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RESEARCH ARTICLE

Antler Collagen/Chitosan Scaffolds Improve Critical Calvarial Defect Healing in Rats

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This study was to develop chitosan scaffolds combined with collagen derived from deer antler and test its *in vivo* bone regeneration capacity in the calvarial defect model. Chitosan was crosslinked with deer antler-derived collagen by chemical reaction and lyophilized to attain porous scaffolds. Untreated chitosan was taken as control. Proliferation rate of rat bone marrow-derived mesenchymal stem cells was measured at 3 or 5 day after seeded on the porous scaffold *in vitro*. Sprague-Dawley rats were subjected to operation to make calvarial bone critical defects. Then the defects were left empty or implanted with chitosan scaffolds, or antler collagen/chitosan (A-collagen/chitosan) scaffolds. Samples were collected for micro-CT then decalcified for histology analysis 8 weeks post-surgery. Cell proliferation was significantly enhanced when they seeded onto the A-collagen/chitosan scaffolds compared with chitosan only scaffolds. The volume of mineralized tissue was also markedly increased in the calvarial defect region when implanted with A-collagen/chitosan compared with chitosan only scaffolds. Histological results also showed more new bone forming beneath the A-collagen/chitosan scaffolds, which was consistent with that of micro-CT analysis. In conclusion, A-collagen/chitosan scaffolds showed promising reparative effect in rat critical-sized calvarial bone defect models.

Keywords: Tissue Engineering, Tissue Scaffolds, Chitosan, Antlers, Skull Injuries.

1. INTRODUCTION

Large segmental bony defects and non-unions of fractures remain tough problems in clinical setting, which also considerably impairs the quality of life of patients.[1] Bone tissue engineering approaches are becoming more effective alternatives to autologous or allogenic bone grafting in the orthopedic, plastic, and reconstructive surgery.[2] One particularly promising strategy involves a biodegradable material and certain bioactive factor (s) in order to heighten tissue responses and accelerate healing.[2]

For the bone tissue engineering, a scaffold should be osteoconductive and highly porous to facilitate bone formation.[3] Different classes of materials have been utilized for scaffold fabrication including a variety of ceramics and polymers. Calcium phosphate-based ceramics are popular scaffolding materials because their chemical structures are similar to the mineral phase of bone.[3] A number of natural and synthetic polymers are also currently being employed in bone tissue engineering,[4–6] especially in the repair of non-weight bearing site, such as cranium.

Chitosan is a naturally occurring polymer, which derived from the shells of crustaceans as a biodegradable and bioactive biomaterial.[7] Contrary to many synthetic polymers, chitosan is characterized by a hydrophilic surface that promotes proliferation as well as mineralization of bone-forming cells.[3] More and more cellular and animal studies reported that chitosan-based scaffolds exhibited

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osteoconductive effect, which make it a promising biomaterial for bone tissue engineering.[8–10] Furthermore, chitosan with a higher degree of deacetylation corresponds to a higher percentage of positively charged primary amines, which can form polyelectrolyte complexes with anionic biological macromolecules. Deer velvet have been used for thousands of years in Traditional Chinese Medicine as an efficient tonic by strengthening musculoskeletal system.[11] A variety of chemical components have been identify, among of which, collagen and growth factors are recognized to be effective in promoting bone health.[12, 13] Collagen is one of the most substantial amount of organic components of deer antler.[11] Scaffolds made from collagen play an important role in tissue engineering, which lead to accelerated bone healing.[14, 15] It is valuable to make full use of this natural collagen for regenerative medicine. In this study, we design a novelty porous scaffold by using chitosan crosslinking with collagen derived from deer antler for the repair of calvarial bone defect. The primary objective of this proof of concept study is to develop chitosan scaffolds combined with collagen derived from antler and test its *in vivo* bone regeneration capacity in the calvarial bone defect model.

2. MATERIALS AND METHODS

2.1. Preparation of Antler Collagen

The extraction of antler collagen is modified from the methods as previously described.[12] Briefly, after removing the skin and flesh, the antlers of fresh Sika deer velvet (100 g) bought from local pharmacy (Jilin Dongfeng Pharmaceutical, Jilin, China) were cut into 1–3 mm^3 cubes. These cubes were then homogenized in distilled water by handheld grinder (Benchmark Scientific, Edison, NJ), and then transferred to Mikro-dismembrator for further homogenizing (B. Braun Biotech International, Melsungen, Germany). Subsequently, the homogenate was incubated in distilled water at 60 °C for 48 h and then centrifuged. The supernatant was dialyzed against distilled water for 24 h (molecular weight cut-offs that range from 8∼15 kDa). The dialysate was lyophilized and stored at −80 °C until further use.

