Melatonin Promotes Differentiation of Human Spermatogonial Stem Cells Cultured on Three-Dimensional Decellularized Human Testis Matrix

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Purpose: The use of 3D (3-Dimensional) culture systems supported cell-to-cell and cell-to-extracellular matrix (ECM) interactions, proliferation, and differentiation of SSCs (Spermatogonial stem cells). The potential advantages of ECM-based scaffolds for in vitro spermatogenesis have been indicated in human and animal experiments. Furthermore, the strong antioxidant and anti-inflammatory activities of melatonin have improved in vitro manipulation of human SSCs in culture conditions.

Materials and Methods: SSCs were isolated from the testis of three dead-brain patients and then propagated for four weeks. The characterization of SSC colonies was done using real-time PCR (Polymerase chain reaction), ICC (Immunocytochemistry), and xenotransplantation to mice model. Decellularization of the human testis was performed using 0.3% sodium dodecyl sulfate (SDS) solution and 1% Triton X-100. Also, various characterizations of DTM (Decellularized testicular matrix) were carried out using histological staining and DNA content analysis. The optimum dose of melatonin was selected by MTT (Methyl thiazol tetrazolium). SSCs were cultured in 4 groups: control, melatonin, ECM, and ECM-melatonin in a differentiation medium for four weeks. The expression of differentiation genes was evaluated by real-time polymerase chain reaction. In addition, the viability of cultured cells was assessed by MTT assay.

Results: The results of ICC and real-time PCR showed the expression of undifferentiated SSC markers (PLZF and GRFA1) in SSC colonies following the 2D culture of isolated SSCs. The presence of testicular ECM components after different staining methods; and the reduction of DNA content confirmed the proper decellularization process. Germ cell apoptosis significantly decreased in melatonin and ECM groups, and the higher viability of SSCs was seen in the ECM-melatonin group. The relative expression of GFRA1 and PRM2 decreased and increased in ECM and ECM-melatonin groups, respectively.

Conclusion: Our study showed that the addition of melatonin to the human naturally-derived ECM scaffold could provide a suitable platform for inducing the differentiation and preserving the viability of SSCs.

Keywords: melatonin; spermatogonial stem cells; 3D culture system; extracellular matrix.

INTRODUCTION

Spermatogonial stem cells (SSCs) are an undifferentiated subpopulation of spermatogonia, which are located on the basement membrane of seminiferous tubules and possess the ability for self-renewal and differentiation [(1)]. SSCs are the foundation of spermatogenesis, and they can differentiate into mature sperm and transmit genetic information to the next generation. Thus, self-renewal, propagation, and differentiation of SSCs are substantial in mammalian fertility preservation[(2)]. SSCs rapidly divide during spermatogenesis, making them highly susceptible to DNA fragmentation induced by cytotoxic chemotherapeutic agents[(3)]. Semen cryopreservation before gonadotoxic treatments is a proven and validated technique for male fertility preservation, although this approach is not appropriate for prepubertal boys who are not able to produce spermatozoa[(4)]. Establishing an efficient in vitro culture system that can mimic the spermatogenesis process has recently attracted a great deal of attention[(5,6)]. 3D culture systems have provided a promising platform to artificially reproduce the in vivo organization and function of the testis seminiferous tubules. 3D culture systems supported cell-to-cell and cell-to-extracellular matrix

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Table 1. The sequence of primers used for real-time PCR.

Gene	Forward 5'-3'	Reverse 5'-3'
BAX	TGTCGCCCTTTTCTACTTTG	GCCCATGATGGTTCTGATC
BCL-2	TGGAGAGTGCTGAAGATTGATG	AGTCTACTTCCTCTGTGATGTTG
PLZF	AATGGCTGTGGCAAGAAGTTC	CGTTGTGCGTTCTCAGGTG
GFRA1	CTGCCTCCTCGCCTACTC	GGTCGTTCCCACTGTTGC
SCP3	GAGAGCCTATGACTTTGAGACTG	TAATGTCAACTCCAACTCCTTCC
PRM2	CTGGAAGTTAAGAGAAAGTCACC	GCTTGAGCATTTGATGTAGGG
GAPDH	GCCACATCGCTCAGACAC	GCAACAATATCCACTTTACCAGAG

(ECM) interactions, proliferation, and differentiation of SSCs in a controlled in vitro environment[7,8].

Previous experiments reported that different 3D culture systems supplemented with naringenin, laminin, growth factors, and melatonin improved the efficacy of in vitro spermatogenesis[9,10]. Melatonin (5 methoxy-N-acetyltryptamine) was introduced in 1958 in the bovine pineal. It is an important bioactive hormone synthesized by vertebrates' pineal glands[11]. Melatonin plays the main role in controlling biological rhythms and physiological reactions all over human reproductive life. In addition, melatonin and its metabolites are considered as multipurpose antioxidants, and they could effectively up-regulate endogenous antioxidant enzymes and scavenge free radicals[12]. There is growing evidence about melatonin's anti-inflammatory and antioxidant functions in various tissues, such as male reproductive system injuries. A remarkable reduction in melatonin concentration in the testis is accompanied by local inflammatory responses and oxidative stress[13]. The addition of melatonin as a sperm cryoprotectant agent was found to have advantageous effects on sperm mitochondrial activity, in vitro embryo development, plasma membrane, and acrosomal integrity[14]. The presence of melatonin in SSCs cryopreservation medium reduced autophagosome formation and mitochondrial apoptosis, regulated SSCs' antioxidant defence system, and increased viability, proliferation, and differentiation of SSCs post-thawing[15]. Researchers have proved that melatonin could improve reproductive function, fertility rate, in vitro colonization, and proliferation of SSCs [10,16,17].

By virtue of the high efficacy of melatonin in the male reproductive system, in the present study, we investigated the differentiation of cultured human SSCs on 3D decellularized human testis scaffolds supplemented by melatonin (**Figure 1**).

MATERIALS AND METHODS

Preparation of human testes tissue

Human testicular samples were acquired from three brain-dead patients, 22, 25, and 28 years old. The organ Procurement Unit (OPU) of Sina University Hospital (Tehran, Iran) received informed consent from their families to donate the whole testes in this experiment. The present study was authorized by the Tehran University of Medical Sciences Ethics Committee (IR.TUMS.MEDICINE.REC.1400.119). Patients who participated in this study had no history of gonadotoxic radiotherapy or chemotherapy, and normal spermatogenesis was proved by histological evaluation. Samples were transferred to the laboratory using a tissue bag containing phosphate-buffered saline (PBS) and 100 U/mL penicillin-streptomycin (Gibco, Life Technologies, Grand Island, NY, USA). Testicular tissues were used for the decellularization process (ECM extraction) or mechanically cut into small fragments to be used for SSCs isolation.

Isolation and dissociation of human testicular cells from testicular tissues

According to the protocol of Jabari et al., 2023, SSCs were separated by two-stage enzymatic digestion[18]. The first stage of enzymatic digestion was initiated by transferring fragmented tissues to a sterile conical tube containing Dulbecco modified Eagle medium (DMEM; Gibco, Life Technologies, USA) supplemented by penicillin-streptomycin, 1 mg/mL trypsin, 1 mg/mL collagenase type I (Gibco, Life Technologies), 1 mg/mL hyaluronidase, and 0.05 mg/mL DNase (Sigma-Aldrich). The conical tube was incubated in a shaker incubator (150 cycles per minute at 37 ^{0}C) for 40 min, and then cell suspension was centrifuged at 1100 rpm for 4 min. The second stage of the enzymatic digestion protocol was done exactly the same as the first stage. The cell pellet was resuspended and filtered through a 40 µm cell strainer, the number of obtained cells was evaluated using a hemocytometer, and 0.04 % trypan blue (Sigma-Aldrich) was used for the determination of cell viability.

Purification and culture of human testicular cells

According to the protocol of Nikmahzar et al., 2023, Differential plating is a widely accepted method for reducing somatic cells (myoid and Sertoli cells) and purification of SSCs, as Sertoli cells can attack the flasks quicker than SSCs[19]. Therefore, suspension of unattached cells was transferred into plates containing proliferation medium containing DMEM/F-12 supplemented with 20 ng/mL glial-derived nerve growth factor (GDNF; Sigma-Aldrich, USA), 10 ng/mL basic fibroblast growth factor (bFGF; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), and 10 ng/mL leukemia inhibitory factor (LIF; Sigma-Aldrich, USA), 100 IU/mL penicillin-streptomycin, 40 µg/mL gentamycin (Invitrogen, USA), 5% knock-out serum replacement (KSR; Invitrogen, USA), and 5% FBS. Cell plates were incubated in an incubator with 5% CO2 at 35 °C for four weeks, and the medium was replaced every three days. An inverted microscope (Zeiss, Germany) was used for evaluating Sertoli cell proliferation and the formation of SSC colonies.

