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The Effect of Silk Nanofibrous Scaffold and Co-Culture with Sertoli Cells on Spermatogonial Stem Cell Proliferation

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ABSTRACT

BACKGROUND AND OBJECTIVE: In vitro proliferation and maintenance of spermatogonial stem cells is one of the treatment options for infertile men. Creating a suitable microenvironment similar to the natural conditions of reproduction of these cells leads to the improvement of culture and spermatogenesis. Therefore, the aim of the present study was to use a 3D silk nanofibrous electrospun scaffold and co-culture with Sertoli cells in order to increase the proliferation of spermatogonial stem cells.

METHODS: In this experimental study, spermatogonial stem cells were isolated from the testes of 50 2-4-day-old mice (5 samples each time) using two-step mechanical and enzymatic digestion within two weeks. After placing 2×10^4 cells on the surface of the silk scaffold, they were exposed to culture medium. The viability of these cells was assessed using MTT assay and their binding was evaluated by SEM microscopy. The studied variables, including Stra8, DAZL and Piwill2 specific genes were analyzed by real time PCR and immunocytochemistry.

FINDINGS: The viability of cultured cells in the presence of scaffold and Sertoli (91±6), (90±2) was higher than the control group (85±2), (83±8). Real time PCR results confirmed a significant increase in the expression of specific markers of spermatogonial stem cells such as Stra8, Piwill2 and DAZL in cells cultured respectively on scaffold (1.47±6) (1.28±5) (1.43±2) compared to the 2D culture group (1.17±5) (1.05±3) (1.13±7) ($p< 0.05$).

CONCLUSION: The results showed that silk scaffold in the presence of Sertoli cell co-culture can increase in vitro proliferation of spermatogonial stem cells and can have a positive effect on the viability and proliferation of these cells.

KEY WORDS: *Scaffold, Silk, Sertoli, Proliferation, Spermatogonia.*

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Introduction

Spermatogonial stem cells are unique cells that process spermatogenesis, which plays a role in transmitting male genetic information to the next generation and is important for the preservation of the species. The process of spermatogenesis in testicular seminiferous tubules is performed by spermatogenic cells and is supported by Sertoli cells (1-3). Research shows that sperm cell transplantation can be used as a treatment option in infertile men. On the other hand, in vitro proliferation of spermatogonial cells before transplantation seems to increase the success rate of treatment in infertile men. Designing a suitable environment that leads to in vitro proliferation of stem cells can be used in assisted reproductive therapies in male infertility (4-6).

Laboratory culture methods have been important for the proliferation and enrichment of spermatogonial stem cells; designing an efficient system has been the main step in achieving the goal from the very beginning. So far, different culture systems have been used to achieve this goal (7-9). Unlike conventional 2D cell culture, using 3D matrices and scaffolds has an effective potential in the process of proliferation and differentiation of germline stem cells, which with the aim of mechanical and chemical manipulation and simulation of germline stem cells (10-12).

Nowadays, nanofilament scaffolds have many applications in reproductive tissue engineering by supporting stem cell proliferation (13, 14). For this purpose, culturing spermatogonial stem cells in the presence of extracellular matrix elements or scaffolds can be used as a suitable culture system to enrich spermatogonial stem cells (15-17). The microenvironment of spermatogonial stem cells, which is located on the basement membrane of the fallopian tubes, is important in their maintenance and proliferation of these cells. Sertoli cells play an important role in the secretion of essential growth factors in this microenvironment, and one of these factors is GDNF, which is important for proliferation and differentiation of spermatogonial stem cells. Without this factor, the proliferation and differentiation of spermatogonial stem cells under in vitro conditions is practically ineffective (18-20).

The 3D silk nanofilament scaffold, and the combination of this scaffold with the Sertoli cell co-culture, has not been used for proliferation of spermatogonial stem cells so far. In this study, it is predicted that the nanofilament structure of silk provides a 3D environment for the proliferation of spermatogonial stem cells. The porosity in the scaffold of this nanofiber facilitates the possibility of nutrition and excretion of cellular waste. On the other hand, the results of previous reports indicate the supportive role of stem cells after co-culture with Sertoli cells (21-23).

As a kind of supporter, Sertoli plays an important role in their nutrition and physical support and induces proliferation and differentiation in them through secretion of growth factor (24-26). Therefore, the aim of the present study was to increase the proliferation and differentiation of spermatogonial stem cells using a 3D scaffold of nano-silk filament and Sertoli cell co-culture. In this study, it is predicted that the nanofilament structure of silk provides a 3D environment for the proliferation and differentiation of spermatogonial stem cells. Due to the native potential of this scaffold in the field of tissue engineering and reproductive biology, this research is accompanied by many innovations and is used as a suitable platform for further research.

