

# Induction of spermatogenesis by grafting neonatal mouse testicular tissue into epididymal fat of castrated adult mouse

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## Research Article

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# Abstract

Chemotherapy treatment used for childhood cancer can cause irreversible infertility in many cancer survivors. Also, it has been known that epididymal fat is necessary for spermatogenesis. In this study, spermatogenesis development was evaluated after grafting of fresh and frozen-thawed neonatal mice testicular tissue fragments to epididymal fat of bilaterally castrated adult mice. Neonatal male mice as the donor and adult male mice as the recipient were used. After bilateral castration of recipient's mice, fresh or frozen-thawed neonatal testis tissue fragments were grafted into recipient epididymal fat. Eight weeks after implantation, grafted testicular tissue were evaluated by hematoxylin and eosin staining, real time PCR, immunofluorescence staining and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). The blood of the recipient mice was collected to measure Testosterone, FSH and LH levels. Eight weeks after implantation, a gradient of different types of germ cells from spermatogonia up to the elongated spermatids were seen. The meiotic and post-meiotic genes and proteins were upregulated in both fresh and frozen grafted groups and they confirmed the meiosis and post-meiotic progression in grafted tissues. The expression of apoptosis and necrosis genes showed no significant differences between the grafted and non-grafted control groups. There were no significant differences in hormonal assessments between control and experimental groups. Epididymal fat is an area with optimal hormonal and temperature conditions, which could support spermatogenesis in grafted immature testicular tissue. This method of grafting may pave a way for fertility preservation in childhood cancer survivors.

# Introduction

Chemotherapy and radiation treatments used for childhood cancer can cause permanent infertility in many cancer survivors. The current survival rate of 80% among these cancer patients (Gatta, Zigon, Capocaccia, Coebergh, Desandes, Kaatsch, Pastore, Peris-Bonet and Stiller) supports higher priority of fertility preservation techniques, as long-term infertility becomes a major issue compromising their quality-of-life (Wyns, Collienne, Shenfield, Robert, Laurent, Roegiers and Brichard 2015, Schover, Brey, Lichtin, Lipshultz and Jeha 2002, Sadri-Ardekani, Akhondi, Vossough, Maleki, Sedighnejad, Kamali, Ghorbani, van Wely, van der Veen and Repping 2013). Grafting the stored immature testicular tissue prior to cancer treatment has the potential to become a method of preserving fertility in these people (Wyns, Curaba, Vanabelle, Van Langendonck and Donnez 2010, Wyns, Curaba, Petit, Vanabelle, Laurent, Wese and Donnez 2011). *In vitro* mammalian spermatogenesis using either isolated cells or *ex-vivo* tissue culture has had limited success due to a lack of niche support (Galdon, Atala and Sadri-Ardekani 2016). Germ cell transplantation to recipient testes was initially developed in rodents (Brinster and Zimmermann 1994, Jiang and Short 1995) and has more recently been applied to larger animal models (Honaramooz, Behboodi, Megee, Overton, Galantino-Homer, Echelard and Dobrinski 2003, Honaramooz, Behboodi, Blash, Megee and Dobrinski 2003, Mikkola, Sironen, Kopp, Taponen, Sukura, Vilkki, Katila and Andersson 2006, Herrid, Vignarajan, Davey, Dobrinski and Hill 2006, Rodriguez-Sosa, Dobson and Hahnel 2006, Hermann, Sukhwani, Winkler, Pascarella, Peters, Sheng, Valli, Rodriguez, Ezzelarab, Dargo, Peterson, Masterson,

Ramsey, Ward, Lienesch, Volk, Cooper, Thomson, Kiss, Penedo, Schatten, Mitalipov and Orwig 2012). However, currently germ cell transplantation does not have high efficiency and is technically challenging in large animal models (Mulder, Zheng, Jan, Struijk, Repping, Hamer and van Pelt 2016, Nagano, Avarbock and Brinster 1999, Dobrinski, Ogawa, Avarbock and Brinster 1999, Shetty, Mitchell, Meyer, Wu, Lam, Phan, Zhang, Hill, Taylor, Peters, Penedo, Hanna, Orwig and Meistrich 2020). Cross-species transplantation of germ cells to the mouse testis was only successful using rats and hamsters as donors, which resulted in complete spermatogenesis (Clouthier, Avarbock, Maika, Hammer and Brinster 1996, Ogawa, Dobrinski and Brinster 1999, Zhang, Renfree and Short 2003). Differentiation of germ cells from non-rodent donors in the mouse testis could not go further than the stage of spermatogonial expansion, presumably due to an incompatibility between the donor germ cells and the microenvironment in the mouse testis (Dobrinski, Avarbock and Brinster 2000, Nagano, Patrizio and Brinster 2002). Xenografting of immature equine testicular tissue under the back skin of castrated male immunodeficient mice maintained the environment of testicular tissue and supported the progression through meiosis with appearance of haploid cells (Shutler, Tsilfidis, Leblond and Korneluk 1991). Studies with nonhuman primates have revealed that fresh prepubertal testicular tissue survives well when xenografted into the back and testis of nude mice, and is able to differentiate to full spermatogenesis (Honaramooz, Li, Penedo, Meyers and Dobrinski 2004, Ntemou, Kadam, Van Saen, Wistuba, Mitchell, Schlatt and Goossens 2019). Successful long-term survival and proliferation of human spermatogonia has been reported after xenografting of cryopreserved immature testicular tissue (Wyns, Curaba, Martinez-Madrid, Van Langendonck, Francois-Xavier and Donnez 2007, Wyns, Van Langendonck, Wese, Donnez and Curaba 2008, Goossens, Geens, De Block and Tournaye 2008). However, these studies have failed to confirm the functionality of Spermatogonial Stem Cells (SSC) in the grafted cryopreserved tissues. In those studies, germ cells could not proceed further than pachytene spermatocyte. Preserving the maximum number of functional SSC in stored testicular tissue and exploiting an optimal transplantation procedure are two key factors to guarantee the success of fertility preservation approaches in childhood cancer survivors. Therefore, selecting the best location for grafting is one of the fundamental factors in transplantation. The majority of studies have grafted testicular tissue into the subcutaneous layer of the back of the mouse, resulting in spermatogenesis arrest in early meiosis (Ntemou, Kadam, Van Saen, Wistuba, Mitchell, Schlatt and Goossens 2019, Wyns, Van Langendonck, Wese, Donnez and Curaba 2008, Jahnukainen, Ehmcke, Hergenrother and Schlatt 2007). It has been known that epididymal fat is necessary for spermatogenesis. Removal of the epididymal fat pad interrupted spermatogenesis and increased Follicle-Stimulating Hormone (FSH) concentration, however it did not affect testosterone production and serum Luteinizing Hormone (LH) concentration (Chu, Huddlestone, Clancy, Harris and Bartness 2010, Hansel 2010). When surface temperature of the scrotum and the back skin were measured with a Variotherm infrared camera, the mean temperature of the scrotum was 5°C lower than the shaved back skin surface (Luetjens, Stukenborg, Nieschlag, Simoni and Wistuba 2008). Due to the appropriate hormonal and temperature conditions of epididymal fat, we hypothesized that grafting of neonatal testicular tissue to the epididymal fat area may be an optimal site to support spermatogenesis. In this study, spermatogenesis development was evaluated after grafting of fresh and frozen-thawed

neonatal mouse testicular tissue fragments to the epididymal fat region of bilaterally orchiectomised adult mice.

