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Research Article
Antioxidant, Anti-Inflammatory and Antiapoptotic Effect of Mirtazapine Mitigates Cyclophosphamide-Induced Testicular Toxicity in Rats

[1,2]Gomaa Mostafa-Hedeab, [3]Muhannad Faleh Alanazi and [4,5]Hassan A. Abdelmawlla

[1]Department of Pharmacology, Medical College, Jouf University, Sakaka 72388, Kingdom of Saudi Arabia
[2]Department of Pharmacology, Medical College, Beni-Suef University, Beni-Suef 6252, Egypt
[3]Department of Internal Medicine, Division of Radiology, College of Medicine, Jouf University, Sakaka 72388, Kingdom of Saudi Arabia
[4]Department of Anatomy, College of Medicine, Jouf University, Sakaka 72388, Kingdom of Saudi Arabia
[5]Department of Anatomy and Embryology, College of Medicine, Beni-Suef University, Beni-Suef 6252, Egypt

Abstract

Background and Objective: Cyclophosphamide (CP) is an anticancer drug that may result in organ toxicities, especially testicular toxicity. Mirtazapine (MTZ) is an antidepressant drug with antioxidant activities. Here in this study, the effect of the MTZ, silymarin or its combination against CP-induced testicular toxicity in rats was examined. **Materials and Methods:** Thirty male rats were classified into five groups: Group (1) represents the normal control group, group (2) represents the CP-treated group, group (3) represents the mirtazapine-treated group, group (4) represents the Silymarin-treated group and group (5) represent the combined mirtazapine and silymarin-treated group. **Results:** Compared to the normal control group, CP induced a significant decrease in testosterone, catalase, GSH, SOD, IL-10, eNOS, HO-1, Ki67 (IHC), Bcl-2 and a significant increase in MDA, ALK, IL-6, IL-1β and TNF-α and iNOS. In addition, it altered the histological structure of the testis. Administration of MTZ, Silymarin, or its combinations significantly reverses CP-induced testicular toxicity and ameliorated histological changes of testis induced by CP administration. **Conclusion:** Results concluded that MTZ can protect against CP-induced testicular toxicity via its antiapoptotic, antioxidant and anti-inflammatory activities.

Key words: Cyclophosphamide, testicular, toxicity, mirtazapine, antioxidant, anti-inflammatory, antiapoptotic

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Corresponding Author: Gomaa Mostafa-Hedeab, Department of Pharmacology, Medical College and Health Science Research Unit, Jouf University, Sakaka 72388, Kingdom of Saudi Arabia Tel: +966534627393

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Various factors affect infertility in males some of them are reversible while others are irreversible disorders age, drugs, surgical history, exposure to environmental pollutants, genetic issues and systemic disorders are all possible factors for male infertility[1], with drug-induced testicular toxicity being an interesting clinical problem that is still being researched[2,3]. Testicular toxicity is caused by oxidative stress, which activates apoptotic pathways that cause testicular damage, which can lead to male infertility. This toxicity could be attributed to the high amount of polyunsaturated lipids in the testicular tissue's cellular membranes[4].

Cyclophosphamide (CP) is a well-known anticancer and immunosuppressive medication that is used to treat a variety of cancers and autoimmune illnesses[3]. Unfortunately, CP is hazardous to normal cells because it activates reactive oxygen species (ROS) and produces nitric oxide, which causes tissue damage[4]. Active metabolites of CP are produced during metabolism which includes phosphoramide mustard and acrolein. Acrolein is linked to harmful consequences such as oxidative stress induction and cell death[5,6]. Furthermore, CP use has been linked to DNA damage and low glutathione levels[7].

The CP causes testicular damage mostly by increasing oxidative stress and by alteration of the gene expression in various spermatogenic cells[8]. Gonadal toxicity caused by CP exposure can result in infertility in treated patients. The CP administration has been shown to have structural and functional effects on the testis, causing a decrease in various spermatogenic cells of seminiferous tubules as well as an increase in the incidence of oligospermia and azoospermia[9]. CP produced degenerative alterations in the testis of treated mice as evidenced by seminiferous tubule atrophy and vacuolization, congestion of the interstitial tissue and exfoliation of germ cells in the tubular lumen[10].

Mirtazapine (MTZ), an antidepressant medicine, may, on the other hand, be a potential treatment for preventing CP-induced testicular damage. The MTZ provides antioxidant and cytoprotective properties by activating enzymatic and non-enzymatic antioxidant processes and inhibiting some harmful oxidants, according to research. Mirtazapine could be employed as a cytoprotective drug based on these findings[11,12]. Mirtazapine's antioxidant capabilities were found to protect ovarian tissue against the damaging effects of CP. Pretreatment with MTZ enhanced nitric oxide (NO), malondialdehyde (MDA) and myeloperoxidase (MPO) levels as well as glutathione peroxidase (GPx) and superoxide dismutase (SOD) activity, which was reduced by CP alone. In addition, MTZ also improved the histological structure of the ovary[11]. Mirtazapine also reduced oxidative stress and testicular tissue injury caused by nitrofurazone. These data imply that mirtazapine could be used to treat tissue injury as a cytoprotective agent[13]. Compounds with antioxidant capabilities could either restore or prevent CP-induced testicular damage[8].

