

# Treatment of Male Albino Rats with 5-fluorouracil and/or Cyclophosphamide Caused Serious Alterations in Sexual Hormones, Histological Structure of the Testis, and Semen Quality

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## Abstract

**Background:** The therapeutic effect of Cyclophosphamide (CPA) is thus attributed to phosphoramidate mustard and acrolein that leads to the formation of high level of reactive oxygen species (ROS), which results in decreased antioxidant activity. Excessive production of ROS could also culminate in oxidative stress.

**Objectives:** The aim of this study is to evaluate the effect of sub lethal dose of the cyclophosphamide, 5-FU combination of 5-FU and CPA on testicular function, and histopathological alterations in male albino rats. These functions were monitored through some selected biochemical parameters and confirmed by DNA, RNA concentration and histopathological examination.

**Materials and Methods:** Twenty-eight male adult rats were grouped randomly into four groups (n=7 each group), Five for biochemical studies and 2 for histological studies. Group I (control): Rats were injected with saline intraperitoneally and at a dose of 1.0 ml/kg b.w. for 14 days. Group II cyclophosphamide (CPA): Cyclophosphamide at a dose of 10 mg/kg day by day through i.p. to rats for 14 days. Group III Fluorouracil (5-FU): 5-Fluorouracil at a dose of 10 mg/kg day by day in saline was given through i.p. to rats for 14 days. Group IV (CPA+5-FU): Rats were given CPA followed by 5-FU at a dose of 10 mg/kg per day (day by day) through i.p. to rats for 14 days. At the end of the experimental period, rats were anesthetized using light ether. Blood and testes tissue samples were taken and prepared for biochemical and histological measurements. Biochemical parameters in rat serum and tissues were evaluated.

**Results:** Treatment of male rats with CPA, 5-FU and their combination caused a significant decrease in sperm count, motility while increased dead and abnormal sperms compared to those of control. Co-treatment of CPA and 5-FU caused a significant decrease in sperm count, sperm motility and increase in abnormal sperms compared to individual treatment of CPA and 5-FU. Levels of testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) were decreased in the CPA, 5-FU and their combination-treated group. Co-treatment of 5-FU and CPA synergistically reduced testicular hormones compared with individual chemotherapy. Cyclophosphamide, 5-FU and their combination decreased testis DNA and RNA concentrations compared to the control. The combination of CPA and 5-FU was more effective to DNA and RNA compared to individual administration of the chemotherapy. Epididymis of the control rats showed normal histological structure with normal sperm density. Large tubules lined by pseudostratified columnar epithelium with abundant stereocilia projection from their surface. Long and dense processes of sperm tails were observed within the Lumina. The epididymis tubules of CPA-exposed rats were smaller and the majorities of them were free from mature spermatozoa. Epithelium contained numerous numbers of sloughed cells with cell debris in Lumina. 5-FU treated rat showed atrophy as well as necrosis, the arrangement of germ cells was disordered. The number of cell layers of the seminiferous tubules was significantly reduced.

Epididymis of CPA+5-FU-treated rats showed necrotic degeneration and atrophy of the germinal cells, decrease spermatid when compared to CPA.

**Conclusion:** It could be concluded that treatment of mammals with chemotherapy is associated with the production of free radicals that lead to hazardous alterations in sexual hormones, DNA, and RNA concentration, and histopathological structure in testes and semen quality of male albino rats. However, 5-FU and CPA combination could produce a serious alteration in these parameters. Future work should consider combined chemotherapy regimens. Toxicological studies must be performed before using drugs as combination before application. Further research is required on toxicological impacts of drugs and pollutants mixtures.

**Keywords:** cyclophosphamide; 5-fluorouracil; sexual hormones; semen quality; histological structure of the testis; male albino rats

## Introduction

Cyclophosphamide (CP) is one of the most invasive chemotherapeutic agents. Clinical evidence showed toxic side effects of CP in multiple organ systems [1,2], reported that administration of 65 mg/kg i.p. CPA to Wistar rats for 5 days induced reproductive toxicity as evidenced by significantly lowered levels of FSH, LH and testosterone, perturbation of sperm characterization. Histopathological examination of the testis showed ballooned seminiferous tubules with loosed connective tissues and vacuolation of testicular interstitium. [1] reported that Serum testosterone concentration was significantly ( $p < 0.01$ ) decreased in adult male Wistar rats treated i.p. with 6.1 mg/kg of CP/day for 50 days. The male patients treated with cyclophosphamide have demonstrated disturbed gonadotrophin secretion associated with testicular damage along with low blood level of testosterone [3].

5-FU is used to treat colorectal cancer as well as other kinds of cancer. Its cytotoxic effects on cancer cells exert through the inhibition of thymidylate synthase and the incorporation of its metabolites into RNA and DNA [4, 5]. 5-FU is an analogue of uracil with a fluorine atom at the C-5 position in place of hydrogen. It rapidly enters the cell using the same facilitated transport mechanism as uracil [6]. 5-FU is converted intracellularly to several active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP) [7]. [5] concluded that a small dose of 5-FU affect the patient fertility by disturbing the testes histology and sperm morphology.

## 2. Objectives

The aim of this study is to evaluate the effect of sub lethal dose of the cyclophosphamide, 5-FU combination of 5-FU and CPA on testicular function, and histopathological alterations in male albino rats. These functions were monitored through some selected biochemical parameters and confirmed by DNA, RNA concentration and histopathological examination.

## 3. Materials and Methods

The present research was conducted in the Environmental Toxicology Laboratory, Department of Environmental Studies, Institute of Graduate Studies and Research, Alexandria University, Egypt.

### 3.1. Chemicals

Reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene, thiobarbituric acid and all other chemicals were purchased from Sigma Chemical Company (Saint Louis, USA). Other chemicals from Sigma or other trusted commercial sources. Cyclophosphamide and 5-Fluorouracil were purchased from

### 3.2. Animals

Twenty eight male adult rats (Sprague Dawley) with average body weight  $180 \pm 10$  g were obtained from Faculty of agriculture, Alexandria, and acclimatized for two weeks prior to the experiment. They were assigned to four groups and housed in Universal galvanized wire cages at room temperature (22-25°C) and in photoperiod of 12h/day. Animals were provided with balanced commercial diet.

