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Evaluation of co-cultured spermatogonial stem cells encapsulated in alginate hydrogel with Sertoli cells and their transplantation into azoospermic mice

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Research Article

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Summary

An *in vitro* spermatogonial stem cell (SSC) culture can serve as an effective technique to study spermatogenesis and treatment for male infertility. In this research, we compared the effect of a three-dimensional alginate hydrogel with Sertoli cells in a 3D culture and co-cultured Sertoli cells. After harvest of SSCs from neonatal mice testes, the SSCs were divided into two groups: SSCs on a 3D alginate hydrogel with Sertoli cells and a co-culture of SSCs with Sertoli cells for 1 month. The samples were evaluated by quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays and bromodeoxyuridine (BrdU) tracing, haematoxylin and eosin (H&E) and periodic acid–Schiff (PAS) staining after transplantation into an azoospermic testis mouse. The 3D group showed rapid cell proliferation and numerous colonies compared with the co-culture group. Molecular assessment showed significantly increased *integrin alpha-6*, *integrin beta-1*, *Nanog*, *Plzf*, *Thy-1*, *Oct4* and *Bcl2* expression levels in the 3D group and decreased expression levels of *P53*, *Fas*, and *Bax*. BrdU tracing, and H&E and PAS staining results indicated that the hydrogel alginate improved spermatogenesis after transplantation *in vivo*. This finding suggested that cultivation of SSCs on alginate hydrogel with Sertoli cells in a 3D culture can lead to efficient proliferation and maintenance of SSC stemness and enhance the efficiency of SSC transplantation.

Introduction

Spermatogenesis is a dynamic process that is controlled by autocrine/paracrine variables in the endocrine system and, locally, by numerous interactions between developing germ cells and the environment (Caires *et al*., 2010; Hai *et al*., 2014; Giudice *et al*., 2017). The spermatogenesis process is extrinsically controlled by testicular somatic cells (Sertoli, myoid and Leydig cells) along with intrinsic regulators that include transcription factors produced by spermatogonial cells (Mei *et al*., 2015). Proliferation and differentiation of spermatogonial stem cells (SSCs) is a complex and tightly regulated process that occurs in the basement compartment of seminiferous tubules (Talebi *et al*., 2019).

Sertoli cells are the major somatic cells of the SSC niche. They provide morphogenetic support through cell–cell interactions as well as biochemical components by secreting lactate, cytokines and hormones. Aside from mechanical and nutritional support, SCs also provide an immune-protective environment for germ cells via the blood–testis barrier (Ni *et al*., 2019). Sertoli cells are thought to be the primary cellular target of testosterone signalling. In the Sertoli cell, testosterone signals can be translated directly into changes in gene expression, or testosterone can activate kinases, which may regulate processes necessary for spermatogenesis to continue (Walker, 2011). During spermatogenesis, Sertoli cells stimulate SSC self-renewal and promote SSC differentiation; they also regulate meiosis in spermatocytes and conversion of round spermatids to spermatozoa (Ma *et al*., 2013; Hai *et al*., 2014). Studies have shown that the overall efficiency and effectiveness of spermatogenesis depend strongly on the existence of the Sertoli cell–SSC niche (Spradling *et al*., 2001).

Different strategies for *in vitro* derivation of male germ cells are mostly based on two-dimensional (2D) cultures, implementation of established medium, and feeder cells (Sousa *et al*., 2002; Riboldi *et al*., 2012; Yang *et al*., 2014). It has been reported that during the SSC differentiation process, the spatial arrangement of testicular cells is incredibly important (Staub, 2001). In the natural environment, meiotic cells are engulfed in Sertoli cells as large

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interconnected clones that have no interaction with the basement membrane; the 2D culture system cannot provide this sophisticated structure (Khajavi *et al.*, 2014). According to previous studies, three-dimensional (3D) culture systems provide more spatial conditions that mimic the architecture of seminiferous tubules (Shams *et al.*, 2017). In a 3D culture, the cells interact with one another, the extracellular matrix (ECM), and the surrounding microenvironment. Proliferation, differentiation, cell morphology, gene and protein expression, and cell response to external factors are influenced by these interactions in 3D structures (Mohammadzadeh *et al.*, 2019). To date, the behaviour of SSCs has been evaluated in cultivation systems that use 3D surfaces such as soft agar (Abu Elhija *et al.*, 2012), collagen (Lee *et al.*, 2007), alginate (Chu *et al.*, 2009; Jalayeri *et al.*, 2017; Pirnia *et al.*, 2017), poly L-lactic acid (PLLA) (Eslahi *et al.*, 2013) and polyamide (Ultra-Web^{TM}) nanofibres (Shakeri *et al.*, 2013). According to previous research, the 3D culture system assisted by somatic cells could provide an enhanced culture system by creating both physical and paracrine support to enable SSCs to enter meiosis (Stukenborg *et al.*, 2009; Khajavi *et al.*, 2014).

