Original

A novel model demonstrates the successful repair of alveolar cleft defects by tooth germ transplants

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Abstract

Cleft lip and palate is generally treated by promoting the growth and development of the maxillofacial region, and alveolar cleft defects are often repaired with artificial bones or cancellous bones harvested from the anterior iliac crest. The purpose of this study was to develop a mouse model to investigate the potential application of using tooth germs from wisdom teeth to repair alveolar cleft defects via their ability to form bone tissue. The upper first molars of 3-week-old mice were extracted under deep anesthesia to produce alveolar cleft defects using a dental engine with a round tungsten carbide burr. Tooth germs from embryonic day 16 mice together with collagen sponges were transplanted into alveolar cleft-like bone defects for the experimental group. Collagen sponges alone were transplanted into alveolar cleft-like bone defects for the control group. Hard tissue recoveries were evaluated from micro-CT images immediately after, and 3 and 7 weeks after the transplantations, and hard tissue volumes were measured at 7 weeks after the transplantation. Further, the alveolar cleft defects were observed histologically. CT-images of the control group showed no newly formed bone in the alveolar defects at any time point, but newly formed bone was observed in the experimental group at 3 and 7 weeks after the transplantations. At 7 weeks in the experimental group, the bone defects were filled with high density hard tissue, and the volume of newly formed bone at 7 weeks was significantly higher than in the control group ($P < 0.05$). Histological observations revealed that newly formed bone was not observed in alveolar cleft defects in the control group at any experimental period but was observed in the defects in the experimental group at 3 and 7 weeks after the transplantation. Further, osteocalcin-positive cells and TRAP-positive cells were observed adjacent to the new bone at 7 weeks after the transplantation. These findings indicate that transplantation of tooth germs with collagen sponge scaffolds is capable of healing large bone defects.

Key words: tooth germ, alveolar cleft, transplantation, bone formation

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Introduction

The jawbone plays important roles in various functions such as mastication, articulation and pronunciation. Jawbone loss can be caused by cleft lip and plate (CLP), peripheral resection of gingival cancers, alveolar bone loss caused by periodontitis, and so on. CLP is a congenital facial defect with an average prevalence of 7.94 per 10,000 live births internationally[1], and it causes dysfunctions such as deglutition and articulation disorders. Reconstruction of CLP is done via alveolar cleft bone grafting procedures using autologous bone, allogenic and xenogeneic bone grafting materials as well as various tissue-engineered bone replacement materials[2-6]. CLP is generally treated by promoting the growth and development of the maxillofacial region, and alveolar clefts are repaired with artificial bone or cancellous bone harvested from the anterior iliac crest[7]. However, those graft procedures have the risk of various complications, including infection, pain, defective bone healing of the local area and postoperative gait disorder from iliac grafting. Although CLP patients usually receive treatment at 7 to 12 years of age, they have normal impacted tooth germs of wisdom teeth in their jaws. In the dental field, stem cells derived from the dental pulp, periodontal ligament and dental follicles have been used for bone regeneration therapy, and third molar teeth or tooth germs are thought to be potential sources of cells and tissues.

There have been a few experimental reports about animal models of alveolar cleft grafting[8, 9]. Those reports evaluated tissue-engineered bone grafts in artificially created bone defects, and obtained predictable models for testing of bone grafting materials.

We considered that the tooth germs of impacted teeth in CLP patients may be useful to repair their bone defects. So, the purpose of this study was to investigate the potential application of tooth germs obtained from mice to repair alveolar cleft defects and their ability to form bone tissue.

Materials and Methods

Animals

C57BL/6 mice (n = 24) and embryonic day 16 mice (n = 6) were purchased from SLC Inc. (Shizuoka, Japan). All experimental protocols involving animals were approved by the Tokyo Dental College Animal Care and Use Committee (Approval #: 293204). All surgeries were performed under general anaesthesia using a combination of three types of anesthetics, which were mixtures of 0.75 mg/kg medetomidine hydrochloride (Nippon Zenyaku Kogyo, Fukushima, Japan), 4.0 mg/kg midazolam (Sandoz, Tokyo, Japan) and 5.0 mg/kg butorphanol tartrate (Meiji Seika Pharma, Tokyo, Japan).

