J Korean Assoc Maxillofac Plast Reconstr Surg 2013;35(6):353-359
http://dx.doi.org/10.14402/jkamprs.2013.35.6.353
pISSN:1225-4207 eISSN: 2233-7296
Original Article

Analysis of Organic Components and Osteoinductivity in Autogenous Tooth Bone Graft Material

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Abstract

Purpose: Extensive research is actively ongoing for development of an ideal bone substitute that meets the gold standard. Tooth was selected as a donor site for evaluation of potentials in bone substitutes based on its similar chemical compositions to alveolar bone. Previous studies have evaluated inorganic components of autogenous tooth bone graft material (AutoBT) and osteoconductivity. In continuation from the previous studies, the current study was conducted for analysis of organic components and evaluation of osteoinductivity of AutoBT.

Methods: Forty-six extracted teeth were collected from actual patients (Korea Tooth Bank, R&D Institute). Extracted teeth were processed into AutoBT and implanted in dorsal subcutaneous muscular tissues of 15 athymic mice. Biopsy samples were harvested at two, five, and eight weeks. The Bradford assay, sodium dodecyl sulphate polyacrylamide gradient gel, and western blotting were performed for investigation of organic contents of AutoBT.

Results: Histology analyses showed signs of new bone formation as early as two weeks. Results of the Bradford assay indicated the existence of noncollagenous proteins (NCP). 0.29% (2.89 mg/g) of proteins were extracted by weight in the root portion of AutoBT; 0.02% (0.029 mg/g) and 1.79% (17.93 mg/g) of proteins were measured by weight in crown and block-form of AutoBT, respectively. However, recombinant human bone morphogenetic protein-2 was not observed in AutoBT.

Conclusion: Within the limitation of the current study, AutoBT induced new bone formation by NCP embedded in dentin.

Key words: Demineralized dentin matrix, Bone substitutes, Proteins, Osteogenesis, Isolation & purification

Introduction

Bone grafting materials are used effectively in dentistry for guided bone regeneration at bone defect sites. Extensive studies have continuously demonstrated improved quality of bone grafting materials with less technique-sensitivity[1]. Among bone substitutes, autograft has been regarded as the gold standard due to its osteoconductivity, osteoinductivity, and osteogenicity. Yet, because of its limited harvest volume and requirement of donor site surgery with potential complications, clinicians have been leaning toward use of allograft, xenograft, or synthetic bone graft materials, and favorable outcomes have been achieved with use of these types of bone graft materials[2].

However, those non-autogenous bone graft materials also have disadvantages. Allografts are expensive and have

RECEIVED August 6, 2013, REVISED August 26, 2013, ACCEPTED November 26, 2013

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potential infection risks. Synthetic bone grafts lack osteogenesis and osteoinduction. Hence, in finding near gold standard grafts, studies were conducted for evaluation of feasibility of use of fresh teeth in the form of demineralized dentin matrix (DDM) as a biocompatible autogenous graft material[3-9].

Tooth was selected as a grafting site because of its similar chemical composition to bone. Dentin has approximately 70% inorganic contents and 20% organic contents in weight volume. In alveolar bone, the inorganic content is 65%, and the organic content is 25%. Besides type I collagen in organic components of teeth, researchers have extracted bone morphogenetic proteins (BMPs) from animal teeth such as bovine, lapine, and murine[4-8,10]. In addition, the dentin-BMPs promote differentiation of mesenchymal stem cells into chondrocytes and thus enhance bone formation[10,11].

Indeed, tooth is composed of both inorganic and organic substances. Yet, we wanted to examine the question of whether autogenous tooth bone graft material (AutoBT; Korea Tooth Bank, Seoul, Korea) still contains inorganic and/or organic minerals after demineralization and fabrication procedures. AutoBT is processed from patients' own extracted teeth that required extraction due to either non-restorability or orthodontic reasons. The processed AutoBT, then, is utilized in the same patient in situations where bone grafting is necessary during implant placement or guided bone regeneration procedures.

The previous study analyzed inorganic components of AutoBT using scanning electron microscopy, energy dispersive x-ray spectroscopy, x-ray diffraction, and histomorphometric analysis. As a result, we found that AutoBT made from the root portion was composed of low-crystalline hydroxylapatite, tricalcium phosphate, amorphous calcium phosphate, and octacalcium phosphate[1]. As a result, AutoBT is able to provide an osteoconductive environment as a grafting material.

