International Journal of Scientific Reports
Anan HH et al. Int J Sci Rep. 2017 Feb;3(2):39-48
http://www.sci-rep.com
pISSN 2454-2156 | eISSN 2454-2164
DOI: http://dx.doi.org/10.18203/issn.2454-2156.IntJSciRep20170356

Original Research Article

Histological and immunohistochemical study of cyclophosphamide effect on adult rat testis

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Received: 22 December 2016
Revised: 02 January 2017
Accepted: 06 January 2017

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ABSTRACT

Background: Nowadays, cyclophosphamide is widely used as anticancer and immunosuppressive agent in various drug regimens in many diseases and in young and old age. The aim of this research is to study the possible histological changes that may occur in the testes of adult male albino rats as a result of chronic exposure to cyclophosphamide and the prognosis of this effect.
Methods: Thirty healthy adult male albino rats were used in this study. They were equally divided into three groups; a control, an experimental and a withdrawal groups. The Animals of the experimental group were treated with daily dose of 5 mg/ kg cyclophosphamide orally for successive 28 days. Animals of the withdrawal group were left without treatment and sacrificed after 28 days from the last dose of cyclophosphamide. At the time of sacrifice, all animals were anesthetized by ether inhalation and their testes were dissected out carefully and processed for light and electron microscope examinations.
Results: Testes of the cyclophosphamide treated group revealed presence of many distorted shrunken seminiferous tubules which appeared with marked reduction in the thickness of the epithelium and wide lumina. Many germ cells with deeply stained nuclei, giant cells in mitosis and intracellular vacuoles were observed. Cross sections in mid pieces of sperms showed marked affection of axoneme, fibrous sheath and mitochondrial sheath. The cytoplasm of the Leydig cells contained mitochondria, dilated SER, Golgi cisternae and RER. Testes of the withdrawal group showed that the seminiferous tubules still had reduced height of their epithelium with wide intercellular spaces. Abnormal stratification and destructed germinal epithelium were evident with desquamated germ cells. Cross sections of mid pieces of the sperms showed distorted axoneme and swollen mitochondrial sheath. The cytoplasm of leydig cells contained many electron dense granules, RER, many dilated SER and mitochondria.
Conclusions: Chronic cyclophosphamide treatment not only produced serious histological changes of the testis but also in its serological parameter. These changes persisted after cessation of cyclophosphamide administration which indicates the cumulative irreversible toxic effect of cyclophosphamide. So, it is advisable to avoid the usage of cyclophosphamide as possible especially in young patients.

Keywords: Cyclophosphamide, Adult, Testis, Ultrastructure, Immunohistochemistry

INTRODUCTION

Cyclophosphamide (CP) is belonging to the class of oxazaphosphorines that considered as an essential component of many effective drug regimens. It is not only used as anticancer chemotherapeutic drug in childhood and adult malignancies but also used as an immunosuppressive agent for organ transplantation. In addition, it is used for treatment of many acute and chronic benign diseases as systemic lupus erythromatosis, multiple sclerosis and nephritic syndrome.[1] Moreover, Senthinkumar added that cyctophasphamide is effectively maintained remission of childhood nephritic syndrome in

patients who were sensitive to steroid and had frequent relapses.[2]

It was mentioned that the cyclophosphamide is therapeutically inactive prodrug that normally requires bio-activation to exert its anti-tumor function in patient. This activation is carried out by hepatic microsomal cytochrome 450 and then numbers of reactive alkylating fragments are produced. So, cyclophosphamide by itself has no alkylating activity and does not have any cytotoxic effect in vitro. So, this drug requires further activation in vivo, to become therapeutically effective.[3,4]

In the last two decades, there is an increasing success of chemotherapy in the treatment of malignancy and immunologic disorders. But with the gratifying results has come, we have recognized a serious long term side effects. Cyclophosphamide has variable hazardous effects especially on the rapidly proliferating tissues. In particular, the gonads are especially vulnerable to the treatment with chemotherapeutic agents.[5,6]

Ghosh et al mentioned that the testicular tissue is highly susceptible to oxidative stress because testicular membranes are highly rich in poly-unsaturated fatty acid. Moreover, the oxidative damage to this poly-unsaturated fatty acid of the cell membrane results in impairment of membrane fluidity and permeability.[7]

As other chemotherapeutic drugs, cyclophosphamide has variable hazards that either persist or relief spontaneously. Few literatures were done upon the effect of cyclophosphamide on the male and female gonads and how long it persists. Therefore, this work was done to throw more light on the possible histological and ultrastructural changes that may occur on the testis of adult male albino rat as a result of chronic exposure to cyclophosphamide and the prognosis of this effect.