Although the results of numerous studies have shown the essential role of somatic testicular cells in the induction of spermatogenesis, the function of these cells in meiotic progression during co-culture with an SSC encapsulated alginate hydrogel 3D culture system remains uncertain.

Taking all this into account, the *in vitro* conditions for full spermatogenesis are far from routine methodology. In this study, we aimed to evaluate a co-culture of SSC encapsulated alginate hydrogel with Sertoli cells and their transplantation into azoospermic mice.

Materials and methods

Isolation and purification of SSCs

The Ethics Committee of Kermanshah University of Medical Sciences in compliance with the guidelines of Kermanshah University of Medical Sciences approved the animal experiments in this research. Testes were obtained from 80 6-day-old male neonatal BALB/c mice for testicular cell isolation and transferred to phosphate-buffered saline (PBS; Gibco, USA). A two-step enzymatic digestion process was used for tissue digestion. Prior to enzymatic digestion, the tunicae albugineae were removed from all testicles. Pieces of minced testes were digested in a solution that contained collagenase type IV (Sigma, 2 µg/ml) and DNase I (5 µg/ml) for 15 min at 37°C in a 5% CO_2 incubator. After centrifugation at 800 *g* for 5 min, the cell pellet was re-suspended in 1 ml trypsin–EDTA (Sigma) and incubated for 5 min at 37°C with gentle pipetting to ensure suspension of the testicular cells. The cell suspension was then centrifuged for 5 min at 800 *g* and the cell pellet was washed in PBS. The SSCs were purified according to the Invitrogen protocol for magnetic activated cell sorting (MACS) (Jalayeri *et al.*, 2017; Pirnia *et al.*, 2017).

Encapsulation of SSCs in alginate

We prepared the sodium alginate solution by dissolving 1.25 g of powdered alginate in 150 mmol NaCl at pH 7.4. This solution was mixed with the cell pellet. Next, the cell alginate solution was added slowly dropwise to 135 mmol/l of calcium chloride, which created a MicroBead cell alginate. After 10 min, the calcium chloride was extracted by washing the MicroBeads with 0.9% NaCl.

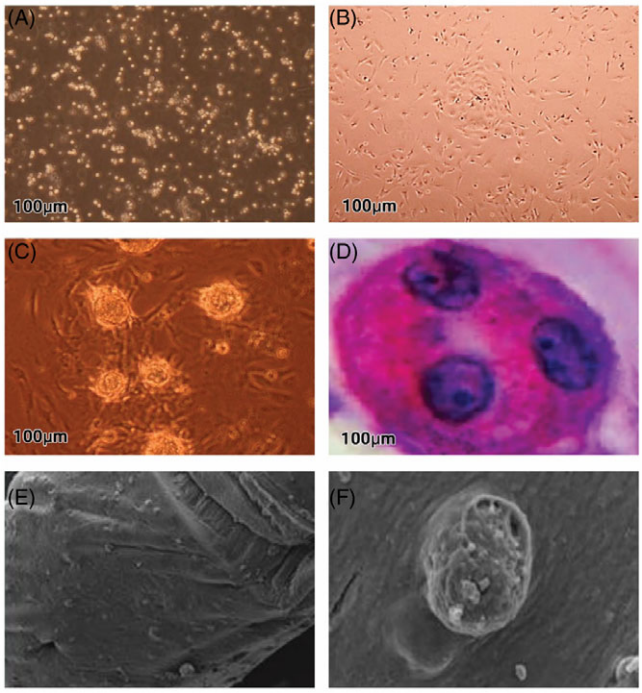

Figure 1. (A) Spermatogonial stem cells (SSCs) after enzymatic digestion using trypsin–EDTA. (B) Morphology of Sertoli cells. (C) SSC colonies. (D) Microbeads after 3D long-term culture stained with haematoxylin and eosin (H&E) dye. Stained sections showed normal spermatogonia morphology. (E, F) Scanning electron microscopy micrographs from SSCs encapsulated in alginate hydrogel.

Depolymerization of the cell alginate solution

We used a solution of 119 mmol/l sodium citrate for depolymerization. The MicroBead solution was placed in an incubator for *c.* 30 min and then centrifuged at 1800 rpm for 8 min. Next, 1 ml of Dulbecco modified Eagle's medium (DMEM) was added to the cell pellet.

