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RESEARCH ARTICLE

Compatibility of Biomimetic Tailor Made Scaffolds Seeded with Bone Marrow Derived Stem Cells: Invitro Study.

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Abstract

Prefabricated porous 3D chitosan based composite scaffolds were characterized physico-chemically and had proved invitro bioactivity in simulated body fluid (SBF) by use of Fourier transfer infrared spectroscopy (FTIR). Stem cells are the body's master cells due to their capacity to grow and mature outside the living body.

Aim: Prove the liability of stem cells to grow and expand on different prefabricated chitosan scaffolds. **Objective:** Investigate the number of cellular expansion for different chitosan based scaffolds of two and three weeks. **Materials and Methods:** Four different chitosan based scaffolds seeded individually in four identical flasks (25 ml volume). The scaffolds compositions were: Chitosan (CH) only, chitosan loaded with gentamycin sulfate drug (CH/S), chitosan with bioactive glass composite scaffold (CH/G) in ratio of 1:2, and chitosan with bioactive glass composite scaffold loaded with gentamycin sulfate drug (CH/G/S). The control flask contained only undifferentiated mesenchymal cells ($UMSC_S$) with no scaffold. All groups were cultured until 15 and 21 days. Under light microscope, the UMSCS were evaluated histologically and the cell counting was done using a hemocytometer. **Results:** Chitosan (CH) scaffolds showed an increase (7×10^5) in cell number in comparison to the control (6×10^5) and to the drug loaded scaffolds at different time intervals. **Conclusions:** The chitosan (CH) scaffold seemed promising for stem cell attachment, expansion, and growth. However, all other scaffold groups showed relatively lesser degree of cellular proliferation. Fortunately; all chitosan based scaffold compositions had been proved non-cytotoxic until 21 days of stem cell culture. Implantation of chitosan-based scaffolds in experimental animals was recommended.

Introduction

Tissue engineering strategies focused on restoration of damaged tissue architecture by seeding UMSCs in suitable three-dimensional scaffolds. Stem cells could be derived and isolated from the early embryo (Embryonic stem cells: ESCs) as well as fetal and adult tissues (somatic stem cells: SSCs). Their capacity to grow and mature outside the living body together with their potentiality to divide and differentiate into various cell types has induced conduction of many researches on the biology, function and potential clinical applications of these strategic cells. [1, 2] UMSCs derived from bone marrow were an obvious source of autologous stem cells of different types like fibroblasts and osteoprogenitors that might be purified and culture expanded from animals and humans. [3-6]

Scaffold design and fabrication are major areas of biomaterial research and they are important subjects for tissue engineering and regenerative medicine research. All scaffold types provided substrate for cellular attachment,

proliferation and differentiation. [7] Chitosan is a natural polysaccharide that had the advantages of biocompatibility, biodegradability, antibacterial nature and its ability to be easily molded into various porous geometries. Therefore, chitosan had been integrated in variety of biomedical applications. [7-10] Moreover, different compositions of chitosan-based scaffolds were considered ideal carriers of antibiotic drugs. [11-13] The chitosan/Ca-P composite scaffold is considered as an ideal bifunctional tissue-engineering scaffold for both bioactivity and drug release ability. [11, 14]

Bioactive glasses were commonly used in tissue engineering scaffolds; as they interact with the surrounding host tissues at the cellular level and they contributed to the formation of a safe strong wonderful biological attachment. [15, 16] The measurements of tensile strength and water-contact angles of bioactive glass composite scaffolds suggested that the incorporation of those glasses improved the mechanical properties as well as the hydrophilicity of scaffolds. [9] However, the insufficient biocompatibility and biodegradability of bioactive glass limited their potential use in the clinical field. [17]

Inspired by the biocomposite structure of human natural bone, [18] combination of degradable (CH) polymers and inorganic bioactive particles represents the approach in terms of achievable mechanical and biological performance. Moreover, these composite structures had decreased the inherent brittleness of bioactive ceramic phase. The chitosan/bioactive composites had the opportunity to provide surface functionality, chemical stability and antimicrobial effects. [19-24]

Gentamycin sulfate (S) is one of the amino-glycosides antibiotics that were used widely and successfully in orthopedic applications. [25] It was effective against both Gram-positive and Gram-negative aerobic pathogenic microorganisms that produce osteomyelitis. [26, 27] Actually, Gentamycin sulfate is characterized by being easily and quickly adsorbed as well as excreted, therefore; it was commonly used to evaluate the drug loading and releasing ability of carrier materials. [28, 29] Furthermore, many researches had confirmed the importance of antibiotics inclusion in biomimetic tissue engineering scaffolds. [30]

