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Histological, ultrastructural and immunohistochemical comparison of immature rat testis cryopreserved with glycerol, propanediol and dimethylsulphoxide

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Abstract
The morphological changes caused by cryopreservation of the testicular tissues with different cryoprotectants have been assessed here. Testes of thirty immature male rats divided into equal five groups; Group (A) as control fresh sample, group (B) with freezing media only. Different freezing protocol were used for other groups; group (C) dimethylsulphoxide (DMSO); group (D) Glycerol and group (E) propanediol (PROH). Light and transmission electron microscope in addition to the Immunohistochemical analysis were carried out. The light and ultrastructure microscopic results showed normal testicular structures were seen in the fresh control group (A), while group (B) showed severe cryoinjury. The severest changes were for propanediol group followed by the glycerol group while DMSO group showed lighter changes. These changes were represented by discontinuity of the cytoplasmic membrane, swelling of the mitochondria and vaculation of the cytoplasm, abnormal nuclei shape with interrupted nuclear membrane and mild clumped chromatin. The results of the DNA fragmentation test revealed the lowest rate of tissue damage in the group (A) and group (C) compared to group (D and E). In conclusion, cryopreservation of immature rat testes by DMSO gave minimum structural alterations of tissue compare with Glycerol and propanediol that did not enhance DNA integrity.

Keywords: immature testicular tissue, Cryoprotectant, cryopreservation, seminiferous tubules

1. Introduction
The focus of recent researches has turned towards preserving gonadal tissue for future use [1]. Cryopreservation of testicular tissue has become a part of gamete preservation of wild animal post-mortem. Genome resource banking is currently urged as an important tool for sustaining the germ line of endangered wild felid species [2, 3]. Testicular sperm are cryopreserved in the form of whole either testicular tissue or sperm suspension [4]. However, freezing as a suspension is less common than freezing as tissue pieces because sperm entrapped in seminiferous tubules are difficultly isolated, resulting in a lower number of sperm retrieval [5, 6]. During freezing/thawing procedure, the extracellular space becomes hypertonic due to the removal of water as ice crystals develop. Intracellular water, therefore, moves outward across the cell membrane due to the differential osmotic gradient, and cells dehydrate and shrink. This is the opportunity when certain cryoprotective compounds come into play permeating the cells and protecting them against high solute concentration or ice crystal damage [7, 8]. For successful cryopreservation of tissue, such as testis tissue, the majority of essential cells need to be viable for the tissue to survive and retain its function. Multiple approaches have been used to assess tissue/cell viability and extent of cryogenic injuries. These approaches commonly include histopathological examination of tissue sections for morphological changes. Using light microscopy, for instance, such objective criteria as seminiferous cord/tubular diameter or cell density within tubule cross sections can be measured, or semiquantitative morphometric analyses applied to subjectively score such criteria as health or integrity of tissue compartments [9, 10]. Transmitted electron microscopy is not widely used, but it can be invaluable in the examination of ultrastrucural components most likely to be affected by testis tissue cryopreservation, including cytoplasm integrity, nuclear membrane, and various organelles. For detection of DNA fragmentation provides insight into the extent of cell damage [11].

The objective of this study was to compare and evaluate three types of cryoprotectants protocols (DMSO, glycerol and propandiol) on the testicular tissue using rats as a model by means of light, transmission electron microscope and immunohistochemical staining.

2. Materials and methods

2.1 Animals

Thirty Immature male rats (*Rattus Rattus Norvigcus*) were used in this experiment. The immature rats were (2-4) weeks old, weighted (30-50) gm. The animals were obtained from the Animal house at the National Center for Drug Control and Research / Ministry of Health. The animals were housed at the animal house in the High Institute of infertility diagnosis and assistant reproductive technology/Al-Nahrain University. They kept under normal environmental condition for one week before euthanized.

2.2 Samples collection and processing

The animals were euthanized by exposure to high dose of ether/chloroform (inhalation). Then the scrotum opened and the testes were collected. The testes weights were recorded after separation of epididymis.

2.3 Experimental design

The testes that collected from (30) immature fertile male rats were divided into five groups:

A- Control group (A): In this group (6 rats) the fresh testicular tissue evaluation assay were done without cryopreservation.

B- Control group (B): In this group (6 rats) the samples were preserved using only freezing media.

C- Cryopreservatives groups (six rats for each): in these groups the samples were frozen six weeks using three different cryoprotectants protocols. Dimethylsulphoxid (Group C), glycerol (group D) and 1, 2, propanediol (group E).

The tissue samples were evaluated by light microscopy, immunohistochemical staining against 8-OHdG antibody, and transmission electron microscopy.

The Cryopreservation of testis occurs in the following steps.

1. The freezing media were prepared as follows: The Hams F-12 culture medium Bottle (Sigma – Aldrich, N6658, USA) at PH range 7.2-7.4 was used. Culture media were filtered by use of 0.22µ Millipore filters (Sartorious). Ten percent fetal calf serum albumen (Sigma-Aldrich, St. Louis, MO, USA) was added to it [12]. Then the cryoprotectant was added at a concentration of 15% for each cryoprotectant DMSO (Sigma), glycerol (BDH), and 1,2 PrOH (Merck). When using DMSO or 1, 2 PrOH as cryoprotectant, the 0.1M of sucrose (BDH) was added to the solution [13].
2. The freezing medium (1.5 ml) was sterilized by using 0.22 µ millipore filters then U.V. light (Daihan Lab Tech) in 1.8 ml cryovial (NUNC).
3. The testis was dissected from rats under dim yellow light and it was washed by using culture media then transported to the cryovial (NUNC) that contains 1.5 ml of freezing media supply with one type of cryoprotectant.
4. The cryovial was placed in the cryovial holder and remained for about 10 minute in the refrigerator when using DMSO as cryoprotectant and at room temperature when using glycerol or 1. 2 PrOH cryoprotectant for equilibration.
5. The cryovial holder that contains cryovial was suspended in the liquid nitrogen vapor for 30 minutes before plunged in the liquid nitrogen, and then it was plunged in liquid nitrogen.
6. After six weeks of cryopreservation. The thawing processes to the sample was done through rapidly transfer cryovial from liquid nitrogen to the water bath (Kotterman) at 37 ºC until melting ice, for at least 5 minutes.
7. The cryoprotectants were removed from sample by descending concentration gradually **(**10%, 5%) of 0.05 M sucrose when use glycerol as cryoprotectant, and by 10% of 0.05 M sucrose when use DMSO or PrOH. Finally, it was washed by culture media only for about 5 minute for each concentration.

2.4 Light microscopy

The specimens were fixed in 10% neutral buffered formalin for overnight and processed with the routine histological procedure for dehydration, clearing, impregnation with paraffin and blocking, sectioning at (5-6 µm) thickness. Finally, the sections were stained with haematoxylin and eosin [14].

The slides were examined under a light microscope (Olympus–Japan), for evaluation of the histological changes. The slides were photographed by light microscope provided with TV-Based computer.

2.5 Transmission electron microscopy

The specimens were fixed in 4% gluteraldyhyde post-fixed in 1% osmium tetroxide and processed with routine histological procedure for the transmission electron microscope [15]. The sections (5-6 µm thickness) were analyzed using a transmission electron microscope (Philips CM-100).

2.6 Immunohistochemical detection kits

Monoclonal mouse Anti- 8-OHdG antibody (manufactured by Santa Cruz Biotechnology), is used for detection of 8-OHdG by immunohistochemistry (including paraffin-embedded sections). Mouse LSAB Staining System (Immunocruz Code sc-2050).

