Original Research

Protective effect of mirtazapine and hesperidin on cyclophosphamide-induced oxidative damage and infertility in rat ovaries

Naglaa Fathi Khedr

Department of Biochemistry, Faculty of Pharmacy, Tanta University, 31527 Tanta, Egypt
Corresponding author: Naglaa Khedr. Email: naglaa_khedr2000@yahoo.com

Abstract

Cyclophosphamide (CP) causes infertility due to ovarian toxicity. The toxicity mechanism suggests oxidative stress. We assessed whether mirtazapine (MTZ) and hesperidin (HSP) could promote ovarian protection against damage due to CP chemotherapy. Female Wistar rats aged 14 weeks were used. Animals were divided into four groups: control vehicle group ($n = 8$); CP group ($n = 8$, rats received 150 mg/kg of CP, single intraperitoneal [i.p.] injection); CP + MTZ group ($n = 8$, rats received same dose of CP + 30 mg/kg of MTZ, orally, daily); and HSP + CP group ($n = 8$, rats received same dose of CP + 100 mg/kg of HSP, orally, daily). After eight days of medication, ovaries were removed and ovarian toxicity was assessed by counting follicles and corpora lutea. Nitric oxide (NO) and malondialdehyde (MDA) levels, myeloperoxidase (MPO), glutathione peroxidase (GPx), and superoxide dismutase (SOD) activities were estimated in ovarian tissue. NO level, MDA level, and MPO activity were increased ($P < 0.001$), while, GPx and SOD activities were lowered significantly ($P < 0.001$) in CP-treated group compared with control vehicle. In addition, ovulation, number of follicles, and ovarian weight were reduced by CP treatment. On the contrary, rats pretreated with MTZ and HSP showed significant decrease in NO, MDA levels, and MPO activity, while, activities of SOD and GPx were increased ($P < 0.001$). Oxidative stress induced by CP in the rat ovary causes infertility in the female rats. HSP and MTZ could reverse this effect and provide protection of fertility against CP-induced toxicity.

Keywords: Cyclophoshamide, mirtazapine, hesperidin, oxidative stress, fertility

***Experimental Biology and Medicine* 2015; 240: 1682–1689. DOI: 10.1177/1535370215576304**

Introduction

The Childhood Cancer Survivor Studies had examined long-term effects of cancer treatments in survivors who were children. One of the primary effects identified in patients treated after age 18 years, when compared with untreated siblings, there was a 13-fold increase in the risk of undergoing premature menopause. Risk was increased with increasing age and increasing doses of radiation or alkylating agents.[1]

Alkylating agents deteriorate reproductive functions by rapid depletion of the oocyte reserve which was mediated by apoptotic cell death and ovarian atrophy with disappearance of resting primordial follicles and growing follicles in humans and resulting in premature menopause.[2]

Cyclophosphamide (CP) is one of the most effective alkylating agents. It is associated with the greatest risk of female infertility. CP causes progressive and irreversible damage to oocytes in a dose-dependent manner. Thereby reduces the number of oocytes in ovaries. With high dose and longer duration, oocyte number is reduced drastically resulting in premature ovarian failure and premature menopause.[1]

This is mostly attributed to ovarian toxicity due to the cumulative doses of CP. CP and its toxic metabolites interfere with intracellular antioxidation systems that play an important role in detoxifying the reactive oxygen species (ROS). The increased production of free oxygen radicals and decreased production of antioxidants may increase proapoptotic signals in antral follicles.[3]

Mirtazapine (MTZ) is an antidepressant drug used in the treatment of major depression. MTZ has antioxidant activity besides its antidepressant effect. Moreover, Altuner *et al.*[4] used MTZ as a cell protector to prevent infertility in rats with oxidative ovarian damage due to cisplatin administration. Thus, the purpose of this study was to demonstrate whether MTZ would be efficacious for preventing infertility in rats with oxidative ovarian damage due to CP administration.

