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Article

5-fluorouracil Improves the Testicular Antioxidant Status and Alleviates Oxidative Stress in Male Albino Rats Treated with Cyclophosphamide

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Abstract: The therapeutic effect of Cyclophosphamide (CPA) is thus attributed to phosphoramide mustard and acrolein leads to the formation of high levels of reactive oxygen species (ROS), which results in decreased antioxidant activity**.** Excessive production of ROS could also culminate in oxidative stress**. Objectives:** This study aims to evaluate the effect of sub-lethal dose of the cyclophosphamide, 5-FU, combination of 5-FU, and CPA on testicular antioxidant status, and oxidative stress in male albino rats. **Materials and Methods:** Twenty-eight male adult rats were grouped randomly into four groups (n=5 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 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 measurements. Biochemical parameters in rat serum and tissues were evaluated. **Results:** Individual injection of CPA and 5-FU to rats were reduced testes TAC, GSH concentration, GR, and CAT activities compared to control. However, the combination treatment of rats with 5-FU and CPA increased the levels of these non-enzymatic and enzymatic antioxidant compared with those treated with CPA alone. Also, results showed significantly increased TBARS and NO concentration in the testes of CPA treated rats when compared to normal ones, while 5-FU increased NO only compared with the control. **Conclusion:** It can be concluded that treatment of rats with CPA is associated with the production of free radicals that leads to hazardous alterations in certain non-enzymatic, and enzymatic functions. The increase in lipid peroxidation probably leads to the intracellular accumulation of ROS with the subsequent development of testes tissue injury. However, 5-FU and CPA combination could produce a significant amelioration in most cases for these changes, and it may be considered as a potentially useful candidate in the combination chemotherapy with CPA to combat oxidative stress mediated non target organs injury even if it was not a complete protection. Future work should consider combined chemotherapy regimens, as two or more mechanisms of action of chemotherapeutic drugs could be more powerful than one mechanism.

Keywords: Cyclophosphamide, 5-Fluorouracil, Combined chemotherapy, Testicular antioxidant, Oxidative stress, Male albino rats

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1. Introduction

The therapeutic effect of CPA is thus attributed to phosphoramide mustard and acrolein is associated with unwanted side effects [1]. Bioconversion of CPA into these metabolites leads to the formation of high level of reactive oxygen species (ROS), which results in decreased antioxidant activity [2]. Excessive production of ROS could also culminate in oxidative stress [3]. The cytotoxic metabolites formed in the liver are distributed to different tissues by systemic circulation. Oxidative stress has been reported to play a role in CPA-induced tissue damage. Free radicals an atom or groups of atoms with one or free radicals. An atom or group of atoms with one or more unshared electrons, which may enter into chemical-bond formation, is called a free radical [4]. They are produced in response to exposure to chemical contaminants that are present in the air, water, and food. Free radicals are usually highly reactive and unstable, free radical molecules a very high level of oxygen which empowers them to destroy unwanted toxins through the process of oxidation. Free radicals have unpaired electrons in the outer shell of the molecules, exposure to certain chemicals helping in the formation of large free radical molecules which are causing serious damage to the cell, Xanthine oxidase, aldehyde oxidase, transition metal ions, drugs, tobacco smoking, gases, chlorinated organics as pesticides, Herbicides, fungicides, insecticides, solvents, and other sources are sources to produce free radicals where these radicals cause oxidative stress, lipid peroxidation, protein damage, DNA damage, affect in Sex ratio and other effects [4].

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 antioxidant status, and oxidative stress in male albino rats.

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, cyclophosphamide, and 5-fluorouracil and all other chemicals were purchased from Sigma Chemical Company (Saint Louis, USA).

3.2. Animals

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

3.3. Experiential protocol

Twenty-eight male adult rats were grouped randomly into four groups (n=5 in 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 II cyclophosphamide (CPA): Cyclophosphamide at a dose of 10 mg/kg day by day through i.p. to rats for 14 days [5]. Group III Fluorouracil (5-FU): 5-Fluorouracil at a dose of 10 mg/kg day by day [6] 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 the rat heart. Tubes were used to compile blood drawn from the heart directly; serum formation, blood was allowed to set for 30 min at 4oC 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 -20oC 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 to the addition of 5-10 ml cold 50 mM potassium phosphate buffer, pH 7.5 containing 1 mM EDTA per gram tissue. Then, the 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 oC for further use.

