Evaluation of Different Cryopreservation Protocols of the Testis Using 8-Hydroxy 2-Deoxyguanosine as Marker of DNA Damage.

Anam R. Al-Salihi[1], Mustafa J. Jalil[1], Estabraq A. Kwaeri[2]
1 High Institute of Infertility Diagnosis and Assistant Reproductive Technology/Al-Nahrain University.
2 Department of chemistry and biochemistry, Collage of medicine/ Al-Nahrain University.
Corresponding author **email: Mostafaalasady_1987@yahoo.com**

Abstract:

Background:
Chemotherapy and radiotherapy can destroy or severely reduce spermatogenesis and thereby jeopardize fertility in the long term. There is still no medical treatment that guarantees fertility preservation after chemo- and radiotherapy. Now with recent improvement of assisted reproductive technologies (ART) and possibility of using testicular spermatozoa or epididymal spermatozoa at *in vitro* fertilization (IVF) by intracytoplasmic sperm injection (ICSI), cryopreservation of testicular tissue is an option in fertility preservation for males who will lose spermatogenic cells as a result of chemo- and radiotherapy and for males with azoospermia.

Objectivs:
This study is an attempt to evaluate oxidative DNA damage in the mice testicular tissue after cryopreservation/thawing cycle when using different types of cryoprotectants as well as to investigate the changes that occur in mice testicular tissue after cryopreservation/thawing cycle as a model for human being.

Methods:
Fifty mature fertile male mice between 8- 12 weeks old were used in the current study. For each mouse, one testis was evaluated histologically and immunohistochemically (using 8-Hydroxy 2>-Deoxyguanosine as marker of oxidative DNA damage) without cryopreservation (control group). The other testis was evaluated histologically and immunohistochemically (using 8-Hydroxy 2>-Deoxyguanosine as marker of oxidative DNA damage) after six weeks of cryopreservation using different types of cryoprotectants (cryopreserved group).

Results:
The microscopically observation of slides obtained from cryopreserved testis differs according to the type of cryoprotectant (glycerol, 1,2 propanediol and dimethylsulfoxide), in the dimethylsulfoxide group, tissue sections showed no major differences versus control group, but in the histological sections obtained from tissue cryopreserved by glycerol as cryoprotectant showed moderate morphological and structural changes as compared with the control group. While, the tissue samples subjected to 1,2 propanediol as cryoprotectant displayed the severest morphological and structural changes as compared with the control group.
Immunohistochemical results of the present study showed a highly significant ($P<0.001$) increase in oxidative DNA damage in the cryopreserved testis (46.78%, 63.45% and 32.59% represent cryoprotectant glycerol, 1, 2 propanediol and dimethylsulfoxide, respectively) compared with control group (19.27%) after six weeks of cryopreservation.

Conclusion:
From the results of the current study, it was concluded that there was alteration in testis histology after cryopreservation/thawing cycle and increase the level of oxidative DNA damage after cryopreservation/ thawing cycle. As well as dimethylsulfoxide cryoprotectant provide good protection for testis histology and DNA.

Keywords: Cryopreservation, Oxidative DNA damage, Reactive oxygen species, 8-OHdG.

Introduction

In contemporary, improvements in treating cancer have enabled many younger persons with cancer to survive (1). However, treatment of these cancers is often highly detrimental to both male and female reproductive function. In male, testis is highly susceptible to the toxic effects of radiation and chemotherapy at all stages of life. The most common strategy to preserve fertility is cryopreservation of sperm before the treatment for later use, but with the introduction of intracytoplasmic sperm injection (ICSI) in 1992 had revolutionized the treatment of severe male factor infertility. Not only ejaculated spermatozoa could be used successfully with ICSI, also epididymal and testicular spermatozoa could be used with great success, so that cryopreservation of testicular tissue became another option for fertility preservation (2 -3).

Despite of cryopreservation 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 the cell structure {e.g., membrane, mitochondria and deoxyribose nucleic acid (DNA)} due to temperature variations and increase the levels of the reactive oxygen species (ROS) generation in spermatozoa (45-). Cryopreservation of testis in mice using different types of cryoprotectants agent (CPA) include: {Propanediol (1,2PrOH), Glycerol and Dimethylsulfoxide (DMSO)}, evaluate oxidative damage to testicular tissue DNA using 8-OHdG level, and put forwards recommendations regarding the optimum type of cryoprotectant which inflects the least DNA damage of testicular tissue during cryopreservation.

Materials and Methods:

- Mice management: Fifty healthy mature fertile male mice of Mus Musculus between 8- 12 weeks old and 25- 35 gm of weight were obtained from the Animal House at the National Center for Drug Control and Research. Room temperature was between 22 -24 °C, the air of the room was changed continuously using ventilating fan. Photoperiod was automatically controlled of 13± 2 hours light from 6 A.M. to 7 P.M. daily regimen.

- Study Design: Mice were scarified by cervical dislocation and testes were excised and rinsed in culture media. For each mouse, one testis was evaluated histologically and immunohistochemically without cryopreservation (control group). The other testis was evaluated histologically and immunohistochemically after six weeks of cryopreservation using different types of CPA (cryopreserved group).

