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Isolation, identification and differentiation of human spermatogonial cells on three-dimensional decellularized sheep testis

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ABSTRACT

Improvement of *in vitro* culture methods of Spermatogonial Stem Cells (SSCs) is known to be an effective procedure for further study of the process of spermatogenesis and can offer effective therapeutic modality for male infertility. Tissue decellularization by providing natural 3D and extracellular matrix (ECM) conditions for cell growth can be an alternative procedure to enhance *in vitro* culture conditions. In the present study, the testicular tissues were taken from brain death donors. After enzymatic digestion, the tissue cells were isolated and cultured for four weeks. Then the identity of the SSCs was confirmed using anti-GFRα1 and anti-PLZF antibodies via immunocytochemistry (ICC). The differentiation capacity of SSCs were evaluated by culture of them on a layer of decellularized testicular matrix (DTM) prepared from sheep testis, as well as under two-dimensional (2D) culture with differentiation medium. After four and six weeks of the initiation of differentiation culture, the pre-meiotic, meiotic and post- meiotic genes at the mRNA and protein levels was examined via qPCR and ICC methods, respectively. The results showed that pre-meiotic, meiotic and post-meiotic genes expressions were significantly higher in the cells cultured in DTM substrate ($P \leq 0.01$).The present study indicated that, the natural structure of ECM prepare the suitable conditions for further study of the spermatogenesis process in the in vitro and contributes to the maintenance and treatment of male infertility.

1. Introduction

Male fertility is dependent on normal spermatogenesis. In this process, spermatogonial stem cells (SSCs) formed mature sex cell (spermatozoa) through a series of mitotic and meiotic cell divisions (Schlatt, 2002). *In vitro* culture of SSCs and spermatogenesis for achieving adult spermatozoa is considered a challenge in a large number of studies. However, good results has been achieved in the generation of mature spermatids in laboratory conditions, which, despite the low fertility rate, is capable of producing normal blastocysts (Tanaka et al., 2003). Spermatogonial cell culture for differentiation to mature spermatids is known to be an effective therapeutic approach for patients recommended for radiotherapy and chemotherapy against cancer, or azoospermia in which there is a condition of maturity arrest in the process of spermatogenesis (Aslam and Fishel, 1998). Preservation and differentiation of spermatogonial stem cells, which are a very small part of testicular cells (2–3 stem cells out of every 10^4 sexual cells), must be carried out in the same conditions as it occurs within the body. Given that the generation of such substrate is not made possible in conventional culture media, cell culture in a 3D substrate has been considered

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more widely (Hofmann et al., 1992; Lee et al., 2006; Stukenborg et al., 2009, 2008). 3D culture with the provision of the necessary conditions similar to that, which occur within the body, is an effective solution for laboratory culture of spermatogonial stem cell for the process of spermatogenesis. With the relevance of 3D SSC culture *in vitro*, selection of a suitable substrate; for this process of cell, differentiation has proven to be very effective. Cells in the body communicate with their surrounding through extracellular matrix. This extracellular matrix (ECM) contains various types of collagen, glycoprotein, proteoglycan and glycosaminoglycan compounds. The molecules of these compounds are intertwined together to form complex structures. However, the ECM protein constituents are different in different types of tissue and organs and in varying amounts (Manabe et al., 2008), and this is dependent upon growth, developmental and pathological conditions (Bonnans et al., 2014; Tom et al., 2009). ECM plays an important role in different mechanical stimulation and factors affecting the functions of the cells, and also providing the required conditions for communication between the cells with respect to the constituent substrate (Hoshiba et al., 2016). Previous studies demonstrated the effect of the ECM on the preservation and differentiation of stem cells (Bi et al., 2007, 2005; Rozario and DeSimone, 2010; Watt and Hogan, 2000). However, it is difficult to obtain the ECM model with the same components and condition to that of the body *in vitro*. Therefore, it is necessary for the adoption of a decellularized tissue as a model for laboratory ECM. Given that, the testicular tissue is rich in ECM due to the type of structure. In this study, we attempted to connect the cells together and differentiate them in vitro through SSCs culture in the decellularized layer of sheep testicular tissue under a condition similar to that occur naturally in the body (Fig. 1).

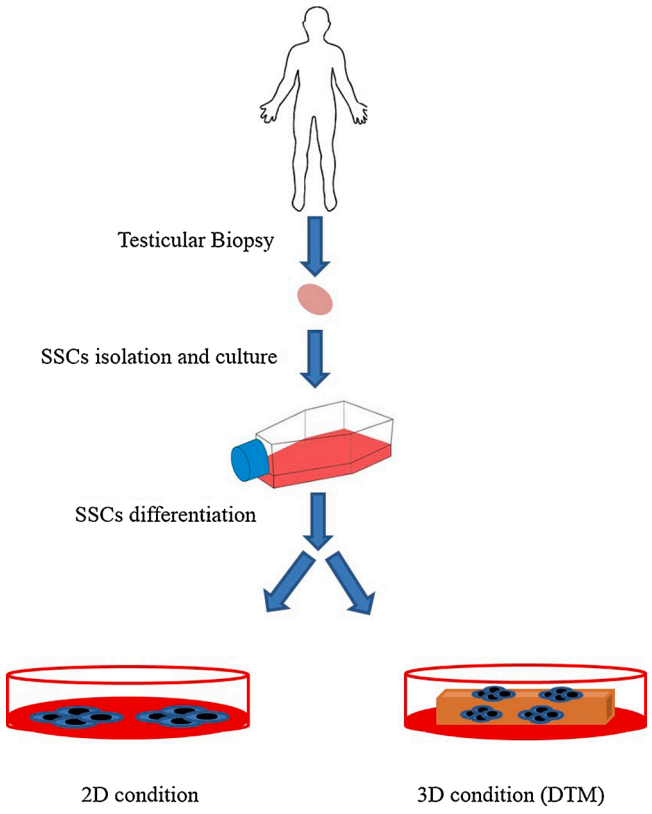

Fig. 1. Schematic presentation of experimental procedures.

