Osteogenic differentiation of encapsulated rat mesenchymal stem cells inside a rotating microgravity bioreactor: in vitro and in vivo evaluation

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Abstract The objective of this study is to evaluate the in vitro and in vivo osteogenic potential of rat bone marrow mesenchymal stem cells (BM-MSCs) using chitosan/hydroxyapatite (C/HAp) microbeads as encapsulation matrix under osteoinductive medium and dynamic culture conditions. The degradation characteristics of C/HAp microbeads were evaluated under in vitro and in vivo conditions for 180 days. BM-MSCs were encapsulated in C/HAp microbeads with > 85% viability, and were cultured in a slow turning lateral vessel-type rotating bioreactor simulating microgravity conditions for 28 days, under the effect of osteogenic inducers. MTT assay showed that the metabolic activity of encapsulated cells was preserved > 80% after a week. In vitro experiments confirmed that the encapsulated BM-MSCs differentiated into osteoblastic cells, formed bone-like tissue under osteogenic microgravity bioreactor conditions. Preliminary in vivo study indicated C/HAp microbeads containing BM-MSCs were able to repair the surgically-created small bone defects in the rat femur.

BM-MSCs-C/HAp composite microbeads may have potential for modular bone regeneration.

Keywords Cell encapsulation · Mesenchymal stem cells · Composite microbeads · Osteogenic differentiation · Microgravity bioreactor · Bone tissue engineering

Introduction

Utilization of engineered osteoconductive constructs is a promising approach for restoring defective bone tissues (Amini et al. 2012). They provide instructive niches for regeneration and a scaffold for vascular ingrowth. Bone constructs are fabricated in different forms, compositions and shapes, depending on their intended clinical applications. Possible forms include sponges, hydrogels, fibers, microbeads, etc. Composite microbeads are drawing interest as cell entrapment vehicles in regenerative medicine, particularly in bone repair due to their ability to fill in defects with their amorphous shapes and large surface areas, easy handling features as well as their controllable sizes (Inanc et al. 2007; Weir and Xua 2010; Hashemibeni et al. 2014).

Microbeads suitable for bone regeneration are usually produced by the combination of synthetic or natural polymers with bioceramics. Composite biomaterials have diverse potential applications for

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combining the advantageous properties of each component. Chitosan (C) is a natural linear biopolyaminosaccharide, obtained by alkaline deacetylation of chitin. It offers a broad range of use for cell-based tissue engineering as it has been proven biocompatible, biodegradable, low-antigenic, non-toxic and biofunctional (Durkut et al. 2006; Cheung et al. 2015). It can interact with anionic glycosaminoglycans, proteoglycans and other negatively charged molecules due to its cationic nature. As a natural component of bone mineral, hydroxyapatite (HAp) is an implantable biomaterial with favorable properties such as high biocompatibility, bioactivity and osteoinductivity (Dorozhkin 2010).

Clinical studies have shown that hydroxyapatite supports and promotes cell attachment, proliferation and differentiation (Amini et al. 2012; Gómez-Barrena et al. 2015). Composite of HAp and chitosan has been studied widely since they have shown osteoinductive, osteoconductive and biocompatible properties, surpassing the chitosan-based scaffold alone (Ding and Teng 2012; Frohbergh et al. 2012). These properties make C/HAp scaffold suitable biofunctional template for growing cells and tissue for various bone tissue engineering (BTE) applications.

To explore the potentials of engineered constructs for biological applications, mesenchymal stem cells, pre-osteoblasts, and osteoblasts are widely employed for the effective treatment of bone injury (Bianco 2015). From the BTE perspective, mesenchymal stem cells (MSCs) derived from different tissues are considered a promising multipotent stem cell source with the ability to differentiate both into multiple mesoderm and non-mesoderm type lineages when induced by the appropriate signals (Grayson et al. 2015; Gugutkov et al. 2017).

BTE bioreactors are usually required for the expansion and differentiation of osteogenic cells on an appropriate support prior to transplantation (Rauh et al. 2011). For this purpose, a number of systems using either compressive, shear, or rotational forces have been developed based on dynamic mixing, agitation, filtration, perfusion, rotating-wall vessel culture, and others (Carpentier et al. 2011). Bioreactors using compressive and shear forces are supposedly more efficient in obtaining biomechanical features in vitro, however, the in vitro graft conditioning step should be subsidiary to subsequent integration of the three-dimensional construct, permitting anastomosis (Demirdögen et al. 2010). Hence,

the body is the most efficient bioreactor for de-novo tissue regeneration.

