

Original Article

Reliability of acellular decalcified and decalcified teeth as bone graft material: an experimental and pathological study in rats

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Abstract: Objective: The present study aimed to investigate the reliability of acellular decalcified teeth in the development of bone scaffolds and in bone regeneration in rats. Methods: (1) Forty-eight human teeth were divided into two groups in vitro: twenty-four were decalcified, while the remaining twenty-four were decalcified and decellularized, following which a conventional scanning-electron microscope analysis was performed. (2) In another experiment, six male SD rats aged 10-12 weeks were selected, then decalcified and acellular decalcified teeth were embedded subcutaneously in the abdomen of the rats. After 4 weeks, the rats were sacrificed for H-E staining and immunohistochemical staining to observe the inflammatory reaction around the two materials. (3) In the ectopic osteogenesis experiment, bone defects were simulated in bilateral craniotectal areas of 12 male SD rats (age 10-12 weeks), following which acellular decalcified teeth were implanted in the right bone defect. The non-implanted left side was used as blank control. At week 4 and week 8, 6 rats were randomly selected for execution, complete specimens were obtained, and micro-CT scan was performed to compare the bone mass from gross morphology. H-E staining was performed at 4 and 8 weeks to observe the surrounding inflammatory response and immunohistochemistry was performed at 8 weeks to observe the degree of new bone formation. SPSS 23.0 software package was used for statistical processing. Results: (1) Under scanning electron microscope, cells in the teeth subjected to acellular decalcification completely disappeared, leaving only inorganic scaffolds. (2) After 4 weeks, the amount of inflammatory reaction in the tissues surrounding acellular decalcified teeth was significantly lower than that in the tissues surrounding decalcified teeth. (3) After four and eight weeks, the amount of bone formation in the bone defects was significantly higher in rats implanted with acellular decalcified teeth than in those in the blank control group ($P < 0.05$). After four and eight weeks, hematoxylin-eosin staining revealed that the degree of inflammatory response was similar around acellular decalcified teeth and blank controls. Immunohistochemistry indicated that the osteocalcin levels were significantly higher around acellular decalcified teeth than that around blank controls. Conclusion: Acellular decalcified teeth show significantly decreased inflammatory reaction, better biocompatibility, better osteogenic potential, and better plasticity than decalcified teeth alone.

Keywords: Cranial defects, decalcified bone matrix, acellular bone matrix, extracted human teeth, rat

Introduction

Bone defects destroy the structural integrity of the bone [1]. The common causes of bone defects include trauma, infection, tumors, osteomyelitis, and various congenital diseases. Compared with traditional autologous bone grafts, allogeneic and xenogeneic bone grafts are obtained from a wider range of sources, decreasing the risk of donor site injury and postoperative infection. Furthermore, allo-

genic and xenogeneic bone materials of a particular shape and size can be selected according to actual clinical needs and are not limited by the shape of donor site bone. Previous studies have reported that the failure rate of allografts due to immune rejection ranges from 10 to 50%, mainly manifesting as delayed healing, non-healing, and fatigue fracture of the bone graft. Notably, complete absorption of the bone graft is also sometimes observed [2].

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Since 90% of the immunogenicity of bone tissue can be attributed to the bone cells, elimination of bone antigenicity is the key to ensure successful integration of allografts [3]. Collagen and inorganic substances are the principal components of acellular bone structures [4]. Some scholars have utilized the acellular bone extracellular matrix (ABECM) method to develop graft materials with adequate biocompatibility that can act as scaffolds for bone deposition. The surface and shape of these materials are conducive to adhesion of cells and growth factors, and they help to avoid disease transmission and immune rejection [5-7]. Other studies have reported that decalcified bone matrix (DBM) is associated with good bone inductivity, indicating that this material is a promising vehicle for bone repair [8-11]. In the early 1960s, Urist et al. [12] reported that DBM is an ideal bone support material possessing a certain amount of plasticity.