2. Methods

2.1. Tissue collection

Human testes samples were obtained from three brain-dead patients of 18, 26, and 38 years old; this work was carried out on the basis of the license of the Medical Ethics Committee of the Tehran University, as well as informed consent from their family for the testing of the testes for research. (IR.TUMS.REC1394.1751).

2.2. Testis dissociation

After the autopsy, the testicular tissues were removed and transferred to the culture lab in a culture medium containing antibiotic at 4 °C. Testicular tissue cells were isolated according to the methods presented by Kanatsu-Shinohara et al. (Kanatsu-Shinohara et al., 2003; Sadri-Ardekani et al., 2009) with slight modifications. In short, after washing the testicular tissue several times in a PBS solution for cell isolation, the testis capsule was first removed, and then testicular tissue was divided into fragments of approximately 0.5–1.0 mm^3 using the mechanical procedure. Thereafter, the dissociated tissues were placed in a 37 °C shaker water bath for 30 min in a solution containing collagenase type I 1 mg/mL (Sigma-Aldrich, St Louis, MO, USA), 1 mg/mL trypsin (Sigma-Aldrich, St Louis, MO, USA) and 0.5 mg/mL DNase (Thermo Fisher Scientific, Massachusetts, USA). The fragments obtained from digestion were centrifuged at 1200 RPM for 3 min and washed in Dulbecco Modified Eagle medium /Nutrient Mixture F-12 (DMEM-F12, Gibco, Grand Island, NY, USA) medium and digested again for 30−45 min in a solution containing collagenase 1 mg/mL, 0.5 mg/mL DNase and hyaluronidase 1.5 mg/m (Sigma-Aldrich, St Louis, MO, USA) in a shaker water bath at 37 °C. After filtration thought a 70-μm cell strainer, the collected cells were washed and prepared for culture.

2.3. SSCs culture and proliferation

The cells harvested from tissue enzymatic digestion were cultured after evaluation and viability analysis using staining with 0.4 % trypan blue solution in a 6-well plate with the culture medium DMEM/F12 containing 10 % FBS (Gibco, Grand Island, NY, USA) with 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Grand Island, NY, USA). In order to isolate SSCs which are normally attached to the bottom of the culture dish later than the somatic cells, the supernatant was removed after 24 h, and centrifuged to isolate the non-attached cells. Then, cells were cultured in DMEM/F12 medium containing 5% KSR (Gibco, Grand Island, NY, USA) and growth factors included 10 ng/mL GDNF (GDNF; G1777, Sigma-Aldrich St. Louis, MO, USA), 20 ng/mL EGF (EGF; E4127, Sigma-Aldrich St. Louis, MO, USA), 10^3 U/mL LIF (LIF; L5283, Sigma-Aldrich St. Louis, MO, USA), 10 ng/mL bFGF (bFGF; F0291, Sigma-Aldrich St. Louis, MO, USA) in 0.2 % gelatin (Sigma-Aldrich, St Louis, MO, USA) coated dishes and then incubated at 37 °C and 5% CO2 for cell proliferation. After elapsing two weeks since the initial culture, SSC colonies were observed. For SSCs proliferation, the cells were cultured in the laboratory for about one month. Then, they were frozen at −196 °C using frozen solution containing 10 % DMSO and 90 % FBS.

2.4. Decellularization of sheep testicular tissue

Sheep testis was prepared from the slaughterhouse and then transferred to the laboratory in PBS solution at 4–8 °C. In the laboratory, the testis was washed several times in a PBS solution to remove the remaining blood. In the next stage, the tissue was transferred to the freezer for about 48 h, and then thin sections of 100-μm diameter were prepared after freezing. For the purpose of decellurization, the sections were transferred to a 1% sodium dodecyl sulfate solution (SDS) and then incubated for 24 h. Then they were placed in a phosphate saline buffer for 2 h. In order to sterilize the scaffolds, the sections were stacked in 70

% ethanol for 1 h. Then, after sterilization with sterile water, the sections were placed in sterile PBS for 2 h. The scaffolds were placed in the culture medium for 24 h before use.

2.5. DNA quantitative assay

DNA was extracted from native tissue and DTM uses QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. The purity and concentration of DNA were determined using the ND-3800 spectrophotometer (Nano-drop Technologies, Hercuvan, Malaysia).

2.6. Study of cytocompatibility of DTM by MTT assay

After the DTM was prepared, its toxicity was checked to ensure that it did not contain any toxic substance during the scaffold preparation and decellularization stages. For this purpose, after the culture of testicular tissue cells with an initial value of 5×10^4 cell in each well of 96-well plate, extract prepared from DTM were added to cells in the culture medium, and after 24 h and 72 h since the inception of the culture, the cells were investigated using 3-(4, 5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT). MTT is metabolically restored using active and living cells and dehydrogenase to produce restored equivalents such as NADH and NADPH. Plate reader (Biotek, Winooski, VT, USA) measured the purple formazan formed during the test steps.

2.7. Histological examination

In histological examination, hematoxylin-eosin (H&E) staining was used to verify the quality of DTM and investigate the migration of the cells to the scaffold after its recellularization. For this staining, the tissue was first fixed in 4 % paraformaldehyde and the sample was placed in paraffin and molded after alcoholic dehydration and immersion in in xylol solution. 5 μm thick sections from the tissue were prepared with the aid of mictrotome, and the standard staining was carried out after paraffin removal with xylol and alcohol rinsing. For more careful and further examination of ECM alterations in the testicular tissue during decellularization and the study of its collagens and glycosaminoglycans (GAGs), Masson's trichrome and Alcian blue staining was performed after tissue preparation and dissection. Lastly, the stained tissues were examined using optical microscope (Olympus, Japan).

