Journal of Controlled Release 254 (2017) 65–74

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

# Effectiveness of tissue engineered chitosan-gelatin composite scaffold loaded with human platelet gel in regeneration of critical sized radial bone defect in rat

Ahmad Oryan[a,*], Soodeh Alidadi[a], Amin Bigham-Sadegh[b], Ali Moshiri[c], Amir Kamali[a]

[a] Department of Pathology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran
[b] Department of Surgery and Radiology, School of Veterinary Medicine, Shahrekord University, Shahrekord, Iran
[c] Razi Drug Research Center, Iran University of Medical Sciences, Tehran, Iran

ARTICLE INFO

*Keywords:*
Chitosan
Gelatin
Platelet gel
Bone tissue engineering
Bone regeneration
Radius

ABSTRACT

Although many strategies have been utilized to accelerate bone regeneration, an appropriate treatment strategy to regenerate a new bone with optimum morphology and mechanical properties has not been invented as yet. This study investigated the healing potential of a composite scaffold consisting of chitosan (CS), gelatin (Gel) and platelet gel (PG), named CS-Gel-PG, on a bilateral critical sized radial bone defect in rat. Eighty radial bone defects were bilaterally created in 40 Sprague-Dawley rats and were randomly divided into eight groups including untreated, autograft, CS, Gel, CS-PG, Gel-PG, CS-Gel, and CS-Gel-PG treated defects. The bone defects were evaluated clinically and radiologically during the study and their bone samples were assessed by gross and histopathology, histomorphometry, CT-scan, scanning electron microscopy, and biomechanical testing after 8 weeks of bone injury. The autograft and CS-Gel-PG groups showed significantly higher new bone formation, density of osseous and cartilaginous tissues, bone volume, and mechanical performance than the defect, CS and Gel-PG groups ($P < 0.05$). In addition, bone volume, density of osseous and cartilaginous tissues, and numbers of osteons in the CS-Gel-PG group were significantly superior to the CS-PG, CS-Gel and Gel groups ($P < 0.05$). Increased mRNA levels of alkaline phosphatase, runt-related transcription factor 2, osteocalcin, collagen type 1 and CD31, vascular endothelial growth factor as osteogenic and angiogenic differentiation markers were found with the CS-Gel-PG scaffold by quantitative real-time PCR in vitro after 30 days of culturing on bone marrow-derived mesenchymal stem cells. In conclusion, the healing potential of CS-Gel scaffold embedded with PG was comparable to autografting and therefore, it can be offered as an appropriate scaffold in bone tissue engineering and regenerative applications.

## Chemical compounds used in this study

Chitosan (PubChem CID: 71853)
Methyl methacrylate (PubChem CID: 6658)
Glutaraldehyde (PubChem CID: 3485)
Acetic acid (PubChem CID: 176)
Glycine (PubChem CID: 750)
Nitric acid (PubChem CID: 944)
Hydrochloric acid (PubChem CID: 313)
Ethanol (PubChem CID: 702)
Formaldehyde (PubChem CID: 712)
Formic acid (PubChem CID: 284)
Calcium chloride (PubChem CID: 5284359)

## 1. Introduction

Tissue-engineered constructs are considered as promising alternatives to bone grafts in regeneration of large bone defects [1–4]. Recently, natural polymers such as gelatin (Gel) and chitosan (CS) have been increasingly proposed as biological materials in designing scaffolds to be applied in bone tissue engineering [3,5–7]. They have several considerable advantages such as high biodegradability, biocompatibility, non-antigenicity, and non-toxicity [5,6,8,9]. Chitosan has a hydrophilic surface that promotes cell adhesion, proliferation and differentiation [2,5]. Since neither CS nor Gel is sufficient to induce bone formation [10,11], incorporation of growth factors within the Gel and/or CS based scaffolds may improve the osteoconductive and osteoinductive properties and enhance new bone formation [1,12,13].

