Effect of NMDA Receptor Agonist and Antagonist on Spermatogonia Stem Cells Proliferation in 2- and 3- Dimensional Culture Systems

Amir Hessam Eskafi Noghani
University of Tabriz
Reza Asadpour (rasadpour4@gmail.com)
University of Tabriz https://orcid.org/0000-0003-4717-9952
Adel Saberivand
University of Tabriz
Zohreh Mazaheri
Institute of Basic Medical Sciences
Gholamreza Hamidian
University of Tabriz

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Abstract

The main purpose of this study was to investigate the effect of D-serine (DS) and Dizocilpine (MK-801or Mk) on the proliferation of SSCs in two-dimensional (2D) and three-dimensional (3D) culture systems. The SSCs of male NMRI mice were isolated by enzymatic digestion and cultured for two weeks. Then, the identity of SSCs was confirmed by anti-*Plzf* and anti-*GFR-α1* antibodies *via* immunocytochemistry (ICC). The proliferation capacity of SSCs was evaluated by their culture on a layer of the decellularized testicular matrix (DTM) prepared from mouse testis, as well as two-dimensional (2D) with different mediums. After two weeks of the initiation of proliferation culture on 3D and 2D medium, the pre-meiotic at the mRNA and protein levels were evaluated *via* qRT-PCR and flow cytometry methods, respectively.

The results showed that the proliferation rate of SSCs in three-dimensional culture with 50 mM glutamic acid and 20 mM D-serine was significantly different from other groups after 14 days treatment. mRNA expression levels of *Plzf* in 3D-cultures supplemented by 20 mM D-serine and 50 mM glutamic acid were considerably higher than the 3D control group ($p<0.001$). The flow cytometry analysis revealed that the amount of *Plzf* in the 2D-culture groups of SSCs with 20mM MK-801 was considerably lower compared to the 2D-culture control group ($p<0.001$). This study indicated that decellularized testicular matrix supplemented with D-serine and glutamic acid could be considered a promising vehicle to support cells and provide an appropriate niche for the proliferation of SSCs.

Introduction

Spermatogonia Stem Cells (SSCs) in the testis have self-renewal, proliferation, and differentiation capacities and provide a spermatogenesis process in the seminiferous tubules [1]. The process of spermatogenesis is impaired by chemotherapeutic agents and radiotherapy procedures in cancer patients [2]. Therefore, in these patients, obtaining cells from testicular tissue and the in-vitro proliferation of SSCs are critical steps to enhance the rate of SSC transplantation in patients [3]. The most critical issue for the proliferation of in-vitro SSCs is to have an environment close to the microenvironment of seminiferous tubules [4]. Some experiments indicated that three-dimensional (3D) culture systems provide an appropriate niche for cells, like seminiferous tubules [5]. Recently, DTM hydrogel scaffold introduces a different approach to the study of SSCs which improves cell-to-cell connectivity and facilitates the propagation of SSCs [6, 7].

N-methyl d-aspartate type glutamate receptor (NMDAR) is an ionotropic receptor consisting of two obligatory GluN1(NR1) subunits, with high binding affinity and agonist effect of glycine or D-serine combined with two subunits of GluN2 and /or two GluN3 subunits that all bind glutamate [8]. Recent studies indicated that several subtypes of ionotropic glutamate receptor (GluRs) are expressed in rat testis [9]. NMDARs were found in germinal epithelium and interstitial spaces [10, 11]. Previous studies have revealed that the agonist NMDA subtype of glutamate receptors directly affects spermatogonia mitotic activity [10, 12], spermatogonia proliferation [13], and spermatozoa maturation via the modulation of testosterone and estrogen amounts in the epididymis [14].

Recently, the D-amino acids were detected in various types of testis cells, including Sertoli cell, Leydig cell, spermatogonia, spermatid, and spermatozoa [15, 16]. It was reported D-serine plays a key role in brain tissue physiology [17], cell development, and cell death signaling [18]. D-serine binds to the NMDA subtype of glutamate receptors and acts like D-aspartate on the hypothalamic-pituitary axis or directly on testicular Leydig cells and promotes testosterone synthesis [13]. Moreover, D-serine may upregulate spermatogenesis by enhancing the activity of NMDA receptors [12].

Dizocilpine (MK-801) is a non-competitive glutamate receptor antagonist which affects the central nervous system. MK-801-induced schizophrenia models describe injuries in the testis in response to reactive oxygen species (ROS) [19]. Moreover, it was shown testosterone declines after the blockade of NMDA receptors by a noncompetitive NMDA receptor antagonist (MK-801), and it is attributed to the reduction of sperm motility [9].

Consequently, cell-scaffold interaction may enhance the cell survival rate. Since there is no available data on the proliferative effect of agonists NMDA receptors on SSCs. The main purpose of this study was to investigate the effect of D-serine (DS) and Dizocilpine (MK-801or Mk) on the proliferation of SSCs in two-dimensional (2D) and three-dimensional (3D) culture systems.

Materials And Methods

Animals and Drugs

Male Naval Medical Research Institute [NMRI) mice were used in this examination. The animals were obtained from Animal Resources Department, University of Tabriz (Tabriz, Iran). The mice were kept in animal houses under standard conditions (at 22 ± 2 °C room temperature, 50–55% humidity, light / dark cycles: 12/12 hours per day). The chemicals that were used in this study were purchased from Sigma–Aldrich unless otherwise mentioned.

