J. of Applied Tissue Engineering
December 2014; 1(1): 14-18

Evaluation of Biocompatible Bioglass/Gelatin Scaffold Enhanced by Mesenchymal Stem Cell for Bone Tissue Regeneration

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Received: 12 October 2013 / Accepted: 15 February 2014

Abstract:

In this study, we are focused on developing cell–scaffold constructs to improve bone growth and bone healing. Rat MSCs were isolated from the femurs of rats. The bone marrow suspensions were cultured in the medium consisted of Dulbecco modified Eagle medium supplemented with fetal bovine serum and penicillin–streptomycin. Bioglassnano-composite (BG) scaffold was made through Sol-Gel methodand was evaluated by in vitro cytotoxicity.Osteogenic materials added tothe culture medium and occurrence of differentiation examined by alizarin red staining. The results revealed that BG scaffolds has no toxicity and is biocompatible, following alizarin red staining thered color demonstrated the mineralizing areas of cultures. The present study demonstrates that rMSCs enable to differentiate to bonecell on the Nano bioglass scaffolds.

Key words: Bioglass, MSCs, scaffold, bone, regeneration

Introduction

Successful repair severe bone defects are a major concern and ongoing clinical challenge. In these cases, autologous bone grafts are best clinical outcome to repair bone deficiencies for its osteogenic and osteoinductive potential [1, 2]. However, its main disadvantages associated with potential donor site morbidity, risk of infectionand nerve damage [3]. Allografts are also used, but have attendant limitations including poor quality and immunogenic response [4]. Due to these drawbacks, tissue engineering seeks to develop strategies to establish an artificial biomaterial scaffold containing regenerating competent cells. The tissue engineered bone complex incorporates osteoconductive scaffolds, cells and osteogenic growth factors [3]. Different parameters for designed and fabricated scaffold like pore size, degradation rate and pore volume were measurement [5]. Moreover, material for fabricate of scaffold should be osteoconductive with three–dimensionally interconnected pores to support cell growth, communication and bone formation [6]. Among bioactive materials, bioactive glasses that are bioactive, biocompatible and osteoconductive, making them candidate materials for tissue engineering [7]. Bone marrow derived mesenchymal stem cells are adherent cells that are capable of self–renewal and can differentiate into several phenotypes, including bone, adipocytes and cartilage [8]. The cellsand biomaterial interactions studies have shown that the implantation of certain progenitor cell–scaffold combination can lead to better results in bone reconstruction than the implantation of the blank scaffolds [6]. In this study, particulate freeze drying techniques was used for the fabrication of 3D and interconnected nano-bioglass/gelatin scaffolds, which mimic both architecture

and composite nature of natural bone. Then, rMSCs were seeded into scaffolds to evaluate the biocompatibility and toxicity of rMSC/scaffold construct as bone graft for bone regeneration.

Materials and Methods

Nanocompositescaffolds preparation

The Nano Bioglass powders (64% SiO_2 , 5% P_2O_5 , and 31 % CaO) (based on mol%), were synthesized by sol-gel technique [9].Based on freeze drying technique, we were able to fabricate gelatin / nano-bioglass scaffolds. Firstly, was provided a homogeneous aqueous solution of microbiology-grade gelatin (GEL) (10 % weight per volume, w/v) (Merck) and added synthesized BG nanopowder to establish a GEL (70)/BG (30) weight composition and homogenized by a stirrer at 400 C for 45 min. Then, a layer of these mixtures were cast into plastic petri dishes and frozen at -200 C for 3 h. The layers were moved to a freeze drier (Christ Beta 2-8 LD plus) for 24 h in order to provide 3D porous structure by sublimation to form a gelatin network matrix on the pore walls and the surface of nanocomposite scaffolds.Then, composite layers were cut at the considered sizes (scaffolds with 5 mm diameter). In the end, nanocomposites were immersed in a cross-linking bathof glutaraldehyde (GA -Merck) (C5H8O2) solution of 1 % (w/v) for 24 h to improve their mechanical properties. Then, to remove the reminding amount of GA, the nanocomposites were intently washed with deionized distilled water.