Development of the alveolar cleft defect model in mice

The upper first molars of 3-week-old C57BL/6 mice were extracted under deep anesthesia, then left for 4 weeks to promote healing of the bone. Briefly, the oral mucosa at the extraction site was incised with a surgical knife approximately 2.0 mm in length. Alveolar cleft model mice were produced using a dental engine with a round tungsten carbide burr (1.0 mm in diameter) (MANI, Utsunomiya, Tochigi) to create bone defects reaching the maxillary sinus and the nasal cavity (Fig. 1a). The oral mucosa was then closed with a 8-0 nylon suture (Natsume Seisakusyo, Tokyo, Japan). C57BL/6 mice 7 weeks after this surgery were used for the alveolar cleft model in this study. We confirmed the prepared clefts using micro-CT (R_mCT; Rigaku, Tokyo, Japan) imaging with an exposure at 90 kV and 150 mA. A micro-CT image of the alveolar cleft model is shown in Fig. 1b.

Isolation of tooth germs

Mice were sacrificed by euthanasia to excise the tooth germs and paraffin sections were cut and stained with H-E (Fig. 2a). Tooth germs were isolated surgically from the mandible of each mouse at embryonic day 16. An example of an isolated tooth germ is shown in Fig. 2b. These procedures were conducted in Dulbecco's

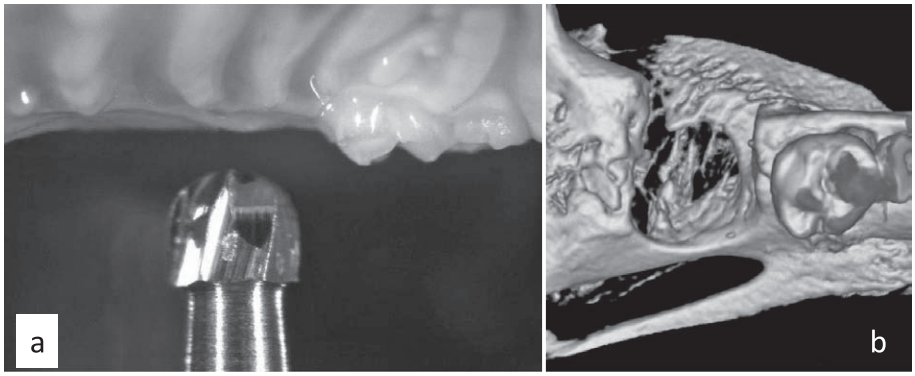

Fig. 1　Macroscopic and CT-images
Macroscopic image of the round burr and the oral mucosa (a) and a representative CT-image of a bone defect (3D image of an occlusal view).

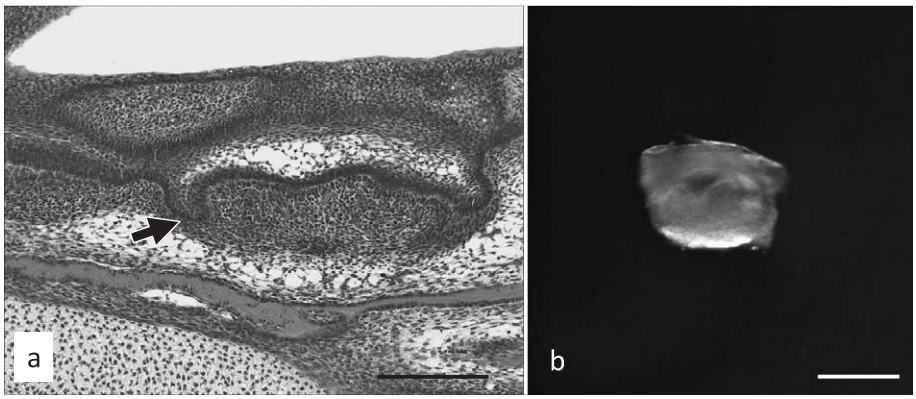

Fig. 2　HE staining and a macroscopic image of a tooth germ
HE staining of a tooth germ (arrow) at embryonic day 16 (a, scale bar: 200 μm) and a macroscopic image of a tooth germ (b, scale bar: 500 μm).

modified Eagle medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Biosera, Nuaille, France), 100 U/mL penicillin and 100 μg/mL streptomycin (Thermo Fisher Scientific).