The rotating wall vessel (RWV) provides the necessary biological, biomechanical and structural cues to regulate the cellular responses. The RWV can be used with scaffolds or microcarriers; thus the most common type is the slow turning lateral vessel (STLV) (Koç et al. 2008). STLV has been successfully used as a bioreactor system that supports and promotes cellular functions on different templates as previously reported (Emin et al. 2008; Inanç et al. 2007).

Previous studies performed in our laboratory reported the use of C/HAp microbeads as carriers for human periodontal ligament fibroblasts (hPDLFs) (Inanç et al. 2007). However, the potential of rat bone marrow mesenchymal stem cells encapsulated C/HAp for BTE is yet to be explored. This study has been designed to evaluate the potential of BM-MSCs encapsulated C/HAp microbeads as a bone tissue engineering construct in three steps: (1) in vitro and in vivo evaluation of the biocompatibility and biodegradability of microbeads, (2) in vitro determination of the growth and osteogenic differentiation ability of the rBM-MSCs in composite microbeads, (3) in vivo determination of bone-forming capacity of the rBM-MSCs containing microbeads in surgically created non-critical size bone defects.

Materials and methods

Materials

Chitosan of medium molecular weight ($M_r = \sim 400,000$; > 85% deacetylation) was purchased from Fluka Chemical Company (Milwaukee, WI, USA). Hydroxyapatite (Type I, in 0.001 M phosphate buffer; pH 6.8), sodium tripolyphosphate (TPP), α -Modified Eagle's minimal essential medium (α -MEM), fetal bovine serum (FBS), ascorbic acid, β -glycerophosphate, dexamethasone, antibiotics, trypsin/EDTA, and all other chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA).

Preparation of C/HAp microbeads

C/HAp microbeads were generated by using the following procedure: 2% chitosan solution was prepared by solubilizing in 2% (w/v) ascorbic acid

solution. Subsequently, hydroxyapatite microparticles were incorporated into the solution with a ratio of 9:1 (w:v), then the slurry mixture was mechanically stirred overnight at room temperature to obtain a homogeneous mixture. After that, the mixture was transferred into an encapsulator system (Nisco, Zurich, Switzerland) and extruded through a 250 µm diameter-nozzle into 0.6% TPP solution (pH 7.6) containing 0.89% NaCl to form spheric microbeads. The resulting microbeads were allowed to harden for 10 min in this solution and subsequently were investigated for in vitro and in vivo degradation properties ($n = 3$). The mean diameters of the microbead groups were measured using images retrieved from inverted microscopy examinations.

In vitro degradation

In vitro degradation of the microbeads was assessed by incubating the samples in 0.1 M phosphate-buffered saline (PBS, pH 7.4) at 37 °C in an incubator up to 180 days. Firstly, the initial dry weight of the microbeads (W_1) was recorded and the samples were incubated in the PBS solution. The medium was replaced with fresh buffer every 3 days. At predetermined time intervals, the samples were removed from the solution, rinsed thoroughly with double-distilled water and weighed (W_2) after drying. The following formula (Eq. 1) was used to assess the dry weight loss (WL) of each sample:

$$WL = [(W_1 - W_2)/W_1] \times 100 \quad (1)$$

Isolation and expansion of BM-MSCs

BM-MSCs were isolated from the femurs of three donor Wistar rats (250 ± 25 g) as previously described (Celebi and Elcin 2009). Animal protocols were carried out according to the international regulations. Briefly, the animals were anesthetized with an intramuscular injection of a solution of avertin (Sigma-Aldrich). The femurs were dissected out aseptically and the bone marrows were collected by flushing the diaphysis using a syringe filled with 5 mL of alpha-minimum essential medium (α -MEM) containing 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin (standard medium). The bone marrows of the three donor rats were pooled together, and the

cell suspension was centrifuged at 700 g for 5 min, the supernatant was removed and cells were re-suspended in culture medium and transferred to culture plate. The cells were cultured in a humidified tissue culture incubator at 37 °C with 95% air and 5% CO₂. Unattached cells were removed from the culture plate by sterile phosphate buffer saline wash (PBS; pH 7.4) at the 4th day of the initial culture. The culture medium was replaced every 3–4 days. The primary culture reached confluence (~ 75 –80%) within 11–12 days. Then, cells were detached from the culture flasks by treating with 0.05% trypsin/0.53 mM EDTA in PBS solution and replated into new culture flasks. BM-MSCs from the second passage were used in the encapsulation experiments.