In continuation from the previous studies[1,12], the current study investigated the existence of organic substances such as BMP and/or other noncollagenous proteins (NCP) and performed experiments in athymic mice *in vivo* to demonstrate osteoinductive potential of AutoBT.

Materials and Methods

1. Sample collection

Human teeth samples were collected from patients who needed extraction due to orthodontic treatment or non-restorability. Informed consents were given and the nature of the research was explained to patients for use of their extracted teeth. Forty-six samples were used in the current study. This study was approved by the Seoul National University Bundang Hospital (Seongnam, Korea) Institutional Review Board (B-1005-049-003) and all participants signed informed consent agreements.

2. Autogenous tooth bone graft preparation

The extracted teeth were stored in a 75% alcohol sample bottle for up to one week. Then, the anatomical crown portion of the tooth was dissected after removal of attached soft tissues. Sample root portions were crushed into a powder between 400 to 800 μm in diameter in size. Remaining soft tissues and contaminants in crushed AutoBT powder were removed with distilled water. AutoBT powder was subjected to dehydration and defatting processes, and then lyophilized. After sterilization with ethylene oxide, the AutoBT powder was packed. A block form was fabricated using the same process used for powder without being crushed. Patent number: 10-1062381 (Korea).

3. *In vitro* study- sodium dodecyl sulphate polyacrylamide gradient gel and western blotting

The dentin matrix proteins (DMPs) in groups A (dried teeth in 25°C) and B (wisdom teeth in fresh state) were extracted with 500 mL of 4 mol guanidine-hydrochloride for 24 hours at 4°C. The extracted protein solution was concentrated using centrifugal filter units (Amicon Ultra-15; Millipore, Billerica, MA, USA). The concentrated proteins were boiled with sodium dodecyl sulphate (SDS) sample buffer for destruction of the three-dimensional structure. The proteins were separated into a 5% to 20% sodium dodecyl sulphate polyacrylamide gradient gel (SDS-PAGE) under the reducing condition. For western blotting, proteins in the gel were transferred to a polyvinylidene difluoride membrane (Immobilon™ transfer membrane; Millipore) by electroblotting. To avoid non-specific binding, the mem-

brane was placed in a dilute solution of protein (Block Ace™; Yukijirushi, Sapporo, Japan). A dilute solution of primary antibody (anti-human BMP-2 mouse monoclonal antibodies; R&D systems, Minneapolis, MN, USA) was incubated with the membrane under gentle shaking. The membrane was rinsed for removal of unbound primary antibody. A dilute solution of the Amershan™ horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody 1:30,000 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was incubated with the membrane under shaking. The membrane was rinsed again for removal of unbound secondary antibody. Then, the membrane was incubated in chemiluminescent HRP substrate (Immobilon™ Western; Millipore) for 5 minutes. The lights of bands on the membrane were detected using an image station (Light Capture; ATTO, Tokyo, Japan) for an appropriate duration.

4. Bradford assay

AutoBT samples (11 crown samples, 11 root samples, and one block sample) were diluted in distilled, deionized water at 121°C for 1 hour in micro-centrifuge tubes at concentrations of 0.1 g/mL; three dilutions of a protein standard were prepared; 100 μL of each standard and sample solution with 5.0 mL of diluted dye reagent (Bio-Rad Laboratories, Hercules, CA, USA) were inserted into each test tube and vortexed. After 15 minutes of incubation at room temperature, absorbance at 595 nm was measured using a Spectrophotometer (Beckman Coulter, Hialeah, FL, USA).

5. *In vivo* study- athymic mouse experiment

Fifteen athymic mice (average weight: 30 g) were used. The animals were divided into three groups (two-week, five-week, and eight-week) of five mice each for this study. General anesthesia was administered with an intraperitoneal injection of sodium pentobartibal (43 mg/kg, Nembutal; Dainabot Co., Osaka, Japan) under sterile conditions. After opening the dorsum of each sample, right and left side subcutaneous tissues (10 mm in depth) were dissected in order to create a hypodermic pouch for AutoBT insertion; 0.3 g of AutoBT was inserted on each side. After insertion of AutoBT powder, wounds were closed with nylon sutures. To protect the wound from infection, antibiotic ointments were applied throughout the study. The Animal Research Committee of the University of Hokkaido, (Hokkaido, Japan) approved all procedures.