## Materials And Methods

### Study design and donor testicular tissue preparation

In the experimental group, six neonatal (3–5 days old) male NMRI (Naval Medical Research Institute) mice as the donors and six adult (6–8 weeks old) male NMRI mice as the recipients were used. As the control, three neonatal and three adult male mice were used. All the animal experiments were carried out in accordance with the Institutional Animal Care and Use Committee (IACUC) of Tarbiat Modares University in Tehran, Iran.

Donor mice testes were removed and immediately transferred to ice-cold Phosphate-Buffered Saline (PBS). The tunica albuginea was removed, and the testes were cut into small fragments (approximately 1 mm<sup>3</sup>). Testis fragments were kept in Dulbecco's modified Eagle's medium (DMEM, USA, Gibco) supplemented with 10% Fetal Bovine Serum (FBS, USA) on ice. Then, testes fragments were divided in two groups as "fresh" or as fragments to be "frozen". A portion of the fresh fragments of donor testicular tissue was fixed as baseline reference (control) histology before grafting (Fig. 1).

### Freeze and thawing of neonate testis tissue

Fragments of testicular tissue were equilibrated in freezing media including DMEM with 5% FBS and 7% glycerol (cat. no. G2025; Sigma, St. Louis, MO, USA); Testis tissue fragments along with 0.45 mL of the freezing medium were packaged into 0.5-mL plastic mini straws at room temperature (one testis tissue fragment per straw). Straws were sealed and loaded into a programmable freezer (IceCube 14S; cat. no. 16821/ 2000; Minitube, Ingersoll, ON, Canada) using the following slow freezing program. Sample and chamber temperatures were monitored during the entire freezing process by inserting a thermocouple in one straw and another in the chamber. The freezing program was developed and modified based on a report on porcine testis tissue cryopreservation (Abrishami, Anzar, Yang and Honaramooz 2010). The process was initiated by maintaining the straws at 22°C for 10 min, cooling to 4°C at - 1°C/min rate, holding at 4°C for 5 min, cooling 0.3°C/min from 4°C to - 8°C, holding at -8°C for 10 min, cooling 0.5°C/min from -8°C to -50°C, then 10°C/min from - 50°C to -90°C, and holding for 10 min at -90°C. At this point, straws were plunged directly into Liquid Nitrogen (2) and stored until analysis or grafting. Prior to analysis or grafting, the cryopreserved testicular tissue fragments were thawed/warmed using the following procedure: Straws containing cryopreserved tissues were transferred from the LN2 tanks and immersed into a water bath (37°C) until the ice melted (approximately 11 sec). The sealed ends of the straws were cut, and tissues drained into 2 mL of the first thawing solution (DMEM with 20% FBS and 0.5 M sucrose) at 37°C and incubated for 1 minute. Tissues were then washed in the second solution (DMEM + 20% FBS) at 37°C for 1 to 2 minutes and kept in this medium on ice until immediate analysis or grafting.

# Grafting of testicular tissue fragments

The recipient mice were anesthetized with an intraperitoneal injection containing a mixture of ketamine (80 mg/ kg) and xylazine (10 mg/ kg) (Upjohn Pharma, Germany) in sterile physiological saline. A ventral medial incision was made in the abdominal skin of the recipient, and the testes were removed. After bilateral orchietomy, four pieces of fresh or frozen-thawed neonatal testis tissue fragments were grafted into recipient epididymal fat next to the testicular artery, and the incisions were closed by a absorbable VICRYL® (Johnson & Johnson Co., USA) suture.

## Histology

The recipient mice were sacrificed 8 weeks after grafting by cervical dislocation. The grafts were dissected from the fat and fixed overnight in Bouin's solution. After washing in 70% ethanol, grafts were embedded in paraffin (for all histological staining) and cut into 5µm sections. The tissue sections were stained with Hematoxylin and Eosin (H&E). The control neonatal testicular tissues (non-grafted) were processed in the same way. Slides were observed under an AxioPlan microscope (Carl Zeiss GmbH, Germany), equipped with an AxioCam camera, to evaluate the level of spermatogenesis and seminiferous tubule morphology.

## Real-time RT-PCR analysis

Total Ribonucleic Acid (RNA) was extracted from the control (non-grafted) and grafted testicular tissues using QIAzol (Qiagen, Germany) according to the manufacturer's recommendations. To eliminate genomic contamination, RNA was treated with Deoxyribonuclease (DNase I) (EN0521; Fermentas, Vilnius, Lithuania). Concentrations of RNA were determined by Ultra Violet (UV) spectrophotometry (Eppendorf, Germany). The cDNAs were synthesized from 500 ng DNase-treated RNA samples with a RevertAid™ First Strand cDNA Synthesis kit (K1622; Fermentas, Germany) by using oligo (dT) primers. Gene expression of PLZF (Zbtb16), Tektin 1 (Tek1), Transition Protein 1 (Tnp1), B Cell Lymphoma-Associated X (Bax), B-cell Lymphoma 2 (Bcl2), Tumor Necrosis Factor Receptor 1 (Tnfr1) and Receptor Interacting Protein Kinase-3 (Ripk3) was analyzed and Beta-actin (Actb) was used as a housekeeping gene. For Polymerase Chain Reaction (PCR) reactions, the primers gene sequences were obtained from the National Center for Biotechnology Information (NCBI) database and the sequence of their exons and introns was determined. Primer design was done using the Primer3 online software. Designed primers are blasted to confirm their accuracy and reproduce only the genes' mRNA sequences and synthesized by Cinnagen company (Table 1). PCRs were performed using Master Mix and SYBR Green I (Cat# S7563, Thermo Fisher) in an Applied Biosystems, StepOne™ thermal cycler (Applied Biosystems, USA). The program started with an initial melting cycle for 5 minutes at 95°C to activate the polymerase, followed by 40 cycles of melting (30 seconds at 95°C), annealing (30 seconds at 58°C) and expansion (30 seconds at 72°C). The quality of the PCR reactions was confirmed by melting curve analyses. Efficiency was determined for each gene by using a standard curve (logarithmic dilution series of cDNA from the testes). For each sample, the reference gene (Actb) and target gene were amplified in the same run. All runs were performed in triplicate. The target genes were normalized to the housekeeping gene and relative expression (before and

after graft of neonatal testis tissue and adult testis tissue) was determined with the  $\Delta\Delta\text{CT}$  method. cDNA from adult testis tissue was used as a positive control.

Table 1

The list and details of RT-PCR primers that were used to evaluate germ cell development, apoptosis and necrosis.