Characterization of the BM-MSCs

The immunophenotype of the pooled cells ($n = 3$) was established by flow cytometric analysis with FACSAria II (BD Biosciences, San Jose, CA, USA) device. The selected cluster differentiation (CD) antigen panel, which is known for mesenchymal stem cells, is composed from CD29, CD31, CD34, CD44, CD73, CD 90, CD54, CD105 and CD106. Morphological characterization of the cells was performed under an inverted light microscope (Nikon TS 100, Tokyo, Japan).

The self-renewal capacity of the cells was morphologically assessed by the colony-forming unit-fibroblast (CFU-F) assay. Briefly, low density cultures (100 cells/cm²) of BM-MSCs in 6-well plates were cultured for 3 weeks without passaging. They were fixed, then stained with crystal violet (1%) and the colonies were visualized under the light microscope.

The multipotency of the BM-MSCs was investigated by conducting osteogenic, chondrogenic, and adipogenic differentiation studies. All differentiation inducers were purchased from Sigma-Aldrich. For osteogenic differentiation, BM-MSCs were cultured in osteogenic differentiation medium (standard medium containing 10 nM dexamethasone, 50 µg/mL ascorbic acid and 10 mM β -glycerophosphate). For chondrogenic differentiation, the cells were cultured in chondrogenic differentiation medium (standard medium containing 10 ng/mL TGF β 1, 50 µM L-ascorbic acid-2-phosphate, 10⁻⁷ M dexamethasone, and 1% ITS solution; i.e. 5 µg/mL insulin, 5 µg/mL transferrin, 5 µg/mL selenous acid). For

adipogenic differentiation, the cells were cultured in adipogenic differentiation medium (standard medium containing 1 mM dexamethasone, 0.5 mM isobutylmethylxanthine, 10 μ M insulin and 200 μ M indomethacin).

The medium was changed every 3 days. At predetermined time points, cells were fixed in ice-cold methanol for 5 min, and then air-dried. Osteogenically differentiated cells were stained using Alizarin Red S (Sigma-Aldrich), chondrogenically differentiated cells were stained using Alcian blue (Sigma-Aldrich), while adipogenically differentiated cells were stained using Oil Red O solution (Sigma-Aldrich).

Cell encapsulation and culture

C/HAp microbeads were prepared according to the method described above. Encapsulation was performed in 3 separate batches. BM-MSCs were trypsinized and the obtained cell pellet was resuspended in 300 μ L medium ($\sim 6.0 \times 10^6$ cells/mL). The cell suspension was mixed with 2.7 mL sterilized C/HAp solution in a sterile syringe and the mixture was transferred into the closed encapsulator chamber (Nisco), and was extruded through the nozzle ($\Phi = 250 \mu\text{m}$) into 0.6% TPP solution containing 0.89% NaCl. Spherical beads formed instantaneously and were allowed to harden for 10 min. The size was controlled by regulating the extrusion flow rate using a syringe pump (Cole-Parmer, Court Vernon Hills, IL, USA). Each microbead contained about 2500 cells. Encapsulated cells were kept in the standard medium for 24 h before evaluating the cell viability. Then, the medium was switched to osteogenic medium (standard medium supplemented with 10^{-8} M dexamethasone, 10 mM Na- β -glycerophosphate, and 50 mg/ml ascorbic acid). The experimental procedures relating to BM-MSC encapsulation and cell culture are summarized in Fig. 1.

MTT assay

The mitochondrial dehydrogenase activity of the encapsulated BM-MSCs was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)-based assay (Sigma). Briefly, 30 μ L of MTT solution (5 mg/mL) was added to 270 μ L α -MEM containing BM-MSCs-encapsulated microbeads, and incubated at 37 °C and under 5% CO₂ for 4 h. At the

end of incubation period, the MTT solution was aspirated and the intracellular insoluble purple formazan product was solubilized in 0.1 N HCl in isopropanol via pipetting upon inverted microscope imaging (Nikon TS 100, Tokyo, Japan) 21 days after encapsulation in osteogenic cultures. The absorbance values of the encapsulated cells cultured in standard medium were quantified by reading optical density values at 570 nm using a spectrophotometer. This assay was repeated for all 3 different encapsulation batches.