-Freezing media and protocol (6,7): Cryomedium consisted of Hams F-12 culture medium (Sigma-ALdrich) supplemented with three different types of cryoprotactent (DMSO, Glycerol and 1,2PrOH). These CPA was added to the culture media at concentration 15% glycerol (BDH), 15% DMSO (BDH) and 15% 1,2PrOH (Merck). When using DMSO or 1,2PrOH as cryoprotectant, the 0.1M/l of sucrose (BDH) was added to the solution.

The cryopreservation of samples was done at the sperm activation and freezing laboratory of Assistant Reproductive Technology (ART) unite in the High Institute of Infertility Diagnosis and Assistant Reproductive Technology/Al-Nahrain University.

After sacrificed male mice by cervical dislocation, the testis was dissected under dim yellow light and it was washed by using culture media then it was transported to the 1.8 cryovial that contains 1.5 ml of freezing media supply with one type of CPA. After that the cryovial was placed in the cryovial holder (NUNC) and remained for about 10 minute in the refrigerator when using DMSO as CPA and at room temperature when using 1,2PrOH and glycerol as CPA for equilibration. Finally the cryovial holder that contains cryovial was suspended in the liquid nitrogen vapor for 30 minutes before plunged in the liquid nitrogen and then plunged in liquid nitrogen.

After six weeks of cryopreservation, the thawing processes to samples were done through rapidly transfer the cryovials from liquid nitrogen to the water bath (Memmert) at 37 ºC until melting ice, for at least 5 minutes, and then the CPAs were removed from sample by descending concentration of CPA gradually as fallowing:

a- 10%, 5% and finally washed by culture media only when use glycerol as CPA for about 5 minute for each concentration.

b- 10% with 0.05M sucrose, 5% with 0.025M sucrose and finally washed by culture media only when use DMSO or PrOH as CPA for about 5 minute for each concentration.

- Histological and Immunohistochemical evaluation of mice testis (8, 9): The samples were fixed with 10% neutral buffered formalin (BDH) for overnight (24 hours). After that, the tissues were embedded in paraffin. The paraffin-embedded samples were sectioned at a thickness of 5 µm. Some sections were stained with hematoxylin and eosin, and the other sections were used for immunohistochemical assay. For immunostaining of 8-OHdG, the slides were placed in a drying oven (hot air oven) at 65°C for one hour. Then the formalin-fixed sections were deparaffinized in washes in xylene through ethanol to distilled water. To block endogenous peroxidase activity, 0.3% hydrogen peroxide was applied to the specimens for 10 min. All slides then were incubated with protein block for 5 min to block nonspecific binding. Primary antibodies were added to the slides and incubated for two hours in a humidified chamber at room temperature. Secondary antibody (biotinylated link) was then applied and incubated for 10 min at room temperature. Enough drops of streptavidin reagent were applied covering the whole section and incubated at room temperature for 10 minutes in humid chamber. Then Substrate-chromogen solution were applied on each section covering the whole specimen and incubated in darkness at room temperature for 10 minutes. After that enough drops of the haematoxylin (as a nuclear counter stain) solution were applied covering the whole section and incubated at room temperature for 1 minute. Finally enough drops of an aqueous-base mounting

medium were applied onto the tissue sections and covered with coverslips and left to dry.

- Assessment of Immunohistochemical Staining: For assessment of Immunohistochemical Staining, image were captured by using Microns microscope contain TV-Based computer with a 40X objective and at least ten images were captured for each sample. Actual assessments of immunohistochemical staining were done by using Aperio positive pixel count algorithms to analyze digital slides. The Aperio positive pixel count algorithm can be used to quantify the amount of a specific stain present in a slide image. This algorithm has a set of default input parameters when first selected. These inputs have been pre- configured for Brown color quantification in the three intensity ranges (weak, positive, and strong). The algorithm is applied to an image by using Image Scope software v10 (Aperio).

Statistical Analysis: Statistical analysis was performed with SPSS V. 17 (statistical package for social sciences) and also Microsoft Excel Work Sheet 2010 programs. Paired sample t- test was applied by use SPSS statistical program to compare between the result of cryopreserved groups and control group. Data were expressed as mean ± standard error of mean. And the values were considered statistically significant when p-value 0.05.

Results

-Results of histological evaluation

In control group, the paraffin section of testis stained with Hematoxylin & Eosin showed normal tissue morphological and structural and showed normal components of germinal epithelium that composed of seminiferous tubules with normal sperrmatogenic lineage cells (spermatogonia, spermatocyte and spermatid) and supporting (Sertoli) cells. Between the seminiferous tubules were seen the interstitial cells (Leydig cells), they are large polyhedral cells that have spherical nucleus (Figure 1).

In cryopreserved groups, different types of CPAs (glycerol. l,2PfOH 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 CPA. In the cryopreserved group, the tissue samples cryopreserved by DMSO as CPA showed no major differences versus control group. These samples contain well-preserved testicular cords and the main prominent histological changes observed after cryopreservation by DMSO as CPA was mild disruption of interstitial tissue and mild increase in the intertubular space. In addition to that, the testicular cords showed mild detachment of cells in the seminiferous tubules from the basement membrane and contained necrotic cells confined to the center of the testicular cords that was not seen in the control group (Figure 2). While in the paraffin sections obtained from tissue cryopreserved by glycerol as CPA showed moderate morphological and structural changes. These changes characterized by moderate disruption of interstitial tissue and moderate increase in the intertubular space. Also, detachment of spermatogonia from the basement membrane of the testicular cords and necrotic cells in the lumen seminiferous tubules were observed (Figure 3).

