Original Article

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Colonization of neonate mouse spermatogonial stem cells co−culture with Sertoli cells in the presence and absence soft agar

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ABSTRACT

Objective: To investigate the effects of soft agar on $in-vitro$ proliferation of neonate mouse spermatogonial stem cells co-cultured with Sertoli cells.

Methods: Tissues of neonate NMRI male mice testes were used for harvesting spermatogonial stem cells and Sertoli cells. After cell harvest, flow cytometry using promyelocytic leukemia zinc-finger (PLZF) protein antibody was used to assess the purity of the cells. The isolated testicular cells were cultured in the absence (the control group) or presence of soft agar-coated dishes (the experimental group) supplemented with leukemia inhibitory factor and glia cell line–derived neurotrophic factor for two weeks. Alkaline phosphatase activity was assessed in the colonies formed after two weeks of culture by alkaline phosphatase staining. On day 14 of culture, the expression levels of DNA-binding protein inhibitor ($ID-4$) and $PLZF$ genes in the undifferentiated cells were evaluated by the detection of PLZF protein antibody using real-time PCR and immunocytochemistry techniques. The number and diameter of the colonies of spermatogonial stem cells were assessed by ImageJ software.

Results: In the experimental group, the number and the diameter of colonies significantly increased as compared with those in the control group ($P<0.05$, $P<0.01$, respectively). In addition, the level of expression of $ID-4$ and $PLZF$ genes in the undifferentiated cells significantly increased in the experimental group as compared with the control group ($P<0.01$). However, the expression of tyrosine-protein kinase kit ($c-kit$) gene in differentiated cells decreased in the experimental group as compared with the control group, but there was no significant difference between the two groups.

Conclusions: Spermatogonial stem cells can be efficiently proliferated by culturing the stem cells in soft agar-coated dishes. The new protocol used in this study can be a valuable method for future studies.

KEYWORDS: Spermatogonial stem cell; Soft agar; Colonization; Proliferation; Mouse

1. Introduction

Spermatogonial stem cells (SSCs) simultaneously expand and differentiate into other cells, leading to sperm production. These cells are considered as the foundation for sperm production and male fertility. However, like tissue-specific stem cells, the number of these stem cells is very low[1]. Many reports have indicated a high percentage of male infertility, particularly in children, after cancer treatment by chemotherapy or radiotherapy[2]. *In vitro* proliferation of SSCs has paved way for investigations into the treatment of male infertility. Sertoli cells play a vital role in the regulation of proliferation and differentiation of SSCs. The presence of Sertoli cells increased the survival rate of SSCs in cultured mouse testis tissue and cell samples[3]. Previous studies investigating the factors associated with the proliferation of cultured SSCs have focused on growth factors such as leukemia inhibitory factor[4], glia cell line-derived neurotrophic factor[5], basic fibroblast growth factor, epidermal growth factor[5,6], and stem cell factor[7,8]. Many studies have confirmed that these factors cause the development of SSCs when applied in different culture systems. One of the most widely used techniques for *in vivo* cellular transformation is the soft agar colony formation assay. Other techniques such as the clonogenic

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assay have also been used by researchers for evaluating the ability of cells to form colonies[9]. Because the soft agar culture provides a suitable environment and similar *in vivo* for cell proliferation and migration. In recent years, this culture medium has been known to identify tumorigenic inhibitors in cancer cells[10].

Previous studies have reported clonal proliferation of bone marrow cells and lymphocytes in soft agar culture and the factors involved in the regulation of proliferation and differentiation in these cells[11,12]; however, not much research has been done in the effects of soft agar on the *in vitro* proliferation of SSCs in two-dimensional culture.

This study was to investigate the *in–vitro* effects of soft agar supplemented with leukemia inhibitory factor and glia cell line-derived neurotrophic factor on the proliferation of neonatal mice SSCs co-cultured with Sertoli cells in two-dimensional culture.

2. Methods and materials

2.1. Animals

SCCs were harvested from 3 to 6-day old NMRI male mice. The mice were kept under standard conditions with free access to food and water. We kept mice at 12 h in the dark and 12 h in the light and at constant temperature and humidity.