Materials and experimental procedures

Materials

Fabricated composite scaffolds were seeded with bone marrow extracted stem cells of dog. The prepared composite scaffolds were formed of chitosan (CH) and bioactive glass (G) and the chitosan scaffolds were included for comparison in the research. The physicochemical characterizations of all fabricated scaffolds were performed and discussed in the previous research. Chitosan (CH) and composite (CH/G) scaffold groups were drug loaded with Gentamicin sulfate (S) as was also interpreted in previous thesis. [31]

The materials used were: Sodium thiopental (Egyptian International Pharmaceutical Industries co(EIPICO) Egypt), heparin (Stem cell technologies, USA), Acid citrate dextrose (ACD) (Sigma-Aldrich Co. USA), phosphate buffered saline (PBS) (Source Bioscience, UK), Ficoll (Biosearch Technologies, USA), flasks (Falcon, Becton Dickinson, USA), DMEM (Gibco, Paisley, UK), FCS (Fetal cuff serum) and Trypsin and PS (Penicillin Streptomycin) (Lonza, Walkersville, MD USA).

Experimental procedures

1. Isolation and culture expansion of dog bone marrow derived stem cells

1.1. Bone marrow aspirate

Bone marrow aspiration was performed under complete aseptic conditions at the Department of Surgery and Anesthesiology, Faculty of Veterinary Medicine, Cairo University. A healthy adult (12 months old) male mongrel dog of average weight of 8-10 kg and complete dentition was selected for the procedure. Animal sedation was carried out through IV administration of Sodium thiopental 2.5% solution; 20-30 mg/kg body weight. Red bone marrow (50 ml) was aspirated from tibial plateau of the anesthetized dog, after shaving and asepsis at the site for puncture using bone trocar and large plastic syringe that was previously heparinized **(Fig 1-A)**. Then, the obtained mixture of heparin and red bone marrow was transported into a tube containing acid citrate dextrose (ACD) to the cell culture laboratory in supper ice powder (Covance gel pack); which was mixed with water for preservation.

Blood layering and separation

Immediately after aspiration, bone marrow extract was diluted with phosphate buffered saline (PBS) in a ratio 1:1; which was called the first wash. Therefore, the volume of obtained diluted cell suspension was 100 ml. Blood layering and separation were performed with Ficoll to separate the extracted marrow blood into: RBC***s***, buffy coat and plasma (Fig 2-A). Four sterile tubes, each containing 10 ml Ficoll were prepared. For each tube, a 20 ml of

diluted bone marrow was carefully layered too slowly and gently on the sidewall of the tube over the Ficoll [Ficoll: blood ratio was 1:2] (Fig. 1-B). Then, at a temperature 20°C, those tubes were allowed to centrifuge (Thermo, Germany) at 2000 rpm for 30 min. All procedures were carried out in laminar airflow (Nuair, USA) for achieving complete aseptic conditions.

1.2. Stem cells isolation /Fishing

After centrifugation, the interface containing undifferentiated mesenchymal stem cells ($UMSC_S$) were expertly collected (observed white ring), that laboratory procedure was called cell fishing **(Fig.2-B)**. That stem cell fishing step was highly critical and sensitive and needed special standardization and training. The collected $UMSC_S$ cells were introduced into a new sterile tube containing PBS that was double its volume in order to grantee cell viability. Then, centrifugation was allowed at 1500 rpm for 10 min. Afterward, The $UMSC_S$ washing was done twice and the supernatant was discarded following each wash for further cell concentration.

1.3. Determination of cell concentration /cell counting

To determine the cell concentration, 10 µl of the UMSCS suspension were collected into a sterile tube containing 90 µl of 5% acetic acid for cell counting with the aid of a hemocytometer (Boeco, Western Germany) under a light microscope (Nikon Ts.100 Japan) **(Fig 3-B).** Once you have obtained the total cell count for the four chambers, cell concentration can be calculated from the following formula:

$$\text{Total cells/ml} = \text{Total cells counted} \times \frac{\text{dilution factor}}{\text{\# of Squares}} \times 10{,}000 \text{ cells/ml}$$

Total cell counts / number of chambers multiplied by 10^4 (No. of counted cells/4 x10^4 in 1 ml). That procedure was regularly repeated at two and three week's intervals for all different scaffold groups to determine the cell expansion.

1.4. Media preparation / cell culture

The total $UMSC_S$ suspension was distributed into three flasks; each of 25 ml volume and loaded with freshly prepared cell culture media. The cell media was elaborated by a mixture of 90 % DMEM (Dulbecco's modified eagle's medium with 4.5 g/L glucose), 9% FCS (Fetal cuff serum) and 1% PS **(**Penicillin Streptomycin). Cell cultures were maintained at 37°C and 5% CO_2 inside special incubator (Water –jacketed US, Nuair, USA**)**, where flasks should be slightly opened to allow gaseous exchange. The culture media were changed twice a week to get rid of the waste products of stem cell metabolism for three successive weeks and cell count was done each time of changing with fresh media.

