Research Article

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In Vivo Therapeutic Effect of a Novel Thiolated Chitosan/Modified Calcium Carbonate Composite Microspheres Scaffold for Bone Repair

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Abstract

In this study, thiolated chitosan (CS-TBA) and modified calcium carbonate microspheres (CCM), were used to fabricate composite scaffolds, and their physical and performances were compared and evaluated in vitro and in vivo. Specimen of the following was prepared as 5 mm diameter, 1 mm thick discs; CS-TBA/CCM group and a control group (blank group). A scanning electron microscope study was conducted. Graft materials were implanted in a 5 mm diameter in the calvarial bone. Rats were sacrificed after four and eight weeks for micro-CT and histological staining, and the findings obtained were used to calculate the bone mineral density (BMD), bone volume/total bone volume (BV/TV), and trabecular number (TB.N). It was found that these three values were significantly higher in the CS-TBA/CCM group than in the control group ($p<0.05$). This study demonstrated an excellent potential of CS-TBA/CCM scaffold as a bone graft substitute.

Keywords: Biomaterial; Thiolated chitosan; Modified calcium carbonate; Scaffold; Bone defect; Bone graft.

Introduction

Cranial bone defects associated with pathology, trauma and fracture nonunion represent a significant clinical problem [1, 2]. Autologous and allograft bone graft represent the ideal graft material as they possess the desirable properties of osteoinductivity and osteoconductivity while existing issues like the limited supply, risk of immune rejection, and chronic immune responses have limited their application, thereby leading to the development of engineered bone substitutes [3-7]. Hence, an improved strategy is urgently needed to better treat the cranial bone defect.

Chitosan has been widely used in tissue engineering, because of its favorable biological properties such as biocompatibility, biodegradability, and non-toxicity [8]. It has been demonstrated that the addition of thiolated groups to the main amino groups of chitosan can improve some properties of chitosan [9]. Unlike chitosan can only be dissolved in acidic medium, while thiolated chitosan has a good solubility under neutral pH condition, thus making scaffold-based thiolated chitosan suitable for the bone substitutes which almost demand the neutral pH.

The main component of coral is calcium carbonate, which has been widely used in clinical bone transplantation [10]. Calcium carbonate has good biocompatibility and bone conductivity, it has been considered as a promising alternative to coral repair bone defect [11]. However, the coral bone grafts are confronted with some barriers such as severe environmental problems, inflammatory reaction risk, and biological variation [12]. Calcium carbonate ($CaCO_3$) being synthesized in the presence of organic additives usually has fantastic architecture and the combined use of thiolated chitosan /modified calcium carbonate has not been evaluated. In this study, a novel CS-TBA / CCM scaffold was used to repair the skull defect of rats with the main goal to verify its osteogenic properties, and providing an experimental basis for further clinical application [13].

Materials and Methods

Preparation and Characterization of Scaffold Based On CS-TBA/CCM

CS-TBA was prepared using 2-iminothiolane hydrochloride according to the method reported by Liu et al. [14]. Modified calcium carbonate ($CaCO_3$) microspheres were prepared by immobilization of Cchitosan onto chondroitin sulfate modified (originally presented by T.M et al. [15]. CS-TBA/CCM was prepared with tripolyphosphate

(TPP) by ionic cross-linking. In brief, a 5 mL of 0.2% (w/v) CS-TBA solution (in water) with 50 mg modified calcium carbonate was added dropwise into 200 ml of 10% (w/v) TPP solution under continuous stirring for 36 h. Afterward, the microspheres were washed several times with distilled insulated water and dried at room temperature. For the scaffold fabrication, pure acetic acid was used to partially dissolve the surface of the microspheres in a 96-well plate as mold and allow them to fuse, thus forming a porous scaffold. The scaffolds and $CaCO_3$ were metalized by sputter-coating with carbon and observed by scanning electron microscope (SEM, JSM-7001F, and Japan).