2.8. Investigating the 3D structure of the DTM with an electron microscope

To investigate the three-dimensional structure of DTM in terms of quality and decellularization and cell migration after recellularization, the scaffold prepared was analyzed using a scanning electron microscope (Seron, South Korea). Thus, the tissues were fixed with 2.5 % glutaraldehyde and then with 1 % osmium tetroxide and dehydrated with increasing degrees of ethanol. After dehydration, the tissues were placed on a grid and covered with gold-palladium coating and the photos were taken from the samples.

2.9. SSCs culturing and differentiation on a DTM layer

For the purpose of cell culture on the scaffold, a layer of DTM was first placed at the bottom of the 24-well plate. Then, 1.5×10^5 cells were added to each well. To examine the effect of the scaffold on the cell differentiation in similar conditions, the cells were placed in a 24-well plate in the absence of DTM, and accordingly were cultured in a differentiation medium whose base was DMEM/F12 medium supplemented with 5 % KSR (Invitrogen, USA), 5 % FBS, 10 μg/mL insulin-transferrin-selenium solution (Gibco,Grand Island, NY, USA), 3.3×10^{-7} M retinoic acid (Sigma-Aldrich, St Louis, MO, USA), 10 μg/mL vitamin E (Gibco,Grand Island, NY, USA), 10^{-4} M vitamin C (Sigma-Aldrich, St Louis, MO, USA), 1 m M pyruvate (Sigma-Aldrich, St Louis, MO, USA), 2.5×10^{-5} U human follicle-stimulating hormone (Merck, Darmstadt, Germany), 10^{-7} M testosteron (Sigma-Aldrich, St Louis, MO, USA), 1X antibiotic-antimycotic solution (Gibco, Grand Island, NY, USA) [18]. The cells were cultured in both substrates for 4 weeks and 6 weeks. During the culture period, the differentiation medium was replaced every 48 h.

2.10. Extraction of RNA and quantitative real-time PCR (qPCR) analysis

In the fourth and sixth weeks of the differentiation culture, the total RNA of the cells cultured in the DTM and 2D substrate was extracted using the Trizol reagent kit (ready Mini KIT, Qiagen, USA according to the manufacturer's instruction. Purity and concentration of RNA was determined using ND-3800 spectrophotometer (Nano-drop Technologies, Hercuvan, Malaysia). For Complementary DNA (cDNA) preparation, 1 μg total RNA was reverse-transcribed using a Prime Script RT reagent kit (Takara Bio Inc, Tokyo, Japan) according to the manufacturer's instructions. The qPCR was performed using a real-time PCR machine (Applied Bio Systems, Foster City, USA) and the SYBR Premix Ex Taq Kit (Tli RNaseH Plus). The qPCR steps were as follows: initial denaturation at 95 °C for 30 s; amplification for 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 20 s, melting curve analysis at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s. The specific primers used to determine the expression levels of pre-meiotic, meiotic and post-meiotic genes via qPCR are listed in Table 1. All samples were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control, and the relative quantification of gene expression was determined using the comparative CT method (ΔΔCT).

2.11. Immunocytochemistry (ICC)

After proliferation of SSCs and before the start of differential culture, the presence of these cells was confirmed by primary antibodies anti-GFRα1 (pa519873; Rabbit polyclonal, 1:100, Thermo Fisher, Altrincham, UK) and anti- PLZF (sc28319; mouse monoclonal, 1:500, Santa Cruz, Houston, USA) which are specific for SSCs. In the cells cultured in a differentiation medium examining anti-Boule (orb155857; Rabbit polyclonal, 1:100, Biorbyt, St Louis, MO,USA) and anti-Scp-3 (ab181746; Mouse monoclonal, 1:200, Abcam, Cambridge, UK) antibodies for spermatocytes, and anti-Protamine-2 (orb103281; Rabbit polyclonal, 1:100, Biorbyt, St Louis, MO, USA), anti- Crem (orb160579; Rabbit polyclonal, 1:100, Biorbyt, St Louis, MO, USA) antibodies for spermatids. For this purpose, the cells were fixed with 4% paraformaldehyde (Merck, Germany). For antigen recovery, 2-chloride (Merck, Germany) chloride acid (Merck, Germany) was added to the cells, and borate buffer was added to neutralize the excess acid after a few minutes. After the cells were washed, a 0.3 % triton solution (Sigma-Aldrich, St Louis, MO, USA) was used to permeate the cell membranes. Then, 10 % goat serum (Sigma-Aldrich, St Louis, MO, USA) was added after washing the cells. In addition, the primary antibody was added to

Table 1
Primer sequences used for mRNA expression level of markers of different human spermatogenic stages.

Stage	Target	Type	Sequences (5′⇨3′)
Pre-meiotic	*OCT4*	Forward	**F**: CTGGGTTGATCCTCGGACCT
		Reverse	**R**: CACAGAACTCATACGGCGGG
Pre-meiotic	*PLZF*	Forward	**F**: GGTCGAGCTTCCTGATAACG
		Reverse	**R**: CCTGTATGTAGCGCAGGT
Meiotic	*SCP3*	Forward	**F**: TGCAGAAAGCTGAGGAACAA
		Reverse	**R**: TGCTGCTGAGTTTCCATCAT
Meiotic	*BOULE*	Forward	**F**: AAGGGTATGGTTTCGTCACTTTT
		Reverse	**R**: GGACCGAAGTTACCTCTGGAG
Post-meiotic	*CREM*	Forward	**F**: ACACCACCTAGTATTGCTACCA
		Reverse	**R**: GGATTGTTCCACCTTGGGCTAT
Post-meiotic	*Protamine2*	Forward	**F**: CAGTCTCACTATAGGCGCAG
		Reverse	**R**: CTTAGTGCCTTCTGCATGTTCTC

the cells, which was placed at 4 °C for overnight. After rinsing, the secondary antibody was added, and incubated in darkness for 1 h and 30 min at 37 °C. Control cells were treated under similar conditions except for the removal of the primary antibodies and instead of the primary antibodies, IgG Isotype was used in negative control cells to assert the specificity of primary antibodies in the immunocytochemical reactions. Further, the cells were then washed and stained with DAPI. Finally, the cells were observed by the fluorescent microscope (Japan, Olympus) equipped with 400 mm lens for the confirmation of the markers.