Characterizations of SSC colonies

1. Immunocytochemistry (ICC)

Cultured human testicular cells were subjected to ICC analysis and fixed with 4% PFA for 30 min at room temperature; 0.5% Triton X-100 was used for the permeabilization of fixed cells. Nonspecific binding sites were blocked with 10% goat serum (Sigma-Aldrich, USA). The cells were incubated with primary antibodies to detect cultured testicular cells: PLZF, GFRA1

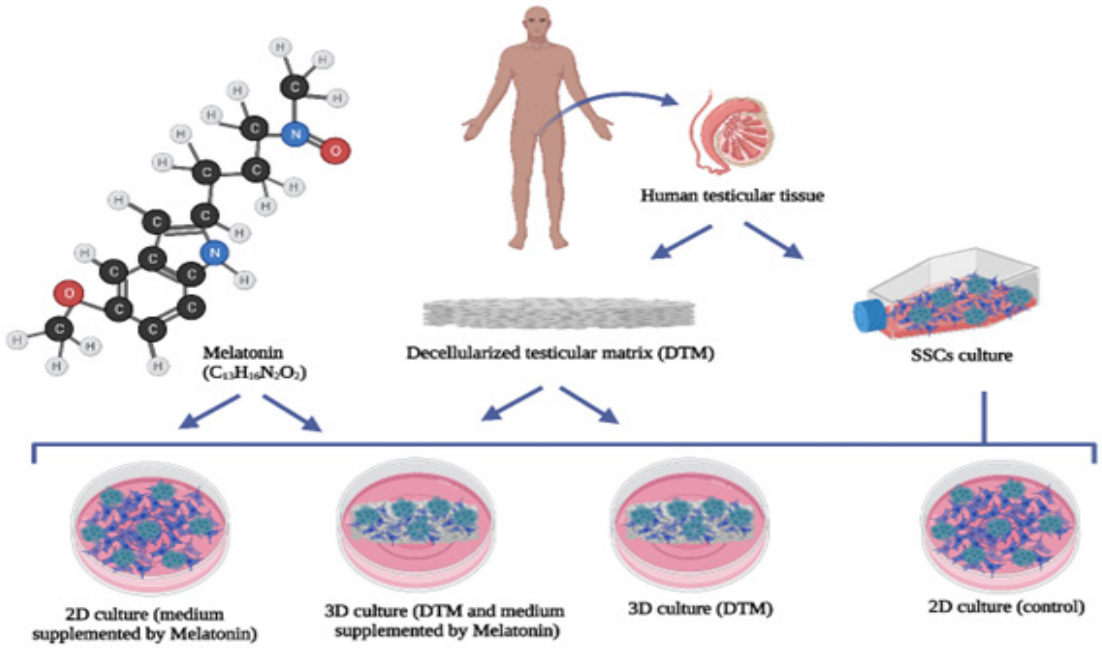

Figure 1. Schematic presentation of experimental procedures.

(undifferentiated SSC markers), SCP3, PRM 2 (meiotic and post-meiotic markers, respectively), and Vimentin (Sertoli cells' marker). Primary antibodies (anti-PLZF antibody, ab104854, 1:100; anti-GFRA1, ab8026 1:100; anti-SCP3 antibody, ab15093, 1:100; anti-PRM2 antibody, ab190791, 1:100; anti-vimentin antibody, ab137321, 1:200) were used for two h at 37°C and followed by secondary antibodies conjugated with FITC or PE (anti-rabbit (FITC), F1262, 1:200, Sigma Aldrich St. Louis, MO, USA, anti-mouse (PE), ab97024, 1:200) for three h at room temperature in the dark. All antibodies were acquired from Abcam (Abcam, Cambridge, MA, USA) unless otherwise specified. The primary antibody was eliminated from control cells, and cell nuclei were detected with 4,6-diamidino-2-phenylindole (DAPI; 1 µg/mL; Sigma-Aldrich St. Louis, MO, USA) staining. Subsequently, the fluorescence images were captured using a fluorescence microscope (Olympus, Tokyo, Japan).

2. Real-time PCR analysis

The relative expression of PLZF, GFRA1, SCP3, and PRM2 markers after enzymatic digestion of testicular tissues and 2D culture of human SSCs for four weeks was evaluated by real-time PCR. RNX-plus reagent (Sinaclon, Iran) was used for total RNA isolation according to the manufacturer's instructions. DNase I treatment (Thermo Scientific, Waltham, MA, USA) was utilized for the removal of genomic DNA. The RNA purity and concentration were calculated using the spectrophotometer (Biochrom). Reverse transcription of total extracted RNA into complementary DNA (cDNA) was carried out with a cDNA synthesis kit (Thermo Scientific, Waltham, MA, USA), and real-time quantitative PCR (RT-QPCR) was performed with specific primers in 35 amplification cycles using SYBR Green I on a Rotor-gene Q device (Qiagen, Germany). Melting curve analysis was used to detect nonspecific PCR products and primer dimers. At least three replicates for each experimental group were considered. Quantitative gene expression data were normalized to the level of glyceraldehyde 3-phosphate dehydrogenase GAPDH mRNA as a housekeeping gene. The relative gene expression was calculated using the $2^{-\Delta CT}$ method. Relative expression levels of apoptotic BAX and BCL2 genes and spermatogenesis-related genes (PLZF and GFRA1, SCP3, and PRM2) were also investigated by real-time PCR after four weeks of differentiation induction, according to the above-mentioned procedure. Sequences of specific primers are shown in **Table 1**.

3. Xenotransplantation of human SSCs in recipient azoospermia mice model

According to the protocol of Bashghareh et al., 2023[20], the functionality of isolated human SSCs was assessed by transplantation into the seminiferous tubules of recipient immunodeficient male NMRI mice (7 weeks old, weighing 27–30 g). Mature mice were obtained from the School of Pharmacology, Tehran University of Medical Sciences, Tehran, Iran. Animals were housed in the animal lab under ad libitum conditions, light-dark cycles of 12:12 h, and standard temperature and humidity. The Ethics Committees of Tehran University of Medical Science approved experimental designs and animal care (IR.TUMS.VCR. REC.1397.1125). A single dose intraperitoneal administration of 40 mg/kg busulfan (Sigma-Aldrich, USA) was carried out for the elimination of mice endogenous spermatogenesis. Hematoxylin and eosin (H&E) staining was performed for confirmation of the azoospermia model. Human SSCs were labelled with fluorescent dye Dil (Molecular Probes™, USA) according to the manufacturer's protocol. The recipient male mice were anesthetized by intraperitoneal injection of ketamine and xylazine (100