Experimental Animals and Study Design

All animals were managed under an approved IRB protocol. In this study, 12 healthy female Sprague–Dawley (SD) rats (average weight 200g), supplied by the Animal Research Center of Guangdong Province were divided into two equal groups such as CS-TBA/CCM scaffolds and negative control (no scaffold implantation) group. Under general anesthesia, the cranium was exposed through a medial incision. Single full-thickness circular defects (5mm in diameter, 1mm in thickness) were generated by a dental bur (Figure 1). The defect was implanted with CS-TBA/CCM scaffold. The control group was left untreated. In all animals, the wound was irrigated, fascia and skin were closed. Post-operatively, three rats were housed per cage in a 12 h day-night rhythm with free cage activity and drinking water. The calvaria were harvested for evaluation after both 4 and 8 weeks of implantation.

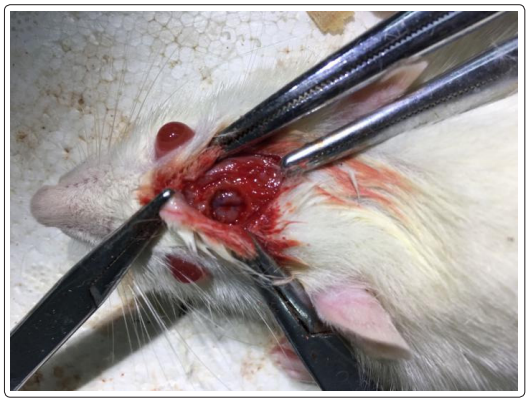

Figure 1: The surgical procedure of the bone defect. A medial incision was made on the calvarial bone, and single full-thickness circular defects (5mm in diameter, 1mm in thickness) was generated by a dental bur

Micro-CT Analysis

The harvested specimens were fixed in 10% (v/v) neutral buffered formalin. For the determination of 3D architecture of the calvarial sample, specimens were analyzed in an advanced μ-CT instrument (ZKKS-MC-Sharp-IV, Zhong Ke Kai sheng Bio, Inc.). Three-Dimensional Reconstruction of the image was performed with a 4 mm region of interest using CTAn and CTVol (Skyscan) software. Histomorphometric parameters, including bone mineral density (BMD), bone volume/total volume (BV/TV) and trabecular number (TB.N) were evaluated.

Immunohistochemical Analysis

After the μ-CT analysis, the harvested specimens were decalcified in neutral 10% EDTA solution for two weeks at room temperature. Samples were dehydrated through an alcohol gradient and embedded in paraffin blocks. The paraffin blocks were sliced into sections (5μm) that were stained with hematoxylin and eosin (HE) or Masson's trichrome staining. The stained sections were photographed digitally under a microscope.

Statistical Analysis

SPSS22.0 statistical software was used for analysis. All quantitative data are presented as mean ± SD. The student's t-test was performed to assess the statistical significance of results between groups. Values of $p < 0.05$ were accepted as statistically significant.

Results

Scaffold Characterization

The scaffold displayed around 5 mm in diameter and 1 mm in height (Figure 2a and Figure 2b). The size of the scaffold is adjustable according to the requirements. The inner structure and morphology of the scaffold were examined by Scanning Electron Microscope (SEM). As shown in Figure 2c and Figure 2d, the scaffold was composed of fused bathyspheres with a diameter of 400-600μm, pore size ranged from 100 to 500 μm. Figure 3 showed the SEM images of the modified calcium carbonate particles obtained in the presence of polymers (or their derivatives)-chondroitin sulfate and carboxymethyl chitosan. Panoramic images showed that all particles exhibit well-defined spherical morphology and the size of the spherical particles is ranged from 50 to 100 μm, indicating that the size of the particles can be well controlled by the two polymers.

