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Osteogenic differentiation of human adipose-derived mesenchymal stem cells on gum tragacanth hydrogel

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ABSTRACT

Currently, natural polymer based hydrogels has attracted great attention of orthopedic surgeons for application in bone tissue engineering. With this aim, osteoinductive capacity of Gum Tragacanth (GT) based hydrogel was compared to collagen hydrogel and tissue culture plate (TCPS). For this purpose, adipose-derived mesenchymal stem cells (AT-MSCs) was cultured on the hydrogels and TCPS and after investigating the biocompatibility of hydrogels using MTT assay, osteoinductivity of hydrogels were evaluated using pan osteogenic markers such as Alizarin red staining, alkaline phosphatase (ALP) activity, calcium content and osteo-related genes. Increasing proliferation trend of AT-MSCs on GT hydrogel demonstrated that TG has no-cytotoxicity and can even be better than the other groups i.e., highest proliferation at day 5. GT hydrogel displayed highest ALP activity and mineralization when compared to the collagen hydrogel and TCPS. Relative gene expression levels have demonstrated that highest expression of Runx2, osteonectin and osteocalcin in the cells cultured GT hydrogel but the expression of collagen type-1 remains constant in hydrogels. Above results demonstrate that GT hydrogel could be an appropriate scaffold for accelerating and supporting the adhesion, proliferation and osteogenic differentiation of stem cells which further can be used for orthopedic applications.

1. Introduction

The healing of the bone loss and damages caused by accidents and a variety of diseases have become one of the biggest problems of hospitals and clinics all over the world. Common surgical treatments for this are bone allograft and autograft [1,2]. Immunological problems and transmission of some diseases such as hepatitis B and C and HIV, prompted the researcher to produce synthetic fibers and synthetic bone tissue engineering scaffolds [3]. Current and new strategy in the treatment of musculoskeletal injuries is tissue engineering, which consists of biodegradable and biocompatible scaffolds, cells and bio-macromolecules [4–7].

Hydrogels are one of the most feasible substrate that has been used as scaffold in tissue engineering as well as in drug delivery [8]. Structural properties of hydrogels are similar to macromolecular components of the tissue and are also considered to be biocompatible [9,10]. Nowadays, several natural polymers have been introduced for tissue engineering application resulting biocompatibility, biodegradability and bioactivity of them.

Gum Tragacanth (GT) is an anionic carbohydrate with three characteristic such as naturally occurring complex, branched and heterogeneous polysaccharide [11]. This gum has been used in pharmaceutical and food industry, indicating safety of its usage [12,13]. In addition, a few studies were focused on the use of this gum in tissue engineering. For instance, in the study conducted by Fattahi et al., was cultured L929 cell line on fabricated GT gel by ionotropic complexation and their results showed biocompatibility of this gel significantly increased viability of cell line in comparison to the tissue culture polystyrene culture plates (TCPS) [14]. Another important component of tissue engineering is stem cell, these cells have the remarkable ability to differentiate into cell types in the body and act as a repair system for the body [15]. Until now, various sources for isolating stem cells via, placenta, adipose tissue, bone marrow and umbilical cord blood. Mesenchymal stem cells (MSCs)

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attracted a lot of attention for therapeutic uses because they have a great ability to differentiate and also regulate the innate immune system [16,17]. Recently lots of research focused on the MSCs isolation from the bone marrow and adipose tissues, in the treatment of bone lesion and osteoporosis [18–21].

In the present study, we cultured adipose derived MSCs (AT-MSCs) on GT hydrogel and its osteoconductivity potential was investigated using ALP activity, calcium content assay and the expression of bone related gene markers. The aim of this work is provide alternative to bone substitute.

2. Experimental section

2.1. Gum tragacanth hydrogel preparation

Ribbon type of GT (from Isfahanian Astragalus gossypinus) was purchased from local herbal market (Tehran, Iran). GT hydrogel preparation was carried out according to the protocol reported by Fattahi et al. [14]. In brief, primary suspension was prepared with 25 mg/ml concentration via dispersed of GT in distilled water, and then it was stirred for 24 h at 37 °C. In the next step, 0.5 M NaOH was added to the solution and the mixture was stirred for 6 h at 4 °C. To neutralize 1 M HCl was added to obtain a final pH of 7.5. After centrifuging for 10 min at 6000 rpm, solution was filtered in sinter funnel to insoluble residue elimination and concentration process was carried out by rotary evaporator at 60 °C under vacuum. Before cell seeding, hydrogels were prepaid via the following steps: 1) placed in 70% ethanol solution for 2 h, 2) placed in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen Co., Carlsbad, CA, USA) supplemented with penicillin, streptomycin, and amphotericin B to prevent from bacteria and yeast growth for 4–6 h, and 3) placed in culture medium (DMEM-10% Fetal Bovine Serum (FBS, Invitrogen Co., Carlsbad, CA, USA)) to ensure sterilization and enhance cell attachment after seeding for overnight.