2.2. Isolation of neonatal testis cells

Testis cells obtained from 3 to 6-day old NMRI male mice after general anesthesia had been induced with chloroform (Sigma St. Louis, USA, C2432). Testicular cells were isolated by two-step enzymatic digestion (5 mice at a time in each group). Testes were removed from the scrotum and transferred to phosphate buffered saline (PBS) (Sigma, Munich, Germany). The testes were cut into small pieces after the removal of tunica albuginea. The minced testicular tissues were transferred to a digestion solution containing collagenase type Ⅳ (1 mg/mL, Sigma, Steinheim, Germany), deoxyribonuclease (10 µg/mL, Sigma, St. Louis, USA), and hyaluronidase (0.5 mg/mL, Sigma, St. Louis, USA) for 20 min at 37 ℃ in a 5% CO_2 incubator. To disperse the tubule cells, pipetting was performed at every 2–5 min until the tubules were separated. The dispersed tubule cells were centrifuged for 5 min at $1\,500 \times g$. The same procedure and enzymes were used for the second step of the enzymatic digestion (15 min). The separated cells were washed with PBS (Sigma)[4,13,14].

2.3. Flow cytometry

After the two-step enzymatic digestion, a single-cell suspension from the samples was obtained. These cells were predominantly SSCs. The cell suspension was centrifuged at $1\,500 \times g$ for 5 min after which the cells were counted. A density of 2×10^5 cells/cm^2 was

transferred into a petri dish and kept for 24 h at 37 ℃ in a 5% CO_2 incubator. Following incubation, the dish was washed and the cells were collected.

Flow cytometry (Thermo Fisher Scientific, US) was performed by using promyelocytic leukaemia zinc finger protein (PLZF) antibody to evaluate the purity of the SSCs. To identify PLZF positive cells, 10 µL primary antibody (Anti-PLZF antibody; Abcam Inc., Cambridge, MA, USA, rabbit polyclonal to PLZF) was added to the cells and incubated for 1 h at room temperature. After permeabilization with 0.4% Triton ×100 (Sigma) and washing in PBS, 10 µL of secondary antibody (Donkey Anti-Rabbit; Abcam Inc., Cambridge, MA, USA) conjugated with fluorescein isothiocyanate was added (1 h, 4 ℃). Cells used as control were not treated with any antibody[15,16].

2.4. Preparation of soft agar–coated dishes

The soft agar coated dishes were prepared as follows: 0.7% (w/v) agar (Fisher Scientific, Loughborough, United Kingdom) was dissolved in distilled water to form a gel. Subsequently, the solution was mixed with the same volume of the basic culture medium (Sigma, St. Louis, USA) to achieve a final concentration of 0.35%. Then, 30 mm dishes were coated with 1 mL of the final soft agar (0.35%) solution and stored in an incubator at 37 ℃ at 5% for 1 h[4,17].

2.5. SSCs culture

SSCs were cultured for two weeks on a 24-well plate (density of 2×10^5 cells/cm^2) in a basic culture medium- minimum essential medium-α (sigma St. Louis, USA) containing 10% fetal bovine serum (Sigma, Deisenhofen, Germany), 1× nonessential amino acids (Invitrogen, Carlsbad, CA, USA), 0.1 mM 2-mercaptoethanol (Sigma, Deisenhofen, Germany), 100 U/mL penicillin, and 100 µg/mL streptomycin (both from Sigma, Deisenhofen, Germany), along with 10^3 U/mL human recombinant leukemia inhibitory factor (B&D, Franklin Lakes, NJ, USA) and 10 µg/mL glial cell line-derived neurotrophic factor (R&D, Emeryville, CA, USA) supplementation in each culture dish. In the experimental group, soft agar-coated dishes were used for the co-culture of SSCs and Sertoli cells, but in the control group, soft agar was omitted. Culture medium replacement was performed every 2 days. Culture dishes were kept in an atmosphere humidified with 5% CO_2 at 32 ℃. Cells were cultured for 7 days after which colony staining was performed by using alkaline phosphatase[3].

2.6. Alkaline phosphatase staining

For assessing alkaline phosphatase activity, the cells were stained by using Fast Red TR/Naphthol AS-MX Tablets (Sigma St. Louis, USA, F4648), according to the manufacturer's instructions. Alkaline

Table 1. The primer sequences of *ID–4*, *PLZF*, *c–kit*, and *GAPDH*.

