

High mannuronic acid containing alginate affects the differentiation of Wharton's jelly-derived stem cells to hepatocyte-like cell

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ABSTRACT

For transplantation of cell into injured tissues, cells should be transferred to the damaged site through an adequate carrier. Nevertheless, the nutrient-limited and hypoxic condition in the carrier can bring about broad cell death. This study set to assess the impact of alginate concentrations on the differentiation and the proliferation of cells encapsulated in alginate hydrogels. Human Wharton's Jelly-derived Mesenchymal Stem Cells (HWJ-MSCs) were encapsulated in two concentrations of alginate hydrogel. Then, the proliferation and the hepatic differentiation were evaluated with an MTT assay and the enzyme-linked immunosorbent assay software and urea production. The results demonstrated that the proliferation of cell and urea production in 1.5% alginate concentration was higher than in 2.5% alginate concentration in the hydrogels of alginate. We deduce that the optimized alginate hydrogel concentration is necessary for achieving comparable cell activities in three-dimensional culture.

Key words: Alginate, cell encapsulation, hepatic differentiation, mesenchymal stem cells, umbilical cord

INTRODUCTION

The adult liver performs various metabolic actions.^[1] Recently, there has been extensive research on the transplantation of living cells into damaged tissues for tissue repair.^[2,3] Stem cells (SCs) are exciting for regenerative medicine applications because of their strong proliferative ability and their capability of multilineage differentiation.^[4] Certain studies have suggested that mesenchymal stem cells (HWJ-MSCs) have a capability for use in cell treatment procedures.^[5] Scaffolds are crucial for the regeneration of

tissue and injectable scaffolds may be utilized in minimally invasive procedures; they fit intimately into complex-shaped defects. Besides scaffolds, SCs are another key element in engineering of tissue.^[4,6] In a three-dimensional (3D) culture of cell, cells are placed in a matrix or scaffold.^[7] Cell encapsulation in alginate hydrogels represents highly investigated approaches for cell therapy.^[2,8] Alginate gels are being actively investigated as a consequence of their function to mediate the regeneration and the engineering of a variety of other tissues and organs.^[9]

The polymer of alginate has been used with various cell types its convenient dissolution of gels for retrieval of cell, its limpidity for microscopic assessment, and its gel pore network, which induces nutrients and waste matters' diffusion. The alginate molecular scaffold includes blocks

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of consecutive G or M monomers (-GGG- or -MMM-) or alternating monomers (-MGMG-). In extreme viscous solutions of alginate, viscosity is affected via ionic power, temperature, and weight of molecules, also at lower concentrations.^[10-12]

In this investigation, it was assumed that the gel properties regulate the cells' proliferation and differentiation which are encapsulated in the alginate beads. To increase cell viability during the encapsulation process, a minimum essential medium (a-MEM) was transferred into alginate beads, a modification that was anticipated to improve the accessibility of nutrients for the encapsulated cell. The proliferation and the differentiation of HWJ-MSCs were obtained *in vitro* to indicate how the alginate gels' microstructure regulated cell fate in the 3D cell culture.

MATERIALS AND METHODS

The materials used in the study included sodium alginate (medium viscosity; mannuronic acid content 50% from Nova Matrix Co.); trypsin/EDTA; fibroblast growth factor 4 (FGF4); hepatocyte growth factor (HGF); phosphate-buffered saline (PBS); glucagon; insulin-transferrin-selenium (ITS) supplement (100X); Dulbecco's modified Eagle's medium (DMEM); oncostatin M; dexamethasone (Dex); dimethyl sulfoxide (DMSO); trichostatin A (TSA) from Sigma; fetal bovine serum (FBS) from Gibco; penicillin/streptomycin; L-ascorbic acid 2-phosphate; enzyme-linked immunosorbent assay (ELISA) kit (Pars Azmun); urea assay kit (Pars Azmun); periodic acid solution; and Schiff (Sigma).

