Impact of Cyclophosphamide on Testis and Sexual Glands of Adult Albino Rats and Probable Recovery After Drug Withdrawal: A Histological and Immunohistochemical study

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Abstract:

Background: Cyclophosphamide is a chemotherapy and an immune-suppressive drug which is used for treatment of malignant and immune – related disease. The current work was conducted to evaluate the histological structure of the male gonad and sexual glands during treatment and after discontinuation of cyclophosphamide.

Material and Methods: Thirty male albino- rats were classified into three groups. The first one was treated by normal saline, the second and third groups received cyclophosphamide 150 mg/kg intra peritoneal every two days for one week. The third group regarded as recovery group and divided into two subgroups with one- or two-months' recovery period. At the accomplishment of the work, blood was drowning for hormonal analysis (testosterone) then the animals were sacrificed. The testis, prostate and seminal vesicle were removed and prepare for histological and immunohistochemical staining.

Results: The cyclophosphamide treated group (GII) showed reduction of serum testosterone level, the testicular and sexual glands sections revealed atrophic and degenerative changes of their lining epithelium with reduction of their function and changes of their Ki67 expression which indicate abnormal proliferative pattern of the cells. However, the histopathological changes of treated group showed no improvement after one or even two months of drug withdrawal.

Conclusion: The present study pointed out the risk of infertility and gonadal toxicity during cyclophosphamide treatment which may be permanent even after termination of the treatment.

keywords: Chemotherapy, testis, sexual glands, infertility, proliferative marker

تأثير سيكلوفوسفاميد على الخصية والغدد الجنسية لجرذان ألبينو البالغة والتعافي المحتمل بعد سحب الدواء: دراسة نسيجية ونسيجية مناعية

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الخلاصة:

السايكلوفوسفامايد هو علاج كيميائي معروف ودواء مضاد للمناعة ويستخدم على نطاق واسع في علاج الأمراض الخبيثة والمتعلقة بالمناعة. تم إجراء العمل الحالي لتقييم التركيب النسيجي للغدد التناسلية الذكرية أثناء العلاج وبعد التوقف عن تناول هذا الدواء.

المواد والطرق: تم تصنيف ثلاثون من ذكور الجرذان إلى ثلاث مجموعات. عولجت المجموعة الأولى بمحلول ملحي عادي، بينما عولجت المجموعة الثانية والمجموعة الثالثة بالسايكلوفوسفاميد 150 مجم / كجم داخل التجويف الصفاقي كل يومين لمدة أسبوع. المجموعة الثالثة اعتبرت مجموعة التعافي وتم تقسيمها إلى مجموعتين فرعيتين مع فترة تعافي لمدة شهرا

وشهرين. في نهاية العمل تم سحب الدم لأجراء فحص هرمون التستوستيرون ثم تم قتل الحيوانات وإزالة كل من الخصية، البروستات والحويصلة المنوية وتحضير العينات للصبغات النسيجية والنسيجية المناعية.
النتائج: أظهرت المجموعة المعالجة (المجموعة الثانية) بالسايكلوفوسفاميد انخفاضًا في مستوى هرمون التستوستيرون في الدم، وكشفت مقاطع الخصية والغدد الجنسية عن تغيرات ضامرة وتنكسيه في الظهارة البطانية مع انخفاض وظيفتها وتغيرات في تعبيرها المناعي مما يشير إلى نمط تكاثر غير طبيعي للخلايا. ومع ذلك، فإن التغيرات النسيجية المرضية للمجموعة المعالجة لم تظهر أي تحسن بعد شهر أو حتى شهرين من سحب الدواء
الاستنتاج: أشارت الدراسة الحالية إلى خطر العقم وسمية الغدد التناسلية أثناء العلاج بالسيكلوفوسفاميد والذي قد يكون دائما حتى بعد انتهاء العلاج.