2.12. Statistical analysis

In this study, the statistical differences in the expression of spermatogenesis genes were evaluated by two-way ANOVA and the Tukey's test. Quantification of DNA in native tissue and DTM and MTT assay were evaluated by Student's *t*-test. The data are presented as mean ± standard deviation (SD). P values <0.05 were considered statistically significant.

3. Results

3.1. Confirmation of identity of cultured SSCs

By culturing the cells obtained from the enzymatic digestion of the testicular tissue and collecting the supernatant after 24 h and re-culturing the cells which were not yet attached to the substrate (given that the SSC cells are later attached to the substrate), an opportunity was created for partial isolation of SSC cells. Approximately two weeks after the culture of these cells in the specific culture medium, the small colonies formed from them were observed in the culture medium, and the number of these colonies increased and their size enlarged after four weeks (Fig. 2). To verify the identity of spermatogonial cells, the colonies were investigated using two specific markers of spermatogonial cells (*PLZF* and *GFRα1*) via immunocytochemistry staining (Fig. 2). The results indicated that the majority of the colonies are positive with both spermatogonial markers. For further confirmation, the gene expression related to SSCs and differentiation was measured by qPCR prior to the initiation of the differentiation culture. The results indicated the presence of pre meiotic genes and the lack of differentiation genes (Fig. 7).

3.2. DNA content analysis

Quantification of remaining DNA in native tissue and DTM Showed that only 1 ± 1 % of the native testicular tissue DNA content remained within the matrix after decellularization ($P = 0.001$) and indicated a significant decrease and almost complete elimination of the DNA content in DTM (Fig. 3).

3.3. Cytocompatibility of DTM

The results of MTT testing after 24 and 72 h from the beginning of the culture indicated that the cells cultured in the presence of DTM were not different in terms of number and viability with the control sample. As a result, the resulting substrate does not exert toxic effects on the cells and benefit from biocompatibility (Fig. 3).

3.4. Histological study of the scaffold prepared by DTM

Maintaining the structure and composition of tissue ECM following decellularization is one of the essential requirements for normal cellular behavior after tissue recellularization. DTM staining with hematoxylin-eosin showed that the DTM structure was well-preserved after decellularization, and the seminiferous tubules were completely depleted from the cells. In addition, several days after DTM-based SSCs culture, DTM was again stained and examined using Hematoxylin-Eosin to ensure the migration of the cells. As illustrated in Fig. 4, the cells can attach and grow on this substrate. In addition, to confirm the quality of the DTM, the total collagen and glycosaminoglycans (GAGs) were examined by Masson's trichrome and Alcian blue staining, respectively, and it was shown that these structures were well maintained in DTM (Fig. 4).

3.5. Examination of the tri-dimensional structure of the testicular tissue after decellularization and recellularization

DTM surface and sections were scanned by using an electron microscope. Based on the results, the tissue and the structure of the

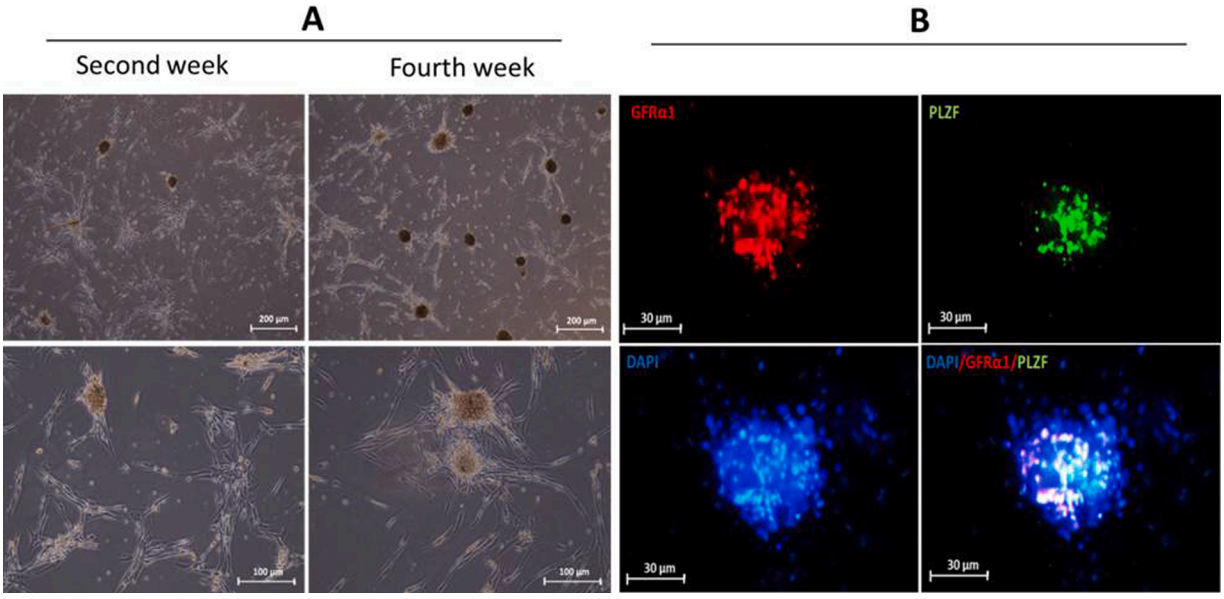

Fig. 2. Light microscopic image of the colonies derived from SSCs proliferation in a specific culture medium during the second and fourth weeks (A) Scale bars = 200 µm and 100 µm. Immunocytochemistry staining of the colonies derived from SSCs proliferation with specific antibodies for GFRα1 (Red) and PLZF (Green) markers, (DAPI color is shown in blue) (B). Scale bars = 30 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