3.3.1. Determination of glutathione concentration

One portion of the testis was homogenized in 5-10 mL of cold buffer (50 mM Tris-HCl, pH 7.5; 5 mM EDTA; 1 mM DTT) per gram tissue. Homogenized tissue was centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was removed and stored on ice. If not assayed on the same day, the supernatant was freezed at -80°C. Reduced glutathione was estimated by the method Moron *et al*. [7].

3.3.1.1. Determination of glutathione reductase activity

Glutathione reductase was determined spectrophotometrically according to the method of Goldberg and Spooner [8].

3.3.2. Determination of catalase activity

Catalase was determined according to Góth [9], using commercial kits obtained from Bio-diagonestic, Egypt.

3.3.3. Total antioxidant capacity

The total antioxidant capacity of testicular tissue was measured by using the ferric reducing antioxidant power (FRAP) assay method [10].

3.3.4. Measurement of NO concentration

Blood samples for the determination of NO concentration were diluted (1:1) (vol/vol) with (0.9%) saline, protein-precipitated 30% $ZnSO_4$, 0.05 ml per ml of blood and centrifuged at 700 g for 10 minutes and frozen at -20°C until the determination of NO level. The NO level in the blood and tissue was determined by measuring nitrite concentrations, a stable metabolic product of NO with oxygen. Conversion of NO_3^{-2} into NO_2^{-2} was done with elementary zinc. NO_2^{-2} concentration in serum and tissue was determined by classic colorimetric Griess reaction [11].

3.3.5. Malondialdehyde concentrations in testicular tissues

The extent of lipid peroxidation was assayed by the measurement of thiobarbituric acid reactive substances (TBARS) according to Yoshioka *et al.,* [12].

3.4. Statistical Analysis

The values are expressed as mean ± SEM. All values are expressed as mean±standard error of the mean (SEM). The Kolmogorov-Smirnov test was used to assess the normality of the distribution of continuous variables. Comparisons between the treatment groups and pathogenic control group were performed by analysis of variance (ANOVA) followed by the Tukey- test. $P<0.05$ was considered as significant [13].

4. Results and Discussion

CPA is an alkylating agent, with its metabolites causing alkyl crosslink within and between DNA strands of dividing cells, causing them to apoptosis [14]. 5-FU has been widely used in the chemotherapy of a variety of human carcinomas including head and neck, gastrointestinal tract and breast cancer, using various schedules [15].

4.1. Antioxidant

Numerous studies have demonstrated that ROS such as superoxide, hydroxyl radical anion, and hydrogen peroxide are important mediators of DNA damage and tissue injury [16]. Glutathione reductase and catalase are important antioxidant enzymes of cell defense against free radical damage. Glutathione reductase also, is important enzyme regenerates GSH by converting GSSG. CAT is a hemoprotein, which catalyzes the reduction of hydrogen peroxides and is known to be involved in the detoxification of H_2O_2 concentrations [17].

Individual injection of CPA and 5-FU to rats reduced testes TAC, GSH concentration, GR and CAT activities compared to control. However, the combination treatment of rats with 5-FU and CPA increased the levels of these non-enzymatic and enzymatic antioxidant compared with those treated with CPA alone (Tables 1, Figures 1-4).

The above data indicate that antioxidant defense mechanisms must take part in the toxicity of cyclophosphamide [18]. They have shown that patients receiving high-dose cyclophosphamide displayed significant reductions of antioxidant parameters in plasma [19]. In the present study, many changes in rat blood serum antioxidative systems also have been observed after cyclophosphamide administration. Cyclophosphamide injection caused a decrease in the activity of antioxidant enzymes: CAT and GR. It is possible that this decrease is a result of protein structure modification through the reactive metabolite acroleine and/or reactive oxygen species generated during cyclophosphamide metabolism as well.

In the present study, the levels of TAC, GSH, GR, and CAT decreased in CPA-treated rats as reported earlier [17, 20], which could be due to the inactivation of cellular antioxidants by the lipid peroxides and ROS that are produced due to CPA intoxication. However, combination of 5-FU and CPA restored the enzyme levels and decreased the formation of lipid peroxidation byproduct MDA. The partial elevation in antioxidant levels inside the testes due to the antagonistic effects of the two chemotherapy.