The study protocol was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (AJUMS), and all procedures were performed according to the ethical committee approval (Ethical code: IR.AJUMS.REC1395.267).

Isolation and culture of human umbilical cord mesenchymal stem cells

The isolation and culture methods were explained briefly as described earlier (Azandeh *et al.*, 2016). The pieces of Wharton's jelly were cultured in the culture containing DMEM (low glucose [LG]) and 2 mM L-glutamine, supplemented with 10% FBS and 100 U penicillin/streptomycin [Figure 1b]. Then, the cells with 80% confluence were trypsinized and transferred into a 25 cm² flask. The cells were passaged every 5 days; the medium was replaced every 3 days and subcultured when they reached a confluence of 80%–90% [Figure 1].^[13]

Phenotypic analysis

The surface antigens of Human Wharton's Jelly-derived Mesenchymal Stem Cells (HWJ-MSCs) were analyzed by flow cytometry technique. Briefly, the cells were incubated with anti-human antibodies against CD105, CD90, CD34,

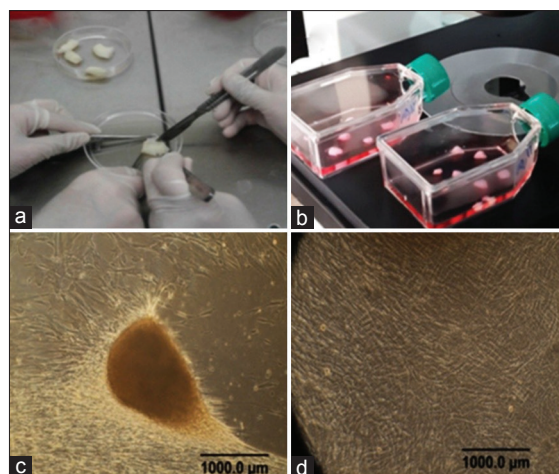


Figure 1: Isolation and culture of MSCs. (a) The umbilical cord, (b) Wharton's jelly cut into 2–5 mm explants, (c) after 7–14 days, hUCMSCs were grown from the explants, and (d) MSCs after two passages

and CD45 for 30 min at 4°C in the dark condition. All antibodies were bought from eBioscience (San Diego, CA). Negative and isotype controls were performed. After cell staining, for each sample, 10,000 events were counted by a Dako Galaxy flow cytometer and data were analyzed using FlowJo version 8.8.7 software (Treestar, OR).

Cell encapsulation

Preparation of alginate solutions of concentrations of 1.5% and 2.5% (w/v) was performed by dissolving alginate into 150 mM of NaCl. The solutions were heated on a hot plate and stirred them thoroughly. Their processes of filtration were sterilized using a 0.22-µm filter.

- CaCl₂ (102 mM) solution: Prepared the solution in dH₂O and sterilized using a 0.22-µm filter
- NaCl (150 mM) solution: Prepared the solution in dH₂O and sterilized using a 0.22-µm filter. Store the working solutions at 4°C.^[13]

The HWJ-MSCs were resuspended in the 1.5% and the 2.5% alginate solutions at 1×10^6 cells/ml. Then, they were mixed by slowly pipetting and drop falls from needle of syringe into the CaCl₂ solution. The alginate beads were incubated at 37°C for 15 min to allow the Ca cations to fully diffuse through alginate and cross-link the alginate cell suspension. CaCl₂ solution was pipetted off; then, the alginate beads were rinsed several times with normal saline and the normal saline was replaced with 3 ml of the culture medium [Figure 2].^[2,14]

After the encapsulation of cells, each of the trials was performed three times.

