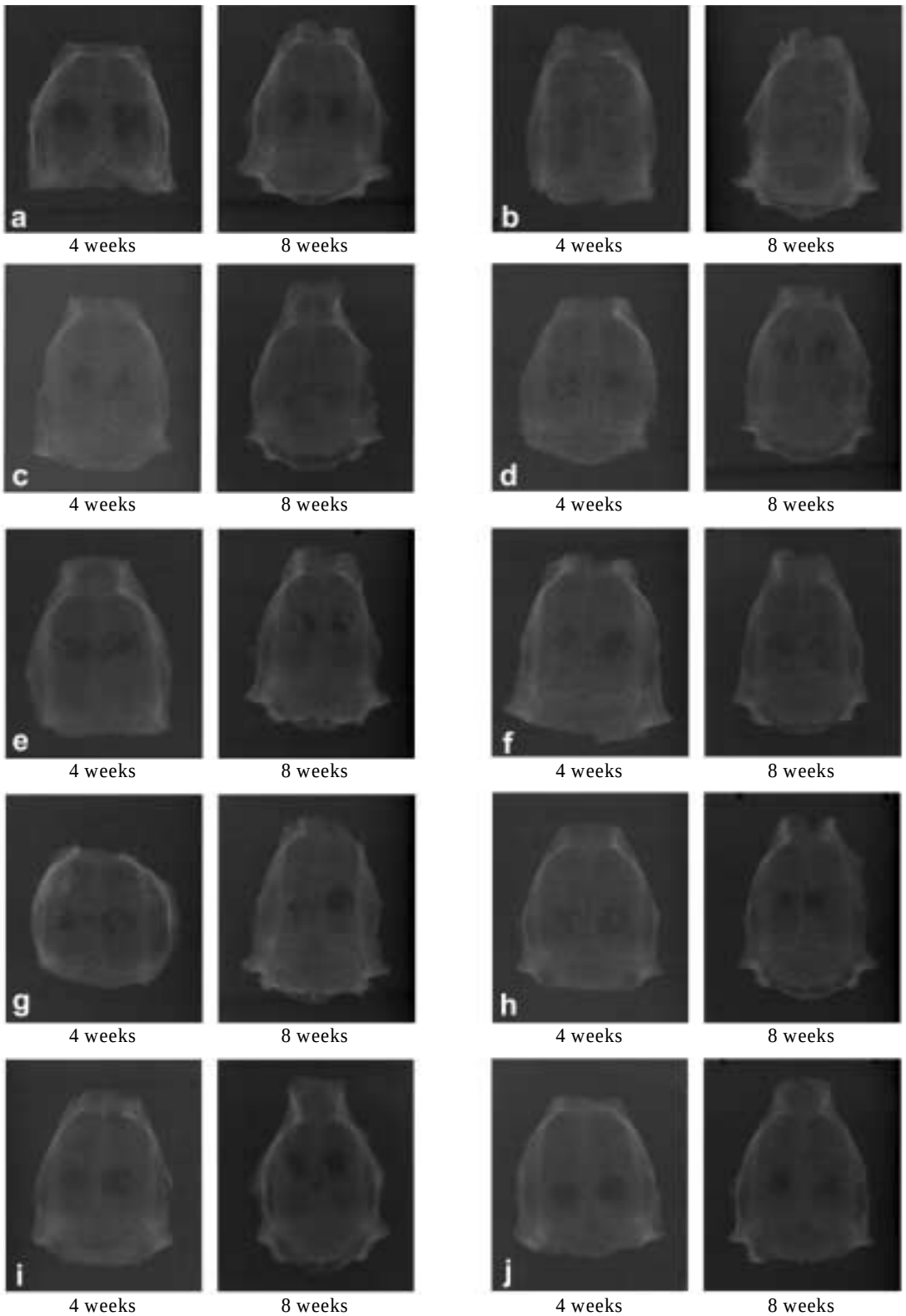

Fig. 5. Radiographic appearance of cranial vault, left 4 weeks, right 8 weeks each.

a. Collagen versus unfilled
b. 100% HA versus 100% HA + Collagen
c. 80% HA +20% TCP versus 80% HA +20% TCP + Collagen
d. 70% HA +30% TCP versus 70% HA +30% TCP + Collagen
e. 60% HA +40% TCP versus 60% HA +40% TCP + Collagen
f. 50% HA +50% TCP versus 50% HA +50% TCP + Collagen
g. 40% HA +60% TCP versus 40% HA +60% TCP + Collagen
h. 30% HA +70% TCP versus 30% HA +70% TCP + Collagen
i. 20% HA +80% TCP versus 20% HA +80% TCP + Collagen
j. 100% TCP versus 100% TCP + Collagen