### 3.3. Experimental protocol

Twenty eight male adult were grouped randomly into four groups (n=7 each group).

- Group I (control): Rats were injected with saline intraperitoneally and at a dose of 1.0 ml/kg b.w. for 14 days.
- Group III cyclophosphamide (CPA): Cyclophosphamide at a dose of 10 mg/kg day by day through i.p. to rats for 14 days [8].
- Group III Fluorouracil (5-FU): 5-Fluorouracil at a dose of 10 mg/kg day by day [9] in saline was given through i.p. to rats for 14 days. .
- Group IV (CPA+5-FU): Rats were given CPA followed by 5-FU at a dose of 10 mg/kg per day (day by day) through i.p. to rats for 14 days.
- At the end of the experimental period, rats were anesthetized using light ether. Blood samples were taken from the vena cava of rat heart. Tubes were used to compile blood drawn from the heart directly; serum formation, blood was allowed to set for 30 min at 4°C to clot, then centrifuged for 5 minutes at 1000 xg. Packed cells were discarded and the supernatant serum samples were decanted and stored into capped sterile polyethylene tubes at -20°C until used (within 24 hours). The abdominal cavity of each rat was opened where the testes were excised. Tissue was blotted on a filter paper to remove excess buffer, and the tissue was weighed prior the addition of 5-10 ml cold 50 mM potassium phosphate buffer, pH 7.5 containing 1 mM EDTA per gram tissue. Then, tissue was homogenized using a glass pestle (glass homogenizer). The homogenate was centrifuged at 10,000 x g for 15 min at 4°C, and the supernatant was collected and stored at -80 °C for further use.

### 3.7. Testicular RNA and DNA concentration

The RNA concentration in the samples was determined by precipitation of the nucleic acids in 0.5 M HClO<sub>4</sub>, after which the RNA was hydrolysed ice by incubation in 0.3 M KOH at 60°C for 1 h. After preremoval of DNA by 0.5 M HClO<sub>4</sub> precipitation, the RNA concentration was determined by boiling the samples for 30 min in 6 M HCl, 0.01% FeCl<sub>3</sub> and 0.3% orcinol. Absorption was measured at 660 nm. RNA concentrations were calculated using a calibration curve with highly purified total adult heart RNA that was isolated by ultracentrifugation through a caesium chloride cushion [10] as a standard.

The DNA concentration in the samples was de- termined by hydrolysing the RNA in 0.1 M NaOH after which the DNA was precipitated with half volume 10% HClO<sub>4</sub>. The DNA was resuspended in 10% 32PHClO<sub>4</sub> and incubated at 70°C to hydrolyse the DNA. After clearing the solution by centrifugation, dip- henylamine and acetaldehyde were added to a final concentration of 2% and 0.01%, respectively. After an overnight incubation at 30°C the absorbance was determined at 560 and 700 nm. The concentration in the samples was calculated relative to the included calibration curve that was prepared using highly Mopurified herring testis DNA [11].

### Histopathological analysis

The method described by [12] was employed in processing testicular samples for histopathological examinations. Bouin-fixed testicular tissues were dehydrated stepwise in graded ethanol and embedded in paraffin wax. A thin section (5-µm thickness) was made from the mid-portion of each sample and stained with hematoxylin and eosin, followed by examination under a light microscope.

### 3.6. Sperm characteristics

The epididymis was placed in a Petri dish containing 1ml of pre-warmed phosphate buffer. The epididymis was cut into small portions to allow sperm to swim out and filtering through a mesh. One drop of sperm suspension was placed on a glass slide, covered with a cover slip. The motility of the epididymal sperm was evaluated microscopically within 2-3 minutes of their isolation from the epididymis and data were expressed as percentage of motile sperm of the total sperm counted.

Sperm count was conducted according the standard procedure with the aid of the Neubauer Haemocytometer under a microscope [13]. The spermatozoa count was obtained by counting the number of sperms in the four WBC chambers using haemocytometer.

The sperm suspension was mixed with one drop of 1% eosin Y and smears were prepared on clean glass slides, slides were viewed by bright-field microscope with magnification of 400x. Two hundred sperms was examined to determine the morphological abnormalities which were classified as amorphous, hook less, coiled, tail less and finally represented as percentage total abnormality [14].

### 3.9. Statistical Analysis

The values are expressed as mean  $\pm$  SE. All values are expressed as mean  $\pm$  standard error of mean (SEM). Comparisons between the treatment groups and pathogenic control group were performed by analysis of variance (ANOVA) followed by Tukey- test.  $P < 0.05$  was considered as significant [15].

## 4. Results

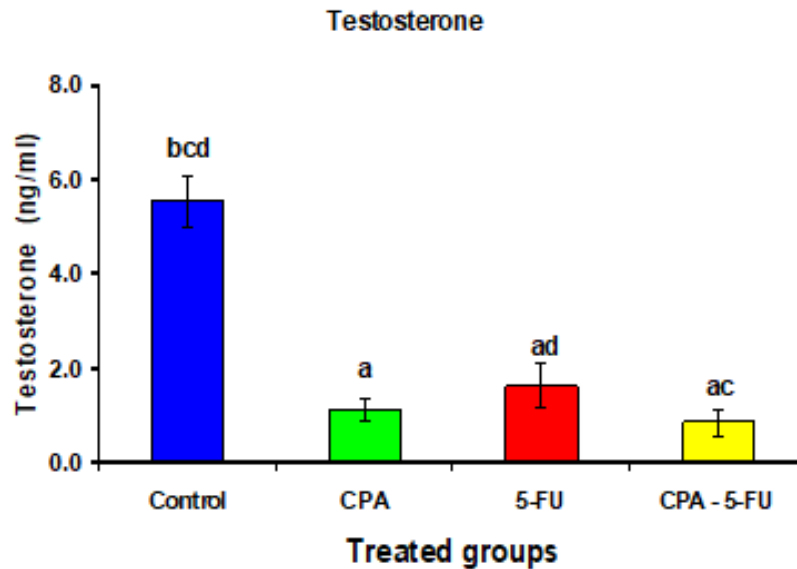
### 4.1. Serum levels of testosterone, luteinizing hormone, and follicle stimulating hormone

A significant decrease was shown in serum levels of testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) in the CPA, 5-FU and their combination-treated group. Co-treatment of 5-FU and CPA synergistically reduced testicular hormones compared with individual chemotherapy (Tables .1 and Figures 1-3).