METHODS

Thirty adult male albino rats (3 months) weighing 180-200 gm were used in this study.[8] They were maintained in room temperature at 23ºc with a 12-hour light-dark cycle. They allowed food and water ad-libitum. They were equally divided into three groups; a control, an experimental and a withdrawal groups. Animals of the experimental group were treated with daily dose of 5 mg/ kg cyclophosphamide orally for successive 28 days. Animals of the withdrawal group were left without treatment and were considered as a follow up group. They were sacrificed after 28 days from the last dose of cyclophosphamide. Blood samples were collected by cardiac puncture without anticoagulant. Serum was separated and stored at 70°c for enzyme immunoassays of testosterone.[4] At the time of sacrifice, animals of all groups were anesthetized by ether inhalation. Their testes were dissected out carefully and processed for light and electron microscope examinations. Specimens for light microscope examination were fixed overnight in Bouin's solution and were processed to prepare 7 µm thick paraffin sections for haematoxylin and eosin stains and immunohistochemical stains for localization of BCL-2 protein (anti-apoptotic marker).[9] Polyclonal rabbit antibody for BCL-2 were delivered from sigma laboratories was used (Code No. 00114386). Universal kit used avidine biotin peroxidase system produced by Novacastra laboratories Ltd., UK. This kit contained: 5 ml of poly-L-lysine solution as tissue adhesive, 2×6 ml of normal rabbit serum as protein blocking reagent, 0.3 ml of biotinylated rabbit anti-mouse secondary antibody, 0.3 ml avidin, 0.3 ml biotinylated horseradish peroxidase and 2 ml of hydrogen peroxide 15 foil wrapped diaminobenzidine (DAB) tablets. Glass slides were primarily coated by adhesive poly-L-lysine before marking the slide with a diamond pencil. Paraffin sections were mounted in the slides and incubated at 65ºc overnight for accurate adhesion. Sections were deparaffinized in xylene, rehydrated in descending grades of alcohol and then immersed in 3% hydrogen peroxide in methanol for 10 min. to block endogenous peroxidase activity. Subsequently, they were washed in phosphate buffer saline (PBS). Then, 10% normal serum was applied for 30 min. to reduce non-specific binding. Excess serum was dried around the sections without wash. The primary antibody was applied overnight then washed in PBS. Sections were covered with biotinylated secondary anti-mouse antibody for 30 min. and then washed in PBS. Avidin biotin reagent were applied for 30 min. then washed in PBS. DAB was added for 4 min. as a chromogen, washed with distilled water followed by Mayer's Haematoxylin as a counter stain. The sections were washed, dehydrated, mounted and examined.[10] This immunohistochemical technique was carried out in National institute of cancer, Cairo University.

Haematoxylin & eosin stained sections and immunohistochemical reaction were morphometrically analyzed using image analyzer computer system in the image analysis unit in the Histology Department, Faculty of Medicine, Cairo University.

Specimens for electron microscope examination were immediately fixed in 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer at pH 7.4 for 2 hours at 4ºc and then washed with phosphate buffer, postfixed in 1% osmium tetraoxide in the same buffer for one hour at 4ºc. After washing in phosphate buffer, specimens were dehydrated with ascending grades of ethanol and then were put in propylene oxide for 30 minutes at room temperature, impregnated in a mixture of propylene oxide and resin (1:1) for 24 hours and in a pure resin for another 24 hours. Then, the specimens were embedded in Embed-812 resin in BEEM capsules at 60ºC for 24 hours.[11] Semi-thin sections (1 µm thick) were stained with 1% toluidine blue for light microscope examination.[9] Ultra-thin sections were obtained using Leica ultracut, UCT and stained with uranyl acetate and lead citrate and were examined with JEOL JEM 1010 electron microscope in Electron Microscope Research

Laboratory (EMRL) of Histology and Cell Biology Department, Faculty of Medicine, Zagazig University.

RESULTS

H&E examination of the control rat's testis revealed testicular parenchyma that consisted of closely packed seminiferous tubules lined by srtatified germinal epithelium. Narrow interstitium inbetween the tubules contained clusters of interstitial cells and blood vessels as shown in Figure 1a. Cyclophosphamide-treated group revealed many distorted shrunken seminiferous tubules with wide interstitium in between. These tubules had marked reduction in the thickness of the germinal epithelium and wide empty lumina. Many germ cells with deeply stained nuclei and giant cells in mitosis detected among the other germ cells as in Figure 1b, 1c. Examination of the withdrawal group revealed that their seminiferous tubules still had reduced height of their epithelium. Some lumina were empty while others had sperm tails. Abnormal stratification and destructed germinal epithelium were evident with desquamated germ cells in the lumen of some tubules. Other tubules showed darkly stained nuclei among their lining germ cells as given in Figure 1d and 1e.

Toluidine blue stained sections showed tubules of control rat's testis were surrounded by single layer of myoid cells and were lined by stratified germinal epithelium that formed of different types of spermatogenic cells and sertoli cells. Sertoli cells with their large nuclei were observed lying on basement membrane as in Figure 2a. In group II many vacuoles of variable size were observed inbetween germinal epithelium Figure 2b. Withdrwal group revealed widening of the intercellular spaces as in Figure 2c.

Immunohistochemical stained sections of control rat's testis showed maximum expression of bcl2 in spermatids and primary spermatocytes with minimum immune-reactions in spermatogonia as in Figure 3a. While, groups II and III showed median expression of Bcl2 was observed in spermatids and spermatocytes with minimal immunoreaction in spermatogonia as in Figure 3b and 3c.