Hesperidin (HSP) is a flavanone glycoside found abundantly in citrus fruits. The antioxidant HSP has the capacity

to sequester 1,1-diphenyl-2-picrylhydrazyl. HSP provides strong cellular antioxidant protection against the damaging effects induced by paraquat and peroxide hydrogen.[5]

HSP is deglycosylated in the gut to its aglycone hesperetin by intestinal microflora. Most studies demonstrated that HSP is inactive or only moderately active. In contrast, hesperetin was shown to be a potent antioxidant, inhibiting Fe^{2+}-induced linoleate peroxidation and autooxidation of rat cerebral membranes, scavenging peroxynitrite, and inhibiting generation of ROS including hydroxyl radical.[6]

The anti-cancer drugs may cause ovarian insufficiency and infertility. Therefore, the present study was conducted to assess whether MTZ and HSP could promote ovarian protection against damage due to CP chemotherapy and to define the association of oxidative stress in ovarian tissues with infertility.

Materials and methods

Chemicals

CP (Cycram)® was purchased from EIMC Pharmaceuticals Co. (Korea). MTZ (Remoron)® was purchased from Organon Pharmaceuticals (NJ). HSP (80% purity), thiobarbituric acid, dithiotheritol, *n*-butanol, and hexadecyltrimethylammonium bromide were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals

The study was performed in accordance with the guidelines for the care and use of laboratory animals was approved by Research Ethics Committee (Faculty of pharmacy, Tanta University, Egypt). Thirty-two female Wistar rats (120–150 g) aged 14 weeks were provided by the National Research Center (Cairo). The animals were kept at room temperature. Food and water were available *ad libitum*. After acclimatization for one week, the experiment was started and rats were divided into four groups:

Control vehicle group: $n = 8$, rats received 1 mL saline orally daily for eight days and 0.5 mL saline single intraperitoneal (i.p.) injection after seven days.

CP group: $n = 8$, rats were scarified after receiving 150 mg/kg of CP single i.p. injection on the eight day of the experiment.[3] It was reported by Ataya *et al.*[7] that i.p. injection of CP (100 mg/kg) at 1, 2, 4, 16, and 24 h decreased serum estradiol level and the number of granulosa cells in rat ovaries. In addition, a single injection of 200 mg/kg CP, in adult rats, destroyed all types of follicles. This destruction was caused by induction of apoptosis in granulosa cells of these follicles.[1]

MTZ + CP group: $n = 8$, received 30 mg/kg of MTZ orally, daily for eight days[4] + 150 mg/kg, CP single i.p. injection on eight day of the experiment. Altuner *et al.*[4] reported that MTZ (30 mg/kg p.o.) for 10 days reverses oxidative stress and infertility induced by cisplatin in the rat ovary tissue.

HSP + CP group: $n = 8$, rats received HSP 100 mg/kg orally, daily for eight days + 150 mg/kg CP, single i.p. injection on eight day of the experiment. It was reported by Hozayen[8] that pre-treatment of male animals with HSP (50 mg/kg) produced a potential increase of glutathione level, glutathione-*S*-transferase, and glutathione peroxidase (GPx) activities in doxorubicin administered rats.

As reported by previous literatures,[3] there are morphological changes in primordial and primary follicles in 24–48 h after exposure to phosphoramide mustard (PM), a toxic metabolite of CP. The biochemical changes in rat ovaries will occur in 24–48 h. Therefore, we decided to sacrifice rats after 24 h of single i.p. injection of CP (150 mg/kg). Rats were scarified with exsanguinations under ether anesthesia. The two ovaries of each rat were removed. The removed right ovaries were weighed and homogenized in specific buffers for the biochemical analysis of malondialdehyde (MDA) level, nitric oxide (NO) level and myeloperoxidase (MPO), glutathione peroxidase (GPx), and superoxide dismutase (SOD) activities. Left ovaries were fixed in 10% formaldehyde and processed for histomorphometric analysis.