Preparation of the Sertoli cell feeder layer

A mouse Sertoli cell line (NCBI code: C513) was purchased from the Pasteur Institute, Iran. Sertoli cells were cultured in a *Datura stramonium* agglutinin (DSA lectin, 10 µg/ml)-coated 25 T flask that contained DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), essential amino acids (EAA), non-essential amino acids (NEAA), and an antibiotic–antimycotic solution (Invitrogen, USA). The flasks were incubated for 2 h at 37°C in a humidified atmosphere of 5% CO_2 in air. When the Sertoli cells reached 90% confluency, they were treated with 10 µg/ml mitomycin C for 3 h. These cells were washed three or four times with DMEM and were subsequently used as the feeder layer (Fig. 1B).

Experimental protocol

Two experimental designs were investigated. In the first group, SSCs obtained by magnetic activated cell sorting (MACS) were cultured in an alginate hydrogel 3D culture with Sertoli cells under 3D culture conditions for 30 days. In the second group, SSCs were co-cultured with Sertoli cells as the feeder layer (2D culture system) for 30 days. The culture medium consisted of 10% FBS and 10 ng/ml glial cell-derived neurotrophic factor (GDNF; Sigma). The SSCs were incubated at 37°C and 5% CO_2 in a humidified atmosphere. The medium was replaced by fresh medium every

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Table 1. Primer sequences used for quantitative RT-PCR

Gene	Primer sequences (5′–3′)	Size (bp)	GenBank reference number
GAPDH-208-F	CAATGTGTCCGTCGTGGATCT	208	NM_008084
GAPDH-208-R	GTCCTCAGTGTAGCCCAAGATG		
Bcl2-mus-F	TAAGCTGTCACAGAGGGGCT	344	NM_007741
Bcl2-mus-R	TGAAGAGTTCCTCCACCACC		
BAX-277-F	CCGGCGAATTGGAGATGAACT	277	NM_007527
BAX-277-R	CCAGCCCATGATGGTTCTGAT		
FAS-314-F	TGCGATTCTCCTGGCTGTG	314	NM_004104
FAS-314-R	CAACCATAGGCGATTTCTGGG		
Nanog-154-F	AGGACAGGTTTCAGAAGCAGAAGT	220	NM_001289828
Nanog-154-R	TCAGACCATTGCTAGTCTTCAACC		
Oct4–170-F	CGGAAGAGAAAGCGAACTAGC	108	~B+ NM_001252452 +B~
Oct4–170-R	ATTGGCGATGTGAGTGATCTG		
PLZF-174-F	CGAGCTTCCGGACAACGA	120	NM_001033324
PLZF-174-R	AAATGCATTCTCAGTCGCAAAC		
Trp53-mus-F	AGAGACCGCCGTACAGAAGA	255	NM_011640
Trp53-mus-R	GCATGGGCATCCTTTAACTC		
Thy1-mus-F	TGCTCTCAGTCTTGCAGGTG	121	NM_009382
Thy1-mus-R	TGGATGGAGTTATCCTTGGTGTT		
integrin alpha 6-mus-F	GGAACTTCTGAACTTCGTGCTGTA	155	~B+ NM_001277970 +B~
integrin alpha 6-mus-R	AGTAGTCTCACACTGGATTGGTCT		
β1-Integrin-mus-F	GCCAGGGCTGGTTATACAGA	226	~B+ NM_010578 +B~
β1-Integrin-mus-R	TCACAATGGCACACAGGTTT		
c-kit-mus-F	CTCACATAGCAGGGAGCACA	123	NM_001122733
c-kit-mus-R	ACAACTCACCCACACGCATA		

3 days. Cell growth and colony formation ability were analyzed under an invert microscope and cell viability was evaluated using the trypan blue assay.

RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted using a SinaPure RNA kit according to the manufacturer's protocol. The concentration of RNA was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). The extracted RNA was used for cDNA synthesis with a Revert Aid First Strand cDNA synthesis kit. qRT-PCR was conducted with specific primers for the *integrin alpha-6*, *integrin beta-1*, *Nanog*, *Plzf*, *Thy-1*, *Oct4*, *P53*, *Fas*, *Bax*, and *Bcl2* genes (Table 1). *Gapdh* was used as a reference gene to standardize the relative qRT-PCR results. Data were estimated using the $\Delta\Delta C_t$ equation.

