

Use of alginate hydrogel to improve long-term 3D culture of spermatogonial stem cells: stemness gene expression and structural features

Research Article

Cite this article: Hemadi M *et al.* (2022) Use of alginate hydrogel to improve long-term 3D culture of spermatogonial stem cells: stemness gene expression and structural features. *Zygote*, **30**: 312–318. doi: [10.1017/S0967199421000551](https://doi.org/10.1017/S0967199421000551)

Received: 25 April 2021

Revised: 11 June 2021

Accepted: 15 June 2021



First published online: 13 October 2021

Keywords:

Alginate hydrogel; Cell culture technique; Encapsulated; Mouse; Spermatogonial stem cells; Stemness genes

Author for correspondence:

Mohammadreza Gholami. Medical Technology Research Center, Institute of Health Technology, Kermanshah University of Medical Sciences, Kermanshah Zip code: 67148-69914, Iran. Tel:/Fax: +98 8334276477. E-mail: rezagholami57@gmail.com

Masoud Hemadi¹, Vahideh Assadollahi², Ghasem Saki³, Afshin Pirnia⁴, Masoud Alasvand² , Abolfazl Zندهدل⁵ and Mohammadreza Gholami⁴ 

¹Cellular and Molecular Research Center, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran; ²Cancer and Immunology Research Center, Research Institute for Health Development, Kurdistan University of Medical Sciences, Sanandaj, Iran; ³Physiology Research Center, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran; ⁴Medical Technology Research Center, Research Institute of Health Technology, Kermanshah University of Medical Sciences, Kermanshah, Iran and ⁵Geriatric Department, Ziyayian Hospital, Tehran University of Medical Sciences, Tehran, Iran

Summary

The quality and quantity of a spermatogonial stem-cell (SSC) culture can be measured in less time using a 3D culture in a scaffold. The present study investigated stemness gene expression and the morphological and structural characterization of SSCs encapsulated in alginate. SSCs were harvested from BALB/c neonatal mice testes through two-step mechanical and enzymatic digestion. The spermatogonial populations were separated using magnetic-activated cell sorting (MACS) using an anti-Thy1 antibody and c-Kit. The SSCs then were encapsulated in alginate hydrogel. After 2 months of SSC culturing, the alginate microbeads were extracted and stained to evaluate their histological properties. Real-time polymerase chain reaction (PCR) was performed to determine the stemness gene expression. Scanning electron microscopy (SEM) was performed to evaluate the SSC morphology, density and scaffold structure. The results showed that encapsulated SSCs had decreased expression of *Oct4*, *Sox2* and *Nanos2* genes, but the expression of *Nanog*, *Bcl6b* and *Plzf* genes was not significantly altered. Histological examination showed that SSCs with pale nuclei and numerous nucleolus formed colonies. SEM evaluation revealed that the alginate scaffold structure preserved the SSC morphology and density for more than 60 days. Cultivation of SSCs on alginate hydrogel can affect *Oct4*, *Sox2* and *Nanos2* expression.

Introduction

Stem-cell therapy is a therapeutic field with the potential for healing a myriad of diseases by transplanting healthy stem cells to restore tissues affected by illness or injury. One suggested treatment is for prepubescent boys who risk loss of fertility due to chemotherapy or radiation treatments for cancer. These patients could recover fertility if their SSCs are isolated before treatment and then transplanted back into the testes after treatment (Brinster, 2007). Because the number of SSCs in the testes is normally very low, a method should be developed to ensure the proliferation and survival of germ cells during culture. The proliferation and enrichment of SSCs *in vitro* could be a decisive strategy for helping to study SSCs in detail to improve the chance of success of *in vivo* SSC transplantation (Jalayeri *et al.*, 2017).

Spermatogenesis is a complex process regulated by the endocrine system and, locally, by multiple interactions between developing germ cells and their surroundings through autocrine and paracrine factors (Caires *et al.*, 2010; Hai *et al.*, 2014; Giudice *et al.*, 2017). Studies have shown that the response of cells in a three-dimensional (3D) culture system is much more similar to the behaviour of cells *in vivo* when compared with the 2D culture system. In a 3D culture, the cells interact with each other, with an extracellular matrix and with the microenvironment around them. These communications in 3D structures affect the proliferation, differentiation, cell morphology, and gene and protein expression as well as the cell responses to external factors (Mohammadzadeh *et al.*, 2019).

Microcapsule technology is now commonly used for drug encapsulation for sustained release, enzyme immobilization, and microbioreactor cell culture. The alginate system has been a focus of attention for microcapsules (Lim and Sun, 1980; O'Shea and Sun, 1986), particularly the sodium alginate microcapsule, because of its ease of production and the efficient recovery of cells. This method has been used to cultivate different types of cells. In addition to mesenchymal stem cells (Bidarra and Barrias, 2019), human neuroblastoma cells (Lewicki *et al.*, 2019), bone

marrow (Barralet *et al.*, 2005) and other SSCs have been evaluated in alginate gel. A previous investigation has identified the antioxidant and non-toxic properties of alginate hydrogel, which forms a scaffold that is applicable for 3D culture and cryopreservation of spermatogonial stem cells (Pravdyuk *et al.*, 2013; Jalayeri *et al.*, 2017; Pirnia *et al.*, 2017).