Transplantation of tooth germs to the alveolar cleft mouse model

Tooth germs isolated from embryonic day 16 mice together with collagen sponges (Olympus Terumo Biomaterials, Tokyo, Japan) were transplanted into the alveolar cleft-like bone defects for the experimental group. Only collagen sponges were transplanted into the alveolar cleft-like bone defects for the control group. After transplantation, the oral mucosa of each mouse was closed with a 8-0 nylon suture (Natsume Seisakusyo).

Micro-computed tomography (micro-CT)

Radiological images were obtained using a micro-CT device (R_mCT) with exposure at 90 kV and 150 mA immediately after, and at 3, 7, 10 and 15 weeks after the transplantation. All mice were placed on the object stage and imaging was performed over a full 360-degree rotation with an exposure time of 2 minutes. CT-images were captured using i-view R software (Morita, Kyoto, Japan).

Hard tissue volume measurement

Hard tissue recovery was evaluated from the micro-CT images. The hard tissue volume was measured using TRI/3D-BON software (Ratoc, Osaka, Japan) immediately after and at 7 weeks after the transplantation. The hard tissue volume at 0 weeks was subtracted from the volume at 7 weeks, and was used to determine the volume

of hard tissue recovery.

Histochemical analysis and immunohistochemistry

Animals were sacrificed and their upper jaws were excised immediately after or at 7 weeks after producing the alveolar clefts, and at 3 and 7 weeks after the transplantation. These tissues were fixed in 20% neutral buffered formalin for 24 hours and then were decalcified in 10% EDTA for 3 weeks at 4℃. Decalcified tissues were paraffin-embedded, sectioned at approximately 3 to 5 micrometers thickness and stained with hematoxylin-eosin (H-E). For immunohistochemistry, sections were prepared and were incubated with anti-osteocalcin (OCN) antibody (5 μg/mL, rabbit; Abcam, Cambridge, MA) as the primary antibody. TRAP staining was performed using a TRAP/ALP stain kit (FUJIFILM Wako, Osaka, Japan).

Statistical analysis

Data are presented as means ± standard deviation (S.D.). We used the Mann-Whitney U