Biochemical analysis of ovarian tissue

GPx activity (EC 1.11.1.9). Small portion of ovary tissue was homogenized in 4–8 volumes (per weight tissue) of cold buffer (50 mmol/L phosphate buffer, 5 mmol/L EDTA, and 1 mmol/L dithiotheritol, pH 7.0). The homogenate was centrifuged at 870 *g* for 15 min at 2–8°C. Then, the supernatant was collected for enzyme assay. GPx activity was measured indirectly[9] using a commercial kit (Biodiagnostic Co., Giza, Egypt). The oxidized glutathione, produced upon reduction of organic peroxide by GPx, is recycled to its reduced state by the enzyme glutathione reductase, which was accompanied by oxidation of NADPH to $NADP^+$. The decrease in the absorbance at 340 nm was monitored for measuring GPx enzyme activity. The molar extinction coefficient for NADPH is 6220 mol/$L^{-1}cm^{-1}$ at 340 nm.

SOD activity (E.C.1.15.1.1). Ovaries were homogenized in 5–10 mL cold buffer (100 mmol/L potassium phosphate and 2 mmol/L EDTA, pH 7.0) per gram tissue. The homogenate was centrifuged at 875 *g* for 15 min at 4°C. The supernatant was collected and stored at −20°C until assay. SOD activity was determined using a commercial kit (Biodiagnostics, Giza, Egypt). The assay was realized on the ability of the enzyme to inhibit the phenazine methosulfate-mediated reduction of nitrobluetetrazolium dye and measured at 560 nm. Enzyme activity was expressed as U/g tissue.[10]

Determination of MPO activity (E. C.1.11.2.2). Ovaries were homogenized in 50 mmol/L phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide (5–10 mL/g tissue). MPO activity was determined using 4-aminoantipyrine/phenol solution as a substrate for MPO-mediated oxidation by H_2O_2.[4] The assay based on oxidative coupling of phenol with 4-aminoantipyrine to

yield 4-*N*-(1,4-benzoquinoneimine)-antipyrine, a chromophore measured at a wavelength 510 nm and the corresponding molar absorptivity equal to 13,900 $mol/L^{-1}cm^{-1}$. One unit of MPO activity is defined as that degrades 1 μmol H_2O_2/min at 25°C. Data were presented as μmol/min/mg protein. Total protein content of ovarian tissue was measured by Folin's reaction according to Lowry *et al.*[11]

Determination of NO level. NO was determined according to the method described by Griess.[12] Samples were treated with copporized cadmium in glycine buffer at pH 9.7 (0.8 g of Cd granules for a 1 mL reaction mixture). Following centrifugation, the sample was mixed with fresh 100 μL Griess reagent and 100 μL of metaphosphoric acid. The Griess reagent consists of 0.1 g sulfanilamide, 2.5 g phosphoric acid, and 0.01 g *N*-(1-napthyl)-ethylenediamine in 100 mL distilled water. A deep purple azo compound occurred and was measured at 540 nm. A standard curve was established with a set of serial dilutions of sodium nitrite. Results were expressed as μmol/L/mg protein.

Determination of malonaldehyde (MDA) level. The degree of ovarian mucosal lipid peroxidation was determined by estimating MDA level using the thiobarbituric acid.[13] Small portion of ovarian tissue was weighed and homogenized on ice with 2 mL of 1.15% potassium chloride solution. A 0.5 mL homogenate was added to a solution containing 0.2 mL of 80 g/L sodium lauryl sulfate, 1.5 mL of 200 g/L acetic acid, 1.5 mL of 8 g/L of 2-thiobarbiturate, and 0.3 mL of distilled water. The mixture was incubated at 98°C for 1 h. After cooling, 5 mL of *n*-butanol: pyridine (15: l) was added. The mixture was vortexed for 1 min and centrifuged for 30 min at 875 *g*. The absorbance of the supernatant was measured at 532 nm. The extent of lipid peroxidation was expressed as MDA (nmol/mg protein) using a molar extinction coefficient for MDA of $1.56 \times 10^5 mol/L^{-1}cm^{-1}$.