Testis dissociation and SSCs isolation

Ten male NMRI mice (6 days old; *n*=10) were euthanized with intraperitoneal injection 100 mg/Kg ketamine and 10 mg /Kg xylazine. Their testis was removed and placed into a petri dish containing culture medium and antibiotic. Testicular tissue cells were isolated with slight modifications according to the methods presented by [20]. In summary, after washing the testicular tissue several times in PBS solution for cell isolation, the testicular capsule was removed, and then the testicular tissue was divided into approximately 1 mm^3 using the mechanical procedure. Thereafter, the dissociated tissues were placed in a solution containing trypsin (0.5 mg / ml, Sigma-Aldrich, St Louis, MO, USA), collagenase I (0.5 mg / mL Sigma-Aldrich, St Louis, MO, USA), and hyaluronidase (0.5 mg / mL, Sigma-Aldrich, St Louis, MO, USA). Then, a cell was centrifuged (1500 rpm for 5 min) and washed in DMEM/F12 (Gibco, Grand Island, NY, USA) medium. Finally, the next digestion was accomplished for 15 min in a solution containing collagenase I (1mg/mL, Sigma-Aldrich, St Louis, MO, USA) DNase (0.5 mg/mL Sigma-Aldrich, St Louis,

MO, USA,), and hyaluronidase (1.5 mg/mL, Sigma-Aldrich, St Louis, MO, USA) in the water bath at 37°C. The obtained cells were washed and prepared for culture after passing via a 70 µm cell strainer.

SSCs culture and proliferation

After enzymatic digestion, cell viability was evaluated with 0.4 % trypan blue solution in a 6-well plate with the culture medium DMEM/F12 containing 10% FBS (Gibco, Grand Island, NY, USA) with penicillin (100 U/mL) and streptomycin (100 µg/mL; Gibco, Grand Island, NY, USA). To isolate the SSCs which usually attach to the bottom of the culture dish later than the somatic cells, the supernatant was removed after 24 hours and centrifuged (1000 rpm for 5 minutes) to separate the non-attached cells. Then cells were cultured in DMEM/F12 medium containing 5 % Knockout serum replacement (KSR) (Gibco, Grand Island, NY, USA) GDNF (10ng/mL, Sigma Alderich St.Louis.MO, USA), LIF (10^3U/mL, Sigma Alderich St.Louis.MO, USA) EGF (20ng/mL, Sigma Alderich St.Louis.MO, USA) bFGF (10ng/mL, Sigma Alderich St.Louis.MO, USA) in gelatin (0.2 %; Sigma Alderich St.Louis.MO, USA) coated dishes, and then incubated at 35°C in a humidified atmosphere with 5 % CO_2. The medium replaces every 48h. After two weeks since the initial culture, SSCs colonies were observed. After two weeks in culture, the *Plzf* protein was traced as a positive marker to confirm the identity of the colonies derived from the SSCs [21].

Immunocytochemistry for characterization of SSCs colonies

To characterize SSC colonies, *Plzf* expression was determined by immunocytochemistry (ICC). After fixation with paraformaldehyde (4%) permeability with 0.4 % Triton X100 (Sigma-Aldrich, St Louis, MO, USA) and blocking with 10% goat serum (Sigma-Aldrich, St Louis, MO, USA), cells were treated with rabbit polyclonal antibody *anti-Plzf* (sc28319;mouse monoclonal, 1:500, Santa Cruz, Houston, USA) and anti-GFR-α1 (pa519873;Rabbit polyclonal, 1:100, Thermo Fisher, Altrincham, UK) for 2 h at 37°C. After washing with PBS, a secondary antibody, Donkey Anti-Rabbit, labeled with fluorescent isothiocyanate (FITC) 1: 100 (Sigma-Aldrich, St Louis, MO, USA), was added for 3 h. Control cells were treated under similar conditions except for the removal of primary antibodies. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, 1µg / mL; Sigma-Aldrich, St Louis, MO, USA) [22].

Tissue decellularization

In this experimental study, testicular scaffolds were generated using the testes obtained from 6 mice models (6-8 weeks old). The tunica albuginea was removed from the testes of the mice using a 29-gage insulin syringe and washed with phosphate buffer saline [PBS, Sigma, USA) to remove the residual blood. The sections of 100 µm diameters of testis were immersed in % 0.5 (v/v) of sodium dodecyl sulfate (SDS, Sigma-Aldrich, St Louis, MO, USA) and % 0.5 (v/v) triton X-100 (TX-100; Sigma-Aldrich, St Louis, MO, USA) and then incubated for 18 h in water. Then, they were placed by PBS for 2 h to remove detergents. After washing, the decellularized tissue sections were sterilized in 70% ethanol for 1 hour and immersed in PBS for 2 hours. Before usage, the scaffolds were immersed in a culture medium for 24 hours [23].

Histological examination

Hematoxylin-eosin (H&E) staining was used to confirm the quality of DTM and evaluate cell migration to the scaffold after its recellularization. Each sample was fixed in formaldehyde [10%), dehydrated through increasing ethanol concentration, after clearing by xylen and embedding in paraffin, 5-µm thick sections were prepared with a microtome (Leica, Germany). Sections were deparaffinized, rehydrated, and stained via Hematoxylin & eosin (all from Merck Germany). Alcian blue staining (Merck, Germany) was conducted to assess the amount of glycosaminoglycan's (GAGs) based on previous described protocols [24]. Masson's Trichrome staining was performed according to guidelines to determine the existence of collagen [25]. Slides were observed under a light microscope (Labomed, USA), and images (nuclei are stained dark blue, cytoplasm are stained bright-red, as well as collagen is stained green or blue) were taken with a Ziess Camera.

