Cryoprotective Effect of Pentoxifylline on Spermatogonial Stem Cell During Transplantation Into Azoospermic Torsion Mouse Model

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Research

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Abstract

Background

Preserving the spermatogonial stem cells (SSCs) in long periods of time during the treatment of male infertility using stem cell banking systems and transplantation is an important issue. Therefore, this study was conducted to develop an optimal cryopreservation protocol for SSCs using 10 mM pentoxifylline (PTX) as an antioxidant in basal freezing medium.

Methods

Testicular torsion - a mouse model for long-term infertility- was used to transplant fresh SSCs (n=6), fresh SSCs treated with PTX (n=6), cryopreserved SSCs with basal freezing medium (n=6) and cryopreserved SSCs treated with PTX (n=6). Eight weeks after germ cell transplantation, samples were assessed for proliferation, through evaluation of Ddx4 and Id4 markers, and differentiation *via* evaluation of C-Kit and Sycp3, *Tnp1*, *Tnp2*, and *Prm1* markers.

Results

According to morphological and flow cytometry results, SSCs are able to form colonies and express Gfra1, ID4, α6-integrin and β1-integrin markers. We found positive influence from PTX on proliferative and differentiative markers in SSCs transplanted to azoospermic mice. In the recipient testis, donor SSCs formed spermatogenic colonies and sperm.

Conclusion

Respecting these data, adding pentoxifylline is a practical way to precisely cryopreserve germ cells enriched for SSCs in cryopreservation, and this procedure could become an efficient method to restore fertility in a clinical setup. However, more studies are needed to ensure its safety in the long term.

Introduction

Considerable number of childhood cancer survivors are at risk of infertility due to the loss of spermatogenic cells after treatment[1]. For adult male cancer patients, fertility can be preserved by cryopreservation of semen[2]. Since spermatogenesis has not commenced in prepubertal boys, cryopreservation of ejaculated or surgically retrieved spermatozoa is not feasible. Therefore there is a need for a clinical application to preserve and restore fertility in these boys[3],[4]. Spermatogonial stem cell auto transplantation (SSCT) is an experimental technique that is still in a preclinical phase[5],[6]. SSCT has proved to restore spermatogenesis in various animal models, including non-human primates[7]. The procedure of treatment requires a testicular biopsy before cancer therapy to preserve the fine SSCs. These cells will be transplanted after successful cancer treatment and proven sterility[8]. Currently, in many centers across the globe, cryopreservation of testicular biopsies is offered to children with cancer[9]. Due to lack of active spermatogenesis at the time of cancer diagnosis, SSC freezing

cannot be helpful by its own; thus, long-term preservation techniques, such as cell culture and cryopreservation in combination with SSC transplantation (SSCT) may be the best strategy for these patients as a possible fertility preservation[9].

The process of cryopreservation results in unfavorable cryoinjuries, which disrupts the normal biological function of cells. Also, previous reports of SSC cryopreservation have indicated that the functional capacity of thawed SSCs is less than ideal. These disruptions include DNA fragmentation, mitochondrial dysfunction, osmotic stress, oxidative stress[10], induction of apoptosis[11] and increased generation of Reactive Oxygen Species (ROS)[12],[13].

Studies have demonstrated that addition of exogenous antioxidant in the freezing extender can improve sperm quality and function and increase the quality and viability of SSCs after thawing, providing indirect evidence that oxidative stress during cryopreservation can be a potential harm to these cells[14],[15]. Pentoxifylline (PTX), a methylxanthine derivate, is known as an inhibitor of cyclic adenosine monophosphate[16]. There are studies indicating that PTX can increase partial pressure of oxygen and pose anti-inflammatory activity, eliminate free radicals, block the expression of NF-κB, macrophagic nitric oxide synthesis induced by and TNF-α mRNA, and reduce cellular apoptosis[17]. Indeed, PTX possesses both antioxidative and ROS scavenging (ROS identification and inhibition) which demonstrates the anti-oxidative activity of PTX properties[18].We hypothesized that transplantation of SSCs treated with PTX may help to improve fertility after testicular torsion (TT). To investigate the effect of PTX, we optimized an infertility model representing an impaired spermatic cord by TT. Next, we transplanted cryopreserved SSCs treated with PTX to pursue its effect on markers of pre-meiotic and post-meiotic spermatogonial stem cells (SSCs) for the first time (Supplementary Fig.1).

Method And Material

Animals and study design

A total number of 10 male NMRI mice (3-6 days-old) underwent testicular surgery and were used as donners for SSC. 6-8weeks-old male NMRI mice (n=66) were used as recipients of SSC transplantation. All NMRI mice were obtained from the Pharmacy Faculty of Tehran University of Medical Sciences. Animals were preserved under standard conditions of 12 hours/12 hours' light/dark. All surgical procedures were done under xylazine/ketamine (10/90 mg/kg, i.p.) anesthesia using sterile conditions. *All procedures in this study were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055, USA), and also this study was approved by the Ethical Committee of Tehran University of Medical Sciences (IR.TUMS.MEDICINE.REC.1397.210). Forty-eight* mice in four testicular torsion transplantation groups (12 mice in each group), 9 mice in the control group and 9 mice in the torsion group were considered as experimental groups. After testicular torsion model was confirmed, transplantation groups were divided as followed. The group that received SSCs (TT+Fresh), the group receiving SSCs after freezing (TT+Cryo), the group which received SSCs treated with PTX after freezing

(TT+Cryo+PTX) and the group receiving SSCs with PTX (TT+Fresh+PTX). Also, control group received DMEM. The TT group had testicular torsion operation but did not undergo transplantation. Two weeks after testicular torsion, 3 mice from each of 6 groups were sacrificed to confirm the torsion by cervical dislocation. Two weeks after transplantation, 3 mice in each transplantation group were sacrificed to confirm the transplantation. Recipient mice were euthanized, and testes were collected and decapsulated 8 weeks after transplantation. Finally, 36 mice in all groups (6 groups of mice, 6 mice in each group) included four testicular torsion transplantation groups, control and torsion group (as a sham-transplanted testes) were used to evaluate the testes at the end of the study which was 8 weeks after transplantation (Supplementary Fig.2).

Testicular surgery, SSC isolation and enrichment

After anesthesia, testes were dissected and transferred into the phosphate-buffered saline (PBS; “Sigma-Aldrich)”. Then SSCs were isolated by a two-step enzymatic digestion according to the protocol of Kanatsu-Shinohara[19]. First, the testes were suspended in 3 mL Dulbecco modified Eagle medium (DMEM) containing 5 µg/mL DNase (Sigma-Aldrich, Germany), 1 mg/mL collagenase type IV (Gibco, CA) and 1 mg/mL hyaluronidase (Sigma-Aldrich). Then, they were transferred to an incubator for 20 min at 37°C until forming a cellular suspension[20]. Interstitial cells were digested after pipetting and centrifuging at 15×g for 5 minutes. In the second step, cellular pellets were enriched by the same medium for 15 minutes. Cell detachment from seminiferous tubules occurred in this step. The suspension was centrifuged at 15×g for 5 minutes, and the cell pellets were transferred to the gelatinized dishes for further pelleting with a duration of 5 hours. For SSC enrichment, the cellular suspension was transferred into gelatin-coated (Sigma-Aldrich) culture dishes for 2 hours at 37°C. The non-adherent SSCs were cultured in minimal essential medium (Gibco-Invitrogen, US) containing 2% Fetal bovine serum (FBS) (Life Technologies), 1000 U/mL Leukemia Inhibitory Factor (LIF; Sigma, Haverhill), 10 ng/mL basic fibroblast growth factor (Peprotech, Rocky Hill, NJ), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), 1% non-essential amino acids (Gibco, Invitrogen, UK), 10 µg/mL glial cell line-derived neurotrophic factor (GDNF; Sigma-Aldrich, USA), 100 U/mL penicillin (Sigma-Aldrich, Darmstadt) and 100 µg/mL streptomycin (Sigma, Germany). Afterward, the cells were transferred into an incubator (5% CO_2, 37°C) for 2 weeks. The medium was changed every two days[21].

