Bull. Egypt. Soc. Physiol. Sci. Vol. (41), Issue (4), 553 -564

Bull. of Egyp. Soc. Physiol. Sci.

(Official Journal of Egyptian Society for Physiological Sciences)

(pISSN: 1110-0842; eISSN: 2356-9514)

Study the Effect of Silymarin on Cyclophosphamide Induced Testicular Damage in Adult Albino Rats

Soad Shaaban Abd El Azeem Ramadan, Nermin Mohammed Madi, Mahmoud Abd El Hamid Elghareeb, Elham Nasif

Medical Physiology department, Faculty of Medicine, Tanta University, Tanta, Egypt

Submit Date: Jan 1, 2021
Revise Date: March 8, 2021
Accept Date: March 19, 2021

Keywords

- Silymarin
- Cyclophosphamide
- testicular damage

Abstract

Background: cyclophosphamide one of toxicants that induce testicular damage. Silymarin has antioxidant and antiapoptotic properties. Aim: assess the effect of silymarin on cyclophosphamide induced testicular damage in male albino rats. Materials and Methods: This study was conducted on 40 adult albino male rats that were divided into 4 groups. control group: were injected intraperitoneally with carboxy-methylcellulose daily for six weeks. Cyclophosphamide group: were injected intra-peritoneally with cyclophosphamide only once, then continuous injection of carboxy methylcellulose for the rest of six weeks. Silymarin group were injected intra-peritoneally with silymarin once per day for six weeks. Cyclophosphamide and Silymarin were injected intraperitoneally with cyclophosphamide only once and then injected with Silymarin for six weeks. Serum testosterone, FSH, LH level, oxidative stress biomarkers, antioxidant enzymes, apoptotic marker, testicular histopathology, sperm count and testicular weights were assessed. Results: Cyclophosphamide significantly decreased levels of measured hormones, antioxidant enzyme, testicular weight and sperm count but significantly increased the oxidative stress biomarkers. Also, it induced degeneration and necrosis of seminiferous tubules. silymarin illustrated a significant increase in levels of measured hormones, antioxidant enzyme, testicular weight and sperm count but a significant decrease in the oxidative stress biomarkers. Normal testicular architecture was noticed with silymarin treatment. Conclusion: Silymarin has antioxidant and antiapoptotic activities that enable it to improve testicular function after its damage by cyclophosphamide.

Corresponding author: Elham Nasif, M.D , Physiology Department, Faculty of Medicine, Tanta University, Tanta, Egypt. Official e elham.nasif@med.tanta.edu.eg .Personal email: elhamnasif2015@gmail.com. Orcid ID: http://orcid.org/0000-0001-6224-6573.Adress: El- Geesh S Faculty of Medicine, Tanta University, Tanta, Egypt Postal Code: 31511 Tel.: +201004329445.Egypt

INTRODUCTION

There are many environmental toxicants that have the capacity to impair human fertility [1]. Cyclophosphamide (CYP) is an alkylating compound [2], and a pro-drug which changed to its active metabolites (Phosphoramide Mustard (PM), and acrolein) [3] in liver. The ordinary use of CYP is treatment of cancer diseases such as leukemia, lymphoma, myeloma, mycosis, adenocarcinoma, retinoblastoma and breast carcinoma [4], also used as an immunosuppressive agent for management of autoimmune diseases as rheumatoid arthritis, nephritic syndrome, and wegeners granulomatosis [5]. However, the clinical utilization of this drug is restricted as a result of its side effects including hepatotoxicity, nephrotoxicity, cardiotoxicity, neurotoxicity, immunotoxicity, alopecia, and bone marrow suppression [6].

Reproductive toxicity, involving azoospermia, oligospermia, histological changes in epididymis and testis, reduction in weight of reproductive organs and impaired fertility and growth, has been demonstrated following CYP administration in humans and experimental animals. The high frequency of cellular division happens in the cells of seminiferous epithelium makes testis highly sensitive to chemotherapeutic drugs [7].

On the other hand flavonoids are natural compounds commonly present in plants, and seeds [8]. Recent study has shown flavonoids relieve unwanted adverse effects of chemotherapy [9].

Silymarin (SMN) is a natural flavonoid which is considered the active component of milk thistle extract [10]**.** Among its properties can point to anti-inflammatory [11], antioxidant [12], anti-cancer and hepatoprotective properties [13].

Silymarin exerts antioxidant effect by scavenging free radical also by increasing levels of glutathione peroxidase [14].

Many authors reported the harmful effect of CYP on the endocrinal function of the testis and its structure [6] [7]. Moreover, it is well known that Silymarin has antioxidant , anti-inflammatory and antiapoptotic effect[11] [12] [13] .

We designed the current study to assess the effect of silymarin on cyclophosphamide induced testicular damage in male albino rats.