In reproductive biology, maintenance of SSC self-renewal is important (Song and Wilkinson, 2014). If SSCs self-renew too frequently, the result will be deficiencies in spermatogenesis. Conversely, insufficient SSC self-renewal will result in progressive loss of germ cells (Meng *et al.*, 2000). *Oct4*, *Nanog*, and *Sox2* are transcriptional regulators involved in the maintenance of self-renewal of embryonic stem cells (Assadollahi *et al.*, 2019a, 2019b). *Bcl6b* has been identified as promoting SSC self-renewal and is highly expressed in germ cells (Song and Wilkinson, 2014). *Plzf* and *Nanos2* are important genes that initiate SSC maintenance (Buaas *et al.*, 2004; Schmidt *et al.*, 2009).

Although SSC transplantation techniques provide quantitative measurements of stem-cell performance in donor cell populations, this approach is technically challenging. It is a retrospective procedure that requires a 2–3 month span of time between transplantation and analysis (Phillips *et al.*, 2010). In an effort to accelerate research in this critical area, several studies have suggested that the quality and quantity of a SSC culture can be measured over a shorter period of time using 3D culture in a scaffold (Yeh *et al.*, 2007). The aim of the present study was to evaluate self-renewal gene expression and provide information about morphological and structural characterization and colony formation of SSCs encapsulated in alginate hydrogel.

Materials and methods

SSC isolation and purification

BALB/c mice were purchased from the Razi Laboratory Animal Center (Tehran, Iran). All procedures were approved by and performed in accordance with the National Research Council guidelines. The testes were extracted from 6-day-old pups and transferred to Dulbecco's modified Eagle's medium (DMEM; Gibco) containing penicillin (100 IU/ml) and streptomycin (100 µg/ml; Gibco). Then the epididymis and tunica albuginea were separated and the seminiferous tubules were extracted to a culture medium containing DMEM, 2 mg/ml collagenase (Gibco) and 200–500 µg/ml DNase I (Gibco). The testicular suspension was held at 37°C and centrifuged at 100 g for 5 min. The cell pellet then was resuspended in 1 ml trypsin/EDTA (Sigma) and gently pipetted to provide a testicular cell suspension, which was filtered through #60 nylon micro-mesh (Gholami *et al.*, 2013a, 2013b, 2014).

Separation and purification with laminin and MACS

Petri dishes containing laminin were washed with phosphate buffered saline (PBS) and then incubated with bovine serum albumin (BSA) to prevent nonspecific binding. For SSC purification, the MACS method was performed using the specific marker for SSCs, Thy1 (CD90.1 MicroBeads, mouse-Miltenyi Biotec, 130-094-523), as a positive control and c-kit (CD117 MicroBeads, mouse-Miltenyi Biotec, 130-091-224) as a negative control (columns: MS, LS, XS, auto MACS columns).

In total, 10⁷ cells were centrifuged at 300 g for 10 min and then were resuspended in 90 µl of PBS with a pH of 7.2, 0.5% BSA, and 2 mM EDTA. The MACS BSA stock solution (#130-91-376) was

diluted with 1:20 auto MACS rinsing solution (#130-091-222) and 10 µl CD90.1 microbeads. The buffer was incubated for 15 min in the refrigerator and centrifuged for 10 min. The cell suspension was loaded onto a MACS column and was placed in the magnetic field of a MACS separator.

SSC encapsulation in alginate scaffold

The SSCs initially were resuspended in DMEM and alginic acid (sodium salt; Sigma-Aldrich, Germany) dissolved in 0.9% NaCl (pH 7.4) to achieve a final concentration of 1%. The SSCs then were resuspended in alginate hydrogel to achieve a final cell density of 10⁶ cells/ml. This cell/scaffold mixture was dripped (10 µl droplets) into 135 mmol/l CaCl solutions for polymerization. The alginate beads were washed twice in sterile 0.9% NaCl (Merck, Germany).

3D cell culture

Encapsulated SSCs were cultured in DMEM containing 10% fetal bovine serum and 10 ng/ml glial cell-derived neurotrophic factor (GDNF; Sigma) for 2 months. The medium was replaced every 48 h.

Haematoxylin–eosin staining

After 2 months, the 3D culture of SSCs in alginate hydrogel microbeads was fixed in formalin. After being embedded in paraffin, the tissue sections were cut and dehydrated and then stained with haematoxylin–eosin to determine their histological structure.

Alginate depolymerization

The alginate depolymerization solution used contained 119 mmol/l trisodium citrate and 0.9% NaCl (pH 7.6) for extraction of the SSCs.

Viability

SSC viability assays were performed after staining with trypan blue (0.4%).

RNA extraction and cDNA synthesis

RNA was isolated using a Qiagen kit (Qiagene, cat. no. PP-2105) following the manufacturer's instructions. cDNA synthesis was performed using a Bioneer kit (Bioneer, cat. no. k-2046) according to manufacturer's instructions.

Real-time PCR

Real-time PCR was performed using a Qiagen Rotor Gene 6000 instrument. Forty-five reactions were considered. Each reaction cycle was performed for 15 s at 95°C and 45 s at 60°C (Gholami *et al.*, 2013a). Table 1 lists the primers.