Alkaline phosphatase staining

Fast-Red TR/Naphthol AS-MX tablets (Sigma-Aldrich) were used for assessment of alkaline phosphatase activity. For this purpose, Tris buffer was added to the Fast-Red TR/Naphthol AS-MX tablet to form an alkaline dye and was observed under an inverted microscope (IX71, Olympus, Japan).

Identification of SSC using flow cytometry

For the identification of SSCs using flow cytometry, Gfra1(1:100; Sigma Aldrich, USA), Id4 (PA5-26976, Invitrogen, USA), rat polyclonal anti-α6-integrin (1:100; Sigma- Aldrich, USA) and rat polyclonal anti-β1-integrin (1:100; Sigma-Aldrich, USA) antibodies were added to a total number of 106 cells. Goat anti-rat

labeled with fluorescein isothiocyanate (FITC) (1:200; Sigma-Aldrich, USA) was used as a secondary antibody for Id4 using flow cytometer (FACSCalibur, BD, Franklin Lakes, New Jersey, USA). The fluorescence intensity was measured between 464 and 601 nm for control-PE and between 515 and 545 nm for control-FITC using flow cytometry.

SSC **Cryopreservation**

Single cells were suspended in 2.5×10^5 cells/mL of freezing medium containing Minimum Essential Medium Eagle - alpha modification (α -MEM) (Invitrogen) with 10% dimethyl sulfoxide (DMSO; 1.4 M, Sigma, Germany), MEM-α (Sigma, Germany), and 10% fetal bovine serum (FBS; Sigma, Germany) without additional supplements (basal freezing medium)[22] or basal freezing medium with the addition of 10 mM PTX (pH = 7.4, osmotic pressure 300 mOsm/kg, Purity(TLC) ≥ 98%, cat.no. P1784, Sigma-Aldrich), and were placed in 1.8-mL cryovials (Corning). Cryovials were frozen in a Nalgene freezing container (cat. no. Z359017, Sigma) at a rate of -1°C per minute to -80°C and stored overnight at -80°C. After overnight storage, cryovials were placed in liquid nitrogen for at least a week of storage. After removal from liquid nitrogen, samples were maintained at room temperature for 30 seconds, and then in a water bath at 37C for 2.5 minutes. The cryovial contents were transferred to a tube with prewarmed medium and diluted 1:10 with α-MEM containing 10% FBS in a drop-wise manner. The cells were washed two times with medium and centrifuged at 1200 × g for 5 minutes[22].

Cell viability assay

After thawing, cell viability was determined using Methyl thiazolyldiphenyl-tetrazolium bromide (MTT; Sigma-Aldrich) assay in control and treatment groups at doses of 5, 10, and 15 mM of PTX. 100 µl MTT reagent (5 mg/ml in PBS, pH 7.6) was added to each well of 96-well plates, and plates were incubated in 5% CO_2 for 2 hours at 37°C. After that, an equal volume of DMSO was added. The absorbance was measured at 570 nm with background subtraction at 630 nm.

Measurement of cellular reactive oxygen species

Cellular ROS was detected using specific ROS probes by flow cytometry. 10000 SSC cells were loading with 50 µm DCFH-DA (ROS Assay Kit; Beyotime, Haimen, Jiangsu, China) according to the manufacture instructions. DCFH-DA-fluorescence was measured using logarithmic amplification in the flow cytometer (Becton Dickinson, USA). Data were reported as peak fluorescence intensity between 500 and 530 nm. Experiments were performed in triplicate and repeated three times. ROS production was calculated as the intensity in the fluorescence compared with the control group. The data was analyzed with Flowjo software [version 7.6.1].

Optimization of the mouse model for infertility

For this purpose, the scrotum was excised through a midline incision. The tunica vaginalis was opened, and the left testis was exposed to the surgical field. The left testis was rotated 720° in a clockwise

direction and maintained in this torsion position by fixing the testicle to the scrotum with a 6-0 silk suture[23]. Animals underwent 2 hours of unilateral testicular ischemia. Then, the suture was removed and the ischemic testis was untwisted and replaced in the scrotum, and the incision area was closed[24]. For confirmation of model, 3 mice in each group (control, torsion and transplantation groups) were sacrificed and excluded from the study in order to examine the seminiferous tubules and confirm the torsion by cervical dislocation. After 2 weeks, TT and the testes of mice were removed, fixed in a Bouin's solution for 48 hours, embedded in paraffin, sectioned at 5-μm thickness and finally stained with hematoxylin-eosin (H&E) and the testis tissue sections were examined using an optical microscope (Nikon, Japan).

Germ cell transplantation

To detect the transplanted cells and purify them from testicular endogenous cells, 2×10^5 /ml cells were exposed to a 2 μg di-alkyl indocarbocyanine (DiI, Eugene.OR, USA) before transplantation for 5 minutes. 1 ml of PBS preservative solution was placed at room temperature and then placed in a dark place for 20 min at 4 °C. After ensuring that the cells were stained under a fluorescent microscope (Japan, Olympus, LX71), the cell surface was washed with PBS and then isolated from petri dish by trypsin enzyme (25%) in 0.1% ethylenediamine tetraacetic acid (EDTA) (Sigma, USA). After washing 3 times in the medium, they were ready to be transplanted into the host testis.

Two weeks after teticular torsion, the recipient mice in four transplantation groups, as described above (n=36) received transplantation. By clipping the abdominal hair and disinfection of the area with cedium chlorhexidini alcoholicus 0.5% (BE351513; Laboratoires Gifrer Barbezat, Décines-Charpieu, France), the surgical area was ready for use. The testes were exteriorized by incision of abdomen. Afterward, the fatty tissue around the efferent ducts was gently removed, and 12 μl of the cell suspension containing 2×10^5 cells/ml was injected into the efferent ducts using a microinjection needle under a stereomicroscope. If the tracking dye (trypan blue at the tip of the pipette) along with the injected solution entered the seminiferous tubules, the transplantation was considered as "successful".