2.2. Preparation of Antler Collagen/Chitosan Scaffolds

Antler collagen or chitosan (75–85% deacetylated, Sigma-Adrich, St. Louis, MA) was dissolved in 0.5% acetic acid (Sigma-Adrich, St. Louis, MA) solution to prepare a 2.0% (w/v) solution, respectively. The collagen suspension was slowly dropped into chitosan solution in the ratio of 1:9 (collagen:chitosan) and homogenized to obtain collagen/chitosan mixture. The chitosan solution without addition was taken as control. After deaerated under vacuum to remove entrapped air bubbles, the solution was transferred into the 24-well culture plates and frozen in 70% ethanol bath at −20 °C for 1 h and then lyophilized for 24 h to obtain a porous chitosan or collagen/chitosan scaffold. The chitosan or collagen/chitosan scaffolds were then trimmed into cylinders with diameter of 5 mm, and thickness of 1 mm.

To improve the biostability, the scaffolds were treated with glutaraldehyde (Sigma-Adrich, St. Louis, MA).[16] All scaffolds were rehydrated in 0.05 M acetic acid (Sigma-Adrich, St. Louis, MA) solutions for 15 min firstly, and then were cross-linked in the 0.25% glutaraldehyde solutions at 4 °C for 24 h. After washed with sterilized Milli-Q water for 5 times, the scaffolds were freeze-dried again to obtain the cross-linking collagen/chitosan scaffolds.

2.3. Cell Culture and Cell Proliferation

Mesenchymal stem cells (MSCs) were isolated from femoral bone marrow of a Green Fluorescence Protein (GFP) positive Sprague-Dawley (SD) rat (male, 4 weeks old), and expanded as previously described.[18] MSCs were cultured in culture medium, α-Minimal Essential Medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1% antibiotics (penicillin/streptomycin/neomycin, Invitrogen Corporation, Carlsbad, CA) at 37 °C with 5% CO_2. The surface phenotypes and multipotent differentiation were characterized as previously described.[17, 18] The cell proliferation was determined using Alamar Blue cell viability reagent (Invitrogen, Carlsbad, CA). The rat MSCs were seeded onto each scaffolds at 20,000 cells/well in a 96-well plate, respectively. At day 3 and day 5, the cells were incubated with Alamar Blue. The metabolic rate of the cells was determined at 570 nm, with reference wavelength at 600 nm by an ELX800 Microplate reader (Bio-Tek Instruments, Winooski, VT).

2.4. Animal and Surgery

All animal experiments were approved by the Academic Committee on the Ethics of Animal Experiments of the Guangdong Medical University, Zhanjiang, China. Animal surgeries were performed according to previous publications.[11, 18] 15 male Sprague-Dawley (SD) rats weighing (412 ± 18) g were anesthetized with katamine and xylazine, then mounted onto a stereotaxic frame for stereotactic control. An approximately 15 mm midline incision extending the length of the skull was made in the sagittal plane across the cranium. Two bilateral symmetric Φ 5 mm full thickness circular bones were removed in each parietal region of the cranium using a hollow trephine bur with a 5 mm outer diameter (Fig. 3). Then the defects were left untreated ($n = 10$, 5 rats) as negative controls or implanted with antler collagen/chitosan ($n = 10$) or chitosan ($n = 10$) scaffolds (Φ = 5 mm, thickness = 1 mm). The periosteum and scalp were closed by suture. Animals were allowed to move following recovery from the anesthesia and were sacrificed by overdose of pentobarbital at 8 weeks post-surgery.

2.5. Micro-Computed Tomography (Micro-CT)

Micro-CT was used for quantitative evaluation of the bone formation.[11] The samples were imaged using a high-resolution 70 kVp scan by microCT machine (μCT40, Scanco Medical, Bassersdorf, Switzerland). The 3D reconstruction was performed using standardized segmentation parameters (sigma: 0.8, threshold: 220–1000), which were kept constant through the scan. Circular contour lines were drawn around the defect area (diameter = 5 mm) excluding the neighboring native bone. The 3D reconstructive images of samples were generated from 2D slices by machine built-in software. The bone volume within the selected circular defect was calculated using the quantitative 3D evaluation program included in the micro-CT software package (μCT40, Scanco Medical, Bassersdorf, Switzerland).