Scanning electron microscopy (SEM)

SEM was used to evaluate of morphology and microstructure and measure of pore size of nanocomposite scaffolds.Drynanocomposite scaffolds were sputter-coated with a thin layer of gold (Au) (EMITECH K450X, England) and then the morphology of them were observed on a scanning electron microscope(SEM-Philips XL30) at accelerating voltage of 15 kV.

Cytotoxicity evaluation

Nanocomposite scaffolds were immersed in70%ethanolfor 1 h for sterilization, then scaffolds were placed inside a standard 6 -well-plate polystyrene and were washed with sterile distilled water, after with phosphate buffer saline (PBS)sterile solution and finally with culture medium. Then, Dulbecco's Modified Eagle Medium (DMEM) (GIBCO) cell culture media containing 15% (v/v) fetal bovine serum (FBS)(Gibco) and 1% (v/v) penicillin/streptomycin (P/S)(Gibco) were added to PS plates. Rat mesenchymal stem cells with a density of 4 ×105 cell mL^{-1} were added to the samples in PS plates, cells were allowed to initially attach for 5 h and maintained in incubator (37 ^{0}C, CO25 %) for 48 h [10,11]. Finally, the samples were fixed in 100 % ethanol for 15 min, and visualized by light microscopy (Nikon Eclipse 50i) [12].

Cell isolation

Bone marrow was obtained from 4-5 week - old wistar rat. The animals were killed by cervical dislocation, and their tibias and femurs were dissected and cleaned of all soft tissue. The epiphysis of each bone was clipped, and the bone marrow was flushed out of the tibia and femur and was suspended in Dulbecco's modified Eagle's medium (DMEM).Then the cell suspensions from all bones were combined and centrifuged at 1200 g for 5 min. The resulting pellet was resuspended in fresh primary medium [DMEM supplemented with 15% fetal bovine serum and 100 U/ml penicillin-streptomycin] and seeded to tissue culture flasks and kept in a humidified incubator at an atmosphere of 5% CO2 at 37 0C . After three days of expansion, the cultures were rinsed three times with PBS for removal of non-adherent cells. The medium was exchanged every three days throughout the studies. For use in the experiments, adherent cells were rinsed thoroughly with PBS and then detached by trypsinization.

Cell seeding

Briefly, nano-bioglass/gelatin scaffolds were sterilized using 70% ethanol, followed by rinsing the scaffold several times with phosphate buffered saline (PBS) [13].rMSCs in the third passage, were released from the culture substratum using trypsin/EDTA (0.25% w/v trypsin, 0.02% EDTA - Gibco) and were suspended in DMEM medium without FBS. Then rMSCs were seeded onto the scaffold by pipetting the rMSCs suspension onto the materials. The construct of nano-bioglass scaffold/rMSCs was incubated for an additional 4 h to allow cell attachment in vitro before implantation.The extent of cell attachment and growth was assessed 24 h and 5 days after cell seeding.The constructs were fixed in 2.5% glutaraldehyde(Merck)for 1 h at room temperature. After thoroughly washing with PBS, the cells adhered to the scaffold section, then were dehydrated in an ethanol – graded series (50-100%) for 5 min each and allowed to dry on a clean bench at room temperature [4]. The samples characterized by scanning electron microscopy (SEM-Philips XL30)after Gold (AU) coating.

Osteogenicdifferentiation of MSCs

Osteogenicdifferentiation was induced by culturing confluent rat MSCs for 3 weeks in inducing medium as previously [14].The inducing medium was a complete medium supplemented with 10 nM dexamethasone (Sigma), 50 µg/ml L-ascorbic acid 2-phosphate (Sigma), and 10 mMβ – glycerophophate(Sigma). Theosteogenic medium was changed every 3 days and the cells were used for study after 21 days.

Alizarin red S histochemical staining

Using Alizarin red S histochemistry, the cultured cells were stained on day 21 for assessing the mineralized matrix. The medium was removed, and the cell layers were rinsed with PBS 3 times and allowed to air dry. The fixed cells were stained with 2%Alizarin red S PH 7.2 (BIO-IDEA – Iran) . After 1 hour, the cell layers were washed with deionized water and observed with the light microscope.