5-Bromo-2-deoxyuridine (BrdU) cell labelling and transplantation into recipient mice

We added BrdU to the medium 24 h before transplantation to label and trace the cells in the recipient mice. This step was performed after depolymerization of the SSC-alginate in the 3D culture and the SSC clusters and underlying Sertoli cells were trypsinized in the 2D culture at the end of 4 weeks.

Adult male (6–8-week-old) BALB/c mice were used for recipient mice. The mice testes were treated with 40 mg/kg busulfan prior to transplantation (Fig. 3A). Then, the spermatogonial cells were transplanted into the seminiferous tubules of the recipient mice. At 6 weeks after treatment, Adult recipient mice were anesthetized with 10% ketamine and 2% xylazine. Approximately 10^5 of the cultured cells in 10 µl DMEM were injected into the seminiferous tubules in one testis per recipient mouse, while the other testis was used as an internal control. The cells were transplanted by retrograde injection through the efferent ducts (Gholami *et al.*, 2014). The recipient testes were collected 6 weeks after transplantation, and 5–7 µm cross-sections were prepared after the cells were fixed in a paraffin block to consider spermatogenesis. We used the BrdU IHC kit according to the manufacturer's instructions to identify the injected SSCs that were labelled with BrdU.

Haematoxylin and eosin staining

At 6 weeks after the SSC transplantation, the mice were killed with a high dose of anaesthetic and their testicular tissues were harvested for examination. After fixation, the left testes were enclosed in paraffin, cut, and dehydrated. These tissues were then stained with haematoxylin and eosin (H&E) to evaluate the histologic changes. The findings were evaluated on the basis of a descriptive procedure. Blinded evaluation of tissue sections inside similarly

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staged seminiferous tubules was carried out by specialists. The results were evaluated according to the method by Johnson (Lewis-Johnes, 1985).

PAS-H staining

Deparaffinized and rehydrated sections were soaked for 10 min in a 0.5% periodic acid solution and rinsed in flowing tap water. The sections were immersed in Schiff's reagent (Merck, Darmstadt, Germany) for 15 min and then treated with sulfur water [12 ml of 10% sodium bisulfite solution, 10 ml of hydrochloric acid (1 N), and 200 ml of distilled water] three times for 3 min each. After rinsing in flowing tap water, the sections were stained with haematoxylin.

Statistical analysis

Data were analyzed and compared using one-way analysis of variance (ANOVA) followed by Tukey's test for post-hoc analysis. Data are shown as the mean ± standard error of the mean (SEM). A P-value < 0.05 was considered to be statistically significant.

Results

Isolation and enrichment of mouse SSCs

Mouse SSCs were isolated by a procedure that involved sequential enzymatic digestion with three enzymes: collagenase IV, DNase I and trypsin. In the current research, SSCs were isolated using anti-Thy-1 antibody and MACS. The viability of the isolated cells was determined by trypan blue staining, which indicated that the vast majority (>90%) of cells were viable (Fig. 1A).

Colony formation of SSCs

The SSCs on the Sertoli cell feeder layer (Fig. 1B) were round or oval bodies with large nuclei and small cytoplasm. At 48 h, the SSCs started to form cell clusters and colonies were visible on the third day (Fig. 1C). We observed that the SSCs in the alginate capsules were circular and not attached to the surface. H&E staining results showed that the encapsulated SSCs had a healthy appearance and a well defined membrane, and nuclei were enclosed in alginate and distributed evenly inside the alginate (Fig. 1D). Electron microscopy examination of the encapsulated SSCs showed that SSCs inside the alginate scaffold formed spherical colonies and had optimal growth (Fig. 1E, F).

Molecular assessment

SSCs on the alginate hydrogel 3D culture with Sertoli cells in the 3D culture had significantly decreased expression levels of the *Fas*, *P53*, and *Bax* genes compared with the SSCs co-cultured with a feeder layer of Sertoli cells ($P < 0.05$; Fig. 2A). The expression levels of *Bcl2*, *integrin beta-1*, *Thy1*, *Oct4*, *integrin alpha-6* and *Nanog* genes at 4 weeks after culture in the alginate hydrogel by Sertoli cells under the 3D culture of SSCs increased significantly when compared with the mSSCs co-cultured with Sertoli cells ($P < 0.05$; Fig. 2A, B). There was less c-*kit* expression by Sertoli cells in the alginate hydrogel under the 3D culture system compared with the 2D culture system ($P < 0.05$; Fig. 2B).