2.6. Histology

The bone samples (two defects remained in one piece of cranial bone, 15 mm × 10 mm approximately) were fixed in 10% formalin solution at room temperature, then decalcified in 9% formic acid (Sigma-Adrich, St. Louis, MA) for 3 weeks at room temperature on a rotating rocker. After gradient dehydration in ethanol, the samples were embedded in paraffin and sectioned (thickness = 5 μm) in the transverse plane. Sections were subjected to hematoxylin and eosin (H&E, both from Sigma-Adrich, St. Louis, MA) staining.

2.7. Statistical Analysis

Data were presented as mean ± SD, and analyzed using SPSS 16.0 software for Windows (SPSS, Chicago, IL, US). The statistical differences among groups were analysed by ANOVA with post hoc Turkey's HSD. Probabilities (P) less than 0.05 were considered significant.

3. RESULTS AND DISCUSSION

Collagen could be successfully extracted from the antler by the methods modified from a previous publication.[12] Glutaraldehyde was used in this study to increase the efficiency of cross-linking between the antler collagen and chitosan after frozen dry (Fig. 1(a)). Previous data also demonstrated that collagen isolated from fresh bovine tendon could be cross-linked to chitosan by glutaraldehyde treatment.[16] Since both collagen and chitosan are positively charged in acidic solution, their mixture in solution is stable and does not precipitate. Therefore, sufficient mixing of these two hydrophilic biomacromolecules in sub-molecular level can be achieved. Figure 1(b) showed the gross view of the chitosan or A-collagen/chitosan scaffolds after cross-linking.

As illustrated in Figure 2, GFP labeled MSCs grows vigorously after seeded onto the scaffolds for 7 days and 14 days, especially on the A-collagen/chitosan scaffolds. Data showed that after 14 days of culture, proliferation rate

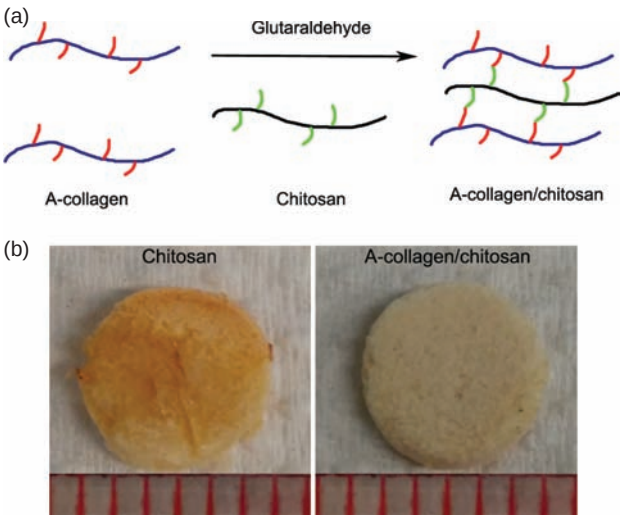

Fig. 1. Schematic diagram of the preparation of A-collagen/chitosan and gross view of chitosan or A-collagen/chitosan scaffold. (a) Schematic diagram of A-collagen cross-linked with chitosan in the presence of glutaraldehyde; (b) gross view of chitosan or A-collagen/chitosan scaffold.

of MSCs was significantly increased by 67.5% ($P < 0.01$) when they seeded on the A-collagen/chitosan scaffolds compared with chitosan scaffolds, indicating that collagen extracted from deer antler may benefit proliferation of MSCs (Fig. 2).

The chitosan and A-collagen/chitosan scaffolds were evaluated in rat critical-sized calvarial bone defect models for their efficacy in new bone generation. The amount of bone tissue regenerated in defects was quantified by analyzing bone volume/total volume (BV/TV). The representative images of micro-CT analysis showed the regenerated bone tissue in the defect area as a result of various treatments. The empty, untreated defect (Blank) displayed minimal irregular, patchy bone formation, whereas the chitosan scaffold exhibited much more organized mineralized regions toward the defect edge (Fig. 4). Interestingly, a remarkable mineralized tissue formation within the defect area was found when implanted with the antler collagen/chitosan scaffold, when compared with the other groups (Fig. 4(a)). Quantitative data showed that the BV/TV was 8.5-fold ($P < 0.01$) and 3-fold ($P < 0.01$) higher in the defects treated with A-collagen/chitosan scaffolds, when compared with empty defect controls and the chitosan scaffold groups, respectively (Fig. 4(b)). The pattern of distribution and the amount of new bony tissue across the defect regions as a result of histology is consistent with that of micro-CT analysis. Histological data showed that unfilled gap remained in the untreated defect regions, whereas more new forming bone was found in the defect area when implanted with chitosan scaffolds than empty controls (Fig. 5). In the defects implanted with A-collagen/chitosan scaffolds, we found new bone formation was significantly enhanced in the defect than the other two groups. And the new forming bony tissue grew from