Hepatic differentiation

This method is explained as briefly described earlier (Yoon *et al.*, 2010). The hWJ-HWJ-MSCs encapsulated in the alginate beads were differentiated by a four-step

protocol; Step 1: DMEM (LG) +10 ng/ml FGF4; Step 2: DMEM (LG) +1% ITS + 20 ng/ml HGF; Step 3: DMEM (LG) +100 nM Dex + 1% ITS + 100 nM glucagon + 10 ng/ml OSM; Step 4: DMEM (LG) +100 nM Dex + 1% ITS + 100 nM glucagon + 10 ng/ml + (1% DMSO or 100 nM TSA). The medium was exchanged every 2 days [Table 1].^[15]

Albumin secretion

To determine the albumin content in the supernatant, the immunosorbent assay ELISAs (Pars Azmun kit) were used based on the instructions of manufacturer (the complex with Bromocresol green). The absorbance was obtained by a photometer tool (Convergys 100, Germany) at 546 nm.

Urea assay

The urea concentration was determined using QuantiChrom™ urea assay (Pars Azmun kit) based the manufacturer's protocol (Berthelot method). The absorbance was measured by a photometer tool (Convergys100, Germany) at 578 nm.

Cell viability assay

The encapsulated hHWJ-MSCs' viability was assessed by the MTT assay that explained earlier by Son et al. Briefly, For MTT assay, the capsules were transferred into a 24-well plate. After adding 1ml of culture medium to each well, the amount of 100 ml of MTT solution was also added and incubated for 4 h at 5% CO₂ and 37 °C incubator. The amount of 100 ml of each well was transferred to a 96-well plate and the absorbance was measured at 570nm.

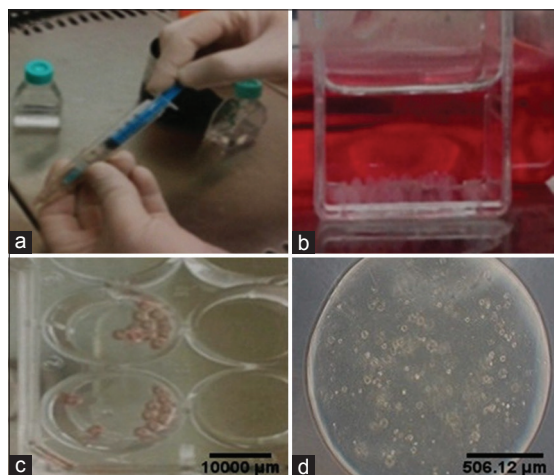


Figure 2: Cell encapsulation. (a) Resuspend MSCs in alginate solution, (b) gel forming in CaCl₂ solution, (c) the alginate beads, and (d) the MSCs in the beads have a round shape

Table 1: A four-step protocol for differentiation period

Step	Step 1 (2 days)	Step 2 (2 days)	Step 3 (2 days)	Step 4 (8 days)
Differentiation factors	FGF4	ITS + HGF	Dexamethasone + ITS glucagon + OSM	Dexamethasone + ITS glucagon + OSM + TSA

TSA: Trichostatin A, ITS: Insulin-transferrin-selenium, HGF: Hepatocyte growth factor, FGF4: Fibroblast growth factor 4, OSM: Oncostatin M

The beads without cells were applied to the control.^[16]

Periodic acid–Schiff Staining

Periodic acid–Schiff (PAS) staining for glycogen: Differentiated cells on day 14 were fixed in paraformaldehyde 4% and were afterward incubated in periodic acid solution 1% for 5 min at room temperature.

The cells are rinsed in PBS followed by in rinsing in distilled water and incubated in Schiff reagent for 10 min.

The images were taken with an Olympus microscope.^[17]

Scanning electron microscopy

The alginate beads were fixed in 2.5% glutardialdehyde and then dried on filter paper.

The beads' morphology and the encapsulated cells were assessed by a scanning electron microscopy.

Statistical analysis

Data are presented as mean-standard deviation from three independent experiments. The statistics generated in this study were performed using the SPSS software (version 22.0; SPSS Inc., Chicago, IL). Significant differences were analyzed by the Mann–Whitney analysis. The results were assumed meaningful when the $P < 0.05$.