Osteogenic bioreactor culture

Rat BM-MSCs encapsulated microbeads were collected and thoroughly washed with medium and then placed in STL V (Synthecon Inc., Austin, TX, USA) with osteogenic medium consisting of α -MEM, 10% FBS, 1% penicillin–streptomycin solution, 10 mM β -glycerophosphate, 10^{-8} M dexamethasone and 50 μ M freshly prepared ascorbic acid for up to 4 weeks ($n = 3$; bioreactors containing cells retrieved from separate encapsulation batches). Throughout the experiment, the speed of rotation was adjusted to suspend the constructs freely in culture medium. Some samples were evaluated by SEM and MTT assays, while others were evaluated by immunohistochemical analyses (IHC).

Scanning electron microscopy

SEM was employed to examine the surface/cross-sectional morphology of the composite microbeads and the distribution of encapsulated BM-MSCs within the polymer matrix. The microbeads were removed from the bioreactor and fixed with 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4) for 24 h. Following the fixation step, the samples were placed through a series of graded ethanol dehydrations and allowed to air-dry. Subsequently, the samples were sputter coated with a 10 nm thick layer of gold/palladium, and then analyzed under a Jeol-JSM electron microscope (Tokyo, Japan) operating at 20 kV, with magnifications between 50 $\times$ and 5000 $\times$.

In vivo studies

Animal experiments were approved by the Institutional Animal Ethics Committee (2016–97; AU,

Fig. 1 Schematic illustration of the experimental procedures related to mesenchymal stem cell encapsulation and culture

Ankara, Turkey), and were carried out in accordance with the international guidelines for the care and use of laboratory animals. A total of 27 adult Wistar rats were used: 3 rats for the isolation of BM-MSCs, 12 rats for the *in vivo* degradation study, and 12 rats for transplantation of C/HAp-encapsulated BM-MSCs.

For the *in vivo* degradation studies, the sterile microbeads were kept in the incubator under standard culture conditions overnight before implantation. Male Wistar rats (15–20 weeks old; 300 ± 50 g; $n = 12$) were anesthetized by a subcutaneous injection of a solution of avertin (300 mg/kg; 1.25%; Sigma-Aldrich). The groin area was shaved and disinfected with isopropanol and iodine. A 2 cm incision was made through the skin on either the left-hand or right-hand groin fasciae. Using aseptic technique, the microbeads (20 each) were implanted into the epigastric fasciovascular flap of male Wistar rats (Linn et al. 2003). This ectopic implantation site harbors the adipose, the muscle tissue, and the blood vessels side by side. The available volume at this subcutaneous site can help preserving the shape of the implanted microbeads. The incisions were closed with 4-0 silk sutures (Ethicon, Somerville, NJ, USA). Rats were sacrificed and the microbeads were excised from the epigastric area together with the surrounding tissue at predetermined time-points (7, 14, 21, 30, 60 and

180 days) after surgery. Samples were analyzed histologically to evaluate *in vivo* degradation and biocompatibility properties of the constructs.

A pilot study involving animal experiments was conducted to examine the bone repair capacity of C/HAp encapsulated mesenchymal stem cells by implanting in an orthotopic non-critical size uncortical femoral rat bone defect model (Keibl et al. 2011). Since our preliminary experience with direct transplantation of MSC suspension to the bone defect did not exhibit any positive outcome (same as untreated defect), we did not use this approach as a control. Male Wistar rats (300 ± 50 g) were used in animal tests ($n = 12$). The surgical procedures were performed in aseptic conditions under general anesthesia. The cells containing composite microbeads were cultured for 7 days in the osteogenic medium before implantation. After shaving and disinfecting the operation site, the diaphyses of the femurs were exposed. A bone defect was created by the help of a minidrill (with sterile cooling saline) in the mid-shaft part of the femur ($\Phi = 1.0$ – 1.5 mm) and then the C/HAp encapsulated cells (about 8–10 each) were implanted into the defect. The samples were stabilized by covering the defect with muscle tissue; the wound was closed with 4-0 silk sutures. Subjects with empty defects and implanted with acellular C/HAp

microbeads served as the controls. Rats were given free access to food, water and monitored for complications. A dental X-Ray equipment (Hilger & Watts, London, UK) was used to assess the restoration capacity of the cell-laden microbeads 45 days after surgery.

Immunohistochemistry

Rat BM-MSCs containing C/HAp microbeads were retrieved at various time-points for IHC analysis. They were rinsed in 0.1 M PBS and fixed with 10% formaldehyde in phosphate buffer at 4 °C. The fixed samples were dehydrated through a series of graded ethanol and xylene. After paraffin embedding, the tissue blocks were sectioned at 5–10 µm using a microtome. IHC staining was carried out according to the manufacturer's instructions using avidin–biotin horseradish peroxidase detection system (HRP-AEC, Lab-Vision, Fremont, CA, USA) to detect osteopontin [OSP (AKM2A1) polyclonal; sc-21742] and osteocalcin [OSC (V19) polyclonal; sc-18319] (Santa Cruz Biotechnology, Dallas, TX, USA) in 1:50 dilutions. Finally, the specimens were visualized under a Leica DM 4000B model digital light microscope (Wetzlar, Germany).