DAPI staining for nuclei staining of decellularized tissue

Both confirmed the intact and decellularization of the testis by 4, 6-diamidino-2- phenylindol (DAPI) staining and DNA quantification. To assess the homogeneity of decellularization, the samples were collected from different sites of the scaffolds. In brief, DAPI solution (1µg/mL; Sigma-Aldrich, St Louis, MO, USA) was pipetted directly to each tissue section after fixing the tissues in formalin. They were kept in the dark room for 15 min. After washing with PBS, the samples were evaluated with an inverted fluorescence microscope.

DNA content in decellularized testicular tissue

For quantitative testing, the total amount of DNA from native tissue and decellularized testicular scaffold were extracted by a genomic DNA purification kit (Qiagen, UK). The purity and concentration of DNA were calculated by Nanodrop spectrophotometers (UV-visible; Thermo Nanodrop ND-1000) at 260 nm and agarose gel [24].

Synthesis of dual-crosslinked hydrogels: Natural ECM of decellularized testis and Hyaluronic acid hydrogel

For composite-cross-linked hydrogels, 10 mg of sodium hyaluronate powder was dispensed into a circular silicone rubber mold. The same volume of lyophilized testicular tissue extract was sandwiched between two rubber molds and tightly secured. Crosslinking solutions consisted of 0.2 M NaOH containing butanediol diglycidyl ether (BDDE). The concentration of cross linkers was measured in units of µL BDDE/g HA [25]. This fluid was inserted into each rubber mold by a syringe equipped with a 25-gage needle. A second needle created an air escape, and caution was considered to stop bubble trapping. A volume of crosslinking fluid was applied to fill each mold. Molds were roughly 100 µL in volume and had an HA concentration of 10%. HA and crosslink solution were then incubated at 40ºC for 8 h. The cross-linked hydrogels were separated from the molds and relocated to a large amount of distilled deionized water or saline (0.9% NaCl) for swelling.

Cytocompatibility of scaffold by MTT assay

Following the scaffold was produced, its toxicity was tested to confirm that it did not contain any hazardous substances during the scaffold preparation and decellularization stages. After the culture of SSCs at a concentration of 5×10^4 in each well of 96-well plate, scaffold extract was added to cells in the culture medium, and after 24 h, 48 h, and 72 h since the inception of the culture, the cell was evaluated using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). In brief, add 200 µl of MTT solution (0.5ng/ml) to each well and incubated for 4 h at 37 °C in a humidified atmosphere 5 % CO_2 incubator, and after that, cells lysed and purple formazan crystal dissolved by adding 400 µL of DMSO (D8418, Sigma-Aldrich, St Louis, MO, USA) in each well for 30 min at room temperature. Finally, the purple formazan formed during the test step was measured at 540 nm with the microplate reader [1].

Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) evaluated the 3D structure of scaffold, quality, decellularization, and cell migration after recellularization. The tissues were fixed with 2.5% glutaraldehyde for 2 h, and then with 1% osmium tetroxide and dewatering by increasing the degrees of ethanol (30 to 100%). After dehydration, the tissues were placed on a grid, covered with a gold-palladium coating, and seen *via* SEM (FEI Quanta 200). The number and diameter of SSCs colonies in each field and pores size of the scaffold were analyzed using SEM [26].

Preparation of DTM and Reconstitution of testicular cells on it

Reconstituted testicular artificial tissue using a hyaluronic gel matrix 2% and 50 mg of Decellularized testes extract (1:1 v/v ratio) were placed in a 24-well culture dish in a thin layer form. Then, 1×10^5 cells were added to each well plate (Nunc, Roskilde, Denmark) to which collagen gel matrix and somatic cells adhere poorly. After gently agitating the gels for 1 minute, they were overlaid with DMEM/F12 medium supplemented with 5 % KSR, 5 % FBS. During culture, the period medium was replaced every 2 days. After two weeks of culture, the matrix gel was re-dissolved with 0.25% trypsin to release the embedded cells, and then cell viability and proliferation were determined after two weeks of initiation of culture [25].

Experimental Design

The culture groups were classified into 2 categories include (1) 2D culture and (2) 3D culture. Each group were defined in a subgroup with different treatments include 1a (2D- D-Serine) or 2a (3D- D-Serine); D-Serine treatment, 1b [2D- Glutamic acid) or 2b (3D- Glutamic acid); Glutamic acid, 1c (2D- MK-801) or 2c; MK-801 (3D- MK-801). D-Serine and MK-801 were used 20 µM, and Glutamic acid was used 50 µM in a final concentration. SSCs cultured in each experimental group for 2 weeks for assessment of SSCs proliferation in different culture medium situations. Two normal groups included 2D and 3D without D-serine, and MK-801 were designed as control groups in the whole study phases.

Gene expression analysis

In the second weeks of proliferation culture, the total RNA of the cells cultured in 2D and 3D culture was extracted using guanidine / phenol solution (Qiazol-Qiagene USA). Then RNA was treated with DNase I (Fermentase, USA) to eliminate genomic contamination. The purity and concentration of RNA were determined using Nano Drop 2000 (Thermo scientific). For Complementary DNA (cDNA) synthesis, 1 µg total RNA was carried out by reverse transcription kit, using a Prime Script RT reagent kit (Hyper script RT-PCR- GeneAll) according to the manufacture instructions. PCR was carried out using mixed Master Mix and SYBR green in the stage one of thermal cycle (ABI stage one, USA). PCR program began with melting cycle of 15 min at 95°C. The stage was followed by 40 cycles: initial denaturation 30s at 95°C, annealing 30s at 60ºC, and extension 30s at 60ºC. The specific primers used to determine the expression levels of pre-meiotic genes including *Plzf* [Forward: 5' AGT GGG ATT GAT GAG GAG ATG G 3' and Reverse 5' AGT GGA GTG TAG GGA GAA GGA 3') as SSCs specific markers and βactin (Forward 5' TCA GAG CAA GAG AGG CAT CC 3'and Reverse: 5' GGT CAT CTTCTC ACG GTT GG 3') as internal control gene. To investigate the presence of non-specific products and primer dimers, a melting curve analysis was performed. Furthermore, this process was replicated in triplicates for target and reference genes. All samples were normalized to the βactin as a reference gene using (ΔΔCT) method [27].