Results

SEM observations

The morphology of scaffolds, revealed by SEM photographs in Fig.1, indicated a network of interconnected pores with a smooth surface morphology and fairly uniform spherical shape in top view. The pores diameter of the nanocompositesamplesranged from 250 to 500 µm which is desirable for bone cell growth [15,16)].

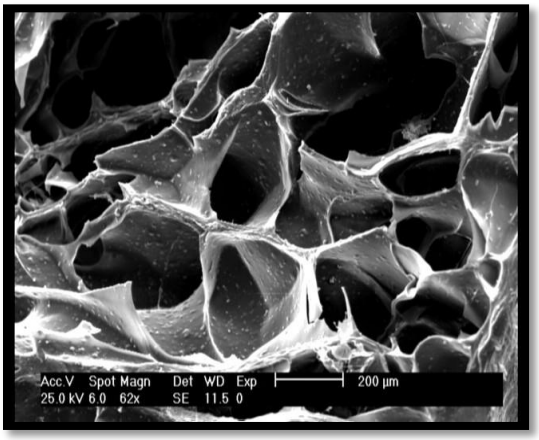

Fig. 1.SEM micrograph of the porous scaffold.

Cytotoxicity evaluation

Indirect cytotoxicity test done with rat mesenchymal stem cells at 48 h after exposing cells to scaffolds. The cytotoxic effects of the scaffolds on rMSCs, is distinguished because of the observation of the cellular attachment, developing filopodias, forming monolayers and spreading. These results indicated that the scaffolds are suitable to support cell growth (Fig. 2).

Cell seeding

The ability of scaffolds to support cell growth and cellular attachment were evaluated by scanning electron microscopy. SEM photographs of rMSCs cultured on the nanobioglass scaffolds showed in Fig. 3. After incubating for 7 days, rMSCs were grew tightly to each other and were suspended among the backbones of scaffolds and grew along the pores of the scaffolds. Moreover, cells reached confluence with abundant fibril networks of extracellular matrix deposited on the scaffolds.These results indicated that the BG nanocomposite scaffold was suitable for support cell growth.

Alizarin red S histochemical staining

After the 28 days of culture in osteoinductive medium, in some areas of the culture plate, nodule – like structures were appeared. But a few cells became detached and floated in the culture. Following alizarin red stainig, the stain for bone nodule formation was positive, and red mineralizing areas of cultures observed (Fig. 4).

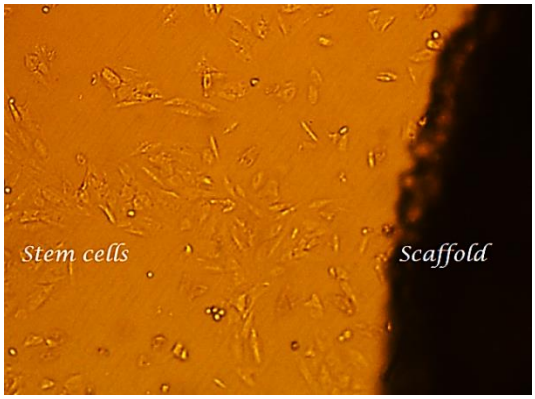

Fig. 2. Micrograph of the mesenchymal stem cells grown on the scaffolds

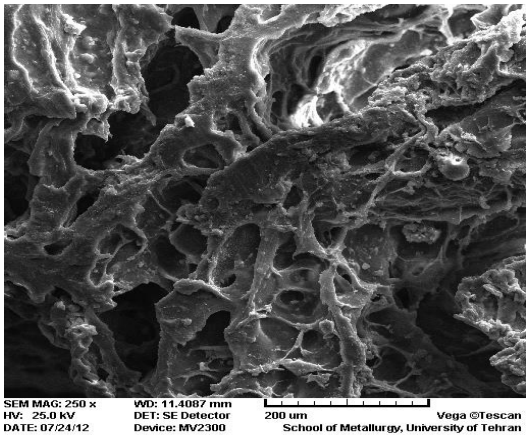

Fig.3. SEM micrographs of rMSC cultured on the scaffolds.