Electron microscope examination of the ultrathin sections of control rat's testes of the showed that seminiferous tubules were ensheathed by flattened myoid cell. Sertoli cells had irregular euchromatic nuclei resting on the basement membrane. Primary spermatocytes had large rounded nuclei with small irregular clumps of heterochromatin. Spermatids had round euchromatic nuclei. Their cytoplasm contained peripherally located mitochondria as shown in Figure 4a. Cross section in the mid piece of sperms showed the central axoneme that formed of nine doublets of microtubules with two central singlets. This axoneme is surrounded by nine electron dense bundles of fibrous sheath and mitochondrial sheath. While in the principle pieces, the axoneme was surrounded by fibrous sheath only. In the end pieces, it was surrounded by cell membrane as presented in Figure 4b. Interstitial cells of Leydig had oval euchromatic nuclei. Their cytoplasm contained lipid droplets, SER and mitochondria as in Figure 4c.

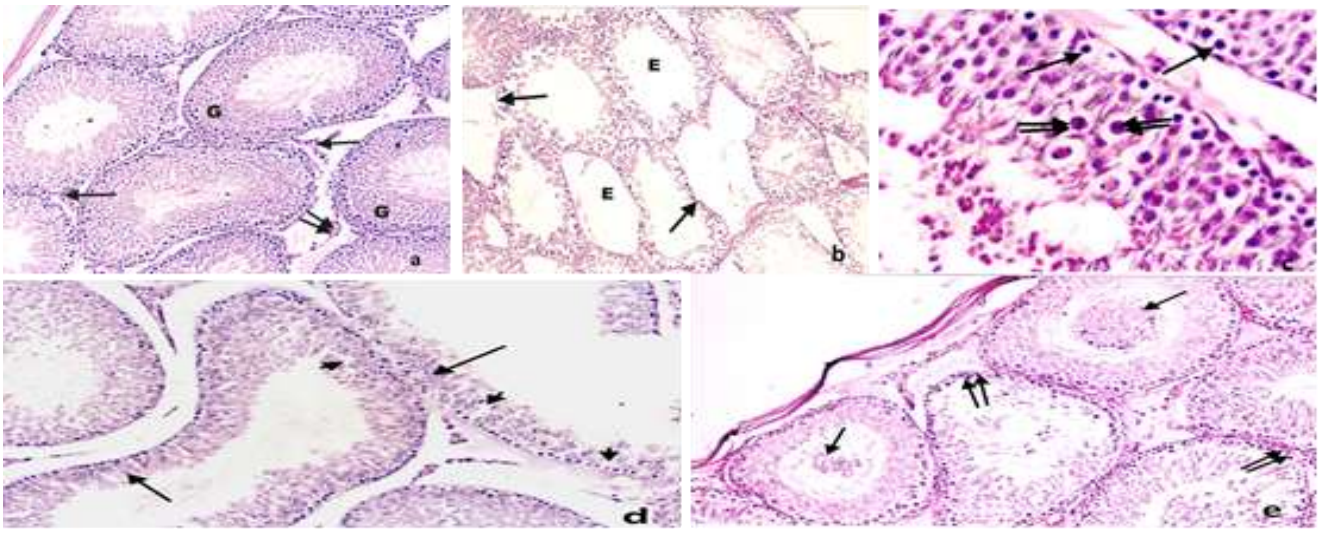

Figure 1: (a): control rat's testis shows many seminiferous tubules lined by stratified germinal epithelium (G). Clusters of interstitial cells (arrows) and blood vessels (double arrows) are present in the interstitium; (b): treated testis shows testicular parenchyma of many distorted shrunken seminiferous tubules (T) with marked reduction in the thickness of the germinal epithelium (arrows). These tubules have wide empty lumina (E); (c): shows many germ cells with deeply stained nuclei (arrows). Giant cells in mitosis (double arrows) are present among the germ cells; (d): testis of a withdrawal group shows seminiferous tubules that still have reduced height of their epithelium (arrows). Wide spaces inbetween germ cells (arrowheads) (H&E 400 X).