Protein evaluation by Flow cytometry

The process of cell staining for flow cytometry was done based on pervious researchers by Kanatsu et al. [28]. Furthermore, the cells fixed at 4% paraformaldehyde and permeabilized at 0.5% Triton X-100 (Invitrogen, UK) for 5 minutes before being blocked by a combination of 10% goat serum and Bovine serum albumin [BSA, 1 mg/ml). The incubation of cells was performed with the primary antibodies, including rabbit anti *Plzf* at 4°C overnight. Cells were incubated with a certain number of secondary antibodies (FITC, conjugated goat anti rabbit IgG) 60 min at 37° C in the dark. After washing with PBS, flow cytometric analysis was accomplished with a Becton Dickinson (BD) and was analyzed with Flowjo 7.6 software.

Statistical analysis

The data presented are expressed as mean ± standard error (mean ± SEM). Kolmogorov-Smirnov test was used to determine the normal distribution of data. An independent sample t-test was used for DNA content and MTT analysis. For multiple comparisons of data, One-way analysis of variance (ANOVA) followed by Tukey's test used. Graph Pad Prism software version 8 (Graph Pad Software San Diego, CA) was used for data analysis. Data with less than < 0.05 were considered statically significant.

Results

SSCs colonies characterization

SSCs were isolated using a protocol involving two enzymatic digestion steps. SSCs proliferating colonies were observed approximately two week after obtaining the culture of cells derived from testicular tissues. The small colonies formed from them were observed in the culture medium, and the number of these

colonies increased. To confirm the identity of SSCs, the colonies were investigated using two specific markers of SSCs (*Plzf* and *GFRα1*) *via* ICC staining (Fig.1 A,B). The results showed that the majority of colonies are positive with both SSCs markers. For further confirmation, the gene expression related to SSCs was measured by qRT-PCR prior to the initiation of the differentiation phase.

Evaluation of decellularization

The first stage of analyzing the effectiveness of decellularization is to validate the removal of cellular components. The histological evaluation indicated that the cells were destroyed with SDS and Triton x100 in the testis. H&E staining demonstrated well preserved structural architecture of the testis with no cell fragments remained in the tissue. The empty seminiferous tubules are clearly distinguishable. The total amount of glycosaminoglycans (GAGs) in Alcian blue and Masson's trichrome staining were tested and showed strong retention in decellularized testis relative to normal tissue (Fig.2,A). The remaining GAG in the scaffold was stained with Alcian blue, indicating that more than 80% of GAG remained. No significant difference was observed in decellularized testis compared to the normal ($p=0.1964$) (Fig.2,C). The amount of DNA was measured to evaluate the efficiency of the decellularization method and compared to DNA amount in native tissue. Analyzing DNA contents showed that DNA was successfully removed from the testis compared to the normal group ($p<0.0001$) (Fig.2, B). The pictures of the SEM assessment were showed a tubular structure in the tissue without any cells. Besides, the structure was remained normal in some parts of tissue (Fig. 2, D).

Proliferation of SSC on scaffold after 14 days culture

MTT results demonstrated that the decellularized scaffolding was non-toxic. SSCs were cultured inside the decellularized testicular scaffold, and cell viability was assessed after 24 h and 48 h. The proliferation rate of SSCs in the three-dimensional culture with 50 mM glutamic acid and 20 mM D-serine was significantly different from the other groups after 14 days of treatment. Nevertheless, the proliferation rate of SSCs in the 3D-culture with MK-801 was significantly lower than in other groups (Fig. 3).

Molecular evaluation of *Plzf* in 3-D cultured SSC

Our results indicated that the expression of *Plzf* gene in the 3D group of spermatogonia stem cell cultures with 20 Mm D-serine and 50 mM glutamic acid was significantly greater than the control group ($p<0.001$). The expression of desired gene in the 2D and 3D group of SSC culture with 20mM MK-801 (MK20) was, decreased compared to each of the control groups, respectively. However, the alteration in gene expression was not statistically significant ($p=0.294$, $p=1.064$ respectively) (Fig. 4).

Protein level PLZF marker in SSCs in different experimental groups

The detection of germ cells at the protein level was performed via one in all experimental groups. The flow cytometric results revealed that the expression of *Plzf* protein in the 3D culture group of SSCs with D-serine and glutamic was substantially more than the 3D culture control group ($p<0.001$). The expression of *Plzf* in the 2D culture groups of SSCs with MK-801 was lower than the 2D culture control group

(p<0.001). No significant difference was observed in the 3D culture of SSC with MK-801 compared to 3D culture control (p=0.187)

Discussion

For the first time, we developed the SSCs culture in the DTM hydrogel scaffold and indicated the role of D-serine, and MK-801 as the agonist and antagonist of NMDA receptors, respectively, in the spermatogenesis process. Decellularized testicular matrix [DTM) hydrogel scaffold is suitable for the surrounded SSCs and Sertoli cells to enhance the proliferation and differentiation of SSCs [29]. Our results, like previous findings, showed that the DTM hydrogel scaffold is appropriate for the proliferation of SSCs [30]. DTM hydrogel scaffolds contain laminin and collagen, which can regulate the proliferation of SSCs and so, the purity of SSCs increases [29, 31]. The other constituents of DTM are GAGs which surround the Sertoli cells and Leydig cells and affect cell adhesion, and proliferation [32]. These results showed that DTM contains some growth factors which can maintain SSCs activity and gene expression.