2.7 Immunohistochemical Detection of 8-OHdG

Immunohistochemical detection of 8-OHdG includes the following steps:

1. Five microns sections were obtained from formalin fixed paraffin embedded tissue blocks and mounted on Fisher brand positively charged slides.
2. Slide baking: the slides were placed in a drying oven (hot air oven) at 65 ^{0}C for one hour.
3. Deparaffinizing and rehydration the slides to distilled water.
4. Peroxidase block: after draining and blotting around the specimen, enough drops of peroxidase block reagent were applied onto the tissue covering the whole section and incubated at room temperature for 10 minutes in a humid chamber, after that the slides were rinsed gently with buffer (BDH) for a minimum of 15 seconds. Then drained and blotted.
5. Protein block: Enough drops of protein block were applied onto the tissue covering the whole section and incubated at room temperature for 5 minutes to block nonspecific background staining, then drained and blotted.
6. Primary antibody: primary antibodies were applied onto each section and incubated at room temperature for 2 hours, in a humid chamber, and then slides were rinsed

with a stream of buffer, and then placed in a fresh buffer bath for 5 minutes. Slides were rinsed again with buffer then drained and blotted gently.

7. Secondary antibody (biotinylated link): secondary antibodies were applied onto the sections and incubated at room temperature for 10 minutes in a humid chamber. Then the slides were rinsed with a stream of buffer and then placed in a fresh buffer.
8. Streptavidin: enough drops of streptavidin reagent were applied to cover the whole section and incubated at room temperature for 10 minutes in a humid chamber. Then the slides were rinsed with a stream of buffer then placed in a fresh buffer bath for 5 minutes.
9. Substrate-chromogen solution: 20 µl of DAB Chromogen were added to 1 ml of DAB Substrate, mix by swirling and applied to each section covering the whole specimen and incubated in darkness at room temperature for 10 minutes. Slides were rinsed with a stream of buffer then placed in a buffer bath for 5 minutes.
10. Enough drops of the haematoxylin solution were applied covering the whole section and incubated at room temperature for 1 minute. The slides were rinsed gently with distilled water then drained and blotted. Enough drops of an aqueous-base mounting medium were applied onto the tissue sections and covered with coverslips and left to dry [16].

2.8 Assessment of Immunohistochemical Staining:

At least ten images were captured for each sample using Microns microscope contain TV-Based computer. Assessments of immunohistochemical staining were done using Aperio positive pixel count algorithms to analyze digital slides. 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).

2.9 Statistical analysis

The mean difference between groups was performed using the one way ANOVA test, through SPSS version 20.

3. Results and discussion

3.1 Histological observations

The control group (A) showed normal tissue morphology. The seminiferous tubules were normal arrangement surrounded by highly vascularized interstitial irregular connective tissue with myoid cells. In addition to the presence of large polyhedral cells (leydig cells) with eosinophilic cytoplasm and spherical nucleus. The seminiferous epithelium composed of spermatogonia and supporting (Sertoli) cells (Figure 1). Few number of Sertoli (sustentacular) cells located at the base of the epithelium with large, basal and oval pale nucleus. The immature cells (spermatogonias) were located at the base of the epithelium as dome-shaped cells (A spermatogonia) with flattened, oval nuclei stained darkly (type A dark spermatogonia) or lightly (type A pale spermatogonia). Whereas type B spermatogonia has round nucleus (Figure 2). These normal histological observations of the testes were similar to the founding in previous researches in rats. [17-19].

In the present work, after six weeks of cryopreservation, the paraffin sections of testis showed morphological and structural alteration in seminiferous tubule and the component of interstitial tissue. However, these alterations differ according to the type of cryoprotectants that used. Studies have been reported that freezing retards autolytic morphological and histological changes caused by autolytic enzymes released from lysosomes [20, 21].

The results in this study showed that the main prominent histological changes observed in group (B) that used only freezing media were the seminiferous tubules presented irregularities and shrinkage, severe disruption of interstitial tissue and increase in the intertubular space. Severe rupture of the layers of the connective tissue fibers was also observed (Figure 3). Furthermore, there were characteristic gaps within the seminiferous epithelium due to the cytoplasmic shrinkage. The testicular cords showed severe detachment of the spermatogonia and spermatocytes from the basement membrane and from the supporting cells. In addition the lumen of the seminiferous tubules contained desquamated cells as noticed in (Figure 4) that was not seen in the control group. These observations after freezing /thawing of testicular samples were reported previously [22]. Whereas, it has been shown that the freezing-thawing procedure had no significant effect on tubular diameter [23]; however, it caused a `folding' of the lamina propria and notable damage to Sertoli cells and spermatogenic cells that displayed occasional nuclear damage, vacuolization, and shrinkage/swelling of the cytoplasm. Regards this phenomena, during freezing samples the extracellular ice formation causes elevated solvent concentrations, it leads to cell dehydration; which can permanently damage cell membranes [24]. The effects of freezing on the live cells are make direct mechanical puncturing of the cell membranes by ice crystals and effects of osmotic changes due to ice formation [25-8],

The histological observation of group (C) samples that cryopreserved by DMSO as a Cryoprotectant, there were no major differences compared with control group (A). These samples contain less damage. They were well-preserved testicular tissue. The main prominent histological changes observed after cryopreservation by DMSO were mild disruption of interstitial tissue and mild increase in the intertubular space (Figure 5). Furthermore, the testicular cords showed mild detachment of cells in the seminiferous tubules from the basement membrane and contained less necrotic cells confined to the center of the testicular cords. However, the other cell components seemed well preserved as noticed in (Figure 6). According to the finding of previous work, some cryoprotectants such as DMSO inhibit nucleation by increasing the high viscosity of intracellular water [26].

In the present study, group (D) that tissue cryopreserved by glycerol as cryoprotectant showed moderate morphological and structural changes. These changes characterized by moderate disruption of interstitial tissue between the layers of the connective tissue fibers, some of the seminiferous tubules presented irregularities and shrinkage (Figure 7). In addition, a detachment of spermatogonia from the basement membrane of the testicular cords and necrotic cells in the seminiferous tubules lumen (Figure 8). On the other hand, glycerol is a better cryoprotectant for pig tissues [9]. These differences may be related testicular architecture, morphology, or lipid composition [27]. However, non-permeating cryoprotectant, increase and promote cellular dehydration by increasing the extracellular solute concentration thereby reducing intracellular crystallization [24].

Based on our observations, group (E) that tissue samples subjected to 1,2 PrOH as Cryoprotectant, they displayed severest morphological and structural changes. There were shrinkage and irregularities of seminiferous tubules in addition to severe disruption of interstitial tissue and increase in the intertubular space (Figure 9). Destruction of the

basement membrane was seen in addition to severe detachment of spermatogonia (Figure 10). Reports had shown that tissue freezing is a cryobiological challenge due to several cell types in the tissue and is influenced by cell-to-cell interaction that limit cell functional integrity [28]. However, these different results of the PrOH compared with the other cryoprotectants may be belong to its biophysical properties and low permeating to the cells and protecting them against high solute concentration [26].

3.2 Immunohistochemical observations

Immunohistochemical procedure were detected the damage in different types of testicular cells that caused by cryopreservation. The results revealed that the positive reaction of 8-OHdG was the nuclei which stained almost homogeneously, the pattern of the stain is diffuse and color of stain is gray to brown. The cells labeled by the antibody display a staining almost entirely confined to the nucleus and with a diffuse pattern. Rarely cytoplasmic staining is observed. In this study appeared that the rat's testicular sections of control and cryopreserved testes were stained with anti-8-OHdG antibody, and in both sections showed variable distribution of 8-OHdG, but with the cryopreserved group displaying significantly higher staining compared to the control group. The statistical analysis to these sections through the Aperio positive pixel count algorithms showed significantly ($P \leq 0.05$) higher value of the group (B) compared with the other groups. In contrast, group (C) represented significantly ($P \leq 0.05$) lower values than cryopreserved groups (D and E), whereas the control group (A) has been the lowest value at all, as shown in figure (11).