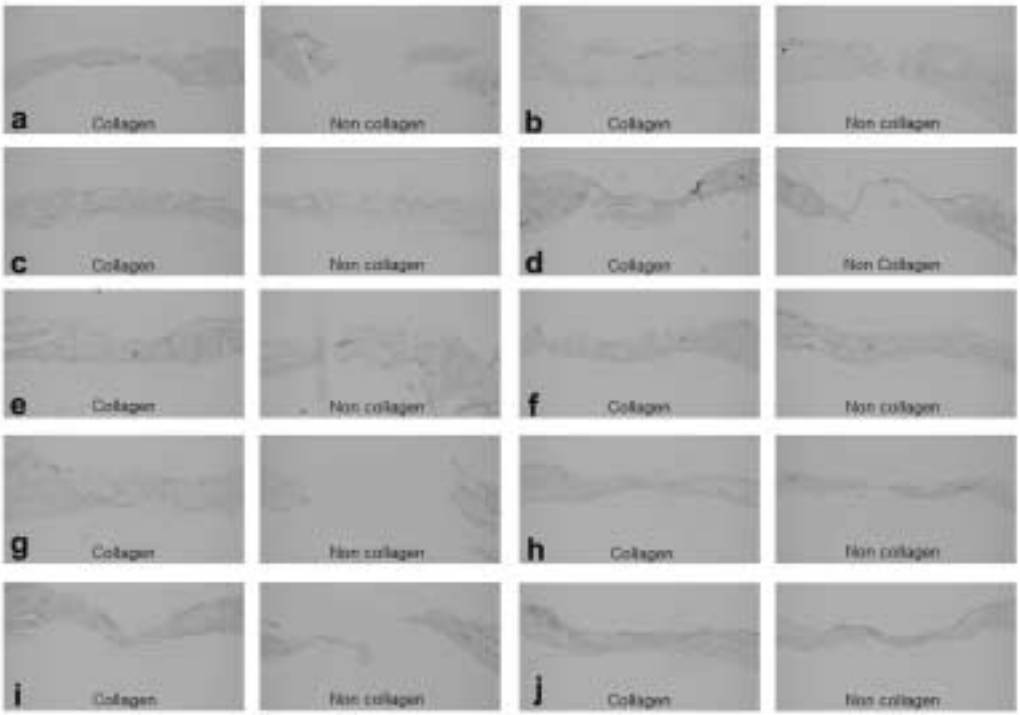

Fig. 6. Histological findings in 4 weeks, H&E stain, × 40.

a. Collagen versus unfilled
b. 100% HA versus 100% HA + Collagen
c. 80% HA +20% TCP versus 80% HA +20% TCP + Collagen
d. 70% HA +30% TCP versus 70% HA +30% TCP + Collagen
e. 60% HA +40% TCP versus 60% HA +40% TCP + Collagen
f. 50% HA +50% TCP versus 50% HA +50% TCP + Collagen
g. 40% HA +60% TCP versus 40% HA +60% TCP + Collagen
h. 30% HA +70% TCP versus 30% HA +70% TCP + Collagen
i. 20% HA +80% TCP versus 20% HA +80% TCP + Collagen
j. 100% TCP versus 100% TCP + Collagen