<i>Gene</i>	<i>Accession Number</i>	<i>Forward</i> <i>Reverse</i>	<i>Product size (bp)</i>
PLZF (Zbtb16)	NM_001033324.2	5'-GCTGCTGTCTCTGTGATGG-3' 5'-GGGCTGATGGAACATAGGGG-3'	154 bp
Tekt1	NM_053285.1	5'-GCTGGCTGAACATCTGG-3' 5'-TTCTTGCTGCGTGATGGC-3'	91 bp
Tnp1	NM_003284.3	5'-TGTGATGCGGCAATGAGC-3' 5'-CGACTGGGATTTACCCACTC-3'	142 bp
Bax	NM_017059.2	5'-TTTGCTACAGGGTTTCATCCAG-3' 5'-GTCCAGTTCATCGCCAATTC-3'	139 bp
Bcl2	NM_016993.1	5'-GAGAGCGTCAACAGGGAGAT-3' 5'-ACAGCCAGGAGAAATCAAACA-3'	169 bp
Tnfr1	NM_001065.4	5'-CCTACTTGGTGAGTGACT-3' 5'-ACCTGGGACATTTCTTTC-3'	134 bp
Rikp3	NM_002415.2	5'-GGAATCAGGGAGATGGAA-3' 5'-CAGTTGTTGAAGACGAGA-3'	158 bp
Actb	NM_001101	5'-TTACTGAGCTGCGTTTTACAC-3' 5'-ACAAAGCCATGCCAATGTTG-3'	90 bp

## Immunofluorescence

In order to evaluate the germ cell development, paraffin blocks fixed in Bouins solution were cut into 5  $\mu\text{m}$  sections and embedded on glass slides. After deparaffinization and rehydration, slides were washed in PBS. Then, antigen retrieval was performed in 10 mM sodium citrate/distilled water (pH 6.0) in an autoclave at 95°C for 30 min. The slides were cooled at room temperature for 30 min. The sections were permeabilized with 0.3% Triton X-100 for 30 min (Sigma, USA) and non-specific binding sites were blocked by incubating in 10% goat serum in PBS for 30 min. Then, slides were incubated with primary antibody PLZF (ab189849, Abcam, Cambridge, MA, USA), Synaptonemal Complex Protein 3 (SYCP3)

(ab97672, Abcam, Cambridge, MA, USA) and Acrosin Binding Protein (ACRBP) (ab211145, Abcam, Cambridge, MA, USA) diluted 1:200 in PBS overnight at 4°C. Following 3 washes in PBS, samples were incubated with Alexa Fluor 488 Donkey Anti-Mouse Immunoglobulin G (IgG) at a 1:500 dilution (Abcam, USA) at 37°C for 1 hour. Nucleus staining was performed using 4',6-Diamidino-2-Phenylindole (DAPI, Sigma). Prepared slides were observed under an inverted fluorescence microscope (Nikon TE 2000, Japan).

## **TUNEL assay**

Apoptotic cells in controls (non-grafted) and grafted tissues were detected by Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay. The sections were stained by using a Roche kit according to the manufacturer's instructions. At first, fixed slides were deparaffinated, dehydrated and permeabilized by 15 µg/ml proteinase K for 30 minutes at 37°C (Roche, Germany). TUNEL reaction mixture was added to slides. The sections were allowed to incubate for 1 hour at 37°C. After 3 PBS washes, the sections were incubated with Converter-POD for 30 minutes at 37°C. Nucleus staining was performed using DAPI. Prepared slides were observed under an inverted fluorescence microscope (Nikon TE 2000, Japan).

## **Endocrine assessment**

For hormonal analysis, the adult recipient mice were divided into 4 groups (non-grafted control, bilateral castrated control, fresh-grafted and frozen-thawed grafted). Eight weeks after grafting, serums were obtained from the blood sample taken during anesthesia of the mice in all 4 groups. The serum sample of each mouse was tested by a commercial kit to measure Testosterone (T) (LS-F10019, USA), FSH (LS-F9659, USA) and LH (LS-F22503, USA).

## **Statistical analysis**

Data were reported as the mean ± standard error. ANalysis Of VAriance (ANOVA) and independent sample t-tests were utilized to compare data by using the Statistical Package for Social Sciences (SPSS) software, Version 18.0 (SPSS Inc., USA). P values less than 0.05 were considered statistically significant (supplementary Fig. 1).

## **Results**

### **Survival of grafted tissue**

The survival and growth of the grafted tissue was easily observed in the epididymal fat of the recipient mice. Figure 1 shows a typical example of the epididymal fat with surviving fresh (1A) and frozen-thawed (1B) testis tissue grafts. Formation of blood supply inside the grafted tissues was obvious (Fig. 1). At 8 weeks after transplantation, 77.8% (14/18) of the fresh grafts and 61.11% (11/18) of the frozen-thawed grafts were recovered. Graft recovery is defined as the detectable graft collected in the 8 weeks after

transplantation. This difference was statistically no significant between the fresh and frozen-thawed groups (Fig. 1C).

## **Histological assessment of spermatogenesis in grafted tissue**

At the time of grafting, the seminiferous tubules of testicular tissue from donor mice only consisted of two types of cells: spermatogonia as the only germ cell type and sertoli cells (Fig. 2A, B). Typical morphology of grafts are shown in Fig. 2 (fresh and frozen-thawed grafts). The seminiferous tubules in the fresh and frozen-thawed groups showed variable degrees of spermatogenic activity and contained different types of cells comparable with the mature testis as a control, ranging from undifferentiated spermatogonia up to elongated spermatid. All the grafts showed some degree of spermatogenesis, as indicated by at least one seminiferous tubule with developing germ cells.

## **Gene expression analyses of grafted testis tissues**

The expression of genes that are important for testis development, apoptosis and necrosis were determined. Expression of PLZF as an undifferentiated spermatogonial cell marker in the adult as a control group was significantly lower in comparison with other groups, either grafted or control (non-grafted) neonatal testes tissues (Fig. 3). The meiotic (*Tekt1*) and post-meiotic (*Tnp1*) genes were upregulated in both fresh and frozen-thawed grafted groups, and they were significantly different from fresh and frozen-thawed control non-grafted neonatal groups (Fig. 3).

The expression of the *Bax* gene as an apoptosis marker in the fresh neonatal group (non-grafted) was significantly lower compared with other groups. The expression of the *Bcl2* gene as an anti-apoptosis marker in the frozen-thawed neonatal group (non-grafted) was significantly lower than other groups (Fig. 4A).

The expression of *Tnfr1* and *Ripk3* genes as necrosis markers was determined in the four groups: fresh non-grafted necrosis model (induced torsion), fresh grafted, and frozen-thawed grafted. The expression of necrosis markers in the frozen-thawed neonatal grafted group was not significantly higher than the non-grafted and fresh grafted groups (Fig. 4B).

## **Immunofluorescence**

The expression of spermatogenesis development stage specific markers, the PLZF (undifferentiated spermatogonial cell marker), SCP3 (spermatocyte marker), and ACRBP (elongated spermatid marker) proteins, were analyzed by immunofluorescence staining 8 weeks after grafting. The fresh non-grafted neonatal and adult testis tissues were used as a control (supplementary Fig. 2). The PLZF marker was expressed in fresh non-grafted neonatal, adult testis, and grafted testis tissues but SCP3 and ACRBP were only expressed in adult and grafted testis tissues (Fig. 5).

## **Apoptosis assay**

TUNEL assay was used for detecting DNA fragmentation and defining the apoptosis level of grafted tissue. The fresh and frozen-thawed non-grafted neonatal testis tissues were used as controls. The frequency of seminiferous tubules with apoptotic cells and number of apoptotic cells per seminiferous tubules in the grafted groups were higher than the control (non-grafted) groups and in the frozen-thawed groups were higher than fresh groups (Fig. 6).

## Hormonal evaluation

Measuring the level of total Testosterone in the mouse blood serum of the grafted and non-castrated groups showed the highest amount, whereas Testosterone level was extremely low in the castrated group (Fig. 7A). Serum FSH level was significantly higher in the castrated group than other groups (Fig. 7B). Serum LH level of the intact group was significantly lower than other groups (Fig. 7C). Also, a tendency for high LH serum level in the castrated group, low serum LH level in the intact group and mid-range serum LH level in grafted groups can be observed.