Immunofluorescent Staining of Testis Cross Sections and Selected Germ Cells

The immunofluorescent analysis was used to explore the expression of Ddx4 and C-Kit in mice testes. After dissection of testes from the mice, testes were fixed in 4% paraformaldehyde for 12 h at 4°C, embedded in paraffin, and sectioned. Sodium citrate buffer (10 mM sodium citrate [pH 6.0]) was used for antigen retrieval by boiling the sections for 15 minutes. Endogenous peroxidase activity was blocked using blocking solution for 10 minutes at room temperature. Sections were then blocked with 10% normal goat serum, followed by incubation with respective primary antibodies of Ddx4 Ddx4 (1:500, ab13840, Abcam, UK) and C-Kit (1:500, ab5506, Abcam, UK) diluted in PBS containing 0.5% Bovine Serum Albumin (BSA) at 4°C overnight, followed by incubation with the appropriate Alexa Fluor dye-conjugated secondary antibodies FITC Goat anti-rabbit IgG H&L (ab6717, Abcam, UK). The sections were washed and incubated with DAPI (4,6-diamidino-2-phenylindole dihydrochloride hydrate) (Vector Laboratories, CA,

USA) in PBS to label the cell nuclei, followed by a fluorescence microscope with magnitude of x40. All image analysis was done by ImageJ 1.5 software.

Western blotting

All of protein was extracted from tissue using a TriPure Isolation Reagent (Roche, Germany). 20 µg of the extracted proteins were loaded on 12% SDS-polyacrylamide gels (BioRad Laboratories, Hercules, CA) and then transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Germany). The nonspecific binding was blocked by PBS buffer containing 0.1% Tween-20, 2% BSA, and 5% nonfat dry milk (Carnation, CA). Afterward, samples were incubated by primary antibodies against Id4 (1:1000, Invitrogen, US), Sycp3 (1:1000, Abcam, UK) and β-actin (1:3000, Abcam, UK) as internal control for an overnight at 4°C, and then incubated using secondary antibodies goat anti-mouse IgG H&L-HRP conjugate (1:5000, Bio-Rad, US) and HRP- conjugated goat anti-rabbit IgG H&L (Abcam, USA). Finally, rates of expression for these proteins were evaluated using enhanced chemiluminescence.

Quantitative real-time PCR

Expressions of *Tnp1*, *Tnp2*, and *Prm1* genes was assessed by quantitative real-time PCR. Extraction of total RNA from tissue samples was performed using a Trizol reagent (Roche, Germany). Reverse transcription of 500 ng of the extracted RNA into complementary DNA (cDNA) was carried out by a cDNA synthesis Kit (PrimeScript™ RT Reagent Kit Fast, RR037A, TaKaRa, Japan). PCR assay was performed using a thermocycler (Bio-Rad Laboratories) and aSYBR Green master mix (SYBR Premix Ex Taq II (Tli Plus), TaKaRa- RR820L). Samples were undergoing an initial melting stage for 5 minutes at 95°C followed by melting stage (40 cycles) for 5 seconds at 95°C and synthesis for 30 seconds at 60°C (n=3). The gene expression cycle threshold ($\Delta\Delta$Ct) values were calculated after normalizing with *Hprt* internal control. Sequences of primers are listed in the Table 1.

Statistical analysis

The normality of the distribution of variances was tested by the Kolmogorov-Smirnov test. Data were presented as the mean ± standard deviation (SD). two-group comparison was analyzed using student t-test. Statistical analysis was performed using GraphPad Prism 8.3.0 (GraphPad Software, Inc. La Jolla, California). Statistical significance was set at P-value less than 0.05.

Result

High expression of Gfra1, Id4, α6-integrin and β1-integrin in SSCs

SSCs depicted distinguish unicellular features having the ability to attach to the bottom of the culture plate. Progression into forming small clusters of cells and then colonies is apparent after extending the time of culture. These colonies are easily seen in red colors after staining with the alkaline phosphatase (Fig.1). Also, expression of markers of SSC, Gfra1, Id4, α6-integrin and β1-integrin were high in the SSCs.

The highest rate was for β1-integrin at 94.20±2.60%. Expression rates for the three markers were 93.70±2.28% (α6-integrin), 90.9±9.13% (Gfra1) and 73.70±8.03% (Id4) (Fig.1).

Cell viability

Statistically significant changes in the cell viability after administration of 10 mM PTX were seen, while there was a decrease in cellular viability after administration of this factor at doses of 5 and 15 mM. Therefore, we used the 10 mM[25] dose of this factor for the subsequent experiments (Data not shown).

Cryoprotective effect of PTX on ROS generation

As shown in the Fig.2, the level of ROS production was measured in 4 groups of SSCs including fresh, fresh+PTX, cryo and cryo+PTX. The results of this experiment showed that ROS production in the cryopreservation group (62.96±3.47%) was higher compared to fresh (12.48±2.58%, $P < 0.001$), fresh+PTX (8.16±1.16%, $P < 0.001$) and cryo+PTX (30.44±5.57%, $P < 0.01$) groups. In particular, ROS production decreased significantly in the cryo+PTX (30.44±5.57%) compared with the cryo group (62.96±3.47%, $P<0.05$); however, the difference between fresh and fresh+PTX groups was not significant ($P>0.05$). We found that PTX decreased SSC intracellular ROS generation related to the cryopreservation.

Optimization of the mouse model for infertility

Our aim was to create a mouse model that can represent the clinical condition for infertility by damaging SSC niche through testicular torsion. The histological findings in the sections are illustrated in the Fig.3 (control and torsion groups). Histological examinations of the control testes revealed seminiferous tubules (Fig.3a). The epithelium of all seminiferous tubules in the ischemic testis was severely disrupted. Most tubules were depleted to the extent that in some of them only the basement membrane was detectable (Fig.3b).

SSC labeled cells were transplanted to confirm the presence of SSCs in cell suspension as well as to assess SSCs colonization in the testis. Eight weeks after transplantation, fluorescent-labeled cells were considered transplanted cells. The labeled cells were localized in the seminiferous tubules of the recipient testes. Eight weeks after transplantation, colonization and proliferation of transplanted cells were observed. Fifteen days after transplantation, SSCs were labeled with DiI, showing the donor-derived origin of germ cells. Red light indicated that the DiI positive cells are localized in the base of seminiferous tubules and they showed homing of the transplanted cells (Fig.4).

Immunofluorescent findings showed that PTX increased expression of proliferation and differentiation markers after transplantation

As expected, expressions of Ddx4 and C-Kit were significant decreased in the torsion (4.11±2.73) vs. control (48.20±1.99) group ($P \leq 0.001$), and importantly, data from double immunofluorescence staining assays revealed that all of the transplantation groups showed a significant increase in the expression of both markers compared to the torsion group (Fig.5A, B, C, D). Similar to immunofluorescence images, the

results of quantitative analysis showed that the percentage of Ddx4 protein expression was 4.11±2.73 (P<0.0001vs control), 21.54±2.26 (P<0.0001vs control), 16.12±1.85 (P<0.0001 vs control), 43.31±1.00 (P<0.0001 vs control), and 35.92±1.28 (P<0.0001 vs control) in the TT, TT+Fresh, TT+Cryo, TT+Fresh+PTX, and TT+Cryo+PTX groups, respectively. The expression of Ddx4 protein was significantly higher in the TT+Cryo+PTX in comparison with the TT+Cryo group ($P \leq 0.001$) (Fig.5A, C). These data showed that cryopreservation leads to the reduction of Ddx4 expression. C-Kit protein almost displayed a similar expression pattern in all groups, and all transplanting groups included TT+Fresh (22.40±2.12, P<0.0001), TT+Cryo (15.24±2.99, P<0.0001), TT+Fresh+PTX (34.46±1.61, P<0.0001), and TT+Cryo+PTX (30.19±2.09, P<0.0001) had higher expression of C-Kit in comparison with the torsion group (2.90±0.60, P<0.0001). C-Kit protein expression displayed a significant increase in the TT+Cryo+PTX group compared to the TT+Cryo group (P<0.0001) (Fig.5B, D). The expression of C-Kit was also higher in PTX-treated donor cells compared with the non-treated groups (P<0.0001) (Fig.5D).