Silymarin (SMN), a polyphenolic molecule found in nature, has antioxidant characteristics because it can react with free radicals and reactive oxygen species (ROS) to transform them into less reactive and harmful chemicals. It can also activate antioxidant enzymes and non-enzymatic antioxidants like glutathione and superoxide dismutase as well as reduce inflammatory responses[14].

Silymarin administration reduced lipid peroxidation and enhanced enzymatic antioxidants, protecting the testicular structure against methotrexate-induced damage[15]. Furthermore, Silymarin, being a powerful antioxidant, reduces the harmful effects of sodium arsenite on the plasma membranes of sperms and the integrity of acrosomes, which may be linked to SMN's capacity to increase sperm antioxidant defense[16].

The most appropriate approach to reduce the toxicity of CP in testis is the combined use of both SMN and MTZ. Keeping in view the above-mentioned facts, the present experimental animal study was designed to explore the precise pathogenic mechanisms by which cyclophosphamide induces testicular toxicity and to investigate the potential ameliorating effects of mirtazapine and silymarin against the testicular damage induced by cyclophosphamide.

MATERIALS AND METHODS

Study area: The study was carried out in the Animal House, Faculty of Medicine, Beni-Suef University, Egypt during the period from January to June, 2022.

Animals: Thirty male Wistar rats weighing 130-250 g were kept in individual cages under a constant temperature of 21°C with alternating 12 hrs light/dark cycles. The study protocol was approved by the Institutional Animal Care and Use Committee, Beni-Suef University (BSU-IACUC)-Egypt under approval number (BSU-IACUC-022-244) which adhere to the provisions of the Declaration of Helsinki in 1995 (as revised in Edinburgh 2000). All animals were kept for 2 weeks for adaptation and then were classified into five groups, each of which consisted of six animals.

Drugs and chemicals: Mirtazapine, cyclophosphamide, Silymarin and ketamine and xylazine (Ketamine hydrochloride/xylazine hydrochloride solution) were purchased from Sigma-Aldrich Company (Sigma-Aldrich, St., Louis, MO, USA).

Experimental design and animal grouping: The animals were given a standard diet and water *ad libitum*. The animals were arbitrarily divided into five groups of 6 rats each:

Group 1 : Was given saline and served as a normal control group

Group 2 : Was given cyclophosphamide only (60 mg/kg/ weeks) for 8 weeks[17]

Group 3 : Received mirtazapine (20 mg/kg/day)[18] and cyclophosphamide (60 mg/kg/weeks) for 8 weeks

Group 4 : Received silymarin plus (200 mg/kg/day)[19] and cyclophosphamide (60 mg/kg/weeks) for 8 weeks

Group 5 : Received mirtazapine (20 mg/kg/day)+silymarin plus (200 mg/kg/day)+cyclophosphamide (60 mg/kg/ weeks) for 8 weeks

Mirtazapine and silymarin plus were given orally through a feeding tube while cyclophosphamide was administered intraperitoneal.

At the end of the 8th week, under ketamine (90 mg kg^{-1}) and xylazine (5 mg kg^{-1}), a retro-orbital blood sample was collected from each animal and serum samples were prepared. The animals were then sacrificed and dissected. The testes were removed and then washed in cold saline. The left testes were kept frozen at -80°C for molecular assessment, the right testes were put in 10% formalin for histopathological study.

Biochemical laboratory investigations: Retro-orbital blood samples were withdrawn in plain microcentrifuge tubes using tin capillaries. A cooling centrifuge from Sigma Aldrich was used to centrifuge blood samples at a speed of 4000 rpm and a temperature of 5°C for 10 min. The separated serum was collected in another microcentrifuge tube for further biochemical analysis examination according to manuals instructions for alkaline phosphatase (ALP) (Catalog #K412-500, BioVision Incorporated, USA) and testosterone (Catalog Number KT-15332 Kamiya Biomedical).

Assay of oxidant parameters levels: One gram was weighted from testicular tissue that was homogenized (Con-Torque Eberbach's Tissue Homogenizer, Michigan) in 1 mL Phosphate Buffer Saline (PBS). A cooling centrifuge (Sigma Aldrich) was used to centrifuge the homogenates at a speed of 10000 rpm and a temperature of 5°C for 10 min. Each supernatant was collected into a new microcentrifuge tube of 1.5 mL. The collected supernatant was used to assess all biochemical tests.

Using ELISA, GSH level (nmol/mL/g tissue) (Catalog Number: GLU39-K01 Eagle Biosciences, Inc., USA) and MDA level (nmol/mL/g tissue) were assessed (Catalog Number: LIP39-K01 Eagle Biosciences, Inc., USA), catalase level (mIU/mL/g tissue) was assessed (Catalog Number CSB-E13439r Cusabio Technology, China) and SOD level (pg/mL/g tissue) was assessed (Catalog Number CSB-EL022397RA Cusabio Technology, China) were assessed in testicular tissues homogenates according to manual instructions.