2-Materials and Methods:

2.1. Chemicals and Reagents:

Silymarin was obtained from New Test Company as dry-frozen powder and dissolved in carboxy-methylcellulose solution to obtain the needed dose (200 mg / kg)

Cyclophosphamide was obtained from Sigma Company as vial and dissolved by addition of normal saline to obtain the needed dose (100 mg / kg).

Carboxy-methylcellulose solution was obtained from Sigma Company and prepared according to the weight of rats to form 10 ml/ kg of 0.5% CMC solution.

2.2. Experimental Animals:

The current work was performed at Tanta Faculty of Medicine, for six weeks starting on February 2019, and the experiment was approved by the Ethical Committee of Medical Research, Tanta Faculty of Medicine, Egypt (approval number: (32871/1/19). Forty adult male albino Wistar rats aged 4 months weighting 200 to 250 grams were purchased from the Experimental

Animal House of Faculty of Science, Tanta University. The rats were housed in animal cages, at room temperature, with free access to water and food.

2.3. Animals grouping and experimental design

The animals were acclimatized for two weeks, and then randomly classified into four equal groups (10 rat /group)as following;

1- control group ; animals were injected intraperitoneally (IP) with 0.5 % carboxy-methylcellulose (CMC) in a dose of (3ml) daily for six weeks.

2-CYP group (Cyclophosphamide group); were injected (IP) with cyclophosphamide, in a dose of (100mg/kg) only once [15], then continuous injection of carboxy-methylcellulose (3ml) for the rest of the six weeks as once daily dose

3-SMN group (silymarin group); were injected (IP) with silymarin, in a dose of (200mg/kg) once per day for six weeks [16].

4- CYP + SMN group IV(Cyclophosphamide + Silymarin group); injected (IP) with cyclophosphamide in a dose of (100 mg/kg) only once and then injected with Silymarin in a dose of (200 mg/kg) once daily for six weeks.

2.4. Blood and tissue sampling:

At the end of the experimental period, all rats were anaesthetized by pentobarbital (50 mg/kg) [17] and blood samples were obtained by cervical decapitation and allowed to clot by leaving it undisturbed at room temperature for 15-30 minutes. Then remove the clot by centrifuge at 1000-2000 r.p.m for 10 minutes. The resulting supernatant is designated as serum. Then testes were dissected for estimation of oxidative stress markers and for histopathological evaluation.

A. Assessment of reproductive hormones:

The serum was used to estimate testosterone hormone, follicle stimulating hormone (FSH) and luteinising hormone (LH) levels using ELISA assay kits according to the methods described by ***Tietz*** [18], ***Gay et al***. [19]**,** and ***Haavisto et al***. [20], respectively and following the manufacturers' instructions.

B. Estimation of testicular oxidative stress and apoptotic markers:

Testes were separated. Each testis was cut transversely into two halves, one half was homogenized in ice-cold sodium potassium phosphate buffer (pH 7.4), centrifuged at 3000 rpm at for 10min and stored at −80 °C for estimation of Testicular MDA, hydrogen peroxide level, GPx activity and Caspase3 (an apoptotic marker) activity using colorimetric assay kits (Biodiagnostic Chemical Company, Giza, Egypt), according to the methods described by ***Ohkawa et al.*** [21], ***Porter and Janicke*** [22], ***Aebi*** 3], and ***Splittgerber and Tappe*** [24] respectively following the manufacturers' instructions.

C. Histopathological examination:

The other half of dissected testes were fixed in 10% buffered formalin for 24 hours then dehydrated in ascending concentrations of ethyl alcohol, cleared in xylene and embedded in paraffin. Thin sections (5µm) were cut and stained with hematoxylin and eosin (H&E) for routine light microscopic examination to assess the structure of seminiferous tubules, integrity of basement membrane and interstitial tissue according to method described by ***Jalali et al*** [25].

D. Sperm count and testicular weight:

The cauda epididymis from both sides were taken out ,minced and incubated in a per-warmed

petri dish 10 ml Hams F10 containing 0.5% Bovine serum albumin and the solution were incubated at 37°C. The spermatozoa were allowed to disperse into buffer ,after 20 min , cauda epididymis removed and suspension was gently shaken to homogenize ,500µl of prepared suspension diluted with formaldehyde fixative ,10 µl from diluted solution transferred into hemocytometer using a Pasteur pipette let to stand for 7 min then the settled sperms were counted and evaluated per 250 small squares of hemocytometer and expressed as million per milliliter of suspension according to method described by ***Torabi et al*** [26]. Also, weight of testis was evaluated according to method described by ***Torabi et al*** [26].

The sacrificed animals were packed in a special package according to safety precautions and infection control measures and sent with hospital biohazard.

2.5. Statistical analysis:

The data obtained was saved using SPSS version 23.0 and then transferred to Excel and edited if necessary. The results were expressed as mean and standard deviation. ANOVA and Tukey tests were used to compare the groups. P value less than 0.05 indicates significance.