The tissue samples subjected to 1,2PrOH as CPA displayed the severest morphological and structural changes. The most typical damage was rupture of the stroma and the cell-cell connections in the interstitial tissue and inside the seminiferous tubules, mainly in the basal compartment. The lamina propria was influenced and showed swelling after cryopreservation with 1,2PrOH. Also, destruction of the basement membrane in addition to sever detachment of spermatogonia from the basement membrane and necrotic cells in the lumen seminiferous tubules were observed (Figure 4).

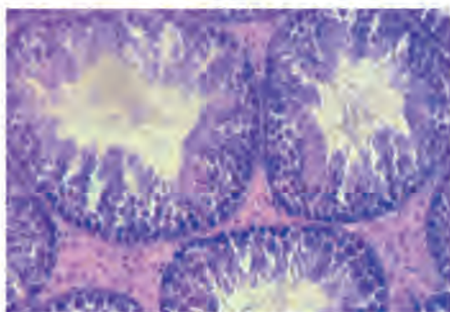

Figure 1: Paraffin section from mice testis of control group show normal tissue structure, this figure showed normal components of germinal epithelium that composed of seminiferous tubules with normal spcrmatogenic lineage cells (spermatogonia, spermatocyte and spermatid) and supporting (Sertoli) cells. Between the seminiferous tubules were seen the interstitial cells (Leydig cells), they are large polyhedral cells that have spherical nucleus. Stained by H&E (40X).

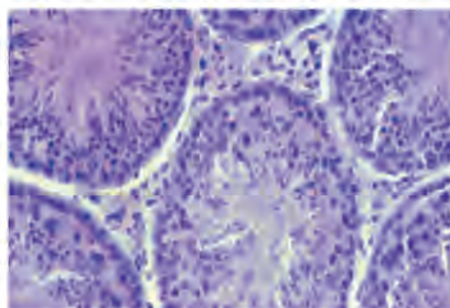

Figure 2: Section from cryopreserved mice testis by DMSO as CPA showing mild disruption of interstitial tissue and mild increase in the intertubular space in addition to mild detachment of spermatogonia from the basement membrane and necrotic cells in the lumen seminiferous tubules, stained with H&E (40X).

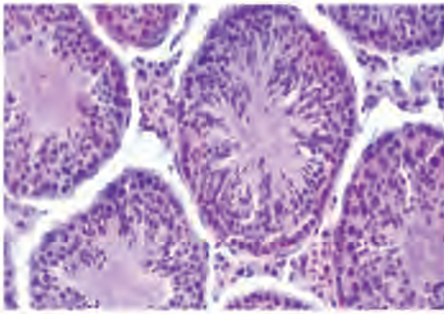

Figure 3: Section from cryopreserved mice testis by glycerol as CPA showing moderate disruption of interstitial tissue and moderate increase in the intertubular space, also detachment of spermatogonia from the basement membrane of the testicular cords and necrotic cells in the lumen seminiferous tubules, stained with H&E (40X).

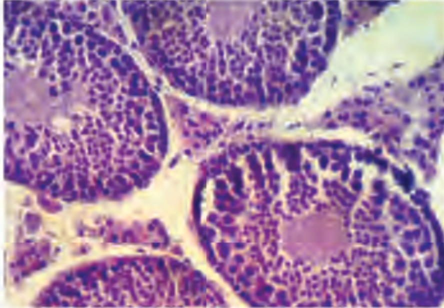

Figure4: Section from cryopreserved mice testis by l,2PrOH as CPA showing rupture of the stroma and the cell-cell connections in the interstitial tissue and inside the seminiferous tubules in addition to swelling of the lamina propria and detachment of the cells (spermatogonia) in the seminiferous tubules from the basement membrane. Also, destruction of the basement membrane and necrotic cells in the lumen seminiferous tubules,stained with H&E (40X).

-Result of immunohistochemical assay:

In this study, we considered the cell positively stained for 8-OHdG when the nucleus was being stained; the pattern of stain is diffuse and color of stain is brown. The cells labeled by the antibody display a staining almost entirely confined to the nucleus and with a diffuse pattern, the color of stain is brown. Rarely cytoplasmic staining is observed, which may represent cytoplasmic synthesis or breakdown.