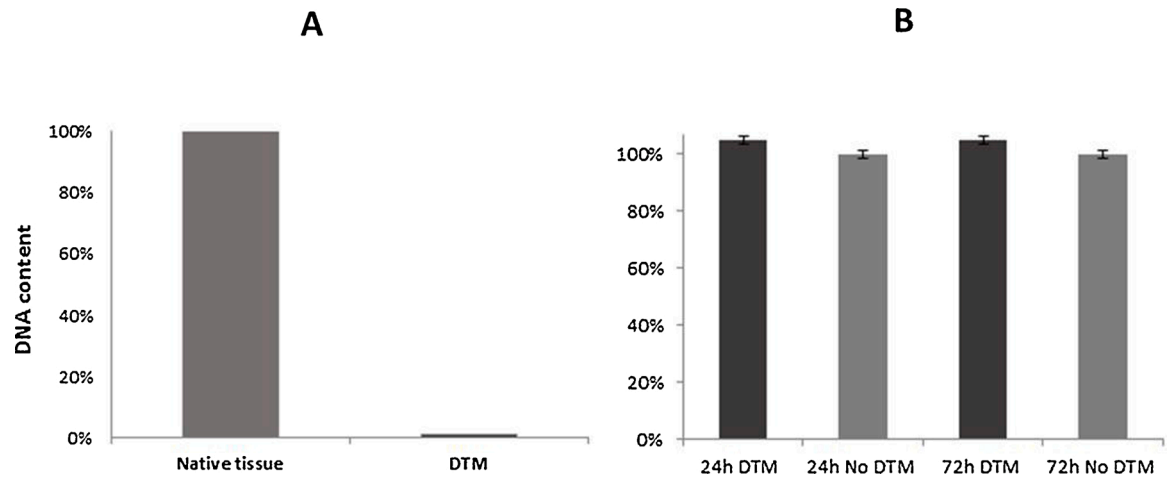

Fig. 3. Quantification of DNA in native tissue and DTM Showed a significant decrease and almost complete loss of the DNA content in DTM. ($P = 0.001$) (A). MTT assay after 24 and 72 h of cell culture indicated that the cells cultured in the presence of DTM were not differ in terms of number and viability with the control sample. The results are expressed as means ± SD (B).

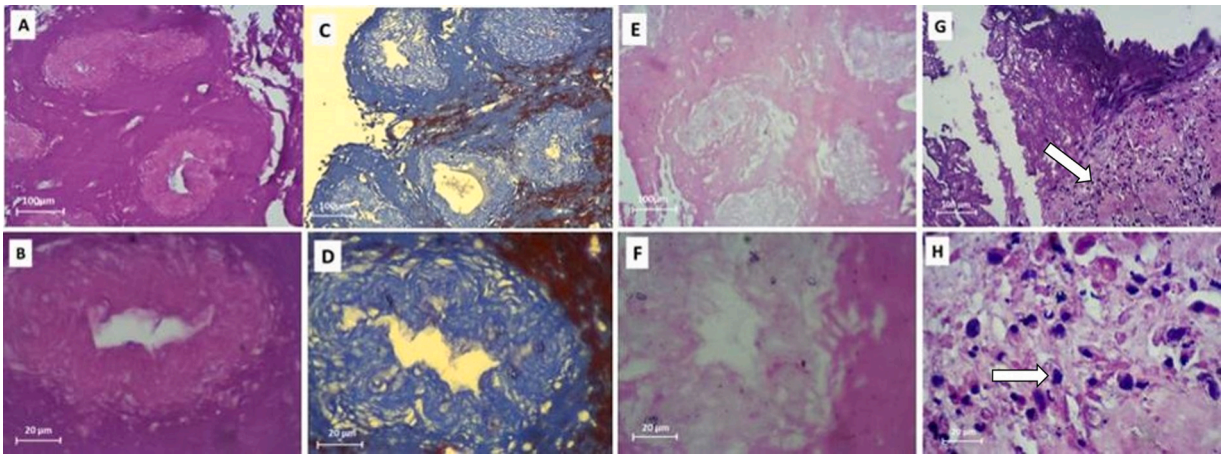

Fig. 4. Histological staining of DTM via H and E (A, B), Alcian blue (C, D) and Masson's trichrome (E, F) evidenced preservation of structure, GAGs and collagen respectively. H and E staining also showed that the cells could attach to DTM after recellularization (Cells attached to the DTM are shown with an arrow). (G, H). Scale bars = 100 μm and 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

seminiferous tubules were preserved well, and the decellularization was completed in the decellularized ECM sample. After the recellularization, the DTM was again examined via an electron microscope and it was observed that the cells were attached to the DTM (Fig. 5).

3.6. Study of differentiation of SSCs after culturing on DTM with immunocytochemistry

SSCs were cultured on DTM in a differentiation medium. After 6 weeks, differentiations of the cells were evaluated by immunocytochemistry method with SCP3, Boule, Crem and Protamine2 antibodies. Based on this the results, the expression of differentiation proteins in DTM is greater than their expression in 2D (Fig. 6).