Moreover, this study's results revealed that the supplementation of a 3D culture medium with glutamic acid improved spermatogonia proliferation. Similar to these results, Santillo et al. revealed that the addition of glutamate to spermatogonia germ cell 1 (GC1) medium increased spermatogonia proliferation [33]. The previous study showed that glutamate-induced the phosphorylation of ERK1/2 protein in spermatogonia GC1 cells [33]. Moreover, a surge of Akt activity in spermatogonia cells treated with glutamate was observed [10]. It has already been shown that extracellular-regulated kinase (ERK1) and Akt regulate various biological functions such as gene expression and cell cycle [34, 35], and play an important role in germ cell proliferation [36]. Besides, it was declared that L-glutamine receptors are functionally expressed in rat testis which might promote stem cell proliferation by increasing glutathione levels inside and outside the cell as well as cysteine/cysteine balance [9, 37, 38]. Another mechanism is that glutamic acid stimulates the proliferation of SSCs via anti-apoptotic pathways and by increasing testosterone in Leydig cells [39].

Based on the results, we found that enriched 3D culture medium with D-serine enhances SSCs proliferation. In agreement with our results, stated that enriched media with D-serine had significantly increased the proliferation and survival of SSC [18]. On the other hand, it was indicated that treatment with D-serine increased the number of germ cells in silkworms via ERK phosphorylation in the testis [39]. After treating testes with D-serine, phosphorylated ERK was identified in spermatocytes and spermatids [40] which is consistent with an earlier finding that the meiosis of rat spermatocytes depended on the ERK activation of rat spermatocytes [41]. Furthermore, agonist NMDA receptors can stimulate the proliferation of stem cells and increase their population in culture by supporting the intracellular antioxidant glutathione synthesis and reducing intracellular ROS [42].

This study's findings revealed that the survival rate of SSCs in the 3D culture decreased after treating 3D and 2D cultures with MK-801. In line with our previous results, the experiments showed that the administration of MK-801 to rat testis resulted in increased oxidative stress in rat testis [43]. Similarly,

Saleh et al. demonstrated that the administration of MK-801 to rabbits increased the atrophy of the tubular structure and the degeneration of germinal cells in the lumen of the seminiferous tubule in histopathological evaluation [44]. Moreover, it was reported that the level of total antioxidant status and sperm kinematics parameters have decreased following the administration of MK-801 [45, 46]. Histological data showed that using MK- 801 as antagonist NMDA, due to the testicular seminiferous tubule atrophy, degenerative changes in the epithelial cells, and a decrease in the number of the spermatozoa and necrotic [47]. MK-801 reduces SSCs proliferation by increasing ROS production and increasing the expression of Bax and Bcl2 in SSCs [18]. These results showed that the presence of D-serine alone or along with glutamic acid in the 3D culture of mouse SSCs creates a testis-like microenvironment.

Conclusions

In summary, this study indicated that the DTM hydrogel scaffold culture system provided a simple and alternative method for culturing SSCs that eliminates potential diversity and contamination caused by feeder cells. Moreover, our results showed that supplemented culture medium with 20mM D-serine and 50mM glutamic acid could be considered a promising vehicle to support cells and provide a suitable niche for the proliferation of SSCs. The results of this study could be a way to further study the process of spermatogenesis in *vitro* and provide novel therapeutics for a higher proliferation rate of SSCs in male infertility to achieve an acceptable SSCs number.

Declarations

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Availability of data and material The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable' for that section.

Authors' contributions Amir Hessam Eskafı Noghani: performed the research and prepared manuscript Reza Asadpour : contributed to study design, wrote the draft, and interpreted the data. AS: contributed in

performing study and revised the manuscript Zohreh Mazaheri: contributed in the construction of 3D culture system, interpret the data, Gholamreza Hamidian: revise the manuscript and interpret histology data; all authors approved the submitted version.

Ethics approval Ethics Committee approved all experimental procedures of the University of Tabriz, Tabriz, Iran, to use animals (Permission No. IR.TABRIZU.REC.1399.045).

Consent to participate Not applicable' for that section.

Consent for publication Not applicable' for that section.