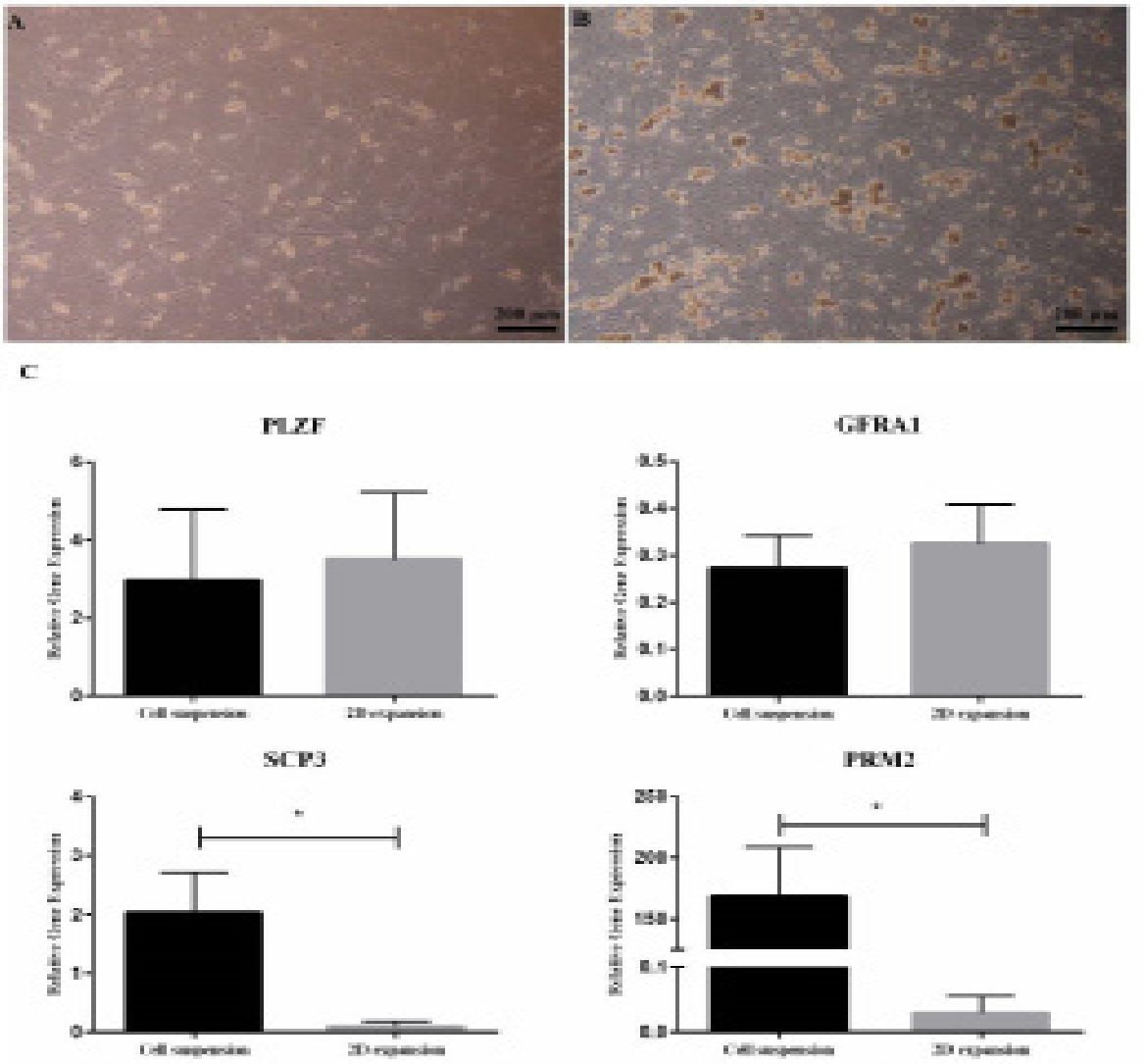

Figure 2. Light microscopic images of human testicular cells (SSCs clusters and Sertoli cells) in 2D culture system in the first week **(A)** and the fourth week **(B)**, Scale bar = 200 µ. Expression of PLZF, GFRA1, SCP3, and PRM2 genes after two-stage enzymatic digestion and 2D culture of SSCs for four weeks **(C)**, Data are shown as mean ± SEM * $P < 0.05$.

mg/ kg and 10 mg/kg, respectively). 105 labeled SSCs in 10 µl DMEM were injected into the left ductus efferents using a microinjection needle under the stereo microscope. The presence of trypan blue dye in the SSCs injection media permits tracking of the seminiferous tubules after injection. The mice were sacrificed by cervical dislocation two months after transplantation, and testicular samples were removed and fixed in 10% neutral buffered formalin solution. Tissue preparation was performed, and serial sections from the specimens were prepared. Obtained sections were stained with DAPI. A fluorescent microscope evaluated the homing of injected SSCs (Olympus BX51TRF, Tokyo, Japan).

Determining the optimal dosage of melatonin

SSCs were cultured in DMEM/F12 medium supplemented with 5% FBS, 5% KSR, 100 IU/mL penicillin, and 100 µg/mL streptomycin containing different concentrations of melatonin (0, 10-6, 10-7, 10-8 and 10-9 µM) and Methyl thiazol tetrazolium (MTT) assay was carried out to obtain the optimum dose of melatonin at days 10 and 15. Cells were incubated in 96-well culture plates in DMEM medium containing 5 mg/mL MTT for four h at 37 ^{0}C in a humidified 5% CO2 atmosphere. The supernatant was discarded, and 100 µl dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added per well for 30 min to solubilize the purple formazan crystals. The optical densities (OD) were measured at 570 nm wavelength using a microplate reader (HTX, BioTek, USA).

Decellularization of human testicular tissues

Human testes were transferred to the laboratory in PBS solution containing antibiotics at 4°C, and the tunica albuginea was removed. According to our previous study [21], the whole testes were cut by four longitudinal sections and then frozen at -80 °C for 48h. Then, sections with 100 µm thickness and about 1×1 cm^2 size were prepared from the testes' longitudinal sections. Testicular sections were treated with 0.3% sodium dodecyl sulfate (SDS; Sigma-Aldrich, USA) in distilled water for 24 hours on an orbital shaker (70 RPM). SDS was re-

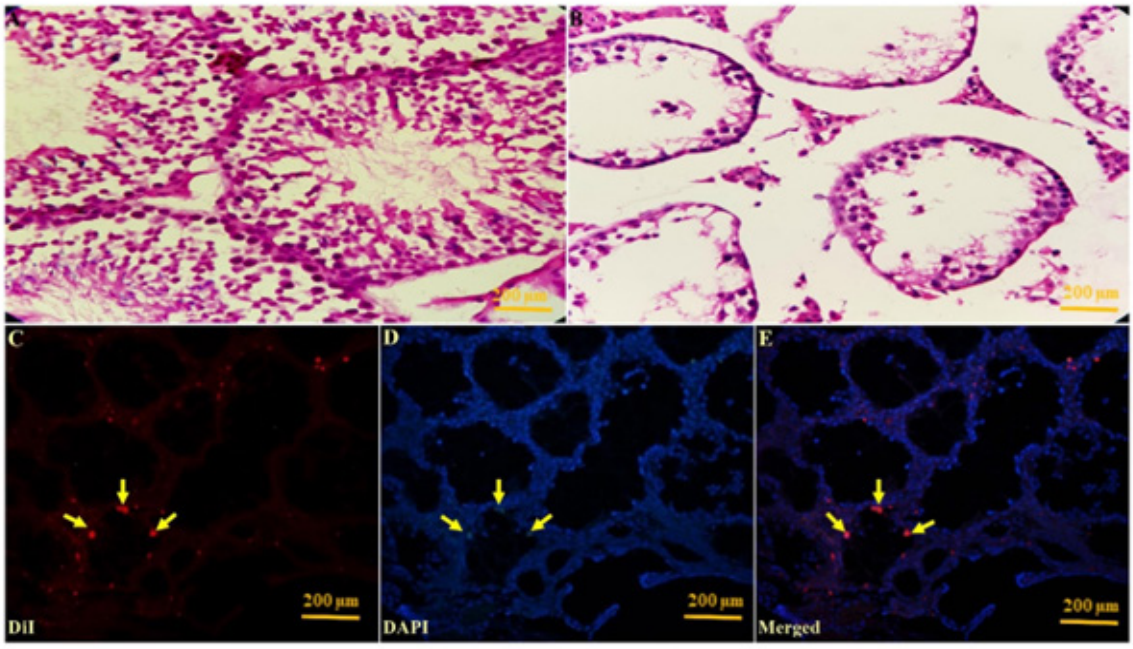

Figure 3. The homing of human SSCs after busulfan treatment. The normal structure of germ cells and the regular construction of the seminiferous tubules can be identified in untreated mice **(A)**. H&E staining of seminiferous tubules in busulfan-treated mice showed a low number of spermatogenic cells in seminiferous tubules and an increased thickness of the germinal epithelium **(B).** Fluorescent images of seminiferous tubules in recipient infertile mice, eight weeks after xenotransplantation. Human SSCs were labeled with DiI and transplanted **(C, D, E)**. Scale bars = 200 µm.

newed every 3 hours with new detergent. After that, tissues were incubated in 1% Triton X-100 solution (Sigma-Aldrich, USA) for 24 hours on an orbital shaker (70 RPM), and Triton X-100 was refreshed every 3 hours. The sections were washed with PBS for 24 h following the decellularization, and PBS was renewed ten times to eliminate the remaining detergents and cellular debris.