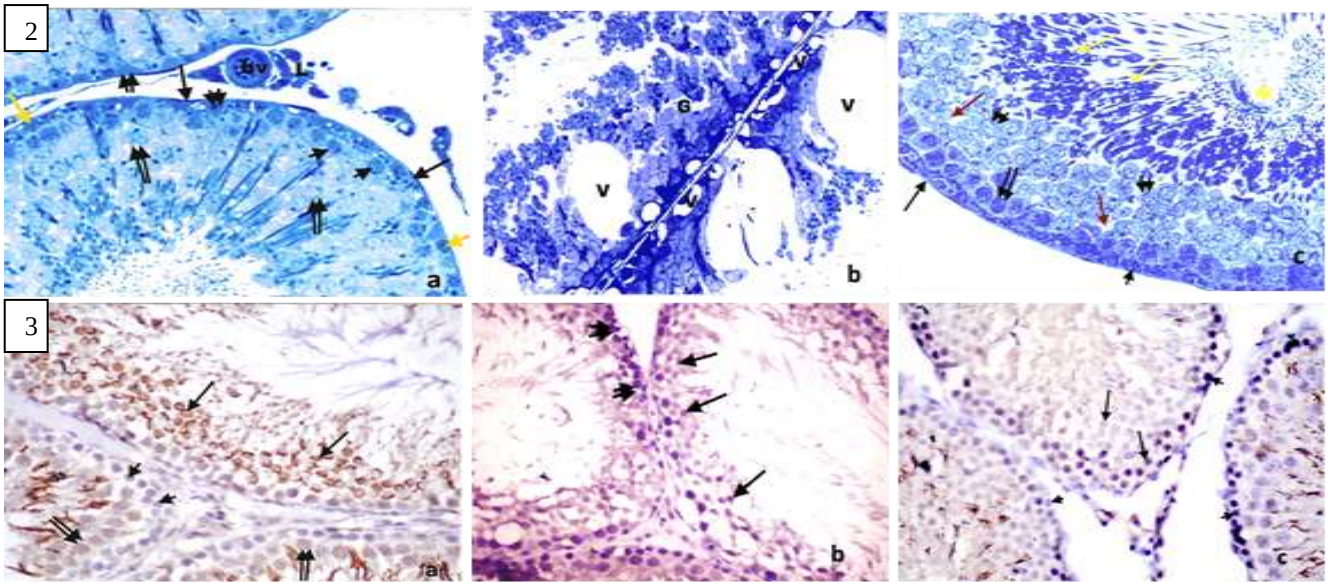

Figure 2: (a): semithin section in the control rat' testis shows seminiferous tubule that is surrounded by single layer of myoid cells(arrows) and is lined by stratified germinal epithelium formed of different types of spermatogenic cells (G) and sertoli cells (S). Sertoli cells with their large nuclei are observed lying on basement membrane; (b): testis of treated rat' testis reveals many vacuoles (V) of variable size inbetween the germinal epithelium; (c): testis of withdrawal group shows widening of the intercellular spaces (arrows) that is still evident (Toluidine blue 400 X).

Figure 3: (a): testis of control rat shows a maximum expression of bcl2 in spermatids and primary spermatocytes (arrows) with minimum immunoreactions in spermatogonia (arrow heads); (b): testis of treated rat reveals median expression of Bcl2 in spermatids and primary spermatocytes (arrows) with minimal immunoreactions in spermatogonia (double arrows); (c): testis of a withdrawal adult group shows median expression of Bcl2 in spermatids and spermatocytes (arrows) with minimal immunoreaction in spermatogonia (double arrows) (Avidin biotin peroxidase system 400 X)

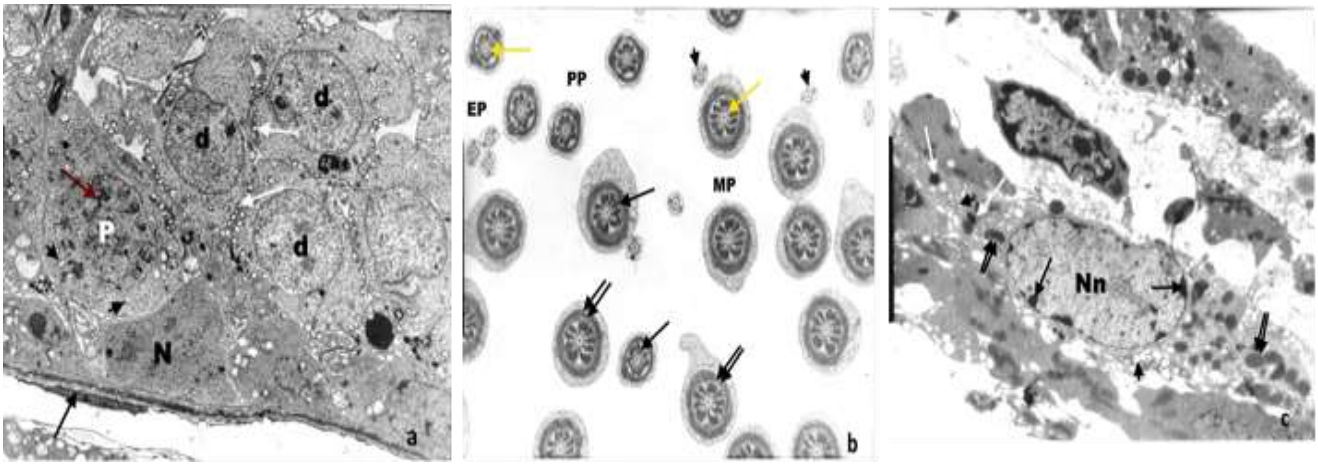

Figure 4: (a): transmission electron micrograph of control rat's testis shows seminiferous tubule that is ensheathed by flattened myoid cell (arrows). Sertoli cell has an irregular euchromatic nucleus (N) resting on the basement membrane. Primary spermatocyte has large round nucleus (P) with small irregular clumps of heterochromatin (red arrows). Spermatids (d) with round euchromatic nuclei are observed. Their cytoplasm contains peripherally located mitochondria (green arrows); (b): reveals cross section in mid (MP), principle (PP) and end pieces (EP) pieces of sperms. In the mid pieces, the central axoneme (red arrows) is formed of 9 doublets of microtubules with 2 central singlets. This axoneme is surrounded by nine electron dense bundles of fibrous sheath (yellow arrows) and mitochondrial sheath (arrows). In the principle pieces, the axoneme is surrounded by fibrous sheath only. While in the end pieces, it is surrounded by cell memberane (green arrows); (c): shows leydig cell that has oval euchromatic

nucleus (Nn) with peripheral clumps of heterochromatin (arrows). The cytoplasm contains lipid droplets (red arrows), SER (green arrows) and mitochondria (yellow arrows).