Gene name	Sequence	Product size (bp)	Annealing temperature (℃)
ID–4	F: 5'- TCCCGCCCAACAAGAAAGTC -3' R: 5'- TCAGCAAAGCAGGGTGAGTC-3'	102	60.54
PLZF	F: 5'- CGTTGGGGGTCAGCTAGAAAG -3' R: 5'- CACCATGATGACCACATCGC-3'	301	57.14
c–kit	F: 5'- AACAACAAAGAGCAAATCCAGG -3' R: 5'- GGAAGTTGCGTCGGGTCTAT -3'	200	57.67
GAPDH	F: 5'-AGCAAGGACACTGAGCAAGAG-3' R: 5'- TCGTTCCTCTGATCGTTTCC-3'	151	60.35

dye was prepared by briefly dissolving a Fast Red TR/Naphthol AS-MX Tablets in 1 mL deionized water. The alkaline dye was added to the cells and incubated at 37 ℃ for 30 min. The stained cells were observed under an inverted microscope (BX51, Olympus, Japan) at ×100 magnification following incubation.

2.7. Immunocytochemistry staining for characterization of SSCs colonies

SSCs colonies were fixed in 4% paraformaldehyde for 20 min, and were permeabilized with 0.4% Triton X100 (Sigma, Germany) and blocked with 10% goat serum (Sigma, Germany). Primary antibody was added to the cells and incubated for 2 h at 37 ℃ with rabbit polyclonal Anti-PLZF antibody (dilution 1:100; Sigma, Germany). Subsequently, the cells were washed with PBS, and the secondary antibody, Donkey Anti-Rabbit labeled with fluorescent isothiocyanate diluted at 1:100 (Sigma, Germany), was added for 3 h. Control cells were not treated with the primary antibody. Nuclei staining was performed by using 4,6-diamidino-2-phenylindole (1 μg/mL; Sigma, Germany).

2.8. Colony assay

The diameter and number of colonies were determined at the end of the second week (on day 14 of culture). After the cultivation of total spermatogonial cells in the 30 mm dishes, colonies were formed on the 4-5th day in the experimental group and on the 7th day in the control group. All colonies in each 30 mm dish were counted for each group. All measurements were repeated three times. We measured the diameter of the colonies by using ocular grid on the inverted microscope used to observe the images. The images were analyzed by using ImageJ software, version 1.6 (National Institutes of Health, Bethesda, MD, USA).

2.9. Real–time PCR

After culturing the cells for 2 weeks, we used the real-time polymerase chain reaction (PCR) technique to evaluate the expression of promyelocytic leukaemia zinc finger protein (PLZF) in the undifferentiated cells, and DNA-binding protein inhibitor (ID-4) and tyrosine-protein kinase kit (c-kit) in the differentiated cells. Following the manufacturer's protocol, the Trizol reagent (Ready Mini Kit, Qiagen, USA) was used for total RNA extraction.

Real-time PCR was performed by using 1 μg of total RNA. The cDNA synthesis kit (Transcript First Strand cDNA Synt, Roche, Indianapolis, IN, USA) was used for this purpose, according to the manufacturer's protocol. Forty PCR amplification cycles were performed for the samples. The results were analyzed by using Applied Bioscience 7500 Fast with SYBR Green detection. Nonspecific PCR products and primer dimers were detected by performing melt curve analysis after each PCR run. The comparative CT method (ΔΔCt) was used for normalizing the samples against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used an internal control. The primer sequences were been shown in Table 1[18].

2.10. Statistical analysis

Results were expressed as mean±standard deviation (mean±SD). Statistical analysis was performed by using unpaired *t*-test for the comparison of the diameter and number of colonies and gene expression studies in the experimental and control groups. *P*<0.05 was considered statistically significant.

2.11. Ethics statement

This study was approved by Ethical Committee of Shahroud University of Medical Sciences on 4 March, 2020 (No. IR.SHMU.REC.1398.166). Moreover, handling of animals was carried out in accordance with the guidelines of the Iranian Council for Use and Care of Animals.