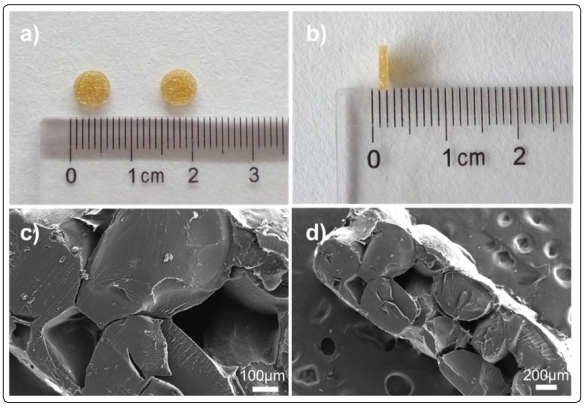

Figure 2: Structure of the CS-TBA/CCM scaffold. (a, b) overall appearance, (c, d) SEM photos of the scaffold.

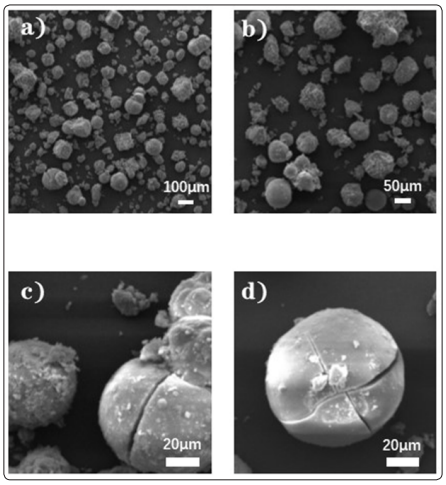

Figure 3: SEM images of the modified calcium carbonate particle (a).100μm (b).50μm (c-d).20μm.

Results of μ-CT Analysis

The 3D reconstruction images in each group are presented in Figure 4. At 4 and 8 weeks after the operation, Micro-CT images showed the minute increment bone formation in CS-TBA/CCM group compared to control group after 4 weeks of implantation, while CS-TBA/CCM group showed the greatest amount of bone compared to control group after 8 weeks of implantation. The BV/TV was 6.47±2.90 in the control group and 13.67±4.25 in the CS-TBA/CCM group at 8 weeks after the operation. The BV/TV of the CS-TBA/CCM group was significantly higher than that of the control group ($p < 0.05$) (Figure 5b). The BMD of the control group, CS-TBA/CCM group were (4W 95.71±50.09, 8W 116.36±20.1) and (4W 189.84±26.42, 8W 222.91±11.79) respectively. The BMD of the CS-TBA/CCM group was significantly higher than that of the control group ($p < 0.05$) (Figure 5a). The Tb.N of the control group, CS-TBA/CCM group were (4W 0.25±0.04, 8W 0.20±0.16) and (4W 0.55±0.16, 8W 0.71±0.19) respectively. The Tb.N of the CS-TBA/CCM group was significantly higher than that of the control group ($p < 0.05$) (Figure 5c).

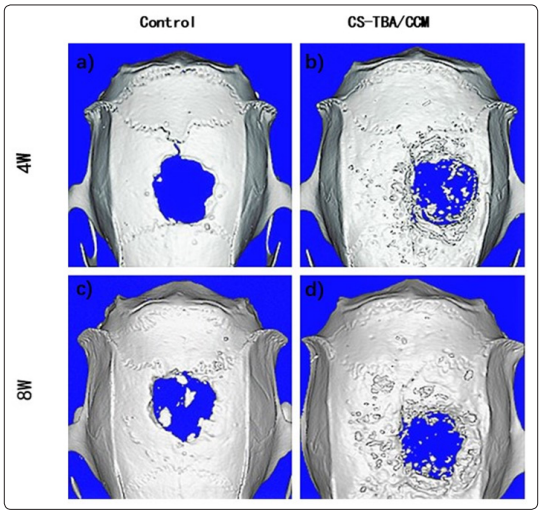

Figure 4: The 3D reconstruction images of microcomputed tomography (μ-CT) in the (a) control group and (b) CS-TBA/CCM group at 4 weeks after the operation, (c) control group and (d) CS-TBA/CCM group at 8 weeks after the operation.