Transplantation

Prior to transplantation, the SSCs were labelled with BrdU to confirm the existence of SSCs in clusters and evaluate the colonization

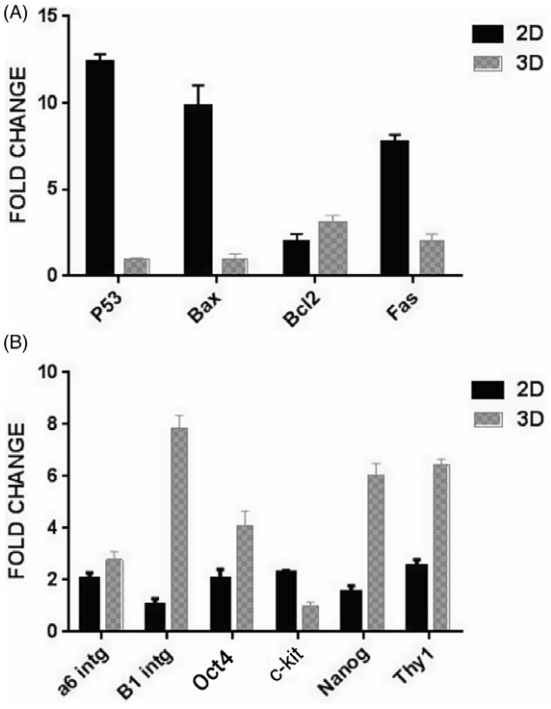

Figure 2. (A) Relative fold change of *Fas*, *P53*, *Bax*, and *Bcl2* gene expression in spermatogonial cells that were cultured in 3D and 2 D systems. (B) Quantitative gene expression analysis by real-time PCR for *integrin beta-1*, *Thy1*, *Oct4*, *integrin alpha-6*, c-*kit* and *Nanog* genes in cultured mouse undifferentiated SSCs in 3D and 2D systems at the 30th day of culture. Values are mean ± standard error of the mean (SEM) and indicated statistically significant difference. Indicated statistically significant difference ($P < 0.05$).

of SSCs in the testis. At 1 month after transplantation, findings of the morphometric studies showed that *c.* 52% of the donor SSCs from the 3D culture combined with BrdU were visible inside the transplanted testis; *c.* 34% of the donor SSCs from the 2D culture combined with BrdU were visible inside the transplanted testis (Fig. 3D). BrdU cell labelling results showed that more 3D cultured cells were located in the basement membrane (Fig. 3B) compared with for 2D cultured cells (Fig. 3C). In sections of ferrous seminiferous tubules, more labelled cells were observed from the 3D culture than from the 2D cultured cells for the central part of these tubes.

PAS

PAS staining of the transplanted samples from the 3D and 2D cultures showed that samples grafted with alginate hydrogel-based 3D cultured cells were more successful in the spermatogenesis process. In the cavities of the seminiferous tubules, the acrosome of sperm differentiated from these cells had distinct staining (Fig. 4A). However, in the samples transplanted with cells obtained from the 2D culture, the cavities of the seminiferous tubules lacked differentiated cells and, compared with the samples transplanted with cells obtained from the 3D culture, the process of cells towards differentiation was slower and the haploid spermatozoa were relatively less formed (Fig. 4B).

H&E staining of the transplanted tissue sections

H&E staining results indicated that the transplanted cells from the 3D and 2D cultures (Fig. 4C, D) showed the successful process of