Statistical analysis

Data from three independent experiments are expressed as mean $\pm$ SD. Unpaired Student's *t* test was carried out to establish the difference for indicated parameters between groups. ($p < 0.05$) was considered to be statistically significant.

Results

In vitro degradation of empty C/HAp microbeads

Empty C/HAp microbeads were fabricated with a mean diameter of 430 ± 30 µm (Table 1). The in vitro weight loss of the composite microbeads was assessed after 180 days of incubation in PBS. Experiments demonstrated gradual degradation of the microbeads. The microbeads lost more than 40% of their initial dry mass after 180 days of incubation in PBS (Figure 2a).

In vivo degradation of empty C/HAp microbeads

The in vivo degradation and the biocompatibility of the C/HAp microbeads were evaluated by implanting the composite microbeads inside the epigastric fasciovascular flap of Wistar rats. In vivo findings containing H&E-stained histology sections retrieved at 7, 14, 21, 30, 60 and 180 days post-operation are presented in Fig. 2b. The implants were well-tolerated by the subjects, and the wounds healed quickly. Histology confirmed that the microbeads exhibited some inflammation especially within 21 days of implantation. We did not observe any sign of fibrous capsule formation. From our qualitative observations, the degree of inflammation decreased by time, in terms of macrophage density. As shown in the micrographs, the morphology of the implants changed and the microbeads disintegrated by time (Fig. 2b). At the first, second and third weeks, the microbeads remained in their original shape. The in vivo degradation of the microbeads started at the tissue-biomaterial interphase after 60 days and the microbeads had been almost completely degraded after 180 days (Fig. 2b).

Culture of BM-MSCs

Rat BM-MSCs were successfully isolated from the bone marrows of three donor rats which were pooled together, and expanded. Immunophenotype analysis of rat BM-MSCs showed that the cells highly expressed multipotent stromal cell-associated surface markers CD29 (99.11%), CD105 (99.37%), CD44 (90.07%), CD73 (89.52%), CD90 (85.90%), CD54 (90.07%) (Fig. 3a). The cells displayed low expression of the surface marker CD106 (14.63%), and did not express CD34 (0%), CD31 (0%), and CD45 (0.2%). These cells formed typical colonies (colony forming unit fibroblasts; CFU-f) in their extended confluent cultures without passaging (Fig. 3b). The chondrogenic differentiation of BM-MSC pellet culture after 28 days was demonstrated by the staining of the glycosaminoglycans by Alcian blue (Fig. 3c). The adipogenic differentiation of BM-MSC culture was demonstrated by the formation of lipid droplets after 21 days (Oil-Red O staining) (Fig. 3d). The osteogenic differentiation capacity of the cells was also confirmed by Alizarin red staining, which showed the accumulation of mineralized extracellular matrix deposits after 28 days (Fig. 3e).

Table 1 Specification of C/HAp microbeads

Type	Chitosan (wt%)	HAp (wt%)	Buffer uptake (%)*	Mean diameter (μm)**	
				Empty	Encapsulated
I	3	1	265.1 ± 7.7	$1050 \pm 110 \mu\text{m}$	$1170 \pm 160 \mu\text{m}$
II	6	1	300.8 ± 17.9	$870 \pm 60 \mu\text{m}$	$1030 \pm 90 \mu\text{m}$
III	9	1	450.5 ± 12.4	$430 \pm 30 \mu\text{m}$	$560 \pm 70 \mu\text{m}$

*Phosphate buffered saline (pH 7.4) uptake of 1.00 g dry empty microbeads after 24 h