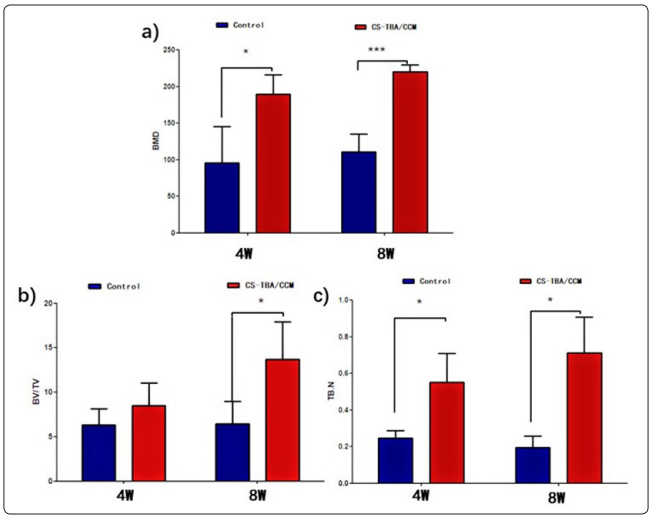

Figure 5: Micro-CT examination of the whole calvaria after 4 and 8 weeks implantation in vivo. (a) Bone mineral density (BMD) (b) bone volume/total volume (BV/TV) and (c) trabecular number (Tb.N). *$p < 0.05$, ***$p<0.001$, CS-TBA/CCM group versus control group.

Results of Histological Staining Analysis

The histological staining showed bone regeneration efficiency after 4 and 8 weeks of implantation (Figure 6). At 4 weeks after the operation, none fiber connection was found in the control group, but the fiber junction was gradually formed in CS-TBA/CCM group, with a large number of a blue nucleus. At 8 weeks after the operation, the fiber junction was gradually formed in control group with few new bone formation at the edge of fiber, while in the CS-TBA/CCM group, there was new bone growth in the fiber space, and obvious vascular formation could be seen on the edge. The histological and Micro-CT analysis results are similar, and besides suggesting that CS-TBA/CCM engineering system should be a favorable candidate for bone regeneration.

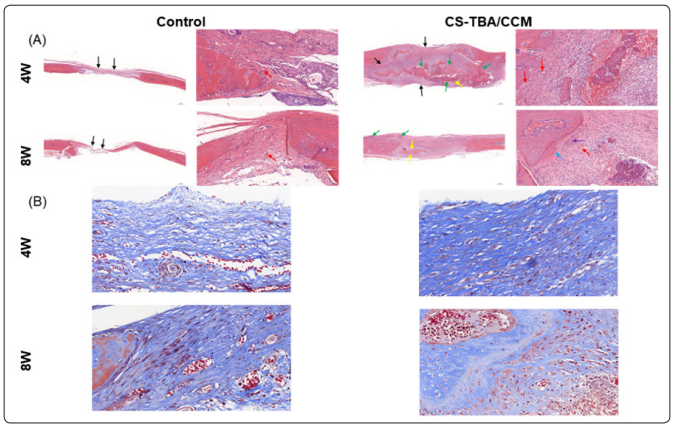

Figure 6: Histology of the cranial specimens at 4 and 8 weeks after implantation in the rat. (A) Hematoxylin and eosin staining in CS-TBA/CCM and control group. New capillaries (red arrow), new bone (blue arrow) and osteoblasts (purple arrow), fiber tissue junction (black arrow), lymphocytes immersion (yellow arrow), fiber connective tissue (green arrow) (B) Masson's trichrome staining in CS-TBA/CCM and control group

Discussion

Regeneration of such segmental bone and large bone defects caused by bone injury, tumor, and other bone diseases has been a difficult problem in clinical treatment [16]. Scaffolds with microstructure simulated host bone for treating bone defects are necessary for bone tissue engineering strategies [17]. In this work, we synthesized CS-TBA/CCM scaffold to verify its osteogenic properties. The extensive characterization showed that the CS-TBA/CCM scaffold is a safe and effective bone graft substitute. Moreover, the superior regenerative capability of the CS-TBA/CCM scaffold in vivo was also confirmed, as shown by the enhanced bone regeneration within a 5 mm rat calvarial defect [18, 19].