Ethical consideration

The study protocol was approved by the Ethics Committee of AJUMS and all procedures were performed according to the Ethics Committee's approval (Ethical code: IR.AJUMS.REC.1395.267).

RESULTS

Morphological characterization of alginate beads

The morphology of hWJ-HWJ-MSCs was changed from a round to a spindle shape after 2D culture in a flask [Figures 1c and d]. The hWJ-HWJ-MSCs in the beads had a round shape [Figure 2]. During the continuation of proliferation, the cells formed gatherings inside the beads. The gatherings were formed early in 1.5% (w/v) beads [Figure 3].

The beads' internal and external morphology was assessed by the scanning electron microscopy [Figures 4a-d] and revealed the full-of-holes structure of the bead matrix, whereby the pore size depended on the initial concentration

of the alginate applied in the preparation; the average pore size for 1.5% (w/v) alginate beads was more than 2.5% (w/v). The scanning electron microscopic images showed that the porous alginate scaffolds were capable of growth, survival, and differentiation of the mesenchymal stem cell protection [Figure 4].

Phenotypic analysis

The cell surface marker expression of HWJ-MSCs was analyzed after passage 3. Results of flow cytometry revealed that many cells expressed high level of CD90 (99.3%) and CD105 (100%) as positive markers for hWJ-MSCs but not expressed CD34 and CD45 as hematopoietic markers [Figure 5].

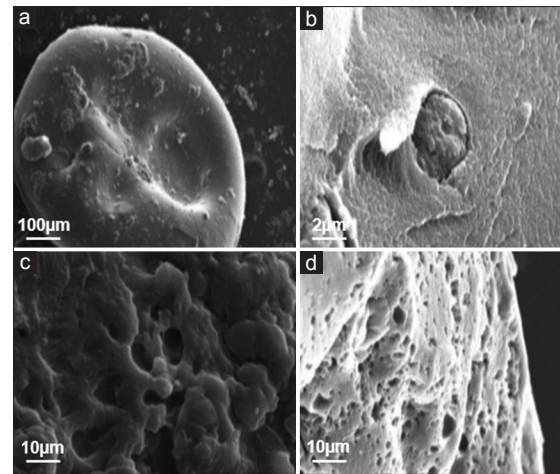


Figure 4: Scanning electron microscopic images of alginate beads formed in calcium solutions. (a) External morphology, (b) cell encapsulated in alginate bead, (c and d) scanning electron microscopic image of 1.5% (w/v) and 2.5% (w/v) alginate structures

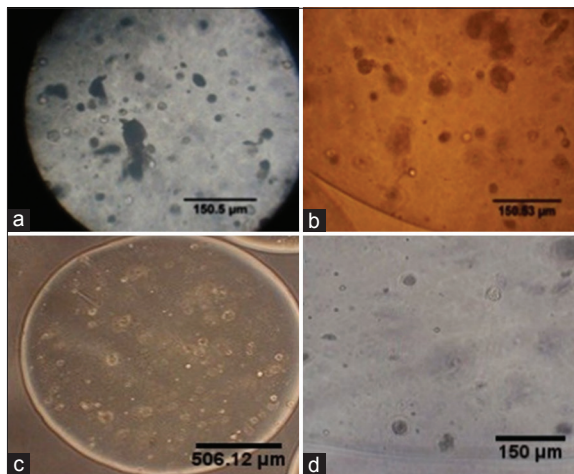


Figure 3: The first cell clusters in Group 1.5% (w/v) and Group 2.5% (w/v) were formed, respectively, on day 8 (a) and day 10 (b). (c and d) Control groups with 1.5 and 2.5% alginate