الكلمات المفتاحية: علاج كيماوي، خصية، غدد جنسية، عقم، علامة تكاثرية

1.INTRODUCTION

Cyclophosphamide is one of chemotherapeutic medication which has been used to treat malignant conditions as lymphoma, breast and ovarian carcinoma, pulmonary tumor, leukemia and multiple myeloma [1]. It is also used as immune suppressor to treat nephritic syndrome, granulomatosis and in organ transplantation [2,3]. It is an alkylating agent and acts by disruption of DNA duplication and RNA creation [4]. Similar to other chemotherapeutic agents, this drug may be associated with many side effects. Some people who use this drug may develop mild side effect as gastritis, loss of hair and low immunity while others developed serious unwanted effect as respiratory fibrosis, hepatotoxicity [5] and cardiotoxicity [6]. However, cyclophosphamide like many other chemotherapies may affect the fertility and induces reproductive toxicity which lead to infertility in both sexes [7]. Ovarian dysfunction with primordial follicle death and loss of follicular reserve was observed in previous study [8] many women treated with this drug developed amenorrhea with depletion of estrogen hormone [9]. In men, changes of sexuality, infertility with reduction of spermatogenesis and abnormal sperm morphology were mentioned by other researchers [10,11]. Some authors indicated that cyclophosphamide has an effect on the protein composition of chromosomes present in sperm [12]. Other workers said that toxicity induced by cyclophosphamide may be caused by oxidative stress and elevation of the free radicles [13]. Prostate and seminal vesicle are sexual glands that have an essential role in sperm activity and affect male fertility [14] . Few studies had been achieved to determine the effect of chemotherapy in general on the structure of the normal male sexual glands. So The present study is aimed to explore the effect of cyclophosphamide administration on the function and structure of the male gonad and the sexual glands (prostate and seminal vesicle) and to detect the chance of reversibility-after-drug-withdrawal

2. MATERIAL AND METHODS

2.1. The drug

Cyclophosphamide (Baxter, Germany) in the form of vial contains cyclophosphamide monohydrate, 500mg / vial was obtained from Pharmaceutical Company (Mosul, Iraq). Each vial was diluted in 5 ml distilled water. It was given as 150mg /kg intra peritoneal (i.p) every two days for one week

2.2. Experiment design

Thirty male albino rats, weighted 200-220 gm and aged 12- 14 weeks obtained from the Animal House of the College of Veterinary Medicine, University of Mosul, Iraq. They were kept in separated cage (each cage contains 10 animals) at a regulated light and dark cycle, 22° C temperature, and accepted humidity. All Animals looked healthy and active, they

were nourished with standard laboratory food and *ad libitum* water. They remained in the laboratory for about one week before the experiment to acclimatize the laboratory condition. This study was done in Mosul university and according to national guidelines and ethical committee of Ninevah university–medical college (the ethical license approval number 103on February 12, 2022).

Each 10 animals were grouped in one cage and treated as follow:

1- Group I regard as control; they received 1,5 ml of physiological saline i.p and used for comparison
2- Group 2 were injected intra peritoneal with cyclophosphamide 150mg /kg every two days for one week
3- Group 3 were treated same as group 2 (injected intra peritoneal with cyclophosphamide 150mg /kg every two days for one week) then they were classified into two subgroups (each one with five animals), the first one left one month without treatment after the last injection while other subgroup remained two months without treatment.

The animals in the 1st and 2nd groups were anesthetized by ethyl ether and killed at about sex days after the last injection. However, five animals from the 3rd group were sacrificed at one month after last injection and considered as Recovery 1 (R1) while the other five were killed at the end of the 2nd month and consider as Recovery 2 (R2). Testes, dorsolateral lobe of prostate and seminal vesicles from all rats were removed and fixed in Bouin's solution. Additionally, blood sample were obtained by heart puncture technique for hormonal estimation.

2.3. Hormonal analysis

Blood samples were centrifuged at 300 rpm for 15 mints then sera were separated and used to assess the testosterone level. Enzyme linked immunosorbent assay (ElISA kit) from Sigma Chemicals Co. USA was used to measure testosterone level in blood of the tested rats. (the reference range of kit for rat is 4.97-6.85 ng/ml)

2.4. Tissue Processing and histological examination

After fixation, the tissues were dehydrated by ascending grad of alcohol, cleared with xylol, embedded in paraffin wax, sectioned by rotary microtome into 5 µm thick sections and stained with:

A- Histological stain: Hematoxylin and Eosin (H&E) to determine the general structure of the cells and to evaluate testicular damage according to Johnsen's testicular score [15].