* Corresponding author at: Department of Pathology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran.
*E-mail address:* Oryan1215@gmail.com (A. Oryan).

http://dx.doi.org/10.1016/j.jconrel.2017.03.040
Received 27 October 2016; Accepted 21 March 2017
Available online 29 March 2017

It is expected that the biological effects of several growth factors such as those present in platelet-rich plasma (PRP), due to their drawbacks, are greater in enhancement of tissue regeneration than a single growth factor such as bone morphogenetic proteins (BMPs) [12,14–16]. Platelets contain angiogenic, mitogenic and osteogenic growth factors in their α-granules [2,12,15,17]. Several studies have demonstrated the effectiveness of PRP in combination with biomaterials in promotion of bone regeneration [17–20]. Nonetheless, it is questionable whether combination with other biomaterials allows the PRP to enhance its biological activity. Overall, composite scaffolds may help bone regeneration in a better way because of the beneficial properties of several biomaterials applied in the scaffold [3,20]. Biodegradable polymers such as CS and Gel have been widely used substrates for cells to attach and grow [5,21]. Moreover, they are able to interact ionically with growth factors having opposite charges [1,18,22]. Therefore, growth factors immobilized in these scaffolds could be released with degradation of the scaffold [18].

Given the above explanations, application of a combination of CS and Gel combined with growth factors appears to provide promising results in bone engineering and regenerative medicine. Therefore, for the first time, this study investigated the role of CS-Gel composite scaffold incorporated with human xenogeneic platelet gel (PG) in bone healing and regeneration of experimentally induced radial bone defects in rats. Although CS and Gel have extensively been used in bone regeneration, the pure effects of CS and Gel, and their combination as CS-Gel, or with human PG as CS-PG and Gel-PG have not been investigated in the previous experimental studies. Thus, we used these five options as our control groups to evaluate the regenerative potential of the CS-Gel-PG scaffold.

## 2. Materials and methods

### 2.1. Preparation of scaffolds

#### 2.1.1. Gelatin scaffold

Acidic Gel from the bovine skin (type B, ~225 g Bloom, isoelectric point ~5; Sigma-Aldrich, Germany) was added into acetone with an equal proportion to precipitate high molecular weight particles. A 10% Gel solution (100 mg/ml) was prepared by adding the residual Gel into deionized water at 45 °C. To crosslink the Gel particles, the solution was then mixed with a 0.25% (v/v) glutaraldehyde (GA, Acros Organics ™) solution in phosphate buffer solution (PBS) at pH = 7.4 by a homogenizer (300 rpm for 15 min). The resultant gel was maintained at − 20 °C for 24 h and then freeze-dried (Christ freeze dryer, ALPHA 2–4 LD plus, Germany) at − 80 °C and a pressure of 1 mBar for 48 h.

#### 2.1.2. Chitosan scaffold

A 2% aqueous solution (w/v) of CS (medium molecular weight, 75–85% de-acetylation degree, Sigma-Aldrich, St. Louis, Germany) was made by dissolving CS into 1% (v/v) acetic acid by stirring at 500 rpm for 5 h to get a perfectly transparent solution. The 0.25% GA solution (v/v) was then added into the CS solution and homogenized to crosslink the solution. The resultant hydrogel was kept at 4 °C for 24 h to allow the gel to be polymerized, then maintained at − 20 °C for 24 h and finally freeze-dried at − 80 °C for 48 h to become porous and ensure complete drying.

#### 2.1.3. Chitosan-gelatin composite scaffold

To prepare the CS-Gel composite scaffold, the 2% CS solution was dissolved in 1% acetic acid (v/v). The Gel (2% w/v) was then added to the CS (2% w/v) solution at 20 wt% and stirred for 12 h at 37 °C to form CS-Gel solution. A 0.25% GA solution was added to the solution as a cross-linker agent. The resultant cross-linked gel was frozen at − 20 °C for 24 h and then freeze-dried at − 80 °C for 48 h.