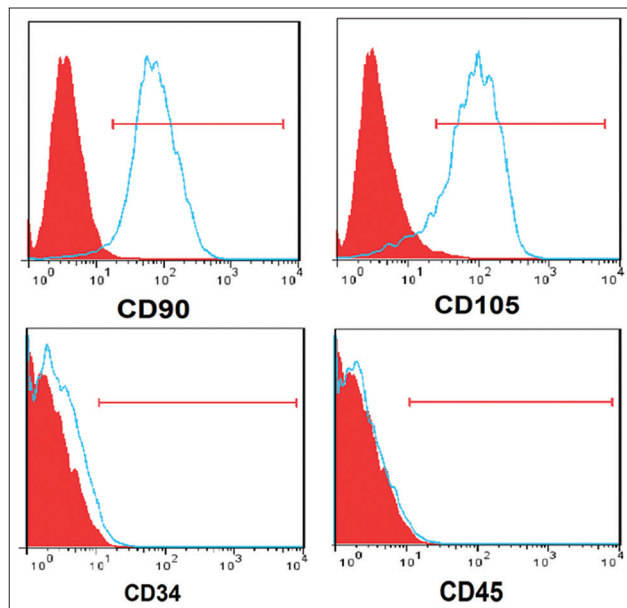


Figure 5: Analysis by flow cytometry shows that mesenchymal stem cells are positive for the expression of CD90, CD105, but negative for the expression of CD34, CD45

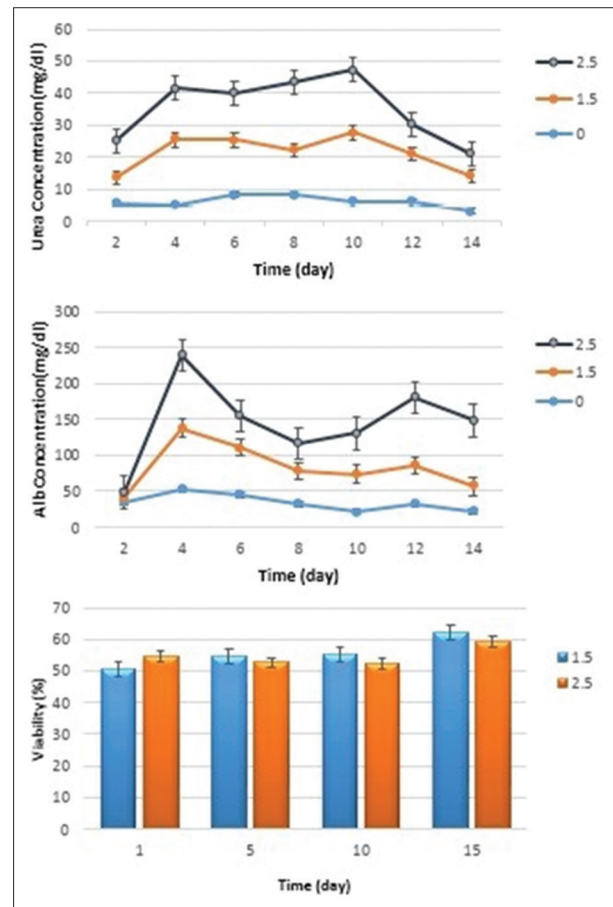


Figure 6: Albumin concentration was measured every two days by using an enzyme-linked immunosorbent assay kit for all the groups (upper graph). The urea synthesis rate during hepatic differentiation was measured every two days (middle graph). The proliferation of the MSCs culture for 15 days in two concentration of sodium alginate was determined by MTT assay on days 1, 5, 10, and 15 (lower graph)

Hepatic differentiation of mesenchymal stem cells

Hepatic differentiation in HWJ-MSCs cultured in beads with 1.5% and 2.5% (w/v) concentrations for the four-step (14 days) protocol was evaluated by the measurement of albumin and urea. When the culture medium was exchanged every 2 days, the previous medium was kept for the measurement of albumin and urea. As shown in Figure 6, albumin concentration was measured every 2 days by using an ELISA kit for all the groups (upper graph). The urea synthesis rate during hepatic differentiation was measured every 2 days (middle graph). In two beads, albumin increased on day 4 by adding HGF, and then, it was decreased [Figure 7]. Once more, it was intermittently increased on days 8 and 10 in beads with concentrations of 1.5% and 2.5% (w/v). The urea synthesis rate in beads with 1.5% (w/v) concentration was higher than 2.5% (w/v) ($P < 0.05$) [Figure 6]. The findings showed that the bead with a concentration of 1.5% (w/v) enhanced the biological performance of urea release in cells of hepatocyte from the HWJ-MSCs.