In control group (A), there were weak positive signals for anti-8-OHdG antibody were detected. However, the positive signals showed variable intensity, weak positive signals appeared in the center of the seminiferous tubule and around numerous germ cells. The positive signals increased intensity in the interstitial tissue, whereas no positive signals were observed in nucleus of the germ cells (Figure 12). In contrast, the results of group (B) showed intensely positive staining, these include the most of the seminiferous epithelium (Figure 13). However, the spermatogonia seemed more resistant to the cryoinjury effects.

In cryopreserved group, the tissue sections displaying significant higher staining intensity compared to the control group (A), variation according to the type of the cryoprotectant used. Tissue cryopreserved by DMSO as cryoprotectant showed varying degrees of positive intensity for 8-OHdG in the seminiferous tubules and the interstitial tissue. The weak positive signals appeared around the germ cells and in the interstitial tissue of the testis. While the nucleus of the germ cells, and spermatocyte showed negative reaction. However, the center of the tubules appeared more intensely positive. But the weak positive signals appeared in the primary spermatocyte in the basal layer (Figure 14). Studies have shown that DMSO is provides a greater protection of DNA integrity than other cryoprotectant in testicular tissue [21]. Because it's has low molecular weight and high tissue penetration offering better results than PrOH [29]. It has been shown that one of the changes is retraction of apical processes of Sertoli cells which affects specialized intercellular junctions and this lead to releasing the germ cells in the tubular lumen [30]. While in present work, tissue samples subjected to glycerol as cryoprotectant displayed greater intensity of the positive signals compared with the other groups. These strong positive signals concentrated mainly in the nucleus of the secondary spermatocyte cells and in the interstitial tissue of the testes (Figure 15). Previously report data supporting that glycerol has lowest rate of tissue survival compared to DMSO [29].

Whereas, when tissue samples cryopreserved by 1, 2 Pr OH as cryoprotectant, the stronger positive signals increased in both seminiferous tubules and the interstitial tissue more than other groups (Figure 16). Generally, the spermatogonia seemed more resistance to the cryodamage than the other types of cells. Studies have been reported that uses the cryoprotectant medium may be not optimal for all types of cells [22]. The optimal conditions to protect a particular cell will also vary and depends on factors such as the molecular weight and permeability of the cell membrane to the compound. Thus an optimal cryoprtectant agent should be defined for each cell type separately. However, it is difficult to find the optimal cryopreservation conditions for all testicular tissues.

3.3 Ultra structural observation

Generally, the ultra-structural analyses of testicular sections of the control group (A) were structurally normal (Figure 17). Clear attachments of spermatogonia and Sertoli cells to the basement membrane. Clear integrity of the cytoplasm membrane and normal distribution of the cytoplasmic organelles including the Golgi apparatus, rough endoplasmic reticulum and the size and shape of mitochondria and its cristae. There were clear dark and light mitochondria in the cytoplasm of the Sertoli cell. Integrity of the nuclear membrane, the nuclei was regular-shaped, normal morphology of euchromatin and heterochromatin, in addition to the normal nucleolus (Figure 17, 18). These normal ultra-structural features of the testicular tissue were seen in rat testes [31]. In addition to the various sizes lipid droplets as a lighter spots found in the cytoplasm of murine Sertoli cells [32]. That was not detected in the present study. Otherwise, sections belong to group (B) demonstrated major types of cryoinjury after freezing-thawing procedure. However, it caused notable damage to Sertoli cells, and spermatogonia. The damage includes intercellular junctions also. The cells loss greater part of the cell membrane and replaced by huge gaps between adjacent cells. The mitochondria showed severe swelling and there were no cristae recognized inside it. Presences of cytoplasm vacuolization or cytoplasmic vacuolation were clearly observed. Greater part of the nucleus membrane had also showed severe disruption. Dense clumped chromatin of the nucleus and missing its marginal arrangement. In addition to the dense clumped nucleoli (Figure 19, 20). These changes correspond to metabolic alterations of the Sertoli cell [30], Whereas the spermatogonia after thawing shows shrinkage due to osmotic damage that lead to loss of membrane integrity followed by cell membrane damage [22].

Minor changes were seen in group (C) at the level of cellular components of the epithelium of the seminiferous tubules. These changes were represented by swelling of the mitochondria. However, some of these had clear cristae. No changes in the cell membrane and cell to cell connection but there was vacuolation of the cytoplasm. In addition to light clumped chromatin in the nucleus. However, they showed mostly normal distribution. Normal nuclei shape with nuclear intact membrane (Figure 21, 22).

Group (D) showed greater changes at the level of cell components compared with the group (C). These changes were represented by discontinuity of the cytoplasmic membrane and clear empty space between neighboring cells.

Swelling of the mitochondria and vaculation of the cytoplasm were seen. Abnormal nuclei shape with interrupted nuclear membrane and mild clumped chromatin (Figure 23, 24). Vacuolization in the cytoplasm of the spermatogenic cells has been reported as in the present work as a type of injury resulting from cryopreservation and it's an early sign of testicular damage [30].

Group (E) were subjected severe ultrastructure cellular changes when compared with the previous cryopreservative groups, including the Sertoli cells and the other spermatogenic cells. These changes were represented by disruption of the cytoplasmic membrane and loss connection between neighboring cells and swelling of the mitochondria. The nuclei are shrinkage with severe interrupted nuclear membrane with dense clumped chromatin (Figure 25, 26). Previous researches have been shown that the degrees of cryoprotection are varying in different cell types depending on the cryoprotectants properties and those of cell membranes [24-7]. And the side effect of the cryoprotectant is additional cytotoxic effects. Thus, tissue tolerance to cryoprotectants is limited and overexposure may cause damage [33].

4. Conclusion

We have demonstrated that dimethylsulphoxid is a well cryoprtatent agent for testicular tissue of immature rats where it showed the lowest affects tissue cells compared with glycerol and propanediol that tissue showed more sensitivity to them.

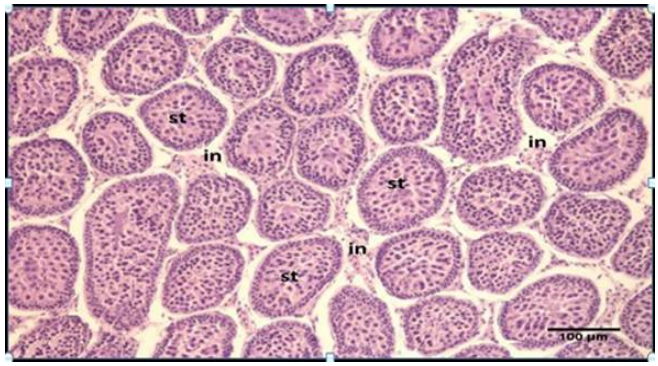

Fig 1: Section of immature fresh rat testis, (Control immature group A) showing normal arrangement of the seminiferous tubules (st) which lined by seminiferous epithelium, interstitial connective tissue (in) with leydic cells (H&E). MP: 100

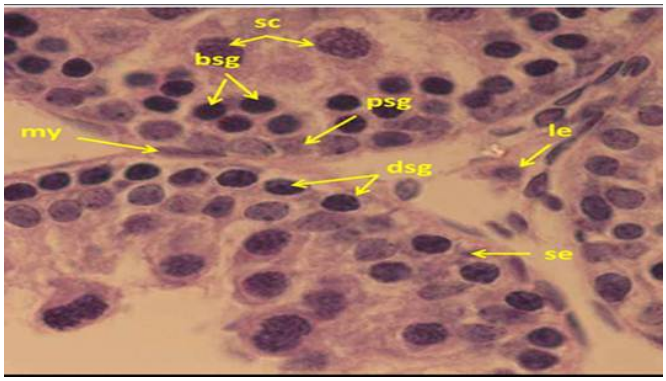

Fig 2: Section of immature fresh rat testis, (Control mature fresh group A) showing normal arrangement of seminiferous tubules which surrounded by connective tissue with leydic cell (le), myoid cell (my) in the basement membrane, dark spermatogonia A (dsg), pale spermatogonia A (psg), spermatogonia B (bsg), spermatocytes (sc) and Sertoli cell (se).(H&E) MP: 1000