## Discussion

Long-term infertility is a substantial side effect of many cancer treatments, as both radiotherapy and chemotherapy have deleterious effects on the differentiating spermatogonia (Howell and Shalet 2001, Krassas and Pontikides 2005). Although adult cancer patients can preserve their semen before starting therapy, prepubertal boys do not have this option, because their spermatogenesis has not yet started. Thus, new fertility preservation techniques are required to be developed for prepubertal boys who need to undergo a gonadotoxic treatment. Storing and transplanting testicular tissue may be a promising strategy that can benefit cancer patients.

Testicular tissue grafting without vascular anastomosis is dependent on the development of new vascular blood vessels to support spermatogenesis activity and reduce ischemic injury (Ntemou, Kadam, Van Saen, Wistuba, Mitchell, Schlatt and Goossens 2019). It has been shown that spermatogenesis is improved and apoptosis is reduced in grafted testicular tissue using various methods including the utilization of factors affecting angiogenesis such as growth factors (Schmidt, de Avila and McLean 2006, Ntemou, Kadam, Van Laere, Van Saen, Vicini and Goossens 2019), hydrogels, or tissue engineering scaffolds (Poels, Abou-Ghannam, Decamps, Leyman, Rieux and Wyns 2016). In the present study, due to adequate blood supply, the apoptosis and necrosis in the grafting group were not significantly different from the non-grafted fresh group.

The site of the graft plays a crucial role in the regeneration process, resulting in the growth of grafted tissue and the improvement of spermatogenesis. Generally, based on previous studies, researchers believe that transplantation to orthotopic sites is more successful than to heterotopic sites. However, due to certain advantages such as easier access to heterotopic sites, attempts to improve methods using this location have continued (Luetjens, Stukenborg, Nieschlag, Simoni and Wistuba 2008). The advantage of orthotopic grafting is that the testicular tissue is transferred into its natural environment including the

presence of high testosterone levels and not being exposed to hyperthermic conditions, which is the case with subcutaneous grafting. The meiotic arrest was reported in ectopic marmoset testicular grafts, whereas complete spermatogenesis was observed in scrotal transplants (Luetjens, Stukenborg, Nieschlag, Simoni and Wistuba 2008). In women, intra-ovarian transplantation has been performed after gonadotoxic treatments with follicular development and restoration of ovulatory cycles (Donnez, Dolmans, Demylle, Jadoul, Pirard, Squifflet, Martinez-Madrid and van Langendonck 2004).

Coulter *et al.* compared the under scrotal skin surface temperature (ectopic transplantation site) with the deep testicular temperature (orthotopic transplantation site). They found a substantial difference of around 5°C between two sites. It has been shown that the temperature of the scrotal surface (30°C) and inside the testis (35°C), are lower than that of the ectopic transplantation site on the backs of the animals (39). This may inhibit the germ cell differentiation. The higher temperature at the ectopic transplantation site on the back of the animals may contribute to the developmental arrest of germ cells. Such impact on germ cell maturation has been reported in multiple species (Jia, Hikim, Lue, Swerdloff, Vera, Zhang, Hu, Li, Liu and Wang 2007, Schwalm, Gauly, Erhardt and Bergmann 2007) and was also used as an experimental method for male contraception. Therefore, various factors are effective in the survival of grafted testicular tissue and the resumption of spermatogenesis, the most important of which is the suitability of grafting site temperature and hormonal conditions. In our study, epididymal fat was used as a graft site. Epididymal fat was considered to be a proper site for transplantation and supporting spermatogenesis with the production of special agent(s) in its place (Chu, Huddleston, Clancy, Harris and Bartness 2010, Hansel 2010), and also having a lower temperature than other parts of the body. Our findings show that testis grafting into epididymal fat, as described here, provides optimal results in terms of graft survival and functionality.

In this study, we evaluated spermatogenesis development after grafting fresh and frozen-thawed neonatal mouse testicular tissue fragments to the epididymal fat region of bilateral castrated adult mice. Our results showed that neonatal mouse testis tissue is able to survive when transplanted into the epididymal fat of castrated adult mice, and that spermatogenesis up to the level of elongated spermatids can be observed after 8 weeks.

In the present research, based on the results obtained from H&E staining, an increase in the size and diameter of seminiferous tubules as well as improvement and the resumption of spermatogenesis in the fresh and frozen-thawed groups were observed. We found an adequate blood supply generated in the grafted tissue, with optimal growth and differentiation of germ cells in seminiferous tubules. In previous studies, an increase in the size and diameter of the seminiferous tubules was observed with growth and differentiation of germ cells up to the stage of spermatocyte, however, meiosis had not been achieved and Jahnukainen *et al.* reported sperm production when prepubescent monkey testis fragments were autologously transplanted in adult castrated host animals (Jahnukainen, Ehmcke, Nurmio and Schlatt 2012, Yamini, Pourmand, Amidi, Salehnia, Ataei Nejad and Mougahi 2016). Van Saen *et al.* found that four months after transplanting the fresh and frozen-thawed human immature testis tissue into the back

skin of the immunodeficient mice, no meiosis occurred of the prepubertal grafted tissues (Van Saen, Goossens, Bourgain, Ferster and Tournaye 2011).

In our study, the expression of the PLZF gene was not significantly different in grafted groups compared with immature groups as the control. Expression in the Tekt1 and Tnp1 genes as meiotic and post-meiotic markers, up-regulated after grafting, comparable with the control adult group. The expressions of the apoptosis and necrosis markers in the grafted groups showed no significant difference in comparison with control groups. According to H&E staining and immunofluorescence results, the presence of SYCP3 and ACRBP cells confirmed the progression of germ cell differentiation in grafted seminiferous tubules. Expression of PLZF as an undifferentiated spermatogonial cell marker stayed stable post-grafting.

In our study, the amount of cell apoptosis in grafted and control groups were evaluated using the TUNEL technique. We found that the number of apoptotic cells was higher in the frozen-thawed group than in the fresh group. Previous studies have also shown higher cell apoptosis in the frozen-thawed group compared to the fresh group (Gholami, Hemadi, Saki, Zendedel, Khodadadi and Mohammadi-asl 2013).

This study showed that the level of serum Testosterone in castrated recipient animals returned to normal either after grafting the fresh or frozen-thawed tissue.

## **Conclusion**

According to the results obtained from this study, spermatogenesis development including growth and differentiation of germ cells was observed in the grafted neonatal testis tissue to epididymal fat of castrated adult mice. The results of this study showed an abundant improvement in spermatogenesis compared to ectopic grafting sites. Therefore, epididymal fat can be used as a suitable site for transplantation and inducing the process of spermatogenesis in future pre-clinical studies to preserve the fertility of childhood cancer survivors.

## **Declarations**

### **Ethics approval and consent to participate**

Animal investigations were approved by the Ethics Committee of Tarbiat Modares University, Iran (IR.MODARES.REC.1398.070).

### **Consent for publication**

This paper is approved by all authors for publication.

### **Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Competing interests

The authors declare that they have no competing interests.

## Funding

Not applicable

## Author's contributions

H.Eyni and Z. Mazaheri designed the study and they were a major contributor in writing, conducting the manuscript and some tests. T. Dickerson and H. Sadri-Ardekani analyzed and interpreted the data. M. Movahedin implemented some tests. All authors read and approved the final manuscript.