Methods

This experimental study with ethics code IR.MAZUMS.REC.1397.1817 was approved by Mazandaran University of Medical Sciences.

Preparation and isolation of spermatogonial stem cells: In this study, testicular cells were isolated from 50 2-4-day old mice. In each stage of isolation, 5 mice were used. After sacrificing the animals by inhaling chloroform and opening lower abdomen, the testicles were removed and were transferred to the culture medium containing the necessary enzymes such as collagenase and trypsin for enzymatic and mechanical digestion. Sediments containing Sertoli and spermatogonial cells were cultured and then separated from each other through differential removal (27, 28).

Spermatogonial stem cell culture: After isolation, the spermatogonial cells were transferred to a cell culture flask in sterile conditions under the hood. Then, the medium containing DMEM (Gibco-Life technologies, Canada) DMEM/F12, Fetal Bovine Serum (FBS) 15%, and 1% nonessential amino acid were added to penicillin/streptomycin to have a 5 ml solution and the cells were then incubated in an incubator (29). Evaluation of spermatogonial colonies: Morphology of cells was evaluated using a reverse

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microscope, and the number of colonies was counted on days 4, 7, and 14. The diameter of the colonies was measured by Image J software (30). The evaluated variables included the number and size of spermatogonial colonies. Cell culture in scaffold: For cell culture in scaffold, 2×10^4 cells were placed on the scaffold surface and then medium containing DMEM (Gibco-Life technologies, Canada) DMEM/F12, and Fetal Bovine Serum (FBS) 15% was added (31).

Evaluation of scaffold toxicity and cell adhesion of spermatogonial stem cells on silk nanofilaments: MTT solution in DMEM culture medium replaced the cell culture medium and then the cells were affected by DMSO and its light absorption was read using ELISA reader. Cell viability rates for each cell were compared with the control group (31).

Evaluation of binding and adhesion strength of spermatogonial stem cells using scanning electron microscope (SEM): SEM images were taken from cells cultured on nanofilament scaffolds to evaluate cell adhesion on this scaffold. For this purpose, the samples were fixed with glutaraldehyde and dehydrated using increasing degrees of alcohol. Cell membranes were fixed using osmium tetroxide and observed by SEM at Amirkabir University (32).

Evaluating the gene expression of spermatogonial stem cells: The cultured spermatogonial stem cells were collected after two weeks. The studied variables included the expression of Stra8, DAZL and Piwill2 genes, which was performed by real-time PCR. Total RNA was extracted using RNA Plus kit (produced by CinnaGen Co.) and after converting the desired mRNA to cDNA, PCR was performed using 50 ng cDNA in 35 cycles, including denaturation at 95 °C (45 seconds) and annealing at 58 °C (45 seconds) and extension performed at 72 °C (30 seconds) at the Corbett Rotor-Gene 3000. After completing each run of PCR, the accuracy of each proliferation curve was confirmed by Melting curve using the specific temperature of Melt of Product, which is specific to the product of each gene (33, 34). The expression of each target gene relative to the reference gene was calculated using the formula $R= 2^{-(\Delta\Delta CT)}$ (Table 1).

Statistical analysis of data: For calculation and analysis of data based on the comparison of the mean of quantitative and qualitative variables between groups, T-test, one-way analysis of variance, Chi-Square, and Tukey test were used. All values are provided based on Mean±SEM. All experiments and measurements were performed in 3 replicates and $p<0.05$ was considered significant.

Table 1. Primer sequences of specific genes of spermatogonial stem cells

Gene	Primer sequence (forward / reverse)	Product size	Significance
Stra8	5- ACGACGCGTCGCTATTCCCTCTCACATCTTC-3 5- AGCGAGCTCGATGCACCTTCGACACTTG-3	441	Spermatogonial marker
Piwill2	5-GCACAGTCCACGTGGTGGAAA -3 5-TCCATAGTCAGGACCGGAGGG -3	681	Spermatogonial marker
DAZL	5-GGAGCTATGTTGTACCTCC-3 5-CCATGTAACTAGATAAGCCAG-3	313	Spermatogonial marker
b actin	5- CTTCTTGGGTATGGAATCCTG -3 5- GTGTTGGCATAGAGGTCTTTAC-3	131	Internal Control

Results

Determining the nature of spermatogonial stem cells

Evaluation of cultured spermatogonial stem cells by phase-contrast microscopy: After 2 days of culture, spermatogonial stem cells were at the bottom of the culture dish and Sertoli cells were attached as a monolayer at the bottom of culture dish. Numerous small colonies consisting of spermatogonial stem cells appeared on the Sertoli layer and gradually increased over two weeks of culture (Figure 1). The number of colonies and their size in the Sertoli co-culture group (9.6±42) (18.27±80) increased during the culture period (8.06±13) (18.27±80).