Testicular sections of mice normal and cryoprescrved testes were stained with anti-8-OHdG antibody. Both control and cryopreserved sections showed variable distribution of 8-OHdG, but with the cryopreserved group displaying significantly higher staining compared to control group. In control group, tissue sections of the testes showed normal tissue morphology and normal components of seminiferous tubule and there are weak positive signals for anti-8-OHdG antibody were detected in the testicular sections (Figure 5-A). Digital image for the testicular sections of control group were captured at 40x objective and analyzed by computer software (Aperio ImageScop program) for quantification of 8-OHdG staining by converting the digital image into pseudo-color markup image as shown in (Figure 5-B). In this figure, the testicular section of control group showed positive signals for anti- 8-OHdG antibody in variable intensity. The weak intensity of positive signals appeared in the center of the seminiferous tubule and around numerous germ cells. But these positive signals increased in intensity in the interstitial tissue of the testis while there are no positive signals were observed in nucleus of the germ cells. In cryopreserved group, tissue sections stained immunohistochemically displaying significantly higher staining intensity compared to control group vary according to the type of the CPA that used. Tissue sections obtained from the samples cryopreserved by DMSO as CPA showed varying degrees of positive intensity for 8-OHdG in the seminiferous tubules and the interstitial tissue of the testis (Figure 5-C). The strong positive signals appeared in the nucleus of the germ cells especially in the spermatogonia, but the weak positive signals appeared around the germ cells and in the interstitial tissue of the testis (Figure 5-D).

Whereas in the tissue samples cryopreserved by glycerol as CPA, the intensity of the positive signals increased in both seminiferous tubules and the interstitial tissue of the testes more than in control and DMSO group (Figure 5-E). These positive signals appeared clearly in (figure 5-F) after performing computer image analysis.

While the tissue samples subjected to 1, 2 PrOH as CPA displayed the stronger positive signals compared with the other groups (Figure 5-F). These strong positive signals concentrated mainly in the nucleus of the germ cells and in the interstitial tissue of the testes. In addition to that, weak positive signals distributed among the germ cells and in the lumen of the seminiferous tubules (Figure 5-G).

Finally, 8-OHdG immunohistochemistry staining in the testicular sections of control and cryopreserved groups were quantified as the percentage of the positive pixels for 8-OHdG in the testicular sections. The Mean + Standard Error (SE) of the percentage of the positive pixels for 8-OHdG in the testicular sections of control group was 0.193+ 0.011 while in cryopreserved groups Mean + Standard Error (SE) of the percentage of the positive pixels for 8-OHdG in the testicular sections were 0.326 ±0.01 , 0.468± 0.011 and 0.635± 0.013 represented CPAs DMSO, glycerol and 1,2PrOH respectively (Figure 6). Statistical analysis showed highly significant increase ($p<0.001$) in mean of percentage of the positive pixels for 8-OHdG in cryopreserved groups as compared with control group as shown in the table (1).