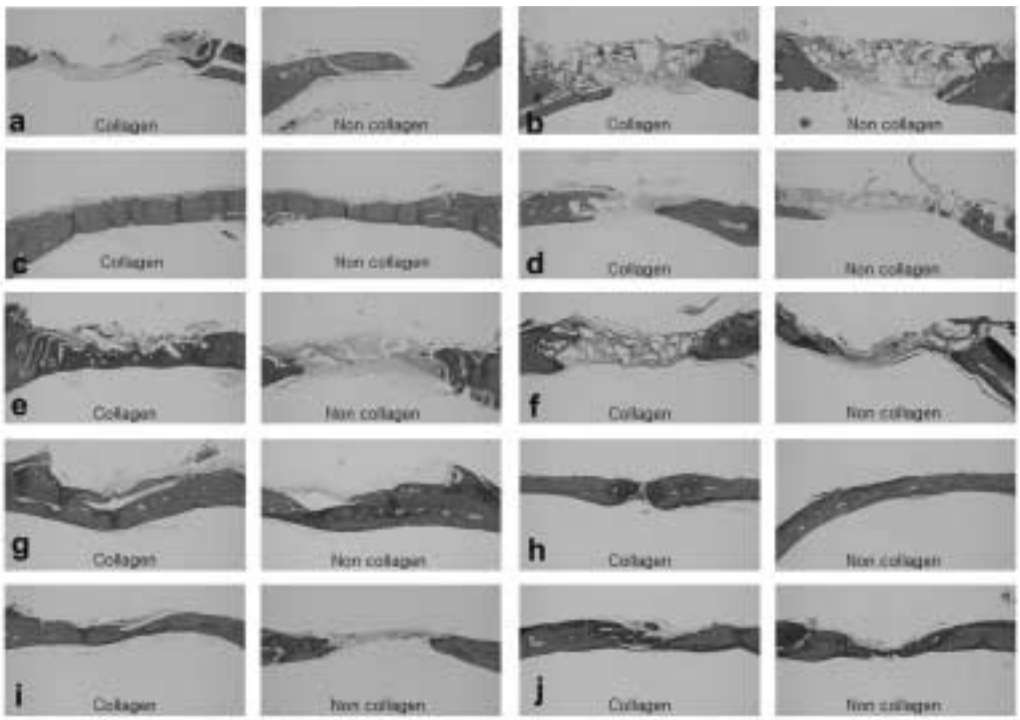

Fig. 7. Histological findings in 4 weeks, MT stain, × 40.

a. Collagen versus unfilled
b. 100% HA versus 100% HA + Collagen
c. 80% HA +20% TCP versus 80% HA +20% TCP + Collagen
d. 70% HA +30% TCP versus 70% HA +30% TCP + Collagen
e. 60% HA +40% TCP versus 60% HA +40% TCP + Collagen
f. 50% HA +50% TCP versus 50% HA +50% TCP + Collagen
g. 40% HA +60% TCP versus 40% HA +60% TCP + Collagen
h. 30% HA +70% TCP versus 30% HA +70% TCP + Collagen
i. 20% HA +80% TCP versus 20% HA +80% TCP + Collagen
j. 100% TCP versus 100% TCP + Collagen

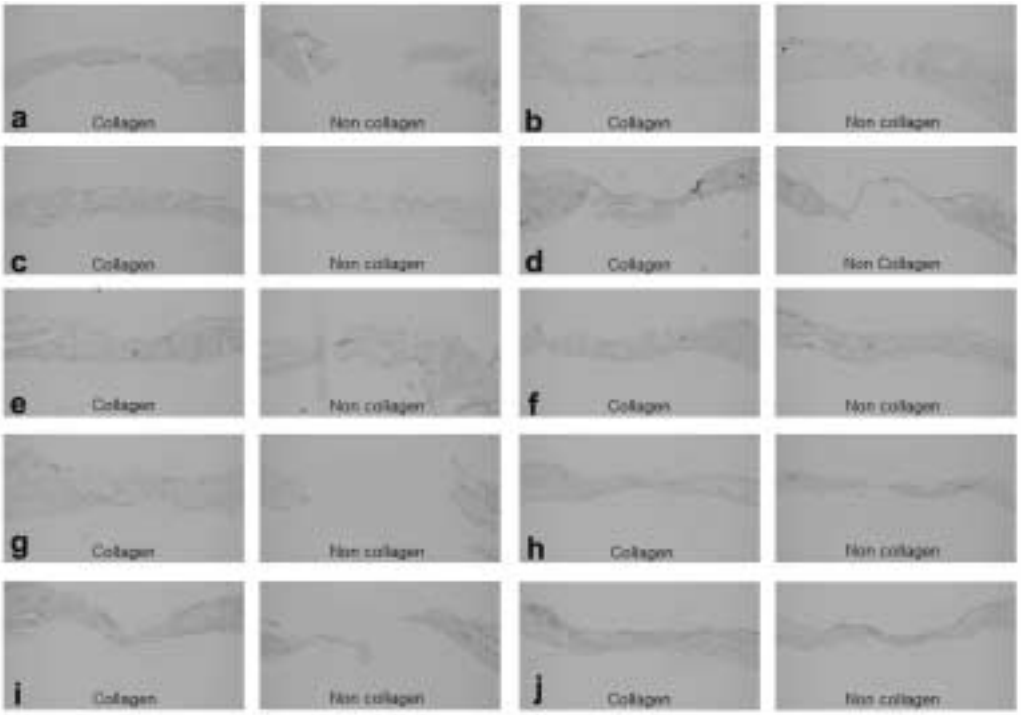

Fig. 8. Histological findings in 8 weeks, H&E stain, × 40.