## Acknowledgements

Not applicable

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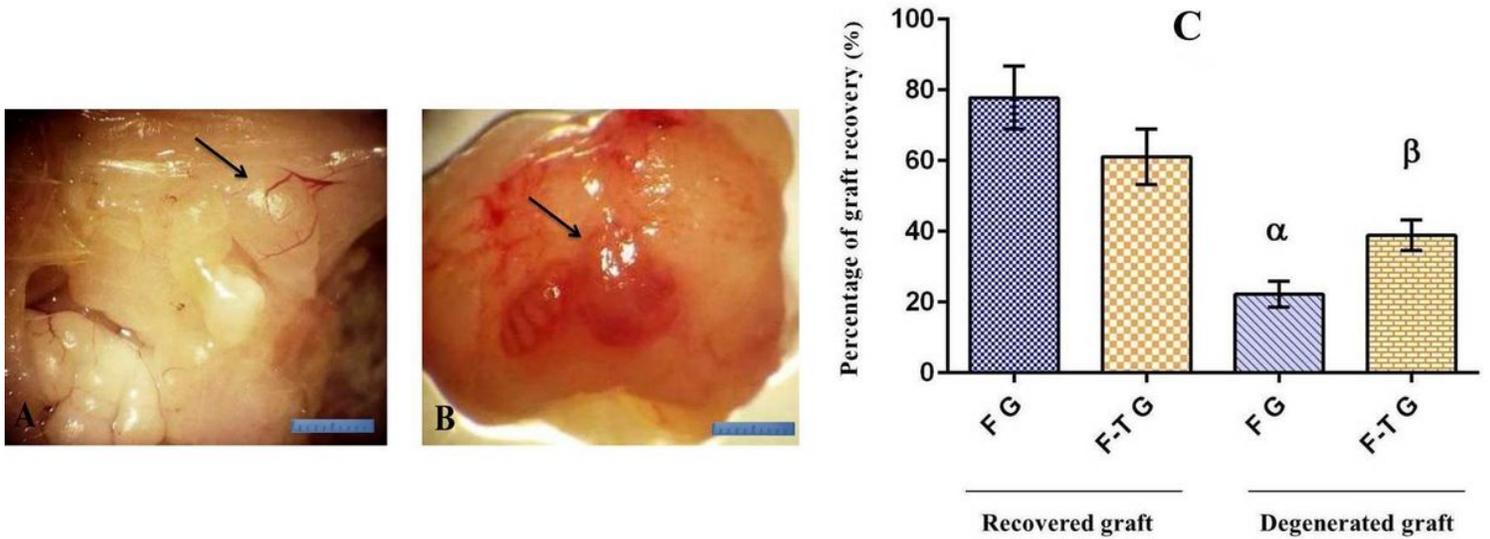
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## Table

**Table 1.** The list and details of RT-PCR primers that were used to evaluate germ cell development, apoptosis and necrosis.

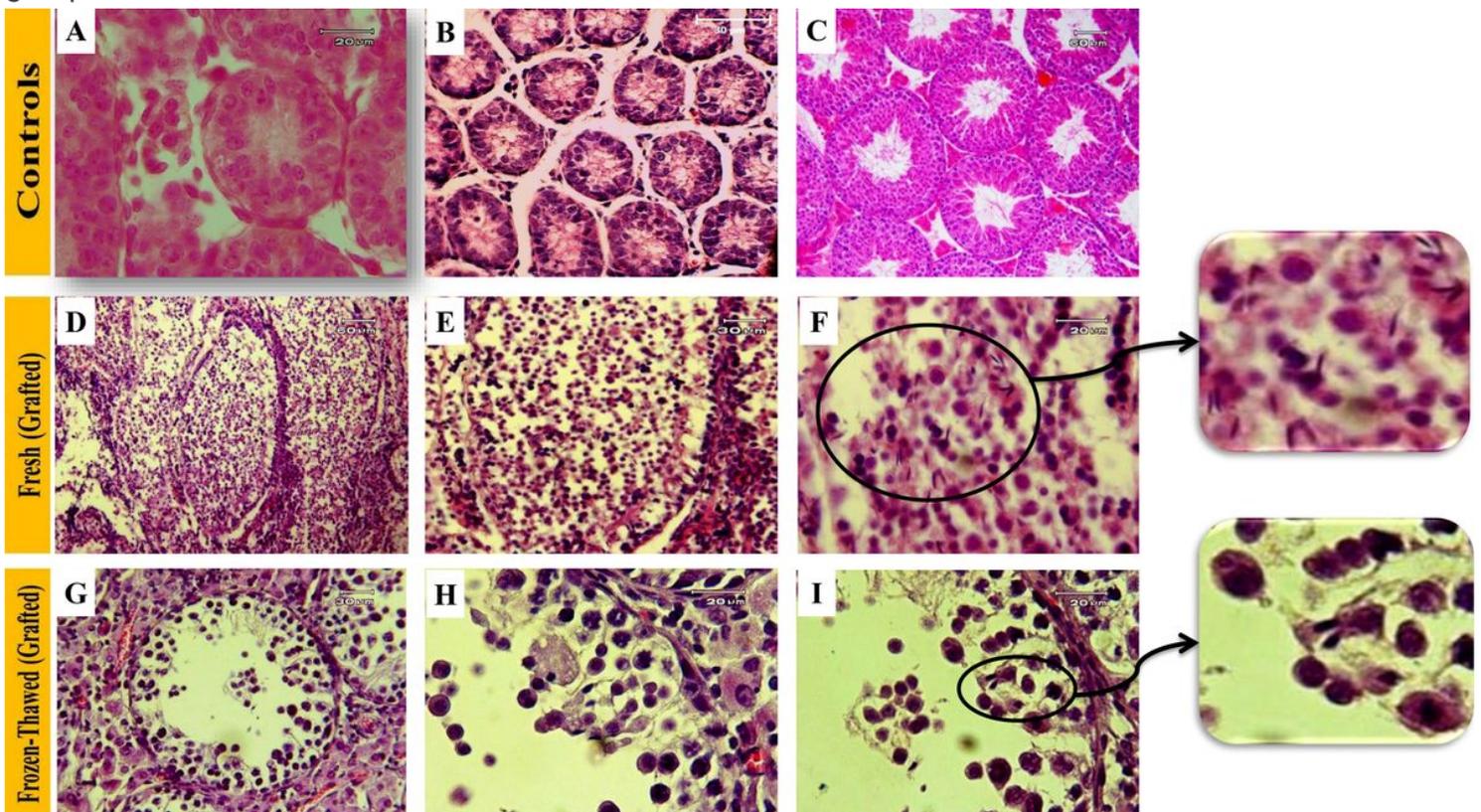
<i>Gene</i>	<i>Accession Number</i>	<i>Forward Reverse</i>	<i>Product size (bp)</i>
PLZF (Zbtb16)	NM_001033324.2	5'-GCTGCTGTCTCTGTGATGG-3' 5'-GGGCTGATGGAACATAGGGG-3'	154 bp
Tekt1	NM_053285.1	5'-GCTGGCTGAACATCTGG-3' 5'-TTCTTGCTGCGTGATGGC-3'	91 bp
Tnp1	NM_003284.3	5'-TGTGATGCGGCAATGAGC-3' 5'-CGACTGGGATTACCCACTC-3'	142 bp
Bax	NM_017059.2	5'-TTTGCTACAGGGTTTCATCCAG- 3' 5'-GTCCAGTTCATCGCCAATTC-3'	139 bp
Bcl2	NM_016993.1	5'-GAGAGCGTCAACAGGGAGAT-3' 5'-ACAGCCAGGAGAAATCAAACA- 3'	169 bp
Tnfr1	NM_001065.4	5'-CCTACTTGGTGAGTGACT-3' 5'-ACCTGGGACATTTCTTTC-3'	134 bp
Rikp3	NM_002415.2	5'-GGAATCAGGGAGATGGAA-3' 5'-CAGTTGTTGAAGACGAGA-3'	158 bp
Actb	NM_001101	5'-T TACTGAGCTGCGTTTTACAC-3' 5'-ACAAAGCCATGCCAATGTTG-3'	90 bp