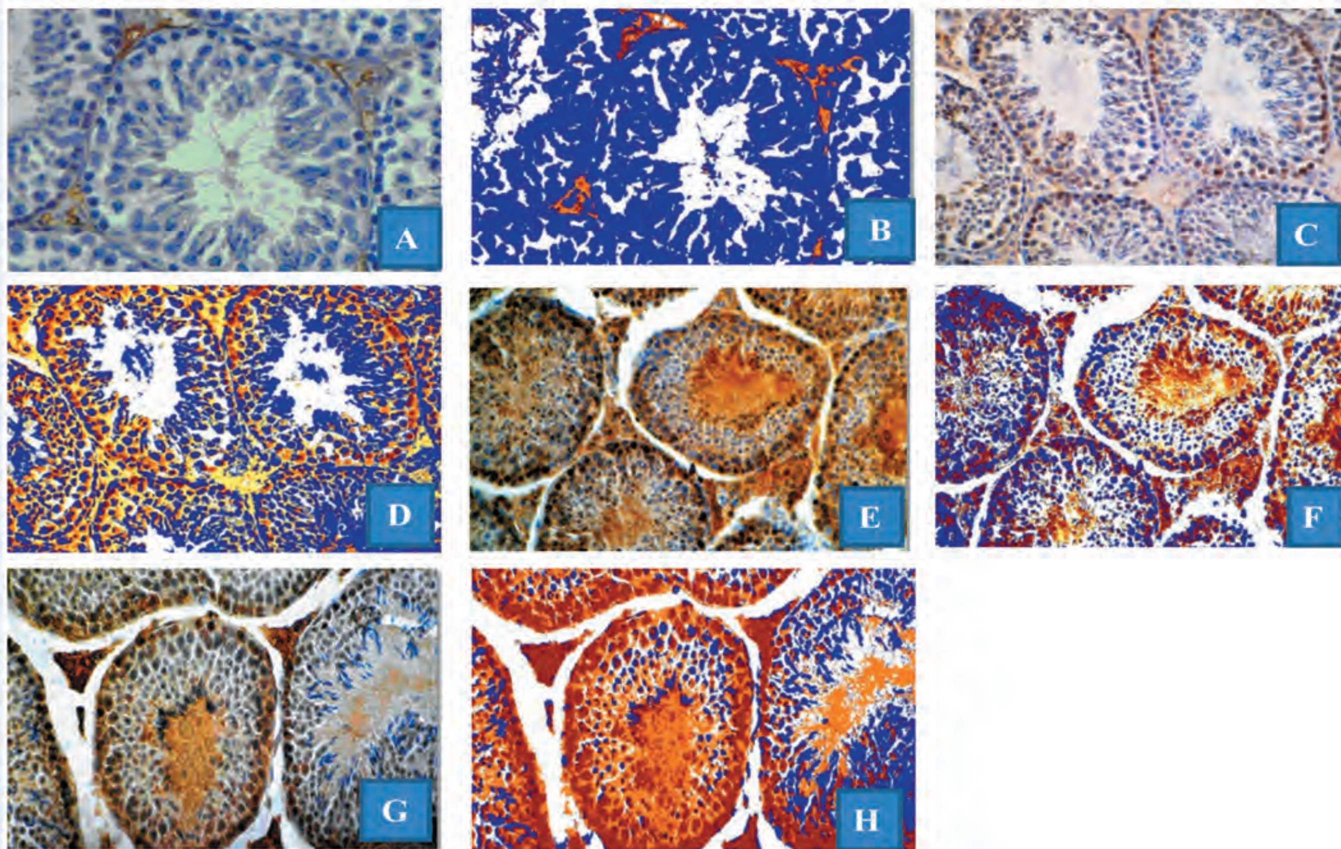

Figure 5: A- Showed the testicular section of control group stained immunohistochemically, a weak positive signal for 8-OHdG appeared in the interstitial tissue of the testis (40X). B- Pseudo-color markup image of testicular section of control group as a result of image analysis. This figure showed positive signals for 8-OHdG in variable intensity (40X). C- Section from cryopreserved mice testis by DMSO as CPA showing varying degrees of positive intensity for 8-OHdG in the seminiferous tubules and in the interstitial tissue of the testis, stained immunohistochemically (40X). D- Pseudo-color markup image after image analysis of testicular section cryopreserved by DMSO as CPA. This figure showed positive signals for 8-OHdG in variable intensity (40X). E- Section from cryopreserved mice testis by glycerol as CPA showing varying degrees of positive intensity for 8-OHdG in the seminiferous tubules and in the interstitial tissue of the testis, stained immunohistochemically (40X). F- Pseudo-color markup image after image analysis of testicular section cryopreserved by glycerol as CPA. This figure showed positive signals for 8OHdG in variable intensity (40X). G- Section from cryopreserved mice testis by I,2PrOH as CPA showing varying degrees of positive intensity for 8-OHdG in the seminiferous tubules and in the interstitial tissue of the testis, stained immunohistochemically (40X). H- Pseudo-color markup image after image analysis of testicular section cryopreserved by I,2PrOH as CPA. This figure showed positive signals for 8-OHdG in variable intensity (40X).

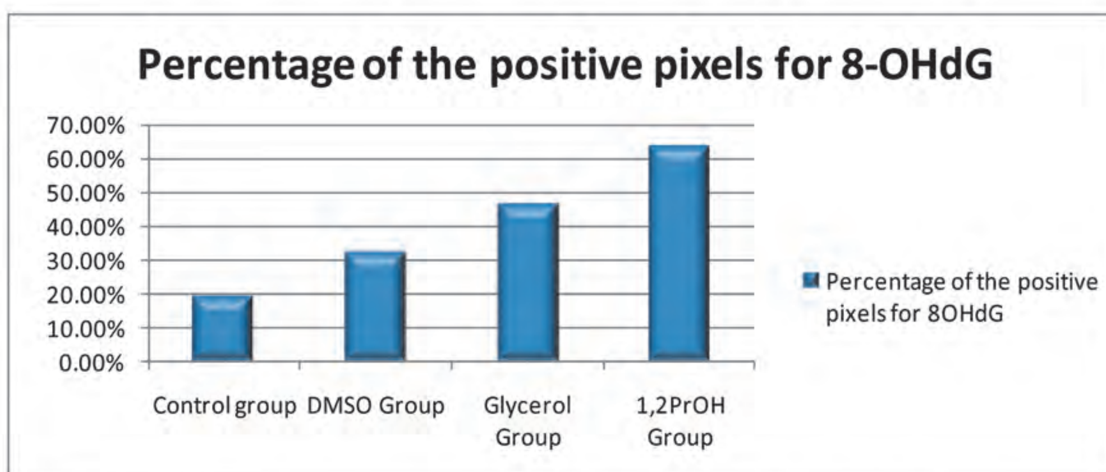

Figure 6: Percentage of the positive pixels for 8OHdG in the testicular sections of control group and cryopreserved groups.

Table 1: Percentage of the positive pixels for 8OHdG in the testicular sections of control group and cryopreserved groups

Groups	Mean + S.E.	S.D	P value	T test
Control	0.193+0.011	0.137	---	
DMSO	0.326+0.010*	0.122	≤0.001	8.267
Glycerol	0.468+0.011*	0.130	≤0.001	16.131
1,2PrOH	0.635+0.013*	0.153	≤0.001	29.238

Paired sample t test: comparison between glycerol, 1,2PrOH and DMSO groups with control group.
* Reveals highly significant differences as compared with control group (10).

Discussion

In the present study, histological paraffin sections showed certain histological and morphological changes that happened after cryopreservation/thawing cycle and these changes differ according to the type of CPA. These changes might be mainly due to ice crystals formation, increase salt concentration, and thermal and osmotic shock. In addition, CPA toxicity might play a role in these changes and finally the cryopreservation process result in increase in level of the ROS which will damage the cells and DNA. One of important pathway leading to that damage occurs by lipid peroxidation.