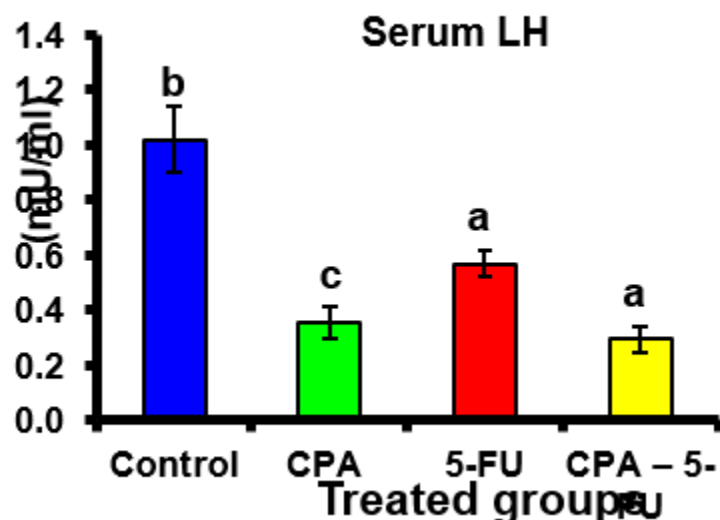
| Parameters                                 | Groups                         |                                |                                |                                |
|--|--------------------------------|--------------------------------|--------------------------------|--------------------------------|
|  | Control                        | CPA                            | 5-FU                           | CPA – 5-FU                     |
| Serum testosterone levels (ng/ml)          | 5.54 $\pm$ 0.55 <sup>bcd</sup> | 1.12 $\pm$ 0.23 <sup>a</sup>   | 1.63 $\pm$ 0.450 <sup>ad</sup> | 0.85 $\pm$ 0.28 <sup>ac</sup>  |
| Serum luteinizing hormone (mU/ml)          | 1.02 $\pm$ 0.12 <sup>bcd</sup> | 0.36 $\pm$ 0.06 <sup>ac</sup>  | 0.57 $\pm$ 0.05 <sup>abd</sup> | 0.30 $\pm$ 0.05 <sup>ac</sup>  |
| Serum follicle stimulating hormone (mU/ml) | 0.40 $\pm$ 0.03 <sup>bcd</sup> | 0.07 $\pm$ 0.01 <sup>acd</sup> | 0.17 $\pm$ 0.04 <sup>abd</sup> | 0.05 $\pm$ 0.01 <sup>abc</sup> |

Significance at  $P < 0.05$ . CPA: cyclophosphamide; 5-FU: Fluorouracil, <sup>a</sup> Comparison of control and other groups; <sup>b</sup> Comparison of CPA and other groups; <sup>c</sup> Comparison of 5-FU and other groups; <sup>d</sup> Comparison of CPA – 5-FU and other groups

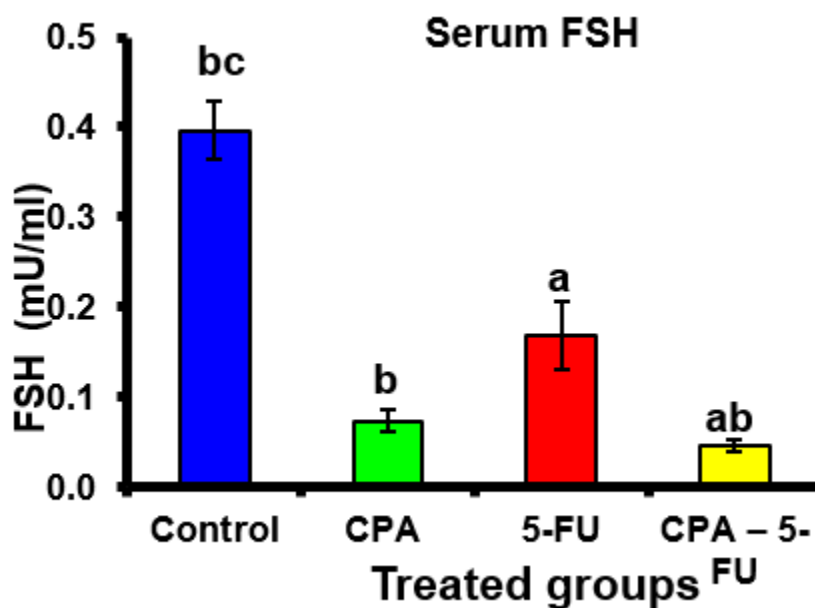
**Table 1:** Serum testosterone, luteinizing hormone, and follicle stimulating hormone levels of rat treated with cyclophosphamide and/or 5-fluorouracil



**Figure 1:** Serum testosterone (ng/ml) of rat treated with cyclophosphamide, fluorouracil and their combination. Significance at  $P < 0.05$ . CPA: cyclophosphamide; 5-FU: Fluorouracil. <sup>a</sup> Comparison of control and other groups; <sup>b</sup> Comparison of CPA and other groups; <sup>c</sup> Comparison of 5-FU and other groups; <sup>d</sup> Comparison of CPA – 5-FU and other groups



**Figure 2:** Serum Luteinizing hormone (LH) (mU/ml) of rat treated with cyclophosphamide, fluorouracil and their combination. Significance at  $P < 0.05$ . CPA: cyclophosphamide; 5-FU: Fluorouracil. <sup>a</sup> Comparison of control and other groups; <sup>b</sup> Comparison of CPA and other groups; <sup>c</sup> Comparison of 5-FU and other groups; <sup>d</sup> Comparison of CPA – 5-FU and other groups



**Figure 3:** Serum follicle stimulating hormone (FSH) (mU/ml) of rat treated with cyclophosphamide, fluorouracil and their combination. Significance at  $P < 0.05$ . CPA: cyclophosphamide; 5-FU: Fluorouracil. <sup>a</sup> Comparison of control and other groups; <sup>b</sup> Comparison of CPA and other groups; <sup>c</sup> Comparison of 5-FU and other groups; <sup>d</sup> Comparison of CPA – 5-FU and other groups