6. Light microscopy

A soft tissue biopsy, including AutoBT in the hypodermic pouch was obtained from each two-week, five-week, and eight-week athymic mouse after sacrificing the animal. A total of 30 biopsies were fixed in 10% formalin for 24 hours. Following decalcification in Calci-Clear Rapid (National Diagnostics, Atlanta, GA, USA) for 12 hours, the specimens were rinsed in distilled water. The specimens were then treated using a Hypercentre XP tissue processor (Themo Fisher Scientific, Waltham, MA, USA), embedded in paraffin, and cut at thicknesses of 4 to 5 μm. Sectioned specimens were prepared for light microscopy after hematoxylin and eosin staining. Images were captured using a MagnaFire digital camera system (Optronics, Goleta, CA, USA).

Results

1. Histology analysis

Histology analysis in two-week samples showed attachment of the lining cells on the surface of the AutoBT (Fig. 1A). Depositions of osteoid in the biopsy area around the AutoBT were detected as well (Fig. 1B). Newly formed collagenous like structures were observed in between AutoBT and native muscle tissues of athymic mice. Signs of cartilage formation started to show at the periphery of AutoBT and advanced toward the inner area with dense fibrous tissues filling AutoBT powder spaces (Fig. 1C). Starting from week 8, the presence of lamellar bone (Fig. 1D) along with the signs of new endocondral bone formation was observed at the edges of AutoBT (Fig. 1E). A continuous cartilage formation replaced empty grafted spaces as AutoBT powder was resorbed in recipient sites.

2. Bradford assay analysis

In order to determine the existence of organic components in AutoBT, the Bradford assay was performed. Embedded proteins in AutoBT were extracted in the AutoBT crown portion, in root, and in block-form. The amount of extracted proteins was 0.29% (2.89 mg/g) in