The significant expression of Ddx4 and C-Kit proteins in the cryopreservation group with PTX indicates the protective effect of PTX on cryopreservation of the SSCs before transplantation as well as its effect on proliferation (Ddx4) and differentiation (C-Kit) markers after transplantation.

PTX increases Id4 and Sycp3 protein expression

Western blot analysis showed significantly reduced expressions of Id4 and Sycp3 in the torsion group (0.41±0.21) compared to the control group (P<0.0001) (Fig.6). Expressions of Id4 showed no significant change between TT+Cryo+PTX (0.42±0.04), compared to the TT+Cryo (0.17±0.10) group during experiment (P>0.05). Analysis of the recipient's samples demonstrated a significant increase in expression of Sycp3 in TT+Fresh+PTX (0.55±0.11, P<0.01) than torsion group (0.22±0.07). The expression of Sycp3 was significantly higher in the TT+Cryo+PTX (0.40±0.08) group compared to the TT+Cryo (0.17±0.10) group (P<0.05).

According to the results, there was no significant change in the expression of Id4 between the cryopreservation group and the cryopreservation group with PTX. Therefore, PTX may play an important role in cryopreservation media by maintaining SSCs before transplantation.

PTX effect on increasing expression of *Tnp1, Tnp2* and *Prm1* genes

Tnp1 exhibited a noticeable reduction in the rate of mRNA expression after exposure to torsion (0.02±0.01, P<0.0001) and TT+Cryo (0.43±0.08, P<0.0001) group, compared with the control group. The rate of expression for this marker in TT+Cryo+PTX (0.73±0.07) group was about significantly higher than TT+Cryo (0.43±0.08) group sample (P<0.001) (Fig.7A).

Tnp2 showed a significant increase in the rate of expression for the TT+Cryo+PTX (0.81±0.03), compared to the TT+Cryo (0.39±0.04) group (P<0.001). The rate of expression for this maker in TT+Fresh (0.77±0.14) and TT+Fresh+PTX (0.92±0.10) groups was almost the same (P>0.05). An increase in the

rate of expression for *Tnp2* in TT+Cryo+PTX group was also noticeable as compared to the torsion (0.03±0.02) (P<0.0001) (Fig.7B).

Finally, *Prm1* showed a noticeable increase in TT+Cryo+PTX (0.84±0.04) group in comparison with the TT+cryo (0.38±0.07, P<0.0001) and TT (0.02±0.003, P<0.0001) group (Fig.7C).

However, there was a significant increase in the expression of these genes in the cryopreservation group with PTX compared to the cryopreservation group (P<0.0001).

Discussion

Azoospermia following cancer therapy, immunosuppressive drugs, genetic factors, environmental toxins, testicular injuries and torsion is considered to be a major factor of quality of life. Given the rising proportion of patients surviving cancer due to improved therapeutic protocols, it is an issue of growing importance. Hence, the efforts to preserve fertility have motivated researchers to develop options for the paediatric population facing fertility-threatening cancer therapies. In prepubertal boys who do not yet produce sperm, cryo-banking of testicular tissue containing spermatogonial stem cells (SSCs) is the only viable option for future fertility preservation. Transplanting the SSCs, or testicular tissue containing SSCs, back to the cured patient appears the most promising strategy[26].

To improve cell survival during freezing and thawing, cryoinjury caused by intracellular ice crystal formation has to be avoided. Addition of cryoprotective agents (CPAs) and controlling freezing and thawing rates are intended to diminish the width of the possible damage window during cryopreservation. Here we demonstrate the effectiveness of PTX as an antioxidant and apoptosis inhibitor on the recovery, proliferation, and differentiation capacity of thawed germ cells enriched for SSCs.

We found that the cells were able to form colonies by expanding the culture time. It means that the cells have self-renewal potential. By assessing Gfra1, Id4, α6-integrin and β1-integrin, the cells were also assayed for their stem cell identity and the high rates of expressions for these markers in the cells indicate that the cells are in their early stages of development in the spermatogenic lineage and do not initiate differentiation processes.

Although the previous studies demonstrate an effective way for cryopreservation, the current techniques can cause cryoinjuries due to the high rate of ROS production. In the present study, our aim was to use PTX as an antioxidant to favorably inhibit ROS production after cryopreservation and testicular torsion. However, some tissues require ROS for self-renewal, in many self-renewing tissues, ROS is harmful to stem cells. It is necessary to be mentioned that the positive role of ROS in self-renewal division has only recently begun to be analyzed. Morimoto et al. showed that in SSCs, ROS amplification plays a critical role in driving self-renewal division[27]. Moreover, excessive ROS production in the cryptorchid condition induces DNA damage in spermatogenic cells, and spermatogonia in SOD1 KO mice showed poor resistance to heat stress[28]. Since ROS can be toxic to germ cells and cause the spermatogenic defect, the success of spermatogenesis seems to be dependent on the delicate control of ROS levels. The

excessive ROS leads to the peroxidation of membrane lipids, DNA damage and protein oxidation, and has an effect on the production of mitochondrial ATP.

The data of this study confirmed a significant improvement in the reduction of ROS after the treatment of the cryopreserved SSCs with PTX for a week. In addition to PTX, the inclusion of DMSO in cryopreservation media has been proven to be beneficial for post-thaw survival and function of various stem cells[29]. To the best of our knowledge, literature has not discussed specifically about the effect of PTX on SSCs culture, the therapeutic efficacy of PTX on spermatozoa has been indicated in some previous studies. In a study conducted by Esteves *et al*[30], the acrosome reaction to ionophore challenge in cryopreserved spermatozoa was improved with PTX treatment before freezing. It has also been shown that treatment of the poor-quality human sperm with PTX may enhance post-thaw sperm fertilizing ability[31].

Therefore, we believe that PTX protects spermatogonia cells as a cryoprotective agent. Although its exact mechanism is not fully understood, two reasons are suggested: (1) PTX may cause changes in the physical state of the freezing medium or minimize cell dehydration by forming a sheath around the cells and (2) PTX can attenuate oxidative metabolism and preserve antioxidant enzyme activities.

Intracellular cAMP concentration, produced by PTX, is effective on in vitro survival, proliferation and differentiation of human germ cells cultured in a system retaining[32]. So, PTX treatment and stem cell transplantation is a hope for significant efficiency of stem cell therapy in some diseases.