**Diameter range of 45 wet microbeads from 3 batches for each experimental group

C/HAp encapsulated BM-MSCs

Cells were successfully encapsulated within C/HAp microbeads. Viability of BM-MSCs after encapsulation was investigated using the MTT assay. The results showed that BM-MSCs had maintained > 85% of mitochondrial dehydrogenase activity after encapsulation and > 80% at the seventh day of culture (Table 2). Mitochondrial dehydrogenase activity of the encapsulated cells was preserved during the osteogenic bioreactor culture. We did not observe significant variability between batches, in terms of cell encapsulation yield and viability (< 2.5% variability), and identity (trilineage differentiation potential). The representative phase-contrast image shows the homogenous distribution of formazan crystals formed at the surface and vicinity of a microbead after 21 days (Fig. 4a), indicating that C/HAp microbeads supported the viability of the cells during osteogenic induction. The surface morphology of the microbeads and the distribution of encapsulated BM-MSCs were evaluated by SEM analysis (Fig. 4b–d). Findings showed the C/HAp microbeads were spherical with a diameter of ~ 250 – $300 \mu\text{m}$, with rough surface characteristics (Fig. 4b). The hydroxyapatite particles were distributed inside the chitosan matrix as seen from the cross-sectional image (Fig. 4c). SEM observations performed on samples on the 14th day post-encapsulation revealed that BM-MSCs had attached to the HAp microparticles via pseudopodia (Fig. 4d). C/HAp microbeads were mechanically stable and practically maintained their size and shape for the duration of the microgravity bioreactor culture (Table 3).

Immunohistochemistry

The osteogenic differentiation ability of encapsulated BM-MSCs was evaluated by immunohistochemistry. Expression of Osteopontin (Fig. 5a) and Osteocalcin (Fig. 5b) was significantly increased in the cellular constructs after 28 days of osteogenic bioreactor culture, while C/HAp microbeads devoid of cells were not immuno-stained. Our findings revealed that BM-MSCs differentiated into osteoblastic cells during the STLV bioreactor culture and formed bone-like tissue structures when encapsulated and cultured within C/HAp microbeads.

In vivo bone repair

The in vivo bone forming/healing ability of the developed BM-MSCs-encapsulated C/HAp microbeads was investigated by conducting a pilot study on a rat femur defect model. All animals survived the surgery with no adverse reactions to the procedure. They returned to weight-bearing activity shortly after surgery. To visualize the bone defect healing process, radiographs were taken 45 days following the surgery and evaluated qualitatively (Fig. 5c–e). After 45 days, the defects were completely filled with newly formed bone-like tissue in the group implanted with BM-MSCs encapsulated in C/HAp microbeads (compare Fig. 5c with d). On the other hand, the defects in the control group did not demonstrate a significant level of bone regeneration (compare Fig. 5c with e), indicating that only cells in microbead structures promote closure of surgically-created small bone defects in the rat femur.

Fig. 2 **a** In vitro degradation profile of C/HAp microbeads in dissolution buffer of PBS at pH 7.4 by time. **b** In vivo degradation of C/HAp microbeads implanted into the epigastric fasciovascular flap of Wistar rats at 7, 14, 21, 30, 60 and 180 days, respectively (hematoxyline and eosin staining). Scale bars: 100 μ m

Discussion

The objective of this study was to evaluate the *in vitro* and *in vivo* osteogenic potential of rat BM-MSCs by using chitosan/hydroxyapatite microbeads as encapsulation matrix under rotating microgravity culture system.

Cell-based bone tissue-engineering approaches are valid options to overcome clinical challenges of bone repair. Several different techniques have been used to produce bone graft substitutes with porous matrices. The porous matrices can be designed in different forms including microbeads, sponges, hydrogels and fibers (Botchwey et al. 2001; Hsu et al. 1999).

In the past few years, some studies have focused on the use of C/HAp composite microbeads due to their useful properties and versatility (Inanç et al. 2007; Ding and Teng 2012). C/HAp composite systems are capable of stimulating cells or immature bone cells to grow and mature by promoting the formation of bonds between tissue and material. In our study, chitosan and hydroxyapatite were chosen as the vehicles to produce composite microbeads although bearing some differences from previous studies, such as the choice of cell source and microgravity cell culture method.

In the present study, C/HAp microbeads were prepared using tripolyphosphate as coagulating agent in an encapsulator system. There are slight ionic interactions between OH^- and triphosphoric ions in the TPP solution and NH_3^+ ions of chitosan. When C/HAp solutions were added dropwise into TPP solution, TPP diffused into C droplets during the aging process resulting in instantaneous formation of microbeads (Granja et al. 2004). Compared with other methods (Qiu et al. 2000; Sivakumar et al. 2002), preparation process for C/HAp composite microbeads was suitable for cell encapsulation purposes, as it was carried out under mild conditions with the absence of organic substances. Moreover, the encapsulator system has a closed container, which supplies a sterile environment for cell culture studies and the size distribution of the resulting microbeads is even. According to SEM images, the C/HAp microbeads were regular and spherically shaped with a diameter of around 250–300 μm . The cross-section images revealed that BM-MSCs attached and were distributed inside the microbeads in a round-shaped morphology. SEM images also showed that the microbeads maintained their initial shape without disintegration, which

suggests that the encapsulator system provided a stable structure and had no adverse effect on the integrity of microbeads and the ratio of C/HAp was suitable for the synthesis of stable microbeads.