B- Immunhistochemical (IHC) stain (was done in Dohuk laboratory) : to evaluate spermatogenesis and cell proliferation activity, Ki 67 expression in germ cells and cells lining of prostate were used as follow : The deparafinzed sections from the testis were treated for 10 mint by hydrogen peroxide 3% in methanol , washed with water then put in microwave in citrate buffer for 15 mint , then preserved in serum rabbit for halve an hour at room (37°C) temperature, then incubated with anti- Ki67 antibody overnight at 4 °C and finally the hematoxylin were used as counter stain [16] . The positive cells appeared as dark brown color of cell nucleus on the tissue sections

All sections from different groups were studied under light microscope (Olympus Optical).

2.5. Morphometrical study

Micro morphometry was done by using the color USB 2.0 digital image camera omax (A3590U) which was provided with image processing software (TuopView 12.5). ten field / section were haphazardly selected to measure the following parameters:

A- To assess testicular damage, Johnsen's testicular score was used for all groups. The Johnsen criteria are into a ten-point scoring system for quantifying spermatogenesis according to the profile of the cells encountered along the seminiferous tubules. A Johnsen score of 10 indicates maximum spermatogenesis activity, whereas a score of 1 indicates complete absence of germ cells.

B- Leydig cells number/ interstitial space

C- A percentage of immuno-labeled cells is calculated by dividing the number of positive cells (brown nucleus) in each field to the number of all cells in that field.

D- Height of prostatic lining epithelium and perimeter of acini were measured in µm

E-Height of seminal vesicle lining epithelium measured in µm

2.6. Statistical Analysis

Graph pad prism (version 8) have been used for statistical analysis by using one-way ANOVA followed by Tukey's multiple comparisons test with the level of significance value at $P \leq 0.05$.

3. RESULT

3.1. Hormonal level

Serum testosterone level in different groups is shown in table 1, figure 1. There is a significant reduction of testosterone hormone in GII and GIII compared to control group.

Table 1: Testosterone level in different classes of animals. (kits reference value is 4.97-6.58ng/ml). Data presented as Mean±SE

Groups / Hormone	Control group (GI)	Cyclophosphamide treated group (GII)	Recovery group (GIII)	
			After one moth R1	After two months R2
Testosterone level (ng /ml)	6.05± 1.38	1.23± 2.39*, [A]	1.56± 1.92*, [A, B]	2.12± 1.75*, [A, B]

* Compare with control group; $P < 0.001$ significant

[A] Compare GII with recovery groups; $P = 0.23$ non-significant

[B] Compare Recovery groups; $P < 0.7$ non-significant.

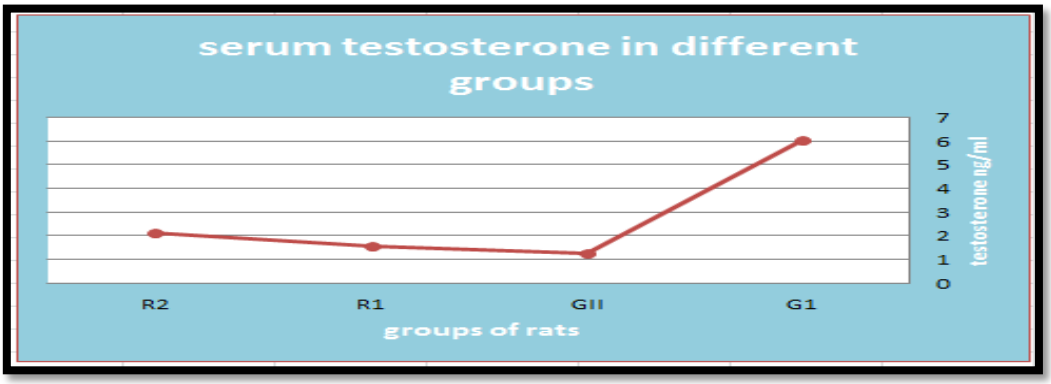

Figure 1: Serum testosterone levels (ng/ml) in groups of animals GI: control, GII: cyclophosphamide treated group, R1: recovery after one month, R2: recovery after 2 months