Cell viability within alginate beads

The encapsulated hWJ-HWJ-MSCs' viability was assessed by an MTT assay. Figure 6 (lower graph) exhibits the proliferation of hWJ-HWJ-MSCs in two concentrations of sodium alginate, which was measured using optical density at 570 nm after MTT treatment. The optical density value was more in 1.5% (w/v) alginate on days 5, 10, and 15 ($P < 0.05$). This result revealed that the hWJ-HWJ-MSCs' cell proliferation in beads with 1.5% (w/v) concentration was higher than that in the 2.5% (w/v) concentration.

Periodic acid–Schiff Staining

PAS staining in cells grown in alginate beads and differentiated from hepatocyte-like cells by the four-step protocol showed that the particles of glycogen were stored in the cell cytoplasm [Figure 7].

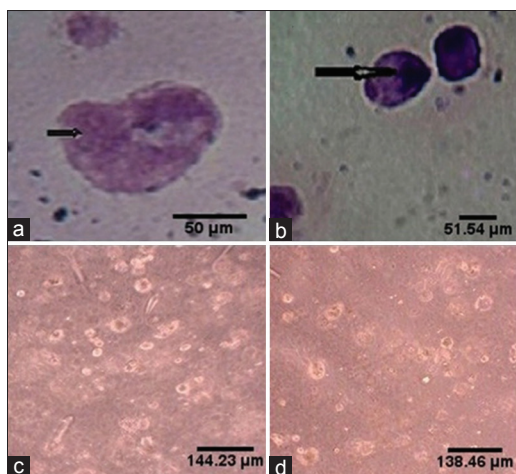


Figure 7: (a and b) Periodic acid–Schiff staining showed that differentiated cells stored glycogen in three-dimensional culture. (c and d): Accordingly, control groups of 1.5% and 2.5% do not show any storage of glycogen in the cell cytoplasm

DISCUSSION

The MS cell proliferation within 1.5% and 2.5% (w/v) alginate was quantified to optimize the encapsulation conditions. Alginate encapsulated cells suggest an adequate 3D environment for a bioartificial liver because they are cryopreservable and manipulable.^[18] Our results showed that cells in alginate, which are 1.5% (w/v) to reproduce their survival levels and ability for proliferation, should be better maintained.

Vecchiatini *et al.* studied the impact of important experimental parameters on microcapsule characteristics and tested the different concentrations of alginate solution ranging from 0.5% to 3.5% (w/v) using concentrations higher than 2.0% (w/v). Their results showed that microbeads produced with an alginate concentration between 1.0% and 2.0% (w/v) were spherical and characterized by a smooth surface. Lowering the alginate concentration down to 0.5% (w/v) caused a partial breaking of the beads, thereby resulting in particles with an irregular shape.^[19]

The data of some studies indicate that 2% (w/v) alginate matrix does not support cell proliferation, whereas 1% (w/v) alginate proved to be an appropriate matrix. The absence of these cells approves that 1% of beads can maintain encapsulated cells in the growth course.^[8] The large initial mesh size of the gel structure in 1% (w/v) concentration of alginate gels supports cell proliferation, while the smaller initial mesh size of the 2% (w/v) concentration of alginate gels supports cell differentiation.^[2]

The albumin gene expression is an early differentiation marker in the liver.