3.Results:

3.1. Serum levels of testosterone (ng/mL), follicle stimulating hormone [FSH (mIU/mL)], and luteinizing hormone [LH (mIU/mL)]:

Serum testosterone level were significantly lowered in cyclophosphamide group when compared with control group ($p < 0.05$). There was a significantly higher level of serum testosterone in silymarin treated group when compared with control group ($p < 0.05$). Cyclophosphamide and silymarin treated group showed significant increase in serum testosterone level when compared to cyclophosphamide group but, showed no significant difference when compared with control group ($p > 0.05$). This result is consistent with those of FSH, and LH hormone **(table 1).**

3.2. Testicular oxidative stress and apoptotic markers::

Testicular MDA levels were significantly elevated in cyclophosphamide group when compared with control group ($p < 0.05$). There was a significantly lower level of testicular MDA in silymarin treated group when compared with control group ($p < 0.05$).Cyclophosphamide + silymarin treated group showed significant decrease in MDA level when compared to cyclophosphamide group but, showed no significant difference when compared with control group ($p > 0.05$). These results is consistent with that of testicular caspase3 activity and testicular hydrogen peroxide levels. Regarding the antioxidants, Gpx activity level was significantly decreased in cyclophosphamide group when compared with control group ($p < 0.05$). There was a significantly higher activity of testicular Gpx in silymarin treated group when compared with control group ($p < 0.05$). Cyclophosphamide + silymarin treated group showed significant augmentation in Gpx activity when compared to cyclophosphamide group but, showed no significant changes when compared with control group ($p > 0.05$) **(table 2) and (figure 1, 2).**

Table 1: showing Serum testosterone, FSH and LH for all groups.

Parameters	normal control group	CYP-treated group	SMN-treated group	CYP + SMN treated group
Serum testosterone (ng/ml)	3.81±0.12	1.22 ± 0.15 [a]	4.08 ± 0.09 [a]	3.75 ± 0.14 [b]
Serum FSH (mlu/ml)	4.13 ± 0.18	3.41 ± 0.23 [a]	4.65 ± 0.19 [a]	3.92 ± 0.19 [b]
Serum LH (mlu/ml)	7.01 ± 0.17	5.93 ± 0.18 [a]	7.61 ± 0.25 [a]	6.83 ± 0.15 [b]

Values are expressed as mean ± SD (n=10). Significance of differences (P < 0.05) is illustrated as [a]versus normal control group; [b] versus CYP-treated group. FSH; follicular stimulating hormone, LH; luteinizing hormone.

Table 2: Showing testicular MDA and H2O2 for all groups.

Parameters	normal control group	CYP-treated group	SMN-treated group	CYP + SMN treated group
Testicular MDA (nmol/gm tissue)	1.26 ± 0.055	1.35 ± 0.042 [a]	0.86 ± 0.024[a]	1.28 ± 0.059 [b]
Testicular H2O2 (nmol/mg protein)	21.93 ± 1.08	37.86 ± 0.88[a]	19.05 ± 0.48[a]	22.16 ± 1.17[b]

Values are expressed as mean ± SD (n=10). Significance of differences (P < 0.05) is illustrated as [a]versus normal control group; [b] versus CYP-treated group. MDA: Malondialdehyde, H2O2; Hydrogen peroxide.

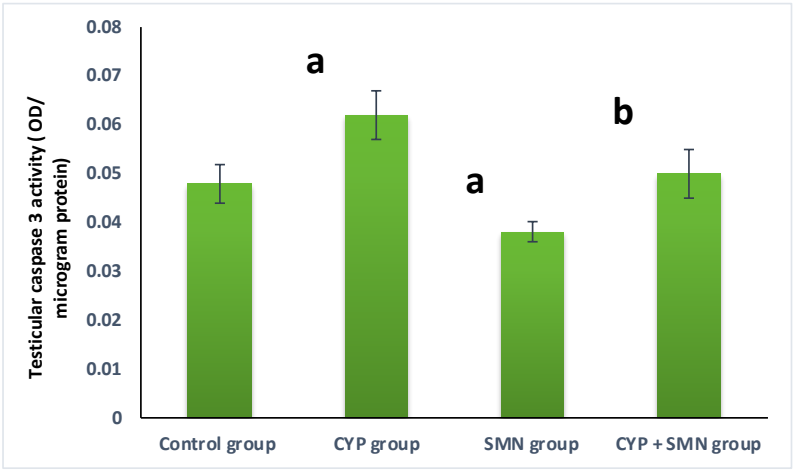

Figure (1): **Testicularcaspase-3 activity (OD/microgram protein):** Values are expressed as mean ± SD (n=10). Significance of differences (P < 0.05) is illustrated as [a]versus normal control group; [b] versus CYP-treated group.