3.7. Determination of differentiation genes expression using real time PCR

To evaluate the effect of DTM on the SSCs differentiation relative to 2D substrate, the cells cultured in both groups were isolated after 4 and 6 weeks since the initiation of culture. After extracting RNA and synthesizing cDNA, the expression of pre-meiotic (*OCT4, PLZF*), meiotic (*SCP3, Boule*) and post-meiotic (*Crem, Protamine-2*) genes, in two groups were measured. The results of this study showed that in the cells cultured on the 3D substrate derived from DTM, the expression of pre meiotic, meiotic and post-meiotic was significantly higher than the 2D sample ($P \leq 0.01$), which indicates the creation of suitable conditions for preservation, proliferation, and differentiation of SSCs in the scaffold prepared. Further, it indicated that the expression of differentiation genes increases with the elongation of culture duration ($P \leq 0.01$) (Fig. 7).

4. Discussion

In vitro proliferation of spermatogonia stem cells (SSCs) and their injection into testicular tissue can be a way to treat azoospermic men. (Mohaqiq et al., 2019; Faes et al., 2013; Takashima and Shinohara, 2018). However, in some men, it is not possible to inject SSCs and differentiate them in the body. Like people whose spermatogenesis has stopped for some reason, and people whose cancer has been treated and the injection of amplified SSCs into the testicular tissue makes it possible for cancer to come back. By producing mature germ cells in the laboratory and injecting them into the cytoplasm of the ovum, laboratory embryos can be produced that can be transferred to the mother's uterus to provide fertility. Researchers differentiated and matured SSCs in mice in the laboratory and injected them into the cytoplasm of the ovum to

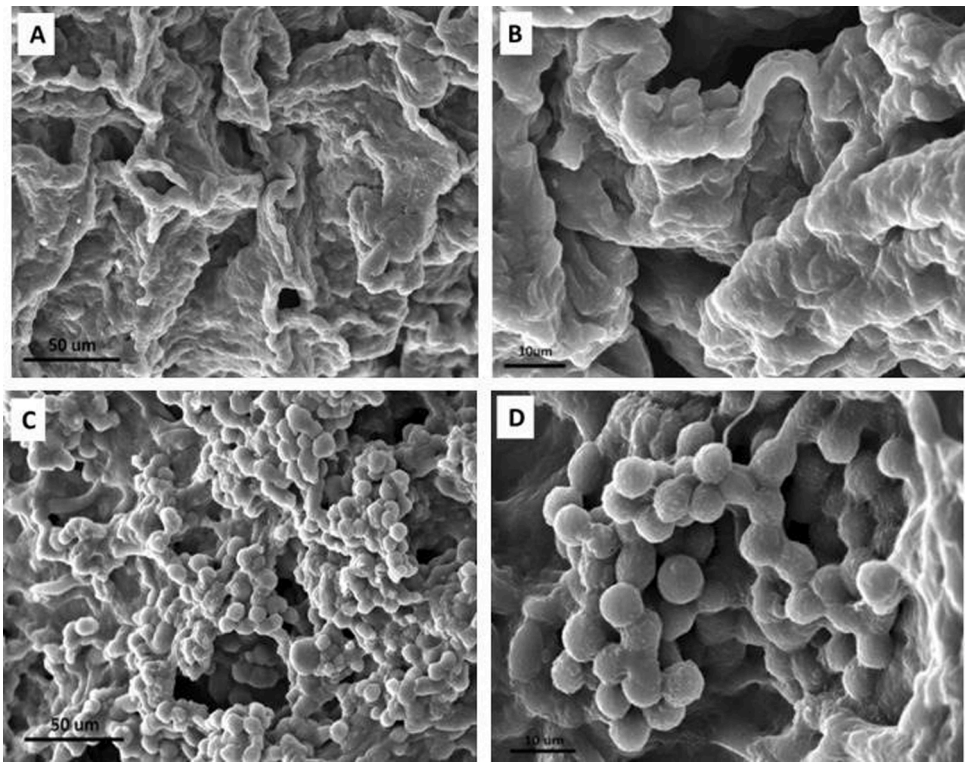

Fig. 5. Scanning electron microscopy for evaluation of the three-dimensional structure of the DTM. The images indicate that the tissue structure and the seminiferous tubules after the decellularization were preserved well (A, B). In addition, the cells can attach to DTM after recellularization (C, D). The number of samples examined for each group (N = 3).The number of technical replicates per group (N = 3). Scale bars = 50 μm and 10 μm.