After thawing, the cells were transplanted to recipient mice and the mice were sacrificed after 8 weeks for evaluation. Our results showed that transplanted SSCs started to express proliferation or premeiotic markers (i.e, Id4 and Ddx4) and differentiation or meiotic markers (i.e, Sycp3 and C-Kit). Interestingly, greater expressions of Id4 and Ddx4 was observed in the presence of 10 mM PTX. As noted in the results, the expression of ID4 and Ddx4 proliferation proteins was markedly reduced in ischemic testes and increased after transplantation. In the transplantation groups, the increase in the expression of these markers was significant in the cryopreservation group with PTX compared to the cryopreservation in the basic medium. The increased expression of these markers indicates an increase in the number and proliferation of SSCs in ischemic testicles. These results confirmed other findings of the present study indicating that the freezing medium containing PTX was able to protect SSCs from cryoinjuries during the freezing-thawing process.

C-Kit is known as an early marker of spermatogonial differentiation[33]. As a marker of spermatogonial (Spg) differentiation, C-Kit functions as an anti-apoptotic factor in primordial germ cells (PGCs), promoting cell replication in PGCs and Spg, and initiating the entry of Spg into meiosis. Activation of early meiotic markers such as Dmc1 and Sycp3 is an outcome of C-Kit activity[34]. Here, we noticed a noticeable increase in protein expression for C-Kit and Sycp3 proteins in the transplanted group with cryopreserved SSCs under the exposure to PTX in comparison with the group with the basal freezing medium.

To have more outlook toward possible influences of PTX on the differentiation of SSCs in transplanted mice, we then tried to assess post-meiotic related factors (i.e, *Tnp1*, *Tnp2*, and *Prm1*). We noticed increased mRNA expressions for *Tnp1*, *Tnp2*, and *Prm1* in transplanted mice with cryopreserved SSCs after exposure to PTX. Increased expressions for *Tnp1* and *Tnp2* in the SSCs have also been reported by others after incubation of the cells with both retinoic acid and stem cell factor [35]. *Prm1* incorporated into chromatin are required to be modified in both transcriptional and posttranscriptional levels[36]. Therefore, it would not be out of expectation to see different functions for PTX on *Prm1* in transcriptional and posttranscriptional levels. This demands ongoing studies to have more accurate interpretations of the possible effects of PTX on SSC *Prm1* expression traits.

The cAMP also appears to play important regulatory roles within germ cells, since post-meiotic germ cell differentiation is disrupted in mice carrying the mutation of the cAMP-responsive element modulator gene. Intracellular cAMP concentration, produced by inhibition of phosphodiesterase with PTX, mimics the effect of FSH on *in-vitro* survival and differentiation of human germ cells[32].

Therefore, our study showed successful spermatogenesis due to the use of PTX for long-term storage of SSCs, which can increase the probability of banking SSCs for infertile men in future recovery of spermatogenesis.

Conclusion

The only option for improvement of fertility in prepubertal boys who suffered from cancer or testicular torsion is preservation of SSCs. The autologous SSC transplantation is a technique with high promise towards a clinical application. The data have demonstrated that the presence of using 10 mM PTX in SSCs basal freezing media containing DMSO significantly improves the post-thaw proliferation capacity of germ cells enriched for SSCs.

Abbreviations

GDNF: Glial cell line-derived neurotrophic factor

Gfra1: GDNF Family Receptor Alpha 1

Id4: Inhibitor of DNA binding 4

Ddx4: DEAD box polypeptide 4

Prm1: Protamine 1

Sycp 3: Synaptonemal complex protein 3

SSCs: Spermatogonial Stem Cells

Tnp: Transition protein

Declarations

Ethics approval and consent to participate

All participants signed informed consent forms approved by the Ethical Committee of Tehran University of Medical Sciences (IR.TUMS.MEDICINE.REC.1397.210).

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that there are no conflicts of interest.

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Authors' contributions

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. MM and TR designed the study, SK supervised the data collection, GS analyzed the data, SS and HT interpreted the data and prepared the manuscript for publication, NT and NK supervised the data collection, analyzed the data, and MS reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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Tables

TABLE 1. Primers and expected length of products: forward and reverse sequences

Primer	Forward sequence	Reverse sequence	Amplicon length(bp)
HPRT	5'-GCAGCGTTTCTGAGCCATTG-3'	3'-TCATCGCTAATCACGACGCT-5'	172
PRM1	5'-ATGGCCAGATACCGATGCTG-3'	3'-GCAGCATCTTCGCCTCCTC-5'	114
TP1	5'-GAGGAGAGGCAAGAACCGAG-3'	3'-CGGTAATTGCGACTTGCATCA-5'	120
TP2	5'-AGCTCAGGGCGAAGATACAAGT-3'	3'-TCCTGTGACATCATCCCAACA-5'	107