3.2. **Histological assessment**

A- Testis in control group (GI): the testicular sections have normal seminiferous tubule with regular outline. The germinal epithelium in different stages of spermatogenesis (spermatogonia, spermatocyte, spermatid and spermatozoa) extend from basal lamina to the lumen with many spermatozoa fill the lumen. Sertoli cells are identified between the germinal epithelium, it has triangular shape nucleus. The seminiferous tubule separated by interstetial connective tissue which contain clump of rounded Leydic cells (figur2A). In cyclophosphamide treated group (GII) the testicular sections show reduction, degeneration, necrosis, disorganization of germinal epithelium and arrest of spermatogenesis at spermatocyte level in many seminiferous tubules with absent of spermatozoa in the lumen. Sertoli cells appeared vacuolated. Interstitial edema and degenerated Leydic cells were observed (figure 2B). The testicular sections in both recovery groups show more or less similar histopathological features to that observed in GII (figure 2, C and D).

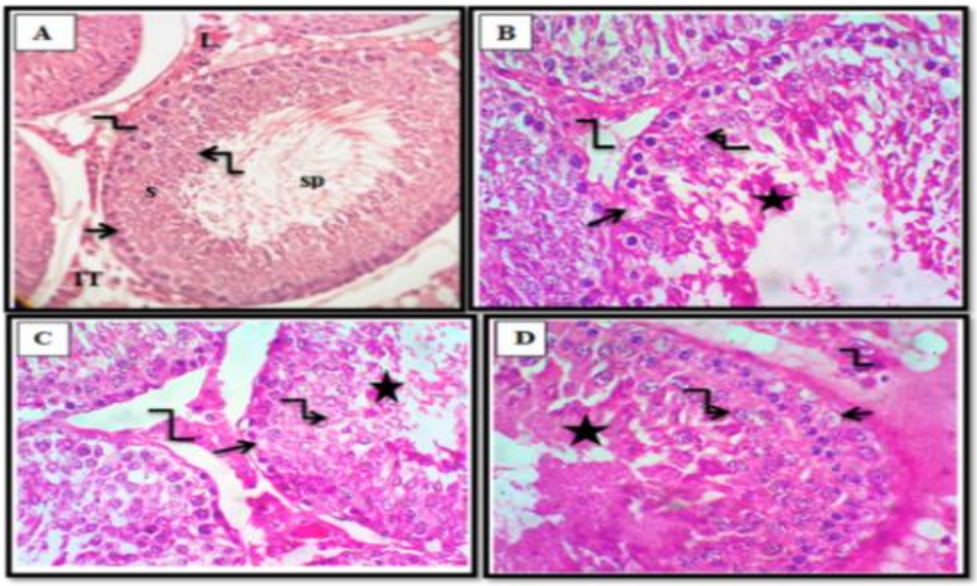

Figure 2: Photomicrograph from testes. A: control group (GI) shows seminiferous tubule with normal germinal epithelium, spermatogonia (arrow) rest on basemen membrane. Spermatocyte (S) , spermatid (curved arrow) and spermatozoa(SP) fill the lumen, sertoli cell (curved line) with triangular nucleus, clump of Leydic cells (L) and normal interstitial space (IT) .B : Cyclophosphamide treated group (GII) shows seminiferous tubule with disorganized germinal epithelium, arrest of spermatogenesis on spermatocyte (curved arrow) , many necrotic depries (star) , no spermatozoa, vacuolated sertoli cell (arrow) and degenerated Leydic cell (curved line). C and D: recovery groups appeared with same histological structure to that of GII. H&E, 400X.

B- Prostate

In control group the dorsolateral lobe of prostate consists of many acini separated by connective tissue stroma. Each acinus is lined by cuboidal epithelium and contains eosinophilic secretion in the lumen (figure 3A). On contrast the prostatic sections from cyclophosphamide treated group showed low height epithelium in some region of the acini and foci of thickening (hyperplasia) in the others with enfolding of germinal epithelium to the lumen which appeared either empty or contain little secretion. Sloughed cells were detected in some lumen. The stroma appears thicker than that of control group and the vessels in it looked

congested with blood (figure 3B). The recovery periods for one and two months induced no histopathological improving from that observed in treated group (figure C &D).