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Figures

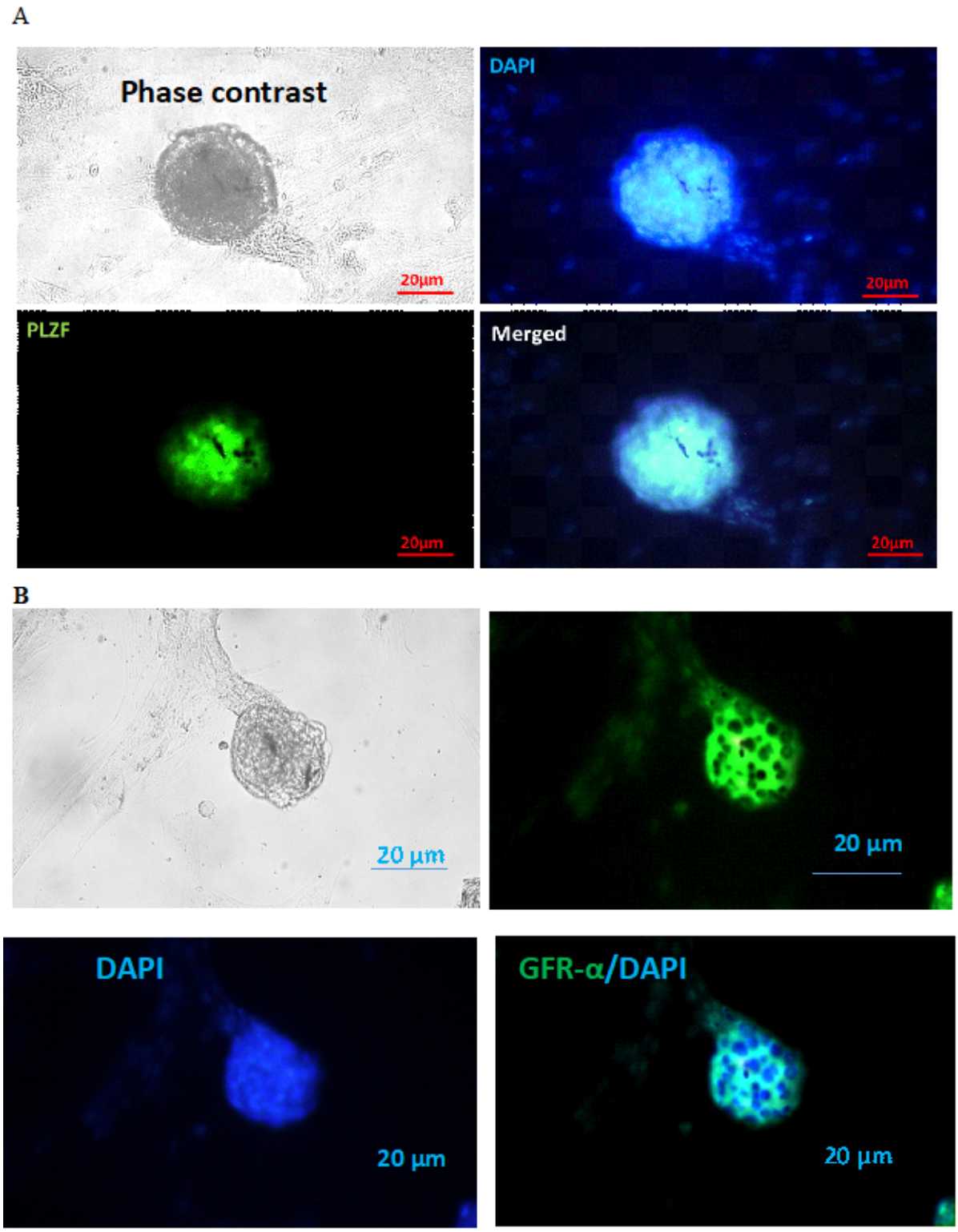

Figure 1

Characterization of SSCs formation. Phenotypic characteristics of SSCs revealed oval shape. Immunostaining of SSCs using Plzf. The ICC results indicated the presence of Plzf (A) and GFRα1 (B) under a fluorescence microscope. Nuclei are stained with DAPI. Scale bar, 20 μm.