Characterization of the decellularized testicular matrix

1. Histological characteristics of human DTM

Hematoxylin-eosin (H&E), Masson's trichrome, Alcian blue, and Orcein staining was performed to confirm the efficacy of the decellularization process and investigate the quality of DTM. The decellularized samples were fixed in Bouin's solution for 24 hours at room temperature. After tissue processing, the samples were molded in paraffin, and sections with five µm thickness were prepared and hydrated. H&E staining was performed for histological evaluation and investigation of remaining cellular structures in the tubular and interstitial parts of the specimens. Alcian blue, Orcein, and Masson trichrome staining were carried out in order to verify the glycosaminoglycan (GAGs), elastin, and collagen content after successful decellularization, respectively. Finally, sections were microscopically evaluated (Olympus BX51, equipped with a DP72 digital camera, Japan).

2. Quantification of DNA content and DAPI staining

DAPI(4′,6-diamidino-2-phenylindole) (Sigma-Aldrich, USA) fluorescent staining was used to ensure the efficiency of nucleus removal after the decellularization procedure. In this regard, DNA remnants in DTM were measured to monitor decellularization efficiency. DNA was extracted using the HiPure Tissue DNA Mini Kit (Magen, Shanghai, China) according to the manufacturer's instructions for quantitative analysis of the DNA content from the native and decellularized tissues. The total DNA concentration was determined using a NanoDrop spectrophotometer (WPA, Biochrom) at 260 nm and 260/280 ratio was used to evaluate the purity of isolated DNA. DNA content was measured and normalized to the weight of freeze-dried samples.

3. Scanning electron microscopy (SEM)

Native and DTM samples were fixed by 2.5% glutaraldehyde (Sigma-Aldrich St. Louis, MO, USA) for two h. Specimens were dehydrated in ascending grades of ethanol (70-100%) for 15 min in each grade. Next, specimens were dried in the air and covered with gold-palladium coating. The specimens were observed under an Ultra 55 field emission scanning electron microscope (VEGA\TESCAN, Czech Republic).

Design of experiments for the culture and treatment of SSCs

The effects of melatonin and DTM on the viability and differentiation of SSCs were assessed for four weeks. First, disinfection of the DTMs was performed in 70% ethanol for one h, then washed with sterile PBS thrice and placed in sterile DMEM for five h. In order to evaluate the viability and differentiation of cells, SSCs colonies were divided into the following groups for further four weeks. Group 1: 2D culture of SSCs in differentiation medium (control group). Group 2: 2D culture of SSCs in differentiation medium supplemented with 10-8 µM of melatonin (melatonin group). Group 3: 3D culture of SSCs on DTM in differentiation medium (ECM group). Group 4: 3D culture of SSCs on DTM in differentiation medium supplemented with 10-8 µM of melatonin (ECM-melatonin group). Cell plates were incubated in an incubator with 5% CO2 at 35 °C for four weeks, and the medium was replaced every three days.

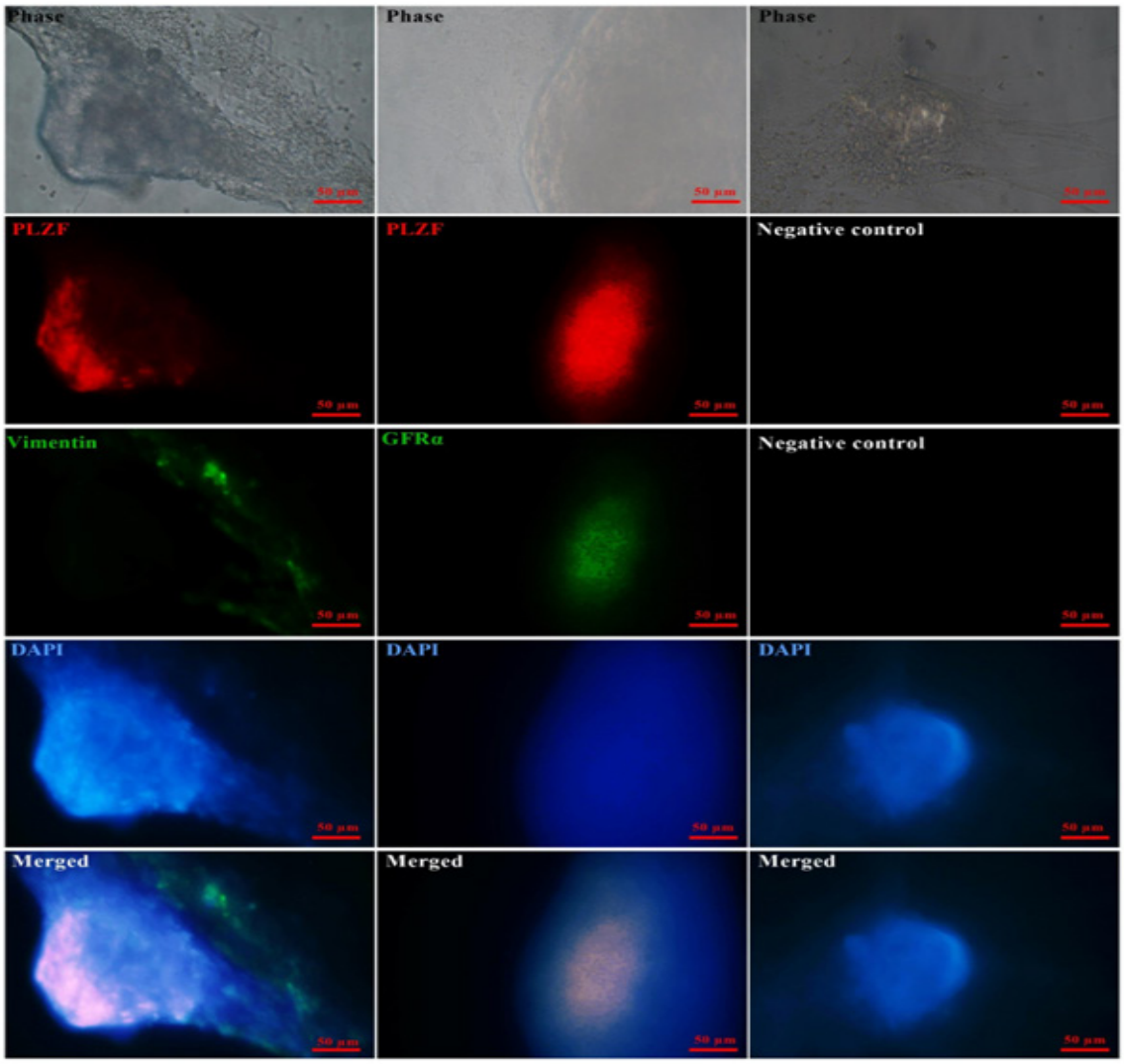

Figure 4. The expression of PLZF, GFRA1, and Vimentin proteins in the undifferentiated culture of SSCs after four weeks of proliferation. In the top row, SSCs colonies were shown using an inverted microscope, the second and third rows were stained with depicted antibodies, the fourth row showed DAPI staining, and the lowest row showed merged images. Scale bars = 50 µm.

Evaluation of human SSCs viability in experimental groups

The viability of human SSCs was determined using MTT assay in different groups after 10, 20, and 30 days. According to experimental groups, human SSCs were cultured in 96-well culture plates at a density of 2×104 cells per well. DMEM/F12 medium supplemented with 5% FBS, 5% KSR, 100 IU/mL penicillin, and 100 µg/mL streptomycin was used for 10, 20, and 30 days of culture. MTT procedure was carried out as mentioned for melatonin optimal dose determination.

Differentiation of SSCs on the DTM

Differentiation medium used for in vitro spermatogenesis included: DMEM/F12 medium supplemented with 5 % FBS, 5 % KSR, 10-7 M testosterone (Sigma-Aldrich, USA), 10-6 M retinoic acid (R2625, Sigma-Aldrich, USA), 2.5 × 10-5 U follicle-stimulating hormone (FSH, Gonal-F, Merck), 100 IU/mL penicillin and 100 µg/mL streptomycin. Each layer of sterile DTM was placed in one well of a 24-well plate, and 50 µL of cell suspension, containing 1.5 × 10^5 cells, was added to evaluate SSCs differentiation. After two h, the differentiation medium was slowly added to the cells on DTM.