1.5. Cell expansion/ trypsinization

When the cell culture flask was near confluence (about 80% of cell expansion), the cells were detached using 0.25% trypsin (2 ml of ethylene-diamine-tetra-acetic acid /flask) for only 1½ min. and a stopwatch was adjusted to avoid risk of cell toxicity. The trypsin effect for cell detachement was checked under the inverted light microscopy. Before trypsinization, the antidote (DMEM 90% and 10% FCS) 18: 2 ml should be readlly prepared in order to save time and to stop the trypsin action promptly. For complete elimination of trypsin effect, the cells were washed twice, centrifuged at 1500 rpm for 2 min. and the supernatant was discarded. Then, the obtained cell suspension was allowed to expand in a new culture flask filled with previously prepared media to start another trait of attachments and 10 µl were gathered for cell counting.

2. Seeding of scaffold groups with the pluripotent $UMSC_S$

The different scaffolds were seeded individually in four identical flasks (25 ml volume) containing the prepared media **(Fig 3-A)**. The scaffolds were: chitosan (CH) only, chitosan with bioactive glass (CH/G), chitosan loaded with gentamycin sulfate (CH/S) and chitosan / bioactive glass loaded with gentamycin sulfate (CH/G/S). The control flask contained only $UMSC_S$ without any type of scaffold. Again; the culture media was discarded and regularly changed twice a week for duration of 21 days.

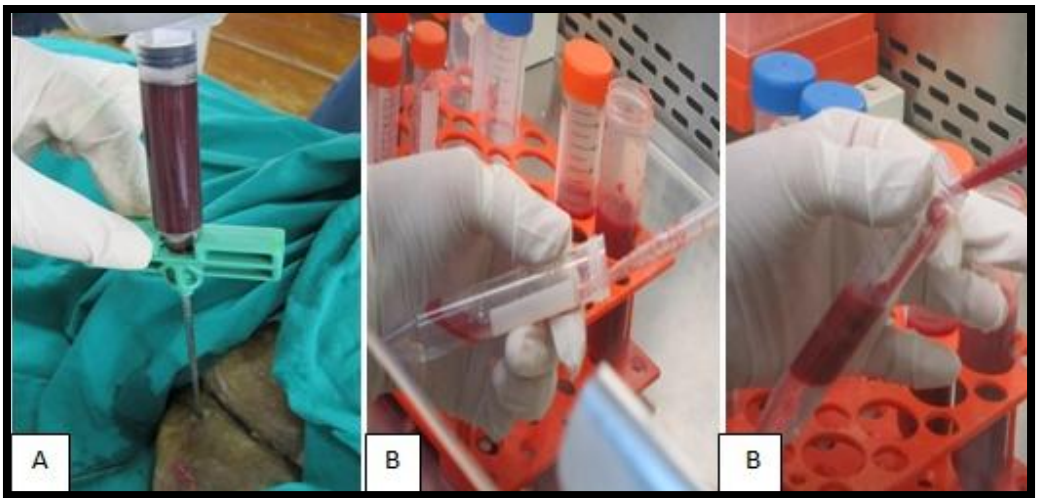

Fig. 1: A-Bone marrow aspirate using bone trocar B/B¯- Bone marrow aspirate layering over Ficoll.

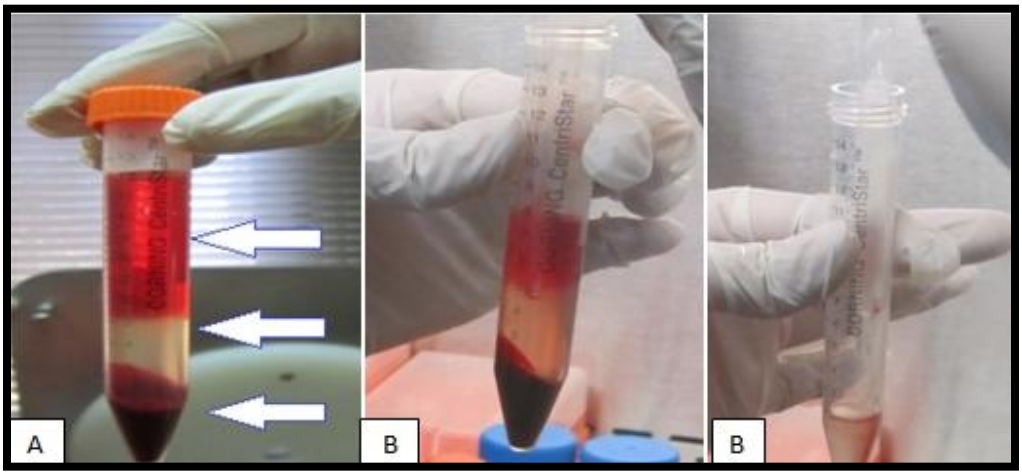

Fig.2: A-Bone marrow blood separation into Plasma, Buffy coat and Red blood cells. B/B¯-Fishing of the Buffy coat with pester pipette into a sterile tube containing PBS.