Therefore, the ideal material for bone grafts should exhibit sufficient biocompatibility, osteogenic ability, bone inductivity, and plasticity. The principal component of human teeth is hydroxyapatite, and the composition of dentin is similar to that of bone, which is composed of 20% collagen, 70% hydroxyapatite, and 10% body fluids [13, 14]. Cells primarily exist in the pulp of teeth, and dentin is a natural mineralized tissue composed of hydroxyapatite. Some studies have reported that the dentin matrix exhibits adequate biocompatibility [15, 16]. However, demineralized dentin matrix (DDM) exhibits adequate biocompatibility as well as bone induction, a property similar to that reported for DBM [17-19]. Dentin has sufficient bone conduction ability [20, 21], and its physical and chemical properties meet the basic requirements for engineered bone scaffold materials. Based on previous research, the present study utilized a combination of methods to prepare a bone scaffold material with adequate biocompatibility, osteogenic ability, osteoinductivity, and plasticity.

Materials and methods

Materials and instruments

Forty-eight extracted teeth (clinical collection) were subjected to decalcification *in vitro* [22]. Teeth were decalcified using 0.5 mol/L hydrochloric acid solution according to a diluted

hydrochloric acid-to-bone weight ratio of 20:1. The container was placed in a thermostatic oscillator set to 20 rpm at 25°C to promote decalcification. The acid was changed after 1 hour. After an additional 1 hour of reaction, the acid was replaced with 20 times the volume of water, and the solution was centrifuged at 1,000 rev/min. The teeth were rinsed with water for 15 min, the supernatant was removed, and water was added five times over a period of 75 min. The decalcified teeth were placed in a sterilized bag immediately after cleaning.

Twenty-four teeth were randomly selected for the preparation of decalcified decellularized teeth [23]. Decalcified decellularized teeth were obtained by alternating immersion in high-hypotonic NaCl solution, digestion in trypsin + EDTA, and immersion in Triton X-100. The decalcified decellularized teeth were obtained by cross-linking with glutaraldehyde.

Eighteen male SD rats (age: 10-12 weeks; weight: approximately 300 g; Shanghai West Poole-Baykay Laboratory Animal Co. Ltd., Shanghai, China) were raised in an environment with a constant temperature (25~27°C), constant humidity (45%~50%), and specific pathogen-free conditions. Micro-CT was performed using a μ CT-100 system (Scanco Medical AG, Switzerland). Human/Rat Osteocalcin MAb (clone190125) and immunohistochemical reagents were purchased from Shanghai Youning Biotechnology Co., Ltd. This study was approved by the Ethics Committee of the Shanghai Jiao Tong University School of Medicine.

Characterization analysis

After decalcification or decalcification/decellularization *in vitro*, specimens from each group were sliced to a thickness of 4 μ m for routine scanning-electron microscope (SEM) analysis.

Subcutaneous embedding

One tooth from each group was implanted under the skin of the abdomen of each rat, following which we compared the degree of inflammatory response and the biocompatibility of the two materials [24, 25]. Six SD rats were anesthetized via an intraperitoneal injection of 3% pentobarbital sodium solution (0.15-0.2 ml/100 g). After successful anesthesia, the

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rats were placed in supine position and were fixed on a fixation plate. Teeth from each group were embedded subcutaneously after making a 1-cm incision in the abdomen of the rats. After wound closure, the incision was disinfected using iodov. Vital signs including respiration and heart rate were monitored during the procedure, following which all rats were labeled with ear tags and kept in cages. After the surgery, they were injected with penicillin 200,000 IU/day for three consecutive days and were sacrificed four weeks later. The embedded teeth were removed and embedded in paraffin. Each specimen was sectioned to a thickness of 4 μ m, and inflammation was analyzed by observing under a microscope by conventional hematoxylin and eosin (H-E) staining and immunohistochemistry.