#### 4.5. Testes DNA and RNA

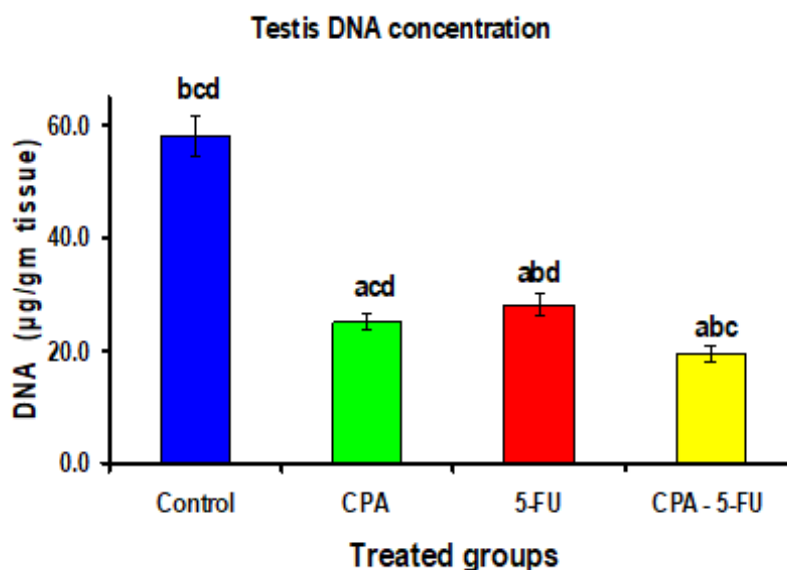
Cyclophosphamide, 5-FU and their combination decreased ( $P < 0.05$ ) testis DNA and RNA concentrations compared to the control. The

combination of CPA and 5-FU was more effective to DNA and RNA compared to individual administration of the chemotherapy (Tables .2; Figures 4, 5).

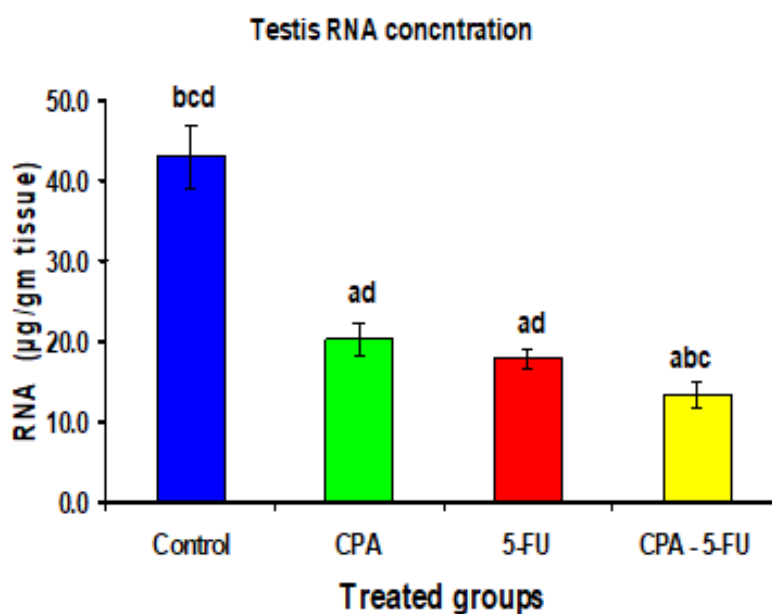
| Parameters                              | Groups | Control                | CPA                    | 5-FU                   | CPA – 5-FU               |
|---|--------|------------------------|------------------------|------------------------|--------------------------|
|   |        | Mean±SE                | Mean±SE                | Mean±SE                | Mean±SE                  |
| Testis DNA concentration (µg/gm tissue) |        | 58±3.41 <sup>bcd</sup> | 25±1.41 <sup>acd</sup> | 28±1.90 <sup>abd</sup> | 19.4±1.36 <sup>abc</sup> |
| Testis RNA concentration (µg/gm tissue) |        | 58±3.41 <sup>bcd</sup> | 25±1.41 <sup>acd</sup> | 28±1.90 <sup>abd</sup> | 19.4±1.36 <sup>abc</sup> |

Significance at  $P < 0.05$ . CPA: cyclophosphamide; 5-FU: Fluorouracil, <sup>a</sup> Comparison of control and other groups; <sup>b</sup> Comparison of CPA and other groups; <sup>c</sup> Comparison of 5-FU and other groups; <sup>d</sup> Comparison of CPA – 5-FU and other groups

**Table 2:** Serum testosterone, luteinizing hormone, and follicle stimulating hormone levels of rat treated with cyclophosphamide and/or 5-fluorouracil



**Figure 4:** Testis DNA concentration (µg/gm tissue) of rat treated with cyclophosphamide, fluorouracil and their combination. Significance at  $P < 0.05$ . CPA: cyclophosphamide; 5-FU: Fluorouracil. <sup>a</sup> Comparison of control and other groups; <sup>b</sup> Comparison of CPA and other groups; <sup>c</sup> Comparison of 5-FU and other groups; <sup>d</sup> Comparison of CPA – 5-FU and other groups



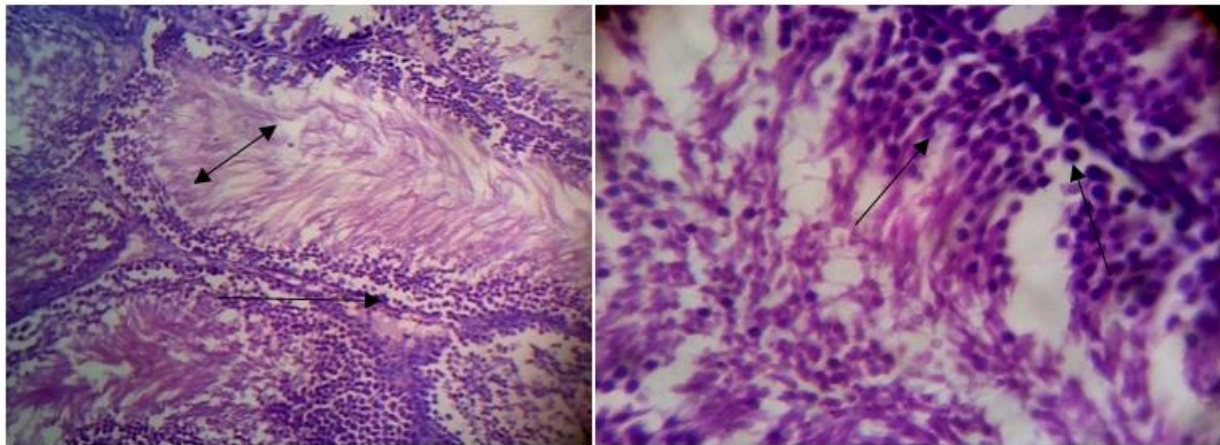
**Figure 5:** Testis RNA concentration (µg/gm tissue) of rat treated with cyclophosphamide, fluorouracil and their combination. Significance at  $P < 0.05$ . CPA: cyclophosphamide; 5-FU: Fluorouracil. <sup>a</sup> Comparison of control and other groups; <sup>b</sup> Comparison of CPA and other groups; <sup>c</sup> Comparison of 5-FU and other groups; <sup>d</sup> Comparison of CPA – 5-FU and other groups