The biocompatibility and degradation properties are crucial to the long-term success of a BTE template. The implantation process of tissue engineered templates is subjected to complications such as inflammation, rejection or pain depending on the composition of the material. The implanted materials that cause low inflammatory response are preferable. The implanted biomaterial should be resorbable through biodegradation processes to replace the natural tissue (Baykan et al. 2014). The early-stage *in vitro* inflammatory response of C/HAp has already been investigated by Przekora and Ginalska (2016). We evaluated the degradation rate of microbeads for up to 6 months. *In vitro* degradation studies showed that the microbeads lost more than 40% of their initial mass after 180 days of incubation in PBS. This degradation rate of C/HAp could help maintain the structure and strength of the microbeads for cell proliferation and tissue formation. To investigate *in vivo* biocompatibility and degradability of composite microbeads, spheres were implanted into the epigastric fasciovascular flap of the male Wistar rats in long-term implantation periods. Throughout the experiment, animals remained in good health without any complications. The primary histological observations demonstrated that the composite microbeads triggered a mild and quite constant inflammatory response and faded within 6-months. In our study, we did not observe any sign of fibrous capsule formation, which indicates the timely removal of the biomaterial. Thus, a slow biodegradation ultimately leads to wall-off the bioabsorbing composition. We conclude that the obtained microbeads are biodegradable and biocompatible, ensuring a favorable environment for remodeling process.

HAp has been recognized as a promising material for bone tissue regeneration due to its bioactive and osteoconductive properties. The addition of HAp into polymers results in the alteration of a number of properties, e.g. porosity, roughness, topography and cell adhesion. The roughness of composite material is a significant engineering factor promoting initial cell attachment by ensuring large surface area to the composite scaffold (Dorozhkin 2010; Kim et al. 2011). In this study, chitosan was used as a polymer matrix.

Fig. 3 Characterization of rat bone marrow mesenchymal stem cells. **a** Immunophenotype analysis was performed by flow cytometry. BM-MSCs highly expressed multipotent stromal cell surface markers CD29, CD105, CD44, CD73, CD90, CD54. CD106 marker was expressed at negligible levels, while CD31 and CD34 markers were not expressed. **b** Crystal violet-stained CFU-f formations (*) due to extended culture without passaging. **c** Chondrogenic differentiation demonstrated by the staining of the glycosaminoglycans after 28 days (Alcian blue staining). **d** Adipogenic differentiation of BM-MSCs demonstrated by the formation of lipid droplets (arrows) after 21 days (Oil-Red O staining). **e** Osteogenic differentiation demonstrated by the accumulation of mineralized extracellular matrix after 28 days (Alizarin red staining). Scale bars: 100 μ m

The hydrophilic nature of chitosan enhances HAP wettability and leads to full dispersion inside the chitosan matrix. The cross-sectional SEM images demonstrated the homogeneous distribution of HAP microparticles inside the chitosan matrix, which possibly improved cell attachment as pointed out by others. SEM images show a direct contact of encapsulated cells with HAP microparticles, which supported and enhanced BM-MSCs attachment, proliferation and osteogenic differentiation.

Mesenchymal stromal cells are found in almost all tissues of the body and they differentiate into various cell types including adipocytes, chondrocytes,

Table 2 Specification of bone marrow mesenchymal stem cells encapsulation in C/HAp microbeads

Parameter	Type III microbeads
Number of microbeads per encapsulation suspension	350 $\pm$ 50
Number of cells per mL encapsulation suspension	$\sim 1.0 \times 10^6$
Cell encapsulation efficiency	87.5 $\pm$ 2.0%
Number of cells per microbead	2500 $\pm$ 350
Viability of encapsulated cells	> 85%
Viability of encapsulated cells after 1 week	> 80%

Fig. 4 **a** Representative phase-contrast image showing formazan crystals formed at the surface and vicinity of a microbead indicating that C/HAp microbeads supported viability of the

cells. **b–d** Scanning electron micrographs of **b**, **c** the surface, and **d** cross-section of C/HAp encapsulated BM-MSCs. Arrows point to cells

Table 3 Stability of C/HAp microbeads (Type III) during the microgravity bioreactor culture