Heterotopic ossification

Rats were prepared to simulate cranio-parietal bone defects to compare the ability of the two materials in bone repair after four and eight weeks. Twelve SD rats were subjected to intraperitoneal anesthesia with 3% pentobarbital sodium solution (0.15-0.2 ml/100 g). After successful anesthesia, they were placed in a prone position and were fixed on the fixation plate. A longitudinal incision of approximately 3 cm was made along the median line of the skull apex, and the skin and subcutaneous tissue were cut successively to carefully separate the periosteum. A planter trephine with a diameter of 5 mm was used to drill and grind the skull carefully on both sides of the middle of the parietal area, and a probe was used to explore the depth of the hole. When the skull was nearly drilled, small forceps were used to pry the area and the bone fragment was removed to avoid damage to the lower dura mater and its surface blood vessels. The right side was implanted with an acellular decalcified tooth, while the left side was used as a blank control (no materials implanted). After wound closure, the incision was disinfected using iodov. Vital signs, including respiration and heart rate were monitored during the procedure. After four weeks, six randomly selected rats were sacrificed, and the remaining rats were sacrificed after eight weeks.

For micro-CT scanning, we extracted the cranial bone and separated the surrounding soft tis-

sues. The scanning parameters were as follows: 70 kV, 114 mA, and slice thickness: 50 μ m. After the three-dimensional reconstruction of craniotectal bone after four and eight weeks, we analyzed the bone mass to quantitatively evaluate the formation of new bone. The craniotectal bone was removed and was embedded in paraffin after four and eight weeks. Each specimen was sectioned to a thickness of 4 μ m for the evaluation of inflammation under a microscope based on conventional H-E staining. Conventional dewaxing treatment for obtaining paraffin section was performed for immunohistochemistry [26]. The presence of osteocalcin was determined under a microscope after eight weeks.

Statistical analysis

All data were analyzed using SPSS 23.0 software (IBM, Armonk, NY, USA). The data were expressed as ($\bar{x} \pm s$). Data were compared using analyses of variance (ANOVA) and t-tests, as appropriate. The level of statistical significance was set at $P < 0.05$.

Results

Characterization analysis

SEM analysis revealed that the average pore size and porosity were similar for decalcified and acellular decalcified teeth. However, dentinal tubules and desiccated dentinal cell protuberances were clearly visible in decalcified teeth, while only dentinal tubules were visible in acellular decalcified teeth (**Figure 1**). Our results indicated that cells in decalcified teeth had been isolated after decellularization.

Subcutaneous embedding

Gross morphology: Four weeks after subcutaneous embedding, all animals exhibited satisfactory wound healing, and no inflammatory manifestations or material exposure were observed. All the embedded teeth were conglutinated with abdominal subcutaneous fat.

H-E staining and immunohistochemical staining after subcutaneous embedding: Obvious differences between acellular decalcified teeth and decalcified teeth were observed under the light microscope. Acellular decalcified teeth were porous, and a significantly less surround-

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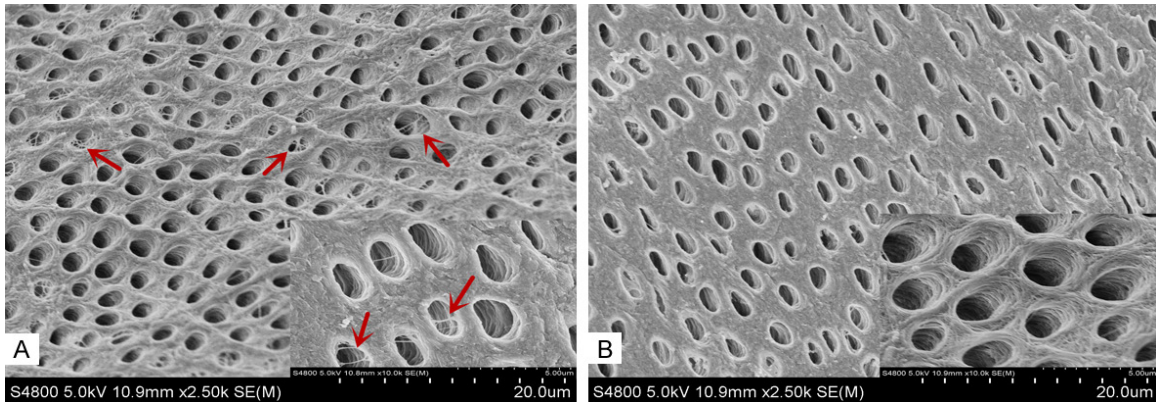


Figure 1. Scanning electron microscopy (SEM) analysis. Arrows represent desiccated dentinal cell processes. The average pore size and porosity of samples (A) and (B) are essentially the same. The dentinal tubules and internal dentinal cell processes of the decalcified tooth samples are clearly visible, while only dentinal tubules are visible in the acellular decalcified tooth samples.