#### 4.6. Histopathological findings

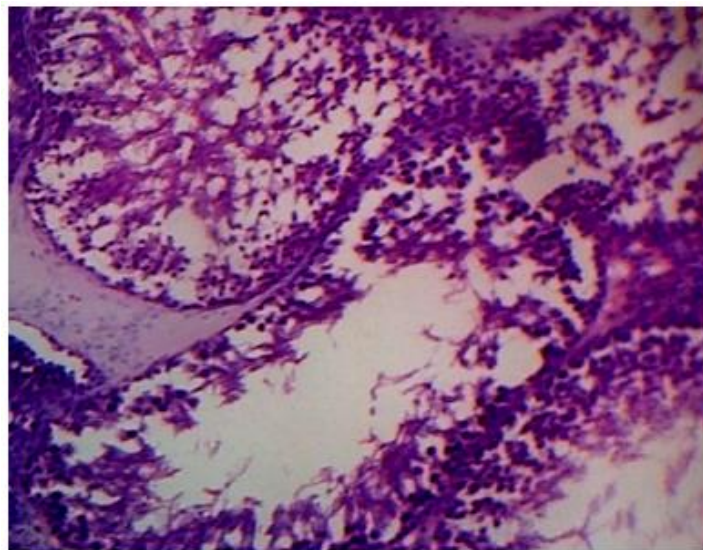
Epididymis of the control rats showed normal histological structure with normal sperm density. Large tubules lined by pseudostratified columnar epithelium with abundant stereocilia projection from their surface. Long and dense processes of sperm tails were observed within the Lumina (Figures .6). The epididymis tubules of CPA-exposed rats were smaller and the majorities of them were free from mature spermatozoa. Epithelium contained numerous

numbers of sloughed cells with cell debris in Lumina (Figure. 7). Photomicrographs of cauda epididymis of 5-FU treated rat showing atrophy as well as necrosis, the arrangement of germ cells was disordered. The number of cell layers of the seminiferous tubules was significantly reduced (Figure .8). Epididymis of CPA+5-FU-treated rats showed necrotic degeneration and atrophy of the germinal cells, decrease spermatid when compared to CPA (Figure .9).