Figures

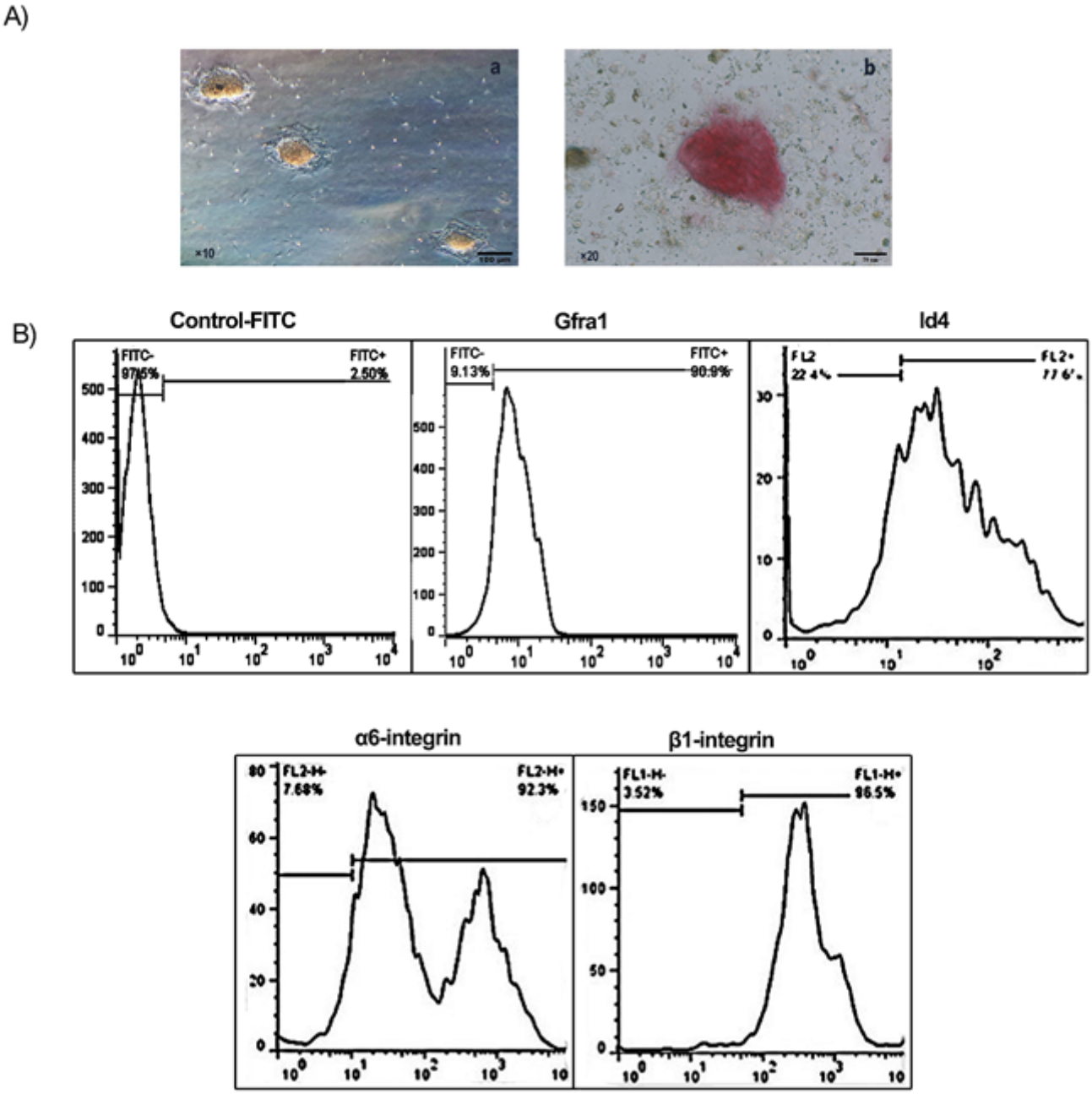

Figure 1

A, Morphological and identity of spermatogonial stem cells (SSCs). Large and dense colonies are visible in an image taken by an inverted microscope at the end of the second week of culture (a) (Scale bar = 100 μm). Results from alkaline phosphatase staining indicate apparent cellular colonies after culturing for 14 days (b) (Scale bar = 50 μm). B, Gfra1, Id4, α6- and β1-integrin were assessed by the flow cytometry to determine whether the isolated cells had SSC identity. As it is evident, these markers were expressed at high percentages in the cells.

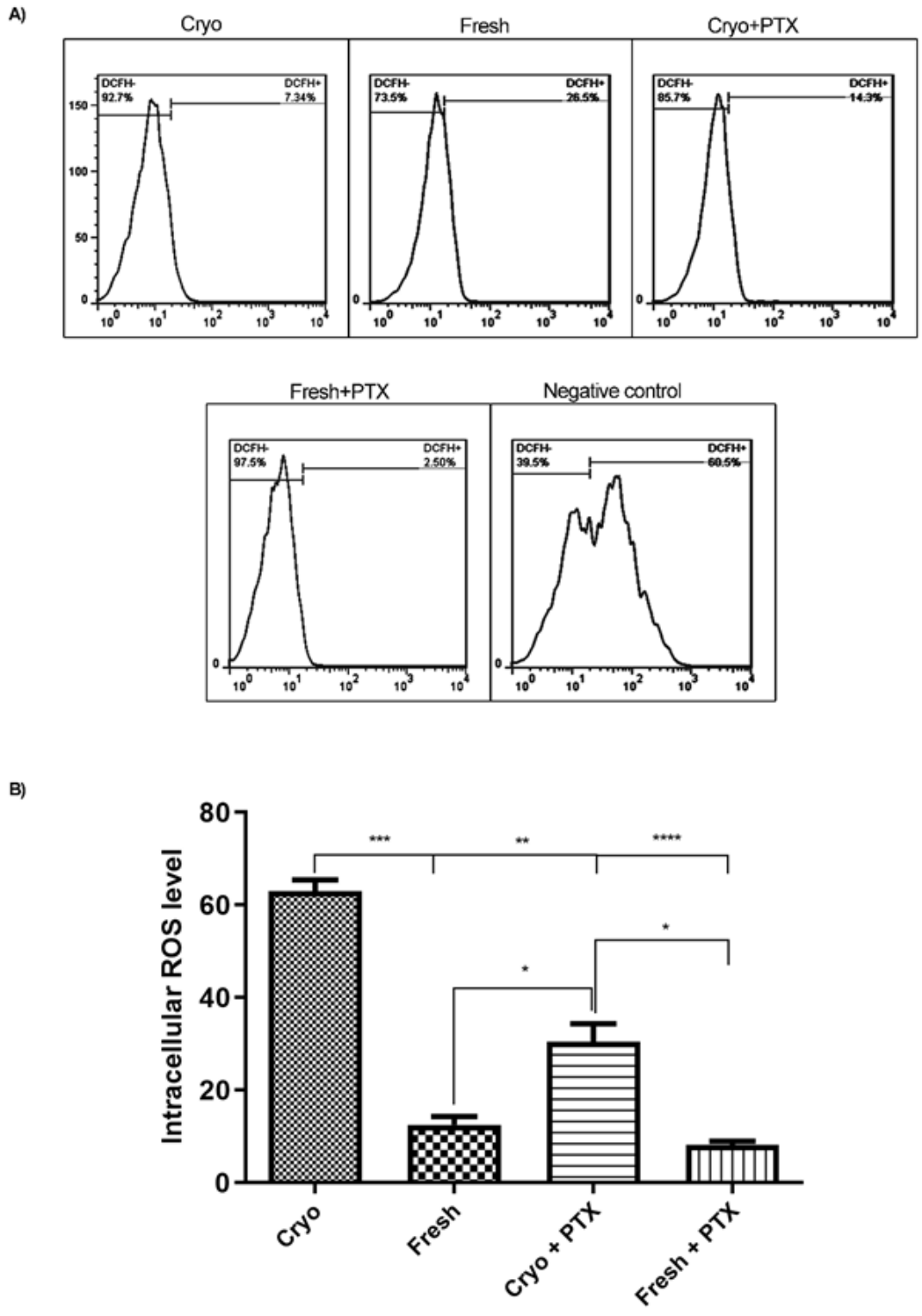

Figure 2

Flow cytometry analysis of reactive oxygen species (ROS) generation assay in spermatogonial stem cells (SSCs). A. comparative graph showing the percentage of ROS positive cells in treatment groups. B. Data are expressed as mean±SD, (*P < 0.05, **P < 0.01, ***P < 0.001)

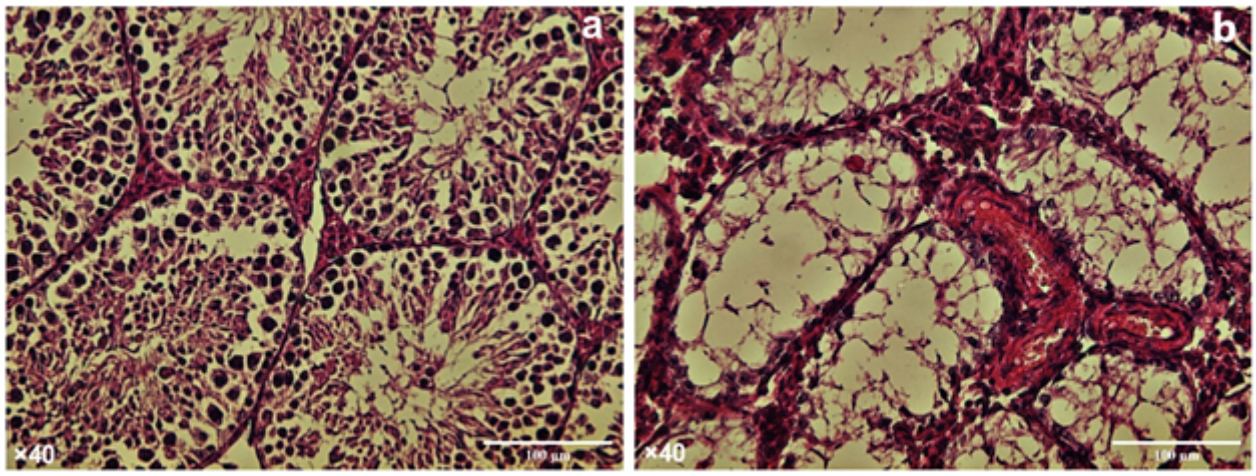

Figure 3

Evaluation of morphological changes of testicular sections by H&E staining. a: The seminiferous tubules in the control group were completely filled due to the presence of spermatogonial cells and the process of spermatogenesis. b: The epithelium of the seminiferous tubules in torsion group was completely disrupted, and most of the empty tubules had a basement membrane and Sertoli cells (scale bar = 100 μm)