#### 2.1.4. Post-fabrication processing of the scaffolds

The active sites or non-reactive sites of GA in the Gel, CS and CS-Gel scaffolds were then deactivated by immersing the scaffolds in the 0.55 milimolar glycine solution for 2 h. All the scaffolds were then washed with distilled water at room temperature at triplicate. The scaffolds were then freeze dried again to remove the solvents without altering the scaffold structure and architecture. The scaffolds were then freeze-dried at − 80 °C for 48 h and sterilized under $^{60}$Co γ-irradiation at a dose of 15 kGy and kept in sterile packs until surgical application.

#### 2.1.5. Platelets

Human platelets were provided from the Shiraz Blood Bank Center. The platelet solution was then immediately freeze-dried at − 80 °C for 48 h and transformed into platelet powder. The powder was then sterilized by $^{60}$Co γ-irradiation at a dose of 15 kGy (λ = 254 nm) for 10 min. Number of platelets in the whole blood and PG was 259.4 ± 41.6 × $10^3$/μl and 1174.3 ± 261.3 × $10^3$/μl (4.5-fold increase), respectively. Health and activity of the platelets were checked and confirmed by the Blood Bank Center.

#### 2.1.6. Preparation of the scaffolds loaded by platelet gel

To prepare the CS, Gel and CS-Gel scaffolds incorporated with PG, the fully dried CS, Gel and CS-Gel scaffolds were suspended in platelet solution (prepared by dissolving the platelet powder into sterile PBS) to absorb the platelets. After absorption of the platelet by the scaffolds, the platelet-scaffold composites were suspended in platelet activator solution (5000 U bovine thrombin + 5 ml of 10% $CaCl_2$) [23]. As a result, PG was formed inside the scaffolds and the PG embedded CS (CS-PG), Gel (Gel-PG) and CS-Gel-PG scaffolds were fabricated. The scaffolds were then stored at − 20 °C for 24 h, freeze-dried at − 80 °C and pressure of 1 mBar for 48 h, and placed in sterile packs until further use.

### 2.2. In vivo biodegradation analysis of scaffolds through subcutaneous implantation

The analysis was performed on five mature Sprague-Dawley rats. After anesthesia of the animals, the fabricated cylindrical scaffolds (CS, Gel and CS-Gel) with dimensions of 10 mm in length and 5 mm in diameter were implanted subcutaneously into the lower back of the rats. The incision sites were then closed in a routine fashion.

### 2.3. Quantitative real-time RT-PCR

Total mRNA was extracted from 3D cultured bone marrow mesenchymal stem cells (BMSCs cellular scaffolds: CS, Gel, CS-PG, Gel-PG, CS-Gel, and CS-Gel-PG), using RNeasy Micro Kit (Qiagen-74004). The cDNA was synthesized from total RNA, using RevertAidTM first strand cDNA Synthesis Kit (Fermentas, k1632) according to the manufacturer's instructions. Osteocalcin (OCN), collagen type 1 (Col1), alkaline phosphatase (ALP), and runt-related transcription factor 2 (Runx2) mRNA levels as osteogenic markers and CD31, vascular endothelial growth factor receptor 2 (VEGFr2) and angiogenic differentiation markers, were measured by real-time RT-PCR (Applied Bio-systems life technologies; ABi step one plus real-time PCR system). The 20-μL Q-RT PCR reaction contained 25 ng cDNA from each sample mixed with 10 μL SYBR® Green Master Mix (Applied biosystems life technologies, Inc., REF 4367659), 10 pmol of each forward and reverse primer and 6 μL RNase/DNase-free water. The Q-RT PCR Thermal conditions were: Pre-heating stage at 95 °C for 10 min followed by cycling stage: 95 °C for 15 s and at 60 °C for 60 s, 40 cycles. The gene expression levels of target genes: OCN, Col1, CD31, VEGFR2, ALP and Runx2 were determined based on the threshold PCR cycle-values (Ct) following the instructions of Applied Bio-systems. The relative quantification was performed, using comparative CT method (also known as the $2^{-\Delta\Delta Ct}$ method), where the amount of target genes normalized to an endogenous control (B2M) and relative to calibrator group (negative control