Assay of inflammatory and anti-apoptotic gene expression levels: The genomic mRNA was extracted from liver and kidney tissues with the mRNA easy extraction kit. The extraction was done as per the recommendations of the manufacturer (Catalogue no. 217004, Qiagen, Valencia, CA, USA). With the help of the reverse transcription (RT) RNA kit, cDNA was made from the mRNAs (Catalogue no. 4427975, ID 000397, Applied Biosystems). TaqMan PCR Master Mix kit was used to amplify the cDNA generated in the previous step (Applied Biosystems, Cat No. 4440040) along with using a Step One System (RQ Manager 1.2, software v 2.1, Applied Biosystem). GAPDH was kept as the normalization control and contamination was excluded by using negative controls. The cDNA was synthesized at 45°C for 15 min. It was followed by the inactivation of reverse transcriptase and polymerase activation for 5 min at 95°C. PCR was amplified for 40 cycles and consisted of 15 sec DNA denaturation at 95°C, annealing of 20 sec primers at 55°C and an amplification step of 30 sec at 72°C. The sequence of the primers for each gene was shown in Table 1.

Histopathological examination: The right testes were taken and fixed in 10% neutral paraffin, dehydrated in ascending grades of ethanol, cleared in xylene and embedded in paraffin wax. Sectioning of paraffin blocks was done to make 5 microns thick sections. These sections were cleared with xylene and then rehydrated with descending grades of alcohol. The sections were finally stained with Harris's hematoxylin and eosin stain and were examined under a light microscope.

Statistical analysis: Data were collected, tabulated and subjected to analysis using SPSS 17.0 for Windows, data were

Table 1: Primer's sequence and annealing temperature specific for each gene

Target gene	Primer sequence: 5'-3'	Gene bank accession number
HO-1	Forward: GGAAAGCAGTCATGGTCAGTCA	NM_012580.2
	Reverse: CCCTTCCTGTGTCTTCCTTTGT	
IL-6	Forward: CCACTTCACAAGTCGGAGGCTTA	NM_012589.2
	Reverse: GTGCATCATCGCTGTTCATACAATC	
IL 1β	Forward: GCTGTGGCAGCTACCTATGTCTTG	NM_031512.2
	Reverse: AGGTCGTCATCATCCCACGAG	
Bcl-2	Forward: GGGAAACACCAGAATCAAGT	NM_016993.1
	Reverse: AGCCAGGAGAAATCAAACAG	
iNOS	Forward: CTGTCACCGAGATCAATGCA	NM_012611.3
	Reverse: CATGAGCAAAGGCACAGAAC	
eNOS	Forward: GGGCAACTTGAAGAGTGTGG	NM_021838.2
	Reverse: AAGAGTTCTGGGGGCTCATC	
TNF-α	Forward: AACTCGAGTGACAAGCCCGTAG	NM_012675.3
	Reverse: GTACCACCAGTTGGTTGTCTTTGA	
GAPDH	Forward: CACCCTGTTGCTGTAGCCATATTC	NG_028301.2
	Reverse: GACATCAAGAAGGTGGTGAAGCG	

presented as Mean±SEM. Groups were compared using the ANOVA Test followed by the Least Significant Differences (LSD) as a *post-doc* Test, considering $p<0.05$ significant.

RESULTS

Mirtazapine improved testosterone and alkaline phosphatase levels in cyclophosphamide-intoxicated rats: Testosterone and alkaline phosphatase (ALK) serum levels were estimated. Cyclophosphamide-induced testicular toxicity group showed significantly increased ALK and decreased testosterone levels contrary to the control group.

The mirtazapine-treated and silymarin-treated groups depicted significantly decreased serum levels of ALK, and increased testosterone compared to the untreated group.

The combined treated group depicted a much more reduction in the ALK and increase testosterone levels as compared to the administration of mirtazapine alone (Table 2).

Mirtazapine attenuated testicular oxidative stress in cyclophosphamide-intoxicated rats: Cyclophosphamide-induced testicular toxicity group depicted oxidative stress as shown by significantly increased malondialdehyde (MDA) and significantly decreased reduced glutathione (GSH), catalase and SOD in contrast to the control group.

Mirtazapine or silymarin administration significantly decreased MDA and increased GSH, catalase and SOD levels in comparison to the cyclophosphamide-treated group. Co-administration of mirtazapine and silymarin significantly restored the antioxidant capacity compared to the cyclophosphamide or mirtazapine-treated group (Table 3).

Mirtazapine mitigated inflammation in cyclophosphamide-intoxicated rats: In the cyclophosphamide-induced testicular toxicity group, inflammatory gene expression (IL-6, IL-1β and TGF-α) levels exhibit a significant increase while IL-10 exhibit a significant decrease in comparison to the control group.

Significant downregulation of IL-6, IL-1β and TGF-α and upregulation of IL-10 gene expression were observed in group 3 and group 4 treated with mirtazapine or silymarin, respectively.

Combined administration of mirtazapine and silymarin in group 5 significantly downregulated the IL-6, IL-1β and TGF-α gene expressions and upregulated IL-10 compared to the untreated group (Table 4).

