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Original Article

Evaluation of the Growth and Differentiation of Spermatogonial Stem Cells on a 3D Polycaprolactone/Multi-Walled Carbon Nanotubes

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Abstract

Introduction: Spermatogenesis is the process that Spermatogonial Stem Cells (SSCs) differentiate to spermatozoa. Effective *in vitro* differentiation of SSCs to sperm can be a promising sign for the reconstruction of spermatogenesis disorders. This research was designed to evaluate the effect of a 3D Polycaprolactone/Multi-Walled Carbon Nanotubes-based microfibrous scaffold on the culture and differentiation of mouse SSCs to germ cells.

Materials and Methods: In this research, by using the electrospinning technique, a microfibrous Polycaprolactone (PCL) scaffold incorporated with Multi-Walled Carbon Nanotubes (MWCNTs) was fabricated. The microfibrous PCL/MWCNTs were assessed using Scanning Electron Microscopy (SEM), Transmission Electron Microscope (TEM), Fourier Transform Infrared Spectroscopy (FTIR), and water contact angle measurements. Then, the isolated SSCs were characterized using flow cytometry. Also, the survival and differentiation of SSCs on the 3D fabricated scaffold and tissue culture plate (2D) were evaluated using MTT and real-time PCR for *PLZF*, *ID4*, *C-Kit*, and *SYCP3* genes, respectively.

Results: Morphological assessment of the scaffold showed that PCL/MWCNTs were randomly oriented as microfibrous. In addition, TEM images indicated the presence of Carbon Nanotube (CNT) into PCL polymer. The characterization result of SSCs indicated that approximately 99% of SSCs were positive for promyelocytic leukemia zinc finger (PLZF). Seeded SSCs on the PCL/MWCNTs scaffold had a high survival rate and differentiation. Accordingly, qRT-PCR results demonstrated that the SSCs on the 3D scaffold overexpressed the *C-Kit* and *SYCP3* genes (Markers of differentiated cells) whereas expression of the *PLZF* and *ID4* genes had no significant difference between 2D and 3D groups.

Conclusions: This research showed the engineered 3D scaffolds can support the proliferation and differentiation of SSCs to germ cells. In addition, this 3D microenvironment could be useful as a new approach in 3D culture systems, especially for culture and the differentiation of SSCs.

Keywords: PCL, MWCNTs, Scaffold, Spermatogonial Stem Cells, Spermatogenesis

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Introduction

About 15% of couples are infertile worldwide,[1] that 7-12% of all men complain of infertility in reproductive ages.[2,3] Azoospermia is an important male infertility cause that approximately 1% of all men or 10-15% of infertile men suffer from it. Sometimes normal volume of ejaculated semen contains no sperm which is called non-obstructive azoospermia.[4,5] Despite being infertile, these patients have a potential to initiate a pregnancy. Actually, their testis biopsy revealed that 30-60% of these men have focal areas of spermatogenesis.[6] Severe male infertility occurs in 2/3 of infertile men that have untreatable testicular disorders and induce spermatogenic failure.[7] Spermatogenesis is cornerstone of male fertility through differentiation of SSCs. These cells are tissue-specific stem cells with self-renewal and differentiation potentials,[8] which could be supported by a long-term culture system.[9]

One of the innovative approaches in medicine to overcome male infertility is *in vitro* spermatogenesis especially in 3D scaffolds. Engineered 3D scaffolds can mimic the native Extracellular Matrix (ECM)[10,11] and provide desired biological niche for stem cells to have self-renewal or differentiation.[12] Being biocompatible, a suitable scaffold meets specific criteria that allow the cells to migrate, attach, proliferate, and differentiate to the desired fate.[13,14] Many polymers can be used to synthesis tissue engineering scaffolds in order to provide the necessary physical and chemical signals for cells to reside and spread in the porous structure.[15] Among polymers, properties of synthetic polymers could be easily tailored to achieve unique architecture and mechanical characteristic for different tissue engineering applications.[16] The most synthetic polymers in tissue engineering are Polylactic Acid (PLA), Poly Glycolic Acid (PGA), Polyurethane

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(PU), polylactic acid, Polycaprolactone (PCL), and poly (l-lactide-co- ε-caprolactone).[17-22] PCL is a linear synthetic biodegradable aliphatic polyester and it is one of the most popular polymers among the researchers for tissue engineering applications. PCL is inexpensive, has a controllable degradation kinetics and mechanical properties and could be easily shaped and manufactured.[23,24] This biocompatible polymer is FDA-approved[25] and exhibits appropriate mechanical, structural[26] and thermal stability. Electrospun PCL scaffold has shown promising results in different tissue engineering applications.[27] Having a porous interconnected structure, microfibrous scaffolds can mimic the filamentous structure of ECM, facilitating optimal cell growth.[27]