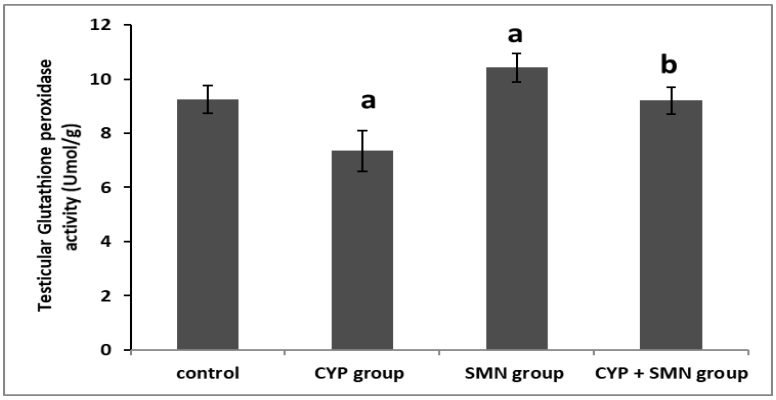

Figure (2): **Testicular glutathione peroxidase activity level (umol/g tissue):**Values are expressed as mean ± SD (n=10). Significance of differences (P < 0.05) is illustrated as [a]versus normal control group; [b] versus CYP-treated group.

3.3. Sperm count and Testicular weight:

As regard to sperm count and testicular weight, there was a significant decrease in cyclophosphamide group when compared to control group ($p < 0.05$). There was a significant higher weight and sperm count in silymarin treated group when compared with control group ($p < 0.05$).Cyclophosphamide +silymarin treated group showed a significant increase both testicular weight and sperm count when compared to cyclophosphamide group who showed no significant changes when compared with control group ($p > 0.05$) **(Figure 3 and 4)**.

3.4. Testicular histopathological observations:

The histopathology of control and SMN group showed normal testicular architecture with normal seminiferous tubules (ST) that appear hexagonal or circular with regular contour, and interstitial tissue (IT) contains a delicate loose CT and blood vessels (arrow heads).The seminiferous tubules surrounded by normal basement membrane (black arrows) show a clear lumen and a normal arrangement of cellular types **(Figure 5)**. CYP treatment caused histological alterations in the form of degeneration and necrosis of the seminiferous tubules (*), interstitial hemorrhage (black arrows), interstitial edema (white arrows) and irregular basement membrane (arrow heads) **(Figure 6)**. These alterations were markedly ameliorated by SMN treatment where histological findings of CYP + SMN treated group came more similar to those of the control group **(Figure 7)**.

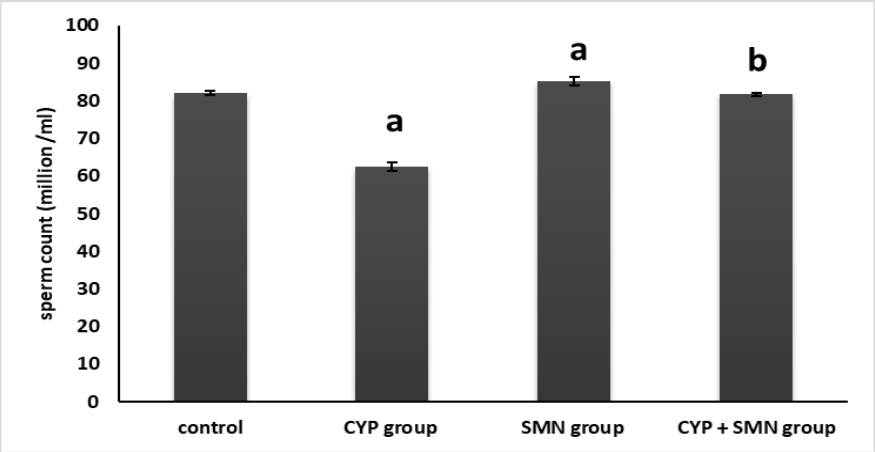

Figure (3): Sperm count (million/ml) in all studied groups. Values are expressed as mean ± SD (n=10). Significance of differences ($P < 0.05$) is illustrated as [a]versus normal control group; [b] versus CYP-treated group.

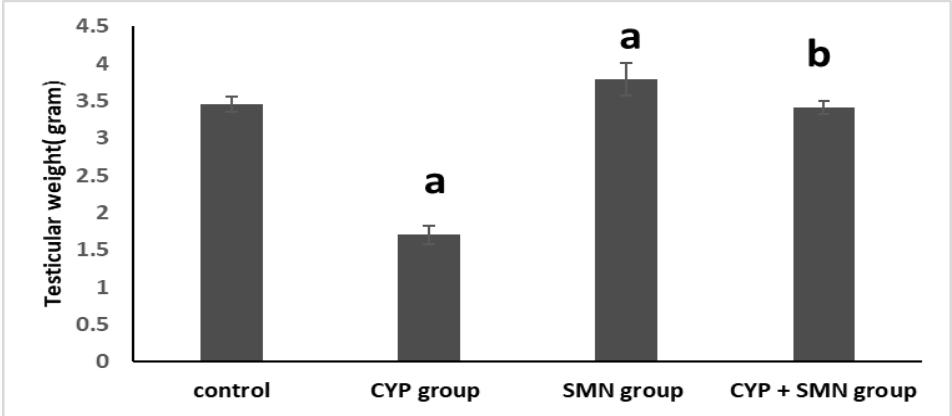

Figure (4): **Testicular weight in gram (g) in all studied group.** Values are expressed a mean ± SD (n=10). Significance of differences ($P < 0.05$) is illustrated as [a]versus normal control group; [b] versus CYP-treated group.