In the current study, cryopreservation of testicular tissue with DMSO as CPA showed no major differences as compared with control group. This result in agreement with study done by Keros *et al.*(11) they are showed no clear structural changes were observed between fresh and cryopreserved testicular tissue after using the protocol developed for adult testicular tissue containing DMSO as CPA.

Also, a study done by Goossens *et al.*(12) showed that cryopreservation of testicular tissue using protocol containing DMSO as CPA provide the best morphology of the basal compartment as compared with another protocol containing ethylene glycol as CPA.

Although 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, but its use for tissue preservation has been limited by its poor penetration qualities (13). In the present study, cryopreservation of testicular tissue using glycerol as CPA showed moderate morphological and structural changes as compared with control group. These results are in agreement with study done by Keros, *et al.*(14) they showed that the testicular tissue samples subjected to glycerol as CPA displayed changes in basal compartment of the tubules. These changes characterized by rupture of the stroma and the cell–cell connections in the interstitial tissue and inside the seminiferous tubules, mainly in the basal compartment, in addition to the detachment of spermatogonia from the basement membrane were observed, and these results supported this study results.

Whereas, cryopreservation of testicular tissue with 1,2PrOH as CPA showed the severest morphological and structural changes. This result in agreement with study done by Travers *et al.*,(15) in which they tried to evaluate several protocols for cryopreservation of rat immature testicular tissue by using DMSO and 1,2PrOH. They found that the cords damages appeared to be less frequent with freezing protocol using DMSO than 1,2PROH in terms of morphological alterations. These damages are characterized by the gap formations and epithelium detachment from the basement membrane, and these results supported this study results.

Also, the results were obtained by Keros *et al.*(14) 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.

The immunohistochemical assay used in this study is adequate to detect significant differences in the oxidative DNA damage between cryopreserved groups and control group by using the 8-OHdG as a biomarker of oxidative DNA damage.

Clear advantages associated with immunohistochemical analysis include the facts that this method is relatively inexpensive, quick, highly sensitive and easy to perform,

uses light microscopy, and is a well-established routine procedure in clinical laboratories (16). As well as, this method is appropriate for a remarkably wide range of applications. Any cell- or tissue-bound immunogenic molecule can, theoretically, be detected in situ using this technique. Immunohistochemical techniques detect antigens in tissues or in cells. Proteins (including immunoglobulins), carbohydrates, nucleic acids, lipids and other compounds can act as antigens (17).

In the present study, percentage of oxidative DNA damage (19.27%) as expressed by immunohistchemical assay was reported among healthy control mice. This is possibly due to the normal existence of 8-OHdG in the nuclear DNA. This fact is supported by study done by Magnus *et al.*, (18) showed that the 8-OHdG "which is a chemical modification of DNA caused by ROS in the cell" seems to exist normally in the nuclear DNA at a level of -0.11- per 105 deoxyguanosines (dGs). Also, a study done by Dai *et al.*, (19) revealed that the level of the 8-OHdG positively correlated with age. They stated that the nuclear 8-OHdG levels in the testis were high (more than two lesions per 106 dGs) from three to 52 weeks of age. In addition, 8-OHdG has been shown to be present in mitochondrial DNA at a level ≈ 16-fold higher than that present in nuclear DNA, an observation consistent with the high levels of ROS generated in this organelle (20).

In the current study, the central issue in the results of immunohistochemical assay showed highly significant increase in the percentage of the oxidative DNA damage ($P<0.001$) in cryopreserved group (46.78%, 63.45% and 32.59% representing CPAs glycerol, 1, 2 PrOH and DMSO; respectively) after six weeks of cryopreservation when compared with control group (19.27%). This study suggests that cryopreservation provoke oxidative stress (generation of ROS overwhelms these antioxidant defenses); this aspect is in agreement with many researchers (21, 22). However, other groups demonstrated an increase in ROS following cryopreservation / thawing cycle due to slow recovery of antioxidant activity (23).

In normal condition excessive production of ROS is limited by various regulatory systems, some enzymatic (catalase, superoxide dismutase, and glutathione reductase) and others, nonenzymatic (vitamin E, vitamin C, taurine, hypotaurine, and pyruvate) (24). It has been postulated that the effects produced by ROS depend on their nature, quantity, length of exposure and on the time length of exposure (25).

As well as attack from ROS on DNA is considered a major source of spontaneous damage to the DNA as well as other macromolecules such as proteins and lipids. There are various intra- and extracellular sources of oxygen radicals. The major intracellular source is believed to be electron leakage from the metabolism process in mitochondria, but the main extracellular source ionizing and ultraviolet radiation (26).

Excessive production of ROS is potentially toxic to sperm quality and function (27). This is because ROS are highly reactive oxidizing agents, among which are included H2O2, $O2^{\cdot}$ and $OH^{\cdot}$ (28). Strong evidence suggests that high levels of ROS mediate the occurrence of high frequencies of single- and double-strand DNA breaks commonly observed in the spermatozoa (29). Furthermore, studies in which the sperm was exposed to artificially produced ROS resulted in a significant increase in DNA damage in the form of modification of all bases, production of base-free sites, deletions, frame shifts, DNA cross-links and chromosomal rearrangements (30).