In tissue engineering, nanocomposites are designed to improve matrix and scaffold's properties. Carbon based nanoparticles such as graphene and CNT are highly versatile in biomedicine and tissue engineering.[28] CNTs are tubular nanoparticles composed of carbon atoms which have specific characteristics such as high mechanical strength and high electrical conductivity.[29] It has been proven that they could support adhesion and proliferation of different cell types such as osteoblasts,[30] neuronal cells[31] and induce stem cell differentiation to different cell lineage.[32] It has been reported that, spermatogonial cells also remained viable and adherent up to 21days when seeded on a CNT-based scaffold.[33]

According to the high efficiency of 3D microfibrous scaffolds in male reproductive systems, a PCL scaffold incorporated with Multi-Wall CNT (MWCNT) scaffold were synthesized using electrospinning technique. Beside morphological and compositional characteristics of the electrospun scaffolds, viability, and spermatogenesis potential of SSCs seeded on the designed scaffold were evaluated compared to the tissue culture plate (2D) (Figure 1)

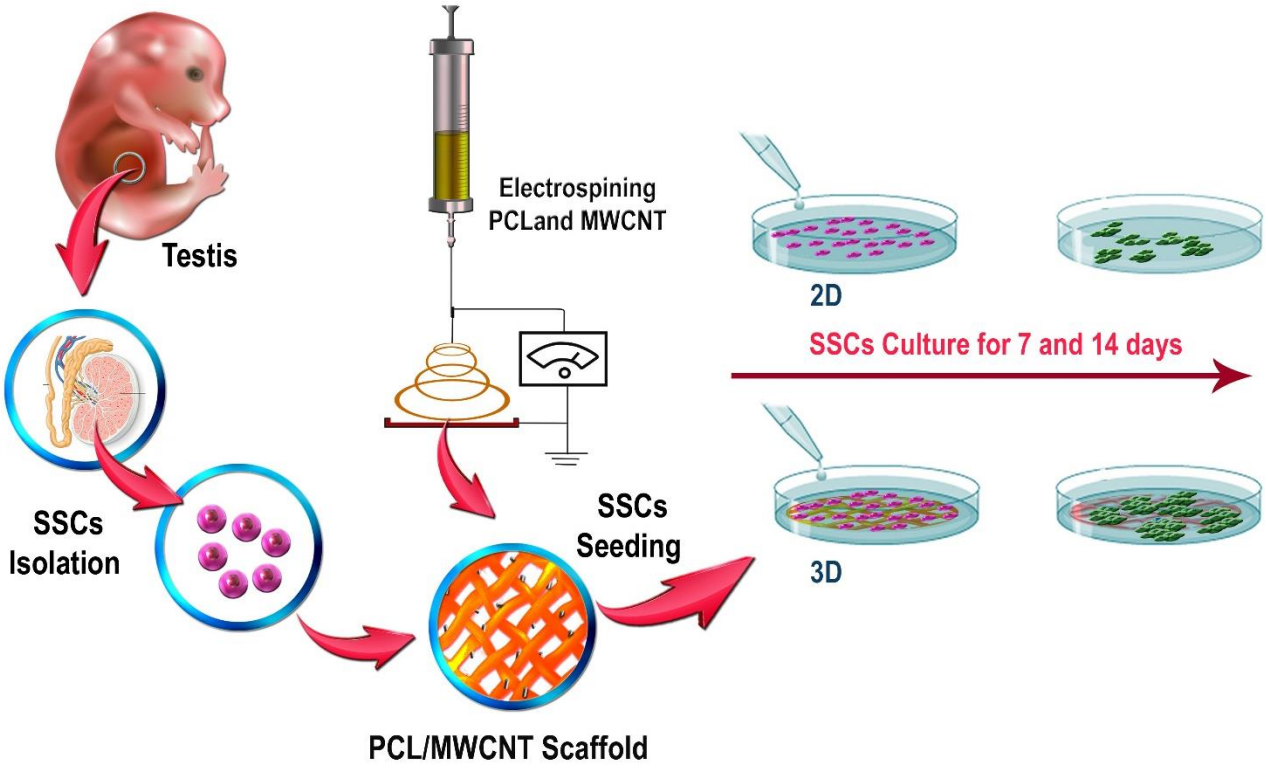

Figure 1. A Scheme of 3D Scaffold Synthesis and Isolation of Spermatogonial Stem Cells (SCCs), Seeding, and Differentiation.

Materials and Methods

Preparation of PCL/MWCNT Scaffold

MWCNTs (Nanocyl Korea Ltd) were prepared using Xiao et al.'s protocol to create active sites on its surface for more reactions.[34] Briefly, MWNTs were dissolved into 70 ml hydrochloric acid (HCl) (36.5 wt%) solution while slowly being stirred until 2 h. Afterward, it was diluted by water, refined by filter, and were dehydrated in vacuum at 40 °C for 12 h. Thereafter, MWNTs were mixed with 50 ml nitric acid (HNO_3) (65 wt%) and warmed up to 140 °C inside nitrogen atmosphere until 4 h and finally cooled at 25 °C.