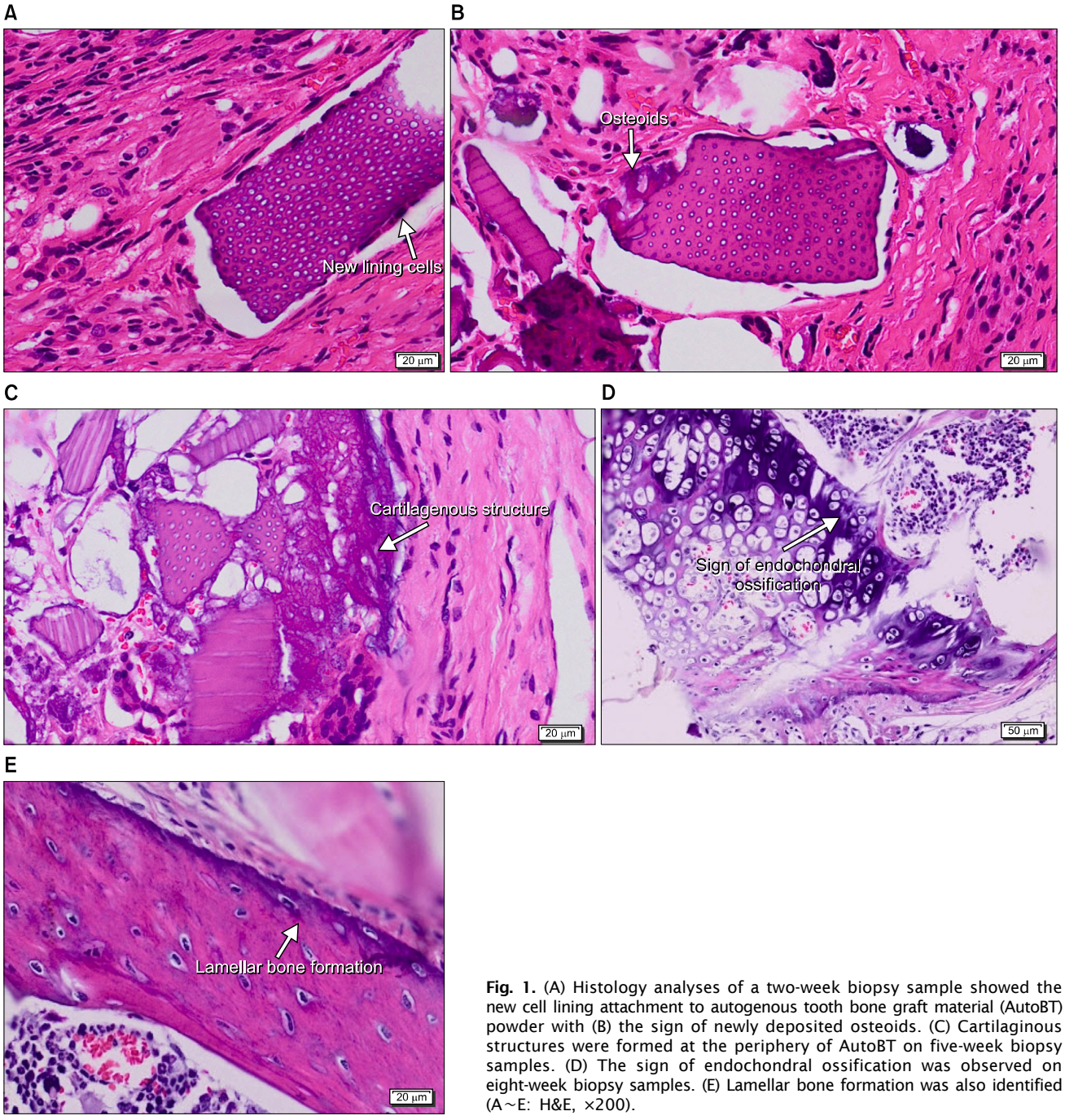

Fig. 1. (A) Histology analyses of a two-week biopsy sample showed the new cell lining attachment to autogenous tooth bone graft material (AutoBT) powder with (B) the sign of newly deposited osteoids. (C) Cartilaginous structures were formed at the periphery of AutoBT on five-week biopsy samples. (D) The sign of endochondral ossification was observed on eight-week biopsy samples. (E) Lamellar bone formation was also identified (A~E: H&E, ×200).

root, 0.02% (0.029 mg/g) in crown, and 1.79% (17.93 mg/g) in block-form by weight, as shown in Table 1.

3. Sodium dodecyl sulphate polyacrylamide gradient gel and western blotting

BMP-2 (17.5 kD) was not observed in all groups of AutoBT samples in electrophoresis. However, minor bands of proteins were observed at 76 kD and 102 kD (Fig. 2).

Table 1. The highest amounts of proteins were extracted in root-form of AutoBT, whereas the lowest amounts were extracted from the crown portion in Bradford assay

Type of AutoBT	Organic component by weight, % (mg/g)
Root portion	0.29 (2.89)
Crown portion	0.02 (0.03)
Block-form	1.79 (17.93)

AutoBT, autogenous tooth bone graft material.

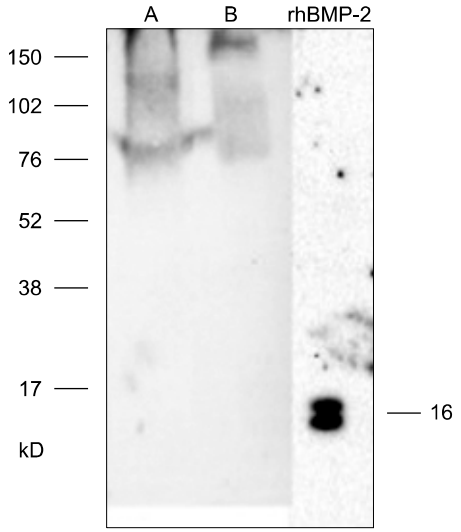

Fig. 2. Sodium dodecyl sulphate polyacrylamide gradient gel of purified fractions from autogenous tooth bone graft material powder fabricated from a dried tooth in 25°C (A), and from wisdom tooth in fresh state (B). rhBMP-2, recombinant human bone morphogenetic protein-2.

Discussion

Finding the most effective and safe bone grafting material has been a continuous subject of study. Due to its osteoinduction, osteoconduction, osteoproliferation, and bone remodeling, autogenous bone graft has been recognized as the gold standard. In fact, because of its similar biological composition to that of alveolar bone, many researchers have examined dentin as a potential carrier for human proteins and as a grafting material[3-6,13]. Both tooth and alveolar bone are derived from neural crest cells and consist of the same type I collagen. In addition, dentin contains BMPs, which induce bone formation and NCP such as osteocalcin, osteonectin, and dentin phosphoprotein[10,14].

Based on previous studies in DDM reported by Urist and colleagues[7-9,14,15] and its potentials, we developed the processing and demineralization technique for fabrication of AutoBT from extracted teeth.