Mirtazapine improved testicular eNOS and HO-1 levels in cyclophosphamide-intoxicated rats: The iNOS, eNOS and HO-1 gene expression were evaluated among the studied groups using RT-PCR. Cyclophosphamide-induced testicular toxicity group depicted significantly decreased eNOS and HO-1 with significant increase iNOS gene expression in comparison to the control group.

Mirtazapine or silymarin or combined treated groups depicted significantly increased eNOS and HO-1 gene expression in contrast to the non-treated group (Table 5).

Mirtazapine prevented testicular apoptosis in cyclophosphamide-intoxicated rats: To determine the anti-apoptotic protective effects of Mirtazapine, we estimated the gene expression levels of the Ki67 (IHC) and Bcl-2 through, the RT-PCR technique. Cyclophosphamide-induced testicular toxicity group depicted significantly decreased gene expression in comparison to the control group.

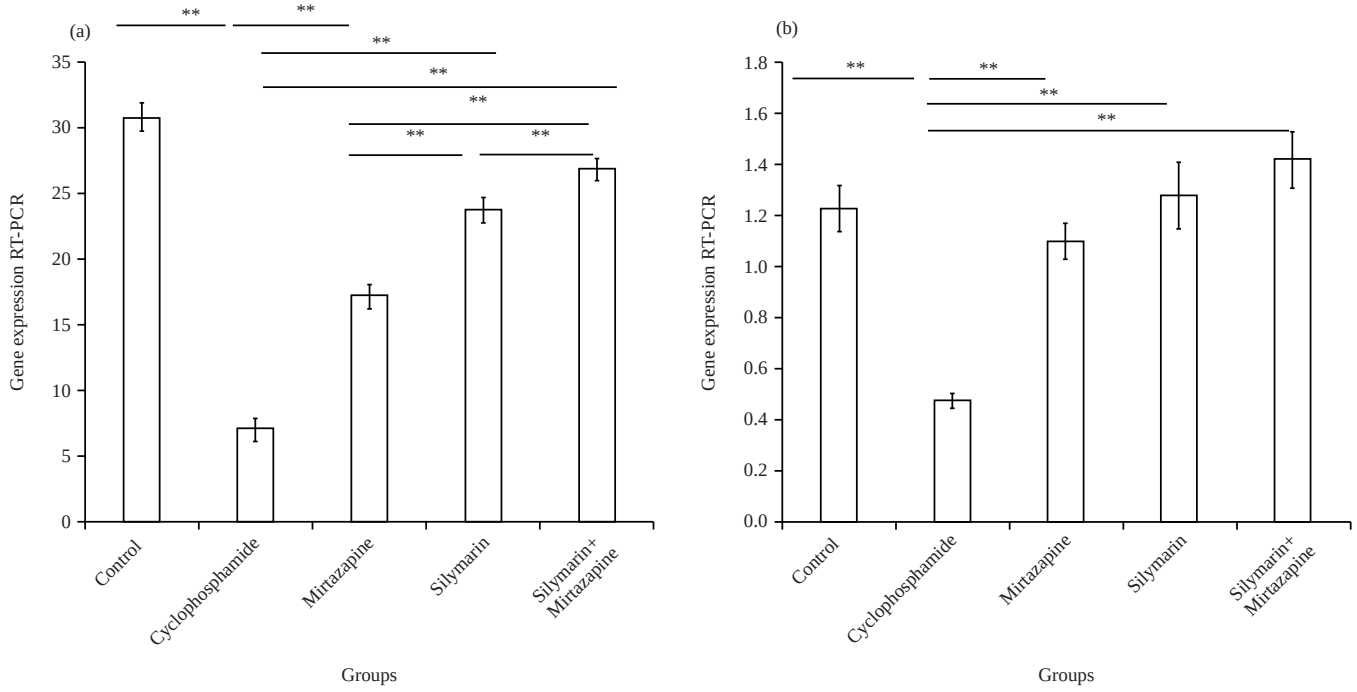

Fig. 1(a-b): (a) Ki67 and (b) Bcl-2 levels among the studied groups

** Significance Difference

Table 2: Testosterone and Alkaline Phosphatase (ALP) levels among the studied groups

Parameters	Testosterone (ng mL^{-1})	ALP (ng mL^{-1})
Control	7.56±0.54	6.65±0.55
Cyclophosphamide group	1.93±0.14*	24.5±2.09*
Mirtazapine	3.67±0.303$^{£}$	11.2±0.74$^{£}$
Silymarin	5.6±0.39$^{£¥}$	8.97±0.67$^{£}$
Silymarin+Mirtazapine	6.3±0.20$^{£¥}$	6.8±0.63$^{£¥}$

*Significant compared to the control group, $^{£}$Significant compared to the cyclophosphamide group and $^{¥}$Significant compared to the mirtazapine group

