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The effects of different type of cryopreservation on testicular tissue of mice: histological changes study

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Abstract

Cryopreservation is a process where cells or tissues are preserved by cooling to low sub-zero temperatures, such as (typically) -80°C or -196°C and this process used extensively in programs of in vitro fertilization (IVF). This study is an attempt to evaluate DNA fragmentation in the cell after cryopreservation/thawing cycle when using different types of cryoprotectant (CPA). Now with the recent improvement of assisted reproductive technologies and possibility of using testicular spermatozoa or epididymal spermatozoa at in vitro fertilization by intracytoplasmic sperm injection (ICSI), the cryopreservation of testicular tissue become widely used in a male who was treated by chemotherapy or radiotherapy because these therapeutic applications can lead to sterility. This study is an attempt to investigate the changes that occur in mice testicular tissue after cryopreservation/thawing cycle as a model for human being after cryopreservation/thawing cycle when using different types of cryoprotectant (CPA). Sixty mature fertile male mice were used in the current study, the mean age of these mice was ten weeks. Fifteen of them were considered as a control group and the rest (forty-five) as a cryopreserved group, this group was divided into three subgroups according to the type of cryoprotectant (glycerol, 1, 2 propanediol and dimethylsulfoxide), each subgroup composed from fifteen mice. The testes were cryopreserved for six weeks then histological evaluation was done by paraffin section. The microscopical observation of slides obtained from cryopreserved testis differs according to the type of cryoprotectant, in the dimethylsulfoxide group the tissue appeared well-preserved cell and mild changes in the interstitial tissue, but the glycerol group shown moderate changes in testicular tissue after cryopreservation such as; rupture of the stroma, detachment the cells form basement membrane in the seminiferous tubules, gaps in germinal epithelium and folding in the lamina propria, while in the 1,2 propanediol group the tissue shows sever changes such as distraction the interstitial tissue, necrotic cells in the seminiferous tubule and difficult to recognize the cells in the seminiferous tubule. From the results of the present study, it was concluded that there was alteration in testis histology in the testicular tissue after cryopreservation /thawing cycle differs according to the type of cryoprotectant and the Dimethylsulfoxide cryoprotectant provides good protection to the testicular tissue and DNA in cryopreservation /thawing cycle than glycerol and 1, 2 propanediol (1, 2 propanediol was not preferable in testicular tissue cryopreservation).

Keywords: Cryopreservation; DNA; Testicular tissue of mice

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Introduction

Chemotherapy, immunosuppressive or cytotoxic therapy, and bone marrow transplantation are widely used to treat patients with aggressive tumors (such as Hodgkin's disease and leukemia) through the ability of this treatment to targets rapidly dividing cells and able to cross blood-testis barrier so that these treatments will be influenced directly on normal spermatogenesis of the testis leading to testicular atrophy, oligozoospermia, azoospermia and infertility [1], under this condition semen cryopreservation and/or testicular tissue cryopreservation is an option to preserving fertility for adult males, but only testicular tissue cryopreservation is an option for conserving fertility in prepubertal boys [2].

Also, testicular tissue cryopreservation was indicated in pre and post-puberty male before direct testicular irradiation for the treatment of testicular cancer or total body irradiation is given prior to bone marrow transplantation [3]. In addition to that, testicular tissue cryopreservation is an option for azoospermic male for diagnostic and treatment purposes. But testicular tissue cryopreservation applied for oligozoospermic male or infertile male who are undergoing a testicular biopsy to avoid repeated testicular biopsies [4, 5]. Moreover, surgical removal for testis, accidental case that may lead to losing the testis and before sterilization (vasectomy) is considering other cause for testicular tissue cryopreservation [6]. Cryostorage has been a topic of interest especially in fertility preservation for which banking of gonadal cells and tissues by cooling to low sub-zero temperatures (-196 °C), is critical for future experiments or diagnoses [7].

In recent years with advanced methods for treatment of malignant disease, these treatments often cause permanent sterility as a result of the loss of spermatogonial stem cells [8], the standard procedure for fertility protection in male cancer patients is cryopreservation of ejaculated spermatozoa, which is well established for adults and sexually mature boys [9], but with the introduction of intracytoplasmic sperm injection (ICSI) in 1992 had revolutionized the treatment of severe male factor infertility. Not only ejaculated spermatozoa can be used successfully with ICSI, but also epididymal and testicular spermatozoa can be used with great success so that cryopreservation of testicular tissue became another option for fertility preservation [10].