In the next step, PCL (Mw 5 100 000, Chemiekas, Vienna, Austria) was mixed (15% w/v) with N, N-dimethyl formamide (DMF) and this mixture was stirred for 6 h at room temperature. For preparing the PCL/MWCNT solution, 3% w/v of MWCNT was mixed with the pure PCL solution and the mixture was ultra-sonicated for 1 h. Finally, for electrospinning, the PCL/MWCNT solution was put in a 10 ml syringe and an 18 gauge metal needle was used. The electrospinning process was done on an aluminum rotating plate as a collector

with the rate of 200 rpm for 8 h and 20 kV voltage, the flow rate of 3 ml/h, and distance of 20 cm.

Morphological Assessment of PCL/MWCNT Scaffolds

The morphological analysis of electrospun scaffolds was done using scanning electron microscopy (SEM, Seron Technology, South Korea). The scaffolds were covered with gold for 3 min by a sputter coater (Quorum Technologies, England) and the SEM results were recorded at 20 kV voltage. An image analyzer (Image J) used for evaluation of fiber diameter. Moreover, Transmission Electron Microscopy (TEM) was used to investigate the incorporation of MWCNTs into PCL fibers. For this purpose, a TEM apparatus (Philips CM-30 TEM operating at a voltage of 250 kV) was used, and the specimen of TEM were prepared by electrospinning PCL/MWCNTs solution on the carbon-coated copper grids attached to the drum for 3 min on the same electrospinning condition.

FTIR Analysis

The scaffolds structure was evaluated using Fourier Transform Infrared (FTIR) spectroscopy (EQUINOX 55, Germany). The scaffolds were grinded with KBr and samples were studied in the 400 to 4000 cm^{-1} wavelength range.

Water Contact Angle Measurement

The water drops contact angle on the surface of fibrous neat PCL and PCL/MWCNTs membranes was measured with the aid of a video contact angle instrument (Sony, model SSCDC318P, Japan) at 10 sec across the surface of the scaffolds to determine the fabricated scaffolds hydrophilicity.

Isolation and Culture of Spermatogonial Stem Cells (SSCs)

All animal studies were conducted with the approval of the Ethical Committee of Baqiyatallah University of Medical Sciences, Tehran, Iran (IR.BMSU.REC.1398.011). Firstly, neonatal mice (3-5 day-old) testis were collected. The testes were de-capsulated and cut into small fragments and then, the testis tissues were washed with Dulbecco's Modified Eagle medium/ Nutrient Mixture F-12 (DMEM/F12; Gibco, UK), containing 100 IU/ml penicillin, 100 µg/ml streptomycin, and 40 µg/ml gentamycin (all from Gibco, UK). The testis pieces were put in DMEM/F12, containing 0.5 mg/ml collagenase/dispase, 0.5 mg/ml trypsin, and 0.08 mg/ml DNase and the suspension was shaken for 60 min at 37 °C. Then, the mixture was washed three times in DMEM/F12 and most of the interstitial cells were removed. Afterwards, DMEM/F12 supplemented with fresh enzymes was added to the seminiferous cord fragments for the second digestion step (45 min at 32 °C). The cells were washed using staining buffer and were then fixed and permeabilized in 4% paraformaldehyde and in 0.5% Triton X-100 (Darmstadt, Germany) respectively. For blocking the nonspecific antibody binding, 10% heat inactivated goat serum with staining solution buffer was used. For each sample 1.5×10^5 cells were utilized. The cells were incubated with primary PLZF (ab189849, Abcam, USA) antibody. The coated cells with species-specific secondary antibodies were put in a staining buffer and they were incubated for 30 min at 4 °C. The flow cytometric analysis was done by a fluorescence-activated cell sorting (FACS, Sysmex Partec CyFlow Space). Finally, 1.5×10^4 cells/cm^2 of the isolated SSCs were seeded on 2D culture vessels (without scaffold) and 3D (with scaffolds) groups. The samples and 2D culture group were incubated in DMEM/F12 containing 10% FBS and 10 ng/ml GDNF, 50 ng/ml BMP4 (both from PeproTech, London, UK) for 7 and 14 days at 34 °C.

Morphological Analysis of SSCs on 3D Scaffolds

The cells were fixed on fibers using 2.5% glutaraldehyde/ PBS solution at 25 °C for 30 min and the seeded cells were analyzed after 7 and 14 days. The samples using a gradient of ethanol (30%, 50%, 70%, and 100% v/v) were dehydrated. Finally, the cells were covered with gold and then the morphological analysis of spermatogonial stem cells was performed using SEM.

Cell Viability

To proliferation assessment of the SSCs on PCL/MWCNTs scaffolds, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (Carl Roth, Germany) assay was performed three times (1, 3, and 7 day). Briefly, after washing the seeded samples by PBS, they were incubated with serum-free DMEM and composed with 5 mg/ml MTT powder at 37 °C in a dark place. The medium was removed after 4 h and for dissolving the formazan crystals, DMSO was added. The Spectrophotometric measurements at 570 nm was performed using a microplate reader (Biochrom, Berlin, Germany).