Scanning electron microscopy

The Santana freeze-dried method was used to prepare the alginate hydrogel microbeads containing encapsulated SSCs for SEM imaging to determine the SSC morphology after 1 month of 3D culture.

Statistical analysis

Gene expression was analyzed using REST-2009 software. Other data were presented as the mean ± standard deviation.

Table 1. Primers used in this study. Sequences and sizes (bp) of primers presented in the table

Primer name	Primer sequence	Size (bp)
<i>Oct4</i>	F GGATGGCATACTGTGGAC	233
	R CTTGGCAAAGTCTCTAGC	
<i>Nanos2</i>	F TGAGGTACCTGTACCCACA	200
	R CGGTCCATGGAGGAAGGGTTC	
<i>Nanog</i>	F GGAGGACTTTCTGCAGCCTT	154
	R ACCTGGTGGAGTCACAGAGT	
<i>Sox2</i>	F CATGGGCTCTGTGGTCAAGT	315
	R TCCTCTTTTTGCACCCCTCC	
<i>Bcl6b</i>	F CCATAATCCTCTACCCGGGC	186
	R GAGGGCCAAGGGACATTCAA	
<i>Plzf</i>	F ACATGGCTGTCTTCTGTCTG	174
	R GGTGGTCACCTGTATGTGAG	

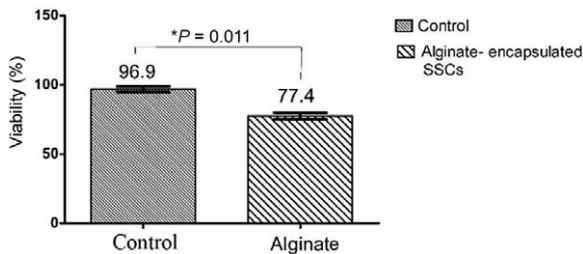


Figure 1. Viability of SSCs encapsulated in alginate after 2 months of culture. Viability of encapsulated SSCs and Fresh SSCs was 96% and 77.4% respectively. * $P = 0.011$.

The Mann–Whitney test was used to detect significant differences between groups. A P -value of 0.05 was considered to be statistically significant. All statistical computations were performed using SPSS 17.0 software (SPSS, USA).

Results

Viability

Figure 1 compares the viability of the 3D alginate culture as the experimental group with the freshly isolated SSC culture as the control group. The viability of the freshly isolated SSCs and 3D cultures was determined after dying with trypan blue. The mean survival rate of the SSCs encapsulated in 3D alginate hydrogel after 2 months was 77.41%, which is significantly lower than the control group at 96.90% ($P = 0.011$; Fig. 1).

Gene expression

The ability of the 3D alginate hydrogel to maintain undifferentiated mouse SSCs was investigated by quantifying the expression levels of *Oct4*, *Sox2*, *Nanog*, *Nanos2*, *Bcl6b* and *Plzf*. These are self-renewing genes specific to undifferentiated SSCs. After 2 months of culture, the expression levels of *Oct4*, *Sox2* and *Nanos2* genes decreased in the alginate-encapsulated SSCs compared with the freshly isolated testicular cells (Fig. 2 and Table 2). qRT-PCR analysis was carried out on day 60 after seeding and showed that there was no significant difference between the

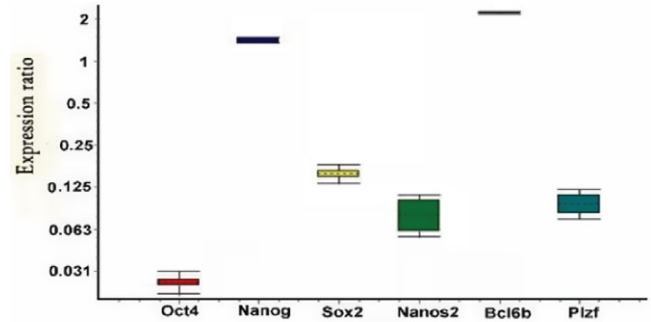


Figure 2. Expression of stemness gene of 6-day-old mice SSCs that were encapsulated in alginate and were cultured for 2 months. *Oct4*, *Sox2* and *Nanos2* decreased, but *Nanog*, *Bcl6b* and *Plzf* did not show significant changes.

relative expression of the *Nanog*, *Bcl6b* and *Plzf* genes in the 3D culture experimental group and the freshly isolated control group. The expression levels of the *Nanog*, *Bcl6b* and *Plzf* genes showed no change (Fig. 2 and Table 2).

Haematoxylin–eosin assessment

The capsules were spherical in shape with a uniform margin and a homogenous distribution of the cells inside the capsule. At 60 days, the capsules had maintained their structural integrity and spherical form. The SSCs in the alginate capsules were circular, but did not bind to the surface. Haematoxylin–eosin examination showed that SSCs with pale nuclei and numerous nucleoli had formed colonies (Fig. 3a–c).

SEM analysis

The SSCs encapsulated in the alginate scaffold were evaluated by SEM. The images showed that the SSCs were in a 3D structure and the formation of the stem cells into colonies was clearly visible. SEM evaluation revealed that the alginate scaffold structure had preserved the SSC morphology and density at 60 days of culture (Fig. 3d).