The first successful cell cryopreservation was started with freezing of fowl sperm. Later on, cryopreservation of bull sperm revolutionized the bovine artificial insemination industries [11]. Since cryopreservation of structurally intact tissues could be more beneficial than cryopreservation of cells, especially when it is equally important to retain all of the tissue's potential. However, providing a robust freezing protocol to maintain all the compartments of the tissue could be more complicated due to highly organized structure of the tissue may vary in its response to cryopreservation; therefore, cryopreservation could be extremely hazardous to the complex tissue structure [12].

The first gonadal tissue cryopreserved successfully was ovarian tissue, resulting in preservation of cell viability with normal function [13]. The history of testicular tissue cryopreservation is short, successful cases have been reported in animal experiments since the mid-1990s.

Now there are two methods of cryopreservation of testicular tissue: one is to freeze a testicular cell suspension by digesting testicular tissue with an enzyme and the other is to freeze testicular tissue after cutting it into squares 1–3 mm in size [14]. Despite Cryopreservation is used extensively in an in vitro fertilization (IVF) programs, but it was known as a cell-damaging procedure and may lead to deleterious

changes in cell structure (e.g., membrane, mitochondria and DNA) due to temperature variations [9, 10]. So that This study is an attempt to investigate the changes that occur in mice testicular tissue after cryopreservation/thawing cycle as a model for humanbeing after cryopreservation/thawing cycle when using different types of cryoprotectant (CPA).

Materials and Methods

Sample collection and processing

Healthy mature fertile male mice of Mus Musculus between 8-12 weeks old and 25-35 gm of weight was obtained from the animal house at the High Institute of Infertility Diagnosis and Assistant Reproductive Technology/Al-Nahrain University. The animals were killed by cervical dislocation. Then they fixed on the dissecting table and make an incision in the scrotal sac and finally, the testis was dissected.

The cryopreservation of samples was done at the sperm activation and freezing laboratory of Assistant Reproductive Technology (ART) unite in the same Institute, while Single Cell Gel Electrophoresis (Comet Assay) application and technical aspect which include equipment and procedure were performed at the biochemistry laboratory of the Medicine College/Al-Nahrain University. While the final photomicrograph was obtained by the fluorescent microscope (Olympus BX41) at the department of physiology of the Medicine College/Al-Nahrain University, and the histological preparation was done at the Department of human anatomy of the Medicine College/Al-Nahrain University.

Experimental design

In the present work, mature fertile male mice were divided into two major groups include:

- The control group (G1): In this group the histological evaluation and comet assay were done to the testes without cryopreservation.
- The group with cryopreservation (G2): In this group the histological evaluation and comet assay were done to cryopreserved testis after six weeks of cryopreservation using different types of cryoprotectants.

The experimental animal protocol was conducted in compliance with humane animal care standards outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental study was approved by the High Institute of Infertility Diagnosis and Assistant Reproductive Technology/Al-Nahrain University

Cryopreservation of testis

The Cryopreservation of testis was performed according to Milazzo et al [15].

The freezing media was prepared as follows:

The Hams F-10 culture medium vial (Biochrom) was dissolved in one liter of distal water. Then 75mg penicillin and 2.6 g NaHCO3 (Merck) was added to the solution after that the solution was filtered by using 0.22 µm millipore filters (Sartorius). The pH was adjusted between 7.2-7.4 using concentrated NaOH (Fluka) or HCL (BDH). Finally, the solution was stored in a refrigerator (Concord) 4- 8 ºC. The cryoprotectant was added to the culture media at concentration 15% glycerol (BDH), 15% DMSO (Sigma) and 15% 1,2PrOH (Merck). When using DMSO or 1,2PrOH as cryoprotectant the 0.1M of

sucrose (BDH) was added to the solution. The freezing medium was sterilized by autoclave (Raypa, AH-75) when using glycerol as cryoprotectant and by using 0.22 µm millipore filters then U.V. light (Daihan Lab Tech) when using DMSO and PrOH as cryoprotectants. The 1.8ml cryovial was filled with 1.5 ml of freezing media.