a. Collagen versus unfilled
b. 100% HA versus 100% HA + Collagen
c. 80% HA +20% TCP versus 80% HA +20% TCP + Collagen
d. 70% HA +30% TCP versus 70% HA +30% TCP + Collagen
e. 60% HA +40% TCP versus 60% HA +40% TCP + Collagen
f. 50% HA +50% TCP versus 50% HA +50% TCP + Collagen
g. 40% HA +60% TCP versus 40% HA +60% TCP + Collagen
h. 30% HA +70% TCP versus 30% HA +70% TCP + Collagen
i. 20% HA +80% TCP versus 20% HA +80% TCP + Collagen
j. 100% TCP versus 100% TCP + Collagen

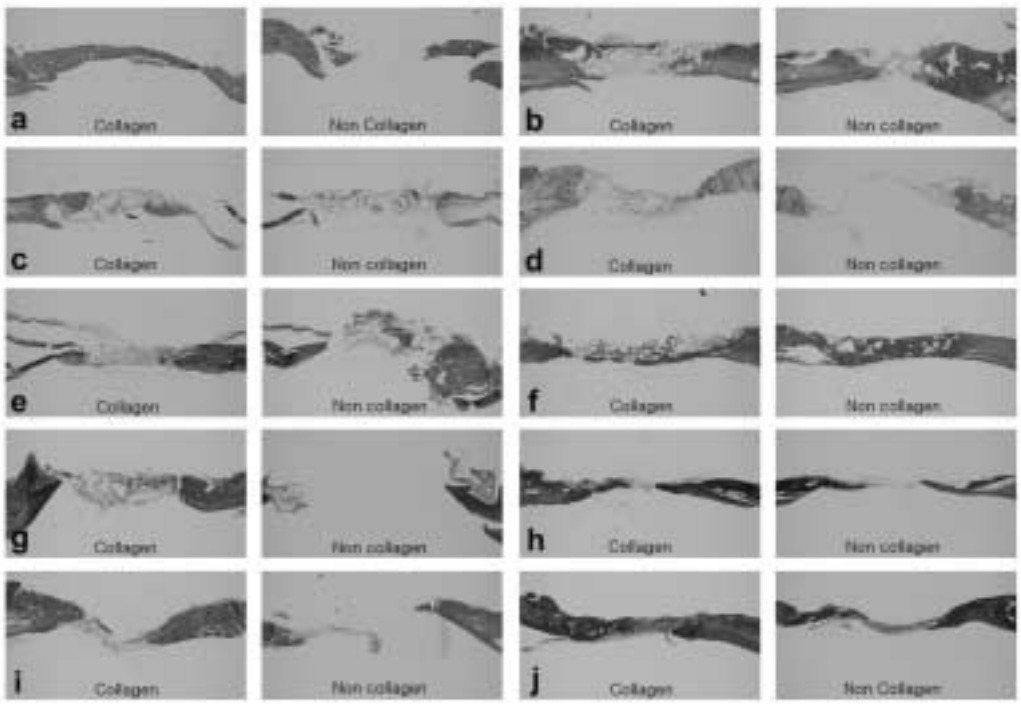

Fig. 9. Histological findings in 8 weeks, MT stain, × 40.

a. Collagen versus unfilled
b. 100% HA versus 100% HA + Collagen
c. 80% HA +20% TCP versus 80% HA +20% TCP + Collagen
d. 70% HA +30% TCP versus 70% HA +30% TCP + Collagen
e. 60% HA +40% TCP versus 60% HA +40% TCP + Collagen
f. 50% HA +50% TCP versus 50% HA +50% TCP + Collagen
g. 40% HA +60% TCP versus 40% HA +60% TCP + Collagen
h. 30% HA +70% TCP versus 30% HA +70% TCP + Collagen
i. 20% HA +80% TCP versus 20% HA +80% TCP + Collagen
j. 100% TCP versus 100% TCP + Collagen

