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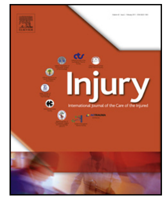

Effectiveness of tissue engineered based platelet gel embedded chitosan scaffold on experimentally induced critical sized segmental bone defect model in rat

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ABSTRACT

Background: Healing and regeneration of large bone defects are a challenging problem for reconstructive orthopedic surgeons.
Purpose: This study investigated the effectiveness of chitosan scaffold (CS), platelet gel (PG) and their combination (CS-PG) on healing process of an experimentally induced critical sized segmental bone defect model in rat.
Methods: Fifty bilateral defects were created in the mid diaphysis of the radial bones of 25 Sprague-Dawley rats. The animals were randomly divided into five equal groups. The bone defects were either left untreated or treated with corticomedullary autograft, CS, PG or CS-PG. Plain radiographs were provided from the radial bones on weeks 2, 5, and 8 after injury. In addition, clinical examinations were done for the healing radial bones. The animals were euthanized after 8 weeks of injury, and their harvested samples were evaluated by gross morphology, histopathology, scanning electron microscopy, CT-scan, and biomechanical testing.
Results: Compared with the defect group, the PG and autograft treated bone defects had significantly superior radiological scored values, bone volume and biomechanical performance which had positive correlation with their superior gross pathological, histopathological and ultra-structural features. Compared with the untreated defects, the PG and CS-PG treated defects showed significantly superior structural and functional properties so that PG had the highest value. In addition, CS had low value in bone regeneration. Although combination of CS and PG improved the healing efficacy of the CS, this strategy reduced the ability of PG to increase osteoconduction and osteoinduction during bone regeneration.
Conclusion: Application of PG alone enhanced bone healing and can be regarded as a promising option for bone tissue engineering in clinical settings. Chitosan was not effective in bone reconstruction surgery and further investigations should be conducted to find a suitable carrier for PG.
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Introduction

Large and critical sized bone defects may not heal via spontaneous healing process [1,2]. Thus, in most cases it is often necessary to reconstruct them with classic grafts to prevent delayed and non-union [3]. Since drawbacks of autograts and allografts including donor site morbidity, disease transmission, and immunogenicity and rejection [2,4,5], bone tissue engineering has offered promising alternatives to the grafts via utilizing scaffolds, growth factors and cells to promote bone regeneration [4]. Chitosan (CS) is a chitin derived polymer produced by deacetylation of chitin and is composed of *N*-acetylglucosamine and glucosamine with well-known biological properties [1,6]. It has been proposed as a potential candidate in drug delivery and tissue engineering applications due to its properties such as bioactivity, biocompatibility, cyto-compatibility, biodegradability, non-immunogenicity, non-cytotoxicity, anti-microbial and anti-fungal properties as well as low cost [1,4,6,7,8]. It is found in various forms including membranes, sponges, paste, microspheres and porous scaffolds [1,7–10], and contrary to many synthetic polymers, it has

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a hydrophilic surface that increases cell adhesion and proliferation [4]. Moreover, CS can be combined with ceramics and other polymers to fabricate composite scaffolds [4,10–12]. Chitosan is positively charged and can produce electrostatic unions with anionic molecules such as growth factors; therefore, it can be applied as an effective carrier vehicle for growth factors [1,6,11,13]. Growth factors can regulate cell differentiation, chemotaxis, adherence, and proliferation as well as angiogenesis [14,15]. A safe and cost-effective approach to achieve high concentrations of growth factors during bone healing process is administration of platelet-rich plasma (PRP) [5,16]. It can be used in different forms such as injectable liquid form, hydrogel, sponge, and platelet gel (PG) [5,15,16,17]. One critical issue regarding PRP is its maintenance in the defect site and rapid release of growth factors [15,16]. Therefore, we assessed the effectiveness of the CS scaffold incorporated with xenogeneic human pure PG in regeneration of critical sized defects created in both radial bones of rats. We hypothesized that since the CS scaffold is biocompatible and biodegradable on one side, and PG is beneficial in bone healing due to its contents on other side; therefore, their combination would have synergic effects on bone healing and regeneration. In such a combination, CS seems to protect PG from acute degradation and inhibit burst release of the growth factors from the PG; therefore, PG affects bone healing probably for a longer time.

Materials and methods

Materials

Chitosan powder with a 75–85% de-acetylation degree and glutaraldehyde (GA, 25 wt, % solution in Na-cacodylate buffer) were purchased from Sigma-Aldrich and Acros Organics, respectively.