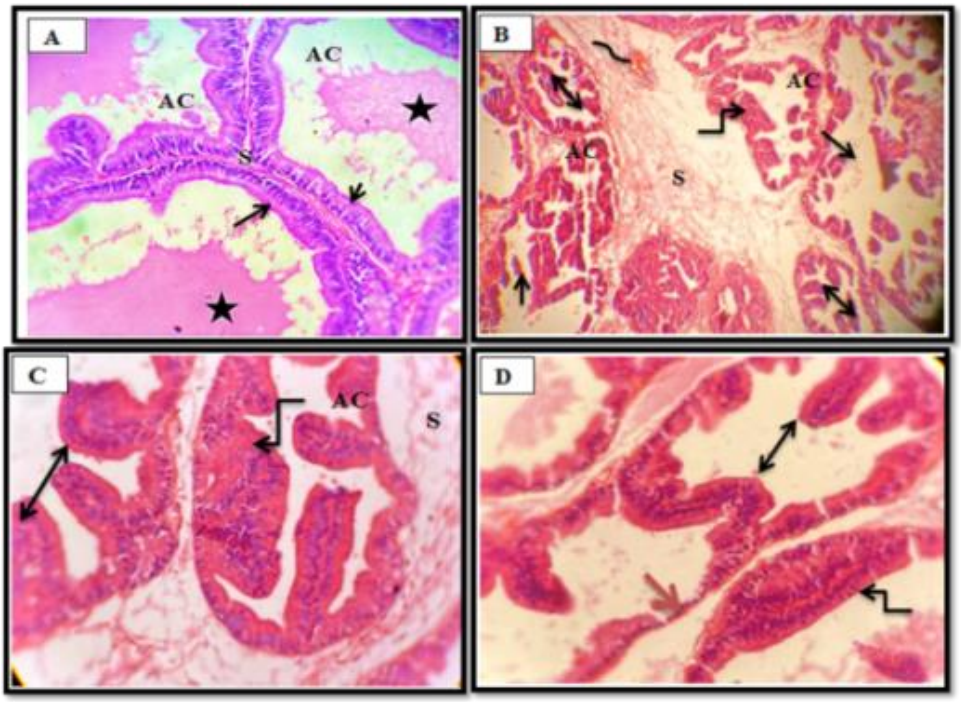

Figure 3: Photomicrograph from prostate. A: Section from control group shows acini (AC) separated by narrow stroma (S) , lined by simple columnar epithelium (arrows) and contain eosinophilic secretion in the lumen (star) H&E,400X. B:Section from Cyclophosphamide treated group (GII) shows acini (AC) with folded epithelium (bihead arrow),low height epithelium (red arrow), foci of epithelial hyperplasia(curved arrow),cellular depries in the lumen (arrows) . The acini separated by thick stroma (S) .H&E,200X. C&D: Sections from recovery groups show acini (AC) with folded epithelium (bihead arrow), foci of epithelial hyperplasia (curved arrow). Thick stroma. H&E, 400X.

C- Seminal vesicle:

In control group the sections show normal folded mucosa (honey-combed like). The lining epithelium is formed of simple columnar- cuboidal cells rest on basement membrane and thin connective tissue lamina properia and muscular stroma, the secretory product is seen in the lumen (figure 3A). While the sections from cyclophosphamide treated group show less folded mucosa with degeneration and rupture of secretory cells and loss of epithelial integrity, the desquamation of necrotic cells is seen in the lumen (figure 3B). However, the recovery periods for one or two months don't produce any improvement of histological changes induced by treatment with cyclophosphamide (figure 3C& D).