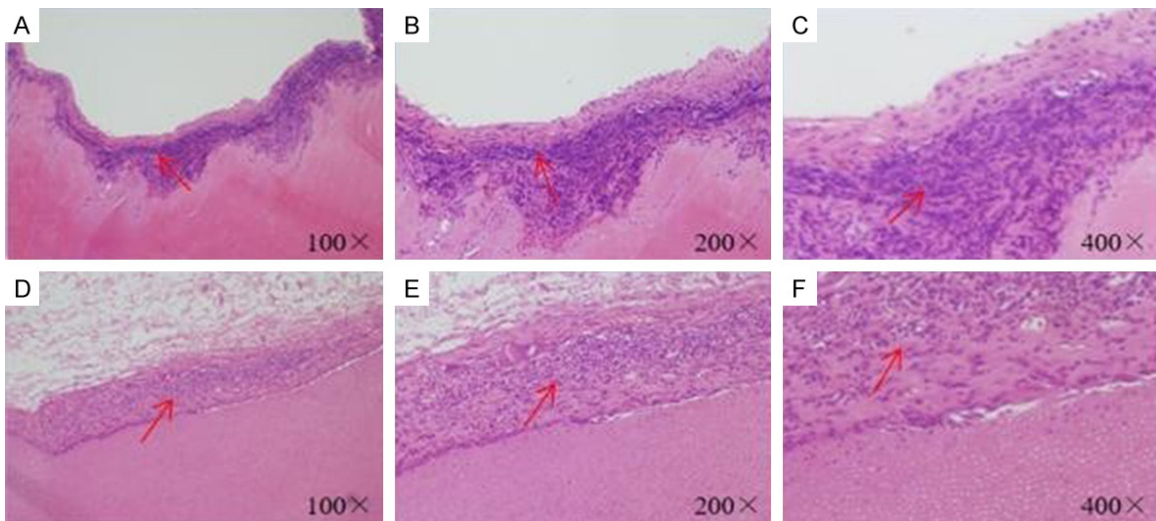


Figure 2. Hematoxylin and eosin (H-E) staining was performed 4 weeks after subcutaneous implantation to examine inflammatory responses. Arrows represent cells associated with the inflammatory response. Optical microscopy analysis revealed that inflammatory responses were less prominent in acellular decalcified teeth (D-F) than in decalcified teeth (A-C).

ing inflammatory response was evident than that for decalcified teeth (**Figures 2** and **3**). It was assumed that after decalcification and decellularization, the antigenicity of xenografts is reduced or eliminated because of the isolation of cells in the teeth, thus, reducing the possibility of allograft failure significantly [1, 20, 21].

Heterotopic ossification

Micro-CT results: All rats exhibited satisfactory wound healing, and all teeth in the experimen-

tal group retained their position. Micro-CT performed after four weeks revealed that the amount of new bone was significantly greater in bone defects implanted with acellular decalcified teeth than that in defects on the blank control side (**Figure 4**). Significant differences in the percentage of new bone volume were also observed between the experimental and blank control groups. After eight weeks, the amount of new bone was significantly more in bone defects implanted with acellular decalcified teeth than in those on the blank control side (**Figure 5**).