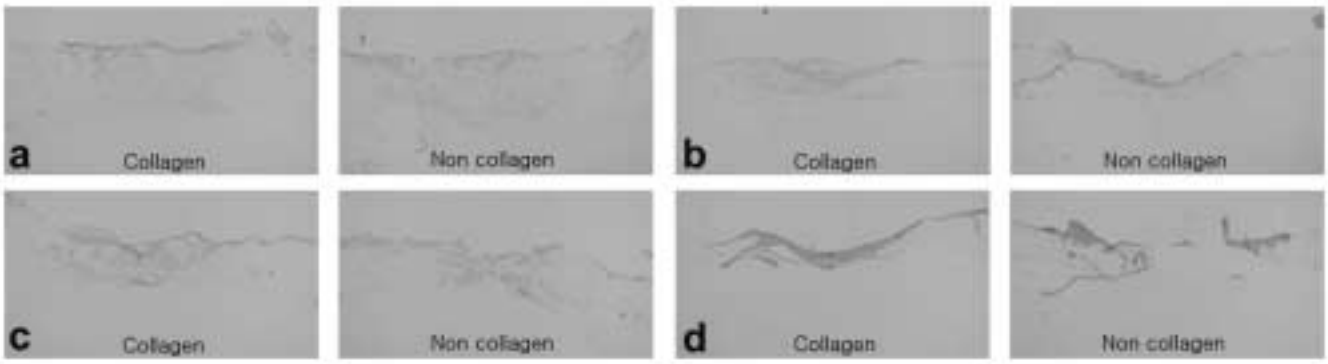

Fig. 10. Immunohistochemical findings, vWF, × 40.
a. 100% HA versus 100% HA + Collagen in 4 weeks
b. 100% TCP versus 100% TCP + Collagen in 4 weeks
c. 100% HA versus 100% HA + Collagen in 8 weeks
d. 100% TCP versus 100% TCP + Collagen in 8 weeks

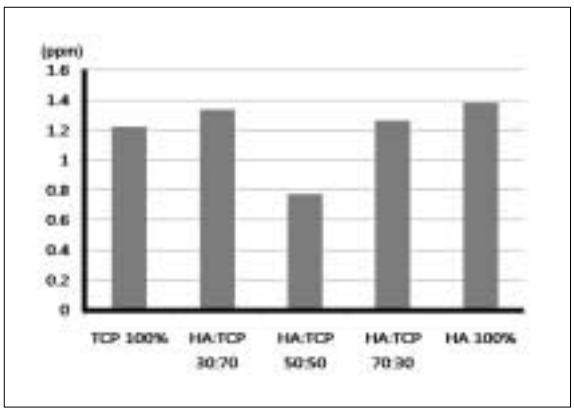

Fig. 11. Calcium analysis with Atomic absorption spectrophotometer (Perkin Elmer AAnalyst 100®).

DISCUSSION

Craniofacial defect repair remains a significant clinical challenge. Although autograft remains the material of choice for defect repair, a variety of synthetic bioimplants have been developed as substitutes.

An adult human has 208 bones amounting to 12-20 % of the body weight. Bone is a highly specialized connective tissue with embedded calcium giving it firmness, enabling it to protect vital organs, and giving the body stability. The chemical structure of an individual bone depends on the type of bone, age, and developmental stage. Adult bone contains a median ratio of approximately 70 % inorganic, which provide firmness, and 30 % organic substances (mainly collagen and 9 % water), which provide elasticity.

In general, we distinguish between connective tissue-based and cartilage-based ossification. While most bone starts out as cartilage (chondral ossification), ossification of skullcap bone, most facial bones, and the clavicle takes place in the embryonal connective tissue (desmoidal ossification). Next to the biomechanical and protective function, bone serves as a buffer for electrolyte balance, maintaining calcium and phosphate homeostasis in particular. In addition, hematogenous bone marrow, responsible for forming almost all human blood cells, is embedded in spongiosa[20].

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is usually specified as an essential bone mineral phase. HA can only serve as a prototype for the bone and tooth minerals[20]. It has been known since the 1920's that bone mineral is, in fact, composed of type B carbonate apatite (carbonate replaces the phosphate groups), also called dahllite[21]. The carbonate content is 4-8 % and can vary depending on age and the type of bone[22-23]. Since additional substances are present in the bone mineral[24-25], dahllite can have various substituents and may be considered structurally defective[26].

The calcium to phosphorus ratio (Ca/P) in bone is approximately 1.3-1.7 on average (stoichiometrically pure hydroxyapatite: Ca/P=1.67). The crystals are narrow, lamellar, and irregularly shaped, measuring approximately 250Å × 25Å × 25Å. With increasing age, the bone apatite becomes more crystalline (i.e. the structure is more ordered and the Ca/P ratio increases), indicating a slow maturation of laminar crystallites[27].

The laminar dahllite in collagen fibrils is bound to acid phosphate and carboxylate terminal groups. The longest side of the crystals lies parallel to the fibril axis. The mineral particles in the 40nm wide spaces between collagen fibrils (the 'gaps') are arranged in a highly organized manner[28-29].

Individual fibrils arrange themselves in higher ordered segments with 'gaps' arranged in a staggered manner. Additionally, these mineralized collagen fibrils arrange themselves into higher order segments depending on the mechanical stress they are subjected to. In the bony callus (newly formed, young bone), collagen fibrils exhibit a minimal order and random distribution. In lamellar bone, collagen fibrils are arranged in a sheet-like manner over large distances. These lamellar surfaces, which are 3?m thick, may be rotated by 90° relative to each other. This rotation results in a so-called 'plywood structure', leading to stability perfectly adapted to the mechanical situation[30].

