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Chitosan/gelatin/platelet gel enriched by a combination of hydroxyapatite and beta-tricalcium phosphate in healing of a radial bone defect model in rat

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ABSTRACT

This study compared the regeneration potentials of the hydroxyapatite (HA) and beta-tricalcium phosphate (β-TCP) alone or in combination with a HA:TCP ratio of 30:70 in the critical-sized radial bone defects of rats. Bilateral 60 radial bone defects created were randomly divided into six equal groups (n = 10 defects/group) including autograft, untreated or defect, chitosan-gelatin-platelet gel (CGP), CGP-HA, CGP-TCP and CGP-HA/TCP. The defects were evaluated by radiography, morphology, histopathology, histomorphometry, CT scan, scanning electron microscopy and biomechanical testing after eight weeks. Compared with the untreated and CGP-HA groups, the CGP and CGP-HA/TCP groups showed significantly higher new bone formation, bone volume, and mechanical properties. The CGP-HA and CGP-TCP scaffolds showed low biodegradability, whereas the CGP scaffolds were completely degraded. Osteoconductivity and osteoinductivity of the CGP and CGP-HA/TCP scaffolds were superior to the CGP-HA ones. The untreated and CGP-HA groups repaired mostly through fibrosis, while there were evidence of higher bone formation in the autograft, CGP and CGP-HA/TCP groups. In conclusion, addition of HA or β-TCP alone into the CGP scaffolds impaired bone regeneration, while bone regeneration with the CGP and CGP-HA/TCP scaffolds was comparable with the autograft. Therefore, the CGP-HA/TCP scaffold can be a possible option to substitute the autologous bone grafting.

1. Introduction

A variety of bone graft materials have been used to fill large bone defects following fractures, tumors, and pathologic conditions [1,2]. Autologous bone is considered as the gold standard due to its superior properties in bone formation, while it has some clinical limitations such as pain, morbidity of the donor site, and additional surgery [3]. Therefore, the researchers have attempted to develop bone substitutes to autografts. Among various bone graft materials used in bone tissue engineering, bio-ceramics including HA and beta-tricalcium phosphate (β-TCP) have been widely used due to their biocompatibility, osteoconductivity and chemical and structural similarity to inorganic component of bone tissue [4–7]. Biocompatible and biodegradable polymers can behave as a carrier for drugs and other biomolecules such as growth factors and hence, they can increase the bioactivity and functionality of the scaffolds [6,8]. In addition to osteoconductivity, the scaffolds should maintain osteoinductivity for a prolonged period [3,9]. Platelets are regarded as an enrich source of osteoinductive growth factors and have high tissue-regenerative ability [10,11]. This study combined biodegradable gelatin (Gel) and chitosan (CS) polymers, which provide controlled release of growth factors in platelet gel (PG), with bioceramics to enhance mechanical strength and osteoconductivity. The aim of the present study was to determine the biocompatibility, biodegradability, and osteoconductivity of a combination of HA and β-TCP in a critical radial bone defect in rat model by comparing with HA and β-TCP alone in a Gel/CS/PG (CGP) base scaffold. It is expected that HA-TCP has more favorable biological and mechanical influence in bone formation compared with HA or β-TCP alone.

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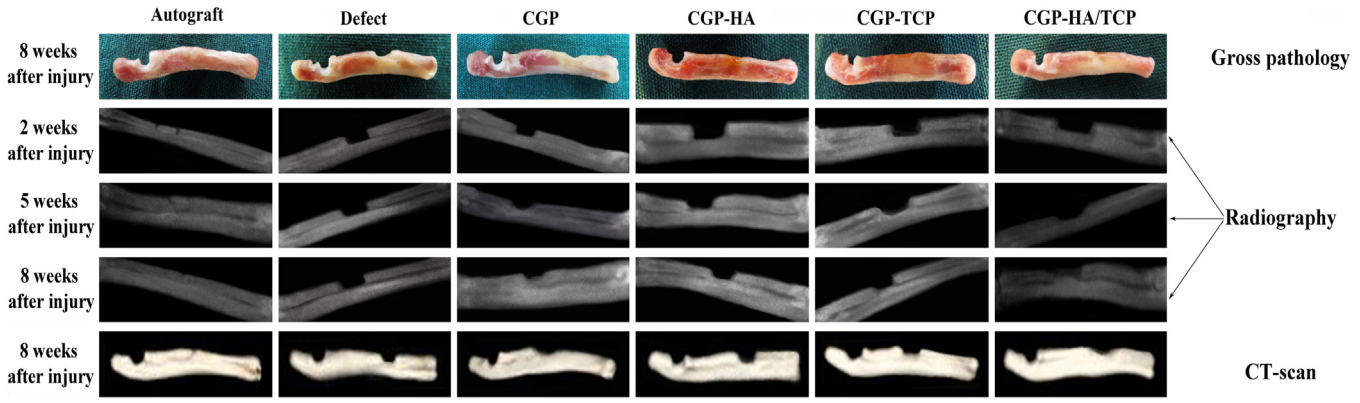