Statistical Analysis

Data were expressed as mean ± standard error of the mean. Statistical analyses were done by Prism software (GraphPad Software, San Diego, CA, United States). In order to determine the normal distribution of data, the Kolmogorov-Smirnov test was used. One-way ANOVA followed by Tukey's test was used for multiple comparisons of data. $P \leq 0.05$ was considered statistically significant.

RESULTS

Identification and morphological evaluation of isolated SSCs

Morphological evaluation of isolated human testicular cells was performed using an inverted microscope after 2 and 4 weeks of 2D culture (**Figure 2**). Sertoli support-

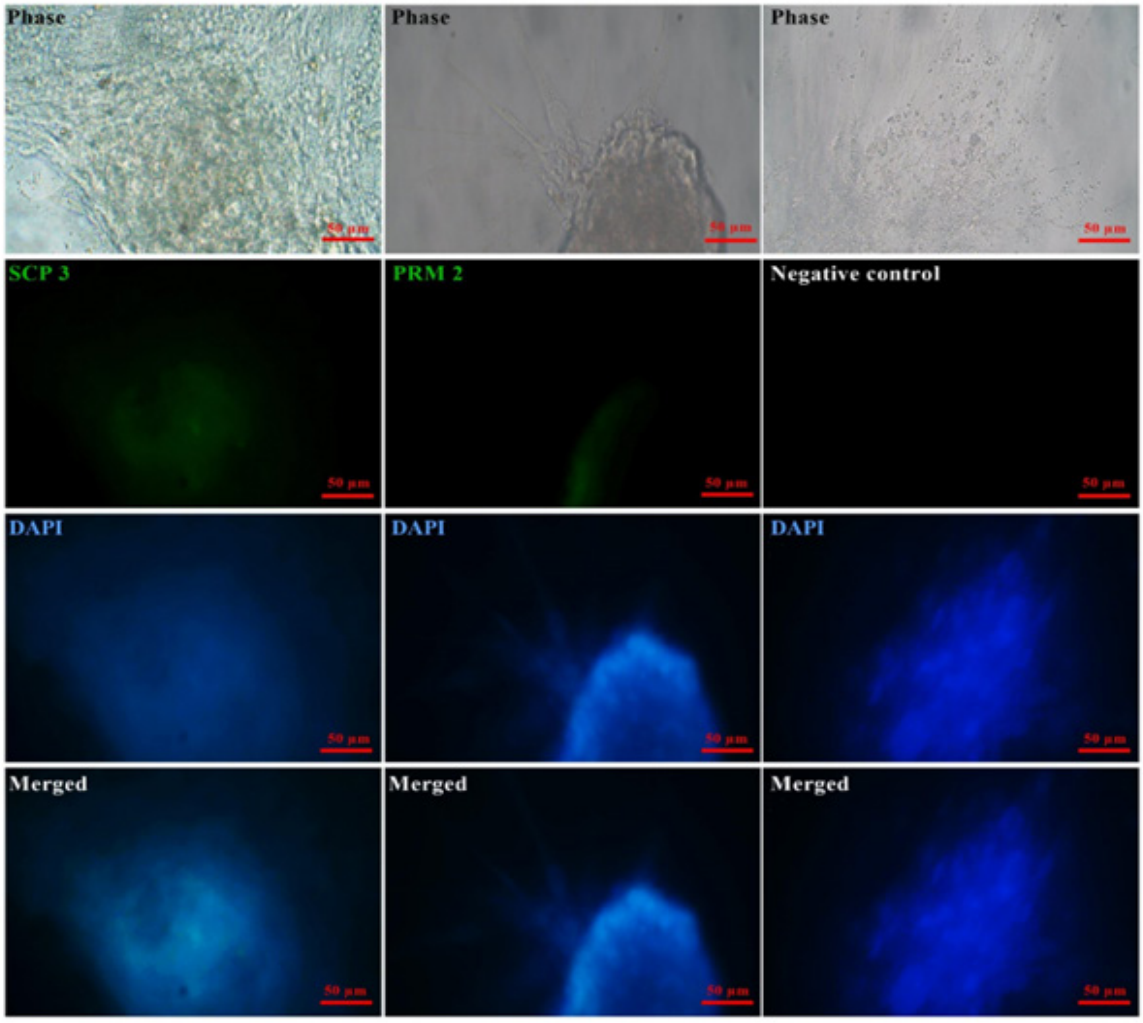

Figure 5. The expression of SCP3 and PRM2 proteins in the undifferentiated culture of SSCs after four weeks of proliferation. In the first row, SSCs colonies were demonstrated using an inverted microscope, the second row was stained with depicted antibodies, the third row showed DAPI staining, and the fourth row illustrated merged images of the second and third row images. Scale bars =50 µm.

ing cells propagated and created a monolayer of cells as a feeder layer. Human SSCs proliferated and formed colonies on the Sertoli feeder surface, which was observed after two weeks (**Figure 2A**). The number and diameter of human SSCs colonies were increased following 2D culture of SSCs in the proliferation medium for four weeks (**Figure 2B**).

Expression of pre-meiotic, meiotic, and post-meiotic genes after enzymatic digestion and 2D culture of testicular cells

The relative expression of GFRA1, PLZF, SCP3, and PRM2 was investigated immediately after two-stage enzymatic digestion and 2D culture of SSCs for four weeks. The relative expression of PLZF and GFRA1 showed no significant difference after four weeks of culture. While the expression of SCP3 and PRM2 significantly decreased after four weeks of 2D culture (p = 0.0469 and p = 0.0243, respectively) (**Figure 2C**).

Evaluation of recipient mice testicular tissue after human SSCs xenotransplantation

Regular organization of germinal epithelium and normal spermatogenesis with the intact location of germ cells was observed in seminiferous tubules of the control testis (**Figure 3A**). In busulfan-induced azoospermia samples, atrophy, vacuolization, and disorganization of seminiferous tubules, reduced number of spermatogenic cells, disintegrity of the tubular basement membrane, and impaired spermatogenesis were observed (Figure 3B). Eight weeks after xenotransplantation, fluorescent microscopic evaluations revealed that DiI-labeled SSCs migrated to the basement membrane of seminiferous tubules in the recipient testes (**Figure 3C, D, E**). Homing of transplanted human SSCs on the basement membrane of seminiferous tubules in azoospermia mice confirmed the functionality of undifferentiated human SSCs after 2D culture.

Immunofluorescent findings for the expression of markers of Sertoli cells, differentiated and undifferentiated SSCs

The expression of PLZF, GFRA-1, SCP3, PRM2, and Vimentin markers was evaluated using immunofluorescence analysis four weeks after the 2D culture of hu-