Gene Expression Analysis

Total RNA was obtained from seeded SSCs on 2D and 3D groups by QIAzol (Qiagen, Germany). To remove genomic contamination, RNA was treated with Deoxyribonuclease (DNase I) enzyme (Fermentas, Vilnius, Lithuania). RNA concentrations were measured by Ultraviolet (UV) spectro-photometry (Eppendorf, Germany). The cDNAs were made from 500 ng DNase-treated RNA samples with a RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Germany) by oligo (dT) primers. *PLZF*, *ID4*, *C-Kit*, and *SYCP3* gene expression was analysed and *GAPDH* was used as a housekeeping gene. C-*Kit* and *SYCP3* genes have a higher expression in differentiated cells. For Polymerase Chain Reaction (PCR), the primers gene sequences were got from the National Center for Biotechnology Information (NCBI) database and their exons and introns sequence was determined and primes were designed using the Primer3 online software.

The primers are blasted to approve their accuracy and reproduce only the genes' mRNA sequences and synthesized by the Cinnagen Company (Table 1). The qRT-PCR were done by Master Mix and SYBR Green I (S7563, Thermo Fisher) in a StepOne™ thermal cycler (Applied Biosystems, USA). The program was initiated with a melting cycle at 95 °C for 5 min to activate the polymerase, followed using 40 melting cycles (30 sec at 95 °C), annealing (30 seco at 58 °C), and expanse (30 sec at 72 °C). The PCR reactions quality was confirmed using melting curve assessments and the efficiency of each gene was determined by a standard curve. The reference gene and target gene for each sample were amplified in the same run. All runs were performed in triplicate. The target genes were standardized with the reference gene and expression of the gen was evaluated with the ΔΔCT method.

Table 1. The Primers Were Used for real-time PCR

Gene	Accession Number	Primer Sequence
PLZF	NM_001033324.2	F: 5'-CCCGTTGGGGGTCAGCTAGAA-3' R: 5'-CTGCAAGGTGGGGCGGTGTAG-3'
Id4	NM_031166.2	F: 5'-GGGTGACAGCATTCTCTGC-3' R: 5'-TTGGAATGACAAGACGAGAG-3'
C-Kit	XM_021163091.1	F: 5'-CTAAAGATGAACCCTCAGCCT-3' R: 5'-GCATAACACATGAACACTCCA-3'
SYCP3	XM_021171638.1	F: 5′-TGTTCAGAGCCAGAGAAT-3′ R: 5′-TCACTTTGTGTGCCAGTAA-3′
GAPDH	XM_021218477.1	F: 5'-CTGCTGGACAAGTGAGTCCC-3' R: 5'-CCAAGTACCCTGGCCTCATC-3'

Statistical Analysis

The results were described as the mean ± standard error. Analysis of variance (ANOVA) was utilized to compare results by using the Statistical Package for Social Sciences (SPSS) software, Version 18.0 (SPSS Inc., USA). The $p<0.05$ was considered statistically significant.

Results

Scaffold Characterization Tests

Morphologic Properties

Based on the SEM images of the PCL/MWCNTs (Figure 2a and b), the randomly oriented microfibrous formed a porous micro and Nano structure. The diameter average of fibers in this scaffold was reported to be 792 ± 37 nm. In addition, TEM was used to investigate the incorporation of MWCNTs into PCL fibers. As shown in Figure 2c and d, the MWCNTs material are in a cylinder like morphology elongated through the microfibrous and the MWCNTs nanoparticles are incorporated in PCL microfibrous which are clearly distinguished (Figure 2c and d).

FTIR Analysis

FTIR is a method to diagnose the chemical groups of the composite fibrous scaffolds. Figure 3a shows PCL/MWCNTs