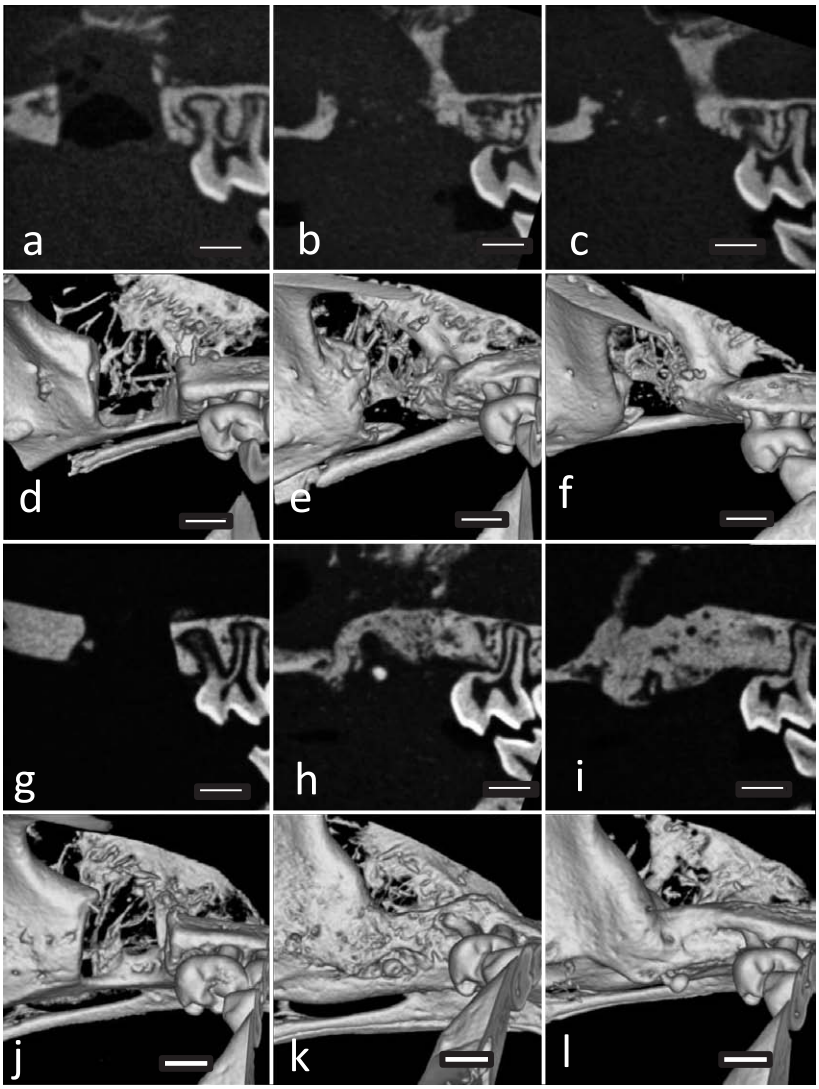

Fig. 3 CT-images of the control (a-f) and experimental (g-l) groups immediately after and at 3 and 7 weeks after the transplantation
a, g: 2D images immediately after the transplantation.
b, h: 2D images at 3 weeks after the transplantation.
c, i: 2D images at 7 weeks after the transplantation.
d, j: 3D images immediately after the transplantation.
e, k: 3D images at 3 weeks after the transplantation.
f, l: 3D images at 7 weeks after the transplantation.

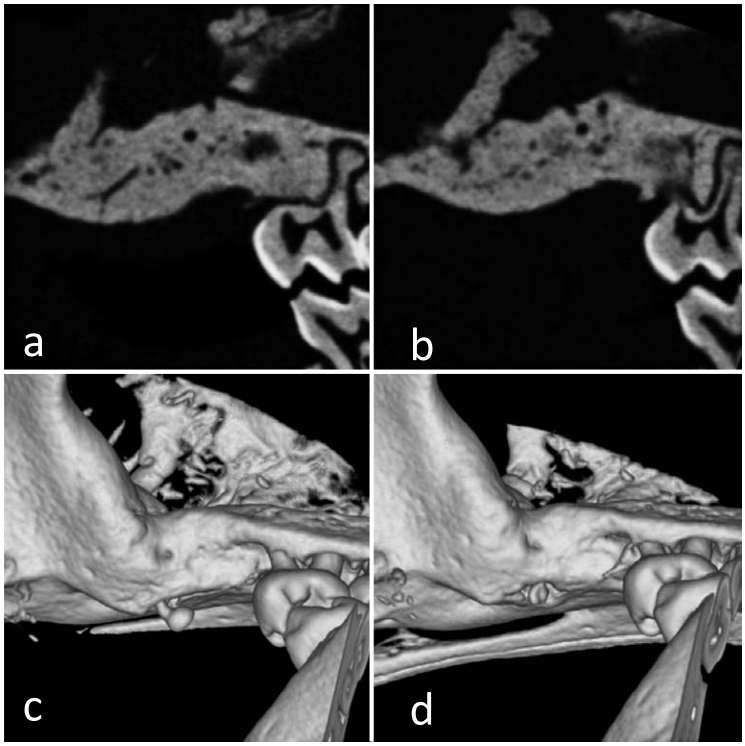

Fig. 4 CT-images of the experimental group at 10 and 15 weeks after the transplantation
a: 2D image at 10 weeks after the transplantation.
b: 2D image at 15 weeks after the transplantation.
c: 3D image at 10 weeks after the transplantation.
d: 3D image at 15 weeks after the transplantation.

test to determine p-values for statistical significance. Analyses were performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA).