3. Results

3.1. Purification of SSCs

To determine the purity of SSCs, we performed flow cytometry by using PLZF antibody after the enzymatic digestion. Of all cells, 72.7% cells expressed PLZF (Figure 1). The other testicular cells isolated from the testes were transferred and co-cultured with the SSCs. The majority of these cells were Sertoli cells which created feeder monolayer cells, and played a vital role in the formation of colonies of SSCs. Following proliferation, SSCs formed prominent colonies on top of the Sertoli feeder layer cells.
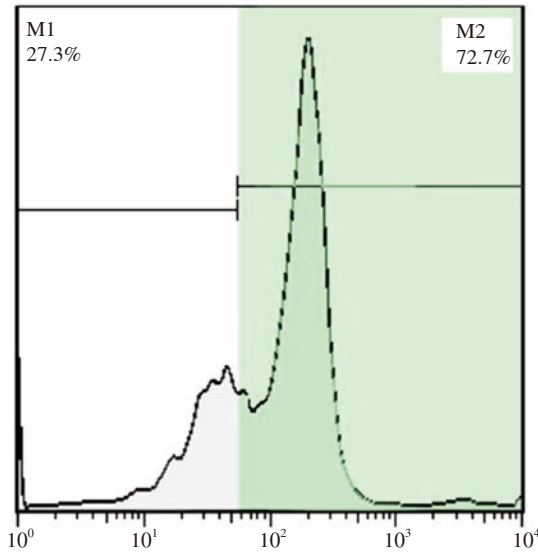
Figure 1. Flow cytometry analysis for the detection of percentage of spermatogonial stem cells purity with promyelocytic leukaemia zinc finger protein (PLZF) marker. M1: without PLZF antibody; M2: with PLZF antibody.

3.2. SSCs culture

The single-cell suspension was cultured for two weeks following the enzymatic digestion. After day 1 of culture, no colonies formed. The cells remained single and attached to the bottom of the dish. Colonies appeared on the 4-5th day of culture. Colony parameters (number and diameter) were measured at the end of the second week (the 14th day after seeding) in the two groups (Figure 2 A, B). SSC colonies formed on the 4-5th day in the experimental group and on the 7th day in the control group. These colonies were positive for the activity of the embryonic stem cell marker, alkaline phosphatase (Figure 2C).

Moreover, the identity of the colonies of SSCs was confirmed by the detection of PLZF protein expression in the nuclei of the SSCs by immunocytochemistry technique. The presence of this protein in the nucleus of clonal cells proved that these colonies were in the stage of proliferation and self-regeneration (Figure 3).

3.3. Results of colony assay

From the colony assay results, the average number of colonies was 4.4±1.9 and the diameter was (257.3±9.1) µm in the control group. In the experimental group, the number and diameter of colonies were 6.5±2.1 and (339.7±15.4) µm respectively at the end of the second week. The number and diameter of the colonies formed in the experimental group were significantly increased as compared with those of the control group ($P<0.05$, $P<0.01$, respectively) (Figure 4).
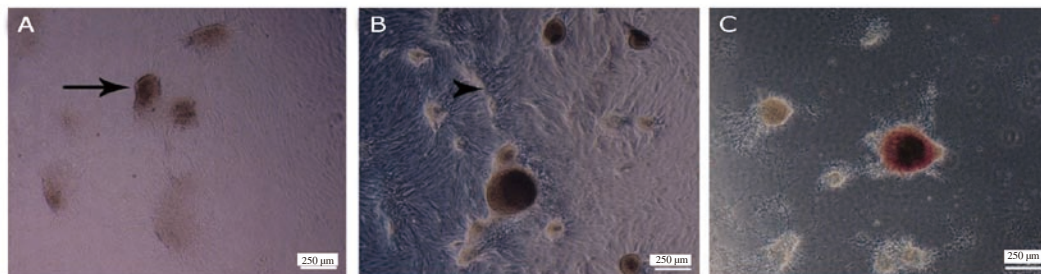

Figure 2. Inverted microscopic morphology of spermatogonial stem cells (SSCs) obtained from 3-6 day old male mice (five mice at a time in each group) (magnification ×100). The size of the colonies at the end of the second week is shown. In the control group (A) and the experimental group (B), Sertoli cells proliferate and create a monolayer of cells as a feeder layer (arrow tip), while SSCs create a prominent colony (arrow) on top of the feeder layer in both groups, with the difference that the experimental group contains soft agar. After cultivation of SSCs, diameter and number of colonies increase in both groups particularly in the experimental group due to the soft agar and they are positive for alkaline phosphatase activity (C).
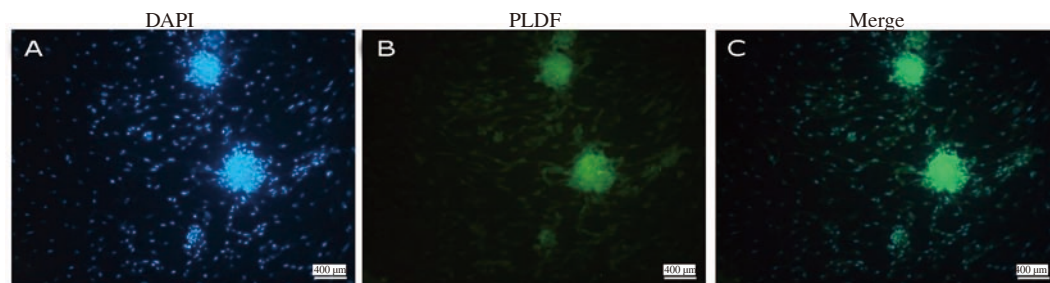