## Figures



**Figure 1**

Morphological study and graft recovery rate of fresh and frozen-thawed grafted testis tissues in epididymal fat region. A: Fresh grafted neonate testis tissue (arrow) in the epididymal fat of mature mouse (8 weeks after graft). B: Frozen-thawed grafted neonate testis tissue (arrow) in the epididymal fat of mature mouse (8 weeks after graft). C: At 8 weeks post-transplantation, the percentage of recovered graft showed no significant difference in the rate of surviving graft harvested from fresh and frozen-thawed tissues. (F G: Fresh grafted, F-T G: Frozen-thawed grafted). α, β : significant difference with other groups. Scale bar: 10mm.



**Figure 2**

Histological appearances of fresh and frozen-thawed grafted testis tissues in epididymal fat region. Hematoxylin and eosin (H&E) staining of fresh and frozen-thawed mouse neonate testis tissue before and after grafting. A: Fresh neonate testis tissue (before graft). B: Frozen-thawed neonate testis tissue (before graft). C: Adult testis tissue. D-F: Fresh grafted testis tissues (8 weeks after graft). G-I: Frozen-thawed grafted testis tissues (8 weeks after graft). Insert shows the area with elongated spermatids.

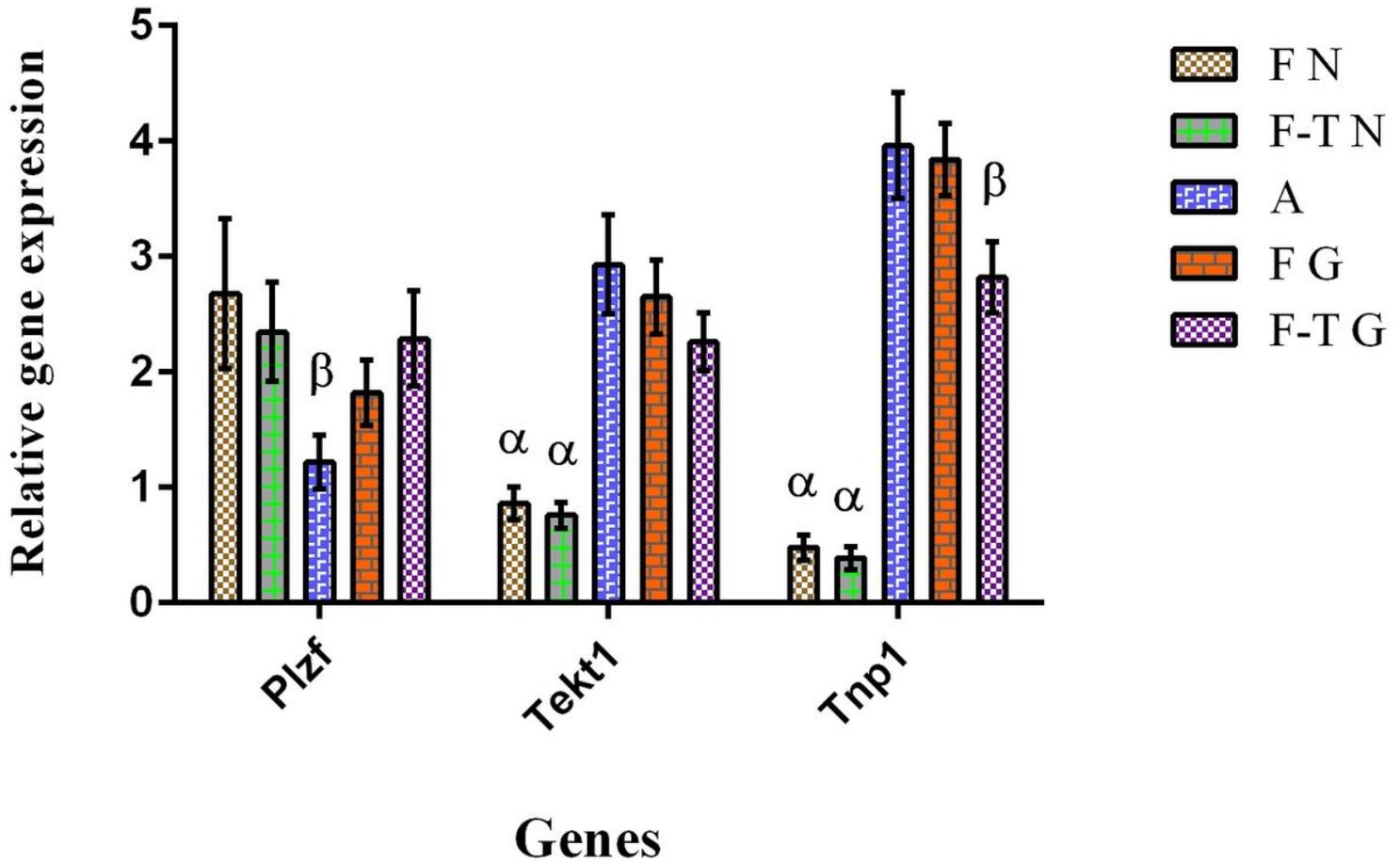
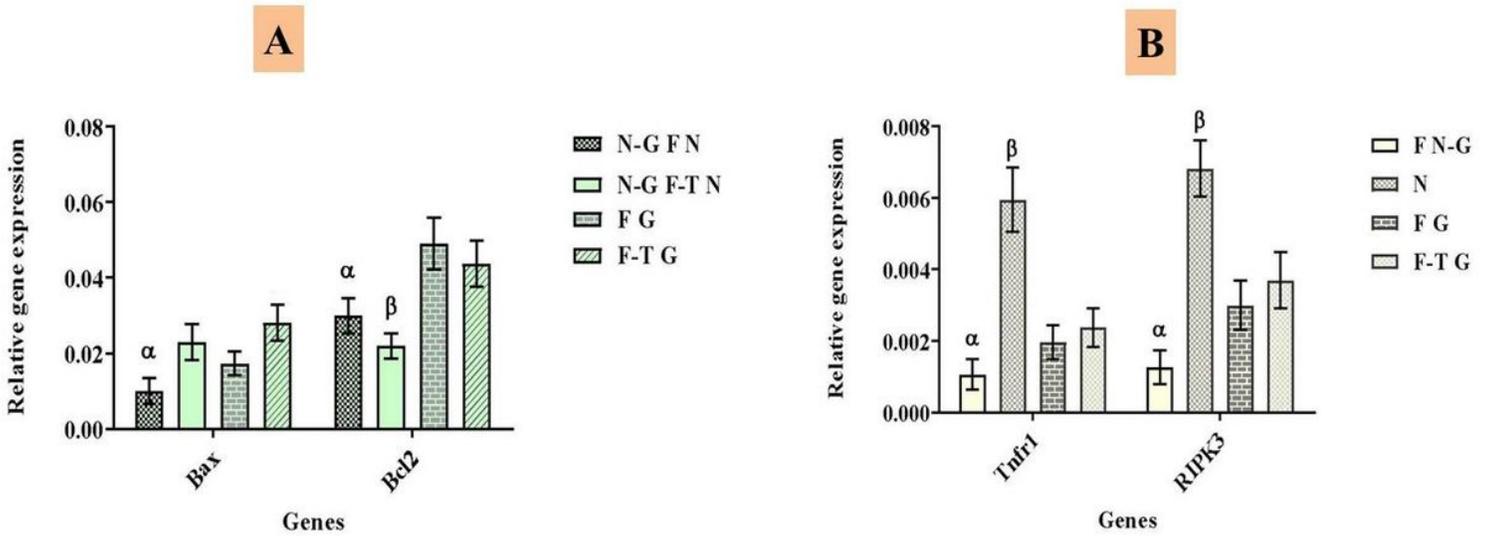