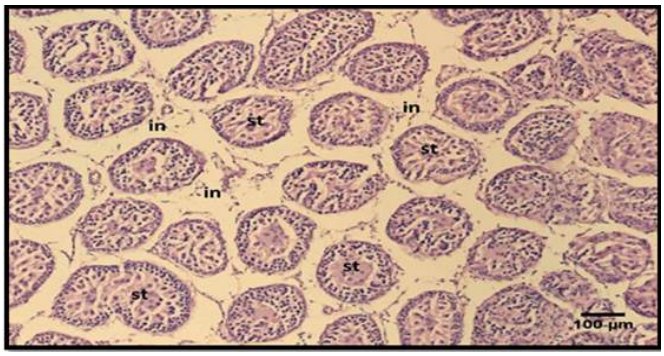

Fig 3: Section of immature rat testis (group B), using freezing media only, showing irregular arrangement and shrinkage of the seminiferous tubules (st), notice intertubular space and gaps between seminiferous tubules (in). (H&E). Mp:100

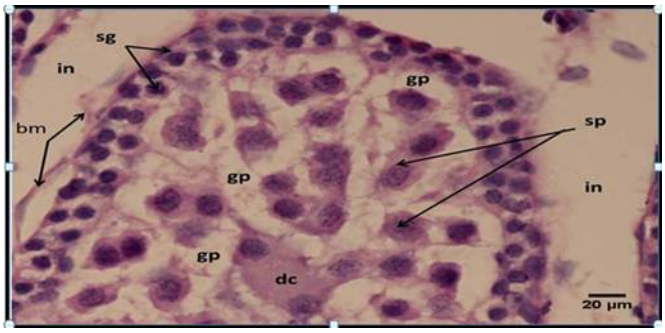

Fig 4: Section of immature testis (group B), using freezing media only, showing disruption of interstitial tissue and intertubular gaps (in), disruption of basement membrane (bm), irregular arrangement of the seminiferous epithelium, Notice: and gaps (gp) between spermatogonia (sg) and also between spermatocytes (sc), desquamated cells (dc). (H&E). MP:1000.

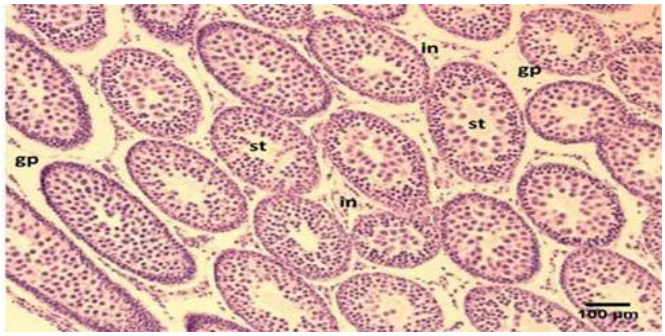

Fig 5: Section of immature testis cryopreserved with DMSO (group C) showing gap (gp) in the interstitial connective tissue (in), light shrinkage in the seminiferous tubules (st). (H&E). Mp:100

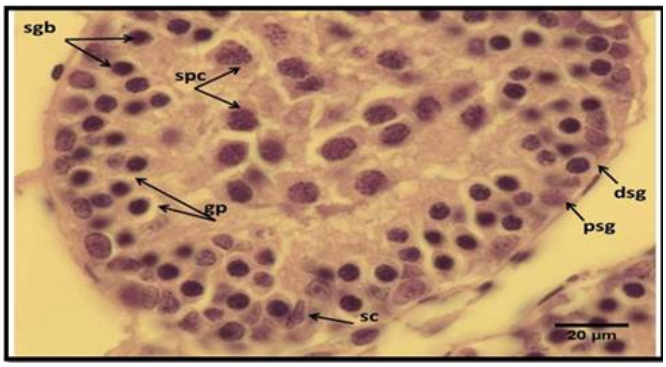

Fig 6: Section of immature testis cryopreserved with DMSO (group C) showing detachment of epithelia of the testicular cord with each other, where pale spermatogonia A (psg), dark spermatogonia A (dsg), spermatogonia B (sgb), Sertoli cell (sc), spermatocyte (spc), gaps between cells (gp). (H&E). MP 1000.

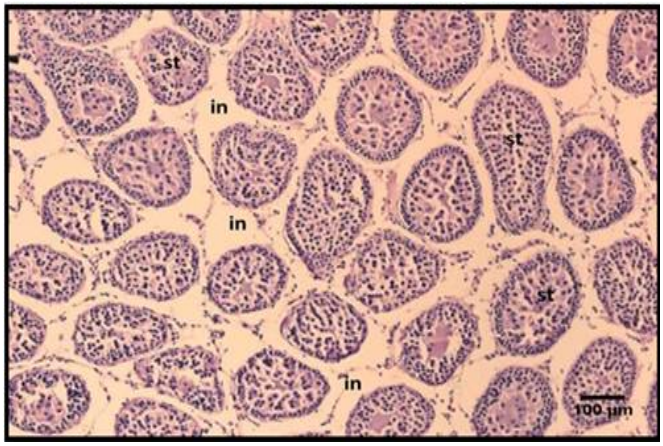

Fig 7: Section of immature testis cryopreserved with glycerol (group D) showing the damage of the interstitial connective tissue and gaps formation (in), shrinkage of seminiferous tubules (st). (H&E). MP:100.

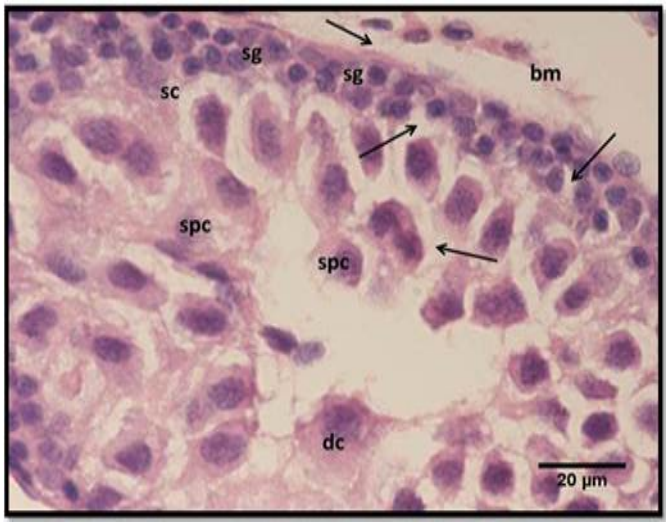

Fig 8: Section of immature testis cryopreserved with glycerol (group D) showing detachment of epithelia of the testicular cord with each other and gaps formation (arrows), where spermatogonia (sg), Sertoli cell (sc), spermatocyte (spc), desquamated cells (dc). (H&E). MP:1000

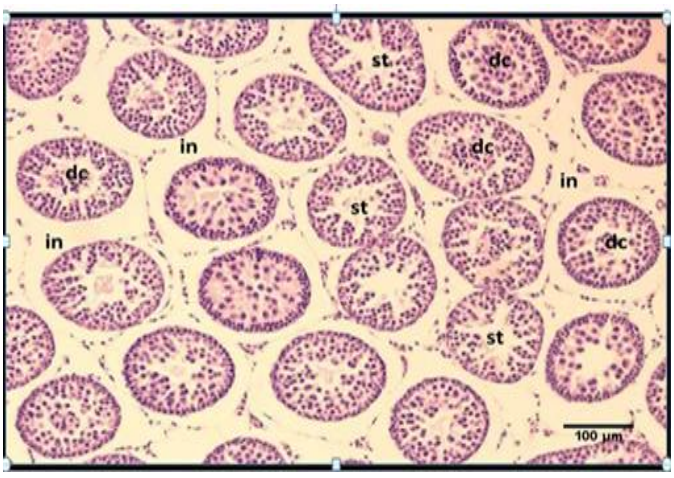

Fig 9: Section of immature testis cryopreserved with 1,2 PrOH (group E) showing sever disruption of the interstitial connective tissue, notice; Clear gaps between seminiferous tubules (in), shrinkage of the seminiferous tubules (st), desquamated cells within tubular lumen (dc). (H&E). Mp:100.