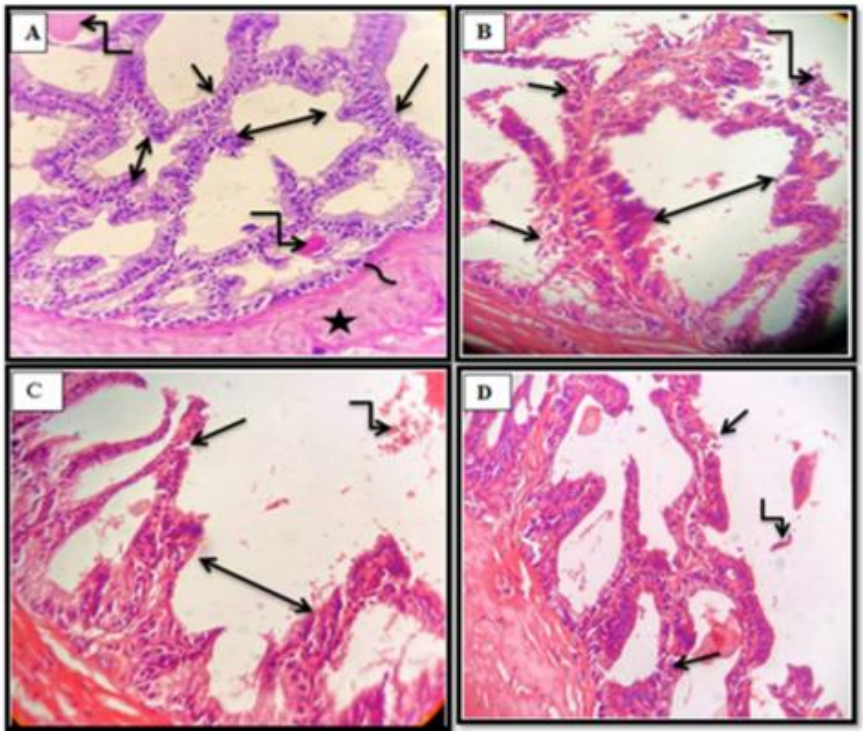

Figure 4: Photomicrograph from seminal vesicle. A: control group (GI) shows normal folded mucosa (bihead arrows), lining epithelium is cuboidal (arrows) , rest on basemen membrane, lamina properia (curved line) and stroma (star) , lumen contains secretary product (curved arrow). B: treated group (GII) shows less folded mucosa (bihead arrows), with degeneration and rupture of secretory cells (arrows), the desquamation of necrotic cells is seen in the lumen (curved arrow). C and D: recovery groups appeared with less folded mucosa (bihead arrows) and degeneration of lining epithelium (arrows) with desquamated cells in lumen (curved arrows). H&E, 400X

3.3. Immunhistochemical (IHC) stain

To evaluate the proliferative activity of germ cells and cells of prostate, Ki67 expression was determined. In the testicular sections, the Immunhistochemical stain indicated that Ki67- positive cells in control group were in spermatogonia and spermatocyte however, the expression of Ki67 were greatly reduced in these cells in both cyclophosphamides treated and recovery groups (figure 5). While in prostatic sections, the Ki67 expresion positive cells were increased in treated and recovery groups compare to that of control group (figure 6).

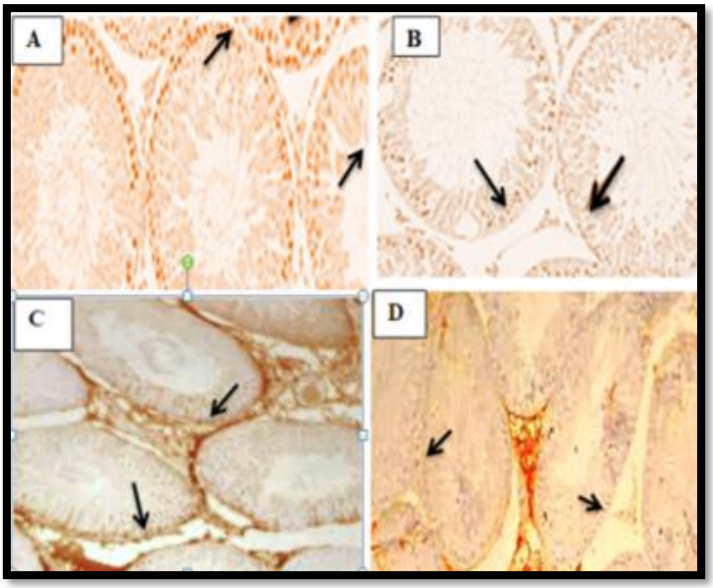

Figure 5: Photomicrographs from testes show Ki67 positive as brown color (arrows) in germinal epithelium. A: control group showing +ve IHC in spermatogonia and spermatocytes (arrows). However, the expression of Ki67 were reduced in these cells in both cyclophosphamides treated (B) and recovery groups (C: Recovery1, D: Recovery2). IHC,200X