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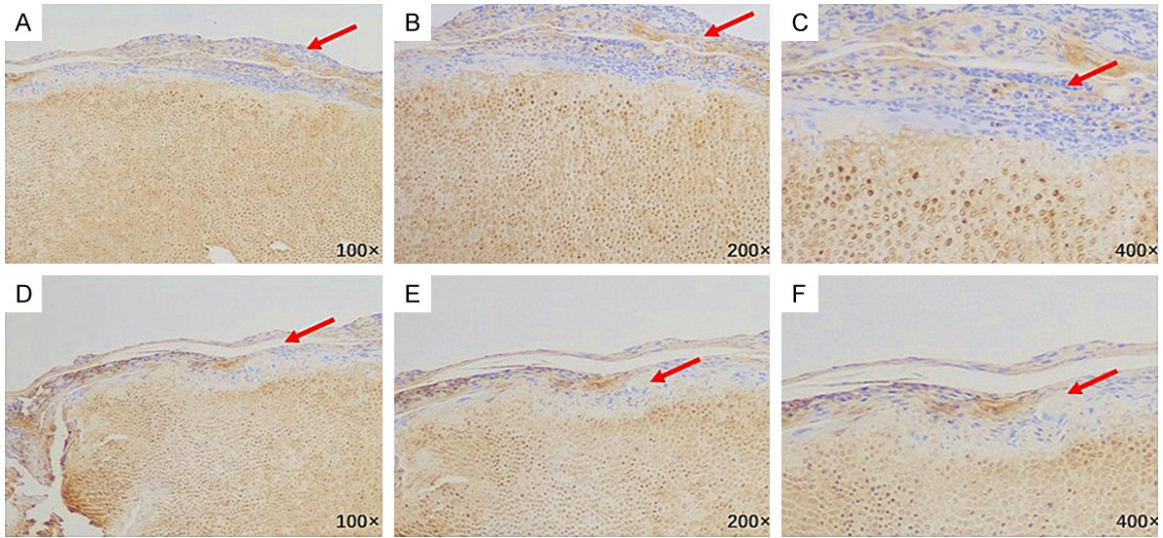


Figure 3. Immunohistochemical staining was performed 4 weeks after subcutaneous implantation to examine inflammatory responses. Arrows represent cells associated with the inflammatory response. Optical microscopy analysis revealed that inflammatory responses were less prominent in acellular decalcified teeth (D-F) than in decalcified teeth (A-C).