Table 1. Testis total antioxidant, glutathione, glutathione reductase, and catalase of rat treated with cyclophosphamide and/or 5-fluorouracil

Groups / Parameters	Control	CPA	5-FU	CPA – 5-FU
	Mean±SE	Mean±SE	Mean±SE	Mean±SE
Testis total antioxidant (U/g tissue)	1.32±0.09 ^{bcd}	0.83±0.12^{acd}	1.04±0.10^{ab}	1.12±0.07^{ab}
Testis glutathione (U/g tissue)	18.05±0.97^{bcd}	9.62±0.75 ^{acd}	13.93±0.82 ^{abd}	11.74±0.84 ^{abc}
Testes glutathione reductase (U/g tissue)	42.10±1.57^{bcd}	24.91±1.86 ^{acd}	32.79±1.45 ^{ab}	33.08±1.27 ^{ab}
Testes catalase (U/tissue)	40.15±1.72 ^{bcd}	23.52±2.69 ^{acd}	32.45±1.17 ^{abd}	35.26±2.00 ^{abc}

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

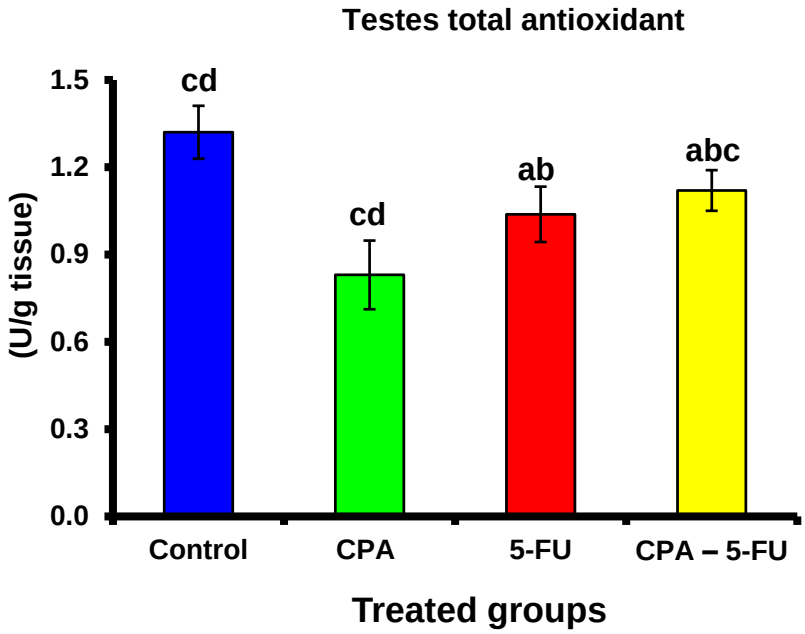

Figure 1. Testis total antioxidant (U/g tissue) of rat treated with cyclophosphamide, fluorouracil and their combination. Significance at $P < 0.05$. CPA: cyclophosphamide; 5-FU: Fluorouracil. [a] Comparison of control and other groups; [b] Comparison of CPA and other groups; [c] Comparison of 5-FU and other groups; [d] Comparison of CPA – 5-FU and other groups

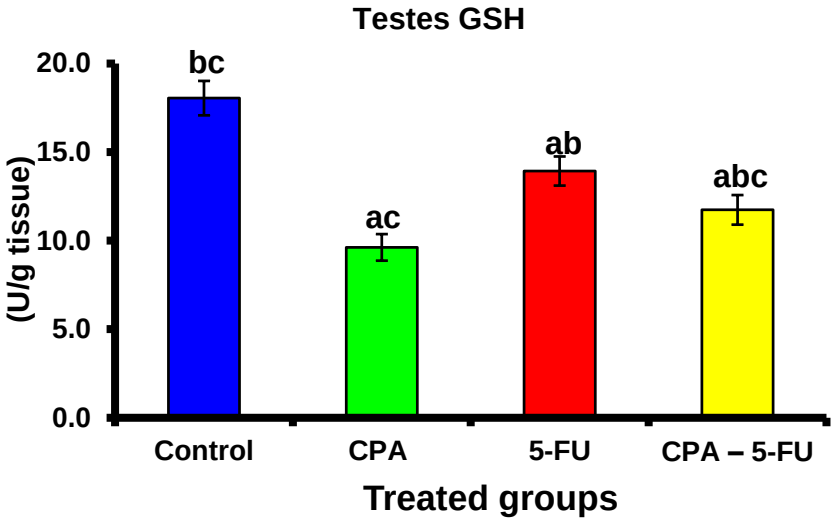

Figure 2. Testes glutathione (U/g tissue) of rat treated with cyclophosphamide, fluorouracil and their combination. Significance at $P < 0.05$. CPA: cyclophosphamide; 5-FU: Fluorouracil. [a] Comparison of control and other groups; [b] Comparison of CPA and other groups; [c] Comparison of 5-FU and other groups; [d] Comparison of CPA – 5-FU and other groups.