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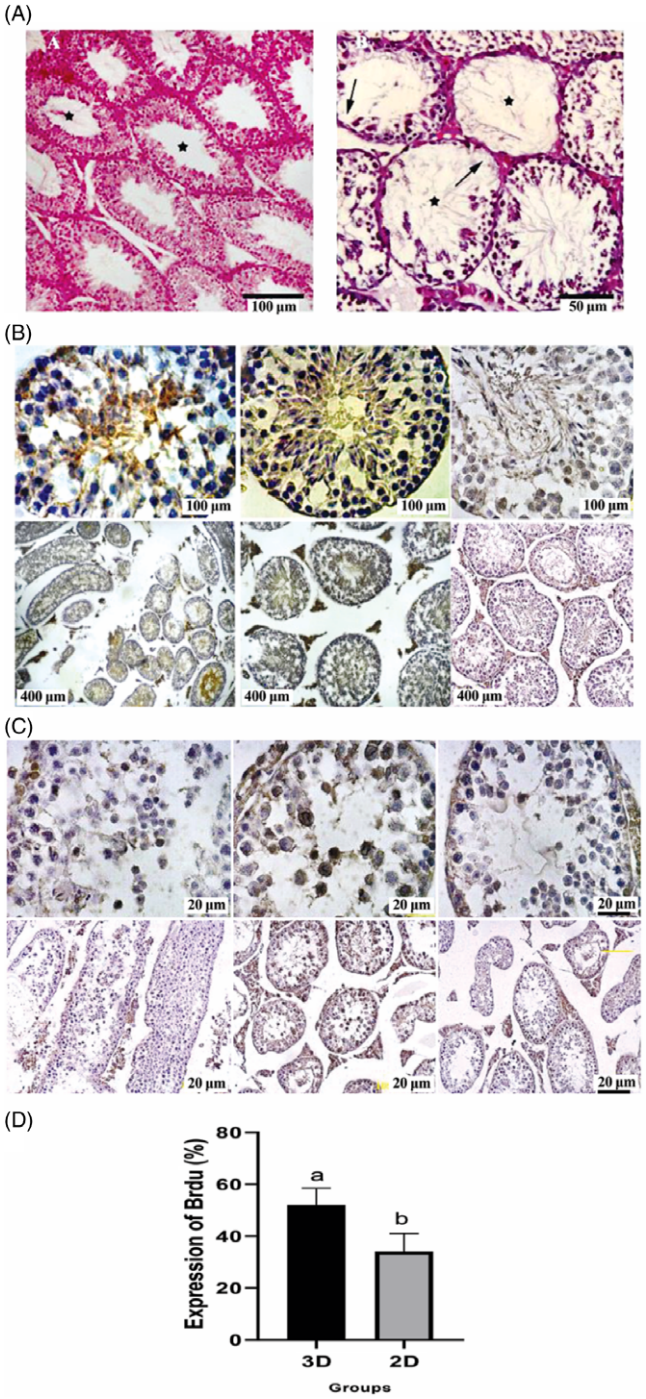

Figure 3. Fertility restoration in busulfan azoospermic mouse model by transplantation with cultured SSCs that were isolated from 6-day immature mice after culture in 3D or 2D systems. (A) Absence of any spermatogonial cells in an azoospermic mouse model seminiferous duct. (B) BrdU staining (brown stain) of 3D cultured SSCs confirms that donor-derived spermatogenesis is reconstituted. (C) BrdU staining of SSCs co-culture with Sertoli cells and transplantation. (D) Comparison of mean percentage of labelled cells of 2D and 3D cultures in transplanted testicular samples using immunohistochemistry.

differentiation of SSCs from the 3D culture and formation of haploid cells in this group of cells compared with 2D culture transplanted cells. Fig. 4(C) shows staining of samples grafted with cells from the 3D culture. The porous compartment of the seminiferous tubules was clearly filled with floating spermatozoa cells. Fig. 4(D)

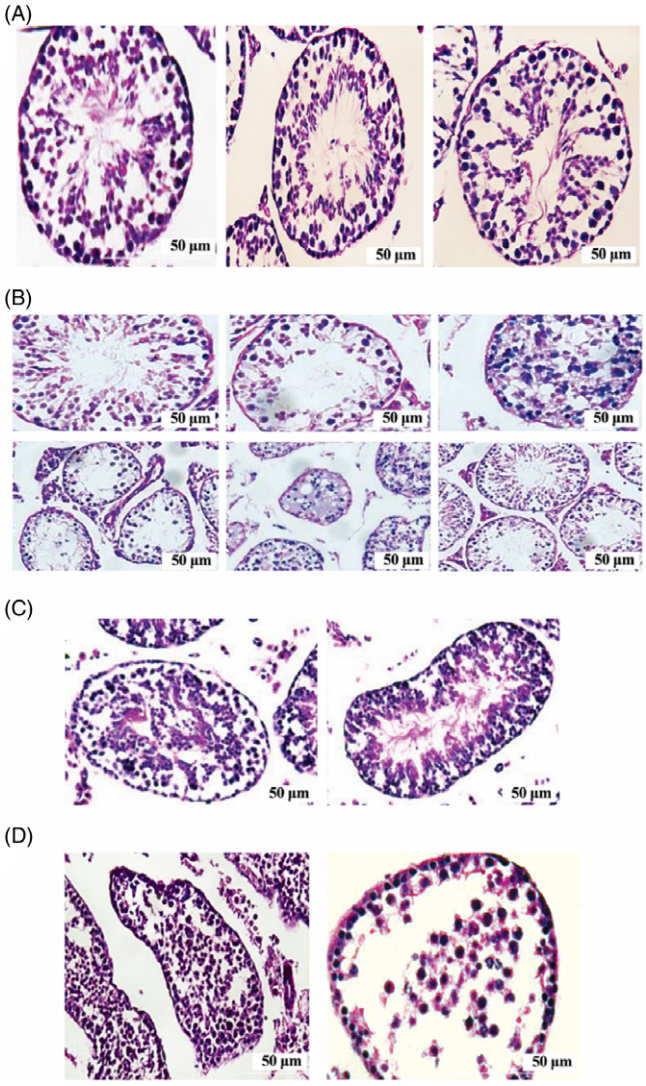

Figure 4. (A, B) PAS staining of testes tissue that received 3D or 2D cultured spermatogonial stem cell transplantation. (C, D) Testis sections stained with haematoxylin and eosin. (C) Samples that received 3D cultured spermatogonial stem cell transplantation. (D) Samples that received 2D cultured spermatogonial stem cell transplantation.