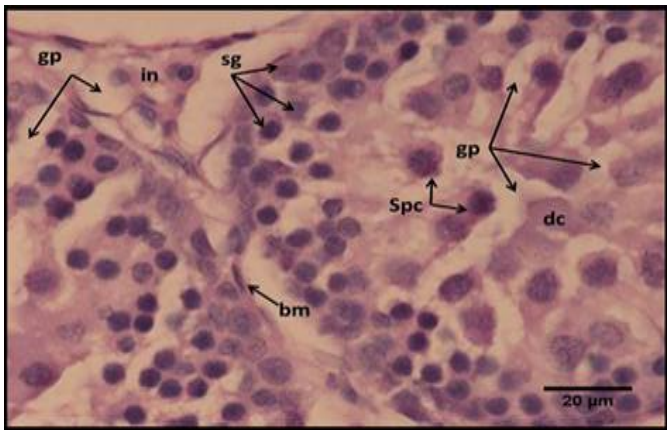

Fig 10: Section of immature testis cryopreserved with 1,2 PrOH (group E) showing sever detachment of epithelia of the testicular cord with each other with clear gap (gp), disruption of the basement membrane (bm), where spermatogonia (sg), Sertoli cell (sc), spermatocyte (sp), desquamated cells (dc). (H&E). MP:1000

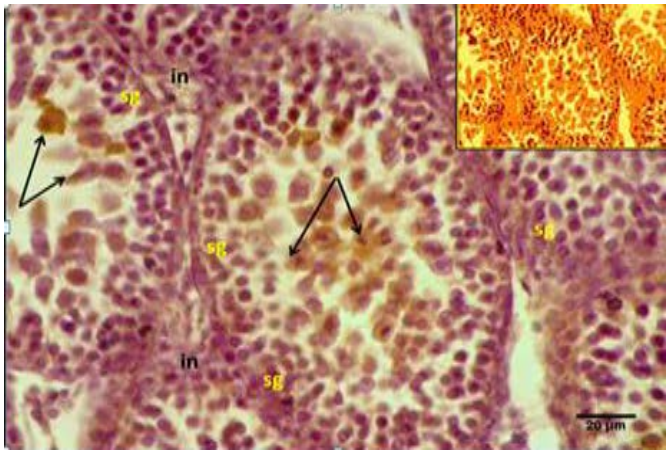

Fig 11: The immunohistochemical micrography of testicular tissues of immature control groups (A), showing slight antigen-antibody reaction as a brown area (arrows) mostly in the spermatocytes and in the tubular lumen, while the spermatogenic cells (sg) showing negative reaction. Inset showing Aperio positive pixel image. MP:400.

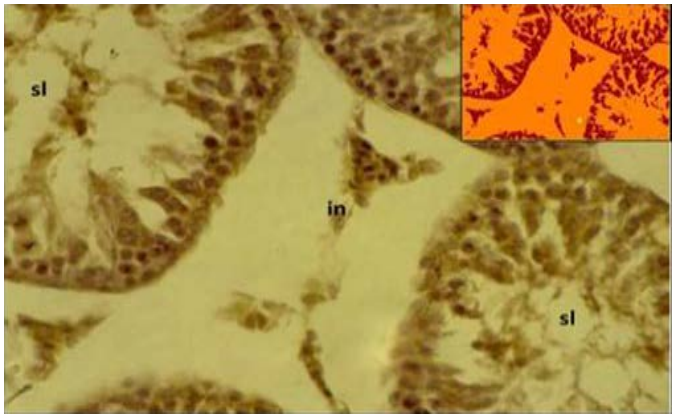

Fig 12: The immunohistochemical micrography of immature testicular tissues preserved with freezing media only (groups B), showing intensely positive staining as a brown area in the most of seminiferous tubule components (sl) and interstitial connective tissue (in) as a positive reaction. Inset showing Aperio positive pixel image. MP:400.

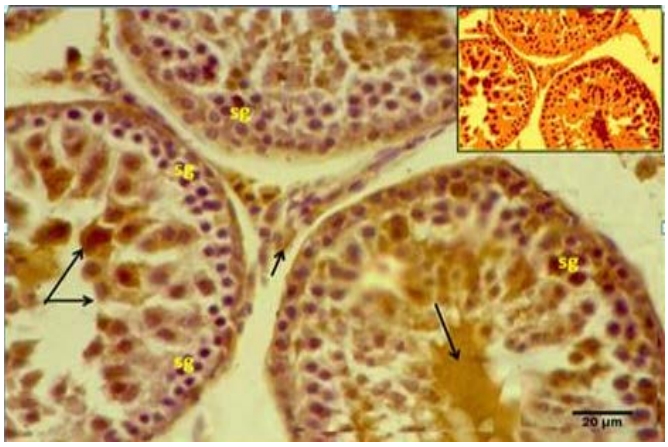

Fig 13: The immunohistochemical micrography of immature testicular tissues cryopreserved with DMSO (groups C), showing positive signals for anti-8-OHdG antibody reaction (arrows) which restricted mostly in spermatocytes, while spermatogonia (sg) showing negative signal. Inset showing Aperio positive pixel image. MP:400.

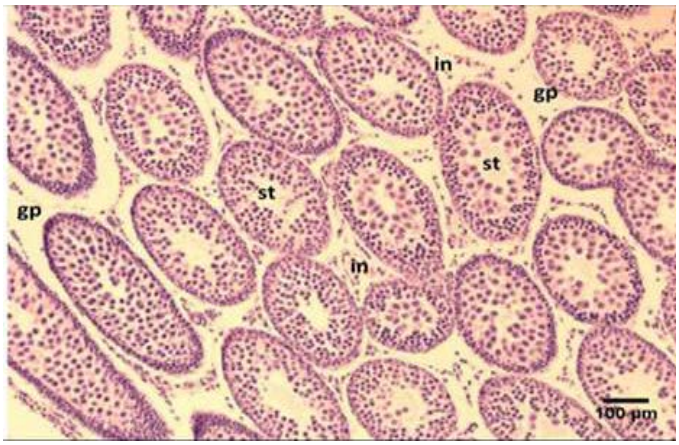

Fig 14: Section of immature testis cryopreserved with DMSO (group C) showing gap (gp) in the interstitial connective tissue (in), light shrinkage in the seminiferous tubules (st). (H&E). Mp:100