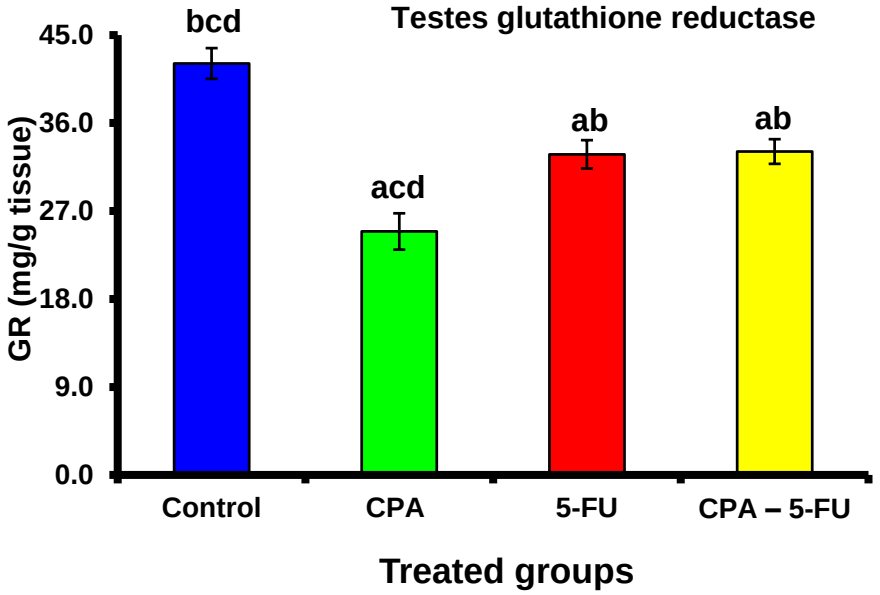

Figure 3. Testes glutathione reductase (U/g tissue) of rat treated with cyclophosphamide, fluorouracil and their combination. Significance at $P < 0.05$. CPA: cyclophosphamide; 5-FU: Fluorouracil. [a] Comparison of control and other groups; [b] Comparison of CPA and other groups; [c] Comparison of 5-FU and other groups; [d] Comparison of CPA – 5-FU and other groups

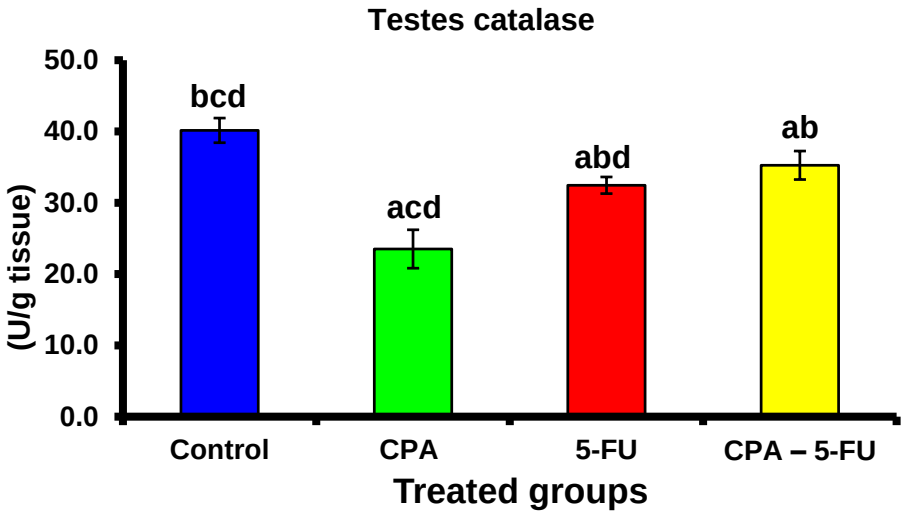

Figure 4. Testes catalase (U/tissue) of rat treated with cyclophosphamide, fluorouracil and their combination. Significance at P > 0.05. CPA: cyclophosphamide; 5-FU: Fluorouracil. [a] Comparison of control and other groups; [b] Comparison of CPA and other groups; [c] Comparison of 5-FU and other groups; [d] Comparison of CPA – 5-FU and other groups.