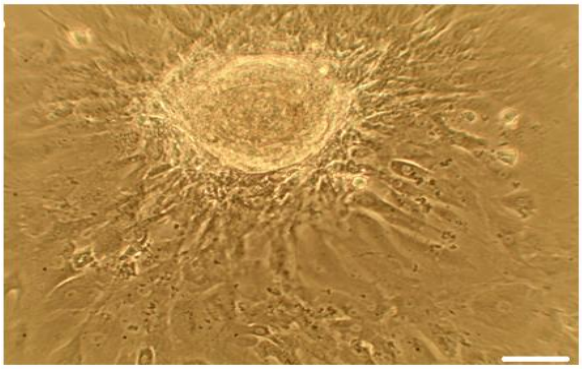

Figure 1. Spermatogonial stem cell morphology in Sertoli coculture group five days after culture (10X magnification)

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Comparison of the number and size of spermatogonial stem cell colonies in co-culture group with Sertoli versus culture without Sertoli cells: Results of counting the number of colonies on days 3, 7 and 14 and measuring the diameter of colonies and their area showed significant increase in the number and size of colonies in Sertoli co-culture group (18.27±80) (28.34±5) compared to control culture group (8.6±77) (19.04±5) ($p<0.05$) (Diagram 1).

Morphological characteristics of spermatogonial stem cells on silk scaffold: SEM evaluation of silk nanofiber scaffold was performed to attach spermatogonial stem cells to the scaffold. In SEM, after the culture of cells in silk nanofibrous scaffolds, the cells had a suitable appearance and became wider on the days of attachment (Figure 2).

MTT test: The results of MTT test showed that on the first day of spermatogonial stem cell culture, cell viability in the control group (96±3) was not significantly different from the group of culture on silk scaffold (97±8). However, on days 7 and 14, a significant difference ($p<0.05$) was observed in cell viability (85±2) (83±8). Cells cultured on silk scaffold (91±6) (90±2) showed a significant increase in viability ($p<0.05$) (Diagram 2).

The effect of silk nanofiber scaffold on expression of spermatogonial stem cell specific genes in culture medium: Expression of Piwill2, DAZL, Stra8 genes in spermatogonial cells cultured in silk scaffold (1.47±6) (1.28±5) (1.43±2) was expressed at a higher level than the control group (1.17±5) (1.05±3) (1.13±7) ($p<0.05$). The expression of the above genes increased in the scaffold group (experimental) during two weeks of culture, while in the control group, it decreased during the culture period (Diagram 3).

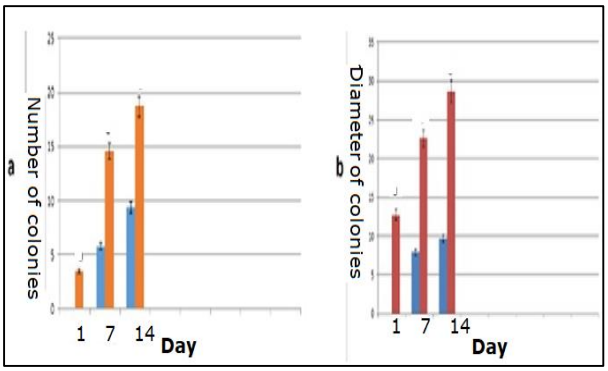

Diagram 1. Comparison of colony number (a) and colony size (b) in spermatogonial stem cells co-cultured with Sertoli cells (red and orange diagram) versus culture without Sertoli cells (blue diagram).

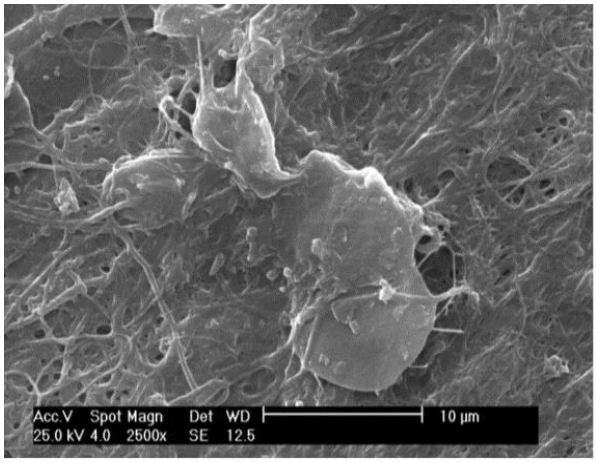

Figure 2. SEM micrograph of spermatogonial stem cells cultured in silk scaffolds. Binding of cultured spermatogonial stem cells on the scaffold.