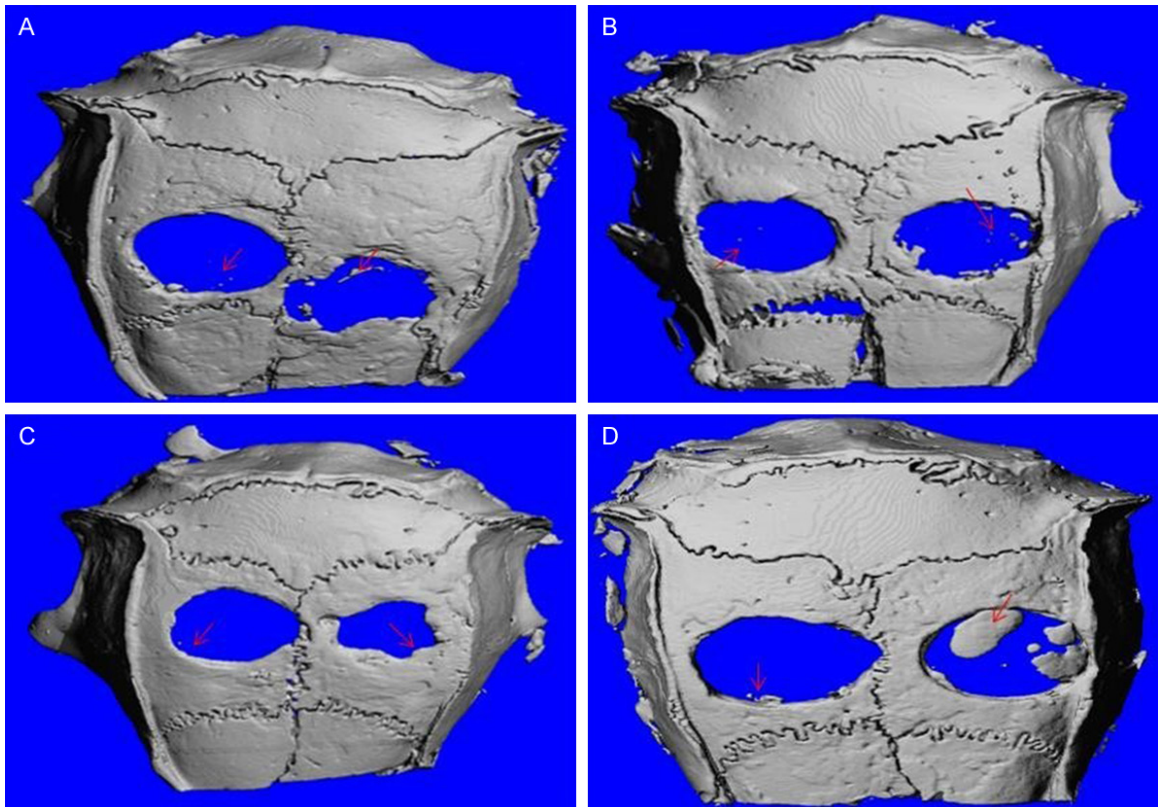


Figure 4. Micro-computed tomography reconstruction of the craniotectic bone after four weeks (A and B) and eight weeks (C and D). Panels represent control studies between the experimental and blank control groups. The arrows represent new bone. The repair of bone defects was significantly better in the experimental group (right side) than in the blank control group (left side) after four and eight weeks.

H-E staining of the calvarial bone: The results of H-E staining after four weeks are shown in

Figure 6 and the results of H-E staining after eight weeks are shown in **Figure 7**. Four weeks

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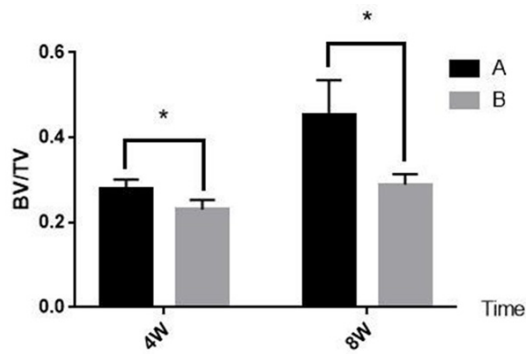


Figure 5. Quantitative analysis of the percentage of new bone volume by micro-computed tomography. A. experimental group (acellular decalcified teeth). B. blank control group (* $P < 0.05$).

in the rat calvaria bone defects of bone experimental inflammatory cells compared with control group, the number of inflammatory cells had no significant difference. The same was true at eight weeks.

Immunohistochemical staining of the cranial bone: After eight weeks, immunohistochemical staining of the cranial bone revealed that osteocalcin levels were significantly higher in the side implanted with acellular/decalcified teeth than that in the blank control side. These findings indicate that more osteoblasts were present on the experimental side than that on the control side [20] (**Figure 8**).

Discussion

In planning the repair of bone defects, autologous bone transplantation is often limited by donor source morbidity and lengthy operation times, which can result in additional trauma and complications. Allogeneic bone grafts often exhibit allogeneic bone resorption and are prone to infection and rejection, while artificial grafts exhibit relatively poor osteogenic capabilities [27].

Alveolar bone defects are the most common type of bone defects encountered in oral and maxillofacial surgery. Although artificial bone powder is commonly used for the repair of such defects, this material is associated with poor plasticity. Therefore, guided bone regeneration (GBR) techniques are often used to prevent the displacement of bone powder and tissue infection. When restoring the vertical bone height, these methods are utilized in conjunction with titanium mesh.

In the present study, we applied conventional methods to isolate teeth for the preparation of DBM and decellularized bone matrix. The method of preparation was consistent with the principles outlined for the preparation of simple DBM and ABECM. Acellular decalcified teeth are primarily composed of collagen, non-collagen, and a low concentration of growth factors. In the subcutaneous-embedding experiment, we observed that acellular decalcified teeth were more porous relative to decalcified teeth. Our results indicated that inflammatory reactions were significantly less prominent for acellular decalcified teeth than those for decalcified teeth. These results demonstrate that decellularization can significantly reduce or eliminate the antigenicity of the teeth and is associated with good biocompatibility.