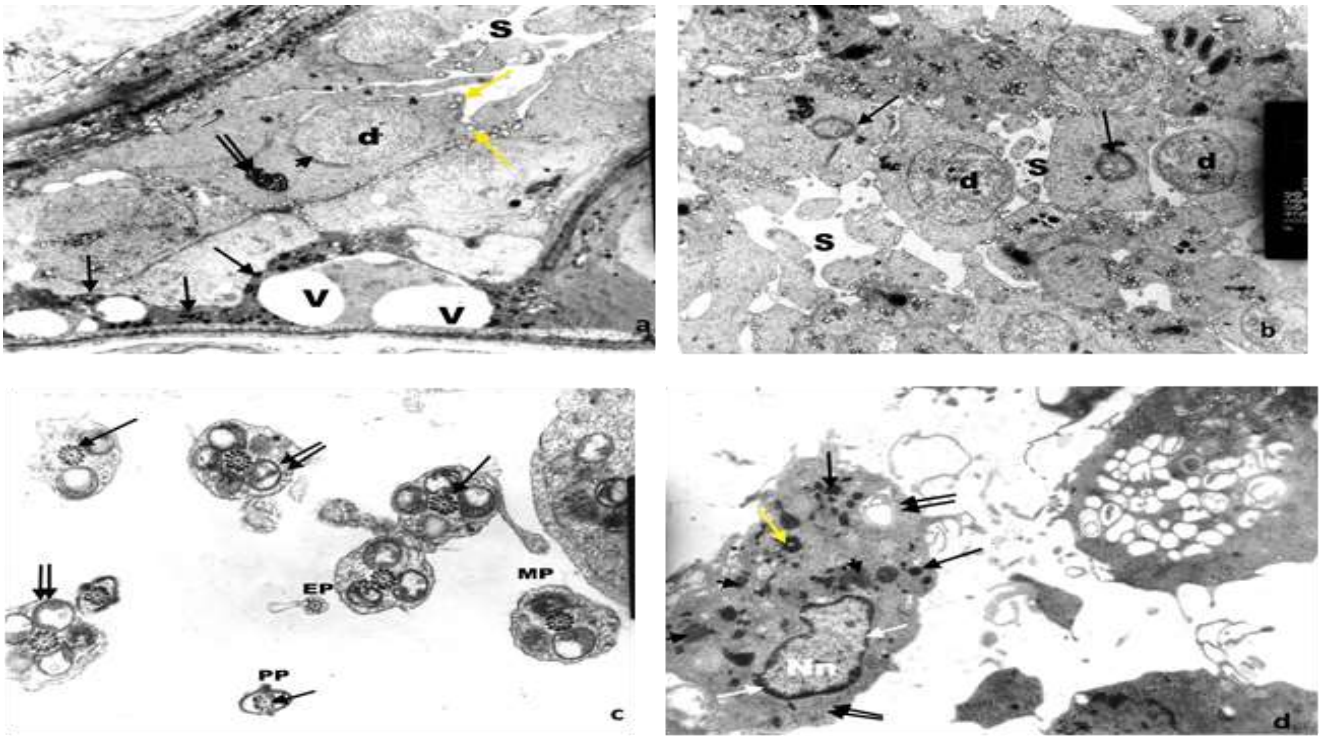

Figure 5: (a): transmission electron micrograph treated rat's testis shows sertoli cells cytoplasm with multiple variable sized vacuoles (V). Spermatids with oval euchromatic nuclei (d) and acrosomal cap (double arrows) are noticed. Their cytoplasm contains residual bodies (red arrows) and peripherally located mitochondria (yellow arrows). Wide intercellular spaces (S) can also see between the cells; (b): reveals spermatids in different stage of maturation. Some of them have shrunken heterochromatic nuclei (red arrows). Spermatids with round euchromatic nuclei (d) and wide intercellular spaces (S) are also observed; (c): shows cross sections in the mid (MP), principle (PP) and end pieces (EP) of the sperm. The central axoneme (arrows) is surrounded by distorted and swollen mitochondrial sheath (double arrows); (d): showing interstitial cells of leydig has large oval euchromatic nucleus (Nn) with peripherally arranged heterochromatin (arrows). Its cytoplasm contains mitochondria (double arrows), dilated SER (red arrows), Golgi cisternae (green arrows) and RER (yellow arrows).