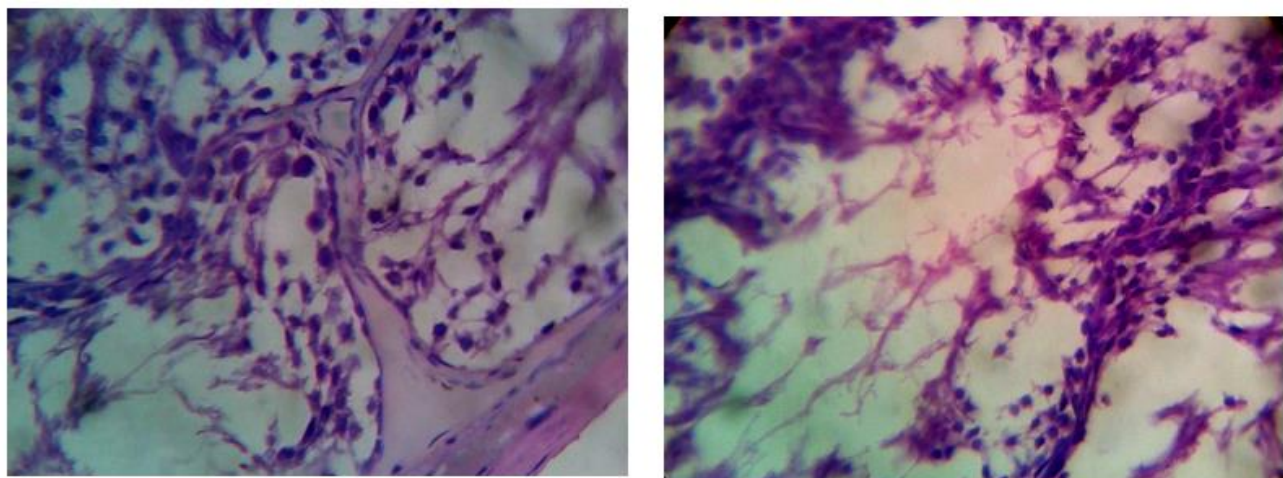




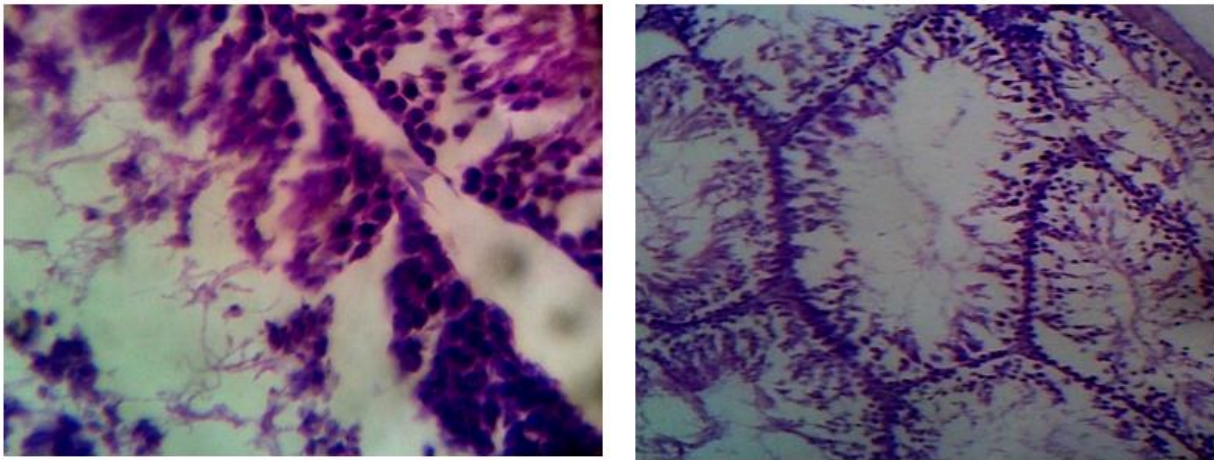
**Figure 6:** Photomicrographs of cauda epididymis of control rat showing normal section in rat testis with normal lyding cells, spermatocytes, seminiferous epithelium, spermatid. Uniform seminiferous tubules lined by normal layers of spermatogenic cells up to mature sperm formation



**Figure 7:** Photomicrographs of cauda epididymis of CPA treated rat showing severe testicular degeneration. Note that sperm density is severely decreased. Some tubules are empty and others have few spermatozoa and LC variable sized and shaped seminiferous tubules lined by few layers of spermatogenic cells with wide lumina



**Figure 8:** Photomicrographs of cauda epididymis of 5-FU treated rat showing atrophy as well as necrosis, the arrangement of germ cells was disordered. The number of cell layers of the seminiferous tubules was significantly reduced.



**Figure 9:** Photomicrographs of cauda epididymis of combined treated rat with 5-FU and CPA showing necrotic degeneration and atrophy of the germinal cells, decrease spermatid.

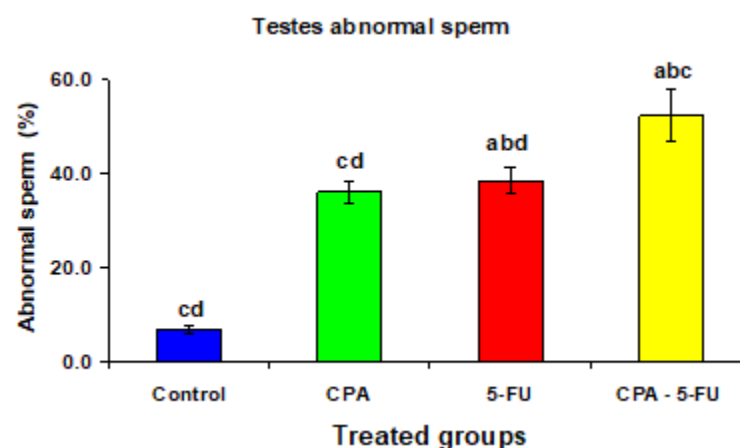
#### 4.3. Sperm characteristics

Treatment of male rats with CPA, 5-FU and their combination caused a significant decrease ( $p < 0.05$ ) in sperm count, motility while increased dead and abnormal sperms compared to those of control. Co-treatment of CPA and 5-FU caused a significant decrease in sperm count, sperm motility and increase in abnormal sperms compared to individual treatment of CPA and 5-FU (Tables 3 and Figures 10-12).

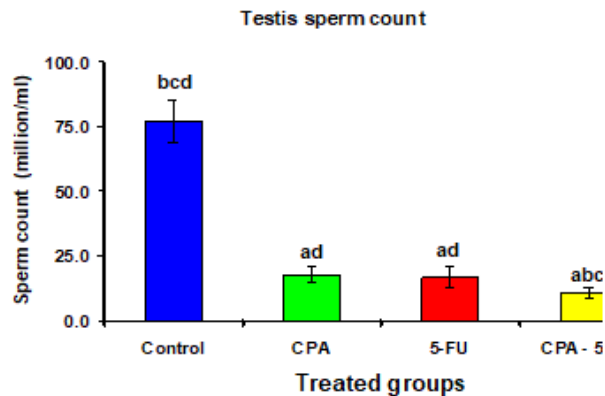
| Parameters                       | Groups | Control                  | CPA                     | 5-FU                     | CPA – 5-FU                |
|----------------------------------|--------|--------------------------|-------------------------|--------------------------|---------------------------|
|                                  |        | Mean±SE                  | Mean±SE                 | Mean±SE                  | Mean±SE                   |
| Testis abnormal sperm (%)        |        | 6.80±0.98 <sup>bcd</sup> | 36.20±2.4 <sup>ad</sup> | 38.60±2.87 <sup>ad</sup> | 52.40±5.50 <sup>abc</sup> |
| Testis sperm counts (million/ml) |        | 77±8.29 <sup>bcd</sup>   | 17.8±2.71 <sup>ad</sup> | 16.6±4.03 <sup>ad</sup>  | 10.4±2.06 <sup>abc</sup>  |
| Testis sperm motility (%)        |        | 81.8±5.12 <sup>bcd</sup> | 17.2±6.46 <sup>ad</sup> | 17±2.68 <sup>ad</sup>    | 10.6±3.32 <sup>abc</sup>  |

Significance at  $P < 0.05$ . CPA: cyclophosphamide; 5-FU: Fluorouracil, <sup>a</sup> Comparison of control and other groups; <sup>b</sup> Comparison of CPA and other groups; <sup>c</sup> Comparison of 5-FU and other groups; <sup>d</sup> Comparison of CPA – 5-FU and other groups

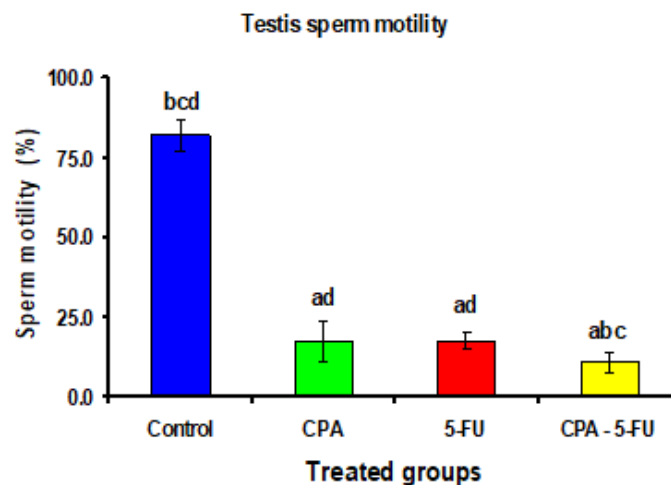
**Table 3:** Testis abnormal sperm, sperm counts, and sperm motility of rat treated with cyclophosphamide and/or 5-fluorouracil