Four and eight weeks after the heterotopic osteogenesis experiment, gross morphologic observation revealed that the implanted teeth retained their position, and that wound healing was adequate, highlighting the plasticity of acellular decalcified teeth. Micro-CT revealed that repair of bone defect was significantly better in the side implanted with acellular decalcified tooth than that in the blank control side, indicative of obvious osteogenic effects. After four and eight weeks, H-E staining revealed similar inflammatory responses for acellular decalcified teeth and conventional tissues. These findings support the notion that acellular decalcified teeth can significantly reduce or eliminate tooth antigenicity, improving biocompatibility and reducing the probability of complete bone graft absorption. After eight weeks, we also observed that osteocalcin content was significantly higher in bone defects implanted with acellular decalcified teeth than in those on the blank control side. Higher levels of osteocalcin are associated with increased production of osteoblasts [28], suggesting that acellular decalcified teeth can induce the production of osteoblasts.

This experiment was intended to explore whether acellular decalcified teeth have the features of a bone-scaffold material. As this was an initial attempt, experiments in the future will be detailed to explore decalcified teeth, acellular teeth, and acellular decalcified teeth, and the advantages and disadvantages of the three groups of materials. Compared

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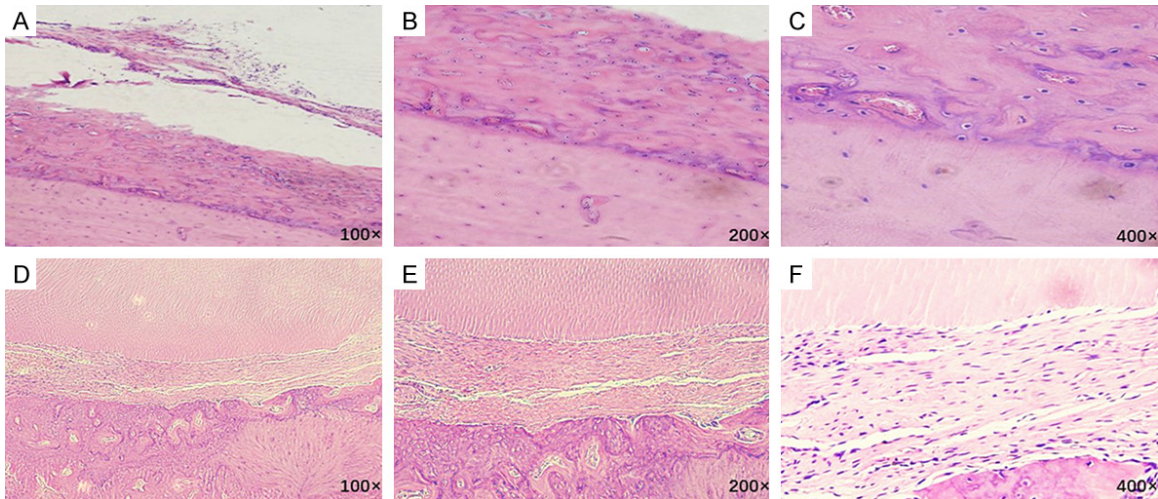


Figure 6. Hematoxylin and eosin (H-E) staining of the rat craniotectal bone after four weeks in the blank control group (A-C) and in the experimental group (D-F). No significant differences in the number of inflammatory cells were observed between the groups.