Preparation of CS, PG and CS-PG

An aqueous 2% CS solution (wt/vol) was prepared by dissolution of the CS flakes in 0.5 mM acetic acid over the magnetic heater stirrer at 600 rpm. A 0.16 wt% of GA solution was added into the CS solution, completely dissolved and homogenized to crosslink the CS solution. The resultant hydrogel was firstly maintained at 4°C for 24 h to let the gel polymerization and crosslinking terminated. The CS gel was then frozen at −20 °C for 24 h and then freeze-dried at −80 °C for 48 h. The freeze-dried CS scaffolds were then treated with 100 mM aqueous glycine solution for 1 h to block the non-reacted aldehyde groups. The scaffolds were then cut in several pieces similar to the rat radial bone defects in size (dimensions: $2 \times 2 \times 5\ mm^3$). Consequently, the scaffolds were washed with deionized water at triplicate for 15 min to remove the residual solvents. Finally, the CS scaffolds were sterilized with Gamma irradiation (^{60}Co γ-irradiation at the dose of 15 kGy) and packed until surgical application.

Human pure platelets contained no red blood cells or white blood cells were provided from the Blood Bank Center, for production of the PG. The platelet solution was freeze-dried to produce a platelet powder and sterilized until further use. The platelet powder was then added to the 0.9% saline solution to produce a platelet solution with desired concentration. The platelets were then activated by adding five ml of 10% CaCL2 combined with 5000 U bovine thrombin [18–21]. After 10 min the platelet solution was transformed into a platelet gel. Platelet counts in the whole blood and PG were $259.4 \pm 41.6 \times 10^3/\mu l$ and $1174.3 \pm 261.3 \times 10^3/\mu l$ (4.5-fold), respectively.

In order to fabricate the PG loaded CS scaffold, the fully dried sterilized CS scaffolds were placed in custom-made rectangular dishes and the platelet solution was loaded into the scaffolds by a syringe pump. After the scaffolds fully absorbed the solution and overhydrated, the platelet loaded scaffolds were transferred into the activator solution. After activation of the platelets, the PG embedded CS scaffold was fabricated. The PG embedded CS scaffolds were immediately implanted in the rat bone defects.

Ethics

All animals received humane care in accordance with the Guide for Care and use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85-23, revised 1985). The study was approved by the local Ethics Committee of “Regulations for using animals in scientific procedures” in School of Veterinary Medicine, of our university.

Animals and surgical procedures

Twenty-five skeletally mature male Sprague-Dawley rats, weighing 250 ± 25 g provided from Razi Institute were used in this experiment. The animals had full access to standard food and water ad libitum during the study. The rats underwent general anesthesia via intramuscular injection of Ketamine hydrochloride (Ketamine 10%, 50 mg/mg), Xylazine (Xylazine 2%, 2 mg/kg) and Acepromazine maleate (1 mg/kg, all purchased from Alfasan Co., Woerden, Holland). Under aseptic condition, two-cm incisions were made over both forearms and the radial bones were exposed. Five-mm bone defects were then created in the diaphysis of the radii, using electrical bone saw (Strong Co., Seoul, South Korea) saline dripping. The bone defects (10 defects/each group) were then either left untreated (defect or untreated group) or implanted with autograft (the radial bone segment removed from the contralateral side of the same rat), CS alone, PG alone or CS-PG. Finally, the soft tissues and skin were approximated in a routine fashion. Analgesia and antibiotic therapy were provided by intramuscular administration of flunixin meglumine (2.5 mg/kg) and enrofloxacin (Enrofan 5%), respectively for five days.

Clinical examination

The animals were blindly evaluated for their clinical behavior and the post-surgical inflammatory signs including edema, swelling, hyperemia, and pain on digital palpation by two veterinary surgeons.

Radiological evaluation

At the 2nd, 5th and 8th weeks after injury, lateral radiographs were provided from the injured forearms. To evaluate the degree of bone healing, each radiograph was blindly scored according to previous studies [22–24].

Macroscopic evaluation

Eight weeks after the operation, the rats were euthanized and their radius-ulna complexes were removed [18]. The bone defects were blindly evaluated for gross signs of bone healing and regeneration and scored [23].

Three-dimensional computed tomography (3D-CT) assay

The bone specimens were scanned at multiple longitudinal and transverse sections with 0.06 mm thickness by CT-scan, using Inveon TM unit (Siemens Healthcare, Inc., PA, USA). Gross profiles of the specimens and their 3-D images were reconstructed via Inveon Research Workplace software (Siemens Healthcare USA,

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