The testis was dissected from mature fertile mice under dim yellow light and it was washed by using culture media then it was transported to the 1.8 cryovial (NUNC) that contains 1.5 ml of freezing media supply with one type of cryoprotectant. The cryovial was placed in the cryovial holder (NUNC) and remained for about 10 minutes in the refrigerator when using DMSO as cryoprotectant and at room temperature when using glycerol and 1, 2 PrOH as a cryoprotectant for equilibration. The cryovial holder that contains cryovial was suspended in the liquid nitrogen vapor for 30 minutes before plunged in the liquid nitrogen then plunged in liquid nitrogen. After six weeks of cryopreservation. The thawing processes to sample was done through rapidly transfer cryovial from liquid nitrogen to the water bath (Kotterman) 37 ºC until melting ice, for at least 5 minutes.

The cryoprotectants were removed from the sample by descending concentration gradually (10%, 5% when using glycerol as a cryoprotectant, 10% DMSO or PrOH with 0.05M sucrose, 5% DMSO or PrOH with 0.025M sucrose and finally washed by culture media only) for about 5 minutes for each concentration. Finally, the comet assay and histological preparation were done to the samples (testis) as the describing below.

Histological evaluation of mice testis [16]

Tissue was fixed in Bouin's solution for 12-16 hours. After fixation, they were washed using many changes of 50%, ethanol to remove the yellow color of picric acid, dehydrated through graded alcohols and cleared using two changes of xylene and embedded in paraffin wax. A serial section of 4-5µm thickness was stained by Harris's hematoxylin and eosin.

Results

The paraffin sections of the testis from control group stained with Haematoxylin & Eosin illustrated normal tissue morphological and structural and showed normal components of germinal epithelium that lining the seminiferous tubules include normal spermatogenic lineage cells (spermatogonia, spermatocyte, and spermatid) and supporting (Sertoli) cells (Figure 1).

The spermatogonia have two types A and B, type A spermatogonia have ovoid nucleus stains darkly (type A dark spermatogonia) or lightly (type A pale spermatogonia) and type B spermatogonia have spherical nucleus, both two types of spermatogonia (A&B) lie attachment to the basal lamina. While the primary spermatocyte larger than spermatogonia occupies the middle zone of the germinal epithelium, and the primary spermatocyte consider the largest germ cells could be seen in the germinal epithelium (Figure 2), but the secondary spermatocytes are about half size of primary spermatocytes, they lie nearer the lumen. The spermatid lies close to the lumen and is spherical or polygonal cells (Figure 3).

Sertoli cells are shown in (Figure 2) are relatively few in numbers, they are spaced along the tubule (tall, pillar) with their bases resting upon the basal lamina of the tubule and the cell outline is irregular, indistinct, have pale and ovoid nucleus is located in a distance above the base of the cell. While the

tubule basement membrane covered by fibrous tissue, which contains numerous connective tissue fibers and some cells with the characteristics of smooth muscle cells called myoid cells (Figure 3). The interstitial cells (Leydig cells) were seen between seminiferous tubules; they are large polyhedral cells that have a spherical nucleus (Figure 2).

In cryopreserved groups, different types of cryoprotectants (glycerol, 1, 2 PrOH and DMSO) were used. After six weeks of cryopreservation, the paraffin sections of testis stained with H&E stain showed morphological and structural alteration in seminiferous tubule component and interstitial tissue. But these alterations differ according to the type of cryoprotectant.

In the cryopreserved group, the tissue samples subjected to glycerol as cryoprotectant displayed moderate morphological and structural changes. The most typical histological changes were noted after cryopreservation by glycerol as CPA was rupturing of the stroma and disruption of the cell-cell connections in the interstitial tissue (Figure 4).

Furthermore, sometimes it was seen a distraction in the basal compartment of seminiferous tubules. Also, detachment the cells (spermatogonia type A, type B, and Sertoli cell) in the seminiferous tubules from the basement membrane was observed (Figure 5), but the most damaged part in the seminiferous tubules after cryopreservation/thawing cycle was basal compartments. In addition to that, it was observed folding of lamina property (Figure 6), gaps in the germinal epithelial (Figure 7) and necrotic cells in the lumen tubule (Figure 4).