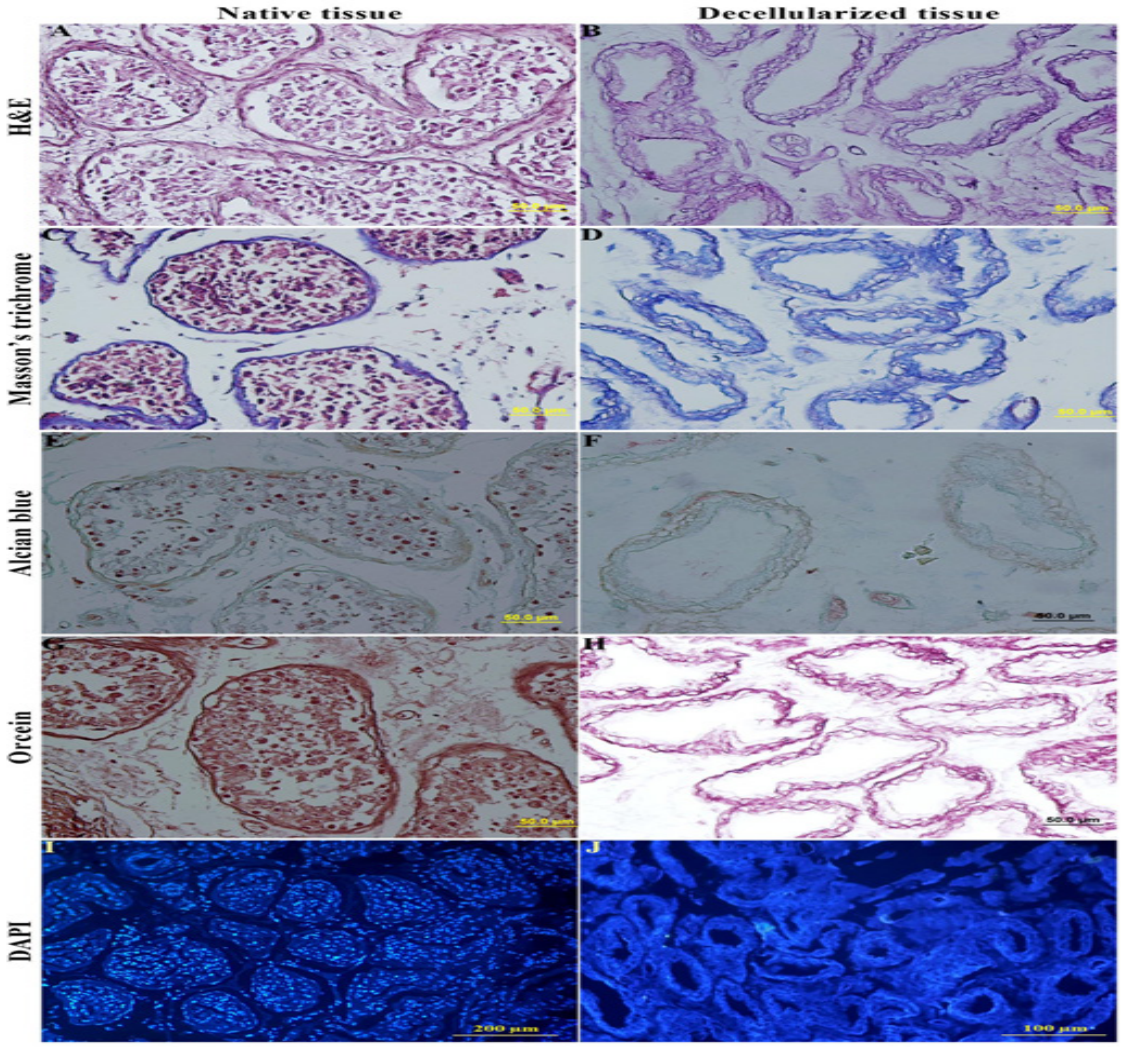

Figure 6. Investigation of the decellularization process and characteristics of ECM components by H & E **(A, B)**, Masson's trichrome **(C, D)**, Alcian blue **(E, F)**, Orcein **(G, H)** and DAPI **(I, J)** stainings in the native and decellularized testis tissue for detection of collagen **(C, D)**, GAGs **(E, F)** and elastin **(G, H).**

man testicular cells. As seen in **Figure 4**, human SSCs colonies were positive for PLZF and GFRA1 proteins. Expression of Vimentin as the specific marker of Sertoli cells was observed in feeder somatic cells (**Figure 4**). Also, immunofluorescent staining results revealed no clear expression of SCP3 and PRM2 in isolated human SSC colonies (**Figure 5**). Furthermore, the expression of proteins of Sertoli cells, differentiated and undifferentiated SSCs was not observed in the negative control group.

Confirmation of the decellularization process and identification of ECM components by histological evaluation

Elimination of cells and cellular constituents was confirmed by DAPI and H&E staining (**Figure 6**). Decellularization process efficiently eliminated nuclear debris and cellular material from testis tissue fragments. H&E and DAPI staining revealed that cell nuclei were absent in DTM, and ECM ultrastructure remained unchanged in decellularized tissue.

Alcian blue, Orcein and Masson's trichrome staining were utilized to evaluate GAGs, elastic, and collagen fibers of testicular ECM, respectively. According to Figure 6, testicular ECM components such as collagen, elastin, and GAGs were visualized after specific staining, and the constituents of testis ECM were properly conserved after the decellularization process. Furthermore, the results of DNA quantification analysis suggested that the decellularization process significantly removed the nuclei and reduced the DNA content of DTM compared to the native testicular tissues ($P < 0.0001$) (**Figure 7A**). Finally, the preservation of the ECM fibers network and elimination of cellular material after the decellularization process (**Figure 7B and C**) and attachment of testicular cells to the ECM after the recellularization were detected using an electron microscope (**Figure 7D and E**).

Dosimetry of melatonin

SSCs were cultured in DMEM/F12 medium supplemented with various doses of melatonin (0, 10-6, 10-7, 10-8, and 10-9 µM), and MTT test was carried out at 10 and 15 days of culture to determine the proper dose

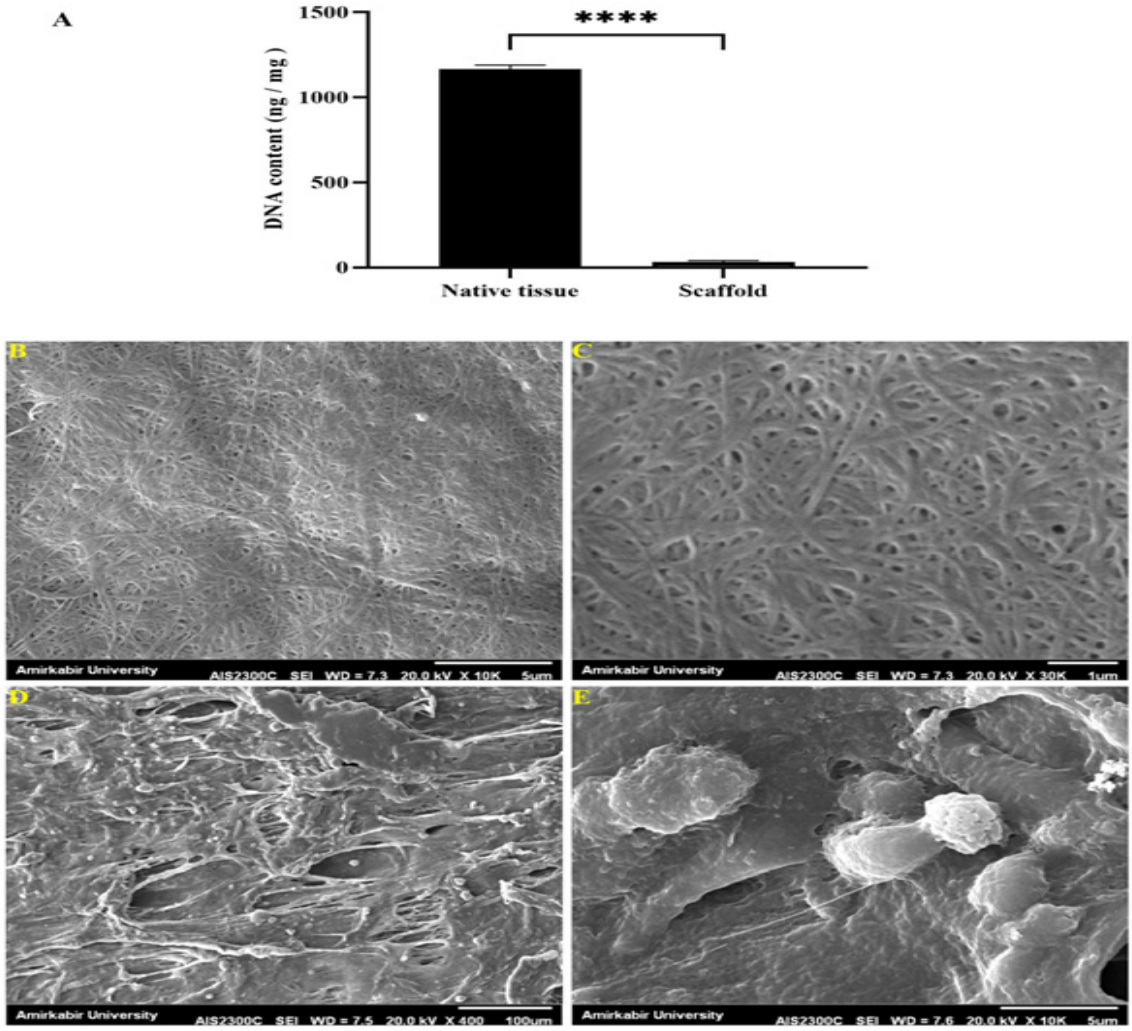

Figure 7. The comparison of DNA content in the native tissue and DTM showed significantly decreased DNA content in DTM. **** *P* <0.0001 **(A)**. SEM analysis for evaluation of the 3D structure of the decellularized testis **(B, C)** and SSC colonies attached to DTM **(D, E).**

of melatonin. The mean absorbance in various doses of melatonin were 0.35 ± 0.052, 0.34 ± 0.068, 0.36 ± 0.089, 0.57 ± 0.12, and 0.41 ± 0.085 (after 10 days from culture) and 0.4 ± 0.1, 0.39 ± 0.097, 0.44 ± 0.14, 0.56 ± 0.17, and 0.43 ± 0.12 (after 15 days from culture) (0, 10-6, 10-7, 10-8, and 10-9 µM, respectively) . Higher viability of SSCs was observed with 10-8 µM of melatonin (Figure 8A). Meanwhile, there was no statistically significant difference in the viability of SSCs cultured in various melatonin concentrations (**Figure 8A**).