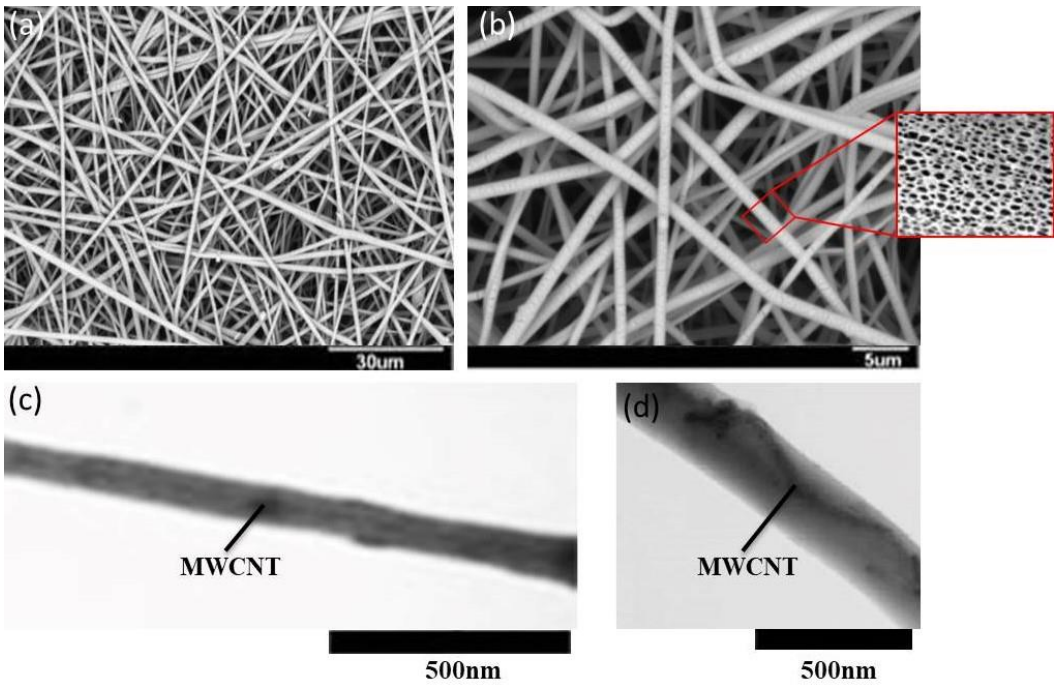

Figure 2. The SEM Image of PCL/MWCNTs Scaffold (scale bars: 5 **(a)** and 30 **(b)** µm). The magnification shows the intra fiber porosity of PCL/MWCNTs scaffold. **c** and **d**) The TEM image of microfibrous of PCL/MWCNTs fibers.

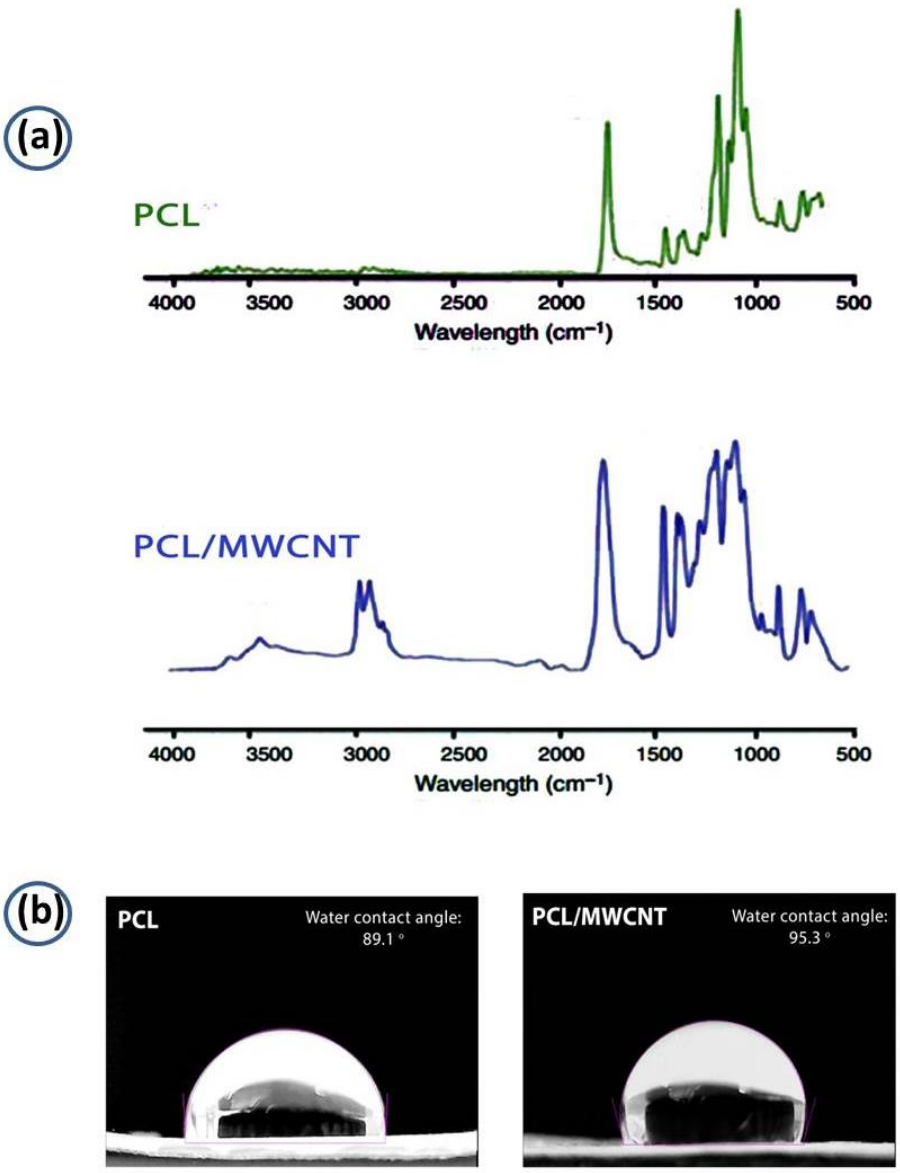

Figure 3. Structural and Hydrophilicity Properties of PCL and PCL/MWNCTs Scaffolds. **a)** Shows the FTIR spectra of PCL and PCL/MWCNTs composite. **b)** The hydrophilicity of the surface of PCL and PCL/MWCNT using water contact angle analysis at 10 sec.