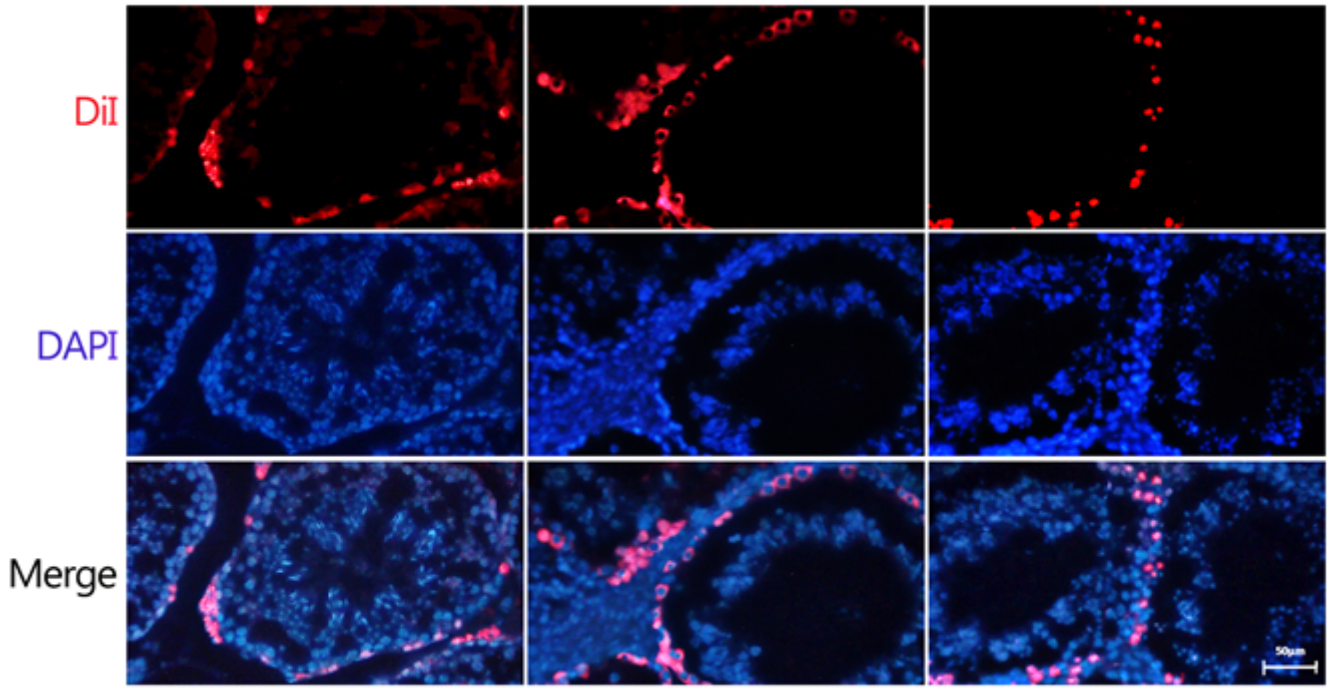

Figure 4

Detection of donor-derived spermatogenesis. The transplantation of cultured DiI+ germ cells from NMRI mice (3-6 day-old) donors allows easy visualization of donor-derived clonal spermatogenesis by

immunofluorescent staining in recipient testes. Donor germ cells form long chain-like structures at the end of week two after cellular transplantation. DiI, di-alkyl indocarbocyanine; DAPI, 4',6-diamidino-2-phenylindole

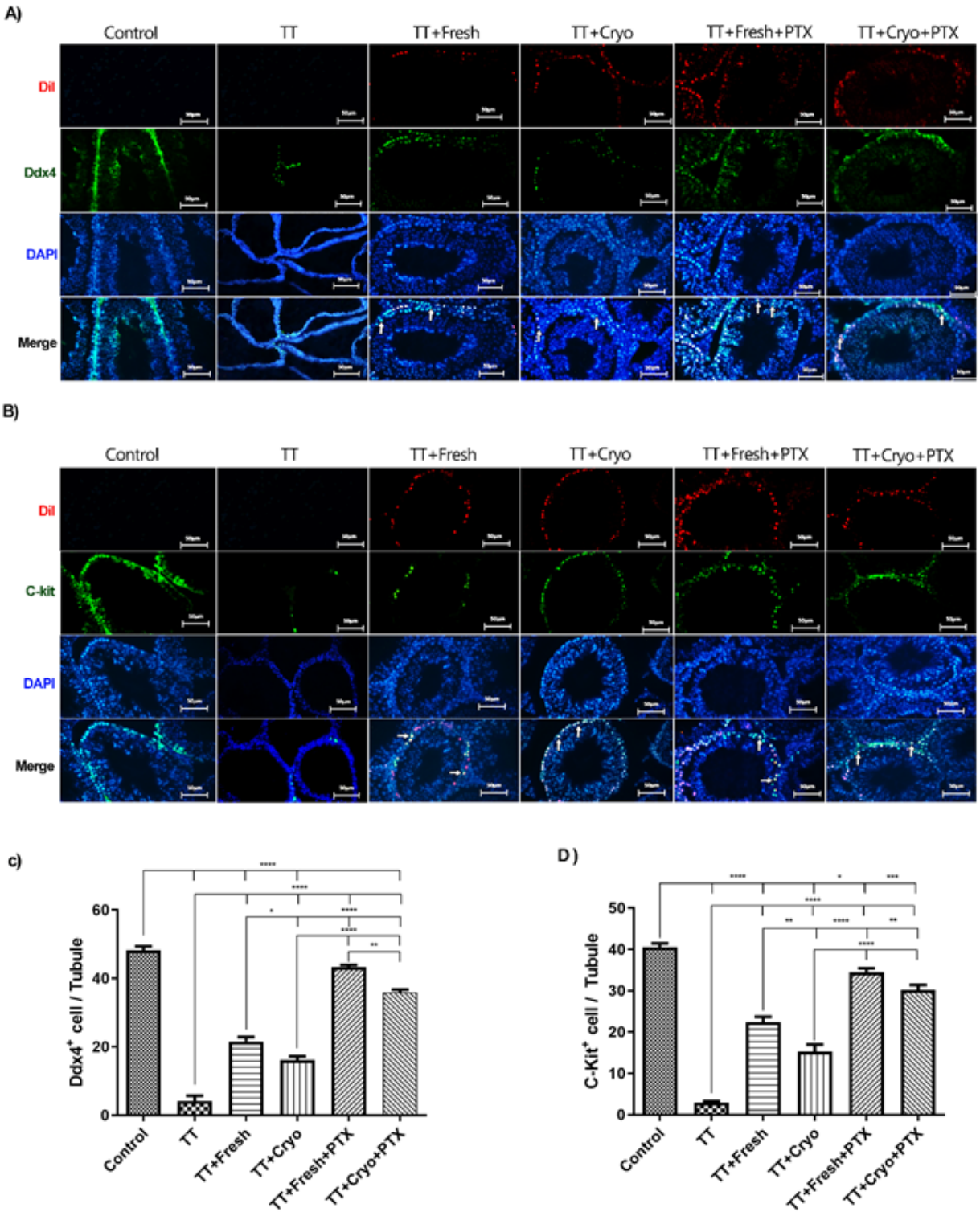

Figure 5

Immunofluorescence staining of spermatogonial stem cells (SSCs) morphology for proliferative (Ddx4) (A) and differentiation markers (C-Kit) (B) assessed 8 weeks after transplantation. Nuclei were stained by