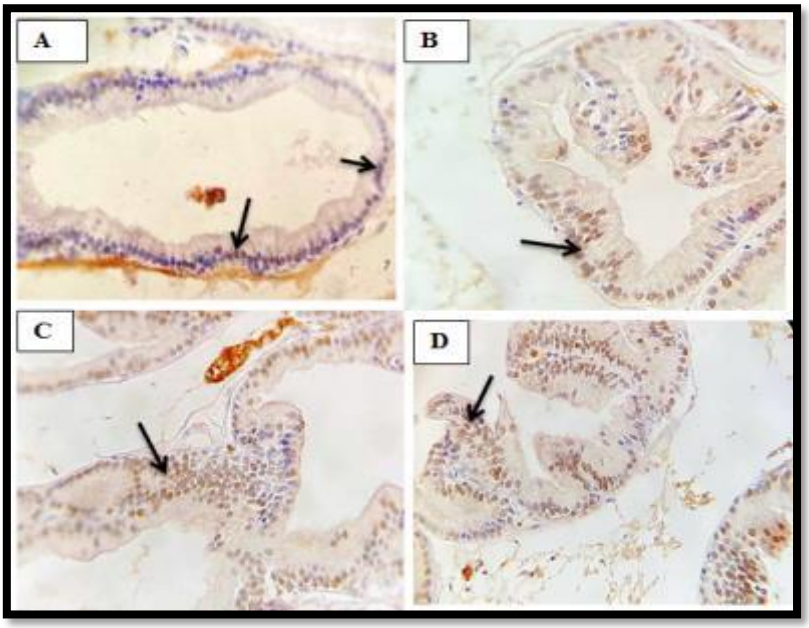

Figure 6: Photomicrograph from prostate show Ki67 positive as brown color (arrows) in prostaic epithelium of A: control. B: Treated. C: Recovery1. D: Recovery2.IHC, 200X

3.4. Morphometrical analysis

Johnsen Score and number of Leydic cells / interstitial space for testicular sections from treated group and recovery groups were significantly ((P <0. 01) reduced compare to that of control (table 2).

The mean percentage area of Ki67 expression area of both testes and prostate of all groups were compared (table2). There was significant reduction of the percentage of Ki67 expression area in testes of treated and recovery groups compare to control

while the percentage area of Ki67 expression in prostate of treated groups were significantly increased (P <0. 01) compare to control.

The mean height of prostatic epithelium was significantly increased in treated (GII) and recovery groups compare to that of control group. On the other hand, the perimeter of acini and the mean height of seminal vesicles were significantly reduced in treated and recovery groups compared to control. Though, there was non-significant variation in these parameters between treated and recovery groups (table 2).

Table 2: Values of Johnsen's score, Leydic cells number, % of Ki67 area in testis and prostate, Height of prostatic and seminal vesicles epithelium, perimeter of acini. Data were expressed as Mean±SE

Parameters / Groups	Johnsen's score	Leydic cells / interstitial space.	% of Ki67 area in testis	Height of prostatic epithelium (µm)	Perimeter of acini (µm)	% of Ki67 area in prostate	Height of seminal V. epith. (µm)
Control (GI)	9.5±2.3	21.3±1.8	57%	16.2±3.1	592±2.1	21.5%	18.1±2.4
treated (GII)	5.3±3.6*^{A}	13.1±2.3*^{A}	20.9%*^{A}	24.2±2.4*^{A}	370±3.1*^{A}	32.4%*^{A}	9.2±1.3*^{A}
Recovery 1	5.9±3.9*^{AB}	14.2±1.3*^{AB}	20%*^{AB}	21.7±1.3*^{AB}	393±1.1*^{AB}	31%*^{AB}	10.4±2.3*^{AB}
Recovery 2	6.2±5.6*^{AB}	16.8±1.8*^{AB}	21%*^{AB}	21.3±2.3*^{AB}	401±3.1*^{AB}	29%*^{AB}	11.0±3.4*^{AB}