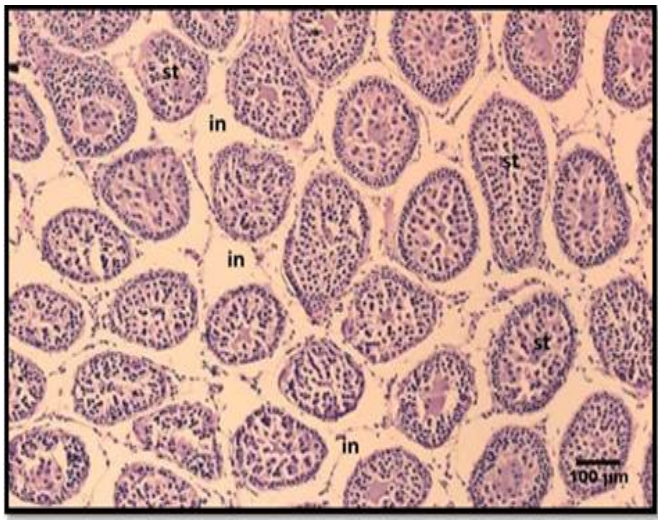

Fig 15: Section of immature testis cryopreserved with glycerol (group D) showing the damage of the interstitial connective tissue and gaps formation (in), shrinkage of seminiferous tubules (st). (H&E). MP:100

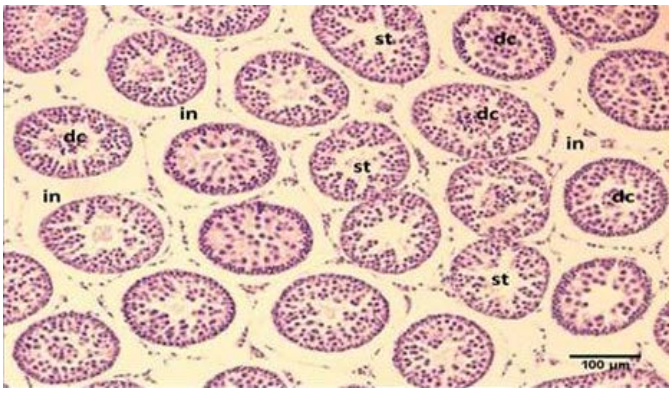

Fig 16: Section of immature testis cryopreserved with 1,2 PrOH (group E) showing sever disruption of the interstitial connective tissue, notice; Clear gaps between seminiferous tubules (in), shrinkage of the seminiferous tubules (st), desquamated cells within tubular lumen (dc). (H&E). Mp:100

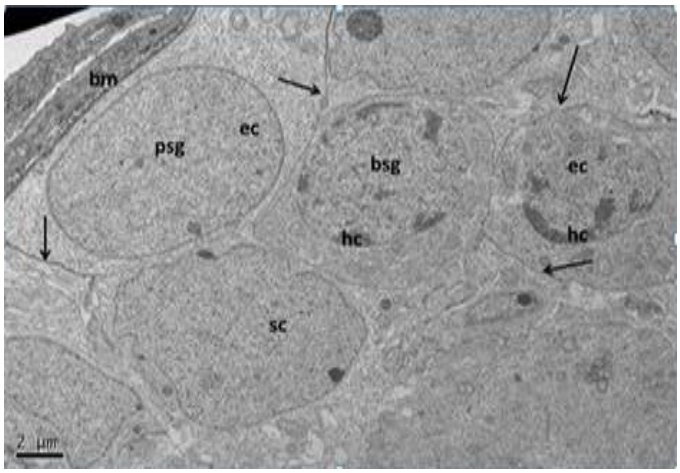

Fig 17: Electron micrograph (TEM) of immature rat testes from fresh control group (A) showing normal cellular components. Notice intact cytoplasmic membrane (arrows), where pale spermatogonia A (psg) with dome shaped nucleus sit on the intact basement membrane (bm), B spermatogonia (bsg) with rounded nucleus, nucleus of sertoli cell (sc) with intact nuclear membrane, normal arrangement of the peripherally heterochromatin (hc), euchromatin (ec). MP: 890.

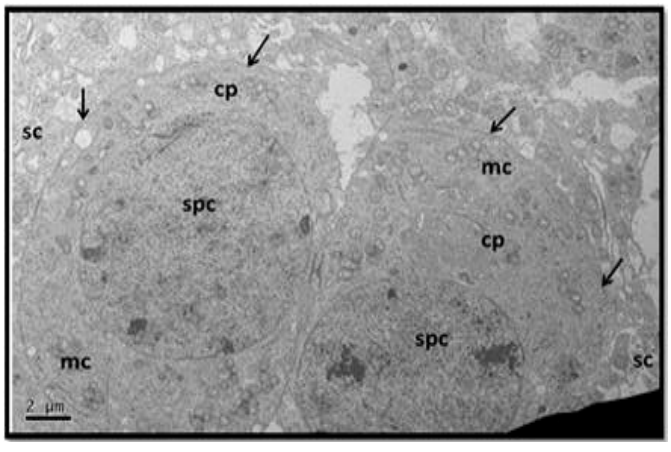

Fig 18: Electron micrograph (TEM) of immature rat testes from fresh control group (A) showing normal cellular components of the spermatocytes (spc). Notice intact cytoplasmic membrane (arrows) surrounded by Sertoli cell process (sc), normal cytoplasmic componment (cp) with normal mitochondria (mc). MP: 890.

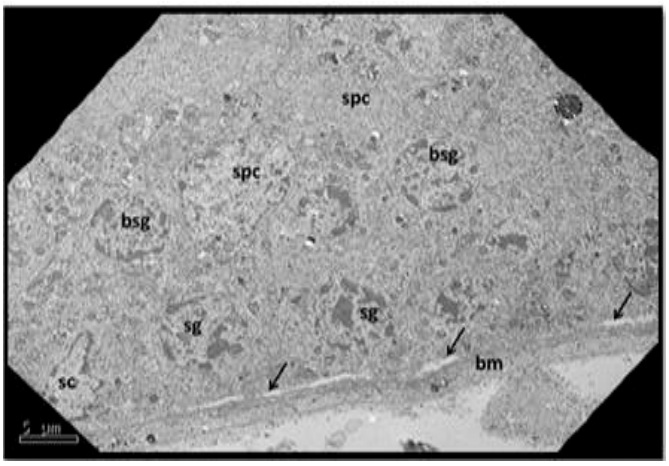

Fig 19: Electron micrograph (TEM) of immature rat testes from group (B) cryopreserved with freezing media only showing sever cellular changes, no clear demarcation between cells due to damage of cell membrane and separation of cells from basement membrane (bm). Notice the cells nuclei had dense clumped chromatin. Where permatogonia A (sg); spermatogonia B (bsg); Spermatocytes (spc); Sertoli cell (sc). MP:440.

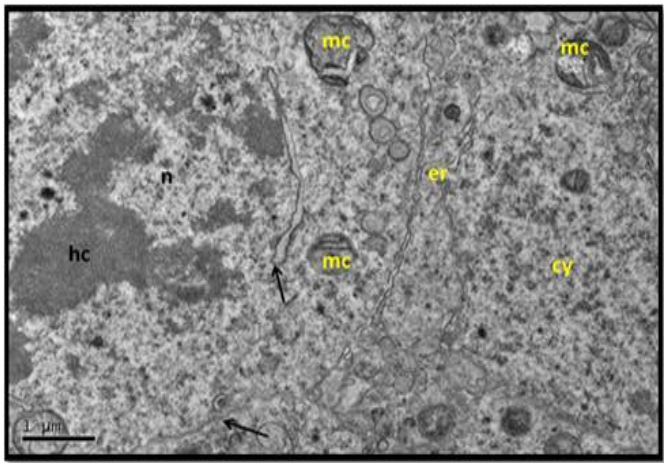

Fig 20: Electron micrograph (TEM) of immature rat testis from group (B) cryopreserved with freezing media only showing; sever cryoinjury of spermatogonia represented by dense clumped chromatin (hc) of the nucleus (n); disruption of the nuclear membrane (arrows); and swelling of the mitochondria (mc), cell cytoplasm (cy) endoplasmic reticulum (re). MP: 2900.