4.2. Biochemical findings of lipid peroxidation and nitric oxide

Cyclophosphamide, one of the most widely drugs in chemotherapy, is a cytotoxic alkylating drug with a high therapeutic index and is effective against a variety of cancers. Although CPA is effective for the treatment of cancer, it induces a wide range of adverse side effects and toxicity, such as nausea, vomiting, and hematopoietic toxicity that restrict the use of this drug in clinic [21]. The pathogenetic pathways may include oxidative damage, release of some inflammatory endocoids such as cytokines and nitric oxide as well as poly (adenosine diphosphate-ribose) polymerase activation [22]. Therefore, it is necessary to search for agents that can reduce the harmful side effects of CPA.

Our results showed significantly increased TBARS and NO concentration in the testes of CPA treated rats when compared to normal ones, while 5-FU increased NO only compared with control (Tables 2; Figures 5, 6). Bhatia *et al.* [23] and Kouidhi *et al.* [24] revealed that CPA-induced hepatotoxicity involves induction of oxidative stress due to excessive formation of ROS which causes lipid peroxidation of the cellular membrane. When rats treated with the combination of CPA and 5-FU a reduction occurred in lipid peroxidation and NO concentration in testes compared with CPA treated rats which could be attributed to the antagonistic interaction of the two chemotherapy (Tables 2; Figures 5, 6). The inhibition in lipid peroxidation may indicate the reduced level of oxidative stress.

Changes in activity of antioxidant enzymes are accompanied by intensification of lipid peroxidation processes, which is confirmed by elevated MDA serum levels that we observed in rats receiving CPA and 5-FU. In quantitative terms, MDA is the most important component among reactive aldehydes originating from lipid peroxidation. For this reason, it is commonly considered as an index of oxidative stress severity [25].

Table 2. Testes TBARS, glutathione and Testes nitric oxide of rat treated with cyclophosphamide and/or 5-fluorouracil

Groups / Parameters	Control	CPA	5-FU	CPA – 5-FU
	Mean±SE	Mean±SE	Mean±SE	Mean±SE
Testes TBARS (U/g tissue)	2.87±0.49 ^{bcd}	10.78±0.64^{acd}	8.08±0.84^{abd}	5.09±0.74^{abc}
Testes nitric oxide (U/g tissue)	443.6±57.14 ^{bd}	727.2±35.63 ^{acd}	460.8±132.26 ^{bd}	558.4±61.51 ^{abc}

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

Increased oxidative stress represents an imbalance between intracellular product of free radicals and the cellular defense mechanisms; notably, MDA is one of the most important markers of oxidative stress [26]. Many researches demonstrated that CPA is a chemotherapeutic agent cause oxidative stress in a dose- and time-dependent manner [27, 28], and increases levels of MDA, depletes GSH [29]. These reports suggested that the generation of oxidative products is mainly related to the DNA damage caused by CPA. In addition, Zhang *et al*. [30] reported that CPA at a dose of 100 and 200 mg kg^{-1}, i.p. significantly caused DNA damages in both mouse bone marrow cells and peripheral lymphocyte cells, and markedly inhibited the activities of glutathione peroxidase and SOD, and increased MDA contents in mouse blood. In our study, the enhanced production of tissue lipid peroxides observed is an agreement with other studies.