scaffold spectra. The PCL FTIR peaks is associated with the existence of C═O, C─O, ─CH3 asymmetric, and ─CH_3 symmetric bonds. The peak of absorbance C═O stretching bonds was detected at 1758 cm^{-1} and stretching absorbance peaks in 1086, 2943, and 2944 cm^{-1} were observed associated with the C─O, ─CH_3 asymmetric, and ─CH3 symmetric bonds in PCL microfibrous, respectively. The peaks in 1455 and 1366 cm^{-1} are related to the presence of ─CH3 asymmetric and symmetric bonds. Furthermore, some MWCNTs characteristic peaks were observable in the spectra and were attributed to the existence of COOH and ─OH bonds. Also, another absorbance peak in 3450 to 3550 cm^{-1} was detected which belong to the stretch bending of O─H of the MWCNTs. Moreover, the peak in 2994 cm^{-1} can be related to C─H stretching vibration in the aromatic structure of MWCNTs (Figure 3a).

Water Contact Angle Measurement

Contact angle is an indicator of hydrophilicity of the scaffolds surface, characterizes the wettability of the substrate and plays an important role in the attachment and fate of cells. The hydrophobicity of the microfibrous was altered by the incorporation of MWCNTs into PCL fibers. The contact angle of PCL was estimated to be 89.1° ± 7° at 10 sec, which increased to 95.3° ± 6° in the PCL/MWCNT sample at the same time (Figure 3b). The numbers of contact angles were statistically significant between PCL and PCL/MWCNT mats (p<0.05) (Figure 3b).

In Vitro Assessments

Morphological and Attachment Analysis and Characterization of SSCs

After two weeks of cell culture, the morphology and attachment

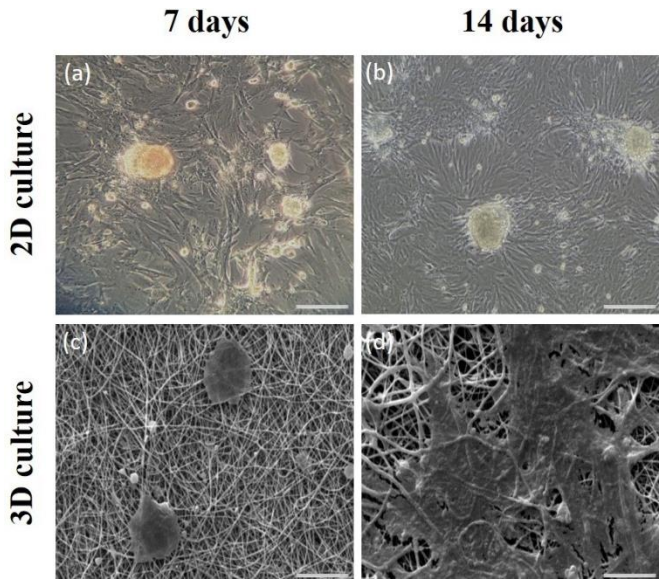

Figure 4. Morphology of Differentiated Spermatogonial Stem Cells (SSCs) on 2D **(a, b)** and 3D **(c, d)** Substrates at 7- and 14-Days after Cultivation (Scale bars: 10 µm).

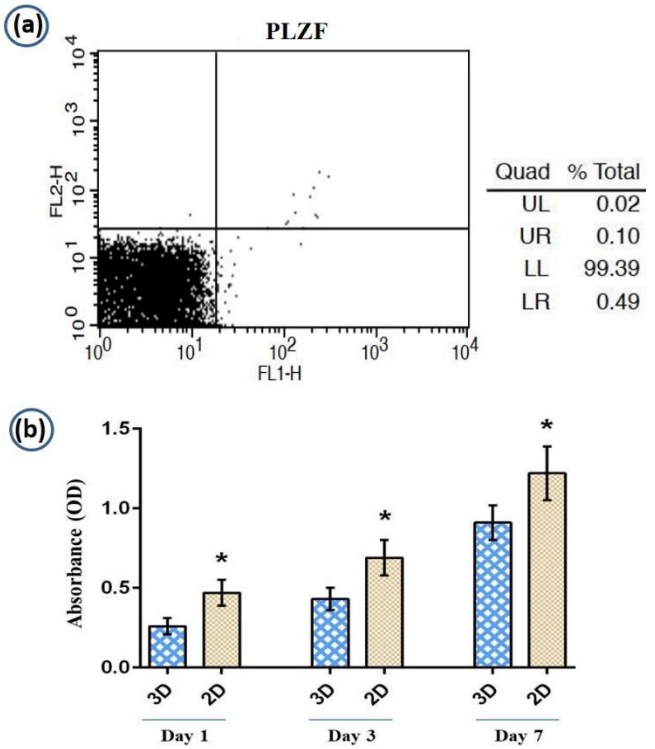

Figure 5. a) Immunophenotyping of spermatogonial stem cells (SSCs) by the flow cytometry using PLZF antibody (N = 3). **b)** Cell viability of SSC on 2D and 3D substrates at different times (Day 1, 3, 7) after cultivation (N = 3).

of SSCs on 2D and 3D substrates was analyzed which presented in Figure 4. The 2D images showed that SSCs had intended to be aggregated and create colony shaped clusters (Figure 4a and b) while the cells were properly spread on the 3D substrate (Figure 4c and d). Generally, PCL/MWCNTs could provide a suitable 3D environment to SSCs prolifration and supported them in culture for 14 days (Figure 4c and d). In addition, to confirm the isolated SSCs, the flow cytometry technique was performed, which indicated 99.39 ± 2.7% of cells expressed PLZF marker (Figure 5a).