**Figure 10:** Testis abnormal sperm (%) of rat treated with cyclophosphamide, fluorouracil and their combination. Significance at  $P > 0.05$ . CPA: cyclophosphamide; 5-FU: Fluorouracil. <sup>a</sup> Comparison of control and other groups; <sup>b</sup> Comparison of CPA and other groups; <sup>c</sup> Comparison of 5-FU and other groups; <sup>d</sup> Comparison of CPA – 5-FU and other groups



**Figure 11:** Testis sperm count (million/ml) of rat treated with cyclophosphamide, fluorouracil and their combination. Significance at  $P < 0.05$ . CPA: cyclophosphamide; 5-FU: Fluorouracil. <sup>a</sup> Comparison of control and other groups; <sup>b</sup> Comparison of CPA and other groups; <sup>c</sup> Comparison of 5-FU and other groups; <sup>d</sup> Comparison of CPA – 5-FU and other groups.



**Figure 12:** Testis sperm motility (%) of rat treated with cyclophosphamide, fluorouracil and their combination. Significance at  $P < 0.05$ . CPA: cyclophosphamide; 5-FU: Fluorouracil. <sup>a</sup> Comparison of control and other groups; <sup>b</sup> Comparison of CPA and other groups; <sup>c</sup> Comparison of 5-FU and other groups; <sup>d</sup> Comparison of CPA – 5-FU and other groups.

## 5. Discussion

A significant decrease was shown in serum levels of testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) in the CPA, 5-FU and their combination-treated group. Co-treatment of 5-FU and CPA synergistically reduced testicular hormones compared with individual chemotherapy. Testosterone is produced by leydig cell in the testes under the influence of LH. Intratesticular testosterone is an absolute prerequisite for normal spermatogenesis. The decrease in the mean testosterone levels of 5-FU and CPA treated rat served as proof for the damage of testis. These results demonstrate that tested chemotherapy caused temporary interference of normal male reproductive system with treatment.

The results revealed that CPA, 5-FU and their combination reduced serum testosterone level, the endogenous antioxidant enzyme activities as well as the non-enzymatic scavenger (GSH) in treated rats. It also suppressed lipid peroxidation and significantly enhanced the lowered TAC and most of the decreased testicular marker enzyme activities. In accordance with previous work [16], CPA administration induced a state of oxidative stress in the rat testis, demonstrated by enhanced lipid peroxidation and compromised antioxidant defense system revealed by decreased TAC and GSH levels with concomitant reduction in GR activities. Oxidative stress is the result of an imbalance between ROS generation and the intracellular capacity for

removing ROS, subsequently leading to excessive damage in the cell [17]. Increase in testicular ROS level was previously reported in CPA-treated rats [18]. Spermatozoa are particularly susceptible to ROS-induced injury and peroxidative damage because of their high concentrations of polyunsaturated fatty acids and low antioxidant capacity [19].

Cyclophosphamide is an alkylating agent commonly used in combination cancer chemotherapy regimens. It is also used alone as an immunosuppressive agent for conditions such as glomerulonephritis [20] and Bechcet's disease [21]. There are a number of reports on the effects of this drug on fertility. Adult patients treated daily with cyclophosphamide (1-2 ng/kg) for more than 4 month showed oligozoospermia or azoospermia [20-22]. This change in spermatogenic function was associated with increases in serum follicle-stimulating hormone (FSH) levels [21]. The return of fertility was unpredictable but in some cases it took a number of years [23].

It is well known that utilization of oxygen represents an efficient mechanism for aerobic organisms to generate energy, but ROS are also produced within the biological systems [24]. Under normal conditions to protect cells against oxidative damage, the potential harmful effects of ROS and free radicals are effectively eliminated by the antioxidant defense systems such as antioxidant enzymes and non-enzymatic factors [25]. Normally, cells possess a well-developed biochemical defense system, comprising low-molecular weight



free radical scavengers, i.e. glutathione (GSH), vitamin C, vitamin E and complex enzymes, including GPx, SOD and CAT [26]. When the balance between the generation and elimination of ROS is broken, as a result of these events, biomacromolecules including DNA, membrane lipids and proteins are damaged by ROS-mediated oxidative stress [27, 28].

[29] had reported that CPA requires microsomal oxidation in the liver to yield its active metabolite, 4-hydroxy-CPA in target cells that spontaneously decomposes to phosphoramid mustard which exerts cytotoxic effect by the induction of DNA single strand breaks as well as crosslinks which result in different types of damage. Also, CPA has been shown to induce apoptosis in the target cells through the modulation of signaling through caspases, Bcl2, Bax, NF-Kappa B, and MAP kinases [30, 31]. [32] had reported that CPA involves inhibition of cell division due to cross-linking of the drug to DNA.

Results of both biochemical and histological investigations showed that CPA, 5-FU and their combination induced a marked reproductive toxicity through induction of oxidative stress. In this study, testicular reduction in testosterone, LH and FSH levels were occurred due to CPA, 5-FU and their combination treatment. Furthermore CPA, 5-FU treatments induced a marked decrease of spermatogenesis, as confirmed by our histological findings of sperm density reduction in epididymis. In explanation to the present finding, it has been reported that antineoplastic agents can disturb Leydig cells directly [33].

Thus, the reduction in circulating testosterone is supposed to be resulting from a direct poisonous effect of CPA and 5-FU on the Leydig cells. Steroidogenesis in the male rats is stimulated by hypothalamic gonadotropin releasing hormone (GnRH) effecting to induce the production and release of LH, which binds to LH receptors on the membrane of Leydig cells to upregulate testosterone production [34]. The reduction in LH level perhaps is result of damage in the negative feedback control of hypothalamic-pituitary axis [35]. In addition, it is likely that the dysfunction of the pituitary in LH releasing was resulted from damage to the cell membrane-mediated signaling mechanisms involved in releasing LH to the blood.