induce fertility leading to birth in mice. Since little research has been done on the differentiation of human SSCs, our aim in this study was to improve the culture conditions and differentiate human SSCs in vitro. (Sato et al., 2011). It is hoped that by conducting similar studies in humans, this can be a step towards treating male infertility. Therefore performing spermatogenesis in vitro is considered as one of the most valuable objectives in the field of reproductive biology. Since cell culture on the 2D substrate cannot create conditions similar to the in vivo microenvironment for cellular growth and differentiation (Hai et al., 2014; Kostereva and Hofmann, 2008; Smith et al., 2014), a large number of studies have been conducted on SSCs culture and differentiation in 3D conditions in recent years (Galdon et al., 2016; Giudice et al., 2017; Reda et al., 2016; Shams et al., 2017; Stukenborg et al., 2009) such as SSCs cultures on a 3-D matrix from a soft agar culture system (SACS) (Jabari et al., 2020; Gholami et al., 2018a, b; Khajavi et al., 2014; Sato et al., 2011; Stukenborg et al., 2008), the substrate of collagen gel (CG) and collagen + Matrigel (CGM) and Methylcellulose Culture System (MCS) (Huleihel et al., 2015) or culture on poly (D, L-lactic-co-glycolic acid) or PLGA (Galdon et al., 2016) and polycaprolactone (PCL) (Orwig et al., 2002; Talebi et al., 2019). To create an ideal culture medium, some attempts have been made to create an *in vitro* culture situation similar to *in vivo* conditions. Therefore, recently, various studies have been conducted to create decellular tissue scaffolds in order to provide optimal 3D culture conditions with ECM preservation, and then pave the way for cell growth and differentiation (Bonandrini et al., 2014; Cortiella et al., 2010; Crapo et al., 2012, 2013; De Waele et al., 2015; Joddar et al., 2014; Navarro-Tableros et al., 2015). V. Navarro-Tableros et al. cultured liver stem-like cells on decellularized liver ECM and differentiated them into hepatocytes (Navarro-Tableros et al., 2015). B. Bonandrini et al. cultured Murine embryonic stem (ES) cells into the decellularized ECM derived from whole kidney, after culture the ES cells lost their pluripotency and differentiated into a mesoendodermal lineage (Bonandrini et al., 2014). S. E. Gilpin et al. cultured pluripotent stem cells on decellularized lung matrix and differentiated them into lung progenitor cells (Gilpin et al., 2014). According to these reports and similar studies, decellularized ECM can be applied to direct stem cell differentiation toward specific lineages. In our study, the DTM-derived scaffolds were used for SSCs culture and differentiation in order to provide a situation like that of *in vivo* situation. The testicular tissue is rich in ECM due to the type of structure. Then, it is an ideal sample for studying matrix compounds, which is an important biomaterial for tissue engineering (Brown and Badylak, 2014).In decellularization process, tissue cells are eliminated while the tissue structure and composition are preserved (Baert and Goossens, 2017; Baert et al., 2014; Vermeulen et al., 2018). Maintaining the structure and composition of ECM after decellularization is one of the essential requirements for normal cellular behavior after tissue recellulaization. In this study, DTM staining with H and E showed that the DTM structure was preserved well and the seminiferous tubules were completely depleted from cells. In addition, Alcian blue and Masson's trichrome staining indicated the presence of GAGs and collagen in DTM. Collagen is the main structure of human testicular tissue (Gulkesen et al., 2002; Laurie et al., 1982; Oğuzkurt et al., 2007), and its maintaining is important for DTM quality. Laminin and fibronectin in ECM act as adhesion molecules. Fibronectin, along with GAGs, plays a role in cell adhesion and migration, indicating the importance of GAGs in the scaffold (Wierzbicka-Patynowski and Schwarzbauer, 2003). In addition, acting as one of the main features of GAGs, water conservation affects the viscoelastic properties of the scaffold. In addition, electron microscope confirmed the quality of DTM using a three-dimensional review. In the present study, after SSCs culture and proliferation in a specific culture medium for one month, the expression of spermatogonial genes including *OCT4, PLZF* and *GFR α1, PLZF* evaluated by using qPCR and Immunocytochemistry, respectively. Then these cells were cultured in the differentiation culture medium on the DTM and 2D substrate. Accordingly, the expression of pre-meiotic, meiotic and post-meiotic genes was investigated using qPCR method after 4–6 weeks since the initiation of culture. Furthermore, Immunocytochemistry investigated the expression of these genes at the protein