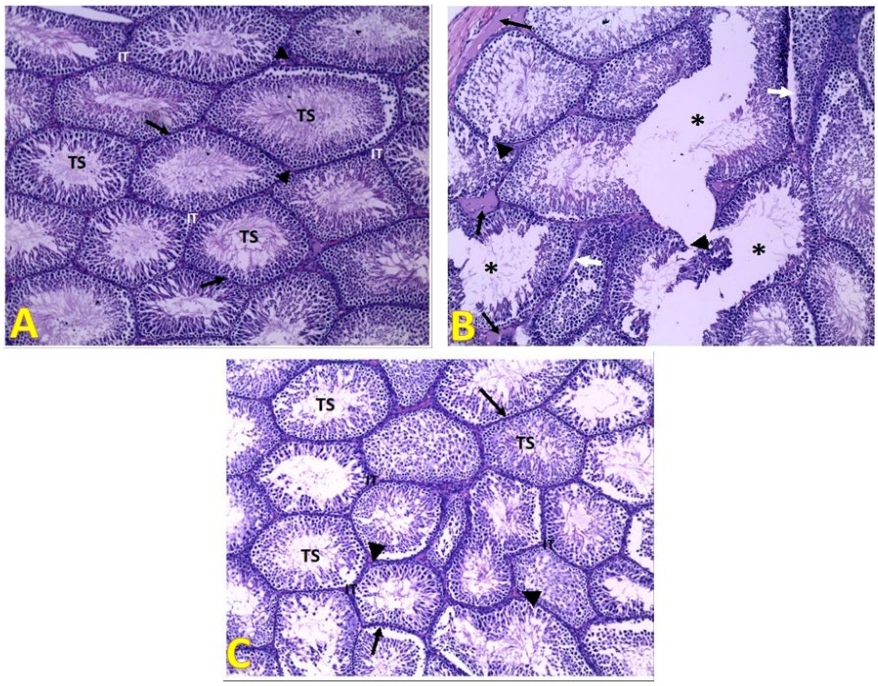

Figure (5): A-Normal testicular tissue (H&E staining 100) IT; interstitial tissue; ST; seminiferous tubules; black arrows; normal basement membrane; arrow heads; delicate loose CT and blood vessels. **B-**Effect of CYP on testicular tissue (H&E staining 100). Black arrows: interstitial hemorrhage ;(*); seminiferous tubules; white arrows; interstitial edema; arrow heads; irregular basement membrane. **C-** Normal testicular tissue (H&E staining 100) IT; interstitial tissue; ST; seminiferous tubules; black arrows; normal basement membrane; arrow heads; delicate loose CT and blood vessels.

4.Discussion:

The results of the current study revealed that, CYP-treated male rats showed low serum concentration of testosterone, together with low serum FSH and LH. CYP causes significant decrease in activity of testicular steroidogenic enzymes which are the key enzymes for biosynthesis of testosterone [27], Also CYP-induced Leydig cell degeneration and lysis with subsequent decrease in testosterone level [25].

The reduction in FSH and LH levels after CYP treatment may explain by the histoarchitecture changes that occurred in the pituitary by CYP [28]. Also CYP administration decreased the membrane fluidity of the pituitary gland affecting the membrane function producing change of receptor binding and secretory mechanisms of pituitary hormones [29].

In accordance with our findings ***Arena et al.,*** [30] and ***Elgazar*** [31], the two studies reported significant lowering of testosterone hormone, FSH, and LH hormone in serum of male rats after CYP treatment.

The result of the present study showed SMN treatment improved the endocrine functions of testis as proved by increase of serum levels of testosterone. Also it increased the level of FSH, and LH hormone in both normal and CYP treated group.

The mechanisms by which the SMN increases testosterone level were described by ***Abedi et al.,*** [32]and ***Oufi et al.,*** [33].SMN is a potent inhibitor to aromatase enzyme which catalyzes the conversion of testosterone to estrogen. By inhibiting this enzyme, the serum level of testosterone is elevated. Also, SMN increased the steroidogenesis process of Leydig

cells causing increase in secretion of testosterone hormone [34]. SMN can exert its action through hypothalamus-pituitary-testis axis. This axis is affected by positive and negative factors. One of these factors is Norepinephrine that increases synthesis of nitric oxide which will increase releasing of GnRH from hypothalamus, LH and FSH hormones from pituitary gland. SMN has increased the concentration of norepinephrine in certain areas of brain of laboratory mice [32].