Table 3: Antioxidant parameters among the studied groups

Parameters	MDA (nmol mg^{-1} protein)	Catalase (mmol mg^{-1} protein)	GSH (nmol mg^{-1} protein)	SOD (U mg^{-1} protein)
Control	0.405±0.037	42.5±2.8	1.62±0.09	6.52±0.27
Cyclophosphamide	2.317±0.14*	7.4±0.3*	0.544±0.07*	1.39±0.19*
Mirtazapine	1.2±0.14$^{£}$	23.9±1.9$^{£}$	1.23±0.08$^{£}$	3.78±0.22$^{£}$
Silymarin	0.563±0.04$^{£¥}$	33.7±1.6$^{£¥}$	1.504±0.07$^{£¥}$	4.98±0.43$^{£¥}$
Silymarin+Mirtazapine	0.52±0.05$^{£¥}$	39.17±3.7$^{£¥}$	1.595±0.07$^{£¥}$	6.65±0.46$^{£¥€}$

*Significant compared to the control group, $^{£}$Significant compared to the cyclophosphamide group, $^{¥}$Significant compared to the mirtazapine group and $^{€}$Significant compared to the silymarin group

Mirtazapine treated group or silymarin-treated group depicted significantly increased Ki67 (IHC) and Bcl-2 gene expression in contrast to the non-treated group. Silymarin treated group showed significant gene expression compared to the mirtazapine-treated group.

The combined mirtazapine and silymarin group depicted significantly increased gene expression of Ki67 (IHC) compared to either them alone or non-treated groups, respectively (Fig. 1a). The combined mirtazapine and silymarin group depicted significantly increased gene expression of Bcl-2 compared to non-treated groups (Fig. 1b).

Mirtazapine improved testicular deterioration in cyclophosphamide-intoxicated rats (histopathological examination results): We evaluated the deleterious and protective histopathological changes induced by cyclophosphamide and mirtazapine/silymarin, respectively.

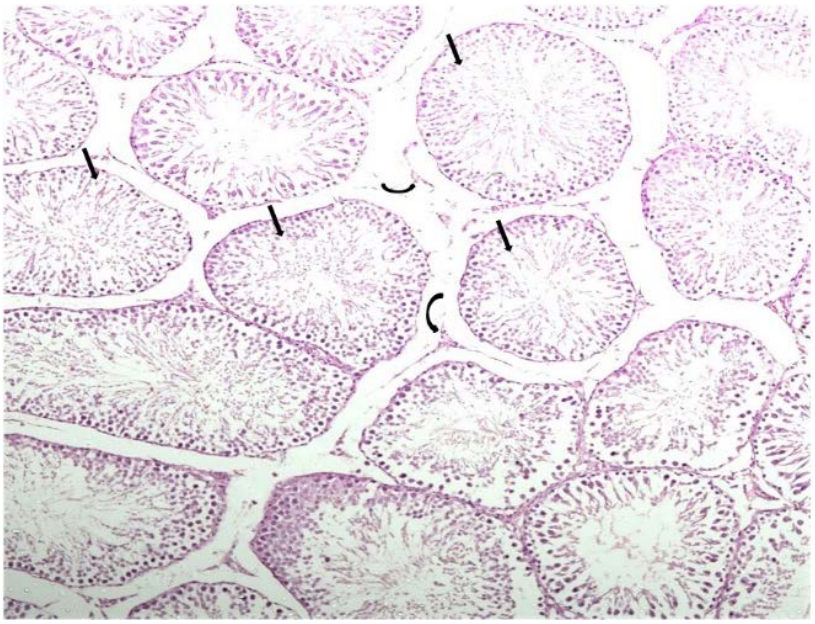

Fig. 2: Photomicrographs of the testicular section from the control group showed normal histological features of seminiferous tubules represented by all the populations of spermatogenic cells (arrows)
Interstitial tissue and Leydig cells depict normal morphology (curved arrows) and (H&E 100X)

Table 4: Anti-inflammatory parameters among the studied groups

Parameters	IL-6	IL-1β	TNF-α	IL-10
Control	1.08±0.04	1.078±0.09	1.14±0.04	1.56±0.13
Cyclophosphamide	2.34±0.05*	2.35±0.03*	3.38±0.11*	0.586±0.03*
Mirtazapine	1.32±0.11$^{£}$	1.38±0.02$^{£}$	2.4±0.12$^{£}$	1.21±0.03$^{£}$
Silymarin	1.24±0.03$^{£}$	1.21±0.03$^{£¥}$	1.37±0.08$^{£¥}$	1.18±0.15$^{£}$
Silymarin+Mirtazapine	1.21±0.05$^{£}$	1.11±0.03$^{£¥}$	1.15±0.03$^{£¥}$	1.67±0.12$^{£¥€}$

*Significant compared to the control group, $^{£}$Significant compared to the cyclophosphamide group, $^{¥}$Significant compared to the mirtazapine group and $^{€}$Significant compared to the silymarin group

Table 5: Nitric Oxide (iNOs and eNOs) and HO-1 levels among the studied groups

Parameters	iNOS	eNOS	HO-1
Control	0.36±0.03	1.23±0.04	1.17±0.03
Cyclophosphamide	1.52±0.06*	0.43±0.14*	0.29±0.03*
Mirtazapine	1.31±0.07	1.02±0.05$^{£}$	0.85±0.09$^{£}$
Silymarin	1.49±0.07	1.09±0.08$^{£}$	1.36±0.11$^{£¥}$
Silymarin+Mirtazapine	1.48±0.11	1.18±0.04$^{£}$	1.53±0.103$^{£¥}$