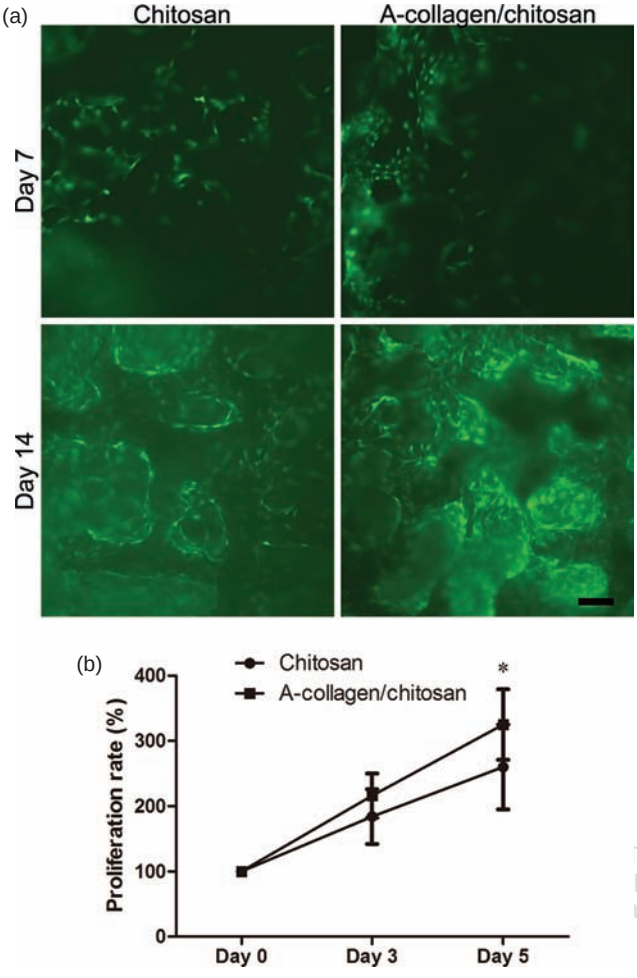

Fig. 2. Proliferation of rat GPF-MSCs seeded on the scaffolds. (a) Fluorescent images taken after 7 days and 14 days from the cell seeding on the chitosan or A-collagen/chitosan scaffolds. (b) Proliferation rate of rat GPF-MSCs seeding on the chitosan or A-collagen/chitosan scaffolds measured by Alarma Blue assay. Scale bar = 50 μm. *, $P < 0.05$ versus Chitosan.

the endocranial to ectocranial and integrated well with the native bone in the calvarial defects when implanted with A-collagen/chitosan scaffolds.

This study provides a comprehensively integrated approach to evaluating skull regeneration in a rat calvarial defect stimulated by chitosan-based scaffolds. It also demonstrated the feasibility and capability of antler-derived collagen/chitosan scaffolds in promoting bone healing. Regarding the biocompatibility and the efficiency in the bone regeneration, the A-collagen/chitosan scaffold may represent a promising advance for clinical application in craniofacial surgery for critical size defects.

Previous data demonstrated that extraction of antler accelerate the proliferation of osteoblast and neurons,[17,18] and promote rat wound healing and hair growth.[13,21] Chemical components of deer antler have been reported previously.[22] Many components, such as collagen, chondroitin sulfate, estrone, estradiol, prostaglandins,

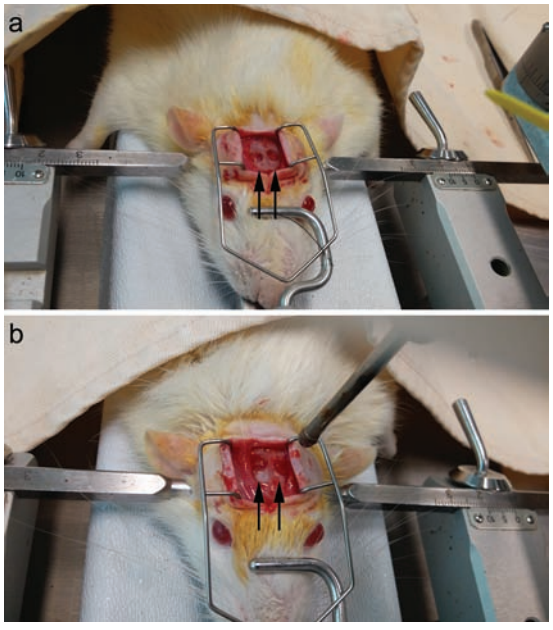

Fig. 3. Surgical approach of implantation in the cranium of rats mounted onto the stereotaxic frame. (a and b) Before and after implantation of scaffold in the defect area. Black arrows point to the defect area.