Fig. 1. Macroscopic and diagnostic imaging findings of the healed radial defects after eight weeks of injury. The defects treated with the autograft, CGP, and CGP-HA/TCP scaffolds were connected to the pre-existing radial bone by hard tissues such as cartilage and bone. The untreated defects remained empty or were filled with soft tissue. The CGP-HA and CGP-TCP treated defects were filled with the scaffold remnants with a soft tissue or cartilage. At the 2nd and 5th weeks post-injury, the autograft group had bone regeneration greater than other groups ($P < 0.05$). Bone union on the radiographs in the CGP and CGP-HA/TCP groups was superior to that in the defect group at the 5th ($P = 0.028$ and 0.047, respectively) and 8th weeks ($P = 0.011$ and 0.034, respectively). The autograft group showed significantly higher bone formation and had the highest radiopacity than the defect, CGP-HA and CGP-TCP groups at the 8th week ($P < 0.05$). The defects treated with autograft had the highest percentage of bone volume. All treated groups had greater bone volume than the defect groups ($P < 0.05$). The bone volume of the CGP and CGP-HA/CTP groups was significantly greater than the CGP-HA and CGP-TCP groups ($P < 0.05$). The difference between the bone volume of the CGP and CGP-HA groups and also the CGP-HA and CGP-TCP was not significant.

2. Materials and methods

2.1. Preparation of the scaffolds

For fabrication of the CGP composite scaffold, the CS-Gel (CG) solution was made by adding a 4% w/v Gel solution (bovine skin, type B, isoelectric point ~5; Sigma-Aldrich, St. Louis, Germany) into 2% CS solution (medium molecular weight, 75–85% deacetylation degree; Sigma-Aldrich, St. Louis, Germany) and it was stirred for 12 h at 37 °C. In order to fabricate the CGP-HA and CGP-TCP scaffolds, 0.4 g of the β-TCP (Merck, Darmstadt, Germany) and 0.4 g of HA powders (Merck, Darmstadt, Germany), respectively, were added into the CG solution under agitation and then treated by sonication until the powder was thoroughly dispersed [7,12]. To make and fabricate the CGP-HA/TCP scaffold, a 70:30 (wt.%) ratio of β-TCP and HA [13,14] was dissolved into the CG solution under agitation and then was sonicated. A 0.25% (v/v) glutaraldehyde (GA, Acros Organics ™) solution in phosphate buffer solution (PBS) was then added to every of the above solution for cross-linking. Finally, all the cross-linked gels were frozen at −20 °C for 24 h and then freeze-dried at −80 °C for 48 h. After freeze-drying, the freeze-dried gels were immersed in the 0.55 mM glycine solution for 2 h for deactivation of the free and non-reactive sites. After washing with distilled water, the scaffolds were freeze-dried again and sterilized under 60 Co γ-irradiation at a dose of 15 kGy and kept in vacuumed packs until surgical application.

After fabrication of the CG, CG-HA, CG-TCP and CG-HA/TCP scaffolds, they were suspended in the platelet solution prepared by dissolving the sterilized platelet powder into sterile PBS, to absorb the platelets in their porous structure. The platelets were absorbed by the scaffolds and the platelet-scaffold complexes were then suspended in platelet activator solution (5000 U bovine thrombin + 5 ml 10% $CaCl_2$) [15]. As a result, platelet gel (PG) was formed inside the scaffolds and the prepared CGP, CGP-HA, CGP-TCP and CGP-HA/TCP composite scaffolds were then lyophilized and kept in vacuumed packs until further use. The number of human platelets (provided from the Shiraz Blood Bank Center) in the whole blood and PG was $259.4 \pm 41.6 \times 10^3/\mu l$ and $1174.3 \pm 261.3 \times 10^3/\mu l$ (4.5-fold in PG compared to the whole blood), respectively.

2.2. Animals and surgical procedures

Thirty adult male Sprague-Dawley rats weighing 250 ± 25 g (8-week-old) were raised on standard laboratory diet and allowed free mobilization. Under general anesthesia (Ketamine hydrochloride 10% (50 mg/kg), Xylazine 2% (2 mg/kg) and Acepromazine maleate (1 mg/kg) (all purchased from Alfasan Co., Woerden, Holland)), 2-cm incisions were aseptically made over the bilateral forearms after shaving and disinfecting with iodine. Fascia and muscles were carefully separated until both the radii were exposed. Afterward, a 5-mm bone defect was created in the middle of each radial diaphysis. The bilateral radial bone defects were randomly divided into six groups (10 radial bone defects/group) according to the implanted materials. The bone defects were left empty (empty or untreated group) or grafted with the cortico-medullary autologous bone harvested from the removed bone segmented from the contralateral side of the radius of the same rat (autograft group). Groups 3–6 were the CGP, CGP-HA, CGP-TCP and CGP-HA/TCP groups, wherein the radial bone defects were implanted with the CGP, CGP-HA, CGP-TCP and CGP-HA/TCP composite scaffolds with the same size and shape as the rat radial bone defects (dimensions = $2 \times 2 \times 5\ mm^3$). After implanting the scaffolds, the operation site was rinsed with diluted iodine and normal saline, and the muscles and skin were then sutured and closed. Flunixin meglumine (Razak Co., Tehran, Iran; 2.5 mg/kg) and enrofloxacin (Enrofan 5%, Erfan, Tehran, Iran) were intramuscularly administered for analgesia and antibiotic therapy immediately after the surgery for 5 days. The animals were euthanized on the 8th week after bone injury and the implantation for analysis. All information regarding animal experiments was approved by the local Ethics Committee of "Regulations for using animals in scientific procedures" in School of Veterinary Medicine of our university. All the rats used in the present experiment received human care in compliance with the National Institutes of Health (NIH publication No. 85-23, revised 1985).

2.3. Clinical examination

Clinical behavior, physical activities, weight bearing capacity with the condition of the operated sites in terms of the presence of pain on digital palpation and inflammatory signs such as hyper-