MTT Analysis

The survival and proliferation of SSCs on the PCL/MWCNTs, was evaluated using MTT after 1-, 3- and 7-days (Figure 5b). The cell viability in a 3D cell culture was less than the 2D cell culture and it was significant ($p \leq 0.05$). This may happen because of the hydrophobicity nature of PCL/MWCNTs scaffolds that induced weak spermatogonial attachment and discarded during medium changing. Based on the MTT results, SSCs cell proliferation on day seven was more than the other days (days one and three) ($p \leq 0.05$). This occurred due to the suitable affinity of SSCs to carbon nanotubes. These results indicated that the PCL/MWCNTs scaffolds have synergetic effects on cell proliferation of SSCs during seven days post-incubation rather than other time points.

Assessment of Differentiation Based on Specific Gene Expression

Differentiation of SSCs to spermatogonia was evaluated using real-time PCR for specific genes expression and compared between 2D with 3D (PCL/MWCNTs) substrates (Figure 6). The gene expression result indicated that the expression pattern of SSC genes such as *ID4* and *PLZF* is similar in 2D and 3D groups and they had no significant differences. The gene expression of *C-Kit* and *SYCP3* as differentiated SSCs-specific genes in 3D group was better than 2D group. In addition, these results revealed the activation of SSCs genes in two weeks of post-culture.

Discussion

Male infertility is an important failure which is associated with the infertility of about half of the infertile couples.[35] There are some techniques that give infertile men a chance to have a healthy offspring, such as microsurgical Testicular Sperm Extraction (m-TESE), Intra-Cytoplasmic Sperm Injection (ICSI), and Round Spermatid Injection (ROSI) which at least the round spermatids are essential for success in these processes.[36] Some of the infertile men suffer from azoospermia in which their semen have no spermatid. One of the useful technique for these men is *in vitro* spermatogenesis.[37] The cancer treatments and chemotherapy are gonadotoxic, the pre-pubertal patient under treatment may wish to sterilize in their future life[38] and sampling before chemotherapy and the culture of testicular tissue fragments is one of the ways to preserve their fertility. This means these men are also another group who take advantage of *in vitro* spermatogenesis.[39] Also, the research

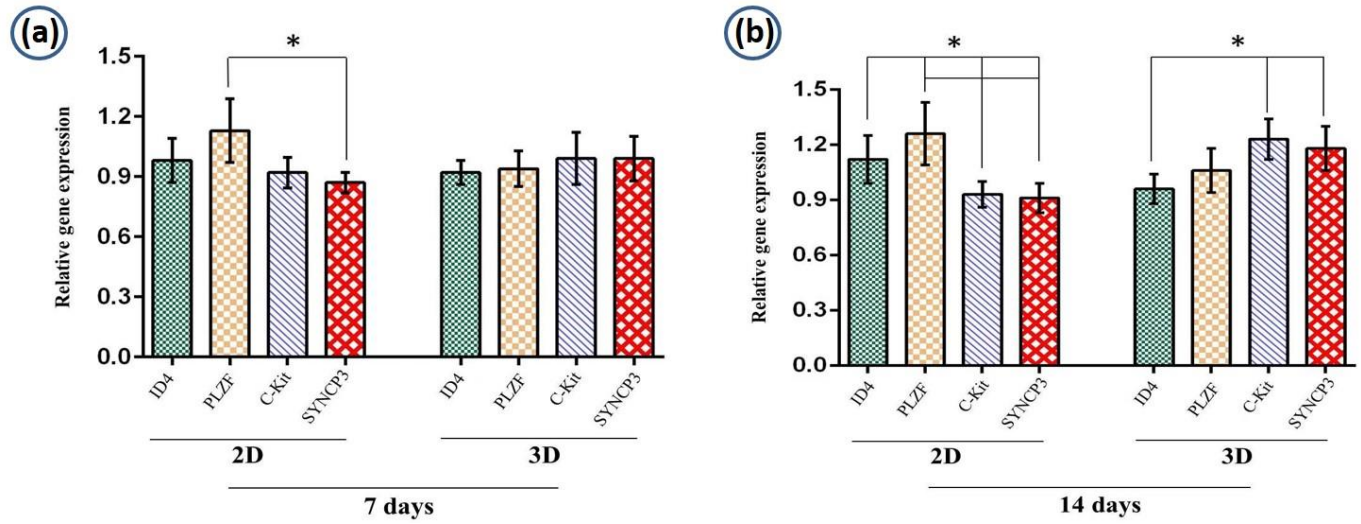

Figure 6. The Quantitative Gene Expression Diagram of *ID4, PLZF, C-Kit,* and *SYNCP3* Genes, Respectively, on 2D and 3D Substrates at Different Times (Day 7, 14) after Cultivation **(a, b)**. All values are presented as mean ± SE.

on spermatogenesis has been hampered by a lack of appropriate *in vitro* methods. To remodel the 3D structure of testicular natural extra cellular matrices, tissue engineering using scaffolds has been developed to mimic a micro-environment for cell growth and divisions. The current research aims to evaluate the effect of Polycaprolactone (PCL)/multiwalled carbon nanotube 3D scaffold on the growth and differentiation of SSCs.