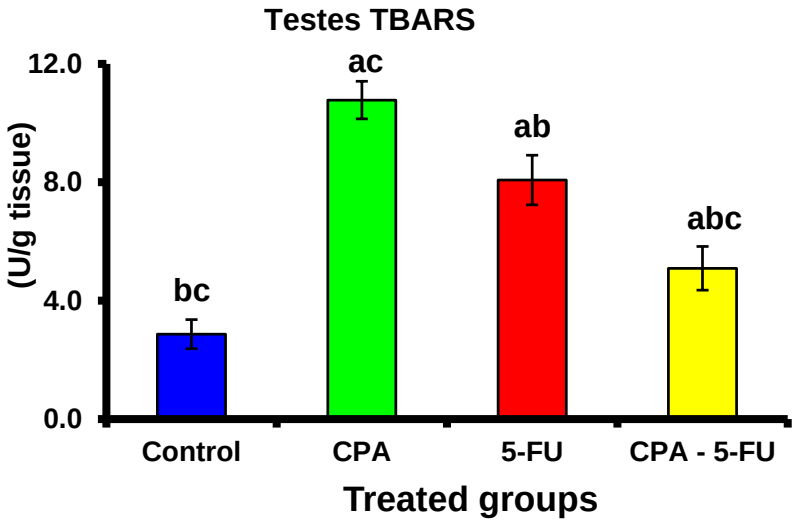

Figure 5. Testes TBARS (U/g tissue) of rat treated with cyclophosphamide, fluorouracil and their combination. Significance at $P < 0.05$. CPA: cyclophosphamide; 5-FU: Fluorouracil. [a] Comparison of control and other groups; [b] Comparison of CPA and other groups; [c] Comparison of 5-FU and other groups; [d] Comparison of CPA – 5-FU and other groups.

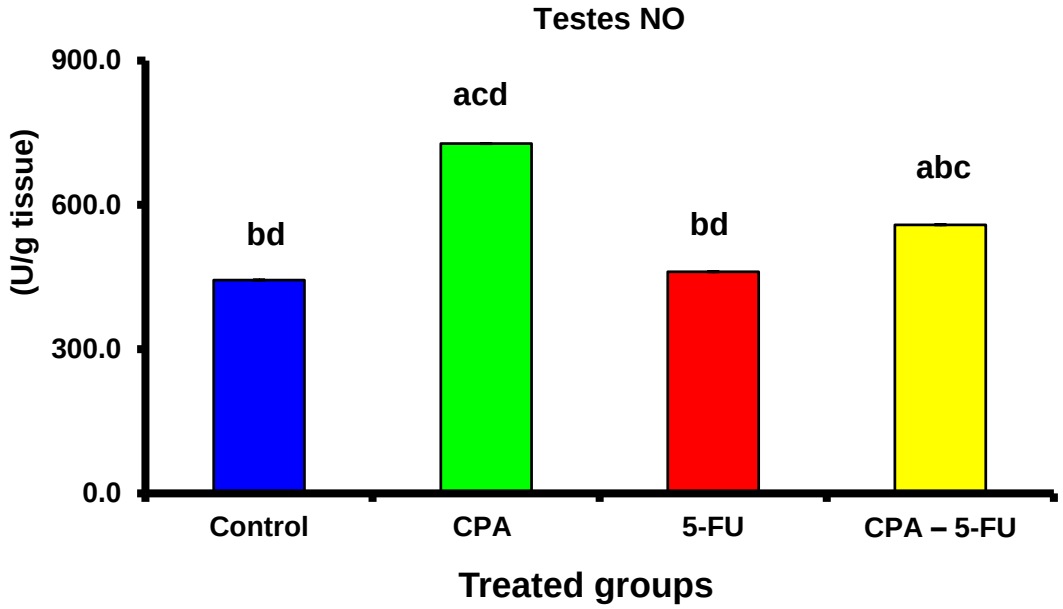

Figure 6. Testes nitric oxide (U/g tissue) of rat treated with cyclophosphamide, fluorouracil and their combination. Significance at $P < 0.05$. CPA: cyclophosphamide; 5-FU: Fluorouracil. [a] Comparison of control and other groups; [b] Comparison of CPA and other groups; [c] Comparison of 5-FU and other groups; [d] Comparison of CPA – 5-FU and other groups.

5. Conclusion

It can be concluded that treatment of rats with CPA is associated with the production of free radicals that lead to hazardous alterations in certain non-enzymatic, and enzymatic functions. The increase in lipid peroxidation probably lead to the intracellular

accumulation of ROS with subsequent development of testes tissue injury. However, 5-FU and CPA combination could produce a significant amelioration in most cases for these changes, and it may be considered as a potentially useful candidate in the combination chemotherapy with CPA to combat oxidative stress mediated non target organs injury even if it was not a complete protection. Future work should consider combined chemotherapy regimens, as two or more mechanisms of action of chemotherapeutic drugs could be more powerful than one mechanism.

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