CYP has decreased the testicular weight as compared to the control group as shown in our study. This can be explained by inhibiting effect of CYP on spermatogenesis and destructing effect on germ cells and Leydig cells.

Rezvanfar et al., [35] showed marked reduction in testicular weight by CYP through diminishing the number of germ cells, significant lower in grate of spermatogenesis and Leydig cells atrophy. Also in agreement with our findings ***Onaolapo et al.*** [36] showed weights of mice's testicles were significantly decreased during treatment in CYP group.

In the present study SMN has increased testicular weight compared to control group and CYP treated group. These findings attributed to the antioxidant effects of SMN by preventing atrophy, necrosis and degeneration of the testicular tissue. SMN has increased the number of germ cells and stimulated the spermatogenesis [32].

CYP-induced oxidative stress, as indicated by elevation of MDA, and H2O2 level and suppression of Gpx activities in testicular tissues of CYP-treated group.

Caspase 3 is an indicator of apoptosis occurs by oxidative stress. Testicular toxicity caused by CYP is due to disturbance of balance between oxidation reduction reactions in tissues resulting in oxidative stress [37]. MDA, an end product of polyunsaturated fatty acids, is commonly used as a biomarker for evaluation of lipid peroxidation which is a well-established mechanism of cellular injury and is used as an indicator of oxidative stress [38]. H2O2 is a reactive oxygen species and its level within the tissue can be used as a marker of oxidative stress [39]. Our results agreed with the findings of ***Novin et al.,*** [40] who noted that CYP-treated group showed a significant rise in the MDA, caspase3 activities, and H2O2 level in testicular tissue when compared with the control group.

SMN has antioxidant effect indicated by suppression of MDA and H2O2 levels and elevation of Gpx activities in the testicular tissues of SMN treated group. It attacks free radicals and reacts directly with cell membrane components to prevent oxidative damages to lipid components of cell membrane which is responsible for maintaining its normal fluidity. This property enables SMN to prevent peroxidation processes [41].SMN is a potent antioxidant regulating the intracellular glutathione peroxidase and stabilizing the cell membrane. Also SMN diminished expression of Caspase-3 in testicular tissue [42]. The antioxidant action of SMN is due to its polyphenolic structure [41].

In the current study, administration of CYP significantly decreased sperm count. This was due to the oxidative effect of CYP, because spermatozoa are highly susceptible to oxidative stress. The seminiferous epithelial vacuolization, spermatogenesis arrest, and germ cells reduction also are in line with decreased sperm count as effects caused by CYP [43].

In accordance with our findings ***Elgazar*** [31] noticed that adult male patients treated with CYP have demonstrated decreased sperm count and absence of spermatogenic cycles in testicular tissue. But SMN has significantly increased sperm count as compared to the control group and CYP treated group. One of the probable mechanisms of SMN effects on increasing sperm count may be its antioxidant properties. During sperm synthesis, lipid peroxidation and ROS production increase and accumulate lead to sperm damage. SMN as ROS scavenging polyphenols can counteract this destructive process [44]. Testosterone is an important factor for spermatogenesis. So, an increase in sperm count was detected as a result of enhancement of level of testosterone by SMN [32]. These results are in accordance with those observed by ***Abedi et al.*** [32], who reported significant increase in average number of spermatid and spermatozoid cells in SMN group compared to the control group. This study has some limitations as Gnrh levels not measured .

In Conclusion**,** taking into consideration findings obtained and related data available in literature we concluded that SMN has therapeutic effect on the testicular damage-induced by CYP via improving the endocrine testicular function and via its antiapoptotic and antioxidant effect.

5. Acknowledgments:

The authors would like to thank Dr. Darin Ali, associate professor of pathology department, Faculty of Medicine, Tanta University for her help in the histopathological part of this study.

6. Conflict of interest:

The authors declare that they have no conflict of interest.

7. Funding:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors

8. References:

1- Foster WG. Environmental toxicant sand human fertility. Journal of Minerva Gynecological 2003;55(5):451-7.

2- Aygun N. Non-BDNA Structures as Candidate Drug Targets against Structural Chromosomal Instability. Journal of Current Pharmacogenomics and Personalized Medicine 2018; 16(2):94-107

3- Netíková IR, PetruželkaL, ŠťastnýM, ŠtenglV. Safedecontamination of cytostatics from the nitrogen mustards family. Part one: Cyclophosphamide and ifosfamide. International Journal of Nanomedicine 2018; 13: 7971-7.

4- Vázquez YG, Cabanillas F, Concepción JR, Miranda OL. Incidence and risk factors for developing herpeszoster among a cohort of patients diagnosed with lymphoma at a community cancer center. Journal of Clinical Lymphoma Myeloma and Leukemia 2019; 19(3): 153-8.

5- Wiertz IA, van MoorselCH, Vorselaars AD,Quanjel MJ, Grutters JC. Cyclophosphamide in steroid refractory unclassifiable idiopathic interstitial pneumonia andinterstitial pneumoniawith autoimmunefeatures (IPAF). EuropeanRespiratory Journal 2018; 51(4):987-91.