*Significant compared to the control group, $^{£}$Significant compared to the cyclophosphamide group and $^{¥}$Significant compared to the mirtazapine group

The control group showed normal histological features of seminiferous tubules, spermatogenic cells, interstitial tissue and Leydig cells (Fig. 2). The cyclophosphamide group depicted distorted seminiferous tubules with impaired spermatogenesis. Seminiferous tubules showed sloughing of epithelial cells into the lumen, decreased layering of the tubules, presence of intracellular vacuolations and edema (Fig. 3a). In addition, haemorrhage and edema were observed in the interstitial tissue (Fig. 3b). Testicular sections from the mirtazapine group showed normal seminiferous tubules. Some abnormal tubules with sloughing of cells were also observed (Fig. 4).

The silymarin group depicted near-normal histological features of the seminiferous tubules. Few tubules were distorted and mild epithelial cell sloughing was also observed (Fig. 5).

Testicular sections from the treated group (cyclophosphamide+mirtazapine+silymarin) showed near-normal histological features of the seminiferous tubules and the interstitial tissue (Fig. 6).

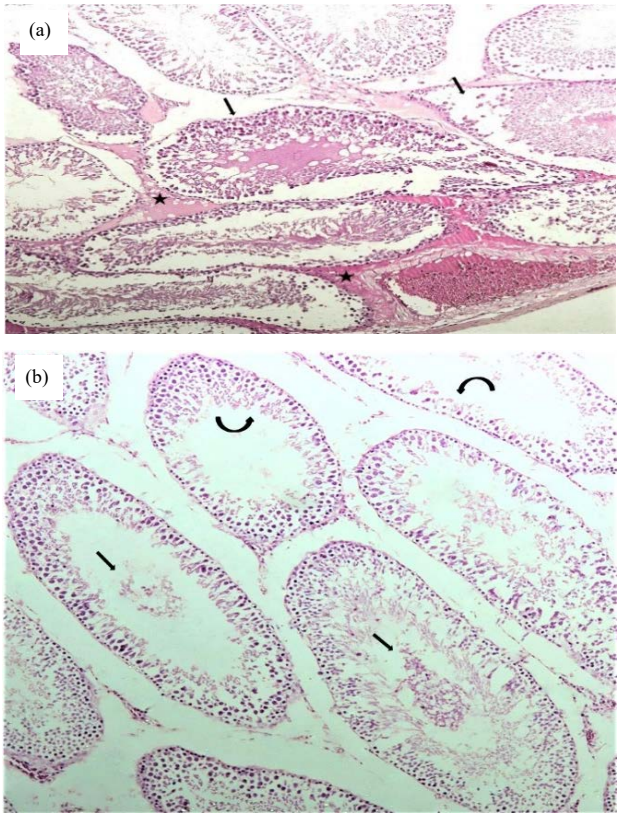

Fig. 3(a-b): Histopathological image of testicular sections, (a) Photomicrograph of the testicular sections from the cyclophosphamide group shows the distortion of the seminiferous tubules with impairment of spermatogenesis. Seminiferous tubules show epithelial sloughing, intracellular vacuolations and edema (arrows). Interstitial haemorrhage and interstitial edema are prominent (star) and (b) Photomicrograph of the testicular sections from the cyclophosphamide group shows distorted seminiferous tubules with sloughing of the epithelial cells into the lumen (arrows) of the tubules along with the decreased layering of the tubules (curved arrows)
(H&E 100X)

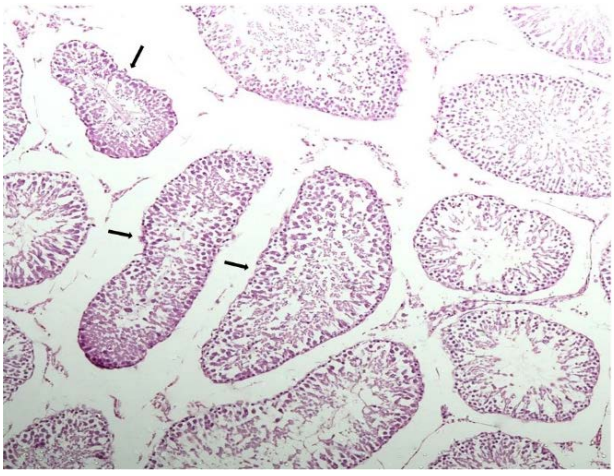

Fig. 4: Photomicrograph of the testicular sections from the mirtazapine group shows normal seminiferous tubules
In addition, some abnormal tubules with sloughing of cells are also seen (arrows) and (H&E 100X)