Discussion

SSCs are undifferentiated spermatogonia that can maintain their capacity for self-renewal and retain their ability to differentiate into dedicated germ cells (Kanatsu-Shinohara and Shinohara, 2013). To maintain the features of SSCs, external factors secreted from surrounding somatic cells and internal factors, such as specific gene expression, play important roles (Orwig et al., 2008). SSCs are an appealing model for the identification of the molecular pathways involved in their regulation of self-renewal and differentiation.

Because of the significant difference in the SSC population in juvenile and adult mice (Aponte et al., 2005; McLean et al., 2003), the testes of 6-day-old mice were used in this study. Evidence also suggested improved spermatogonial viability and differential capacity in immature mice (Nagano et al., 2003). In general, because of the limited number of SSCs and their lack of unique cell-surface markers, it is extremely difficult to isolate a purified population of SSCs (Khajavi et al., 2014). The MACS method is the most appropriate technique and causes minimal stress to the SSCs during isolation (Oatley et al., 2007). A cell-surface marker that is expressed exclusively on undifferentiated SSCs produced successful MACS isolation (Sofikitis et al., 2005).

Table 2. Analysis results of expression of stemness gene. Results showed that *Oct4*, *Sox2* and *Nanos2* decreased, but *Nanog*, *Bcl6b* and *Plzf* did not show significant changes. The results were produced by Rest 2009 software. This software compares gene expression between samples, SSCs were encapsulated for 2 months in alginate, with control, fresh SSCs, in the presence of *Gapdh* (housekeeping gene known as the reference gene)

Gene	Type	Reaction efficiency	Expression	Standard error	95% C.I.	P (H1)	Result
<i>Gapdh</i>	REF	0.735	1.000				
<i>Oct4</i>	TRG	0.525	0.026	0.023–0.029	0.022–0.031	0.000	Down
<i>Nanog</i>	TRG	0.6575	1.412	1.342–1.485	1.342–1.485	0.339	–
<i>Sox2</i>	TRG	0.85	0.157	0.143–0.172	0.136–0.181	0.000	Down
<i>Nanos2</i>	TRG	0.7025	0.078	0.058–0.105	0.056–0.109	0.000	Down
<i>Bcl6b</i>	TRG	0.7675	2.210	2.148–2.274	2.148–2.274	0.169	–
<i>Plzf</i>	TRG	0.7125	0.095	0.078–0.115	0.075–0.120	0.339	–

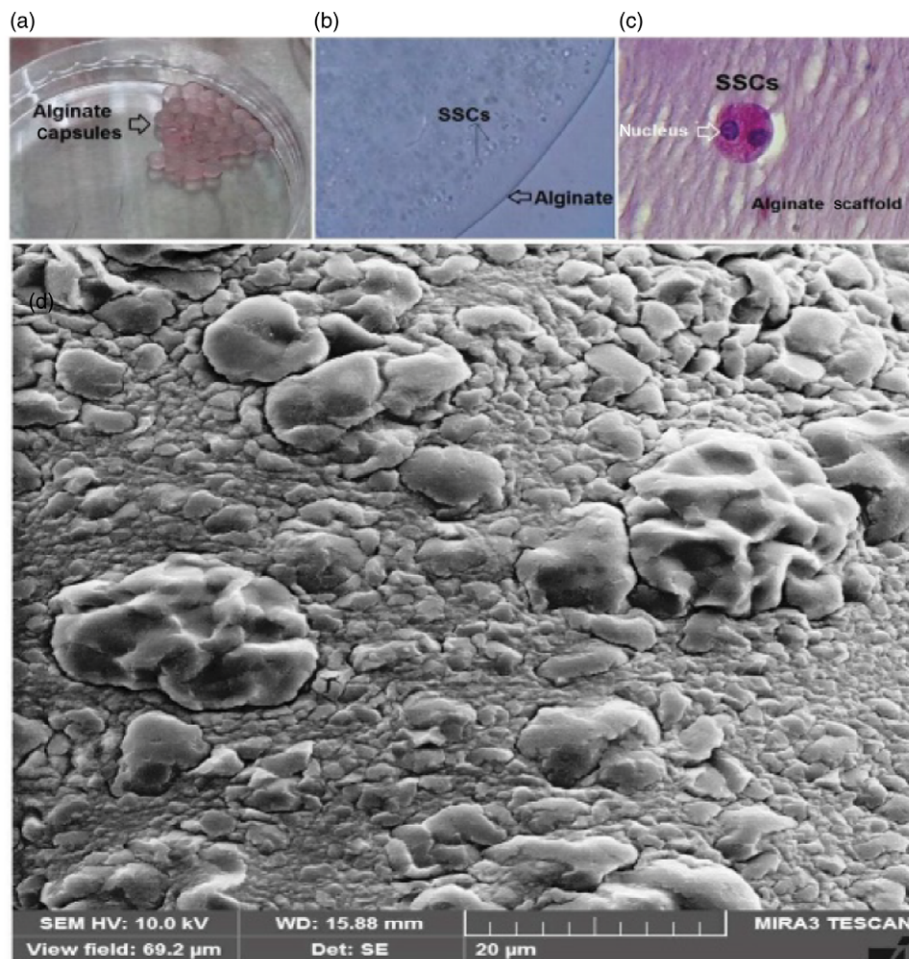


Figure 3. (a, b) SSCs encapsulated alginate microbeads, 3D culture of encapsulated. (c) Microbeads after 3D long-term culture stained with haematoxylin and eosin (H&E) dye. Stained sections show normal spermatogonia morphology. The image shows SSCs mitosis in the alginate scaffold. (d) SEM micrograph from SSCs encapsulated in alginate hydrogel. SSCs encapsulated in alginate scaffold can be seen attached to the surface of the hydrogel scaffold 2 months after culture.