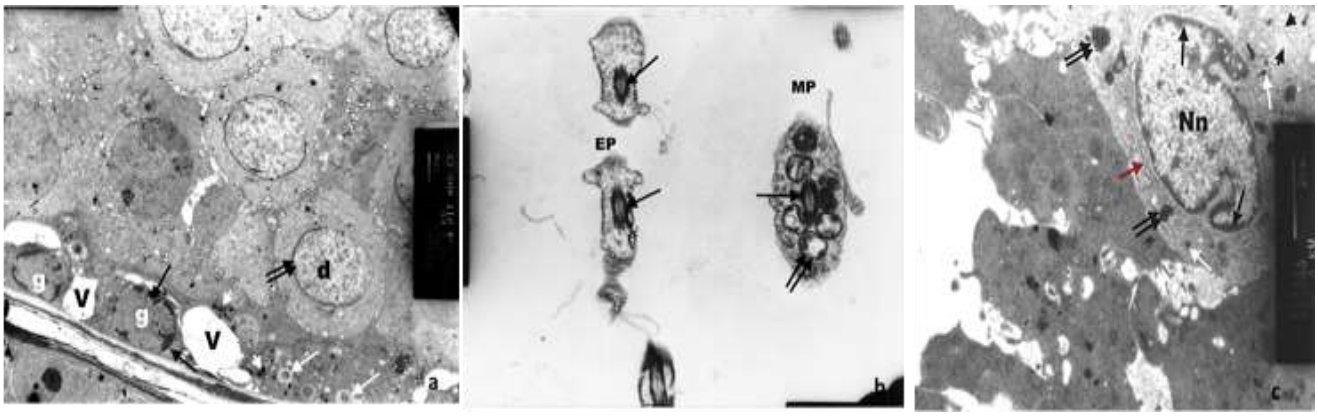

Figure 6: (a): transmission electron micrograph of withdrawal group shows multiple variable sized vacuoles (V) that are still present in between germinal epithelium. Spermatid (d) with round euchromatic nuclei and acrosomal cap (double arrow) is seen near to the basement memberane; (b): showing cross section of mid pieces (MP) with distorted axoneme (arrows) and swollen mitochondrial sheath (double arrows). Abnormal end pieces (EP) are also seen; (c): showing leydig cell has indented euchromatic nucleus (Nn) and peripherally located heterochromatin (arrows). Its cytoplasm contains many electron dense granules (double arrows), RER (red arrows), many dilated SER (yellow arrows) and mitochondria (double red arrows).

While, group II showed sertoli cells with multiple variable sized vacuoles in their cytoplasm. Spermatids in different stage of maturation were detected. Some of them had shrunken heterochromatic nuclei. Wide intercellular spaces were observed between the germ cells as shown in Figures 5a and 5b. Cross sections in the mid pieces of sperms showed marked affection of axoneme, fibrous sheath and mitochondrial sheath as in Figure 5c. Interstitial cells of leydig have large oval euchromatic nuclei with peripherally arranged heterochromatin. The cytoplasm contained mitochondria, dilated SER, Golgi cisternae and RER as given in Figure 5d. The withdrawal group revealed multiple variable sized vacuoles that still present in between germinal epithelium as in Figure 6a. Cross sections of mid pieces of the sperms showed distorted axoneme and swollen mitochondrial sheath as shown in Figure 6b. The leydig cell had indented euchromatic nuclei with peripherally located heterochromatin. The cytoplasm contained many electron dense granules, RER, many dilated SER and mitochondria as seen in Figure 6c.

Statistical analysis of the morphometrical results of the diagonal diameter of the seminiferous tubules using T test revealed that there was a highly statistical difference between cyclophosphamide and control group (P <0.001). Similar finding was observed on comparing between withdrawal and the control group (P <0.001) as in Table 1.

Table 1: Mean (X) and standard deviation of diagonal diameters among the different studied groups.

	$\bar{X}$±SD (range)	T	P
Control	303.4±51.1 (211.73-721.75)		
Cyclophosphamide treated	253.4±39.7 (158.73-335.02)	5.45	0.001**
Withdrawal	239.04±41.1 (164.08-319.53)	6.93	0.001*

T test: compare control with other groups. P = 0.001**

Table 2: Mean (X) and standard deviation of epithelial height among the different studied groups.

	$\bar{X}$±SD (range)	T	P
Control	73.7±15.0 (42.51-114)		
Cyclophosphamide treated	45.45±8.1 (35.09-78.79)	11.66	0.001**
Withdrawal	42.81±8.6 (22.38-69.03)	9.77	0.001**

T test: compare control with other groups. P = 0.001**

Regarding the epithelial height of the seminiferous tubules, there was a highly statistically significant difference between cyclophosphamide-treated and control group (P <0.001). Similar results were observed on comparing between withdrawal and the control group (P <0.001). But there was a non-statistically significant difference between cyclophosphamide-treated and withdrawal group (the P value >0.05 was non-significant) as given in Table 2. As regards optical density of Bcl-2 immunoreaction of the germinal epithelium of seminiferous tubules, a highly statistically significant deference was observed between cyclophosphamide-treated and control group (P <0.001). There was a statistically significant deference in the optical density of Bcl-2 immunoreaction in the withdrawal group in comparing with the control group (P <0.001) as presented in Table 3. Regarding the level of serum testosterone hormone, There was a statistically significant difference between cyclophosphamide-treated group and the control group (P <0.001). However, there was a highly statistically significance difference between withdrawal and the control group (P <0.001) as in Table 4.

Table 3: Mean (X) and standard deviation of optical density among the different groups (Bcl_2).