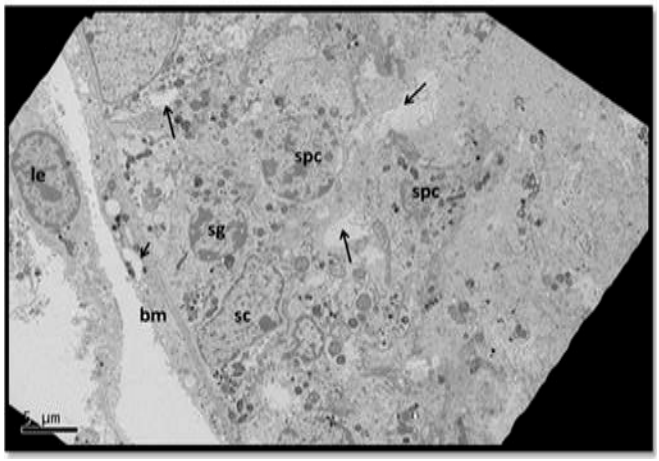

Fig 21: Electron micrograph (TEM) of immature rat testes from group (C) cryopreserved with DMSO showing Light cellular changes of the spermatogonia (sg), spermatocyte (spc), and sertoli cell sited on the basement membrane (bm), notice normal shaped nucleus and intact nuclear membrane with light clumped chromatin, and vacuolated cytoplasm (arrows). MP: 440.

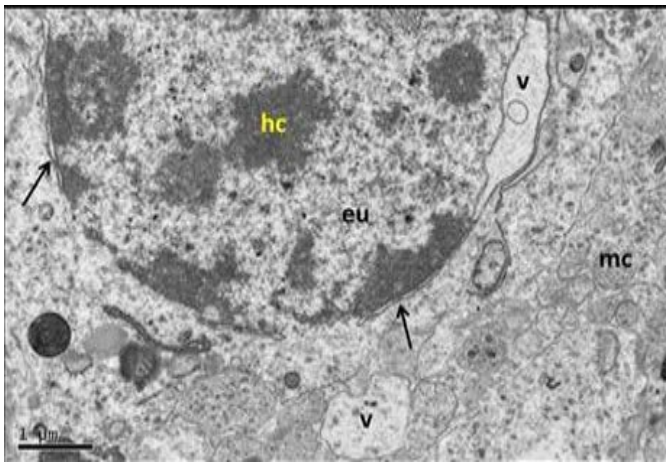

Fig 22: Electron micrograph (TEM) of spermatocyte from immature group (C) cryopreserved with DMSO. Showing; clumped heterochromatin (hc) and euochromatin (eu) surrounded by nuclear membrane (arrows), vacuoles (v) in the cytoplasm and normal mitochondria (mc). MP: 2900.

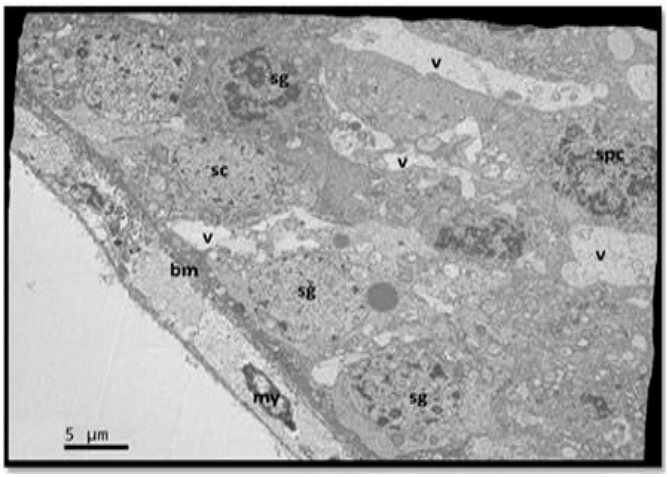

Fig 23: Electron micrograph (TEM) of immature rat testes from group (D) cryopreserved with glycerol showing cellular changes. Notice; no clear cell boarder and gaps formation between two adjacent cells (v) and vacuolation of the cytoplasm, shrinkage of the some nucleus of spermatogonia (sg), spermatocytes (spc) and Sertoli cell (sc), myoid cell (my) where heterochromatin had clumping appearance. MP: 440.

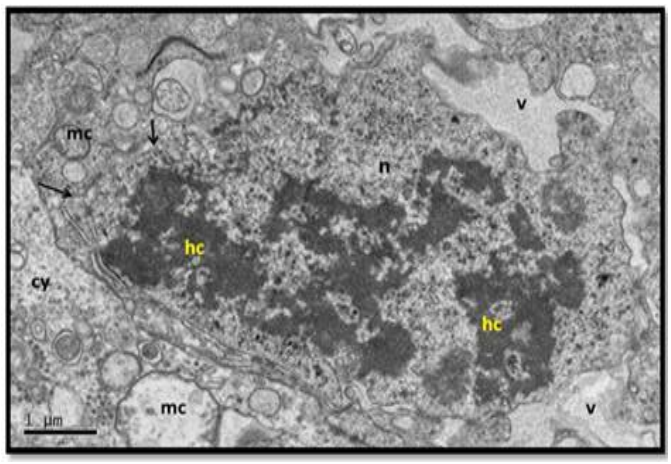

Fig 24: Electron micrograph (TEM) of spermatogonia of immature rat testes from group (D) cryopreserved with glycerol showing sever cellular changes. Notice shrinkage of the nucleus (n), clumping chromatin (hc), discontinuity of nuclear membrane (arrows), the cytoplasm (cy) showing vacuolation, swelling mitochondria (mc). MP: 2900.

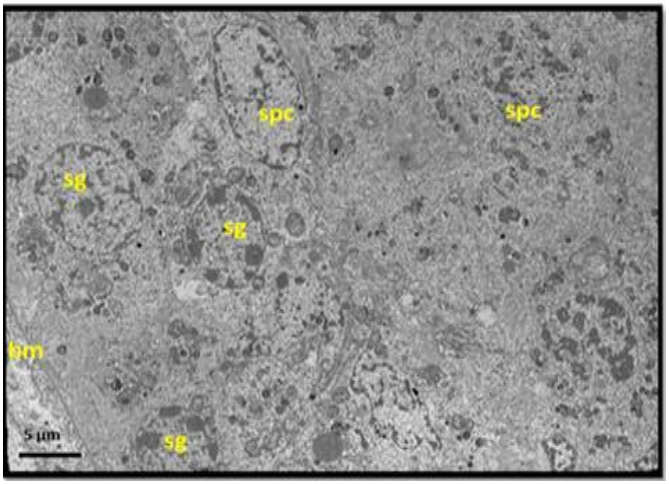

Fig 25: Electron micrograph (TEM) of immature rat testes from group (E) cryopreserved with 1,2 PrOH showing cellular changes. Notice sever damage to the cell membrane (no clear demarcation between cells); shrinkage of the nucleus with clumping chromatin of spermatogonia (sg), and spermatocyte (spc), basement membrane (bm). MP: 440

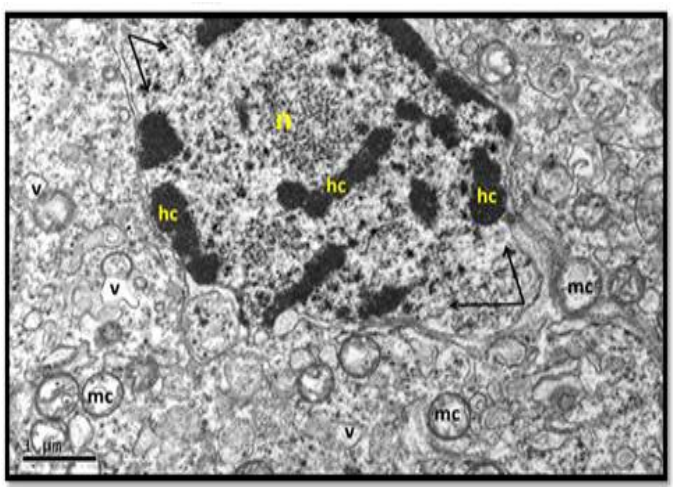

Fig 26: Electron micrograph (TEM) of immature rat testes from group (E) cryopreserved with 1,2 PrOH showing spermatogonia. Notice shrinkage of the nucleus (n) with clumping chromatin (hc), disruption of nuclear membrane (arrows), swelling mitochondria (mc), vacuolated cytoplasm (v). MP: 2900.

