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Fabrication and characterization of platelet-rich plasma scaffolds for tissue engineering applications

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ABSTRACT

Platelet-Rich Plasma (PRP), as a rich source of growth factor, can form a fibrin gel that recapitulates the extracellular matrix of the tissues. The aim of this study was to evaluate the effects of different concentrations of $CaCl_2$ on the PRP scaffold structure which in turn could change the cell's behavior.

PRP was mixed with 2.5, 5 and 10% (*w*/*v*) $CaCl_2$. Then, the tensile strength, biodegradability and water content of the scaffolds were evaluated. We also performed immunostaining for assessment of the actin stress fiber orientation and SEM for detecting the cell phenotype and physical properties of the fibers. Cell viability, attachment and migration were also evaluated.

The highest cell attachment and short term proliferation rate was observed on the scaffolds with 2.5% $CaCl_2$. The cells cultured on the scaffold with higher $CaCl_2$ concentration had fusiform phenotype with few cell processes and parallel arrangement of stress fibers while those cultured on the other scaffolds were fibroblast-like with more processes and net-like stress fibers. The scaffolds with 10% $CaCl_2$ demonstrated the highest osmolarity (358.75 ± 4.99 mOsmole), fiber thickness (302.1 ± 54.3 nm), pore size (332.1 ± 118.9 nm^2) and the longest clotting time (12.2 ± 0.776 min) compared with the other scaffolds. Water content, branching angle, porosity, orientation and tensile strength did not change by gelation with different $CaCl_2$ concentrations.

In conclusion, the cell shape, viability and proliferation were modified by culturing on the PRP scaffolds prepared with various concentrations of $CaCl_2$, and as a result, the scaffolds showed different physical and biological properties

1. Introduction

An ideal scaffold recapitulates the chemical and mechanical properties of tissue specific extracellular matrix (ECM). It should also provide a high surface area for cell attachment [1] and an appropriate microenvironment to keep naïve cell phenotype. Besides, it should support cell proliferation, migration and differentiation. 3D versus 2D culture conditions have been shown to modify the differentiation capacity and gene expression pattern [2]. The biodegradation rate of the scaffolds should be customized to match the rate of naïve matrix formation [3].

Fibrin is a bio-scaffold formed by polymerization of the fibrinogen monomers through the action of thrombin and calcium [4]. In vivo, formation of fibrin clot helps the tissue to regenerate by providing a temporary matrix for cell attachment and migration during tissue healing process [5]. Fibrin is a biocompatible and biodegradable hydrogel with high water content [6] and porosity [7] which promotes the cell viability, attachment and proliferation [8], and also induces angiogenesis [9]. Fibrin provides an appropriate scaffold for wound sealing [10], heart valve replacement [11], drug delivery [12] and fabrication of many tissues such as skin [13], vessels [14], cartilage [15] and bone tissues [16]. However, the presence of bovine thrombin for polymerizing fibrinogen increases the risk of immunological reactions. Human thrombin and supplementary growth factors are expensive and these are disadvantages of using commercial fibrins in regenerative medicine [17].

Autologous fibrin scaffold can be fabricated through the activation of fibrinogen molecules in platelet-rich plasma (PRP) that is the concentrated platelets in a small volume of blood plasma [16]. PRP has been demonstrated to play an important role in tissue repair due to its rich source of growth factors and cytokines such as transforming growth factor-beta (TGF-β), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF) [18], basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) [19], and epithelial cell growth factor (ECGF) [20]. Providing a xeno-free environment with high growth factor content without the potential risk of immunological reactions or viral contaminations are the main advantages of PRP.

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Various factors such as thrombin concentration [21], calcium ions [22], pH and ionic strength [23] determine the way of polymerization of fibrinogen to fibrin. Calcium, as an important factor in clot formation, acts as a cofactor of thrombin. It has critical roles in modulating fibrin structures such as the length, branching, clot stability [24], and accelerating fibrin formation [25]. A correlation has been detected between the fibrin scaffold structure and its mechanical properties. Biodegradability of the fibrin scaffold is also influenced by the mechanical properties such as fiber thickness and pore size [26]. Cell adhesion, morphology, proliferation, migration and differentiation have been reported to be influenced by mechanical properties of the fibrin scaffold, such as fiber diameter and porosity [27]. For instance, fibroblasts and endothelial cells cultured on fibrin clots with thicker fibers produce more prefibrinolytic and antithrombotic agents than those cultured in clot with thinner fibers and as a result, the cells migrate faster inside the clot. Besides, it has been reported that more endothelial cells oriented along the thicker fibers [26,28] and as a result it impacts the rate of the angiogenesis [29]. The pore size also influences the cell attachment, migration, differentiation and angiogenesis.

PRP was used previously as a serum source in tissue culture and also in stem cell differentiation protocols [30]. PRP supplementation has been shown to change the differentiation capacity of Mesenchymal stem cells [31]. Wharton's Jelly-derived Mesenchymal stem cells (WJ-MSC) were considered as a non-invasive cell source for clinical applications with the capability to differentiate into several cell lineages [32]. The behavior and phenotype of these stem cells in the fabricated scaffold are important for choosing the best differentiation strategies. With regard to these considerations, the objective of the current study was to find out the mechanical and physical properties of PRP scaffolds prepared with various concentrations of $CaCl_2$.