Results

CT images of the control group showed no newly formed bone in the alveolar defects at any evaluation time point but newly formed bone was observed in the experimental group at 3 and 7 weeks after the transplant (Fig. 3). At 7 weeks in the experimental group, the bone defects were filled with high density hard tissue. At 10 and 15 weeks in the experimental group, the bone defects had been completely replaced with radio-opaque hard tissue similar to the surrounding bone (Fig. 4). The volume of newly formed bone in the experimental group at 7 weeks was significantly higher than in the control group ($P < 0.05$) (Fig. 5).

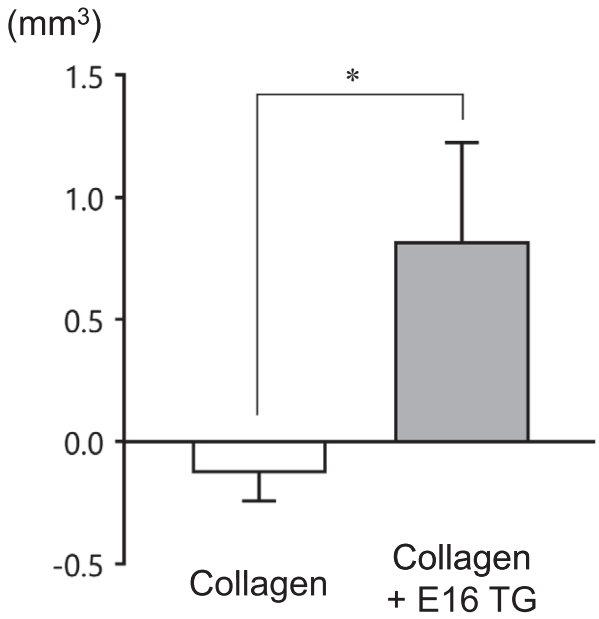

Fig. 5 Graph of bone volume
Newly formed bone volume in alveolar cleft defects at 7 weeks after the transplantation (*, $P < 0.05$)

Histological observations revealed that newly formed bone was not observed in the alveolar cleft defects in the control group at any experimental time point, but was observed in the defects in the experimental group at 3 and 7