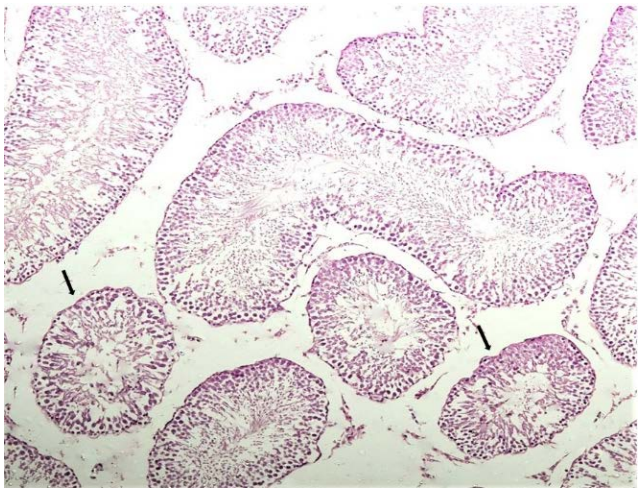

Fig. 5: Photomicrograph of the testicular sections from the silymarin group shows near-normal histological features of the seminiferous tubules
Only some tubules are showing some distortion and mild epithelial cell sloughing (arrows) and (H&E 100X)

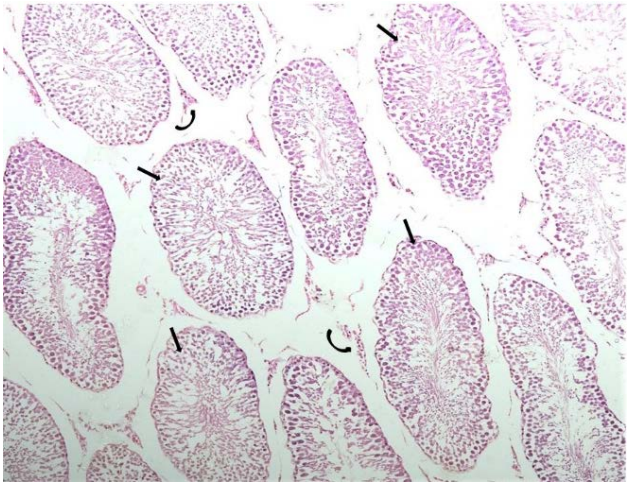

Fig. 6: Photomicrograph of the testicular sections from the treated group
(Cyclophosphamide+mirtazapine+silymarin) shows near-normal histological features of the seminiferous tubules (arrows) and the interstitial tissue (curved arrows) and (H&E 100X)

DISCUSSION

The current study revealed that CP induced marked oxidative stress which was revealed by significantly declining levels of catalase, GSH, SOD and increased levels of MDA which was consistent with earlier research[20]. Despite being used as a chemotherapeutic drug for the treatment of tumours and autoimmune diseases, CP causes testicular toxicity and hormonal disturbances. An imbalance in the generation and absorption capacity of reactive oxygen species (ROS) in cells causes oxidative stress, which leads to tissue damage. It has been discovered that oxidative stress increases ROS and lipid peroxidation by inactivating microsomal enzymes[21]. In oxidative stress, CP is implicated in the generation of free radicals and the decreasing of the content of antioxidants[22].

The present study showed that silymarin and mirtazapine had a protective effect on CP induced testicular damage. Treatment with silymarin and mirtazapine ameliorated this oxidative stress and restored these parameters to near-normal levels. This effect was more profound when both drugs were used together indicating the additive antioxidant effects of

SMN and MZN. The SMN has antioxidant characteristics that work by directly scavenging free radicals, so, it prevents free radical generation by blocking specific ROS-producing enzymes or increasing mitochondrial integrity in stressful situations and both pathways appear to be important. In addition, the fundamental driving force of SMN's antioxidant action is presumably maintaining an optimal redox equilibrium in the cell by activating a variety of antioxidant enzymes and non-enzymatic antioxidants, mostly via Nrf2 activation. Moreover, it inhibits NF-κB pathways to reduce inflammatory responses and is an emerging mechanism of SMN's beneficial benefits in liver damage and other liver disorders. Also, it activates vintages, which are responsible for the production of protective proteins[14].

There were many studies in the literature which have established the role of CP in increasing oxidative stress by generating free radicals. The metabolic products of CP include 4-hydroxycyclophosphamide, aldophosphamide mustard and acrolein. These metabolic products inhibit cellular DNA synthesis in fast-dividing cells and cause cell death. The therapeutic action of CP is due to its alkylating activity on tumour cell DNA. Acrolein, on the other hand, causes oxidative stress, which causes DNA damage in normal cells and toxicities in a variety of target organs. Acrolein enters the cell quickly and triggers intracellular reactive oxygen species, causing damage to the cell's lipids, proteins and DNA. Also, acrolein disrupts the GSH redox pathway, boosting the formation of free radicals in cells[23].

Mirtazapine (MTZ), an antidepressant drug, may be a promising agent for the avoidance of CP-induced testicular toxicity. MTZ provides antioxidant and cytoprotective properties by activating enzymatic and non-enzymatic antioxidant processes and inhibiting some harmful toxic oxidants. So, MTZ could be employed as a cytoprotective drug based on these findings[12].

Inflammation is one of the key etiological causes of infertility. The current investigation showed that administering CP significantly increased IL-6, IL-1β, iNOS and TNF-α levels and decreased levels of IL-10, eNOS and HO-1. This study also showed that cotreatment with SMN and MZN helped in the restoration of these inflammatory biomarker parameters near normal levels. Through, the MAPK signalling pathway, SMN as a natural flavonoid, exhibits anti-inflammatory properties by interfering with the generation of inflammatory mediators such as IL-6, TNF- and IL-1 in numerous cell lines. Therefore, inhibiting MAPK signalling cascade molecules like p38 or ERK1/2 has been shown to regulate TNF- and iNOS production[24].