6- Ma F, Kouzoukas DE,Siegler KL, Hunt DE, Leng L, Bucala R. MIF mediates bladder pain, not inflammation,in cyclophosphamidecystitis.Journal of Cytokine 2019; 1(1):543-6.

7- SalimnejadR, RadJS, Nejad DM. Protectiveeffect of ghrelin on oxidative stress and tissue damages of mice testes followed by chemotherapy with cyclophosphamide. Crescent Journalof Medical and Biological Science 2018; 5: 138– 43.

8- Uyar A, Yener Z, DoganA. Protectiveeffects of Urtica dioica seed extract in aflatoxicosis: histopathological and biochemical findings. Journal of British Poultry Science 2016; 57(2):235-45.

9- Caglayan C, Temel Y, Kandamir FM, Yildirim S, KucuklerS. Naringin protectsagainst cyclophosphamid-induced hepatotoxicity and nephrotoxicity through modulation ofoxidative stress, inflammation,apoptosis, autophagy, and DNA damage. Environmental Science Pollutional Research 2018;25(21): 20968–84.

10- Mohammadi M, Rostamzadeh A, Moayeri A, Allahveisi A, Mohammadi HR, Rezaie MJ. Role of Silymarin in regeneration and treatment of skin disorders; progress in signaling pathways. Research Journal of Pharmaceutical, Biological and Chemical Sciences 2016; 7(5) :1844–9.

11- GinwalaR,BhavsarR,ChigbuDG,JainP, Zafar KK. Potential Role of Flavonoids in Treating Chronic Inflammatory Diseaseswith a Special Focus on the Anti-Inflammatory Activity of Apigenin.Journal of Antioxidants 2019; 8(2): 35-63

12- Siva K, Ramnath V, Jeyaprakash K. Effect of Silymarin on Cadmium induced toxicity in rats. World Journal of Pharmaceutical **Research 2018; 6:543-9.**

13- OkaiyetoK, Nwodo UU, Mabinya LV, OkohAL. A review on some medicinal plants with hepatoprotectiveeffects. Journal of Pharmacognosy Reviews 2018; 12(24):186-99

14- Vivekanandan l, Sheik H, SingaravelS, Thangavel S. Ameliorative effect of silymarin against linezolid-induced hepatotoxicity in methicillin-resistant Staphylococcus aureus (MRSA)infected Wistar rats. Biomedicineand Pharmacotherapy 2018;108: 1303-12

15- Xie R, ChenL, WuoH, ChenT, WangF, ChenX, Sun H, Li X. GnRH antagonist improves pubertal cyclophosphamide-inducedlong-term testicular injury in adult rats. International Journal of Endocrinology 2018:88-92

16- Yaman T, Uyar A, KayaMS, KelesÖF, Uslu BA, Yener Z. Protective effects of silymarin on methotrexate-induced damages in rat testes. Brazilian Journal of Pharmaceutical Sciences 2018; 54(1):454-9

17- AktasA,Tuncer MC,Yildrm A, NergizY, Akkus M. Protective Effects of Melatonin on TesticularTorsion and Detorsion Damage in Sprague-Dawley Rats. International Journal of Morphology2011; 29(1):7-15.

18- Tietz NW. Clinical guide to laboratory tests. Journal of Philadelphia 1995; 7:578- 80.

19- GayVL, Midgley AR Jr, Niswender GD. Patterns of gonadotropin secretion associated with ovulation. Journal of Federation proceedings 1970; 29:1880-7.

20- HaavistoAM, PetterssonK, Bergendahl M, PerheentupaA, RoserJF, Huhtaniemi I. A supersensitive immunofluorometric assay for rat luteinizing hormone. Journal of Endocrinology 1993; 132(4):1687-91.

21- Ohkawa H, OhishiN, YagiK. Assay for lipid peroxides in animal tissues by thiobarbturic

acid reaction. Anal Biochem 1979; 95(2): 351–358.

22- Porter AG, Janicke RU. Emerging role of caspase-3 in apoptosis, Cell Death Differentiation 1999, 6 (2): 99-104.

23- Aebi H. Catalase in vitro. Journal of Methods in enzymology 1894; 105:121-126.

24- Splittgerber AG, Tappel AL. Inhibition of glutathione peroxidase by cadmium and other metal ions. Journal of Archives of biochemistry and biophysics 1979; 197(2):534-42.

25- Jalali AS, HasanzadehS, Malekinejad H. Crataegus monogyna aqueous extract ameliorates cyclophosphamide-induced toxicity in rat testis: Stereological Evidences. Journal of Acta Medical Iranica 2012;50(1):1-8.