	$\bar{X}$±SD (range)	T	P
Control	0.98±0.03 (0.93-1.01)		
Cyclophosphamide treated	0.9±0.04 (0.83-0.96)	4.95	0.001**
Withdrawal	0.87±0.015 (0.84-0.89)	11.29	0.001**

T test: compare control with other groups. P = 0.001**

Table 4: Mean (X) and standard deviation of the level of serum testosterone hormone among the different studied groups.

	$\bar{X}$±SD (range)	T	P
Control	12.6±2.0 (10.3-16.9)	6.03	0.001**
Cyclophosphamide treated	6.84±3.7 (3.5-14.0)	0.56	0.58 NS
Withdrawal	6.0±2.8 (3.5-13.5)		

T test: compare control with other groups. P = 0.001**

DISCUSSION

Cyclophosamide is a cytotoxic agent that has been extensively used for the treatment of various diseases.[12,13] Due to its extensive use in clinics, it possesses occupational exposure to the health care professionals.[14]

Fukushima et al reported that many alkylating agents; chemotherapeutic drugs have been found to produce infertility as a result of impaired sperm production.[15] This

infertility may be inevitable side effects. It was added that the testicular dysfunction is a common long term sequel of cytotoxic chemotherapy used in the treatment of many malignancies .So, the present work was carried out to study the possible changes that may occur during and following the treatment with one of these cytotoxic agents (CP).[16]

In the present work, examination of the testes of the rats treated daily with cyclophosphamide orally for 28 days showed that the testicular parenchyma was formed of many distorted shrunken seminiferous tubules with wide interstitium inbetween. These tubules had marked reduction in the thickness of the germinal epithelium and wide empty lumina. This reduction in the number of stratified epithelial layers attributed by Jedlinska - Krakowska et al to inhibition of B - spermatogonia mitosis which denote elongation of G1 phase of cell cycle growth cycle.[17] Furthermore, Haubitz added that this thinning of the epithelium lead to defect in sperm production. Clinically, this sperm defect manifested by oligospermia or azospermia which cause infertility.[18]

Moreover, the current study reported two types of cells among the germinal epithelium. One of them was cells with darkly stained nuclei and the others referred as giant cells. Yamazaki et al described the first cells as apoptotic cells that increased in number under the effect of cyclophosphamide treatment.[19] Apoptosis constitutes a cellular mechanism which allows the seminiferous tubules to control the number of germ cells.

Sakamaki added that apoptosis can be triggered through different mechanisms.[20] One of the most important and widely known regulatory system of apoptosis is Bcl2 family which composed of anti and pro-apoptotic proteins. These proteins regulate apoptosis by controlling the release of cytochrome c and other mitochondrial changes. It was proofed that from the balance between anti-apoptotic and pro-apoptotic members of Bcl2 family, we can suspect the fate of the cells.[21]

But giant cells that encountered in this work among the germ cells were detected.[22,23] They referred them as immature spermatocytes that appeared hypertrophied and multinucleated or as degenerated cells. In contrary, these cells described by other researchers as apoptotic spermatids.[24]

In this work, parts seminiferous tubules showed many vacuoles of variable size inbetween the germinal epithelium. It was reported that atrophy of the seminiferous tubules was manifested by intraepithelial vacuoles. [25] These atrophied tubules were lined by single layer of germ cells and sertoli cells. Monika et al added that the thinning of germinal epithelium reduce the sperm production.[26] However, the reduction of the sperm formation depends on the dose and the period of treatment by cyclophosamide.

Immunohistochemically, median expression of Bcl2 were observed in spermatids and primary spermatocytes with minimal immunoreactions in spermatogonia. It was mentioned that Bcl-2 is an anti-apoptotic protein that resides in the outer mitochondrial membrane and the membrane of the endoplasmic reticulum.[27] Bcl-2 is known to block cytochrome c (apoptogenic substance) release from the mitochondrial matrix possibly through the inhibition of other pro-apoptotic family; Bax and Bak. Also, the spermatogonia in seminiferous epithelium was considered as stem cells and their population serve as supporting cells providing metabolic and physiologic functions. Degeneration of these cells was an integral and important part of normal spermatogenesis.[21]

Meehan et al reported that the differentiated spermatogonia were the most sensitive cells to any drugs. This sensitivity was related to their short mitotic cycle involving DNA synthesis and cell division.[28] In comparison, spermatids and primary spermatocytes appeared to be more resistant to apoptosis by these drugs. However, the primary spermatocytes are considered the most susceptible cell to the process of apoptosis.[22,29] They attributed this to overexpression of Bcl2w in these cells. So, the apoptotic frequency was found higher in spermatocytes compared to spermatids with some in spermatogonia. They added that the depletion of these cells was responsible for atrophy of the testes.

It was postulated that positive staining of Bcl_2 sub member was preferentially localized to the cytoplasm of spermatogonia as well as anti-apoptotic form expressed in the most tubular cells with preferential expression in the cytoplasm of differentiating spermatid close to luminal surface. They attributed these preferential expression in cell close to the tubular lumen to these proteins may be involved in the interaction between the luminal environment and spermatocytes and spermatids.[30]

Electron microscope examination of the ultrathin sections of the testes of the same group showed that sertoli cells cytoplasm had multiple variable sized vacuoles. Krishnamoorthy et al found large vacuoles in sertoli cells and dilatation of the extracellular spaces.[31] They explained these results by defect in sertoli cells function which were not undergo apoptosis.