While in the tissue samples cryopreserved by 1, 2 PrOH as cryoprotectant showed severest morphological and structural changes characterized by complete destruction in the stroma and interstitial tissue. Sometimes it was observed destruction the seminiferous tubules with some necrotic cells in the lumen seminiferous tubules (Figure 8). In addition, it was noted sever destructive in the basement membrane and the cells detached from its (Figure 9). Moreover, it was impossible to recognize the cells of seminiferous tubules. But in the paraffin sections obtained from tissue cryopreserved by DMSO as cryoprotectant showed no major differences versus control and given slightly morphological and structural changes, and the main prominent histological changes observed after cryopreservation by DMSO as CPAs was mild disruption of interstitial tissue (Figure 10). Moreover, the lamina propria of seminiferous tubules was shown mild folding, but sometimes it was not seen obvious changes (Figure 11). While the cells of seminiferous tubules slightly influenced and showed morphology similar to that presented in the control group, and it was noted mild detachment of cells in the seminiferous tubules from the basement membrane (Figure 12). In addition, it was observed some necrotic cells in the lumen of the seminiferous tubules (Figure 13).

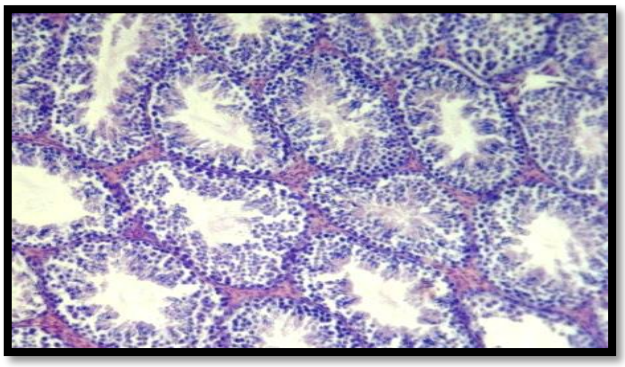

Figure 1.

Paraffin section from mice testis (control group) show normal tissue structure, stained by H&E **(**10X).

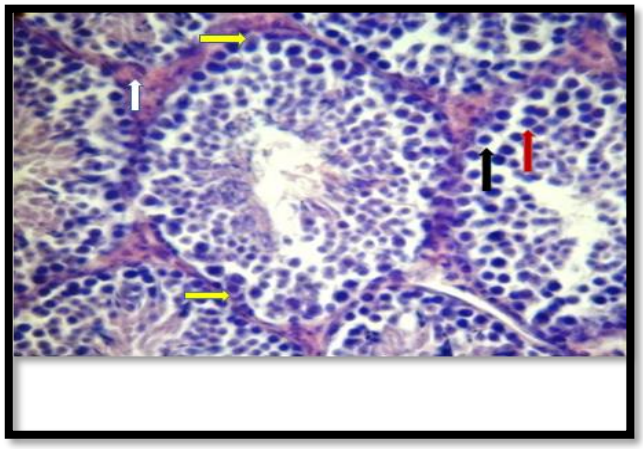

Figure 2.

Section from normal mice testis show normal cells; Sertoli cell (yellow arrow), interstitial cells (white arrow), spermatogonia (black arrow), spermatocyte (red arrow), stained by H & E (40X).

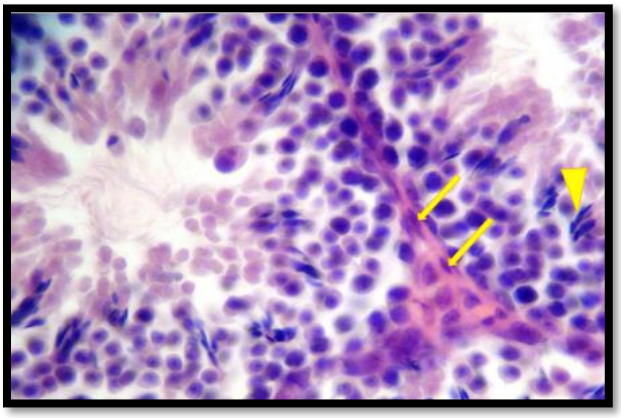

Figure 3.