Histomorphometry of ovarian tissue. Rats have a reproductive strategy that allows them to ovulate and conceive every 4–5 days. During each estrous cycle a cohort of 'resting' primordial follicles starts to develop into primary follicles; this process occurs until the formation of early tertiary follicles. A small number of tertiary follicles enter a preovulatory stage and converted into Graafian follicle. Following extrusion of the secondary oocyte from the Graafian follicle, the granulosa and thecal cells of the follicle remnant undergo hypertrophy and hyperplasia. This process is called *luteinisation* that occurs under the influence of luteinising hormone and prolactin. Luteinisation results in mature *corpus luteum*.[14]

Eight μm sections were prepared and stained with haematoxylin (H) and eosin (E).[3] Ovarian follicles were counted in each section. The follicles were classified into five types according to classification of Erickson[15]: a) primordial – containing an oocyte surrounded by a single layer of flattened cells, b) primary – characterized by a single layer of cuboidal pregranulosa cells, c) secondary – containing 2–5 complete layers of granulosa cells, d) tertiary – containing multiple layers of granulosa cells with some small antrum and e) Graafian – with the cavity occupying most of the total follicular volume. Each follicle was counted once; finally, the total number of each follicle types was found out.

Statistical analysis

Statistical analysis was performed with the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL), version 16.0.[16] Data were presented as mean ± standard deviation (SD). Significance was set at $P < 0.05$. Group means were compared using Student's *t*-test. Biochemical data were analyzed using a one-way analysis of variance (ANOVA) and Tukey's *post hoc* test.

Results

Effect on ovarian weight, NO and MDA levels

Figure 1(a) shows that mean ovarian weight was significantly different among studied groups; CP group showed insignificant decrease (31.11 ± 9.2, $P > 0.05$) compared with control (36.08 ± 6.9). On the contrary, HSP + CP and MTZ + CP groups showed significant increase of 61.11 ± 16.5 ($P < 0.001$) and 44.58 ± 6.16 ($P < 0.05$) in ovarian weight, respectively, compared with CP-treated group; HSP-CP showed the marked increase in ovary weight among treated groups.

Figure 1(b) shows that NO level was significantly elevated in CP group from 7.36 ± 2.1 to 12.08 ± 3.2 compared with control vehicle group. Treatment with MTZ and HSP reduced NO level to 3.2 ± 0.84 ($P < 0.001$) and 4.59 ± 0.78 ($P < 0.01$), respectively compared with CP group.

MDA level (marker of lipid peroxidation) in ovarian tissue was significantly ($P < 0.001$) elevated to 152.9 ± 36.45 compared with control group (30.44 ± 6.23), while MTZ and HSP reduced the elevated MDA level to 60.3 ± 18.5 ($P < 0.001$) and 33.77 ± 9.1 ($P < 0.001$), respectively, compared with CP-treated group, Figure 1(c).

Effect on antioxidant enzymes

Biochemical analysis of ovarian tissue for antioxidant enzymes showed a significant decrease in SOD and GPx activities in CP-treated group compared with control group (Figure 1d and e); GPx was reduced from 58.4 ± 13.09 to 39.21 ± 10.1 ($P < 0.001$), SOD was reduced from 10.36 ± 0.73 to 3.1 ± 1.1 ($P < 0.001$) compared with control. Pre-treatment with MTZ and HSP increased the activity of GPx and SOD compared with CP-treated group; MTZ increased GPx to 103.67 ± 36 ($P < 0.001$) and SOD to 43.95 ± 18.8 ($P < 0.001$). HSP increased GPx to 110.55 ± 39.04 ($P < 0.001$) and SOD to 56.37 ± 13.06 ($P < 0.001$) compared with CP group.

MPO activity was increased after single dose CP from 19.64 ± 3.01 to 58.76 ± 11.27 ($P < 0.001$) compared with control group. Both MTZ and HSP reduced MPO activity in rat ovaries, to 23.88 ± 7.1 ($P < 0.02$) and 26.5 ± 3.23 ($P < 0.001$) respectively, compared with CP-treated group (Figure 1f).