	Day 0		Day 28	
	Mean diameter *	Shape	Mean diameter *	Shape
Empty	430 ± 30 µm	Spherical	480 ± 60 µm	Spherical
Encapsulated	560 ± 70 µm	Spherical	540 ± 50 µm	Spherical

*Diameter range of 45 wet microbeads from 3 batches for each experimental group

Fig. 5 **a, b** Immunohistochemical analysis of C/HAp encapsulated rat BM-MSCs under osteogenic induction culture after 28 days in STLV bioreactor: **a** Anti-rat osteopontin, **b** anti-rat osteocalcin stainings, Scale bars: 200 µm. Insets: IHC images of controls; C/HAp devoid of cells. **c, e** Representative radiographs

from the pilot in vivo study. Bone defect filled with C/HAp encapsulated BM-MSCs: **a** on day 0, and **b** after 45 days. **c** is the control group which did not receive cell-encapsulated microbeads after 45 days

osteocytes, and cardiomyocytes when induced with appropriate cues in vitro or in vivo. While direct delivery of MSCs appears to be safe, osteogenic biomaterials as artificial extracellular templates assures the localized transplantation of the MSCs in the vicinity of the bone defects (Ponce et al. 2006; Han

et al. 2012). In this study, C/HAp microbeads were utilized as an artificial template to provide a biomimicking microenvironment for BM-MSCs. Cell encapsulation has an enormous clinical potential for the treatment of a variety of diseases such as osseous tumours and trauma (An et al. 2015). However, one

concern is that diffusion limitations within especially larger beads may be inconvenient for supplying sufficient nutrients and oxygen to the cells in the interior of beads. It is essential to provide optimal cell culture conditions for the encapsulated cells. Several bioreactors have been developed for the culture of the cell-laden biomaterials. In this study, encapsulated cells were cultured in the turning lateral vessel-type bioreactor. STLV bioreactor provides a high mass transfer of culture medium to the cells and with low shear force. In addition, the medium is recycled continuously, which promotes cell viability and bioactivity of cells. Findings showed that STVL bioreactor contributed to the expression of osteopontin and osteocalcin, providing a more controllable movement and medium exchange to the cells encapsulated in C/HAp microbeads. The results of our study are consistent with the previous literature (Emin et al. 2008; Inanç et al. 2007).

To induce differentiation into bone nodules, encapsulated BM-MSCs were cultured in the presence of in vitro inducers of osteogenesis, ascorbic acid, β -glycerophosphate and dexamethasone. The osteogenic differentiation of BM-MSCs is generally characterized with osteoblastic cell morphology and the expression of osteoblastic markers.

The osteogenic capacity of cells containing composite microbeads was evaluated by the analysis of osteogenic markers, osteopontin and osteocalcin which have various biological functions in bone formation. OPN is a noncollagenous extracellular matrix (ECM) protein that regulates early stages of biomineralization and osteoclast activity. OC, also known as bone Gla protein, is secreted by osteoblasts. OC plays an important role in the process of ossification for bone formation process. Immunohistochemistry findings suggest that the BM-MSCs encapsulated C/HAp microbeads expressed specific bone tissue markers OPN and OC, pointing out the maintenance of metabolic activity of cells and in vitro bone-like tissue formation.

The bone formation performance of the developed microbeads was also investigated. The initial in vivo studies showed that C/HAp microbeads containing BM-MSCs were able to positively influence the restoration of surgically-created small bone defect of the rat femur.

The combined results of in vitro and in vivo assays clearly showed successful entrapment of BM-MSCs

and differentiation into osteogenic cells, while maintaining an optimal proliferation trend in the C/HAp microbeads. Our findings point out that the composite microbeads provide a convenient vessel for the cell-based tissue engineering applications of the bone.

Conclusion

In this study, we have presented the efficient use of biocompatible C/HAp microbeads as a cell encapsulation matrix. We have shown that C/HAp encapsulated BM-MSCs cultured under osteoinductive medium and rotating culture conditions differentiated into osteogenic cells and formed bone-like modular tissue in vitro. We have evaluated the bone repair potential of C/HAp encapsulated BM-MSCs in a pilot study using non-critical size femoral defect model in rats. Nevertheless, further work will be needed to evaluate the bone repair capacity of C/HAp encapsulated BM-MSCs on a critical-size segmental defect by using biomechanical, radiographic and histological analysis.

Compliance with ethical standards

Conflict of interest YME is the founder and shareholder of Biovalda Health Technologies, Inc. (Ankara, Turkey). The authors declare no competing financial interests in relation to this article.

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