Section from normal mice testis show normal cells; myoid cell (arrows), spermatids (arrowhead), stained by H&E(100X).

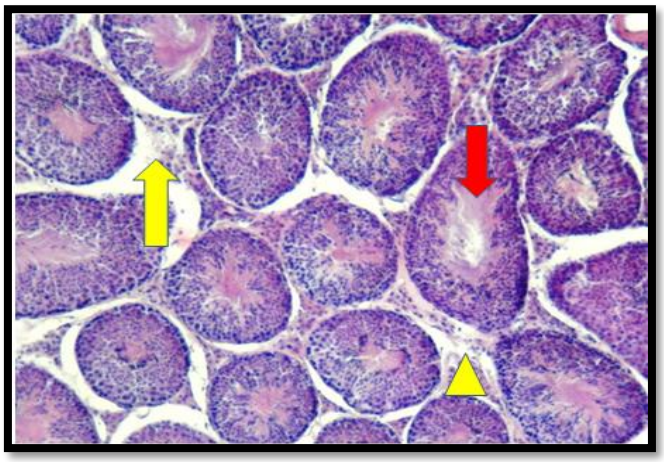

Figure 4.

Section from cryopreserved mice testis by glycerol as CPA showing increase intertubular space (yellow arrow), damage in interstitial tissue (arrow head) and necrotic cells in the lumen of tubules (red arrow), stained by H & E (10X).

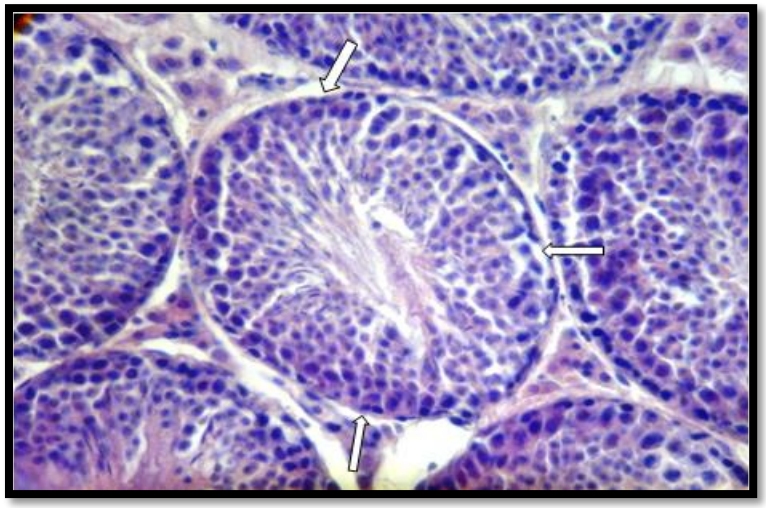

Figure 5.

Section from cryopreserved mice testis by glycerol as CPA showing detachment of cells of germinal epithelium from basement membrane (arrows), stained by H & E (40X).

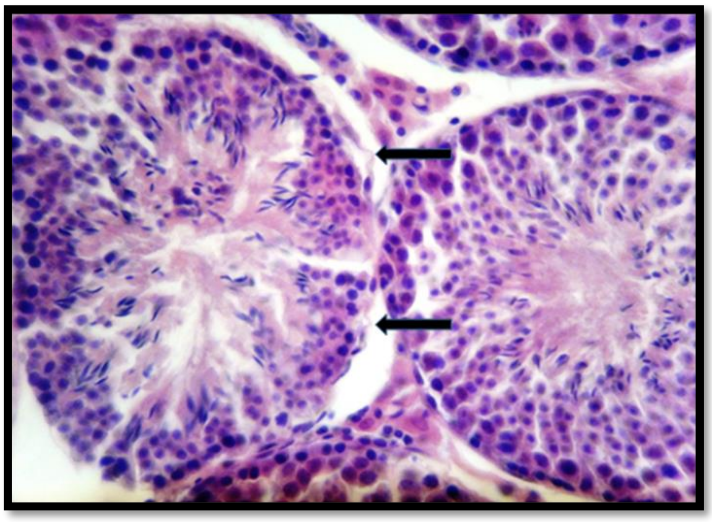

Figure 6.