shows the sample obtained from transplantation with 2D culture cells. The luminal part of the seminiferous tubules did not contain any differentiated cells.

Discussion

Long-term SCC culture under laboratory conditions can result in the loss of specific properties of these cells. A culture system that can establish conditions that more closely resemble the body can be effective in preserving, proliferating and differentiating SCCs. Various factors, including physical interaction of these cells with adjacent cells and unique molecules present in the environment, can lead to the maintenance and induction of SSCs. Therefore, neighbouring cells, growth factors and ECM compounds will modulate and regulate the differentiation, division, and apoptosis of stem cells at each time interval (Berná *et al.*, 2001). A significant body of research has been carried out on the culture, differentiation and cryopreservation of SSCs in 3D alginate hydrogel substrates

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(Chu *et al.*, 2009; Jalayeri *et al.*, 2017; Pirnia *et al.*, 2017). The outcomes of these experiments demonstrated the role of the 3D substrate in maintenance of SSCs. In view of the lack of cytotoxicity and antioxidant properties of the alginate hydrogel, this 3D scaffold could be used for sperm stem cell culture (Jalayeri *et al.*, 2017). Alginate can mimic ECM for SSCs and to promote the capacity for stemness during the cryopreservation process and to restart spermatogenesis after transplantation (Pirnia *et al.*, 2017).

In combination with somatic testicular cells, embedding SSCs in a 3D culture system can provide a structure that mimics the complex structure found in living testes. In an alginate hydrogel, somatic testicular cells and SSCs might create proper contact with the cells and stimulate the differentiation of germ cells in the culture system. In addition, alginate hydrogel and ECM similarities can provide sufficient access for cells to structural proteins and biological molecules. Alginate hydrogel in a 3D culture system can also retain growth factors that are secreted by Sertoli cells (Sargus-Patino, 2013). Tesarik and colleagues (Tesarik *et al.*, 2000) and Minaee Zanganeh and co-workers (Minaee Zanganeh *et al.*, 2013) have demonstrated that a SSC–Sertoli co-culture can advance spermatogenesis during short-term cultivation and provide conditions that might allow the successful *in vitro* differentiation of SSCs. Sertoli cells provide the critical factors required for the efficient differentiation of SSC cells into sperm cells, and their endocrine or paracrine factors play an important role in maintaining sperm cell viability and inducing animal meiosis *in vitro* (Sofikitis *et al.*, 2005). Our data showed that the presence of an alginate scaffold in a culture system compared with the SSCs co-cultured with Sertoli cells increased the mRNA levels of *integrin alpha-6*, *integrin beta-1*, *Nanog*, *Plzf*, *Thy-1* and *Oct4*. *Oct4* and *Nanog* are transcription factors essential for the preservation of embryonic stem cell pluripotency and self-renewal (Assadollahi *et al.*, 2019a, 2019b). The *Oct-4* gene, a premeiotic specific marker, is expressed before spermatogenesis in male mice and is confined to spermatogonia type A (Khajavi *et al.*, 2014). Studies have shown that expression of *Nanog* might be the cause of PTEN and TRP53 suppression (Kuijk *et al.*, 2010; Azizi *et al.*, 2017). *Thy-1*, *integrin alpha-6* and *integrin beta-1* could be considered as markers of SSCs (Oatley *et al.*, 2010; Wang *et al.*, 2014). *Plzf* is active in the maintenance of stem cells. Loss of *Plzf* function changes the balance between self-renewal of spermatogonia stem cells and differentiation towards the cost of self-renewal, which leads to an increase in post-meiosis apoptotic cells (Buaas *et al.*, 2004; Costoya *et al.*, 2004). *Plzf* directly suppresses c-*kit* transcription (Filipponi *et al.*, 2007). c-*kit* is a marker for the lost pluripotency of SSCs and its expression continues until the initiation of meiosis (Zhang *et al.*, 2013). In this study, we analyzed c-*kit* gene expression and observed a decreased level of expression of this gene in cells cultured on alginate hydrogel with Sertoli cells under 3D culture compared with the 2D culture system in SSCs co-cultured with Sertoli cells. Increased expression of undifferentiated spermatogonial cell markers indicated that the culture conditions on the scaffold were appropriate for the expansion of SSCs; decreased expression of the c-*kit* gene also indicated the beneficial effects of scaffolding on self-renewal of SSCs (Talebi *et al.*, 2019).