The treatment and repair of bone defects have always been one of the important clinical problems [20]. It is known that the capacity to promote osteogenesis is the key to the application of synthetic biomaterials in bone defect reconstruction [7]. A study reported by F. He and al found that calcium carbonate is the main component of coral, which has been widely used in clinical bone transplantation [7]. Fupo He and al. found that calcium carbonate is osteoconductive and not osteoinductive [21]. Therefore, in this study, we synthesized

calcium carbonate composite microspheres by using calcium chloride and ammonium carbonate as raw materials and modified with Carboxymethyl chitosan and Chondroitin Sulfate.

Chitosan is biocompatibility, biodegradability, and non-toxicity with almost all the tissues [22]. It has been reported that chitosan displayed significant osteoconductivity, but minimal osteoinductive performance [23]. However, the solubility of chitosan on physiological pH is limited [22]. Some studies stated that the inherent shortcoming of chitosan has been overcome by the formation of derivatives such as acylated, carboxylated or thiolated chitosan [24, 25]. Among these various chitosan derivatives, sulfhydrylation technology has the advantage of high stability [26]. Additionally, the usefulness of thiolated chitosan was mostly used in hydrogel and drug release [27, 28]. However, the potential application of this material for bone tissue engineering scaffold was rarely investigated. In this work, we synthesized CS-TBA /CCM scaffold to verify its osteogenic properties.

In our analysis, we found that the structure of the CS-TBA/CCM composite microsphere scaffold was revealed by SEM observations (Figure 2), which was composed of fused bathyspheres with pore size ranged from 100 to 500μm. Pore volume is one of the important features that determine the success of the implanted scaffolds owing to its role in support. A study reported that pores greater than 20-100μm were beneficial to cell infiltration, and the neovascularization was improved significantly after exceeding 100μm [18]. Some data from literature highlighted that pores more than 300μm promote direct osteogenesis, and pores below 300μm promote cartilage osteosis. As well, it is known that high porosity usually means high surface area/volume ratio, which is beneficial for other substances to adhere to the scaffold and promote bone tissue regeneration [29]. To evaluate the ability of CS-TBA/CCM scaffold and promote bone growth in vivo, we surgically created critical-sized cranial bone defects in SD rats. Our μ-CT analysis showed that the CS-TBA/CCM group had more effective bone regeneration ability compare to the control group (Figure 3). Similarly, the BV/TV, BMD and Tb.N of the CS-TBA/CCM group were significantly higher than that of the control group (Figure 5). Additionally, the 3D reconstruction image (Figure 4) of the CS-TBA/CCM group showed more bone formation compared with the control group (Figure 2). Our vivo studies showed that the new bone-like tissue determined by micro-CT combined with histology examination were significantly increased in the CS-TBA/CCM group than in the control group in vivo (Figure 4-6). The model of a full-thickness cranial defect in vivo showed that CS-TBA/CCM group was superior to the control group in promoting bone regeneration [24]. This change regarding bone regeneration in the experiment group can be explained by the modified/$CaCO_3$, which has high negatively charged groups such as carboxylate and sulfate groups. These two groups have been confirmed to be involved in the control of bio-mineralization [21].

Our study also has several limitations. Firstly, the BMSCs combined with CS-TBA/CCM scaffold may have a more superior osteogenic effect. Secondly, the CS-TBA/CCM scaffold may carry biological factors and drugs. Thus, further investigation is necessary to fully illustrate the CS-TBA/CCM scaffold combined with other substances in bone regeneration in vivo.

Conclusions

In this study, a synthetic CS-TBA/CCM scaffold using CS-TBA/ modified calcium carbonate was successfully made through the ionic cross-linking technique. In vitro study, SEM revealed that the scaffold with a diameter of 400-600μm and pore size varied from 100 to 500μm. Our in vivo study showed a remarkable increasing bone formation in CS-TBA/CCM group compared to the control group 8 weeks after the implantation. This novel CS-TBA/CCM scaffold might be clinically fit as a potentially promising new bone substitute material.

Acknowledgement

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