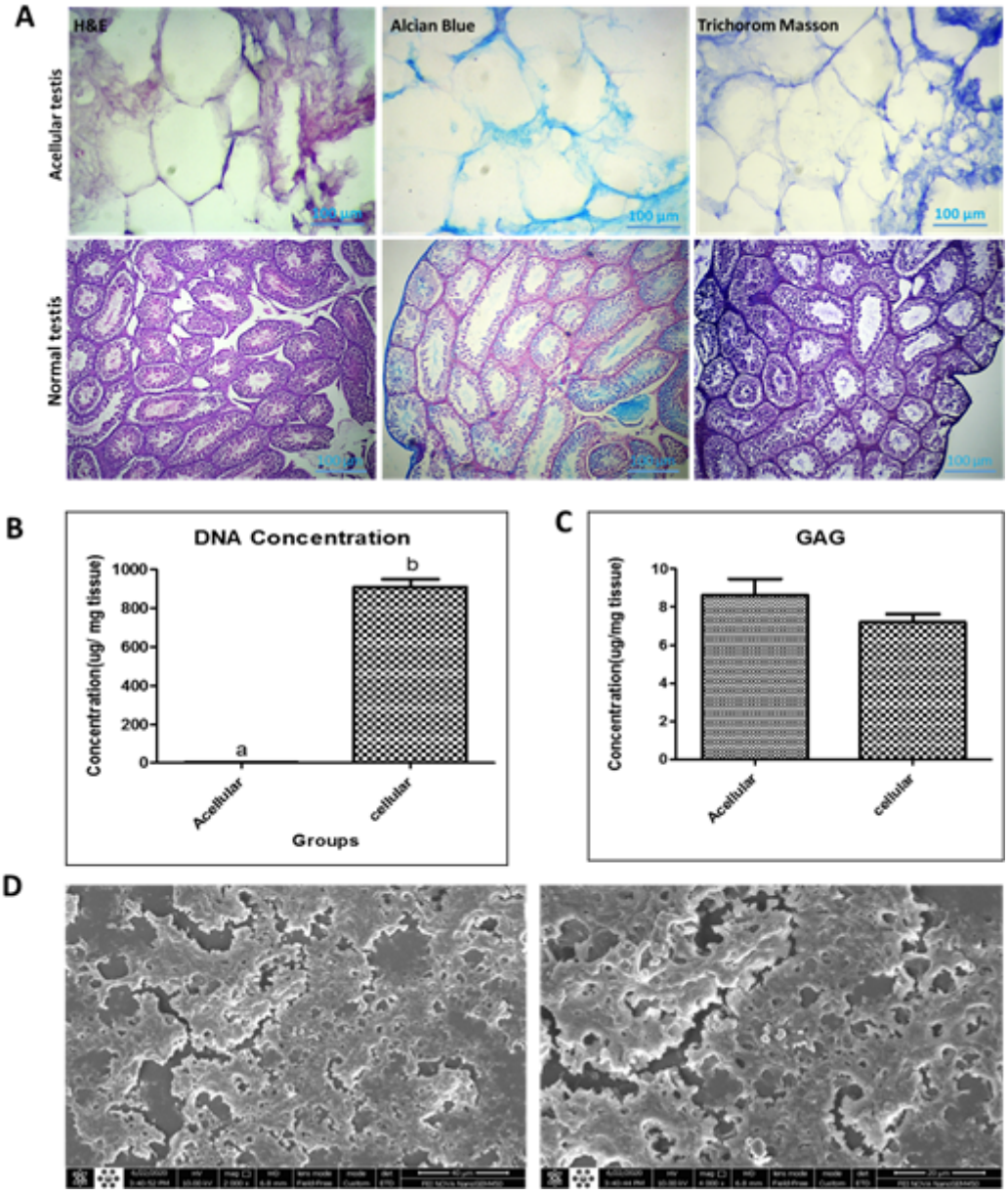

Figure 2

Histological evaluation of the testis after SDS-based decellularization process. A) H&E staining evidenced preservation of structure. Alcian blue and Masson's trichrome staining showed the presence of GAGs. Scale bar, 100 µm. B) DNA quantification manifested remarkable cell eliminated by decellularization procedure compared to the normal testis. C) GAGs concentration of decellularized testicular tissue was not significantly altered in comparison with normal tissue. D) SEM assessment of decellularized testicular tissue. The results are presented as means ± SD. different letters: Significant difference with other experimental group ($p<0.05$).

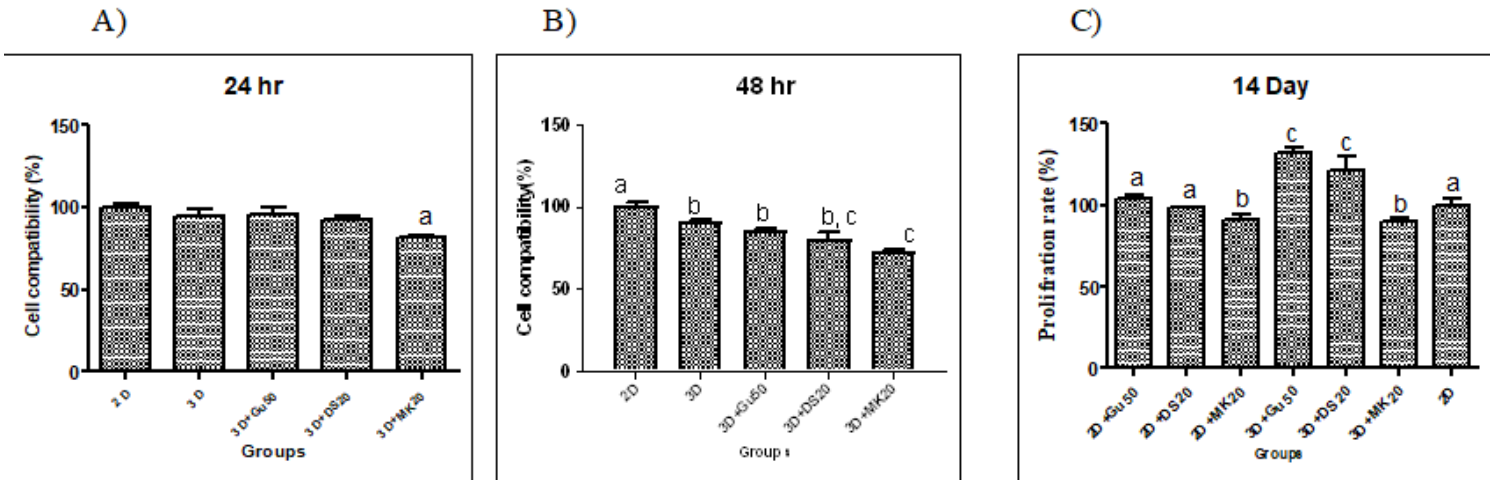

Figure 3

Cell compatibility assay of SSCs in different experimental groups after A) 24 h. and B) 48 h. C) Proliferation rate of SSCs on 3D and 2D culture after 14 days cultivation in different culture media. MTT assay represents the toxicity effect of scaffold during 24 & 48 h culture of SSCs. Forth teen days after SSCs cultivation in 2D and 3D condition into different medium indicates a cell proliferation in 3D culture medium treated by Gu50 and DS20. Although, the results showed a proliferation rate decrease in 3D culture medium treated by MK20. SSC: Spermatogonial Stem Cell; DS: D-Serine; Gu: Glutamic acid; MK: MK-801. Different letters: Significant difference with other experimental groups ($p<0.05$).