Section from cryopreserved mice testis by glycerol as CPA showing folding of lamina properia (arrows), stained by H&E (100X).

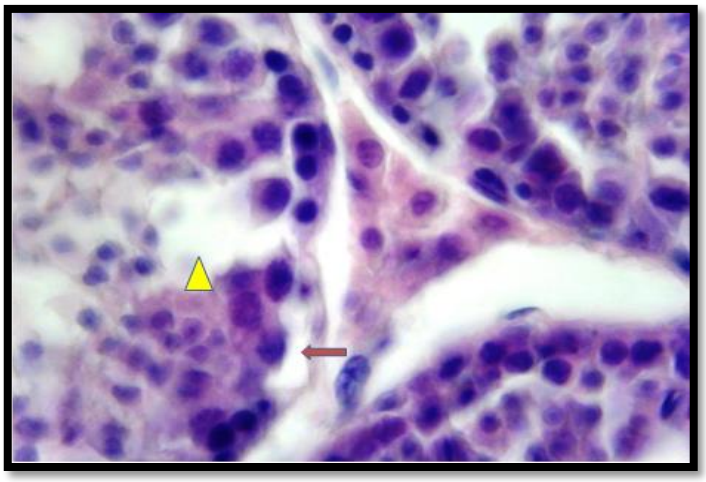

Figure 7.

Section from cryopreserved mice testis by glycerol as CPA showing detachment of cells from basement membrane (arrows) and gaps in the germinal epithelium (arrow head), stained with H&E (100X).

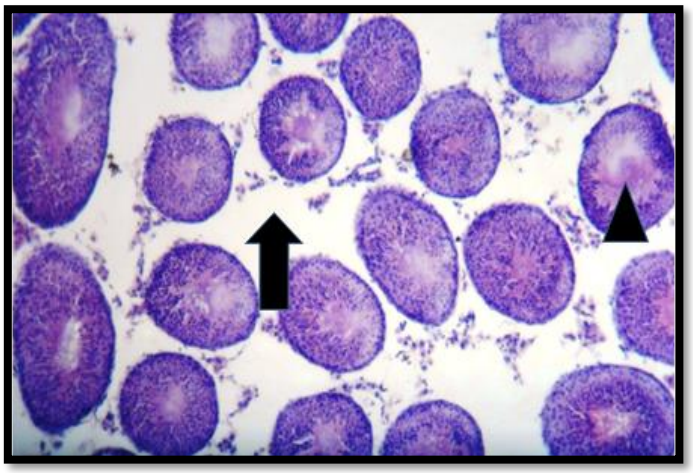

Figure 8.

Section from cryopreserved mice testis by 1, 2 PrOH as CPA showing sever damage in interstitial tissue (arrow) and necrotic cells in the lumen of tubules (arrow head), stained with H&E, (10X**).**

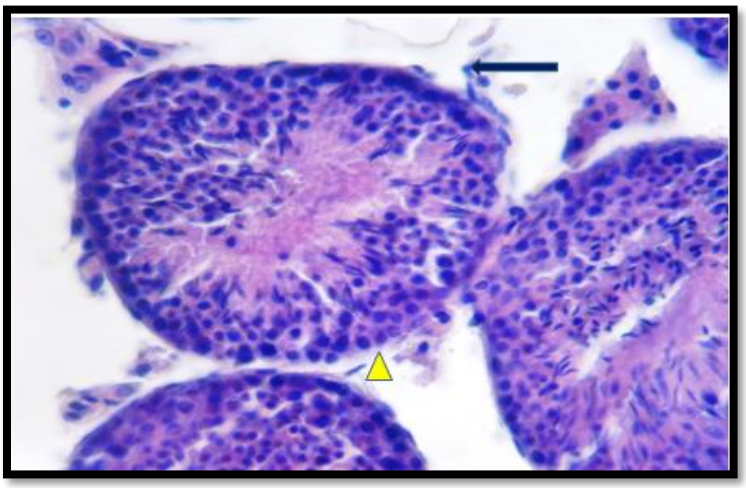

Figure 9.