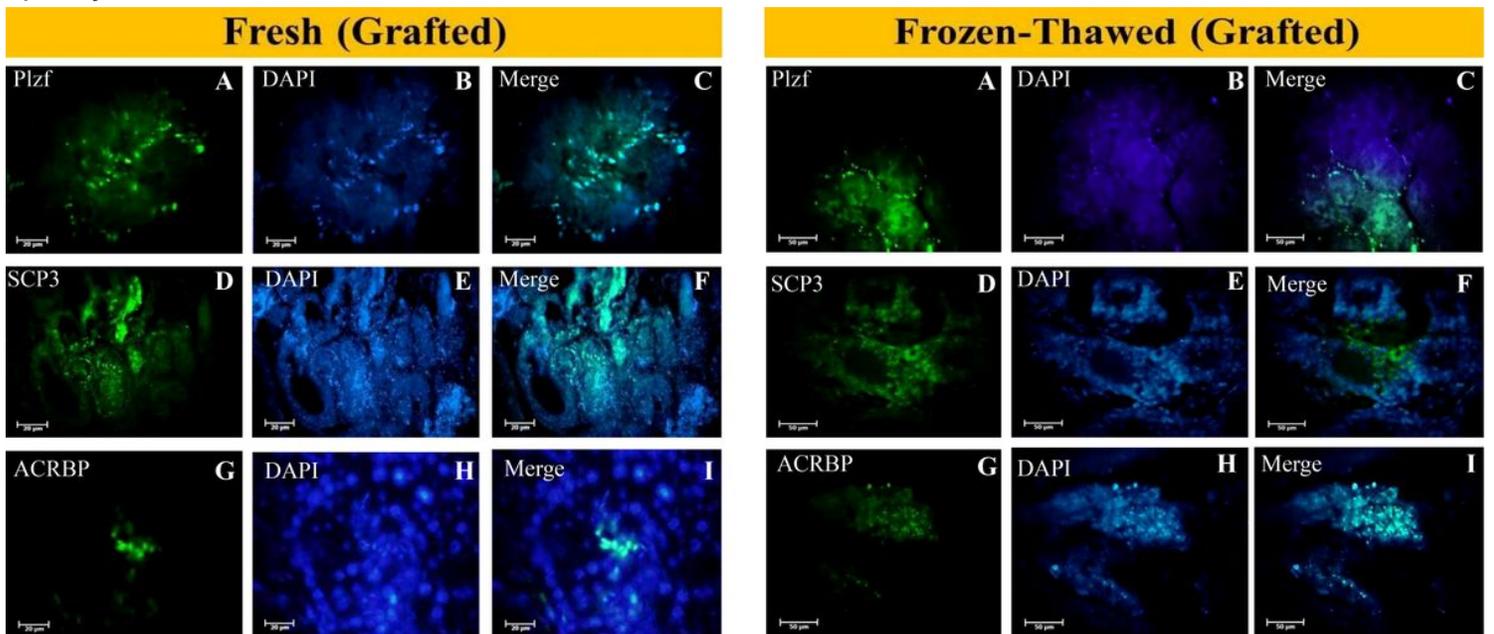
Figure 3

Pre- and post-meiotic germ cell development. Real time-PCR analysis of spermatogenesis genes expression. F N: Fresh neonate, F-T N: Frozen-thawed neonate, A: Adult, F G: Fresh grafted and F-T G: Frozen-thawed grafted. mRNA levels were normalized with respect to Actb, as an internal control. Bar graphs show mean expression values ( $\pm$  SEM, n=3; P < 0.05).  $\alpha$ ,  $\beta$  : significant difference with other groups in the same gene.