Thy1 (or CD90) has been identified in rodents, non-human primates and cattle as a surface marker of SSCs (Oatley *et al.*, 2010). Thy1⁺ testis cell fraction isolation has resulted in the enrichment of SSCs. The cultivation of Thy1⁺ mouse germ cells under conditions supported by GDNF showed increased SSC numbers over prolonged periods of time (Kubota *et al.*, 2004). In the current study, purified populations of Thy1⁺ SSCs were isolated using the MACS method.

The ability of SSCs to maintain spermatogenesis homeostasis is necessary for treatment of infertility by *in vivo* transplantation (Kanatsu-Shinohara and Shinohara, 2013). However, because of the limited number of SSCs (Khajavi *et al.*, 2014), the development of efficient culture systems capable of maintaining SSC self-renewal and promoting their differentiation has attracted attention in the stem-cell research community (Ziloochi Kashani *et al.*, 2020). In the current investigation, after isolating mouse neonatal

testicular cells by enzymatic digestion (Jalayeri *et al.*, 2017; Pirnia *et al.*, 2017) to evaluate the effect of alginate hydrogel on SSC proliferation and self-renewal, isolated mouse testicular cells were cultured in the presence of GDNF.

GDNF is considered to be the most important factor in the proliferation (Hofmann, 2008), survival (Oatley *et al.*, 2010) and self-renewal (Oatley *et al.*, 2006) of SSCs. It is produced by Sertoli cells after birth and is currently the only recognized paracrine factor in the testes that is directly responsible for *in vivo* and *in vitro* maintenance and self-renewal of SSCs (Hofmann, 2008). In the present study, cell viability analysis was determined after staining with trypan blue and revealed significant differences between freshly isolated SSCs and those in the 3D alginate culture system. Although the percentage of viable cells in the 3D alginate culture was lower than in the freshly isolated SSCs, the mean survival rate of SSCs in the alginate hydrogel after 2 months was high. This result is similar to those of Jalayeri and colleagues (Jalayeri *et al.*, 2017). They showed that, after 60 days of encapsulation of SSCs, the viability of these cells remained high at *c.* 74%. Wang and co-workers also showed that embryonic stem cells in alginate encapsulation had survival rates of *c.* 72% after 10 days (Wang *et al.*, 2009).

Alginate hydrogels are non-adhesive, highly hydrophobic and hydrated 3D networks. They are similar to extracellular matrices, cross-linking processes that do not damage the cells and are highly porous structures. Moreover, alginate hydrogel does not require toxic activators or a temperature change to promote cellular activity and metabolism. The antioxidant properties of the alginate that enhance cell survival and facilitate the exchange of ions and other nutrients from cells to the culture medium are important properties of alginate hydrogel that make this scaffold a promising candidate for 3D cell cultures (Azevedo *et al.*, 2014; Sarker *et al.*, 2015; Jalayeri *et al.*, 2017). In the present study, SEM micrographs showed that the cell morphology and their spread and proliferation had been preserved after 60 days of culturing.

Oct4 is expressed in undifferentiated spermatogonia of newborn, juvenile and adult mouse testes (Pesce *et al.*, 1998) and plays a role in the self-renewal of SSCs (Ketkar and Reddy, 2012). In the present study, the results showed that expression of *Oct4* was significantly lower in SSCs encapsulated in alginate scaffold after 60 days of culturing. It is possible that the cells in this group withdrew from pluripotency and were differentiated (Afsartala *et al.*, 2016). Although the expression of *Oct4* has been demonstrated by Wu and colleagues it is not necessary for *in vitro* maintenance of SSCs (Wu *et al.*, 2010).

Sox2 as an important transcription factor involved in embryogenesis, stem-cell pluripotency and primordial germ cell proliferation (Assadollahi *et al.*, 2019a). Kanatsu-Shinohara and colleagues showed that *Sox2*, like *Oct4*, was already being expressed at low levels in cultured SSCs (Kanatsu-Shinohara *et al.*, 2008) and appeared to be an important factor in their expression (de Oliveira *et al.*, 2019). The results of the current study showed that *Sox2* and *Nanos2* expression, similarly to *Oct4*, decreased in the SSCs encapsulated in the alginate scaffold for 60 days. *Nanos2* is primarily expressed in specific types of undifferentiated A-spermatogonia (Ishii *et al.*, 2012). It has been shown that the expression level of *Nanos2* is heterogeneous and that this heterogeneity at the *Nanos2* expression level has practical significance.