and growth factors including insulin-like growth factor-1 (IGF-1),[23] and epidermal growth factor (EGF) are believed to be bioactive.[24] Actually, the collagen extracted from antler in this study was a compound rather than specific pure collagen, which may contain other

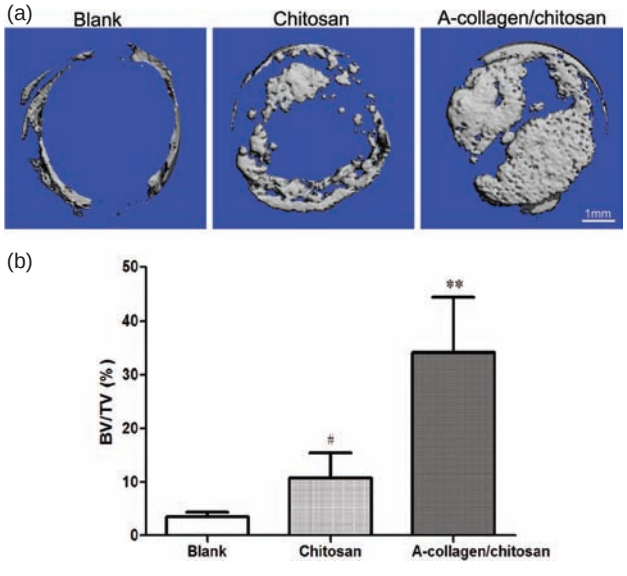

Fig. 4. New bone formation in the calvarial defect area left untreated (blank) or implanted with chitosan scaffolds or A-collagen/chitosan scaffolds after 8 weeks of surgery. (a) Representative micro-CT images showing the regenerated bone tissue; (b) assessment of regenerated bone volume/tissue volume fraction (BV/TV) in defects treated with different groups. Scale bar = 1 mm. #, $P < 0.05$ versus blank; **, $P < 0.01$ versus Chitosan.

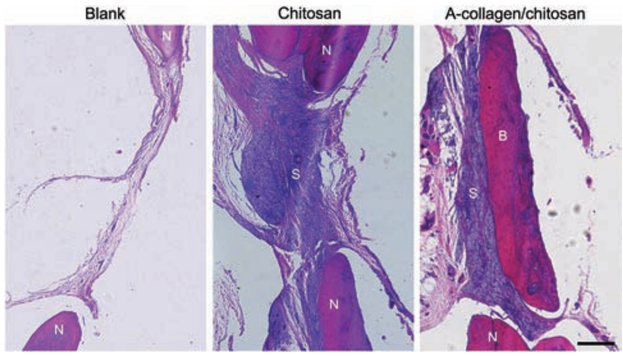

Fig. 5. Representative histological images of the extent of new forming bone in the defects left untreated at blank controls, or implanted with chitosan or A-collagen/chitosan. N, native bone; S, scaffold; B, new forming bone. Scale bar = 500 μm.

components, such as growth factors. These growth factors may partially contribute to the increasing proliferation rate and osteogenic differentiation of MSCs, indicating a possible role in cell regeneration and healing processes in humans.

In the present study, we demonstrated that collagen extracted from deer antler combined with chitosan may benefit the healing of calvarial defect in the rat model, indicating a clinical implication in the treatment of trauma-related large calvarial defects. Previous reports, as well as our study observed the new bony tissue ingrowth from the edges of the defect within the implant region, when implanted with scaffold without stem cells.[11,25] In this case, bone regeneration in cranium is depends on the periosteum, in which the periosteal progenitor cells may migrate to the defect regions and develop into osteoblasts that are essential to the healing of bone.[26] Results from several studies using scaffold combined stem cells demonstrated that, bone formation progressed in both the endocranial to ectocranial and ectocranial to endocranial direction into the scaffold, eventually formed a thickness comparable to the uninjured left parietal bone with time.[27,28] It is believed that a variety of important growth factors that drive rapid regeneration of velvet antler may be associated with its growth-promoting activity.[13] In Traditional Chinese Medicine, velvet antler can be regarded as an effective promoter of health, which promotes rapid growth and regeneration by tonic action.[22] However, the detailed underlying mechanism remained less understood. Further studies are still needed to justify the substance base which may be account for the clinical and medicinal efficiency.

Conflict of Interest

All the authors declare that there is no conflict of interests.

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