* Compare with control group; P < 0.0001 significant

^{A} Compare GII with recovery 1; and recovery 2, P≤ 0.1 non-significant

^{B} Compare Recovery groups; P =0.68 non-significant

4.Discussion

Chemotherapies disturb the growth of uncontrolled proliferation of cells and eliminate the abnormal malignant cells [17]. Cyclophosphamide is an anti-malignant and immune-suppressor drug that has been used widely [18] but it may destroy the normal cells in the body causing unwanted effects [19]. The results of current work suggested that cyclophosphamide caused gonadal dysfunction represented by decreased serum testosterone level and arrested spermatogenesis which confirmed by decline of Johnsen's score and absent of spermatozoa in the lumen of many seminiferous tubules. These findings were in an agreement with that obtained by other studies [20,21]. Ceribaşi *et.al* reported that cyclophosphamide enhances the sperm abnormality and may arrest spermatogenesis in a dose- related manner [22]. . In male, the main function of the testes is secretion of male hormone (testosterone) and production of male gamete (sperm) so, testosterone level is regarded as a marker for the optimum function of the testis [23]. The reduction of testosterone level in this work may be resulted from the degeneration and reduced number of Leydic cells [24]. Sertoli cells play a crucial role in the blood-testis barrier and in regulation of spermatogenesis [25]. consequently, vacuolar degeneration of sertoli cells observed in this experiment may be responsible for arrest of spermatogenesis. Gonadal dysfunction is one of common problem accompany the use of chemotherapeutic agents [26,27]. The high

proliferative activity of reproductive tissues makes them susceptible to toxicity by many drugs including cyclophosphamide [28] which suppressed the replication of DNA leading to apoptosis and inhibit the proliferation of many cells [29]. Gonadal toxicity and infertility is regarding as an important challenges factor in chemotherapy and restrict the efficacy of these drugs [30, 31]. The adverse reactions of chemotherapy depending on dose of drug, duration of use, present of disease and the age of the patients [32].

The harmfulness effect of cyclophosphamide may be related to its metabolites as Acrolein which are produced by the enzymatic action of cytochrome p450 on the drug [33]. Acrolein increases the free radicles production and initiate the oxidative stress which interfere with organs activity [34]. Unwanted effect of chemotherapy could be due to its effect on the apoptotic cascade pathway[35]. Spermatogenesis is a programmed process of germinal cells proliferation and apoptosis. The present data showed that percentage of Ki67-positive cells was decreased in cyclophosphamide and recovery groups compared to that of the control which indicated reduction of cell proliferation. Ki67 protein is expressed mainly in spermatogonia and spermatocytes particularly the primary one [36]. It regards as an ordinary marker for spermatogenic cells proliferation in the testis because it presents in the cell nucleus and takes part in mitotic division and protection of DNA construction [37]. Zhan *et.al* assessed spermatogenic proliferation by measuring the proliferative cell nuclear antigen in different periods of using cyclophosphamide treatment, they found that extensive damage of testis occurs during second weeks of treatment [38]. However, cyclophosphamide doesn't that prostate of cyclophosphamide treated groups had reduction of its secretion with destruction of lining epithelium and compensatory foci of epithelial hyperplasia, additionally the diameters of prostatic acini decreased and the percentage area of Ki67 expressions was increased while the seminal vesicles showed degeneration and rupture of lining epithelium with reduction of their height. These findings indicated hypo activity of these glands. The integrity of glandular epithelium of sexual gland depends on the testosterone level [39]. So the reduction of testosterone level observed in the present study may be responsible for morphological changes of these glands. On the other hand, the spermatogenesis and sperm functioning can be disrupted by unhealthy sexual glands. Olukole *et.al* said that epithelial hyperplasia and reduced acinar lumen reflect the incapability of gland to function well [40]. The structural changes in the sexual glands that appropriated by cyclophosphamide were not reversed after drug withdrawal for one nor even for two months. This indicate that sterility induced by cyclophosphamide treatment may be permanent or need longer time to be recover.

CONCLUSION

This work concluded that cyclophosphamide had serious effect on the male fertility, it could be induced testicular and sexual glands toxicity due to its cytotoxic effect and this effect may be permanent and irreversible even after drug withdrawal. For this reason, patients, especially young people, must be monitored during cyclophosphamide therapy to avoid permanent infertility.

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Competing interest statement

The authors declare no conflict of interest.

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