The *Nanog* transcription factor homeobox plays a crucial role in embryonic development/proliferation and maintenance of SSCs by interacting with *Oct4* and *Sox2* (Patra *et al.*, 2018), although no *Nanog* or *Sox2* protein expression has been detected in these cells

(Kanatsu-Shinohara *et al.*, 2008). Unlike *Oct4* and *Sox2*, the expression of the *Nanog* gene did not change significantly in the SSCs encapsulated in an alginate scaffold for 60 days in the present study.

Plzf (also called ZBTB16 or ZFP145) is a transcription factor that inhibits the differentiation of stem cells and maintains spermatogonia in an undifferentiated state (Jalayeri *et al.*, 2017). Recent studies have shown that *Bcl6b* is involved in spermatogenesis and essential for the maintenance of self-renewal of SSCs *in vitro* (Pesce *et al.*, 1998; Afsartala *et al.*, 2016). Even though *Bcl6b* overexpression has been shown to cause germ cell tumours (Pesce *et al.*, 1998) and based on the findings obtained using siRNA, spermatogenesis degeneration was observed in the *Bcl6b*-null testes (Oatley *et al.*, 2010). Similarly, *Nanog*, *Plzf* and *Bcl6b* genes did not change in expression in the encapsulated SSCs in the alginate scaffold in this study.

The comparison of differentiated cells and SSCs has shown that SSCs express genes that have different functions. Previous investigations have reported that there are 1133 genes in the SSCs with expression levels that are more than twice that of differentiated cells. Among the 1133 genes, 900 gene sequences encode known proteins and more than 200 genes are unknown functionally. Also, 50 genes such as *Nanos*, *Lin28*, *Oct4* and *Plzf*, in SSCs have expression levels of greater than five-fold. In contrast, 462 genes have expression levels of greater than five-fold. This evidence suggests that regulation of gene expression can play a key role in determining the properties of pluripotent cells (Yang *et al.*, 2013).

The male germ cells are located in the intact testes in the 3D structure of the seminiferous tubules. It has been argued that the 3D culture of SSCs could provide an environment that more closely resembles *in situ* conditions (Huleihel *et al.*, 2015). It also has been suggested that 3D systems can possibly provide niches that replicate, to some degree, the microenvironment and spatial structure of the *in vivo* conditions in the seminiferous tubules, in which germ cells are embedded in Sertoli cells (Sharpe, 1994).

Cell-cell interactions are essential for clonal expansion and differentiation of germ cells, therefore 3D culture systems could provide an enhanced structural environment. In addition, these systems may enable germ cells to organize into densely packed clusters that facilitate the delivery of oxygen, nutrients, and other factors, thereby allowing their survival and proliferation to be maintained. These clusters could also provide and maintain contacts between germ cells, which are required during differentiation (Mahmoud, 2012). Despite the decrease in some SSC stemness genes in this study, additional research in the use of alginate scaffolds could find them a prospective candidate for long-term culture.

Acknowledgements. The author would like to thank the research affairs of Ahvaz Jundishapur University of Medical Sciences which funded the project (no. 1793).

Funding. Ahvaz Jundishapur University of Medical Sciences (no. 1793).

Competing interests. The authors declare that they have no competing interests.

Data availability. Data sharing is not applicable to this article as no new data were created or analyzed in this study.