HA and TCP have been used as bone graft substitutes because of their biocompatibility and osteoconductive properties. Beta tricalcium phosphate (β-TCP) was one of the earliest calcium phosphate compounds to be used as a bone graft substitute. In 1920, Albee and Morrison reported that the rate of bone union was increased when β-TCP was injected into the gap of a segmental bone defect. β-TCP is available in porous or solid form as either granules or blocks[31]. Structurally porous β-TCP has a compressive strength and tensile strength similar to cancellous bone[32]. Like other calcium phosphate preparations it has been found to be brittle and weak under tension and shear but resistant to compressive loads[33]. Typically it has been used in the granular porous form. Porous granules tend to migrate less than solid granules due to earlier fixation by fibrovascular ingrowth[34]. β-TCP undergoes reabsorption via dissolution and fragmentation over a 6-18 month period. Unfortunately, β-TCP is not replaced by bone in an equitable way. Thus, there is always less bone produced than the volume of β-TCP reabsorbed[35]. For this reason, β-TCP has been used as an adjunctive with other less reabsorbable bone graft substitutes or as an expander for autogenous bone graft.

The next calcium phosphate preparation to become available was the synthetic HA in the 1970s. Hydroxyapatite $C_{10}(PO_4)_6(OH)_2$ forms the principal mineral component of bone. Synthetic HA comes in ceramic or non-ceramic forms as porous or solid, blocks or granules. Ceramic refers to the fact that the HA crystals have been heated (sintered) between 700 and 1300 °C to form a highly crystalline structure. Ceramic HA preparations are resistant to reabsorption in vivo, which occurs at a rate of 1-2 % per year[36]. Conversely, non-ceramic HA is more readily reabsorbed in vivo and is also available in a self-setting cementable form. Synthetic HA has a good compressive strength but is weak against tension and shear. It is brittle and prone to fracture upon shock loading. Synthetic HA in solid block form is difficult to shape, does not permit fibro-osseous ingrowth, and has a much higher elasticity modulus than bone. Synthetic HA has been successfully used to coat metal implants to enhance osteointegration[37-39]. In porous granular form HA has been used alone or with bone grafts to fill voids[40].

Many types of artificial bone have been used as bone graft materials, with different ratio of HA and TCP. For example, Osteon® (Osteoguid™, KOREA) is mixture of HA and TCP at a ratio of 70:30. And MBCP® (Biomatlante Co., France) is mixture of HA and TCP at a ratio of 60:40. Besides them, despitie of many kinds of these products, most of them were only inorganic materials without including organic comonents.

To create a natural bone structure, the necessary substances must be made available to the body in the quantity required. Through local use of a biocompatible material in the trauma region or defect, bone regeneration can be accelerated. The implanted material must be absorbed at the same rate at which bone formation occurs. Simultaneously, the material must provide a guide rail along which collagen fibrils can form the mineralized structure. Thus, the authors suggest that type I collagen is efficient in promoting bone repair. Recently, Reddi and coworkers observed that type I collagen binds bone-promoting growth factors of the transforming growth factor (TGF) family[41-43].

In this study, we tried to create a defect (5 mm in diameter) in each parietal bone of the calvaria while avoiding the midsagittal suture. The skull sutures are fibrous joints that serve as growth centers within which osteogenic cells are usually found. Thus, injury to a skull suture may expose these cells. Furthermore, by preparing bilateral defects, the present model allowed us to compare the efficacy of two different biomaterials within the same animal. We compared type I collagen to improve bone healing and provide a favorable environment for osteoconduction in bone defects. This component may mimic the bone extracellular matrix, which is responsible for cell migration, differentiation, and proliferation in natural bone. It provides the benefits of a synthetic graft containing inorganic, such as HA and TCP, and organic components that together may improve bony healing. In this study, the unfilled defects in group

1 did not heal. New bone only formed at the margin of the defect and the rest of the defect was filled with fibrous tissue. The amount of bone did not change between 4 and 8 weeks. Defects filled with collagen healed more than unfilled defects. In contrast, HA, TCP, and collagen filled defects with new bone. The amount of new bone and marrow present in the defects was significantly greater than in unfilled defects. A higher ratio of HA resulted in more new bone. In our study, type I collagen may provide a favorable environment for osteoconduction in bone defects. We didn't perform quantitative analysis in immunohistochemical staining, because immunohistochemical findings of BMP-2, -4, and VEGF were not compared markedly. But, vWF staining was especially strong in endothelial cells in newly formed bone margins. Due to the individual differences of calvarial size between each experimental animal, representative histologic or radiographic comparisons were not possible.