26- Torabi F, Shafaroudi MM, Rezaei N. combined protective effect of zinc oxide nanoparticles and melatonin on cyclophosphamide-induced toxicity in testicular histology and sperm parameters in adult wistar rats. International Journal of Reproduction BioMedical 2017;(15)7: 403

27- Bakhtiary Z, Shahrooz R, Ahmadi A, Soltanalinejad F. Ethyl Pyruvate Ameliorates The Damage Induced by Cyclophosphamide on Adult Mice Testes. International Journal of Fertility and Sterility 2016; 10(1):79-86.

28- AyokaOA,Ojo OE,Imafidon EC,Ademoye KA, Oladele AA. Neuro-endocrine effects of aqueous extract of Amaranthus viridis (Linn.) leaf in male Wistar rat model of cyclophosphamide-induced reproductive toxicity. Journal of Toxicology reports 2016; 3:608-19

29- Lafuente A. The hypothalamic-pituitary-gonadal axis is target of cadmium toxicity, An update of recent studies and potential therapeutic approaches. Journal of Food Chemical Toxicology 2013;59:395-404.

30- Arena AC, Jorge BC, Silva MC, de Barros AL, Fernandes AA. Acrocomia aculeataoil: beneficial effects on cyclophosphamide-induced re-productive toxicity in male rats. Journal of Andrologia 2018; 50(6):1187-92

31- Elgazar AF. Protective Role of Walnut Seeds Extract and Vitamin E against Testicular Toxicity Induced by Cyclophosphamide in MaleRats.British Journal of Medicine and Medical Research 2016; 18(10): 1-0.

32- Abedi H,Jahromi SK, Amin SM, Jashni HK, JahromiZK, Pourahmadi M. The effect of silymarin on spermatogenesis process in rats. International Journal of Medical Research and Health Sciences 2016;5 (6): 146–50.

33- Oufi HG, Al-Shawi NN, Hussain S. What Are The Effects of Silibinin on Testicular Tissue of Mice?.Journal of Applied Pharmaceutical Science 2012; 2 (11): 009-013.

34- Glade MJ, Smith K. Oxidative Stress. Nutritional Antioxidants, and Testosterone Secretion in Men. Nutrition Disorder and Therapy 2015; 2(1): 1019-23

35- Rezvanfar MA, SadrkhanlouRA, AhmadiA, Shojaei-SadeeH, Rezvanfar MA. Protection of cyclophosphamide-induced toxicity in reproductive tract histology, Sperm characteristics, and DNA damage by an herbal source; evidence for role of free-radical toxic stress. Journal of Human and Experimental Toxicology 2008; 27(12): 901–10

36- OnaolapoAY, Oladipo BP, Onaolapo OJ. Cyclophosphamide-induced male subfertility in mice: An assessment of the potential benefits of

Maca supplement. Journal of Andrologia 2017; 50(3):233-7

37- Sadek EM, AbdelAzizDH, Embaby AS, Genedy GF. Histological Comparative Study between the Possible Effect of Stem Cells and α-Lipoic Acid on Rat Testes Treated by Cyclophosphamide. Egyptian Journal Of Histology 2019;42(3):686-98

38- Rahal A, Kumar A, Singh V, Yadav B, Tiwari R, Chakraborty S. Oxidative stress, prooxidants, and antioxidants: the interplay. Biomedical Research International 2014; 2014:365-9

39- Sidorkiewicz I, Zareba K,Wołczynski´ S, Jan Czerniecki J. Endocrine-disrupting chemicals Mechanisms of action on male reproductive system. Journal of Toxicology and Industrial Health 2017; 33(7):601–9.

40- NovinMG, GolmohammadiMG, SaghaM, Ziai SA, AbdollahifarMA, Nazarian H. Protective effect of gallic acid on testicular tissue, sperm parameters, and DNA fragmentation against toxicity induced by cyclophosphamide in adult NMRI Mice. Urology Journal 2020;17(1):78-85.

41- Faraji T,Momeni HR, Malmir M. Protective effects of silymarin on testis histopathology, oxidative stress indicators, antioxidant defence enzymes and serum testosterone in cadmium treated mice. Journal of Andrologia 2019; 51(5):61-6

42- HeidariKhoei H, Fakhri S, Parvardeh S, Shams Mofarahe Z, Ghasemnejad-Berenji H, NazarianH. Testicular toxicity andreproductive performance of streptozotocin-induced diabetic male rats, the ameliorating role of silymarin as an antioxidant. Journal of Toxin reviews 2019;38(3):223-33.

43- Yuan D, Wang H, HeH, Jia L, HeY,Wang T. Protective Effects of Total Flavonoidsfrom Epimedium on the MaleMouseReproductive System Against Cyclophosphamide Induced Oxidative Injury by UpRegulating the Expressions of SOD3 and GPX1. Journal of Phytotherapy Research 2014;28(1):88-97.

44- HamidAK, AhmedMA, Tayawi HM. Silymarineffect as anantioxidant to improve damages induced by CCl4on some characteristics of male rats reproductive system. Journal of Pure Science 2018; 23 (2): 60-5.