5. References

1. Bahadur G, Ralphm D. Gonadal tissue cryopreservation in boys with paediatric cancers. Human Reproduction. 1999; 14:11-17.
2. Tsutsui T, Wada M, Anzai M, Hori T. Artificial insemination with frozen epididymal sperm in cats, Journal of Veterinary Medicine Science. 2003; 65:397-399.
3. Chatdarong K, Axne´r E, Manee S, Thuwanut P, Linde C. Pregnancy in the domestic cat after vaginal or transcervical insemination with fresh and frozen semen, Theriogenology. 2007; 68:1326-1333.
4. Crabbe E, Verheyen G, Tournaye H, Steirteghem V. Freezing of testicular tissue as a minced suspension preservessperm quality better than whole-biopsyfreezing when glycerol is used as cryoprotectant. International Journal of Andrology. 1999; 22:43-48.
5. Silber SJ, Van Steirteghem AC, Liu J, Nagy Z, Tournaye H, Devroey P. High fertilization and pregnancy rate afterintracytoplasmic sperm injection with spermatozoa obtained from testicle biopsy. Human Reproduction. 1995; 10:148-152.
6. Allan JA, Cotman AS. A new method for freezing testicular biopsy sperm: three pregnancies with sperm extracted from cryopreserved sections of seminiferous tubule. Fertil Steril. 1997; 13:639-645.
7. Fuller B, Paynter S. Fundamentals of cryobiology in reproductive medicine. Reprodactive Biomedicine Online. 2004; 9:680-91.
8. Pegg DE. Principles of cryopreservation. Methods in Molicular Biology. 2007; 368:39-57.
9. Abrishami M, Anzar M, Honaramooz A. Cryopreservation of immature porcine testis tissue to maintain its developmental potential after xenografting into recipient mice. Theriogenology. 2010; 73:86-89.
10. Travers A, Milazzo JP, Perdrix A, Metton C, Bironneau A, Mace B *et al*. Assessment of freezing procedures for rat immature testicular tissue. Theriogenology. 2011; 76:981-990.
11. Keros V, Rosenlund B, Hultenby K, Aghajanova L, Levkov L, Hovatta O. Optimizing cryopreservation of human testicular tissue: comparison of protocols with glycerol, propanediol and dimethylsulphoxide as cryoprotectants. Human Reproduction. 2005; 20:1676-1687.
12. Shinohara T, Inoue K, Ogonuki N, Kanatsu-Shinohara M, Miki H, Nakata K *et al* Birth of offspring following transplantation of cryopreserved immature testicular pieces and in-vitro micro insemination. Hum. Reprod. 2002; 17:3039-45.
13. Hovatta O. Cryopreservation of human ovarian tissue using dimethyl sulphoxide and propanediol-sucrose as cryoprotectant. Human. Reproduction. 1996; 11(6):1268-1272.
14. Bancroft JD, Stevens A. Tissue processing. In: Theory and practice of histology techniques. Bancroft, J.D. (Ed), 2nd edition. Churchill Livingstone. Edinburgh. 1982, 41-121.
15. Shaklai M, Tavasoli M. A modified technique to obtain uniform precipitation of lanthanum tracer in the extracellular space. Journal of Histochemistry and Cytochemistry. 1977; 25:1013-5.
16. Ishikawa TO, Griffin KJ, Banerjee U, Herschman HR. The zebrafish genome contains two inducible, functional cyclooxygenase-2 genes. Biochemical and Biophysical Research Communications. 2007; 352(1):181-187.
17. Cartner LP, Hiatt JL. color text book of His toloy".Saunders,3rd edition. 2007, 489-499.
18. Naraghi MA, Abolhasani F, Kashani I, Anarkooli IJ, Hemadi M, Azami A. The effects of swimming exercise and supraphysiological dose of nandrolone decanoate on the testis in adult male rats: A transmission electron microscope study. Folia Morphologica. 2010; 69(3):138-146.
19. Nosseir NS, Ali MH, Ebaid HM. A Histological and Morphometric Study of Monosodium Glutamate Toxic Effect on Testicular Structure and Potentiality of Recovery in Adult Albino Rats. Research Journal of Biology. 2012; 2:66-78.
20. Zdravkovic M, Kostov M, Stojanovic M. Identification of postmortem autolytic changes on the kidney tissue using pas stained method. Medicine and Biology. 2006; 13:181-184.
21. Thuwanut P, Chatdarong K. Cryopreservation of Cat Testicular Tissues: Effects of Storage Temperature, Freezing Protocols and Cryoprotective Agents.

Reproduction in Domestic Animals. 2012; 47:777-781.
22. Nogueira D, Bourgain C, Verheyen G, Van Steirteghem AC. Light and electron microscopic analysis of human testicular spermatozoa and spermatids from frozen and thawed testicular biopsies. Hum Reprod. 1999; 14(8):2041-9.
23. Jezek D, Schulze W, Kalanj-Bognar SZ, Milavec-Puretic V, Krhen I. Effects of various cryopreservation media and freezing±thawing on the morphology of rat testicular Biopsies. Anrologia. 2001; 33:368-378.
24. Fuller BJ. Cryoprotectants: the essential antifreezes to protect life in the frozen state. Cryo Letters. 2004; 25:375-88.
25. Mazur P. Equilibrium, quasi-equilibrium, and nonequilibrium freezing of mammalian embryos. Cell Biophysics. 1990; 17:53-92.
26. Bagchi A, Woods EJ, Critser JK. Cryopreservation and vitrification: recent advances in fertility preservation technologies. Expert. Rev. Med. Devices. 2008; 5:359-70.
27. Keros V, Hultenby K, Borgstrom B, Fridstrom B, Fridstrom M, Jahnukainen K *et al*. Methods of cryopreservation of testicular tissue withviable spermatogonia in prepubertal boys undergoing gonadotoxic cancer treatment. Hum. Reprod. 2007; 22:1384-95.
28. Woods EJ, Benson JD, Agca Y, Critser JK. Fundamental cryobiology of reproductive cells and tissues. Cryobiology. 2004; 48:146-156.
29. Yildiz C, Mullen B, Jarvi K, McKerlie C, Kirk C. Effect of different cryoprotectant agents on spermatogenesis efficiency in cryopreserved and grafted neonatal mouse testicular tissue. Cryobiology. 2013; 67(1):70-75.
30. Penna-Videau S, Bustos-Obregón E, Cermeño-Vivas JR, Chirino D. Malathion Affects Spermatogenic Proliferation in Mouse. International Journal of Morphology. 2012; 30(4):1399-1407.
31. Shokri S, Hemadi M, Aitken RJ. Transmission Electron Microscopy for the Quantitative Analysis of Testis Ultra Structure, The Transmission Electron Microscope, Dr. Khan Maaz (Ed.), 2012.ISBN: 978-953-51-0450-6, InTech, Available from: http://www.intechopen.com/books/the-transmissionelectron- microscope/transmission-electron-microscopy-for-the-quantitative-analysis-of-testis-ultra-structure.
32. Chui K, Trivedi A, Cheng Y, Cherbavaz D, Dazin P, Huynh A *et al*. Characterization and Functionality of Proliferative Human Sertoli Cells. Cell Transplantation, 2010, 1-46.
33. Pegg DE. The history and principles of cryopreservation. Seminars Reproductive Medicine. 2002; 20:5-13.