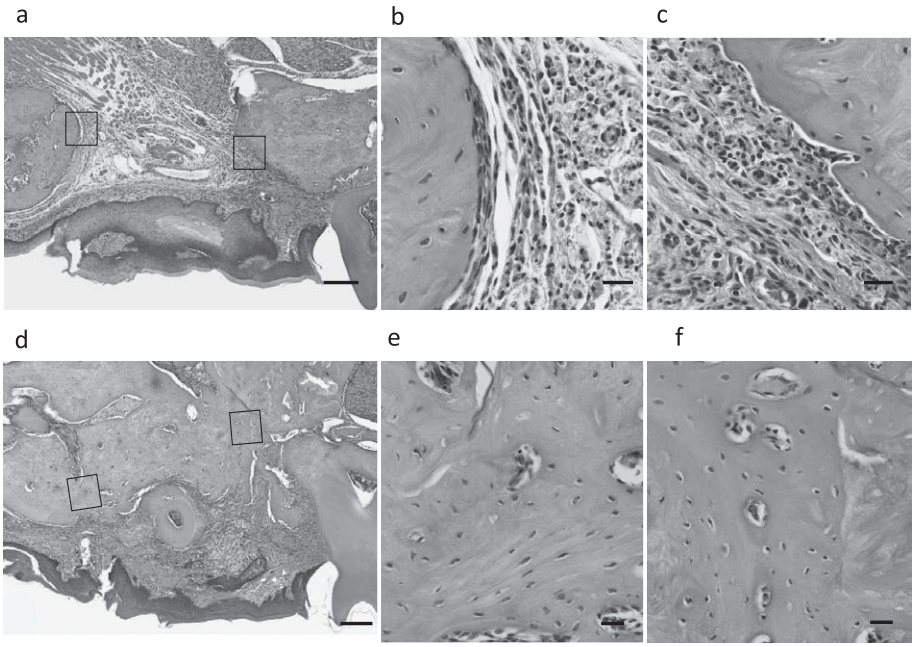

Fig. 6 HE staining
a-c: Control group at 7 weeks after the transplantation.
Fibrous connective tissue invaded the alveolar cleft defects.
d-f: Experimental group at 7 weeks after the transplantation.
Newly formed bone filled in the alveolar cleft defects.
Boxes in panels a and d indicate areas shown at higher magnification in panels b & c and d & f, respectively. Scale bars in panels a and d: 200 μm, in panels b, c, e and f: 20 μm.

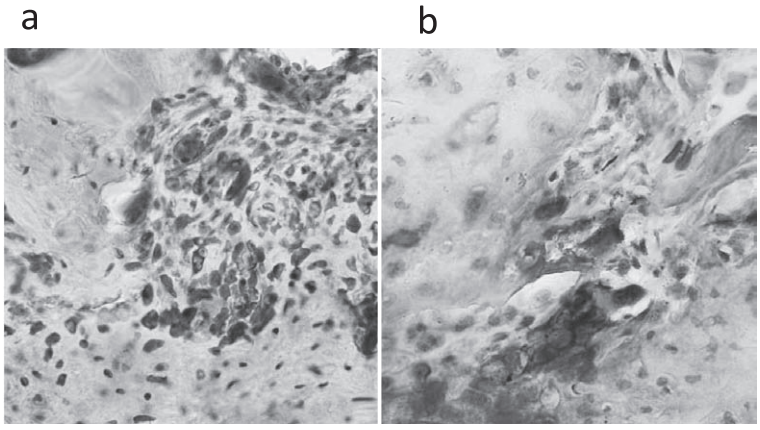

Fig. 7 Immunohistochemical staining of anti-osteocalcin (a) and TRAP staining (b)

weeks after the transplantation (Fig. 6). Immunohistochemically, many osteocalcin-positive cells were observed in osteoblasts located adjacent to the new bone at 7 weeks after the transplantation (Fig. 7a). Some TRAP-positive cells could be seen adjacent to the new bone at 7 weeks after the transplantation (Fig. 7b).

Discussion

Patients with CLP often undergo alveolar bone grafting to stabilize the dental arch and to enable the eruption of permanent teeth into the grafting area[10]. Our experimental design utilized the tooth germ and a collagen gel scaffold to encourage bone development in the CLP area.

Experimental animal models of CLP and cleft alveolar osteoplasty have been reported[8, 9]. Kamel et al. created alveolar clefts in rabbits following the existing anatomy, extracted a central incisor tooth, modified the extraction socket by extending it to the nasal mucosa, and applied a simple bone wax and oxidized cellulose material to help modulate the healing phase in the cleft area and avoid rapid bone generation and filling of the defect[8]. In our study, alveolar clefts between the maxillary sinus and nasal cavity were created after wound healing of an extraction socket without any materials to avoid bone regeneration. Therefore, our study model avoids the influence of such artificial materials. Korn et al. attempted to create a cleft model in rats and reported the usefulness of hydroxyapatite-tricalciumphosphate scaffolds[9]. The innovative idea of our study was derived from the use of extracted isolated tooth germs into artificially created alveolar clefts instead of bone grafting materials. In our study, we observed newly formed bone in alveolar bone defects in a tooth germ transplantation alveolar cleft model. Further, osteocalcin-positive cells and TRAP-positive cells were identified adjacent to the new bone, which revealed that the new bone remodels after producing new bone.

From this study, we conclude that our alveolar cleft mouse model is appropriate because the bone defects did not heal naturally. These findings indicate that the transplantation of tooth germs with a collagen sponge scaffold is capable of healing large bone defects. This study shows the potential of a novel approach for the treatment of alveolar clefts.

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