Figure 3. Immunofluorescent staining of colonies of spermatogonial stem cells (SSCs) (magnification ×100). Nuclei are stained with 4,6-diamidino-2-phenylindole (DAPI) (blue color) (A); SSCs are positive for promyelocytic leukaemia zinc finger protein (PLZF) in the nuclei (green color) (B), and a merge of A and B in C.
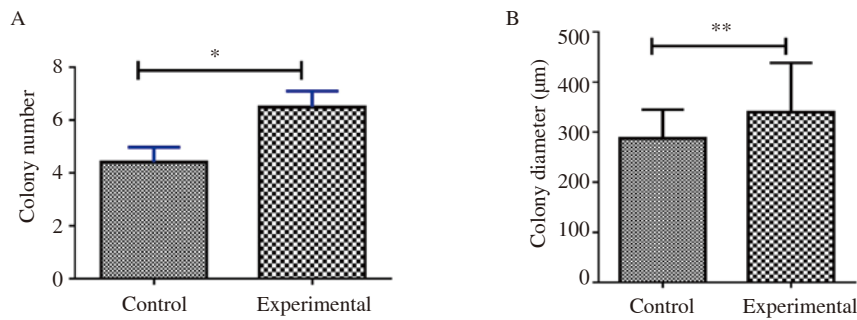
Figure 4. Comparison of colony number (A) and diameters (B) between the control and experimental groups in the second week. $^*P<0.05$, $^{**}P<0.01$.
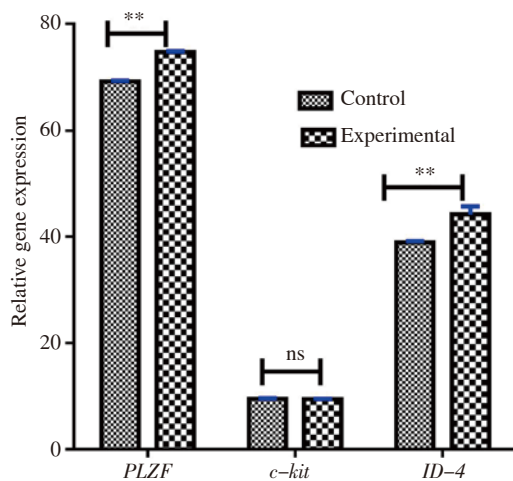

Figure 5. Expression pattern of *c–kit*, *PLZF*, and *ID–4* genes after two weeks of culture analyzed by real-time PCR. Levels of *ID–4* and *PLZF* in the experimental group have an increased value as compared with the control group, but the level of *c–kit* in the experimental group has no significant difference as compared with the control group. Y-axis shows $\Delta\Delta$Ct values of genes. $^{**}P<0.01$. ns: no significant difference.

3.4. Gene expression

Expression levels of *PLZF*, *ID–4*, and *c–kit* genes in the SSCs co-cultured with Sertoli cells were studied by using real-time PCR. The expression levels of *PLZF* and *ID–4* genes were significantly higher in the experimental group when compared with the control group ($P<0.01$). The level of expression of c-kit gene was lower in the experimental group as compared with the control group, however, the difference was not significant ($P>0.05$) (Figure 5).

4. Discussion

In this study, soft agar coated dishes were used to investigate SSCs co-culture with Sertoli cells. Sertoli cells secrete glia cell line-derived neurotrophic factor which plays a critical role in the regulation of the self-renewing and survival of SSCs. Leukemia inhibitory factor also has a pivotal role in the regulation of the function of Sertoli cells and SSCs. Previous studies have demonstrated that the presence of leukemia inhibitory factor in a culture medium inhibits the differentiation of embryonic stem cells[19] and SSCs[3]. The effects of these growth factors on these cells have been studied by many researchers[14,20,21].