Despite the importance of CPAs in the cryopreservation of tissues and cells, but they differ in their suitability and protection for the DNA, as the study showed 63.45% when using 1, 2 PrOH compared to 46.78% and 32.59% when using glycerol and DMSO respectively. DMSO provided superior cryoprotection compared to 1,2PrOH and glycerol. This observation is in agreement with study done by Keros *et al.*, (14) they reported better testicular tissue cryopreservation with DMSO as CPA. Moreover, Loskutoff *et al.*, (31) reported that epididymal sperm had better post-thaw motility when frozen with DMSO rather than with glycerol, ethylene glycol or propylene glycol. These results may be due to the low molecular weight and penetration ability of DMSO (14). In addition to that, the DMSO has ability to scavenge the hydroxyl radicals ($OH^{\cdot}$), one of the most reactive and damaging free radicals (32).

Although the glycerol is a natural metabolite and it is considered a safe option for the cells; metabolically and genetically, but showed 46.78% of oxidative DNA damage when using it as a CPA. This is in agreement with study done by Keros *et al.*, (14) they reported that glycerol led to a lower protection than DMSO for adult testicular fragments. These results could perhaps be attributed to slowly movements of glycerol across cell membranes and the cells may be damaged by osmotic shock during its removal. Therefore, the purported toxic effect of glycerol may be caused by osmotic shock (33). Furthermore, study done by Guidet and Shah (34) showed that glycerol increase in H2O2 generation, which is one of the reactive free radicals. Whereas, cryopreservation of testicular tissue with 1,2PrOH showed highly percentage of oxidative DNA damage (63.45%). This high percentage might be due to the presence of hydroxyl groups in their structure (35), which are highly reactive species and a powerful initiator of lipid peroxidation (36). In addition to that, 1,2PrOH might be responsible for the development of intermediate toxic metabolites, as is the case for the closely related ethylene glycol, whose catabolism leads to formation of toxic aldehydes (37). Hovatta *et al.* and Hreinsson *et al.*, (3839-) supported the results of this study when referred that cryopreservation method optimized for human ovarian tissue using 1,2PrOH and sucrose not to be optimal for cryopreservation of testicular tissue. In addition, Szurek *et al.* (40) showed that DMSO and ethylene glycol are safer to use for oocyte cryopreservation than 1,2PrOH.

References:

1.Blatt J. Pregnancy outcome in long-term survivors of childhood cancer. Med Pediatr Oncol. 1999; 33: 29–33