In both groups, concentrations of 1.5% and 2.5% (w/v) by adding the HGF on day 4 resulted in a sudden increase in albumin produced by the cells, and then, gradually, the albumin level decreased. In addition, to study the HWJ-MSCs differentiation in hepatocyte-like cells, the urea metabolic rate was measured. The mean urea produced was 1.5% (w/v) higher than in 2.5% (w/v) concentration; therefore, it corresponded with the MTT test results.

The cells in 1.5% (w/v) group produced higher rate of urea in comparing with other groups with regard to the generation of albumin and urea; different results were obtained in different researches. The greatest amount of urea was seen on days 8–10 (Azandeh *et al.*, 2016). The increase of urea was seen in day 3 and albumin secretion was observed from day 12 after starting research.^[20] Kang *et al.* investigated production of albumin from day 16 of differentiated time.^[21]

In the process of embryogenesis, the progenitor cells' differentiation into mature hepatocytes depends on the

creation of complicated pathways through multiple signs from nearby cells. Variations in the concentration and the timing of the cell signs are essential for regulating especial transcription parameters that arranges this transition into mature cells.^[22]

At almost 3 weeks of human gestational age, the pancreas progenitor and liver cells, in three isolated areas of the endoderm, start to differentiate. This is known as "specification."

Afterward, two lateral progenitor areas proceed ventral-medially to create the hepatic endoderm^[16] [Figure 1a]. Then, the repression of the mesodermal Wnt and the FGF4 are essentially needed for hepatic induction. DMSO, which alters histone acetylation, has an inductive role with regard to hepatocyte lineage specification and has been used in certain protocols.^[22]

In maturation, differentiated cells show polyhedral hepatocyte morphology with distinguished circular nuclei possessing cytoplasmic vacuoles. Maturation of hepatocytes is affected by growth factors during development of liver. Generally, sequential and various HGF and/or OSM or Epidermal Growth Factor (EGF) levels with insulin and glucocorticoids, present in optimized hepatocyte culture media, are utilized.^[15,22]

CONCLUSION

This study evaluated the influence of the concentration sodium alginate, which is β -D-mannuronic acid (M) and α -L-guluronic acid (G) monomers' combination on differentiation and the mesenchymal stem cells' proliferation capacity obtained from the umbilical cord human tissue *in vitro* conditions. Indicators such as scanning electron microscopy showed that porous alginate scaffolds protection could grow, survive, and differentiate HWJ-MSCs. It seems that combining alginate concentrations 1.5% (w/v), which is biocompatible and biodegradable, and has good porosity, facilitates cell access toward nutrient disposal and creates a favorable condition for the mesenchymal stem cells' differentiation and proliferation into hepatocyte-like cells in the culture.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