Histologic observation in the current study showed evidence of osteoinductivity with new bone formation. Starting from the attached cells lining the surface of AutoBT (Fig. 1A), the first sign of osteoinduction was noted by osteoid formation along the AutoBT at two weeks (Fig. 1B) when grafted into muscular tissues of athymic mice. The attached lining cells could have developed from surrounding perivascular mesenchymal cells. In addition, chemotactic agents from dentin particles in AutoBT would make the attachment of lining cells possible. Perhaps, there might be some endogenous growth factors embedded in AutoBT dentin particles. These endogenous growth factors differentiated osteoblasts resulting in pre-mature collagen deposition on two-week and five-week biopsy (Fig. 1C). As AutoBT resorbed within eight weeks, new lamellar bone was formed adjoining to the remaining AutoBT particles with continuous formation of cartilage (Fig. 1D, 1E). In addition, the remaining AutoBT particle was osseointegrated with new bone.

Next, the Bradford assay was performed in order to determine the existence of organic substances in AutoBT. The block-form AutoBT showed the largest amount of organic contents because it consists of both dentin and cementum. Cementum contains many organic substances, including growth factors like transforming growth factor-beta, immunoglobulin G1, and platelet-derived growth factor-BB[16]. A block form AutoBT was fabricated using the same process used for powder without being crushed. Thus, the original shape of the extracted root structure remained. The lowest amount of organic proteins was found in the AutoBT made from a crown portion of the extracted tooth, because the crown consisted mainly of enamel, which only contains 5% organic substances by weight. Proteins suspended in AutoBT in all three types (root, crown, and block form) could be categorized as NCP.

According to SDS-PAGE and western blotting *in vitro* studies, recombinant human bone morphogenetic protein-2 (rhBMP-2) was not detected in AutoBT powder (Fig. 2). However, electrophoresis showed minor bands from 76 kD to 102 kD. These minor bands could be partial degradations of BMPs during isolation or purification. There are a few possibilities in undetected rhBMP-2 *in vitro*. First, the use of 10% H_2O_2 during the oxidation treatment in a vital tooth could have denatured the remaining dentin collagens and NCP. Second, if a particular AutoBT was prepared by an endodontic treated tooth, growth factors including rhBMP-2 were already inactivated during previous root canal treatment with sodium hypochlorite. Finally, the quantity of endogenous BMPs in dysfunctional sample teeth was small or nil from the beginning so that

it could not be extracted. However, in the current study, unfound rhBMP-2 can be considered as one of the limitations.

Unfound rhBMP-2 in electrophoresis does not discourage in evaluation of osteoinductivity of AutoBT. The new bone formations in muscular subcutaneous tissues of athymic mice *in vivo* with a support of NCP existence from the Bradford assay were sufficient for evaluation of the osteoinductivity of AutoBT. Ninety percent of organic components in dentin are type I collagen, and the rest are composed of NCP, biopolymer, lipid, citrate, lactate, and so on. Studies have shown that proteins that are categorized in NCP, such as phosphophoryn, sialoprotein, glycoprotein, proteoglycan, osteopontin, osteocalcin, DMP-1, osterix, and Cbfa1 (also known as Runx2) have osteoinductive potentials[17,18].

In addition, in histologic analysis, attached lining cells with osteoid formations to the exposed AutoBT powder were suggestive of sites of endogenous growth factor activity. Thus, based on the current study, we can carefully conclude that AutoBT has osteoinductivity through NCP embedded in AutoBT dentin particles.

Based on previous research and with limitations in AutoBT, the current study evaluated human tooth as a safe, yet simple, autogenous bone grafting material with demonstration of osteoinductivity. At the same time, this study draws some limitations, including short duration of incubation (eight weeks), and a small number of samples (n=46) in the experiment. Conduct of more in-depth studies will be needed in the future with sample variations, such as examining the quality of AutoBT with the use of vital and non-vital teeth, carious teeth, and periodontally involved teeth. In addition, conduct of clinical trials using AutoBT in guided bone regeneration, socket preservation, and repair of bone defects is needed.

Conclusion

In this study, AutoBT induced active new bone formation by NCP embedded in dentin. Within the limitations of the current study, we may conclude that AutoBT is a simple and safe bone substitute with maintenance of its osteoconductivity and osteoinductivity. Therefore, it can be a considerable option in selection of bone graft materials when tooth extraction is necessary.

Acknowledgements

This study was supported by a grant from the Korea Healthcare Technology R&D Project, Ministry of Health and Welfare, Republic of Korea (No. A102065) and a Grant-in-Aid for Scientific Research in Japan (No. C22592190).

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