In previous studies, Sertoli cells have been utilized as a feeder layer consisting of a row of monolayer cells on the bottom of the dish on which SSCs are colonized[14,21–23]. This finding is in agreement with our results in which the presence of soft agar in the culture dishes helped to keep the feeder layer, thereby contributing to the growth and proliferation of SSCs.

Colony assay has been used as an *in vitro* morphological criterion to evaluate the development of SSCs in many studies[14,23,24]. According to our results, the number and diameter of SSCs colonies increased at the end of the second week of culture. Razi *et al* also reported an increase in the number and diameter of colonies of SSCs when stem cells were co-cultured with Sertoli cells in collagen-coated dishes in the presence of growth factors[21]. In other studies, colony evaluation has been used as a criterion in the proliferation and self-regeneration of SSCs. In these studies, evaluation of SSC colonies has been evaluated[14,24].

In the soft agar colony formation assay, cell clusters were gently dispersed by pipetting into a single cell suspension and were cultured in the presence of Sertoli cells acting as a feeder layer or in a medium that contains growth factors. Since the formation of colony of tumors in a two-dimensional culture medium is very slow and difficult, the soft agar colony formation assay is one of the most suitable techniques for colony production[10,26].

In recent years, three-dimensional soft agar culture system has been suggested as an efficient method for the proliferation of SSCs *in vitro*. This culture system is composed of two layers: soft layer and solid layers. The main disadvantage of this method is the inability to

isolate live cells[4,17,25,26].

Apart from the studies of Razi *et al* and Barralet *et al*, there is a general lack of research on the appropriate dish content which is a valuable source for the development of SSCs[21,27]. In this study, we used PLZF antibody to evaluate the purity of SSCs, and we achieved a purity of 72.7%. Many studies have demonstrated the importance of PLZF antibody in the purification and identification SSCs colonies[23,28]. Our results show that soft agar has a positive effect on the expression of *ID–4* and *PLZF* genes, and a negative effect on the expression of *c–kit* gene. The results of this study indicate a positive correlation between the proliferation of SSCs and culture in soft agar medium.

C–kit gene is expressed in the early stages of meiosis, so decreased expression of this gene confirms increased proliferation of SSCs. This key gene has been studied in many studies for lack of differentiation in these cells[29]. *PLZF* is expressed in the SSCs of A_{al}, A_s, A_p and plays an essential role in regulating the proliferation of SSCs[3]. Therefore, the use of PLZF in both real-time PCR technique for gene analysis and immunocytochemistry staining for protein expression proves that these SSC colonies did not enter the differentiation stage. We also used alkaline phosphatase staining, a common technique for proving the presence of colonies. Other studies have used different genes such as *Stra8* to study the formation of SSC colonies[14].

ID–4 is one of the genes that is expressed in the early stages of proliferation of most stem cells. In most studies, it is evaluated as a key gene in stem cells[30]. There are many reports that are consistent with the results of our research[4,14,21,23,31].

In recent years, the soft agar in the three-dimensional model has been mentioned to improve the culture and differentiation of SSCs. But, there is no access to living cells in the three-dimensional model of soft agar[4,32,33]. This shows the superiority of our research.

Spermatogenesis in men begins at puberty, therefore, chemotherapy and radiotherapy in children with cancers can cause loss of spermatogonial stem cells, leading to infertility. So, improving SSCs culture can provide a valuable tool for preserving fertility before cancer treatment. In recent years , stem cells, including SSCs[34] have been used to treat many diseases[16,18,35–37]. Culturing techniques provide a suitable method for the proliferation of stem cells.

Even though culturing stem cells have a number of advantages, the method has few drawbacks. There is a possibility of contamination due to the use of soft agar in the culture medium such. Also, the separation of colonies from the soft agar can a daunting task.

In conclusion, our findings indicate that SSCs obtained from neonatal mouse testis can be successfully cultured in soft agar-coated dishes. The culture protocol used in this study can increase the expansion of SSCs and can be a valuable method for future studies.

Conflict of interest statement

The authors declare no conflict of interest in any form.

Authors' contributions

Shadan Navid conceived of the study, performed animal treatments, collected and analyzed specimen and analyzed/interpreted data. Mehdi Abbasi, Shadan Navid and Ali Talebi analyzed real time PCR. All authors conceived of and oversaw the study, interpreted data, assisted with figure preparation, and wrote the manuscript. Yumi Hoshino, Maryam Borhani and Shadan Navid read and approved the final manuscript.

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