2.2. Scanning electron microscopy (SEM)

Scanning electron microscope (SEM, S-4500; Hitachi, Japan) was used to hydrogels morphological study. After hydrogel fabrication, they were lyophilized, fixed on a brass stub, and sputter coated with gold and then were examined at an accelerating voltage of 20 kV.

2.3. MTT assay

MTT assay was used to biocompatibility evaluation of hydrogels and also viability of MSCs seeded on hydrogels in comparison with TCPS. Stem cells were seeded with a cell density of 5×10^3 cells per cm^2 on sterilized hydrogels after placed in a 24-well culture plate and TCPS. Every day during 5 days after cell seeding, 50 μl of MTT solution (5 mg/mL in DMEM) was added to each well ($n = 3$). Then, the plate was incubated at 37 °C and after 3.5 h supernatant was removed and Dimethyl sulfoxide (DMSO) solvent was added to plates. A micro-plate reader (BioTek Instruments, USA) was used to read their optical density at a wavelength of 570 nm.

2.4. Cell culture

AT-MSCs were purchased from the cell bank of Stem Cell Technology Research Center. These stem cells were isolated and characterized previously [22]. Prior to hydrogel cell seeding, AT-MSCs were cultured and maintained in DMEM supplemented with 10% FBS and incubated with 95% air and 5% CO_2 at 37 °C for expansion and obtain passage 3 cells.

2.4.1. Cell seeding and osteogenic differentiation

Passage 3 AT-MSCs were seeded on the tragacanth and collagen hydrogels and TCPS as control under the basal medium (DMEM with 10% FBS) overnight for attachment. For osteogenic differentiation induction, medium was removed and DMEM supplemented with 10% FBS, 3 mM β-glycerophosphate, 50 μg/mL ascorbic acid and 10^{-9} M Dexamethasone was added as osteogenic induction medium on all groups. The medium was replaced every two days during 21 days.

2.5. Osteogenic differentiation evaluations

2.5.1. Alizarin red staining

Alizarin red staining was used to evaluate the calcium deposited by differentiated cells after the period of study. All samples were fixed in cold 4% paraformaldehyde for 20 min at 4 °C after removed the medium and washed with PBS respectively. Then 2% Alizarin red S at pH 7.2 (Sigma) was added to the samples for staining. After 10 min incubation at room temperature, the samples were washed with PBS two times and studied by invert light microscopy. Then morphology of stained cells was quantified by image j software (http://rsb.info.nih.gov/ij).

2.5.2. Alkaline Phosphates Activity and Calcium content assay

To alkaline phosphatase (ALP) activity measurement, total protein was extracted from differentiated stem cells in all groups at 7, 14 and 21 days after osteogenic induction using 200 μL of RIPA lysis buffer. Then lysate was centrifuged at 15,000 RPM at 4 °C for 15 min to sedimentation of cell debris. Supernatants were collected and ALP activity was measured using Pars Azmoon ALP activity kit (Iran) and the enzyme activity level (IU) was normalized against the total protein which measured by Pars Azmoon total protein kit (Iran).

To measure the amount of calcium deposited on all groups Cresolphthalein Complex one method was used. Medium was removed and after washing by PBS, 0.6 N HCL (Merck) was added to the hydrogels for homogenization followed by shaking for 4 h on ice. After that Pars Azmoon calcium content kit (Iran) reagent was added to the calcium solutions and the optical density of samples was measured at 570 nm in a micro-plate reader (BioTek Instruments, USA).

2.5.3. Gene Expression Analysis

Real-Time RT-PCR was used to the evaluation of the four important bone-related genes expression such as runt-related transcription factor 2 (Runx2), collagen type one (Col-1), Osteocalcin and Osteonectin at 7, 14 and 21 days after initiate osteogenic differentiation process. RNeasy kit (Qiagen, Germantown, MD) was used to the total RNA extraction according to manufacturer's protocol. Synthesize complementary strands was also carried out using RevertAid First Strand cDNA synthesis kit (Fermentas, Burlington, Ontario, Canada). Parameters that used to the PCR procedure were included denaturation at 95 °C for 3 min, then 40 cycles at 95 °C for 20 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s. Real-Time RT-PCR was performed using SYBR Premix Ex Taq (Takara, Japan). Rotor Gene 6000 (Corbett, Concorde, NSW, and Australia) was used to the gene expression levels quantification. All primer sequences were used in PCR and Real-Time RT-PCR procedures are displayed in Table 1.

2.6. Statistical analysis

All experiments were conducted for three times. Data were reported as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare the results. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were conducted with SPSS software (SPSS, Chicago, IL, USA).