In the present study, epididymal sperm count decreased by CPA treatment while the number of dead and abnormal sperms increased and motility did not show significant difference. [36] showed that the incidence of male infertility following CPA chemotherapy resulted from changes in sperm parameters. Germ cells in testes are vulnerable to DOX-induced oxidative stress [37]. Anthracyclines like DOX exert their antitumor properties as well as other organ toxicity by intracellular producing of free radicals and ROS accompanied by intercalation with DNA and consequent inhibition of topoisomerase [38].

This increased oxidative stress effects on the sperm membranes, proteins and DNA [39-41]. Therefore, DNA damage may be liable for the increased level of abnormal spermatozoa forms. As confirmed by acridine orange staining, treatment with DOX causes single/double strand breaks in sperm DNA. In the case of the adult rat testis, the gonadotoxic drug DOX induces programmed cell death in meiotic spermatocytes and type A and intermediate spermatogonia by intercalating into DNA to create strand breaks and by preventing topoisomerase II activity [42]. These genotoxic alterations up-regulate expression of p53, an essential mediator of cell cycle stop considered to inhibit DNA replication in the presence of DNA damage, resulting apoptosis and finally drop in sperm counts [42].

In response to some anticancer drugs like anthracyclines, the number of male germ cells undergoing apoptosis increases several folds. Because the most sensitive cells to CPA and 5-FU are the early spermatogenic cells and primary spermatocytes, treatment with chemotherapy may lead to the loss of proliferating immature germ cells and finally of mature spermatozoa [43]. Therefore, a possible reason for the disruption of spermiogenesis in the CYT-treated rats is failure of testosterone dependent attachment of spermatids to Sertoli cells [44].

In a normal status, secretion of GnRH from hypothalamus stimulating the anterior pituitary gland to produce FSH and LH which in turn bring about gonadal hormones secretions (testosterone and estrogen) in testes. Reduced hypothalamic sensitivity to negative feedback effects of androgens increases GnRH secretion and consequently the amount of gonadotropin and androgen

secretion increases which cause testicular growth and the incidence of other secondary sex characteristics. GnRH analogues with continuous stimulation of GnRH secretion, inhibits the decrement in secretion of LH and FSH hormones [45]. A GnRH analogue increases the secretion of LH and FSH in first injection, but thereafter, despite the presence of GnRH, gonadotropin secretion decreases [46]. Gonadotropin receptors are located on the testis and seminiferous tubules, decreased spermatogenesis which is caused by the chemotherapy agent disrupts the pituitary gonadal axis. In the rat chemotherapy the level of FSH is controlled based on spermatogenesis. Because the inhibition of meiosis by the chemotherapy agent, the spermatogenesis phase is quite impossible to observe [47, 48]. In the present study the levels of FSH and more distinctly LH are reduced, consequently the spermatogenesis negatively affected by chemotherapy. According to Brinkworth [49] cyclophosphamide-induced genetic damage to cells causes sexual harm.

According to Masta *et al.* [50] cross connections in the active parts of chromosomes are damage in chemotherapy. Evidences show that, the damages to the DNA of the male sex cells caused by chemicals and drugs lead to gene mutations and consequently congenital malformation which are transferable [49]. Men treated with anticancer drugs are more likely to experience permanent infertility and defects in the gonads [51]. Since the male sex cell division rate is very high, thus it is sensitive to anti-cancer or antimetabolic agents. The results of this study confirmed that tissue damages in testicular tissue are higher in cyclophosphamide and 5-FU treated group than in the group treated with 5-FU plus CPA. The reason for reduction in the effect of combined chemotherapy is due to antagonist action leads to increase in non-enzymatic and enzymatic antioxidant and reduction in oxidative damage.

Testicular toxicity by chemotherapeutic agents is considered as inevitable side effect of cancer treatment in male patients. A strategy to diminish the side effects of anticancer drugs with preservation of chemotherapeutic efficacy is necessary. The present study reconfirmed the testicular toxicity of cyclophosphamide.

Cyclophosphamide has alkylating properties that result in nucleotide base mispairs and DNA/DNA or DNA/protein cross-linking that lead to major disruptions in nucleic acid function and the inhibition of DNA synthesis [52]. Cyclophosphamide undergoes biotransformation by hepatic microsomal cytochrome P450 isoenzymes to aldophosphamide, which is spontaneously broken down to produce phosphoramid mustard and acrolein. Phosphoramid mustard is responsible for anti-cancer effects, while acrolein is associated with the toxic effects observed during CPA therapy [53]. As CPA is an alkylating agent that causes more damage into rapidly dividing cells, the drug is expected to affect the seminiferous epithelium and reduce the number of spermatozoa produced. The number of spermatozoa in the cauda epididymis provides a good estimate of spermatozoa reserves.

Cyclophosphamide treatment has also been shown to cause oxidative stress [54]. Mammalian spermatozoa are rich in polyunsaturated fatty acids and thus very susceptible to ROS attack [55]. Excessive ROS increases germ cell apoptosis and detrimental effect on spermatozoa resulting in decrease in sperm viability, sperm motility and increase in morphology defects with deleterious effects on sperm capacitation and acrosome defects. Lipid peroxidation (LPO) destroys the structure of lipid matrix in the membranes of spermatozoa, and it is associated with loss of motility and defects of membrane integrity [56].

Therefore stress could play an important role in the induction of sperm abnormalities, namely higher susceptibility of sperm DNA to denature and fragment [57]. Cyclophosphamide and 5-FU treated rats in the present study had a significant increase in testes LPO and a significant drop in antioxidant levels suggesting the presence of free-radical toxic stress within the testes. Lipid peroxidation serves as an index of intensity of oxidative stress

## 6. Conclusion

It could be concluded that treatment of mammals with chemotherapy is associated with the production of free radicals that lead to hazardous alterations in sexual hormones, DNA, and RNA concentration, and

histopathological changes in testes and sperm quality of male albino rats. However, 5-FU and CPA combination could produce a serious alteration in these parameters. Future work should consider combined chemotherapy regimens. Toxicological studies must be performed before using drugs as combination before application. Further research is required on toxicological impacts of drugs and pollutants mixtures.

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