Viability of cultured human SSCs in experimental groups

The viability of human SSCs was investigated in different experimental groups following 10, 20, and 30 days of culture using MTT assay. The mean absorbance were 0.33 ± 0.003, 0.48 ± 0.042, 0.51 ± 0.023, and 0.67 ± 0.051 (after 10 days from culture), 0.21 ± 0.031, 0.39 ± 0.039, 0.27 ± 0.016, and 0.38 ± 0.012 (after 20 days from culture), 0.22 ± 0.026, 0.36 ± 0.012, 0.36 ± 0.011, and 0.43 ± 0.026 (after 30 days from culture) (in control, melatonin, ECM, and ECM-melatonin groups, respectively). As seen in Figure 8B, the viability of SSCs in the ECM-melatonin group significantly increased compared to the control group after 10 and 30 days of culture (p = 0.0067 and p = 0.0064, respectively). Furthermore, the higher viability of SSCs was observed in the melatonin group compared with the control group after 20 days of culture (p = 0.0382). Generally, the highest and lowest number of colonies were observed in ECM-melatonin and control groups, respectively (**Figure 8B**).

Real-time PCR results for expression of different genes in experimental groups

The relative expression of BAX and BCL2 as specific apoptotic genes was assessed by real-time PCR after four weeks of differentiation. A general downregulation trend of BAX was noticed in experimental groups compared to the control group. Furthermore, results revealed that the relative expression of BAX was significantly decreased in the ECM group compared to the control group (p = 0.0435). Although the relative expression of BCL2 was higher in the melatonin group, the relative expression of BCL2 showed significantly increased levels in melatonin group compared to the

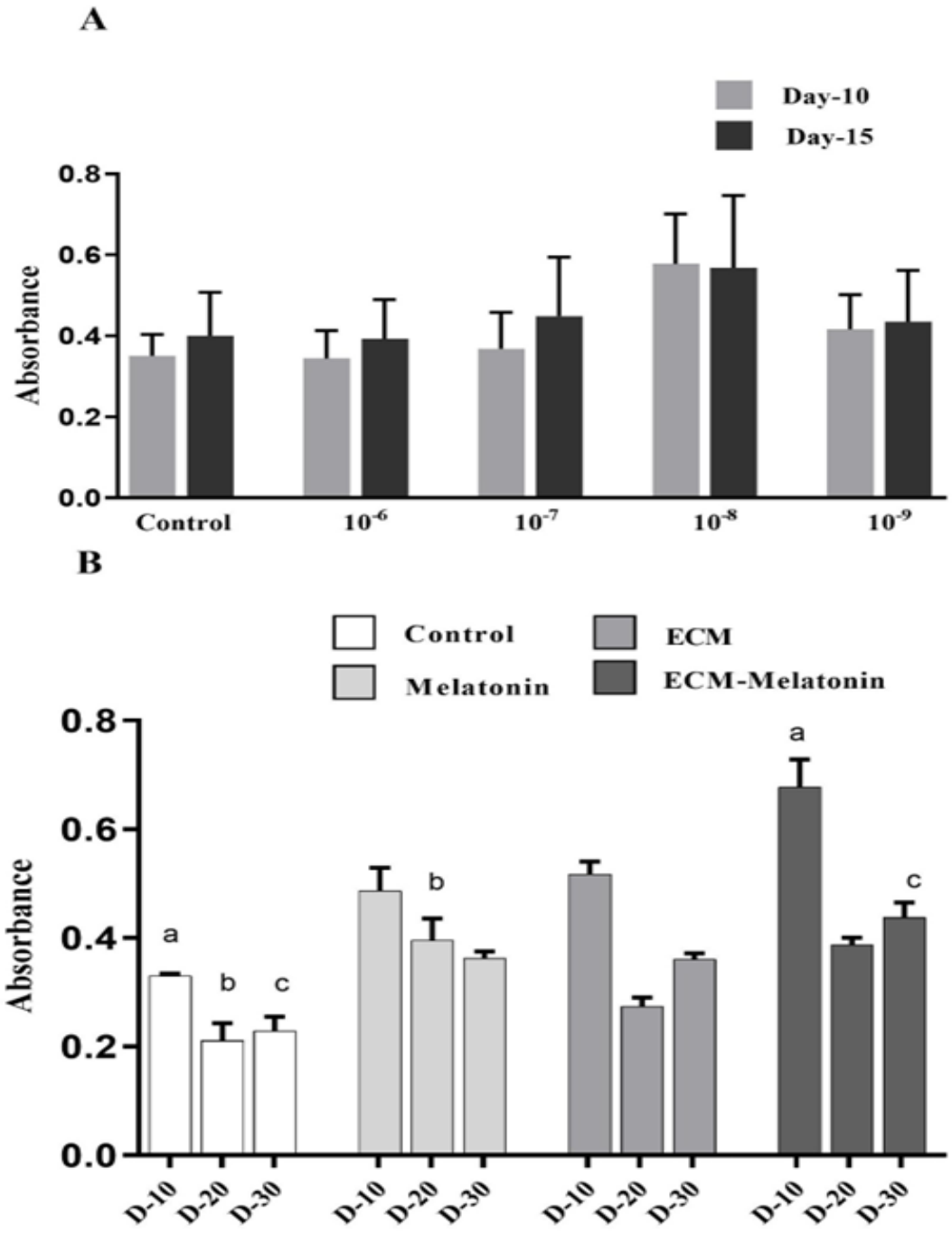

Figure 8. The effects of various doses of melatonin (0, 10-6, 10-7, 10-8, and 10-9 µM) on the viability of SSCs following 10 and 15 days of culture **(A).** The evaluation of SSCs viability after 10, 20, and 30 days of culture in the experimental groups. The highest cell viability was observed in the ECM-melatonin group after 10 days of culture **(B)**. Columns with the same letters are significantly different * P < 0.05.

control group (p = 0.279). The relative expression of PLZF gene showed no significant difference between experimental groups; however, a higher expression level of PLZF was observed in the ECM group. The expression of GFRA1 was enhanced in the control and melatonin groups; however, the relative expression of GFRA1 was only significantly increased in the control and melatonin groups compared to the ECM group (p = 0.0358, and p = 0.0225, respectively). Our findings revealed that there was no statistically significant difference in the expression of SCP3 gene in different groups, although the highest expression of SCP3 was observed in melatonin and ECM-melatonin groups. Increased expression of PRM2 in ECM and ECM-melatonin groups was observed; however, a significant increase was noticed in ECM-melatonin group in comparison to melatonin group (p = 0.0275) (**Figure 9**).

DISCUSSION

Spermatogenesis is a complicated process of self-renewal and differentiation. Self-renewal capacity preserves the stability of the germ cell pool, while differentiation gives rise to the formation of haploid mature spermatozoa. The balance between the propagation and differentiation of SSCs in adult testes plays an important role in male fertility[22]. SSCs are the main off-targets of chemotherapy and radiotherapy used for cancer treatment[23]. The gonadotoxic potential of these approaches may deplete the differentiating spermatogonia pool. With regard to scarce populations of SSCs and their fundamental role in spermatogenesis, any alterations in their genome disturbed SSCs' self-renewal and differentiation ability, which resulted in defective spermatogenesis and male infertility[24,25]. Cryopreservation, in vitro culture, proliferation, and transplantation of SSCs have been widely used for the long-term preservation of SSCs and fertility restoration[26]. SSCs grow in a compact 3D multicellular structure within the testis, which permits close cell-to-ECM and cell-to-cell interactions. These communications are requisite for proper spermatogenesis[27]. Many experiments have recently utilized different scaffold-based procedures to assist such communications and establish this 3D culture condition[28-34]. Decellularized ECM-based scaffolds with high similarity to the native niche of SSCs are appreciable microenvironments in tissue engineering, and they were found to be more efficient approaches for in vitro spermatogenesis[35,36].