Apoptosis is a type of programmed cell death in which a highly complex and orderly series of biochemical (Goodman, 2007) apoptosis pathways are intrinsic (*Bax*, *Bcl2*, *P53*, *caspase3* genes) and extrinsic (*Fas*, *Fas-L* genes) (Singh *et al.*, 2019). The expression levels of apoptosis genes (*Bax*, *P53*, *Bcl2*, *Fas*) were assessed by qRT-PCR. We observed significantly lower expression levels of apoptosis genes in the group of alginate hydrogel encapsulated cells cultured with Sertoli cells compared with the group of SSCs co-cultured with Sertoli cells. Studies have shown that, by its anti-apoptotic properties, alginate prevents cell death by preventing oxidation (Król *et al.*, 2017). Long-term encapsulation of cells in alginate microcapsules makes them non-permeable and decreases the release of insulin and cell death. Encapsulation of SSCs helps cells to detect the external environment and release small proteins such as growth factors and it does not allow large proteins such as antibodies to enter the cell. Alginate appears to provide higher biocompatibility through proper distribution of oxygen and other nutrients (Jalayeri *et al.*, 2017).

Transplantation of SSCs into an azoospermic testis model has been performed on different animal models (Uchida and Dobrinski, 2018). It was found that transplantation of seminiferous SSCs could contribute to the restoration of spermatogenesis (Brinster and Avarbock, 1994). The niche of the SSCs contains the essential structural and basement membrane of the seminiferous tubule, the Sertoli cell, peritubular myoid cells, and Leydig cells. In this research, the azoospermia model was established 2.5 months after busulfan injection (Gholami *et al.*, 2014). Histological examination of the testes revealed that more tubules were devoid of germ cells and often contained a single, basal row of Sertoli cell nuclei. The Sertoli cells were naked and accessible in this state. Vacuum spaces between Sertoli cells act as hosts for transplanted SSCs and provide a suitable niche for homing cells (Rahmani *et al.*, 2019). In the current research, the findings of histological studies and the trace of BrdU in the host testes 6 or 8 weeks after transplantation suggested that the transplanted SSCs localized in the base membrane of the seminiferous tubules and showed the progression of spermatogenesis to the cells in the lumen centre. The progression of spermatogenesis of transplanted SSCs was significantly higher in cells cultured on the alginate hydrogel 3D culture by Sertoli cells under 3D culture conditions than in mSSCs co-cultured with Sertoli cells. Taken together, it could be said that alginate hydrogel 3D culture by Sertoli cells under 3D culture conditions enhanced transplantation efficiency.

Our findings showed that spermatogonial cells seeded on a scaffold had a higher progression in spermatogenesis, which could be correlated with the existence of more functional Sertoli cells. The results of other studies indicated that the presence of somatic cells in the culture system increased the spermatogenesis differentiation stage (Stukenborg *et al.*, 2008; Minaee Zanganeh *et al.*, 2013; Talebi *et al.*, 2019). Finally, the potential for the alginate hydrogel with Sertoli cells to support the spread of SSCs and *in vitro* spermatogenesis has been demonstrated in this study. Therefore, it is possible to use the findings of this study for scientific and clinical applications.

In conclusion, we have shown that SSCs can survive and proliferate on alginate hydrogel 3D culture by Sertoli cells under 3D culture conditions up to 30 days. On this 3D alginate hydrogel with Sertoli cells under 3D culture conditions, self-renewal, colonization, and viability were improved compared with the 2D culture system in SSCs co-cultured with Sertoli cells as the feeder layer. Our study also demonstrated that transplantation of SSCs cultured in an alginate hydrogel 3D culture by Sertoli cells under 3D culture improved the quality of spermatogenesis *in vivo*. The process and findings of this study are new ideas for male infertility research, and they have the potential for use in the treatment of male infertility and reproduction of rare species. We hope to achieve better results in the future by applying modifications and improving culture conditions.

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Data availability. Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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Conflicts of interest. The authors declare they have no competing financial interests.

Ethics approval and consent to participate. All stages of this experiment were in accordance with the ethical standards of the Ethics Committee of Kermanshah University of Medical Sciences, Kermanshah, Iran (Approval ID: IR.KUMS.REC. 1397.811).

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