- Kinoshita T, Sekiguchi T, Xu MJ, Ito Y, Kamiya A, Tsuji K, *et al.* Hepatic differentiation induced by oncostatin M attenuates fetal liver hematopoiesis. *Proc Natl Acad Sci U S A* 1999;96:7265-70.
- Lee BH, Li B, Guelcher SA. Gel microstructure regulates proliferation and differentiation of MC3T3-E1 cells encapsulated in alginate beads. *Acta Biomater* 2012;8:1693-702.
- Zhou H, Xu HH. The fast release of stem cells from alginate-fibrin microbeads in injectable scaffolds for bone tissue engineering. *Biomaterials* 2011;32:7503-13.
- Tang M, Chen W, Weir MD, Thein-Han W, Xu HH. Human embryonic stem cell encapsulation in alginate microbeads in macroporous calcium phosphate cement for bone tissue engineering. *Acta Biomater* 2012;8:3436-45.
- Ghasemzadeh-Hasankolaei M, Eslamnejad MB, Batavani R, Sedighi-Gilani M. Comparison of the efficacy of three concentrations of retinoic acid for transdifferentiation induction in sheep marrow-derived mesenchymal stem cells into male germ cells. *Andrologia* 2014;46:24-35.
- Ye JH, Xu YJ, Gao J, Yan SG, Zhao J, Tu Q, *et al.* Critical-size calvarial bone defects healing in a mouse model with silk scaffolds and SATB2-modified iPSCs. *Biomaterials* 2011;32:5065-76.
- Andersen T, Auk-Emblem P, Dornish M. 3D cell culture in alginate hydrogels. *Microarrays (Basel)* 2015;4:133-61.
- Wang N, Adams G, BATTERY L, Falcone FH, Stolnik S. Alginate encapsulation technology supports embryonic stem cells differentiation into insulin-producing cells. *J Biotechnol* 2009;144:304-12.
- Lee KY, Mooney DJ. Alginate: Properties and biomedical applications. *Prog Polym Sci* 2012;37:106-26.
- Trouche E, Girod Fullana S, Mias C, Ceccaldi C, Tortosa F, Seguelas MH, *et al.* Evaluation of alginate microspheres for mesenchymal stem cell engraftment on solid organ. *Cell Transplant* 2010;19:1623-33.
- Usov AI. Alginic acids and alginates: Analytical methods used for their estimation and characterisation of composition and primary structure. *Russian Chem Rev* 1999;68:957-66.
- Mandal S, Kumar SS, Krishnamoorthy B, Basu SK. Development and evaluation of calcium alginate beads prepared by sequential and simultaneous methods. *Braz J Pharm Sci* 2010;46:785-93.
- Younesi E, Bayati V, Hashemitabar M, Azandeh SS, Bijannejad D, Bahreini A, *et al.* Differentiation of adipose-derived stem cells into Schwann-like cells: Fetal bovine serum or human serum? *Anat Cell Biol* 2015;48:170-6.
- Azandeh S, Mohammad Gharravi A, Orazizadeh M, Khodadi A, Hashemi Tabar M. Improvement of mesenchymal stem cell differentiation into the endoderm lineage by four step sequential method in biocompatible biomaterial. *Bioimpacts* 2016;6:9-13.
- Yoon HH, Jung BY, Seo YK, Song KY, Park JK. *In vitro* hepatic differentiation of umbilical cord-derived mesenchymal stem cell. *Process Biochem* 2010;45:1857-64.
- Son YJ, Yoon IS, Sung JH, Cho HJ, Chung SJ, Shim CK, *et al.* Porous hyaluronic acid/sodium alginate composite scaffolds for human adipose-derived stem cells delivery. *Int J Biol Macromol* 2013;61:175-81.

17. Asgari S, Moslem M, Bagheri-Lankarani K, Pournasr B, Miryounesi M, Baharvand H, *et al.* Differentiation and transplantation of human induced pluripotent stem cell-derived hepatocyte-like cells. *Stem Cell Rev* 2013;9:493-504.
18. Selden C, Hodgson H. Cellular therapies for liver replacement. *Transpl Immunol* 2004;12:273-88.
19. Vecchiatini R, Penolazzi L, Lambertini E, Angelozzi M, Morganti C, Mazzitelli S, *et al.* Effect of dynamic three-dimensional culture on osteogenic potential of human periodontal ligament-derived mesenchymal stem cells entrapped in alginate microbeads. *J Periodontal Res* 2015;50:544-53.
20. Khoramroodi A, Nasri S, Amiri I. The *in vitro* assessment of biochemical factors in hepatocyte like cells derived from umbilical cord blood stem cells. *Armaghane Danesh* 2009;14:23-34.
21. Kang A, Park J, Ju J, Jeong GS, Lee SH. Cell encapsulation via microtechnologies. *Biomaterials* 2014;35:2651-63.
22. Duan Y, Ma X, Zou W, Wang C, Bahbahan IS, Ahuja TP, *et al.* Differentiation and characterization of metabolically functioning hepatocytes from human embryonic stem cells. *Stem Cells* 2010;28:674-86.