In the present study, we evaluated in vitro differentiation using 3D culture of SSCs on DTM supplemented with the proper melatonin concentration. Human DTM is predominately composed of ECM proteins such as collagen, GAGs, laminin, and fibronectin, as shown in our results. Components of ECM are essential for the restructuring events of spermatogenesis in the seminiferous tubules[37]. Human DTM structurally and biochemically imitates the environment of native ECM. In addition to its structural activity, the transportation of bioactive molecules is another principal function of human testicular ECM. These features are responsible for the viability and differentiation of germ cells by communication between the various cellular compartments[38]. Similarly, our results showed that the relative expression of apoptosis-related genes, meiotic and post-meiotic markers was changed after 3D culture of SSCs on DTM. The present experiment has described a simple way to create a cytocompatible human testicular acellular matrix. SDS and Triton X-100 have been used to decellularize human testicular tissues.

According to previous reports, SDS and Triton X-100 are suitable detergents for efficient decellularization of the human[39], pig[40], sheep[41], and mouse testis[42]. Destructive effects of SDS on ECM and its dose-dependent cytotoxicity were reported in previous studies [43,44]. Therefore, optimal concentration and complete removal of SDS after decellularization of tissue are very important. Triton X-100 is widely utilized for the decellularization of various tissues, protein extraction, and as a lysis buffer in DNA extraction. Triton X-100 is safer and has high cellular biocompatibility compared with SDS. This nonionic detergent is often applied after incubation with SDS for improvement of the decellularization process, SDS removal, and washing of the decellularized tissues[45,46]. The study of Baert et al. 2015 was one of the pioneering works in the characterization of the human testis scaffold[39]. In agreement with their study, other reports have described that SDS detergent has more effective decellularization outcomes than Triton X-100[47-49]. Conservation and organization of ECM components after decellularization process are principal parameters for normal cell physiology and functionality after recellularization. In support of previous observations[39,40,50,51], our histological evaluations displayed well-preserved structural integrity of the testis and elimination of nuclear debris and cellular material following acellularization. Similarly, mice testicular fragments were decellularized using 1% SDS for 18 h. The relative expression of meiotic and post- meiotic markers of mice SSCs cultured on DTM significantly increased in agreement with our findings[52]. It could be concluded that the culture condition on DTM scaffold is able to make a suitable microenvironment for SSC proliferation and differentiation.

The relative expression of early germ cell markers (PLZF and GFRA1) and meiotic (SCP3) and post-meiotic (PRM2) markers was evaluated following four weeks of culture in our experiment. We observed that the relative expression of PLZF remained unchanged after the culture period in the ECM-melatonin group, similar to the previous findings in the 3D culture of SSCs[50,53]. In a study by Navid et al., the effect of melatonin was evaluated on the colonization of mouse SSCs on a soft agar culture system (SACS) using a basic culture medium for four weeks. They reported that the expression level of PLZF significantly increased in SACS supplemented by melatonin compared to a control group, in disagreement with the results of the present study[54]. This difference could be attributed to the presence of testosterone, retinoic acid, and FSH in our culture media, which stimulated SSCs differentiation. In our study, the highest expression of GFRA1 as a marker of undifferentiated spermatogonia was detected in control and melatonin groups (2D culture system), and the lowest expression of GFRA1 was observed in the ECM and ECM-melatonin groups (3D culture system). Our results demonstrated the progress of human SSCs differentiation cultured on DTM supplemented with proper melatonin concentration. The expression level of PRM2 remarkably increased in ECM-melatonin group. Protamine 1 and protamine 2 (PRM1, PRM2) are under translational regulation in round and elongated spermatids[55]. Their expression is required for normal spermatogenesis and fertility since protamines adjust sperm DNA condensation during the final steps of spermatid differentiation[56]. Thus, the 2D culture system increased the expression of the undifferentiation gene (GFRA1), and the 3D culture system enhanced the expression of the differentiation gene (PRM2). Also, the melatonin induced a significant increase in the expression of PRM2 in the 3D culture system (ECM-melatonin group). SCP3 is an element of the synaptonemal complex; it is considered a meiosis-specific gene and necessary for the synapsis of homologous chromosomes. Interestingly, the expression level of SCP3 showed no significant difference in different experimental groups conforming with previous investigations[42,57]. These findings could be attributed to the prior results; they have displayed a possible bottleneck in transmission from mid to late pachytene cells[50,53,57]. On the contrary, 3D culture of mice testicular cells on collagen gel matrix in the study of Khajavi et al. illustrated that the expression of SCP3

has been increased following coculture with testicular somatic cells[58]. Also, the expression level of SCP3 increased after the culture of human testicular cells on SACS for four weeks[59]. Therefore, the signaling pathway involved in the expression of SCP3 gene needs to be evaluated in future studies. Generally, our results showed that the effect of melatonin on the expression of BCL-2, GFRA1, and PRM2 genes in 2D culture system (melatonin group) and 3D (ECM-melatonin group) is different. Melatonin in 2D culture system increased the expression of anti-apoptotic genes, while in 3D culture system, it increased the expression of differentiation genes. In support of this finding, many studies have demonstrated the differential effects of growth factors (LIF, GDNF, FGF, Retinoic acid, testosterone, and BMP4, …) on the expression of apoptotic, proliferation, and differentiation genes in 2D and 3D culture systems[60].

Generation of reactive oxygen species (ROS) and high mitochondrial oxygen consumption were detected during repeated cell divisions and the proliferation process of spermatogenesis[61]. Production of ROS is associated with oxidative damage, cell injury, and reduction in SSCs' functionality[62]. As a result, it seems necessary to develop an optimal culture condition to alleviate these injuries. Therefore, the present study evaluated the efficiency of a 3D culture system containing melatonin as a factor with diverse supportive roles for male fertility[63-66]. Melatonin has stimulated cellular defense and scavenged free radicals by increasing the production of the main antioxidant enzymes[67]. So it could regulate cell responses to apoptotic signals. In agreement with our results, the addition of melatonin protected mouse SSCs against apoptosis induced by chromium, and the protein levels of BAX and BCL2 were changed after melatonin administration[68]. They concluded that melatonin could maintain male fertility by inhibiting germ cell apoptosis and improving the histological structure of seminiferous tubules[68]. Another research showed that melatonin could improve colonization of neonate mouse SSCs after two weeks of culture in a basic proliferation medium. The mean number and diameter of SSC colonies and expression of undifferentiated spermatogonia genes were significantly increased after melatonin administration[17]. Melatonin improved goat SSCs propagation by increasing GDNF production in supporting Sertoli cells. Phosphorylation of the AKT and ERK pathways was activated by attachment of GDNF to GFRA1-RET, which resulted in SSCs proliferation and self-renewal[69]. Regulation of apoptosis plays a key role in cellular physiological metabolism. Our findings revealed that the expression of BAX as a pro-apoptotic gene was decreased while the expression of BCL-2 as an anti-apoptotic gene was increased in the melatonin group. Consistent with the previous reports, it was shown that melatonin is involved in cell apoptosis by regulating anti-apoptotic and pro-apoptotic markers. In addition, higher viability was seen in ECM-melatonin group after 30 days of culture. It could be concluded that the application of human DTM supplemented by melatonin provided a suitable culture condition for in vitro proliferation and differentiation of human SSCs. Furthermore, the addition of melatonin to the freezing medium of neonatal mice diminished the production of intracellular ROS and apoptosis in the frozen-thawed SSCs. The expression of pre-meiotic, meiotic, and post-meiotic proteins was remarkably increased after transplantation of frozen-thawed SSCs to azoospermia mice model induced by busulfan[70].

CONCLUSIONS

We briefly disclosed in the present study that decellularized ECM-based scaffolds could successfully improve spermatogenesis by providing a suitable 3D microenvironment similar to the intact testis. The presence of intact ECM components, and cell-to-cell and cell-to-ECM interactions are the main parameters in the efficacy of ECM-based scaffolds for the proliferation and differentiation of human SSCs. Also, the addition of an appropriate concentration of melatonin to ECM-based scaffolds could remarkably protect human SSCs by inhibiting intracellular ROS and decreasing germ cell apoptosis. The application of human ECM-based scaffolds supplemented with effective antioxidants could provide a foundation for the clinical management of male infertility in prepubertal cancer survivors.

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