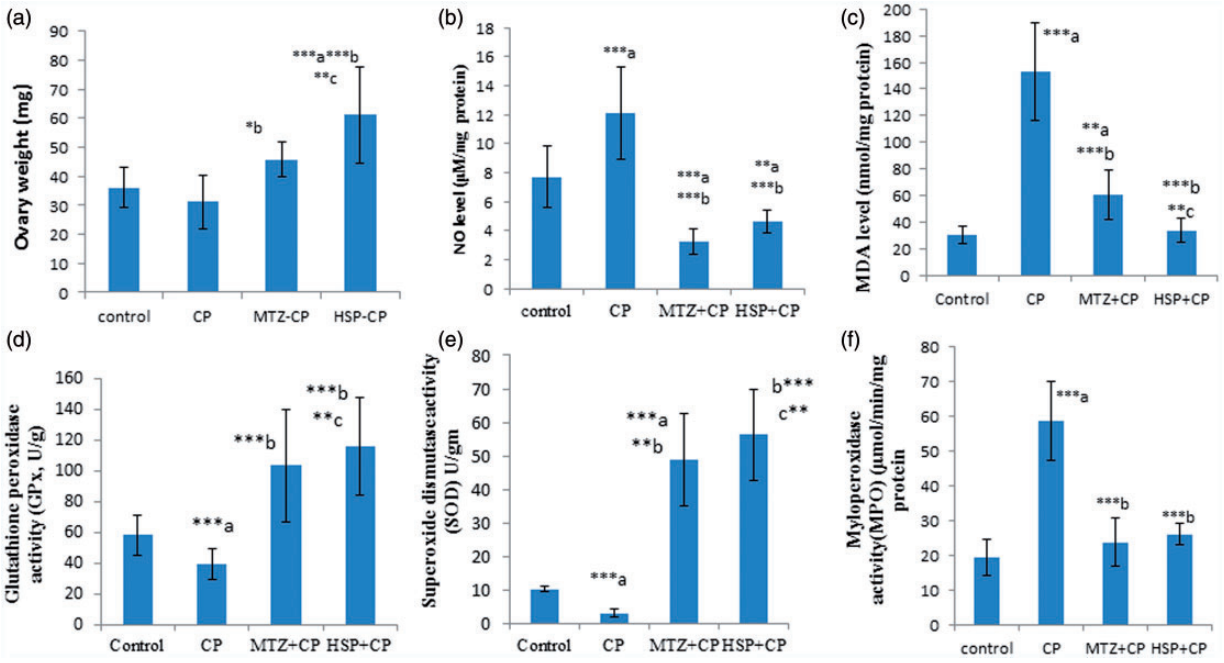

Figure 1 Ovary weight, NO level, MDA level, MPO, SOD, and GPx activities in ovarian tissue of studied groups. Values are mean ± SD, significance was set at $P < 0.05$; a: significant vs. control, b: significant vs. CP, c: significant vs. MTZ + CP. *$P < 0.05$, **$P < 0.01$, ***: $P < 0.001$). CP: 150 mg/kg of cyclophosphamide, single i.p. injection, MTZ + CP: 30 mg/kg of mirtazapine orally, daily for eight days + 150 mg/kg of CP single i.p. injection on eighth day. HSP + CP: 100 mg/kg of hesperidine orally, daily for eight days + 150 mg/kg, CP single i.p. injection. GPx: glutathione peroxidase, i.p.: intraperitoneal, MDA: malondialdehyde, MPO: myeloperoxidase, NO: nitric oxide, SOD: superoxide dismutase. (A color version of this figure is available in the online journal.)

Effect on histopathology and histomorphometry of rat ovaries

Control vehicle group showed normal morphology of the ovary with normal ovulation, normal ovarian architecture and normal count of follicles as reported by previous literatures.[3] The number of primordial follicles (21.66 ± 2.9) and secondary follicles (SF) (6.3 ± 0.8) are normal. Oocyte (O) is surrounded by a single or two layers of granulosa cells. Preovulatory (Graafian) follicles (GF) (3.0 ± 0.9) are present with and without free-floating primary oocytes within the follicular lumina. Mature corpus lutea (CL) (3.5 ± 0.54) are observed (Figure 2 and Table 1).