During mirtazapine medication, a study found a link between antidepressant response and a decrease in peripheral inflammatory markers. This finding suggested that alterations in inflammatory responses may play a role in antidepressant action and the antidepressant effects of mirtazapine effect are most likely due to their anti-inflammatory properties[25].

Immunohistochemical observations have revealed that CP caused decreased expression of Ki-67. The antigen Ki-67 is a nuclear protein linked to cellular proliferation. It is also linked to the transcription of ribosomal RNA. Inactivation of the antigen Ki-67 causes ribosomal RNA production to be inhibited. As a result, CP suppressed cell proliferation and increased apoptosis[26]. In the current study, Ki-67 expression was elevated with SMN administration which was consistent with the previous findings that indicated that SMN therapy increased Ki-67 expression, thereby implying that it has beneficial effects on the regeneration of injured cells[27]. Similar results were obtained with MZN administration. Previous reports also found that the enhanced cell proliferation due to mirtazapine was indicated by increased Ki-67 reaction[28].

In this study, CP administration caused a reduction in Bcl-2 level which is improved by SMN and MZN administration. As it upregulates the expression of Bcl-2, SMN has antigenotoxic properties and modulates the expression of genes involved in cell defense against DNA damage[29]. Engel *et al.*[30] found that mirtazapine can upregulated the anti-apoptotic protein, Bcl-2 which results in modification of the apoptotic activity and enhanced plasticity and cell survival in depressive patients.

The evaluation of the hormone assay showed that CP treatment had negative effects on plasma testosterone thereby significantly decreasing its levels. This result was consistent with the previous finding that CP treatment resulted in a reduction in testosterone levels[31]. A direct toxic effect of chemotherapy on the leydig cells or an indirect toxic effect via germinal epithelial damage may lead to dysfunction of the leydig cells. In addition, a reduction in testosterone concentrations may affect epididymal function. The result of the present study showed that SMN and MZN treatment improved the endocrine functions of the testis as proved by an increase in serum levels of testosterone. The aromatase enzyme, which catalyzes the conversion of testosterone to estrogen, is inhibited by SMN. So, the serum level of testosterone is raised by blocking this enzyme[32]. The SMN also boosted the steroidogenesis process in Leydig cells, increasing testosterone hormone secretion[33].

Also, CP treatment induced a significant elevation in alkaline phosphatase level which is ameliorated by SMN and MZN administration. The ALP is involved in spermatogenesis and is necessary for sperm survival and motility. Its activity has previously been linked to testicular degeneration[34]. The reduced level of this enzyme as a result of SMN administration might probably be, in part, due to the presence of chemical constituents in the extract[35].

In the present study, results revealed that treating rats with CP induced many histological alterations in the testis and these alterations included a distortion of the seminiferous tubules with impairment of spermatogenesis. Seminiferous tubules showed epithelial sloughing, intracellular vacuolations and edema fluid. Interstitial haemorrhage and edema were prominent. Current findings of testicular toxicity in CP-treated rats were in concordance with the results of previous studies[31]. CP may cause testicular tissue damage by inducing lipid peroxidation and oxidative stress and as a result, alter the structure and function of the testis[36].

The cytoprotective effects of SMN and MTZ against testicular damage caused by CP administration improved the histological structure of the testicular tissue. Our findings in the SMN and MTZ treatment harmonized with several other studies that established SMN and MTZ cytoprotective benefits against CP-induced tissue damage[15,28]. The protective action of silymarin is attributed to its ability to protect the cell membrane from oxidative stress. The antioxidant properties of silymarin may be attributed to its constituents, which include silybin, silydianin, silychristin and flavonolignans[37]. Similarly, mirtazapine appears to work by activating antioxidant pathways which include both enzymatic and non-enzymatic pathways. Furthermore, mirtazapine raises serotonin levels which have antioxidant properties against lipid peroxidation and oxidative stress[38].

The testicular protective effect of mirtazapine represents a promising effect of mirtazapine use, however, further experimental and clinical examination may be needed.

CONCLUSION

The CP is a widely used drug for chemotherapy and is known for its potential to generate free radicals and induce male reproductive toxicity, therefore, the compounds having antioxidant potential will be beneficial to prevent CP-induced toxicity. This study revealed that supplementation of SMN and MTZ has the potential to ameliorate the male reproductive toxicity induced by CP through, a wide range of mechanisms that include suppression of lipid peroxidation, augmenting antioxidant activities and increasing reproductive hormone levels and preservation of the testicular histological features.

SIGNIFICANCE STATEMENT

The use of cyclophosphamide is limited by its side effects, especially testicular toxicity. The current work showed the possible protective effects of mirtazapine against cyclophosphamide-induced testicular toxicity in rats. These protective effects may be through mirtazapine antioxidant activities, amelioration of the inflammatory process and apoptosis.

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