2. Material and methods

2.1. Isolation of human Wharton's Jelly MSCs (WJ-MSCs)

The umbilical cords were collected from normal full-term infants (39–40 weeks) born through cesarean section at Hafez Hospital affiliated to Shiraz University of Medical Sciences after taking the parents' informed consent. Then, the umbilical cords were immediately transferred to the laboratory in phosphate buffer saline (PBS). The blood was flashed by PBS through the umbilical vein and the amnion was removed by scraping in sterile condition. The umbilical cords were cut into 1 cm length pieces. Then, the lumen of the vein was cut longitudinally to scrap the endothelial cells, the arteries were removed and the remainder tissue, Wharton's jelly, was cut into the small pieces, approximately 1 to 2 mm^2. The explants were put into a tissue culture flask. Then, they were covered completely with alpha-Minimal Essential Medium (α- MEM) (Gibco, containing 10% Fetal Bovine Serum, 1% L-glutamine and 1% penicillin/streptomycin) and incubated at 37 °C in humidified incubator under 5% CO_2. The cells expanded out of tissue fragments up to 70% confluency. Cells at the third passage were used for subsequent experiments.

2.2. Osteogenic and adipogenic differentiation procedures

To evaluate the pluripotency, the differentiation potential of the WJ-MSCs toward several lineages including osteoblasts and adipocytes was examined. For osteogenic differentiation, 5×10^4 WJ-MSCs were plated in each well of a 24 well plate in the presence of the commercial NH-OsteoDiff Medium (MACS, Germany) for four weeks. Then, the cultures were fixed with ethanol and stained with 0.5% alizarin red S in PBS to detect the calcium deposition by newly differentiated osteoblasts.

To test the adipogenic potential of WJ- MSCs, about 5×10^4 cells were plated in each well of a 24 well plate in Dulbecco Minimal Essential Medium (DMEM) containing commercially prepared human adipogenic stimulatory supplements (Stem Cell Technologies Inc., Canada) for three weeks. The cells were then stained with Oil Red O to detect lipid droplets produced by differentiated adipocyte. Leica inverted microscope was used for imaging (Leica, Solms, Germany).

2.3. Phenotype analysis

To characterize WJ-MSCs, the expression pattern of the surface CD markers was evaluated by flow cytometry. The samples were permeabilized by the buffer containing tween 20 and goat serum. Then, the cells were treated with FITC- conjugated anti-CD44, CD144 and CD90, PE-conjugated anti-CD106, CD34 and CD73, and pre-CP-conjugated anti-CD105 antibodies (all from Abcam, UK, Cambridge). The cells were fixed with 4% paraformaldehyde and the percentage of the positive cells for each CD marker was evaluated by flow cytometry. Non-specific binding sites were excluded by corresponding isotype controls. A four color FACSCalibur flow cytometry machine with CellQuest pro software for data acquisition was used to analyze the positive-reacting cells to various antibodies. The flowjo software, version 10.1.1 was used to depict the graphs.

2.4. PRP preparation

Platelet- rich plasma (PRP) was collected from the whole blood of healthy volunteers by Iran blood transfusion organization, Shiraz. The PRP was achieved in a bag containing CPDA as anti-coagulant. The platelet count was assessed by an automated counter (Sysmex, XS-800i, Kobe, Japan).

To prepare PRP scaffold, we mixed it with 2.5, 5 and 10% (*w*/*v*) $CaCl_2$ (Merck) at 9:1 ratio. The mixture was incubated in 24 well plates at 37 °C and 5% CO_2 for 15 min to form the gel.

2.5. Osmolarity assessment

To determine the osmolarity, we exposed the culture media to the PRP scaffolds prepared with different $CaCl_2$ concentrations. After 1 h, they were removed and the osmolarity was evaluated by osmometer (Gonotec, Germany, Berlin) calibrated with distilled water.

2.6. Cell seeding on PRP scaffold

A population of about 5000 cells/200 mL of culture medium (α-MEM containing 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin) were seeded on each PRP scaffold and incubated at 37 °C and 5% CO_2 for 5 or 7 days to perform further analyses. The culture media were replaced every two days.

2.7. Scanning electron microscopy (SEM)

The PRP scaffolds with or without cells were prepared for SEM. PRP scaffolds were fixed with 2.5% glutaraldehyde (Sigma-Aldrich, USA) in PBS at pH 7.4 for 2 h. The samples were dehydrated in gradually increasing ethanol concentration for 15 min intervals. Then, they were dried by ethanol/Hexamethyldisilazane (HMDS, Merck, Germany) mixture and pure HMDS. After gold coating, the scaffolds were visualized using an SEM (VEGA\\TESCAN-XMU) with an accelerating voltage of 20 kV.

SEM micrographs were analyzed by the free software Image j (http://imagej.nih.gov/ij/index.html) [33] for angles of fibrin fiber branches, diameter of fibers, orientation, surface porosity, and pore size.

2.8. Cell attachment assay

The cells were seeded at a density of 10^5 cell/mL both on the PRP scaffolds and the floor of a 24-well plate as the control for 160 min. Thereafter, the culture media were removed, centrifuged at 290g for 5 min and the cell palate resuspended in 250 μL medium. The number