In cyclophosphamide- treated rats, spermatids in different stage of maturation were detected. Some of them had shrunken heterochromatic nuclei. Their cytoplasm contained residual bodies and peripherally located mitochondria. Wide intercellular spaces were observed between the germ cells. These results were explained by oxidative stress that occur by cyclophosphamide and release of reactive oxygen species which lead to cellular injury via several mechanisms including lipid peroxidation and oxidative damage of protein and DNA. This oxidative stress is recognized as strong mediator of apoptosis.[32]

In this work, cross sections in the mid pieces of sperms showed marked affection of axoneme, fibrous sheath and mitochondrial sheath. The sperms abnormalities were attributed to defect in sperm production due to abnormal spermatogenesis. In contrast, failure in detection of these remarkable changes might be attributed to point of time of observation. Furthermore, DNA microarray analysis might utilize to reveal these changes and to detect alteration of the gene expression which might lead to anomalous spermatogenesis.[15]

In this study, interstitial cells of leydig revealed large oval euchromatic nuclei and peripherally arranged heterochromatin. The cytoplasm contained mitochondria, dilated SER, Golgi cisternae and RER. The same finding reported by Hutson who explained these findings by defect in leydig cells function as secretory cells.[33] They added that this improper function of the cells was manifested by reduction in the testosterone level in the serum. The work emphasized in this report by detection the diminution in the level of this hormone in the serum.

Light microscope examination of the transverse sections of the testes of the adult male albino rats of the withdrawal group revealed that the seminiferous tubules still had reduced height of their epithelium. Some lumina were empty while others had sperm tails. Abnormal stratification and destructed germinal epithelium were evident with desquamated germ cells in the lumen of some tubules. Other tubules showed darkly stained nuclei among their lining germ cells. These results were emphasized by authors who stated that the ability to regain spermatogenesis after chemotherapy depending on the types of medication used and the period of treatment.[15,34] In contrary, Rezvanafar et al reported that the use of lower doses of alkylating agents for decreased numbers of cycle could reduce the gonadotoxicity.[35]

Under an elevated oxidative stress status, the reactive oxygen species cause cellular injury via several mechanisms.[36] These mechanisms included lipid peroxidation and oxidative damage of proteins and DNA. The latter could lead to condensation of the nuclear chromatin. On the other hand, Bhatia et al reported that cyclophosphamide may reduce apoptosis if it administrated long term in low doses.[36] In addition, cyclophosphamide, as a powerful alkylating agent, would alkylate a range of cellular macromolecules. These molecules include the mRNA and proteins that mediate the apoptotic responses. Thus, poisoning the machinery of apoptosis in susceptible germ cells might occur. Alternatively, cyclophosphanide may damage the DNA of genes required for germ cell apoptosis.

Electron microscope examination of the ultrathin sections of the testes of the withdrawal group revealed multiple variable sized vacuoles that still present in between germinal epithelium. Spermatids were located abnormally near to the basement membrane and the cross sections of the sperms still showed distorted axoneme. These results were in agreement with some authors who reported that the abnormal spermatids in late stage of apoptosis migrate deep within the epithelium near to the limiting memberane of the tubules.[37] Also, they added that all apoptotic germ cells were engulfed by sertoli cells. So, these cells were characterized by multiple digestive vacuoles which appeared in treated groups.

In the interstitium, leydig cell had indented euchromatic nuclei with peripherally located heterochromatin. The cytoplasm contained many electron dense granules, RER, many dilated SER and mitochondria. These findings were correlated with the results reported by other researchers who observed that the mitochondria were prominent in the leydig cells of the testes even after treatment by chemicals. These mitochondria were apparently normal and remain predominantly tubular. However, the lipid droplets were generally extracted and variable stages of lysosome were detected as many vacuoles.[38]

Regarding the level of serum testosterone hormone, there was a statistically significant difference between cyclophosphamide-treated group and the control group. Ghosh et al stated that the difference in the testosterone level between cyclophosphamide-treated and control animals served as evidence for the damage of the testes.[7] Also, these data were strengthen the idea about the inhibitory effect of cyclophosphamide on testicular steroidogenesis and spermatogenic disorder indicated by diminution in the number of different generation of germ cells. However, there was a highly statistically significance difference between withdrawal and the control group. Some report postulated that the effect of cyclophosphamide on the testis is irreversible if it is left without supplementation by any of its antagonist.[39]

In conclusion, the present work showed that chronic cyclophosphamide treatment not only produced serious histological changes of the testis but also in the serological parameter. These changes persisted after cessation of cyclophosphamide administration. These finding indicate the cumulative irreversible toxic effect of cyclophosphamide. So, it is advisable to avoid the usage of cyclophosphamide as possible especially in young patients.

Funding: No funding sources
Conflict of interest: None declared
Ethical approval: The study was approved by the institutional ethics committee

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Cite this article as: Anan HH, Wahba NS, Abdallah MA, Mohamed DA. Histological and immunohistochemical study of cyclophosphamide effect on adult rat testis. Int J Sci Rep 2017;3(2):39-48.