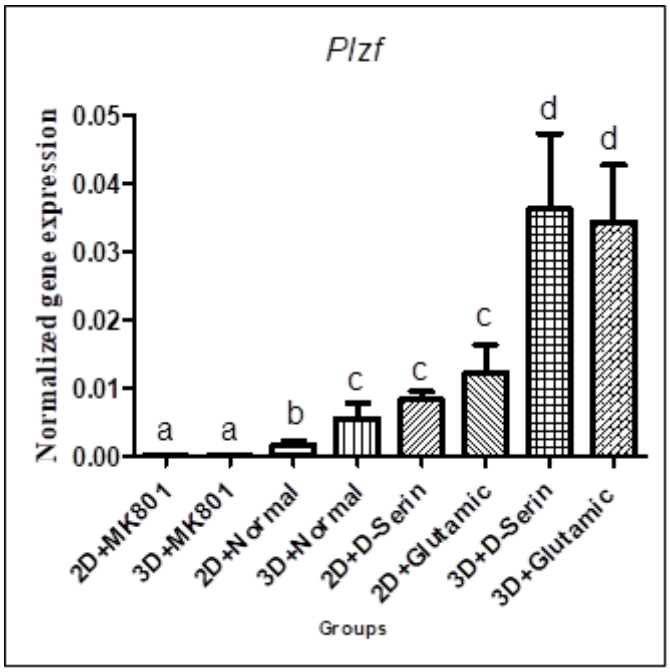

Figure 4

Expression level of Plzf in 2D and 3D cultured SSCs with 20 mM D-Serine and 50 mM glutamic acid or MK-801. The real time PCR results were normalized to a reference gene (β-actin gene). The letters shown in the figure 4 have statistically significant differences ($p<0.001$). SSCs: Spermatogonial Stem Cell; MK: MK-801. The results are presented as means ± SD.

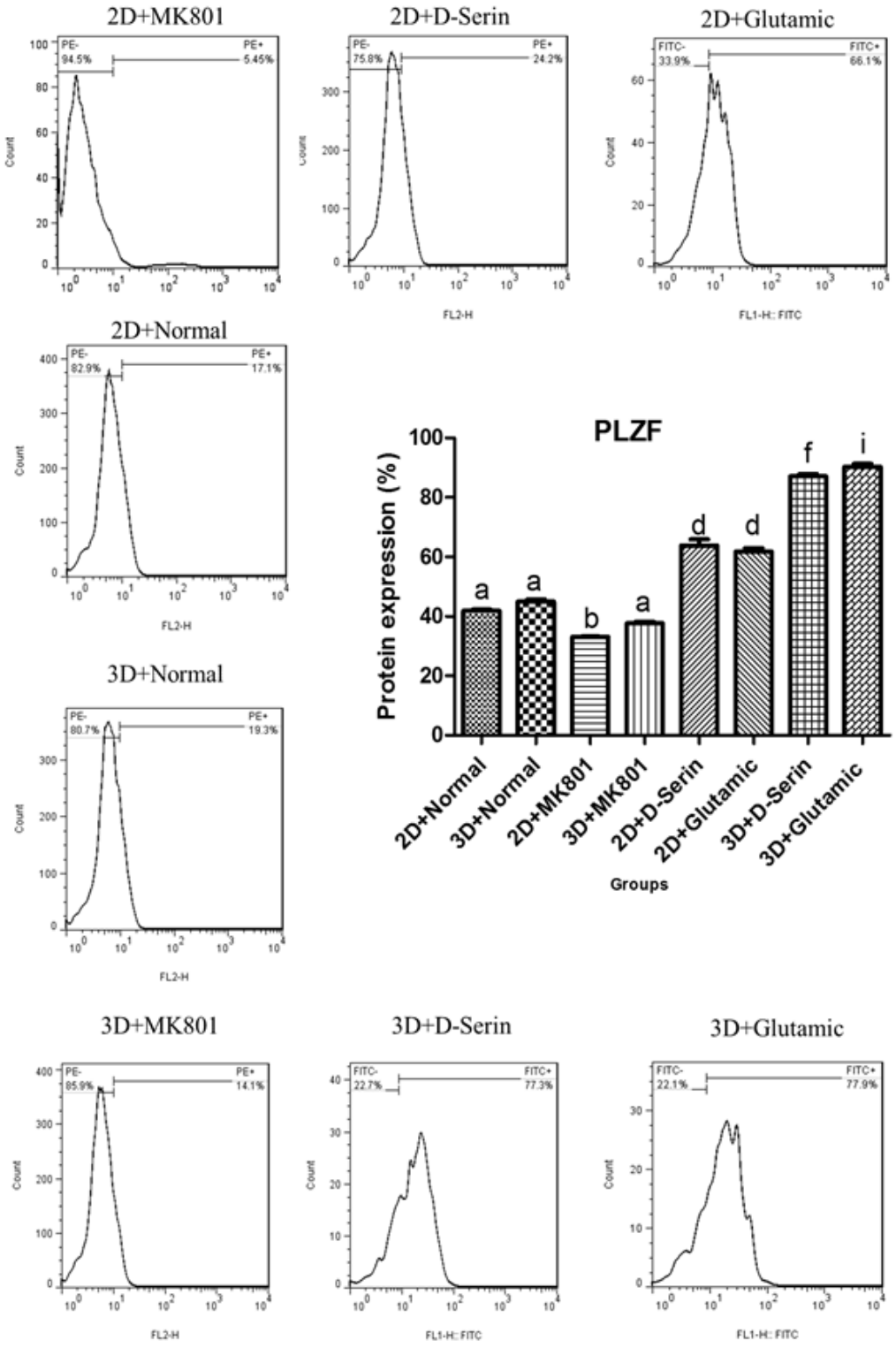

Figure 5

Protein level of Plzf in 2D and 3D cultured SSC with 20mM D- serine and 50 mM glutamic acid or MK-801. Intensity of FITC dye was used for analyzing of Plzf protein in comparison with isotype control sample by flow cytometric assay. The different letters shown in the figure 5 have statistically significant differences ($p<0.001$). SSCs: Spermatogonial Stem Cells; DS: D-Serine; MK: MK801. The results are presented as means ± SD.