Ovarian toxicity by CP was additionally confirmed by histomorphometry of ovarian tissue. In CP-treated group, ovary sections show an obvious reduction in the number of follicles and corpora lutea; this characterizes ovarian atrophy. There is a significant reduction ($P < 0.01$) of primordial (16.75 ± 1.6) and maturing follicles (3.1 ± 0.83) as well as GF (1.5 ± 0.5) and CL (1.6 ± 0.74) compared with control group (Figure 3 and Table 1).

The ovarian toxicity induced by CP was significantly reversed by MTZ and HSP. Both MTZ and HSP show increase in the number of maturing follicles ($P < 0.001$) compared with CP-treated group, which was confirmed by increase in the ovary weight (Table 1). In MTZ + CP-treated group, the normal morphology of the ovary with multiple mature CL (6.6 ± 0.91) is observed. In HSP + CP-treated group, ovary shows normal ovulation; PF (12.3 ± 1.2), SF (5.9 ± 0.8), mature CL (5.8 ± 0.78), and mature corpus albicans (CA) are observed (Figures 4, 5 and Table 1).

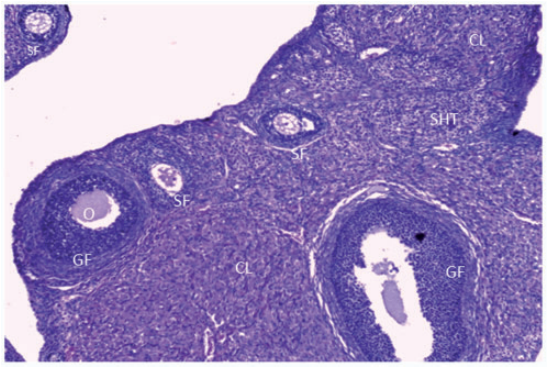

Figure 2 Photograph of ovarian tissue from control vehicle group (hematoxylin and eosin [H&E], 200X). The normal morphology of the ovary with normal ovulation shows the presence of normal ovarian architecture; secondary follicles (SFs), the oocyte (O) is surrounded by a single or two layers of granulosa cells. Preovulatory (Graafian) follicles (GF) are present with and without free-floating primary oocytes within the follicular lumina. Mature corpus luteum (CL) is observed. (A color version of this figure is available in the online journal.)

Discussion

This biochemical and histologic studies examined the effect of MTZ and hesperidin on CP-induced ovarian toxicity. The loss of reproductive function is one of the most important adverse effects of chemotherapy. CP is highly reactive alkylating agent that forms covalent bonds with critical cellular macromolecules through adduct formation, as well as intrastrand and interstrand DNA cross-links and blocking DNA replication. Alkylating agents are highly correlated with arrested menstruation and ovarian failure.[1]

Table 1 Follicle count in rat ovaries of studied groups.

Groups	Primordial follicles	Primary follicles	Secondary follicles	Graafian follicles	Corpus lutea
Control ($n=8$)	21.66 ± 2.9	10.0 ± 2.4	6.3 ± 0.8	3.0 ± 0.9	3.5 ± 0.54
CP group ($n=8$)	$17.75^{a***} \pm 1.6$	$8.37^{a*} \pm 1.18$	$3.1^{a***} \pm 0.83$	$1.5^{a***} \pm 0.5$	$1.6^{a***} \pm 0.74$
MTZ + CP group ($n=8$)	$20.8^{b**} \pm 2.4$	9.1 ± 0.9	$5.25^{b***} \pm 0.9$	1.5 ± 0.5	$6.6^{b***} \pm 0.91$
HSP + CP group ($n=9$)	$22.23^{b***} \pm 1.56$	$12.3^{b***} \pm 1.2$	$5.9^{b***} \pm 0.8$	2.0 ± 0.8	$5.8^{b***} \pm 0.78$

Data presented as mean ± SD, $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.
CP: 150 mg/kg of cyclophosphamide, single i.p. injection, MTZ + CP: 30 mg/kg of mirtazapine orally, daily for eight days + 150 mg/kg of CP single i.p. injection on eighth day, HSP + CP: 100 mg/kg of hesperidine orally, daily for eight days + 150 mg/kg, CP single i.p. injection.