Atomic absorption spectrometry (AAS) is an analytical technique that measures the concentrations of mineral elements, with the principles of absorbing characteristic wave lengths of light by atoms of different elements. In this study, calcium content of 8 weeks specimens determined by elementary analysis was not significant statistically because the number of specimens was not enough and dissoluted bony tissues were not chilated completely.

Many types of artificial bone have been used as bone graft materials, but most of them were inorganic materials. By adding organic type I collagen to the inorganic HA and TCP mixture, the effect of bony regeneration is improved. These new artificial bone graft materials, which are under strict storage and distribution systems, will be suggested to be available to clinical dentistry demands in the sooner future.

CONCLUSION

The purpose of this study was to evaluate new bone formation and healing in rat calvarial bone defects using HA and TCP mixed at various ratios. Additionally, this study evaluated the effects of type I collagen as a basement membrane organic matrix.

1. Unfilled defects of group 1 did not heal. New bone only formed at the margins of the defects, with the rest of the defect being filled fibrous tissue.
2. Defect filled with collagen was more bony healing than unfilled defect.
3. Mixture of HA and TCP with type I collagen were almost bridging with new bone. So, significantly greater than in the unfilled defects.
4. The higher ratio of HA, the more new bone was showed.

Many kinds of artificial bones have been used as the bone graft materials. But, most of them were applicable as the inorganic materials. By adding the organic type I collagen to the inorganic HA and TCP mixtures, the improved bony regeneration effect can be confirmed in this study. Therefore, these new artificial bone graft materials under strict storage and distribution systems will be able to be suggested to the further demands in the clinical dentistry.

REFERENCES

1. Citardi MJ, Friedman CD: Nonvascularized autogenous bone grzafts for craniofacial skeletal augmentation and replacement. Otolaryng Clin N Am 1994;27:891-911.
2. Lekholm U, Wannfors K, Isaksson S, et al.: Oral implants in combination with bone grafts. Int J Oral Maxillofac Surg 1999;28:181-187.
3. Triplett RG, Schow SR: Autologous bone grafts and endosseous implants: complementary techniques. J Oral maxillofac Surg 1996;54:486-494.
4. Hill NM, Horne JG, Devane PA: Donor site morbidity in the iliac crest bone graft. Aust N Z J Surg 1999;69:726-728.
5. Broecke DGA: Neurotmesis of the lateral femoral cutaneous nerve when coring for iliac crest bone grafts. Plast Reconstr Surg 1998;102:1163-1166.
6. Forrest C, Boyd B, Manktelow R, et al.: The free vascularized iliac tissue transfer: donor site complication associated with eighty-two cases. Br J Plast Surg 1992;45:89-93.
7. Bruchardt H, Jones H, Glowczewskie F, et al.: Freeze-dried allogenic segmental cortical-bone grafts in dogs. J Bone Joint Surg 1978;60:1082-1090.
8. Buck BE, Malinin TI, Brown MD: Bone transplantation and human immunodeficiency virus. An estimate of risk of acquired immunodeficiency syndrome (AIDS). Clin Orthop 1989;240:129-136.
9. Perry CR: Bone repair technique, bone graft, and bone graft substitutes. Cli Orthop Rel Res 1999;360:N71-86.
10. Ohbayashi Y, Miynoru M, Nagahata S: A long-term study of implanted artificial hydroxyapatite particles surrounding the carotid artery in adult dog. Biomaterials 2000;21:501-509.
11. Ono I, Tateshita T, Nakajima T: Evaluation of a high density polyethylene fixing system for hydroxyapatite ceramic implants. Biomaterial 2000;21:143-151.
12. Breitbart AS, Staffenberg DA, Thorne CCHM: Tricalcium phosphate and osteogenin: a bioactive on lay bone graft substitute. Plast Reconstr Surg 1995;96:699-708.
13. Fortunato G, Marini E, Valdinucci F, et al.: Long-term of hydroxyapatite-fibrin glue implantation in plastic and reconstructive craniofacial surgery. J Craniomaxillofac Surg 1997;25:124-135.
14. Andrade JCT, Camilli JA, Kawachi EY, et al.: Behavior of dense and porous hydroxyapatite implants and tissue response in rat femoral defects. J Biomed Master Res