2.Bahadur G, Chatterjee R and Ralph D. Testicular tissue

cryopreservation in boys. Ethical and legal issues: case report. Human Reproduction. 2000; 15: 1416–1420.
3.Wyns C, Curaba M, Vanabelle B, *et al.* Options for fertility preservation in prepubertal boys. Human Reproduction Update. 2010; 16 (3): 312-328.
4.Ganadini L, Lambordo F, Lenzi A, *et al.* Cryopreservation and sperm DNA integrity. Cell tissue bank. 2006; 7(2): 918-.
5.Mazzilli F, Rossi T, Sabatini L, *et al.* Human sperm cryopreservation and reactive oxygen species (ROS) production. Acta Eur Fertil. 1995;26:145–148.
6.Milazzo JP, Travers A and Bironneau A, *et al.* Rapid screening of cryopreservation protocols for murine prepubertal testicular tissue by histology and PCNA immunostaining. Andrology. 2010; 31(6): 617-630.
7.Sherman J, Cryopreservation of human semen. In Handbook of the Laboratory Diagnosis and Treatment of Infertility, Keel B and Webster BW (Eds.), CRC Press, Boca Raton, Fla, USA, 1990; 229-260.
8.Bancroft JD and Stevens A. Tissue processing. In: Theory and practice of histology techniques. Bancroft, J.D. (Ed), 2nd edition. Churchill Livingstone. Edinburgh. 1982; 41-121.
9.Ishikawa T, Fujioka H, Ishimura T, *et al.* Increased testicular 8-hydroxy-2'-deoxyguanosine in patients with varicocele. BJU international.2007; 100: 863-866.
10.Sorlie D E. Examination and board review: Medical Biostatistics and Epidemiology. (1sted). Appleton and Lang, Norwalk, Connecticut. 1995; 47-88.
11.Keros V., Hultenby , Borgstro B, *et al.* Methods of cryopreservation of testicular tissue with viable spermatogonia in pre-pubertal boys undergoing gonadotoxic cancer treatment. Human Reproduction. 2007; 22(5): 1384-1395.
12.Goossens E, Frederickx V, Geens M, *et al.* Cryosurvival and spermatogenesis after allografting prepubertal mouse tissue: comparison of two cryopreservation protocols. Fertility Sterility. 2008; 89(3):725-727.
13.Newton H, Aubard Y, Rutherford A, *et al.* Low temperature storage and grafting of human ovarian tissue. Hum Reprod. 1996; 11: 1487-91.
14.Keros V, Rosenlund B, Hultenby K, *et al.* Optimizing cryopreservation of human testicular tissue: comparison of protocols with glycerol, propanediol and dimethylsulphoxide as cryoprotectants. Human Reproduction. 2005; 20(6):1676-1687.
15.Travers A, Milazzo JP, Perdrix A, *et al.* Assessment of freezing procedures for rat immature testicular tissue. Theriogenology. 2011; 76: 981-990.
16.Penault-Llorca F, Adelaïde J, Houvenaeghel G, *et al.* Optimization of immunohistochemical detection of ERBB2 in human breast cancer: impact of fixation. J Pathol. 1994;173:65-75.
17.Beesley JE. Immunocytochemistry. A practical approach. Oxford, United Kingdom: Oxford University Press. 1993.
18.Magnus Z, Tim H, Jean C, *et al.* 32P-postlabeling high-performance liquid chromatography (32P-HPLC) adapted for analysis of 8-hydroxy-2>-deoxyguanosine. Carcinogenesise.1999; 20:1241-1245.
19.Dai N, Hiroyuki A, Hideki K. *et al.* Age and organ dependent dpontaneous generation of nuclear 8 hydroxydeoxyguanosine in male fischer 344 rats. Laboratory investigation. Technical Methods and Pathology. 1999; 80: 249-261.
20.Shigenaga M K, Gimeno, C J and Ames B N. Urinary 8-hydroxy-2>-deoxyguanosine as a biological marker of in vivo oxidative DNA damage. Proceedings of the National Academy of Sciences of the United States of America; 1989; 86: 9697-9701.
21.Thomson LK, Fleming SD, Aitken RJ, *et al.* Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. Hum Reprod. 2009; 24(9): 2061-2070.
22.Zribi N, Chakroun NF, El Euch H, *et al.* Effects of cryopreservation on human sperm deoxyribonucleic acid integrity. Fertil Steril. 2008; November 20 (Epub ahead of print).
23.Wang AW, Zhang H, Ikemoto I, *et al.* Reactive oxygen species generation by seminal cells during cryopreservation.Urology. 1997; 49: 921–925.
24.Griveau JF and Le Lannou D. Reactive oxygen species and human spermatozoa: Physiology and pathology. Int Androl. 1997; 20 (2):61-69.
25.Aitken RJ and Fisher H. Reactive oxygen species generation and human spermatozoa: The balance between benefit and risk. Bioessays. 1994; 16(4): 259-267.
26.Friedberg EC, Walker GC, Siede W, *et al.* DNA repair and mutagenesis. Chapter one. ASM Press: Washington, D.C. 2006; 14.
27.Saleh R and Agarwal A. Oxidative stress and male infertility: from research bench to clinical practice. J. Androl. 2002; 23: 737-752.
28.Warren JS, Johnson KJ, and Ward PA. Oxygen radicals in cell injury and cell death. Pathol. Immunopathol. Res. 1987; 6: 301-315.
29.Aitken RJ and Krausz C. Oxidative stress, DNA damage and the Y chromosome. Reprod.2001; 122: 497-506.
30.Duru NK, Morshedi M and Oehninger S. Effects of hydrogen peroxide on DNA and plasma membrane integrity of human spermatozoa. Fertil. Steril.2000; 74: 1200-1207.
31.Loskutoff NM, Simmons AH, Goulding M, *et al.* Species and individual variations in cryprotectant toxicities and freezing resistances of epididymal sperm from African antelope. Anim. Reprod. Sci. 1996; 42: 527-535.
32.Babbs CF. Scatchard analysis of methane sulfinic acid production from dimethyl sulfoxide: a method to quantify hydroxyl radical formation in physiologic systems. Free Radical Biology and Medicine. 1989; 6(5): 493-503.
33.Agca Y, Gilmore J, Byers M, *et al.* Osmotic characteristics of mouse spermatozoa in the presence of extenders and sugars, Biol. Reprod. 2002; 67: 1493–1501.
34.Guidet B. and Shah SV. Enhanced in vivo H2O2 generation by rat kidney in glycerol-induced renal failure. Physiol. 1989; 257 (3 Pt. 2): 440-445.
35.Van der Elst J, Van den Abbeel E, Nerinckx S, *et al.* Parthenogenetic activation pattern and microtubular organization of the mouse oocyte after exposure to 1,2-propanediol. Cryobiology. 1992; 29: 549–562.
36.Baumber J, Barry A, Jennifer J, *et al.* Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. Andrology. 2003; 24(4): 621-628.
37.Klug S, Merker HJ and Jackh R. Effects of ethylene glycol and metabolites on *in vitro* development of rat embryos during organogenesis. Toxicology *In Vitro*. 2001; 15(6): 635-642.
38.Hovatta O, Silye R, Krausz T, *et al.* Cryopreservation of human ovarian tissue using dimethylsulphoxide and propanediol–sucrose as cryoprotectants. Hum Reprod. 1996; 11(6):1268-1272.
39.Hreinsson JG, Scott JE, Rasmussen C, *et al.* Growth differentiation factor-9 promotes the growth, development, Cryopreservation of human testicular tissue and survival of human ovarian follicles in organ culture. Endocrinol and Met. 2002; 87(1): 316-321.
40.Szurek EA and Eroglu A. Comparison and avoidance of toxicity of penetrating cryoprotectants. J Pone. 2011; 6 (11):19-24