DAPI (blue) and SSCs labled with DiI(red). Green stained SSCs represent the expression of Ddx4 and C-kit markers in control, TT, TT+ Fresh, TT+ Cryo, TT+ Fresh+ PTX, TT+Cryo+ PTX groups, respectively. (Scale bars = 50 μm) C, D, Graphs are indicative of the quantified area stained fluorescently for the markers (n = 6; *P < 0.05, **P < 0.01, ****P < 0.0001).

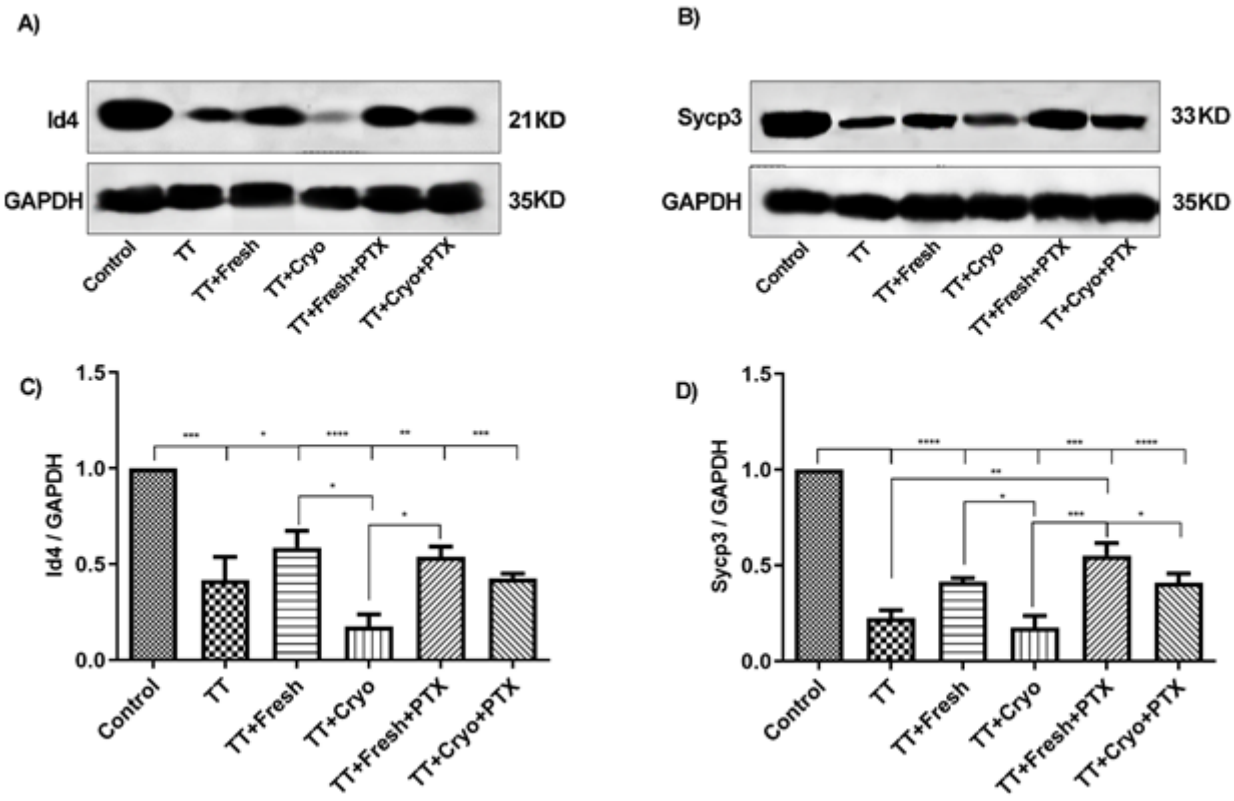

Figure 6

Western blot assay for evaluation of protein expressions for proliferative (Id4) (A) and differentiation (Sycp3) (B) markers in spermatogonial stem cells (SSCs) assessed at 8 weeks after transplantation. GAPDH was used as an internal control. C, D, Graphs are indicative of the ratio for normalization of the density of the markers to the GAPDH. (n=6; *P <0 .05, **P < 0.01, ***P < 0.001, ****p<0.0001)

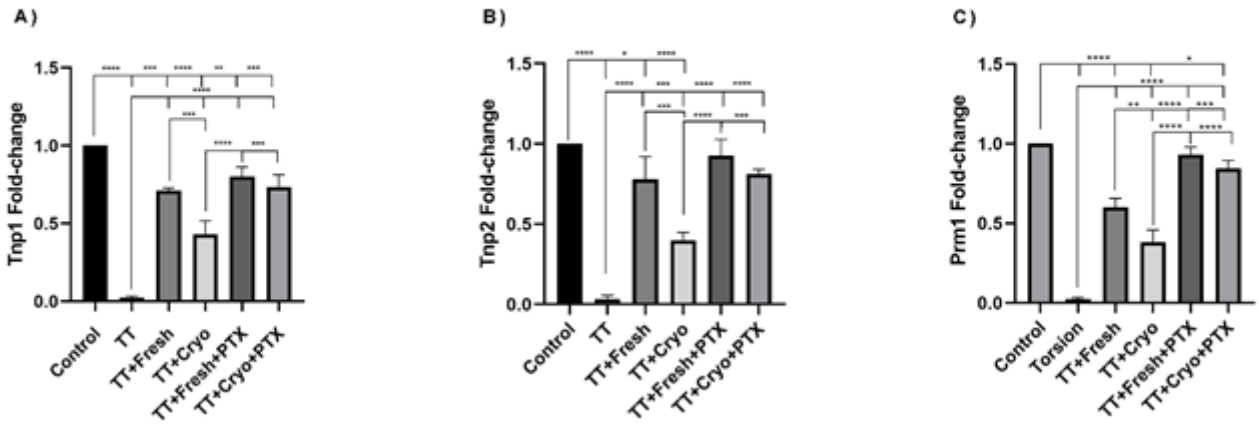

Figure 7

Evaluation of the gene expressions for Tnp1(A), Tnp2(B), and Prm1(C) by real-time PCR. Results were normalized at first to the HPRT and then to the control (n = 4,0 *p < .05, **P < 0.01, ***P < 0.001, ****p<0.0001); PCR, polymerase chain reaction

Supplementary Files

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- SupplementaryFigure.docx