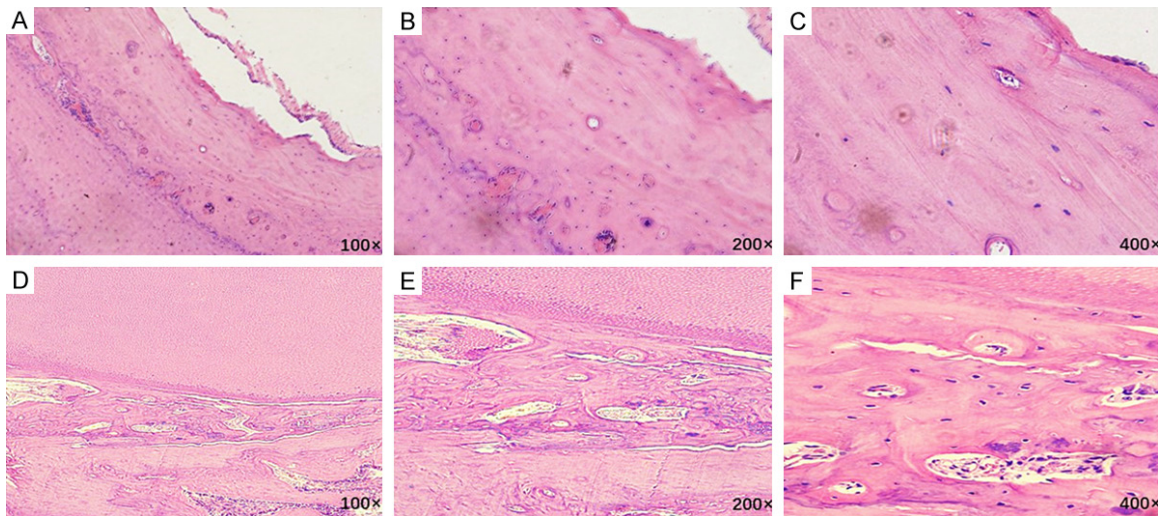


Figure 7. Hematoxylin and eosin (H-E) staining of the rat craniotectal bone after 8 weeks in the blank control group (A-C) and in the experimental group (D-F). No significant differences in the number of inflammatory cells were observed between the groups.

with the bone powder materials commonly used in the market at present, the advantages and disadvantages of teeth as bone-scaffold materials were further explored. In addition, the materials used in this study are the parts of dentin as seen on the longitudinal section of teeth, and the components of teeth are mainly dentin. No meticulous study on the osteogenic ability of enamel, dentin, and cementum has been conducted. These deficiencies should be addressed further by follow-up experiments.

To summarize, acellular decalcified teeth elucidate a lower inflammatory response, better plasticity, and better osteogenic potential than decalcified teeth alone and show potential of being a more reliable bone graft material than decalcified teeth.

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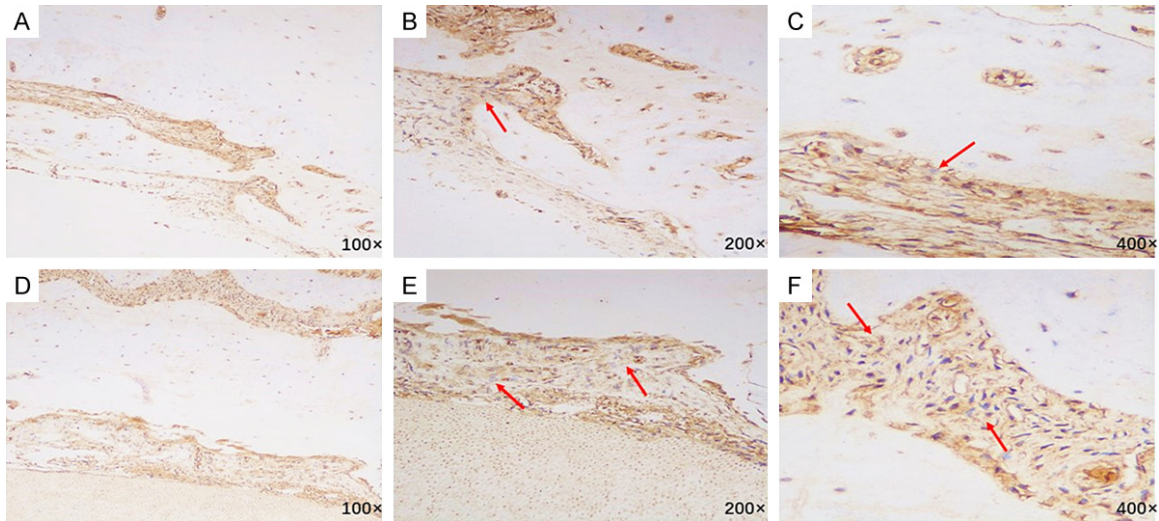


Figure 8. Immunohistochemical staining for osteocalcin in the rat craniotectal bone after 8 weeks. A-C. Blank control. D-F. Acellular/decalcified teeth. Arrows represent osteocalcin. Optical microscopy analysis revealed that levels of osteocalcin were significantly lower on the blank control side than on the side implanted with acellular decalcified teeth.

analysis, and interpretation of data and writing the manuscript.

Disclosure of conflict of interest

None.

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