Discussion

Osteoconductive scaffolds and osteoprogenitor cells are the two main factors for bone tissue regenerating.The main challenge in the repair and reconstruction of bone defects is the search for biocompatible and functionally proven graft materials [17]. The aim of current research was to study the ability of gelatin/nano-bioglass scaffold to support the differentiation and viability of rat mesenchymal stem cells. The BG nanocomposite scaffolds are biocompatible, biodegradable and osteoconductive which performed the role of a temporary matrix for cells to grow and begin to differentiate [3]. Mesenchymal stem cells derived from bone marrow are adherent cells of non-hematopoietic origin that have a strong regeneration potential, multilineage differentiation potential and immunosuppressive properties that are important for cell therapy and allografts [14]. In this study, by layer solvent casting combined with freeze drying, BG nanocomposite scaffolds were fabricated. SEM was used to observe the average pore diameter of the nanocomposite scaffolds. It is very important that the pore size in the engineered scaffold be greater than 100 µm to allow cellular migration, tissue ingrowth and eventually vascularization [18)]. SEM images showed well-interconnected pore network structure that the diameters of these pores range between 250 and 500 µm

which are suitable for cell migration, growth and differentiation in vitro and in vivo [10,19,20]. The cellular response and bioactive potential of scaffold specimens was tested using culturing rat mesenchymal stem cells on the scaffolds which were crosslinked with 1% GA.

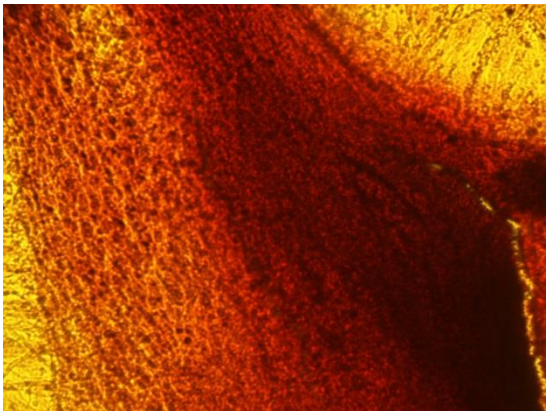

Fig. 4. Alizarin red staining showing red mineralizing of cultures (100x)

The results indicated that the scaffolds could be a perfect tissue engineering scaffold, because of observation of the cellular attachment, proliferation of cells on the surface and surroundings of the scaffolds and developing filopodias. [10].Thus, the general morphology and level of growth observed for cultured cell proves that they could survive and function normally beside scaffolds. The results obtained from Cytotoxicity evaluationshowed that BG nanocompositescaffoldswerebiocompatible and not toxic for living cells and posed as good candidates to be used as bone scaffolds [21].In the present investigation, the mesenchymal stem cells with multilineage differentiation capability were isolated from rat bone marrow. Certain features of the cells having been isolated, ensured us that they were MSCs. In addition, the most important properties of these cells were their multilineagemesenchymal differentiation in appropriate medium that were demonstrated by alizarin reds staining [22].To induce osteoblastic differentiation, confluent rat MSCs were further cultured in osteoinductive medium, as previously described [13]. In osteoinductive cultures, there was bone nodule formation and red mineralizing areas of cultures appeared, following alizarin red staining [7,22]. To evaluate the ability of the scaffolds to support cell growth, cellular attachment and interaction within 3D scaffolds, we seeded rMSCs in the BGscaffold and evaluated by scanning electron microscopy. The BG scaffolds have pores with a suitable diameter for cell seeding and growth, also cells after being cultured in vitro, were found attached along the material surface and actively secrete extracellular matrix [3,7].

Conclusion

In summary, the present research demonstrates that the combination of Nanobioglass/gelatin scaffolds with rMSCs further enhancedcell differentiation to bone cells and may have superior potential of bone regeneration.

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