Section from cryopreserved mice testis by 1, 2 PrOH as CPA showing the structure take fuzzy form, damage in basement membrane (arrow), detachment the cell from basement membrane (arrowhead), stained with H&E (40X)**.**

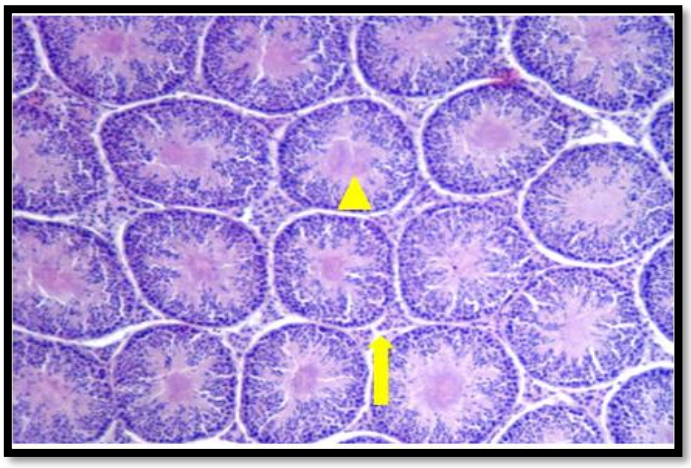

Figure 10.

Section from cryopreserved mice testis by DMSO as CPA showing mild damage in interstitial tissue (arrow) and necrotic cells in the lumen of tubules (arrow head), stained with H&E (10X).

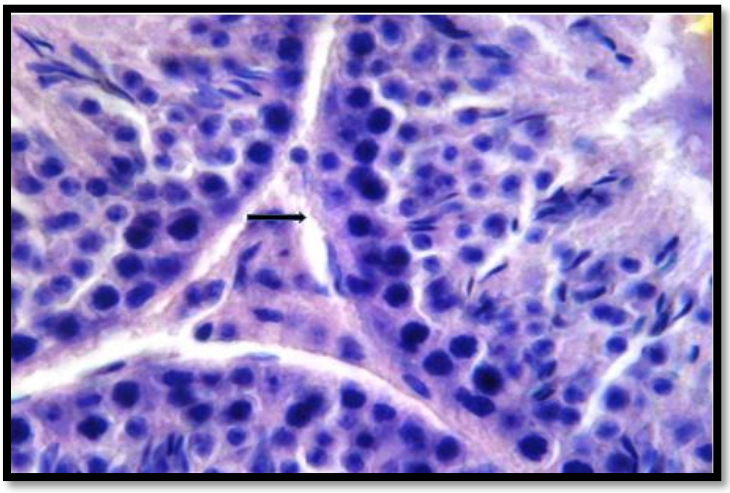

Figure 11.

Section from cryopreserved mice testis by DMSO as CPA showing mild folding of lamina propria (arrow) stained H&E stain (100X).

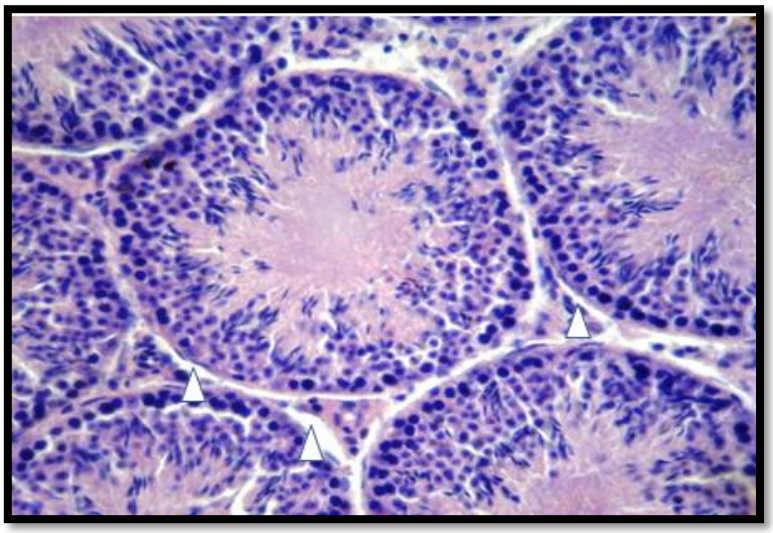

Figure 12.