2002;62:30-36.
15. Thomas MS, Lynne AO, John AP: Repair of critical size rat calvarial defects using extracellular matrix protein gels. J Neurosurg 1995;83:710-715.
16. Barrack RL, Cook SD, Patron LP, et al.: Induction of bone ingrowth form acetabular defects to a porous surface with OP-1 ○R. Clin Orthop 2003;417:41-49.
17. Caplan AI: New logic for tissue engineering: multifunctional and biosmart delivery vehicles. In: Davies JE, ed. Bone Engineering. Toronto: Em squared, 2000:441-446.
18. Hollinger JO, Kleinschmidt JC: The critical size defect as an experimental model to test bone repair materials. J Craniofacial Surg 1990;1:60-68.
19. Hing AH: Bone repair in the twenty-first century: biology, chemistry or engineering? Phil Trans R Soc Lond A. 2004;362:2821-2850.
20. Posner AS: The mineral of bone. Clin Orthop 1985;200:87-99.
21. Eisenberger S, Lehrman A, Turner WD: Basic calcium phosphates and related system, Subpart A. Carbonate-apatite versus hydroxyapatite. In: Chemical review Vol. 26, Johnson Reprint Corporation New York 1940:285.
22. Legros R, Balmain N, Bonel G: Age-related changes in mineral of rat and bovine cortical bone. Calcif Tissue Int 1987;41:137-144.
23. Peters F, Schwarz K, Epple M: The structure of bone studied with synchrotron X-ray diffraction, X-ray absorption spectroscopy and thermal analysis. Thermochim Acta 2000;361:131-138.
24. Posner AS, Betts F: Synthetic amorphous calcium phosphate and its relation to bone mineral structure. Accts Chem Res 1975;8:273-281.
25. Tadic D, Peters F, Epple M: Continuous synthesis of amorphous carbonated apatites. Biomaterials 2002;23:2553-2559.
26. Le Geros RZ: Effect of carbonate on the lattice parameters of apatite. Nature 1965;206:403-404.
27. Lowenstam H, Weiner AS: On biomineralization. Oxford University Press New York Oxford. 1989.
28. Mann S: Biomimetic materials chemistry. VCH publishers Inc. Weinheim, New York Cambridge. 1996.
29. Weiner S, Arad T, Sabanay I, Traub W: Rotated plywood structure of primary lamellar bone in the rat: Orientation of the collagen fibril arrays. Bone 1997;20:509-514.
30. Weiner S, Wagner HD: The material bone: structure-mechanical function relations. Annu rev Master Sci 1998;28:271-298.
31. Albee FH, Morrison HF: Studies in bone growth, triple calcium phosphate as a stimulus to osteogenesis. Ann Surg 1920;71:32.
32. Jarcho M: Calcium phosphate ceramics as hard tissue prosthetics. Clin Orthop 1981;157: 259.
33. Geesink RGT, De Groot K: Bonding of bone to apatite coated implants. J Bone Joint Surg 1988;70:17.
34. Byrd HS, Hobar PC: Augmentation of craniofacial skeleton with porous hyroxyapatite granules. Reconstr Surg 1993;91:15-22.
35. Hollinger JO, Brekke J: Role of bone substitutes. Clin Orthop 1996;324:55-65.
36. Constantino PD, Freidman CD: Synthetic bone graft substitutes. Otolaryngol Clin North Am 1994;27:1037-1073.
37. Havelin LI, Engesaeter LB, Espehaug B, Furnes O, Lie SA, Vollset SE: The Norwegian Arthroplasty Register: 11 years and 73,000 arthroplasties. Acta Orthop Scand 2000; 71:337-353.
38. Bellemans J: Osseointegration in porous coated knee arthroplasty. The influence of component coating type in sheep. Acta Orthop Scand Suppl 1999;288:1-35.
39. Strnad Z, Strnad J, Povysil C, Urban K: Effect of plasma sprayed hyroxyapatite coating on the osteoconductivity of commercially pure titanium implants. Int J Oral Maxillofac Implants 2000;15:483-490.
40. Oonishi H, Kushitani S, Yasukawa E, et al.: Particulate bioglass compared with hydroxyapatite as a bone graft substitute. Clin Orthop 1997;334:316-325.
41. Paralkar VM, Nandedkar AKN, Pointer RH, et al.: Interaction of osteogenin, a heparin binding bone morphogenetic protein, with type IV collagen. J Biol Chem 1990;265:17281-17284.
42. Sampath TK, Muthukumaran N, Reddi AH: Isolation of osteogenin, an extracellular matrix-associated, bone-inductive protein, by heparin affinity chromatography. Proc Natl Acad Sci 1987;84:7109-7113.
43. Vukicevic S, Paralkar VM, Cunnungham NS, et al.: Autoradiographic localization of osteogenin binding sites in cartilage and bone during rat embryonic development. Dev Biol 1990;140:209-214.