References

- Afsartala Z, Rezvanfar MA, Hodjat M, Tanha S, Assadollahi V, Bijangi K, Abdollahi M and Ghasemzadeh-Hasankolaei M (2016). Amniotic membrane mesenchymal stem cells can differentiate into germ cells *in vitro*. *In Vitro Cell Dev Biol Anim* **52**, 1060–71.
- Aponte PM, Van Bragt MP, De Rooij DG and Van Pelt AM (2005). Spermatogonial stem cells: characteristics and experimental possibilities. *APMIS* **113**(11–12), 727–42.
- Assadollahi V, Fathi F, Abdi M, Khadem Erfan MB, Soleimani F and Banafshi O (2019a). Increasing maternal age of blastocyst affects on efficient derivation and behavior of mouse embryonic stem cells. *J Cell Biochem* **120**, 3716–26.
- Assadollahi V, Hassanzadeh K, Abdi M, Alasvand M, Nasser S and Fathi F (2019b). Effect of embryo cryopreservation on derivation efficiency, pluripotency, and differentiation capacity of mouse embryonic stem cells. *J Cell Physiol* **234**, 21962–72.
- Azevedo MA, Bourbon AI, Vicente AA and Cerqueira MA (2014). Alginate/chitosan nanoparticles for encapsulation and controlled release of vitamin B2. *Int J Biol Macromol* **71**, 141–6.
- Barralet JE, Wang L, Lawson M, Triffitt JT, Cooper PR and Shelton RM (2005). Comparison of bone marrow cell growth on 2D and 3D alginate hydrogels. *J Mater Sci Mater Med* **16**, 515–9.
- Bidarra SJ and Barrias CC (2019). 3D culture of mesenchymal stem cells in alginate hydrogels. *Methods Mol Biol* **2002**, 165–80.
- Brinster RL (2007). Male germline stem cells: from mice to men. *Science* **316**(5823), 404–5.
- Buaas FW, Kirsh AL, Sharma M, McLean DJ, Morris JL, Griswold MD, de Rooij DG and Braun RE (2004). Plzf is required in adult male germ cells for stem cell self-renewal. *Nat Genet* **36**, 647–52.
- Caires K, Broady J and McLean D (2010). Maintaining the male germline: regulation of spermatogonial stem cells. *J Endocrinol* **205**, 133–45.
- de Oliveira TV, Tesser RB, Nunes N, Stumpp T (2019). Dynamics of Sox2 expression during rat germ cell development and its relationship with emergence of spermatogonia. bioRxiv doi: <https://doi.org/10.1101/558015> [preprint]
- Gholami M, Hemadi M, Saki G, Zendedel A, Khodadadi A and Mohammadi-Asl J (2013a). Does prepubertal testicular tissue vitrification influence spermatogonial stem cells (SSCs) viability? *J Assist Reprod Genet* **30**, 1271–7.
- Gholami M, Saki G, Hemadi M, Khodadadi A and Mohammad-di-Asl J (2013b). Effect of melatonin on the expression of apoptotic genes in vitrified-thawed spermatogonia stem cells Type A of 6-day-old mice. *Iran J Basic Med Sci* **16**, 906–9.
- Gholami M, Saki G, Hemadi M, Khodadadi A and Mohammadi-Asl J (2014). Melatonin improves spermatogonial stem cells transplantation efficiency in azoospermic mice. *Iran J Basic Med Sci* **17**, 93–9.
- Giudice MG, De Michele F, Poels J, Vermeulen M and Wyns C (2017). Update on fertility restoration from prepubertal spermatogonial stem cells: how far are we from clinical practice? *Stem Cell Res* **21**, 171–7.
- Hai Y, Hou J, Liu Y, Liu Y, Yang H, Li Z and He Z (2014). The roles and regulation of Sertoli cells in fate determinations of spermatogonial stem cells and spermatogenesis. *Semin Cell Dev Biol* **29**, 66–75.
- Hofmann MC (2008). Gdnf signaling pathways within the mammalian spermatogonial stem cell niche. *Mol Cell Endocrinol* **288**(1–2), 95–103.
- Huleihel M, Nourashrafeddin S and Plant TM (2015). Application of three-dimensional culture systems to study mammalian spermatogenesis, with an emphasis on the rhesus monkey (*Macaca mulatta*). *Asian J Androl* **17**, 972–80.
- Ishii K, Kanatsu-Shinohara M, Toyokuni S and Shinohara T (2012). FGF2 mediates mouse spermatogonial stem cell self-renewal via upregulation of Etv5 and Bcl6b through MAP2K1 activation. *Development* **139**, 1734–43.
- Jalayeri M, Pirnia A, Najafabad EP, Varzi AM and Gholami M (2017). Evaluation of alginate hydrogel cytotoxicity on three-dimensional culture of type A spermatogonial stem cells. *Int J Biol Macromol* **95**, 888–94.
- Kanatsu-Shinohara M and Shinohara T (2013). Spermatogonial stem cell self-renewal and development. *Annu Rev Cell Dev Biol* **29**, 163–87.
- Kanatsu-Shinohara M, Lee J, Inoue K, Ogonuki N, Miki H, Toyokuni S, Ikawa M, Nakamura T, Ogura A and Shinohara T (2008). Pluripotency of a single spermatogonial stem cell in mice. *Biol Reprod* **78**, 681–7.
- Ketkar A and Reddy KVR (2012). Effect of Oct-4 silencing on proliferation and differentiation of mouse undifferentiated Type A spermatogonial cells. *J Cell Sci Ther* **3**, 131.
- Khajavi N, Akbari M, Abdolsamadi HR, Abolhassani F, Dehpour AR, Koruji M and Habibi Roudkenar M (2014). Role of somatic testicular cells during mouse spermatogenesis in three-dimensional collagen gel culture system. *Cell J* **16**, 79–90.
- Kubota H, Avarbock MR and Brinster RL (2004). Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proc Natl Acad Sci USA* **101**, 16489–94.
- Lewicki J, Bergman J, Kerins C and Hermanson O (2019). Optimization of 3D bioprinting of human neuroblastoma cells using sodium alginate hydrogel. *Bioprinting* **16**, e00053.
- Lim F and Sun AM (1980). Microencapsulated islets as bioartificial endocrine pancreas. *Science* **210**(4472), 908–10.
- Mahmoud H (2012). Concise review: spermatogenesis in an artificial three-dimensional system. *Stem Cell* **30**, 2355–60.
- McLean DJ, Friel PJ, Johnston DS and Griswold MD (2003). Characterization of spermatogonial stem cell maturation and differentiation in neonatal mice. *Biol Reprod* **69**, 2085–91.
- Meng X, Lindahl M, Hyvönen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, Sarma M and Sariola H (2000). Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science* **287**(5457), 1489–93.
- Mohammadzadeh E, Mirzapour T, Nowroozi MR, Nazarian H, Piryaei A, Alipour F, Modarres Mousavi SM and Ghaffari Novin M (2019). Differentiation of spermatogonial stem cells by soft agar three-dimensional culture system. *Artif Cells Nanomed Biotechnol* **47**, 1772–81.
- Nagano M, Ryu BY, Brinster CJ, Avarbock MR and Brinster RL (2003). Maintenance of mouse male germ line stem cells *in vitro*. *Biol Reprod* **68**, 2207–14.
- O'Shea GM and Sun AM (1986). Encapsulation of rat islets of Langerhans prolongs xenograft survival in diabetic mice. *Diabetes* **35**, 943–6.
- Oatley JM, Avarbock MR, Telaranta AI, Fearon DT and Brinster RL (2006). Identifying genes important for spermatogonial stem cell self-renewal and survival. *Proc Natl Acad Sci USA* **103**, 9524–9.
- Oatley JM, Avarbock MR and Brinster RL (2007). Glial cell line-derived neurotrophic factor regulation of genes essential for self-renewal of mouse spermatogonial stem cells is dependent on Src family kinase signaling. *J Biol Chem* **282**, 25842–51.
- Oatley JM, Kaucher AV, Avarbock MR and Brinster RL (2010). Regulation of mouse spermatogonial stem cell differentiation by STAT3 signaling. *Biol Reprod* **83**, 427–33.
- Orwig KE, Ryu BY, Master SR, Phillips BT, Mack M, Avarbock MR, Chodosh L and Brinster RL (2008). Genes involved in post-transcriptional regulation are overrepresented in stem/progenitor spermatogonia of cryptorchid mouse testes. *Stem Cell* **26**, 927–38.
- Patra SK, Vemulawada C, Soren MM, Sundaray JK, Panda MK and Barman HK (2018). Molecular characterization and expression patterns of *Nanog* gene validating its involvement in the embryonic development and maintenance of spermatogonial stem cells of farmed carp, *Labeo rohita*. *J Anim Sci Biotechnol* **9**, 45.
- Pesce M, Wang X, Wolgemuth DJ and Schöler HR (1998). Differential expression of the Oct-4 transcription factor during mouse germ cell differentiation. *Mech Dev* **71**(1–2), 89–98.
- Phillips BT, Gassei K and Orwig KE (2010). Spermatogonial stem cell regulation and spermatogenesis. *Phil Trans Roy Soc Lond B* **365**(1546), 1663–78.
- Pirnia A, Parivar K, Hemadi M, Yaghmaei P and Gholami M (2017). Stemness of spermatogonial stem cells encapsulated in alginate hydrogel during cryopreservation. *Andrologia* **49**, e12650.
- Pravdyuk AI, Petrenko YA, Fuller BJ and Petrenko AY (2013). Cryopreservation of alginate encapsulated mesenchymal stromal cells. *Cryobiology* **66**, 215–22.