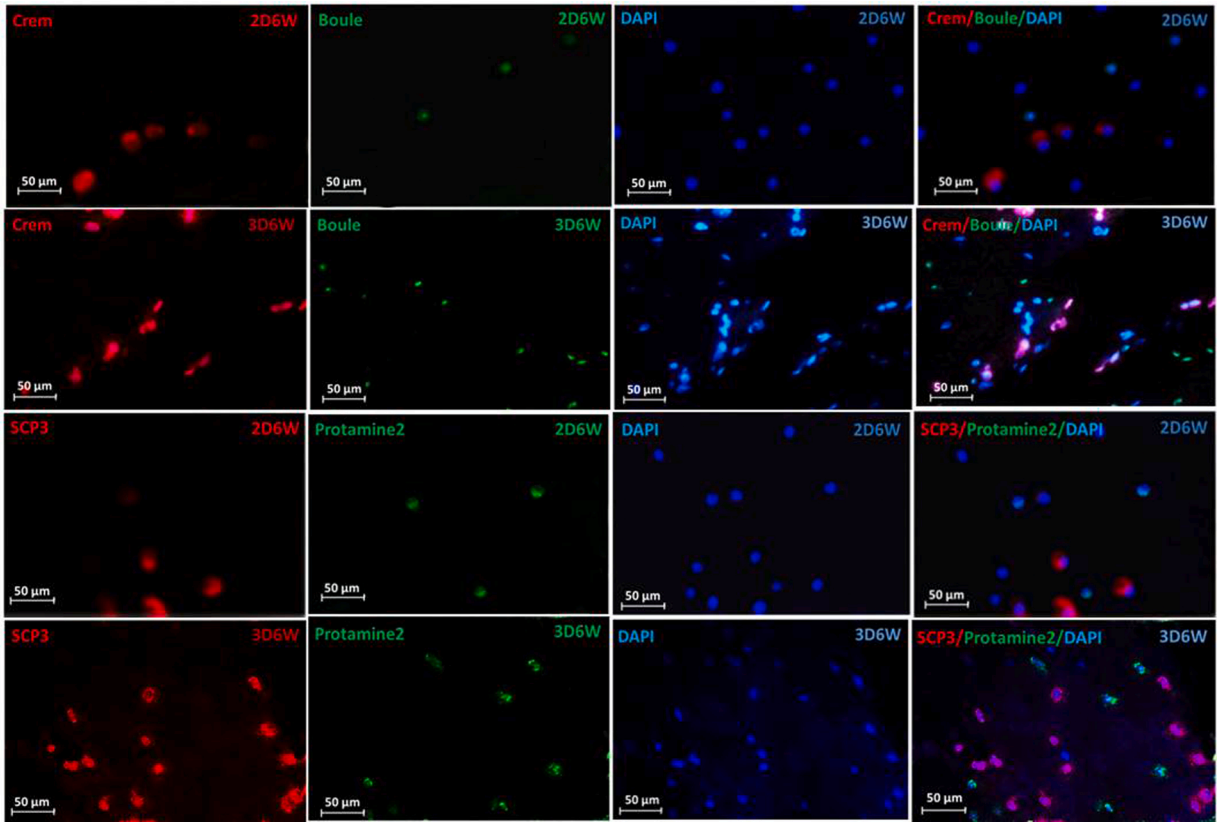

Fig. 6. Immunocytochemical staining of the cells were cultured in differentiation medium in a 2D substrate for 6 weeks (2D6W) and in DTM-based 3D substrate for 6 weeks (3D6W) regarding a review of the expression of spermatocyte markers (SCP3 (Red) & Boule (Green)) and spermatid markers (Crem (Red) & Protamine 2 (Green)) with their specific antibodies, (DAPI color is shown in blue). Scale bars = 50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

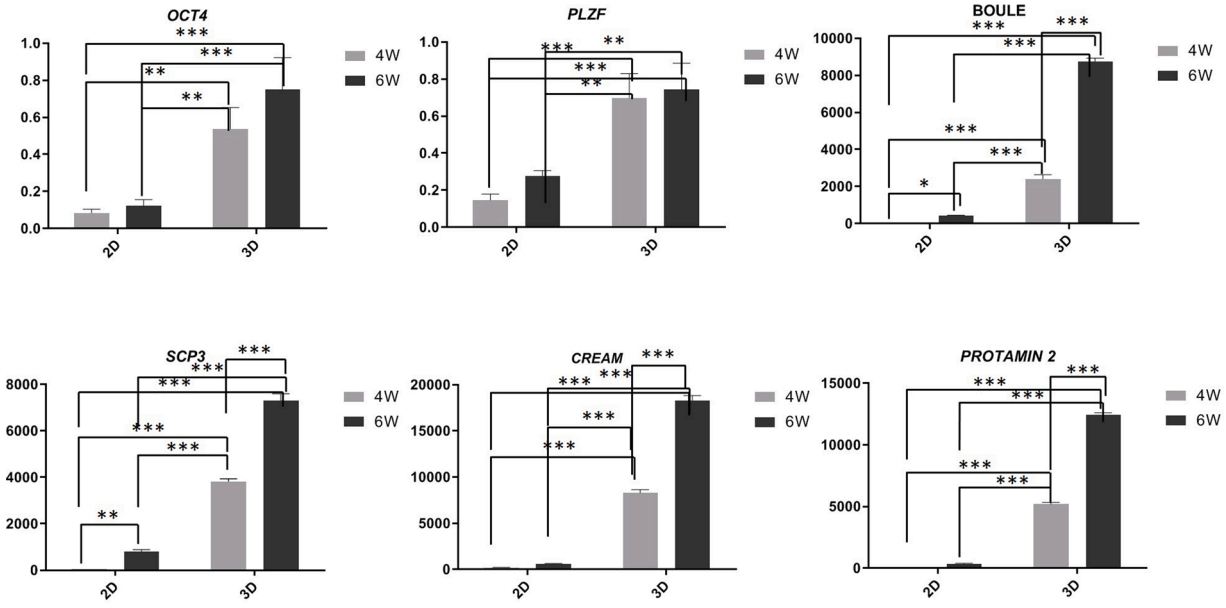

Fig. 7. Expression of spermatogenesis genes (pre-meiotic (OCT4,PLZF), meiotic (SCP3, Boule) and post-meiotic (Crem, Protamine2) genes), and in a 2D substrate for 4 and 6 weeks (2D-4W),(2D-6W) and in cells cultured in DTM-based 3D substrate for 4 and 6 weeks (3D-4W),(3D-6W)analyzed by real-time PCR. The results showed that the expression of pre meiotic, meiotic and post-meiotic genes were significantly greater than the expression of the genes in the 2D conditions in DTM-based culture. The number of samples examined for each group (N = 6 wells).The number of technical replicates per group (N = 3). Data are shown as means ± SD ***P≤0.001.

level. The results of these studies showed that the expression of pre meiotic, meiotic and post-meiotic genes were significantly greater than the expression of the genes in the 2D conditions in DTM-based culture, which can be related to the proper conditions prepared by DTM at the time of the formation of cells and their reciprocal contact. In addition, such condition not only leads to more preservation of early cells, but also provides suitable conditions for their proliferation and differentiation. Also, the results of the previous studies confirmed the mutual contact of cells with each other and with extracellular matrix in preserving and surviving cells (Amsterdam et al., 1999; Fiorini et al., 2008; Makrigiannakis et al., 2000; Peluso, 1997; Pendergraft et al., 2017; Perrard et al., 2016). Finally, the elongation of the differentiation period, compared to the fourth and the sixth weeks of differentiation culture, indicated that the increased elongation of the culture period contributes to an increase in the expression of the differentiation genes, as well.

In conclusion, three-dimensional culture of SSCs on DTM by creating natural ECM around these cells can provide suitable conditions for their preservation and proliferation. The results of this study could be a way to further study the process of spermatogenesis in vitro as well as hope for the treatment of infertility in men.

Ethical approval

The study protocol was approved by the Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.REC1394.1751) and all procedures were performed in accordance with the university's guidelines.

CRediT authorship contribution statement

Sepideh Ashouri Movassagh: Conceptualization, Methodology, Writing - original draft. **Sanaz Ashouri Movassagh:** Data curation, Investigation. **Mehdi Banitalebi Dehkordi:** Investigation. **Gholamreza Pourmand:** Data curation. **Keykavos Gholami:** Data curation. **Ali Talebi:** Investigation. **Ayob Jabari:** Investigation. **Azam Samadian:** Formal analysis. **Mehdi Abbasi:** Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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