Section from cryopreserved mice testis by DMSO as CPA showing detachment cells of germinal epithelium from basement membrane (arrow heads), stained with H&E (40X)**.**

Discussion

Even there is no comprehensive study of the effects of freezing/thawing on the morphology of testicular tissue using different cryopreservation media. In the present study by histological paraffin sections was observed certain histological changes that happened after cryopreservation/thawing cycle and these changes differ according to the type of cryoprotectant. There are three main suggestions to explain these changes in the tissue:

- It can be supposed that freezing and thawing, together with the cryopreservation medium, cause the formation of ice crystals, increase salt concentration, thermal and osmotic shock.
- Increase in oxygen-free radicals which will damage the cells and DNA. And one important pathway occurs by lipid peroxidation.
- Cryoprotectant toxicity

The results were obtained by Keros et al., [17] under light microscopic include complete disruption in the interstitial tissue, difficult to recognize between cells inside the seminiferous tubules and detachment of cells from the basement membrane after cryopreservation/thawing cycle by 1, 2 PrOH and sucrose for cryopreservation of testicular tissue, it goes with result of this study. Hovatta et al. and Hreinsson et al., supported this study results when referred that cryopreservation method optimized for human ovarian tissue using 1, 2 PrOH and sucrose not to be optimal for cryopreservation of testicular tissue [18,19].

Glycerol has been used as a reliable agent in lowering the freezing point of intracellular water, and widely used for cryopreservation of spermatozoa and suspensions of testicular cells. However, it has been recognized that glycerol has certain toxic effects on spermatozoa, causing hyperosmotic injury in frozen/thawed ejaculates (20). In a morphological study by light microscopic and ultrastructural

analyses were performed in order to assess the influence of freezing and thawing on the seminiferous epithelium, using glycerol as a cryoprotectant. Nogueira et al., demonstrated that the parts of the seminiferous tubules most affected by freezing-thawing are the basal tubular compartment and cells such as spermatogonia, Sertoli cells, and spermatocytes, while round and elongated spermatids/spermatozoa are much less exposed and therefore maintain their characteristic structure [15].

Also folding of the lamina propria and gaps between the basal and the apical tubular compartments reported by Nogueira et al., [21], and these results supported this study results. Another cryoprotectant agent, dimethylsulfoxide (DMSO) has been routinely and widely used in cell culture and freezing because of its low molecular weight and good penetration characteristics [22]. In addition to that, DMSO has been tested for possible freezing and thawing of human embryos [23]. DMSO with its low molecular weight and high tissue penetration provides superior cryoprotection especially in solid tissues [17]. DMSO also provides superior cryoprotection in cell suspensions [24].

Goossens et al., supported this study results when tested two cryopreservation protocols for banking testicular tissue: one protocol using ethylene glycol (EG) and another one using DMSO as cryoprotectant and provide the best morphology of the basal compartment was obtained when the cryoprotective medium contained DMSO and also found DMSO a better than EG [25].

The results of the current study are in agreement with the result of Honaramooz et al., who investigated the effect of cooling or cryopreservation with DMSO on the testis fragments of pigs before grafting and founded the tissue maintained the potential to develop complete spermatogenesis and maintained steroidogenesis when grafted onto mice [26]. Also Jezek et al. reported both glycerol and DMSO, when applied at moderate concentrations (6-25%) preserved the structure of the seminiferous epithelium and no significant effect on tubular diameter; however, it caused a `folding' of the lamina propria and mild to moderate damage to Sertoli cells, spermatogonia, and spermatocytes. Round and elongated spermatids and spermatozoa displayed occasional nuclear damage, vacuolization of the cytoplasm [27]; these results corroborate results of the present study.

Conclusion

From the results of the present study, it was concluded that there was alteration in testis histology in the testicular tissue after cryopreservation /thawing cycle differs according to the type of cryoprotectant and the Dimethylsulfoxide cryoprotectant provides good protection to the testicular tissue and DNA in cryopreservation /thawing cycle than glycerol and 1, 2 propanediol (1, 2 propanediol was not preferable in testicular tissue cryopreservation).

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