- Sarker B, Rompf J, Silva R, Lang N, Detsch R, Kaschta J, Fabry B and Boccaccini AR (2015). Alginate-based hydrogels with improved adhesive properties for cell encapsulation. *Int J Biol Macromol* **78**, 72–8.
- Schmidt JA, Avarbock MR, Tobias JW and Brinster RL (2009). Identification of glial cell line-derived neurotrophic factor-regulated genes important for spermatogonial stem cell self-renewal in the rat. *Biol Reprod* **81**, 56–66.
- Sharpe R (1994) Regulation of spermatogenesis. In *The Physiology of Reproduction* (Knobil E and Neill JD eds). Raven Press: New York. pp. 1363–434.
- Sofikitis N, Pappas E, Kawatani A, Baltogiannis D, Loutradis D, Kanakas N, Giannakis D, Dimitriadis F, Tsoukanelis K, Georgiou I, Makrydimas G, Mio Y, Tarlatzis V, Melekos M and Miyagawa I (2005). Efforts to create an artificial testis: culture systems of male germ cells under biochemical conditions resembling the seminiferous tubular biochemical environment. *Hum Reprod Update* **11**, 229–59.
- Song HW and Wilkinson MF (2014). Transcriptional control of spermatogonial maintenance and differentiation. *Semin Cell Dev Biol* **30**, 14–26.
- Wang N, Adams G, Buttery L, Falcone FH and Stolnik S (2009). Alginate encapsulation technology supports embryonic stem cells differentiation into insulin-producing cells. *J Biotechnol* **144**, 304–12.
- Wu X, Oatley JM, Oatley MJ, Kaucher AV, Avarbock MR and Brinster RL (2010). The POU domain transcription factor POU3F1 is an important intrinsic regulator of GDNF-induced survival and self-renewal of mouse spermatogonial stem cells. *Biol Reprod* **82**, 1103–11.
- Yang L, Wu W and Qi H (2013). Gene expression profiling revealed specific spermatogonial stem cell genes in mouse. *Genesis* **51**, 83–96.
- Yeh JR, Zhang X and Nagano MC (2007). Establishment of a short-term in vitro assay for mouse spermatogonial stem cells. *Biol Reprod* **77**, 897–904.
- Ziloochi Kashani M, Bagher Z, Asgari HR, Najafi M, Koruji M and Mehraein F (2020). Differentiation of neonate mouse spermatogonial stem cells on three-dimensional agar/polyvinyl alcohol nanofiber scaffold. *Syst Biol Reprod Med* **66**, 202–15.