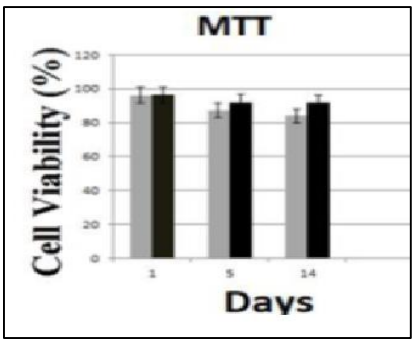

Diagram 2. MTT test showing cell viability in silk scaffolds. Dark color is related to culture on scaffold, which shows a significant increase in spermatogonial stem cell viability during two weeks of culture compared to monolayer culture (light color).

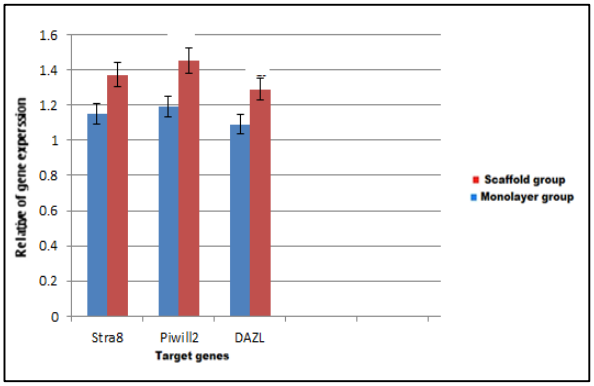

Diagram 3. Significant increase in gene expression of Stra8, Piwill2 and DAZL specific markers in spermatogonial stem cells cultured on silk scaffold compared to monolayer group in two weeks (letter a indicates a significant increase in gene expression compared to the control group).

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Discussion

The use of scaffold silk in the present study led to improved cell viability compared with 2D culture. In fact, by meeting the cellular needs in 3D culture conditions in the presence of silk scaffold, this scaffold leads to the spatial orientation of cultured spermatogonial cells and morphological changes during culture. The 3D structure and the size of the extracellular matrix filaments are designed by nanofiber filaments. These scaffolds are a 3D structure for inducing the necessary signals for cultured cells, and their cellular phenotype is more effective than 2D cultures (35-38).

The scaffold provides the right physical environment for cells to proliferate and the space needed for tissue remodeling. Recently, electrospun nanofibrillar scaffolds have been used for cell culture. Previous studies confirm the protective role of scaffolds in the proliferation of spermatogonial stem cells; Koruji et al. stated that the Polyl-Lactic Acid (PLLA) filament scaffold increased the proliferation and colonization of frozen-thawed spermatogonial stem cells and culturing spermatogonial stem cells on this scaffold leads to the production of cellular and colonic masses in frozen-thawed spermatogonial stem cells (11).

Lee et al. also concluded the positive effects of biodegradable poly(lactic acid)-based scaffold on spermatogonial stem cells (12). Other researchers cultured embryonic stem cells in nano-fibrous scaffolds and confirmed their proliferation, increase, and viability. During cell culture, the number of clusters in frozen-thawed groups and also frozen testicular samples were lower compared to the control group (39). In the present study, the relationship between Sertoli somatic cells and spermatogonial stem cells was provided by a silk electrospun nanofibrillar scaffold. This culture system seems to be effective in the reconstruction of 3D tubular structures of testicular seminiferous tissue (6-8). Similarly, some researchers have reported that the use of Sertoli cells along with the matrix is effective in regenerating the fallopian tubes. Sertoli cells are somatic cells that are in direct contact with differentiated germ cells. Spatial cell arrangement between Sertoli and differentiated germ cells is one of the most complex examples of intercellular exchange (40, 41). Molecular studies showed that the expression of spermatogonia-specific genes such as Stra8, piwill2, DAZL was higher in the scaffold group compared to 2D culture. These results are similar to those of other groups that studied and reported changes in the expression of these genes during spermatogonial stem cell culture (5).

The nature of spermatogonial stem cells in this study was identified, evaluated and confirmed using various criteria. In fact, the expression of a high percentage of specific spermatogonial markers such as Stra8, piwill2, DAZL confirmed the essential nature of these cells. The use of these markers to confirm the essential nature of cell in culture medium is very common (4).

The results of previous research show that gene expression in spermatogonial stem cells as a surface marker is predominantly specified. Gene expression also depends on the presence of scaffold and Sertoli. The results of the present study show that scaffolds can be used in reproductive tissue engineering and can support the process of cell proliferation. The viability and proliferation of spermatogonial stem cells increased in the designed culture system, making this system unique in the proliferation and differentiation of these cells for clinical applications, tissue engineering applications, tuberculosis therapy, and tissue regeneration.

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