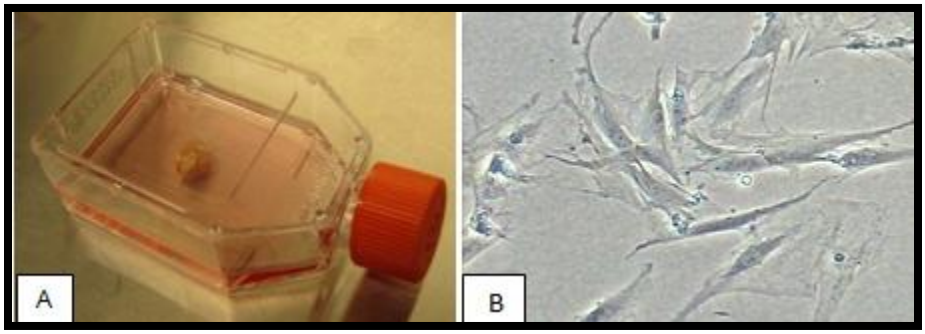

Fig. 3: A- flask containing culture media with the scaffold. B- Stem cells under the light microscope for cell counting procedure.

Results

The base line cell concentration was (4×10^5 / ml). After 15 days, the CH scaffolds showed highest number of $UMSC_S$ (7×10^5) of all groups, which was also more than the control one (6×10^5). The CH/S and the CH/G/S scaffolds (5×10^5) revealed lowest number of cells that was even less than that of the control group **(Table: 1)**.

After 21 days, again the CH scaffolds showed highest number of $UMSC_S$ (7×10^5) of all groups, which was also more than the control one (6×10^5). The CH/S scaffolds (4×10^5) revealed lowest number of cells that was even less than that of the control group (6×10^5). The CH/G scaffolds had lesser number of $UMSC_S$ than it was after 15 days, however; the CH/G/S scaffolds showed same rate of cell proliferation as that after 15 days **(Table: 1)**.

Table 1: UMSCS proliferation rate for different scaffold groups.

Time "Days"	Control	CH	CH/S	CH/G	CH/G/S
Zero	4×10^5 / ml				
15	6×10^5	7×10^5	5×10^5	6×10^5	5×10^5
21	6×10^5	7×10^5	4×10^5	5×10^5	5×10^5

Discussion

The chitosan (CH) scaffolds showed highest and maintained rate of cell proliferation (7×10^5 / ml) of all groups over time; which confirmed that chitosan provides a 3D non-protein matrix that stimulates cell proliferation and histoarchitectural tissue organization[32]. This was in agreement with *Talebian S.et al* who found that above 90% of UMSCs maintained its viability on all chitosan scaffolds. The cell counts into those scaffold constructs were similar at 72 h, 10 days and 21 days. Those findings were in consistent with *I. Saygun*, who concluded that chitosan scaffolds represented promising potential implementation in tissue engineering. [9]

However; the CH/S (drug loaded) scaffold potential for cellular activity seemed to be lesser (5×10^5) than that of the CH scaffolds and it had also declined over time; which might be attributed to the incorporation of Gentamicin sulfate antibiotic particles in that drug loaded scaffolds that hindered the beneficial cell proliferation effect of chitosan. [33- 35] The high hydrophilicity of Gentamicin sulfate and dissemination of its particles might interpret the marked decrease in cell proliferation after 21 days (4×10^5). [36, 37]

In comparison to the CH scaffolds, the CH/G composite scaffolds had relatively lower (6×10^5) rate of cell proliferation (but similar to that of normal control cells), which was higher than that of the CH/G/S scaffolds (5×10^5). Those later drug loaded CH/G/S scaffolds showed maintained (5×10^5) rate of cell proliferation; which might suggest a chemical cross linking between the Gentamicin sulfate and the CH/G composite scaffold constituents (chitosan and bioactive glass). [38, 39]

Conclusions

The fabricated Chitosan /Bioactive glass composite scaffolds are promising tissue engineering scaffolds, which are recommended for further experimental trials. Gentamicin sulfate drug loading for these prepared bioactive composite scaffolds is not cytotoxic to the cells and implantation of the CH/G/S scaffolds in experimental animals bone will give a reliable issue of biocompatibility.

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