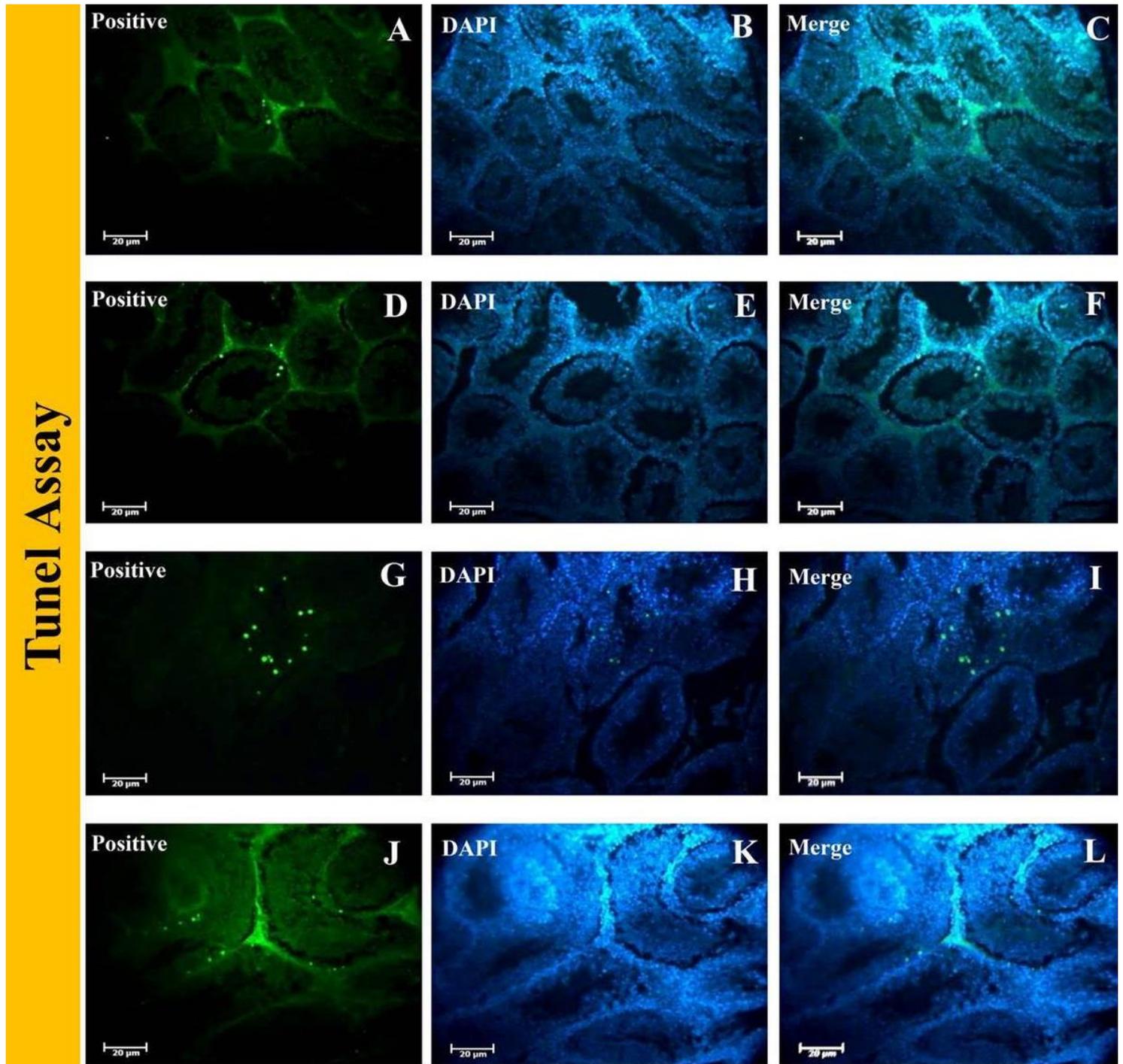
**Figure 4**

Cell apoptosis and necrosis evaluation of grafted testis tissues in epididymal fat region. A) Real time-PCR analysis of apoptosis genes expression. N-G F N: Non-grafted fresh neonate, N-G F-T N: Non-grafted frozen-thawed neonate, F G: Fresh grafted and F-T G: Frozen-thawed grafted. B) Real time-PCR analysis of necrosis genes expression. F N-G: Fresh non-grafted, N: Necrosis model (induced torsion\*), F G: Fresh grafted and F-T G: Frozen-thawed grafted. mRNA levels were normalized with respect to Actb, as an internal control. Histograms show mean expression values ( $\pm$  SEM, n=3; P < 0.05).  $\alpha$ ,  $\beta$ : significant difference with other groups in the same gene. \*: Testicular torsion was achieved via low, ventral midline incision. Each testis was exposed through the incision, and the gubernaculum and the avascular epididymo-testicular membrane were incised. The testis was rotated 720° for 2 hours.



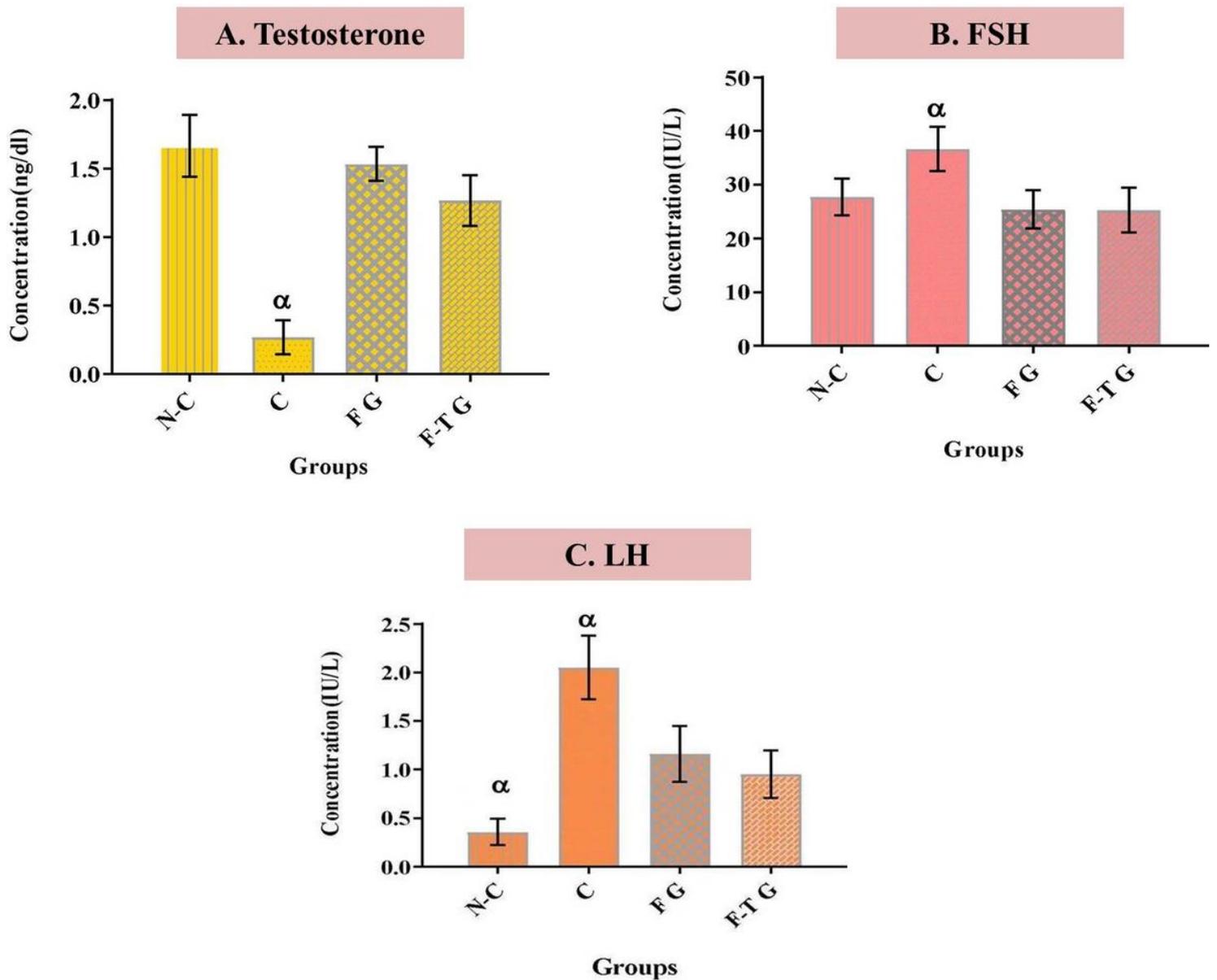
**Figure 5**

Different types of germ cells in grafted fresh and frozen-thawed neonatal testis tissues in epididymal fat region. Immunostaining of fresh and frozen-thawed grafted testis tissues groups for PLZF (Zbtb16), SCP3 and ACRBP. A-C: PLZF, D-F: SCP3 and G-I: ACRBP. Nuclei were stained with DAPI.



**Figure 6**

DNA integrity of grafted testis tissues in epididymal fat region. Tunel assay of control and experimental groups. A-C: Fresh non-grafted neonate testis tissue, D-F: Fresh grafted neonate testis tissue, G-I: Frozen-thawed non-grafted neonate testis tissue and J-L: Frozen-thawed grafted neonate testis tissue. Nuclei were stained with DAPI.



**Figure 7**

Serum Testosterone, FSH and LH Levels. N-C: Non-castrated adult mouse, C: Castrated adult mouse, F G: Fresh grafted and F-T G: Frozen-thawed grafted. Histograms show mean expression values ( $\pm$  SEM, n=3;  $P < 0.05$ ).  $\alpha$ : significant difference with other groups in the same hormone.

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