The cells distributed uniform in 3D culture. However, in the 2D culture, we observed the SSCs formed the colonies, moreover 3D scaffold induced better cell differentiation. Research have revealed that the 3D culture systems could increase the stem cells differentiation to different cells such as osteoblast,[40] hepatocyte[41] and neurons.[42] It may be due to several factors such as the structural similarity of scaffold to ECM which induce the suitable environment for e tgrowth and differentiation of cells.[43] Among numerous natural or syntheticderived materials, PCL has been widely explored as a bio-scaffold because of its impressive biological features, excellent mechanical structure, and minimal inflammatory reaction. In addition, another important factor can be the MWCNT stimulation effect on cells differentiation.[44] CNTs are viewed as a class of materials in nanoscale with a great potential for various biomedical applications because of their unique characteristics such as cellular binding, ease of cellular uptake, and electrical conductivity, which can be effective in the growth and differentiation of different types of stem cells. The cell viability in 3D scaffold was approximately similar to 2D culture system and the 3D scaffold enhanced the SSCs proliferation, which could be due to the electrical and mechanical properties of CNTs.[29] Actually, as it has been demonstrated they could increase the proliferation in different cell lines.[45,46] Generally, in the first days of culture, 2D culture systems maintain the cell viability more than 3D scaffolds, but 3D scaffold increases the cell proliferation compared to 2D culture systems.[47] There is evidence about the positive impacts of PCL scaffolds on spermatogenesis.[48] Moreover, Rafeeqi et al. have reported that the presence of CNTs in media as a scaffold is likely to enhance the SSCs proliferation.[33]

The salient importance of electrospun scaffolds in terms of porosity and crosslinking in cell survival, proliferation and migration has been emphasized in other studies.[49] It can be concluded that the porous topographic structure of PCL/ MWCNT scaffolds provides sufficient dimensions for cell infiltration. In addition, PCL wettability decreased after MWCNT integration, which was related to the lower OD of cells implanted in the scaffold in MTT assays. SEM cultured SSCs showed excellent adhesion, penetration and aggregation of these cells on scaffolds. The results clearly showed that the applied scaffolds not only provide good support for cell homing, but also increase the diffusion and differentiation of SSCs.

The *PLZF* is essential for the normal function of SSCs and indirectly controls the earliest cell fate decisions in spermatogenesis,[50] and the imbalance of *PLZF* gene expression impairs the self-renewal and SSCs differentiation.[51] Helix-loop-helix protein *ID4* gene expression have an important role in SSCs pool maintenance.[52] Our results demonstrated that the expression of *ID4* and *PLZF* gene in 2D and 3D culture system was similar which means none of them induce SSCs damage. Ghorbani et al. revealed that the spermatogonial genes expression in cells that cultured in PLLA/MWCNTs scaffold were decreased,[11] but another study indicated that the PCL 3D culture increased the level of *PLZF* gene expression but had no effect on *ID4* gene expression.[53] *C-kit* is a marker of spermatogonial differentiation and have no direct effect on SSCs survival and proliferation.[54]

Synaptonemal Complex Protein3 (SYCP3) is a meiosis and germ cell differentiation marker.[55] In the present study the gene expression of these two differentiation SSC markers in 3D scaffold were more than 2D culture system which has been confirmed by the morphological assessment of the present study. Other studies revealed that 3D culture systems enhance the expression of differentiation SSCs genes.[11,53] This betterment may be due to scaffold affinity to ECM that could promote a good environment for cells and the scaffold materials that stimulate cells to be differentiated,[43,44] so the 3D culture systems improve the cells proliferation and differentiation.

Conclusion

In this study, electrospun PCL/MWCNT was synthesized and it was shown that these 3D-like electrically conductive scaffolds could support SSCs attachment and proliferation also could maintain the cell survival like 2D culture system. Also, 3D engineered scaffold and SSCs in culture are likely to construct a testis-like microenvironment. Thus, this study suggests that PCL/MWCNT scaffold can be useful as a new approach in 3D culture system and it opens up a promising field in the study of *in vitro* spermatogenesis using 3D scaffold, guiding the stem cell differentiation toward elongated spermatid in the near future.

Authors' Contributions

MG and MRN designed the study and they were a major contributor in writing and conducting the manuscript, and also carrying out some tests. HA and VG analyzed and interpreted the data. All authors read and approved the final manuscript.

Ethics Approval

This study was approved by the Ethics Committee of Baqiyatallah University of Medical Sciences, Isfahan, Iran with code of IR.BMSU.REC.1398.011.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

Acknowledgment

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