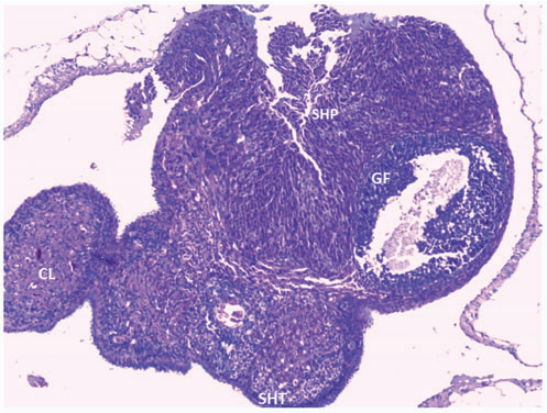

Figure 3 Photograph of ovarian tissue from CP-treated group (hematoxylin and eosin [H&E], 200X). Ovary section shows an obvious reduction in the number of follicles and corpora lutea; this characterizes ovarian atrophy. Hyperplasia (SHP) and hyperthecosis (SHT) of stromal interstitial cells are the common features of ovarian atrophy. CP: 150 mg/kg of cyclophosphamide, single i.p. injection. (A color version of this figure is available in the online journal.)

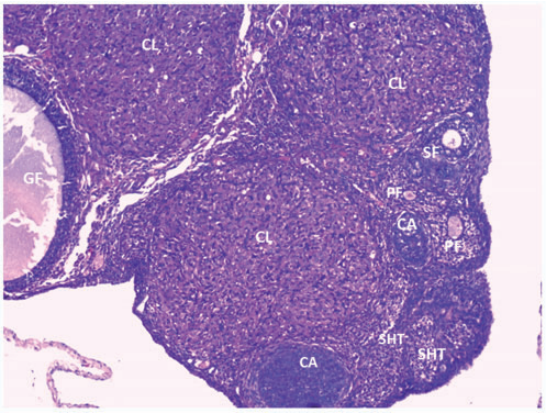

Figure 5 Photograph of ovarian tissue from HSP + CP-treated group (hematoxylin and eosin [H&E], 200X). Ovary section shows normal ovulation; primary follicles (PFs), secondary follicles (SFs), mature corpus luteum (CL), and mature corpus albicans (CA) are observed. Hyperthecosis of stromal intestinal cells is also observed. HSP + CP: 100 mg/kg of hesperidine orally, daily for eight days + 150 mg/kg of CP single i.p. injection on eighth day. (A color version of this figure is available in the online journal.)

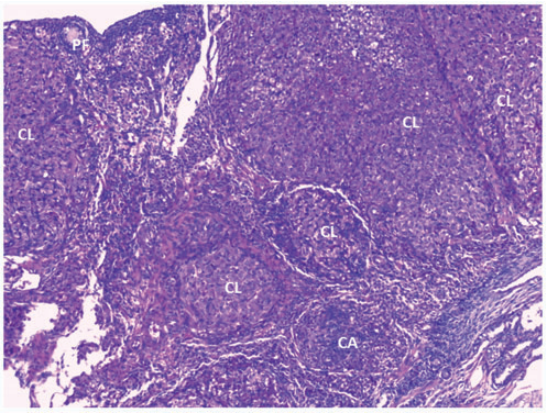

Figure 4 Photograph of ovarian tissue from MTZ + CP-treated group (hematoxylin and eosin [H&E], 200X). The normal morphology of the ovary with multiple mature corpus lutea (CL) is observed. Also, congestion of stroma is observed. MTZ + CP: 30 mg/kg of mirtazapine orally, daily for eight days + 150 mg/kg of CP single i.p. injection on eighth day. (A color version of this figure is available in the online journal.)

CP is a prodrug that is activated by oxidation at C-4 position by the hepatic cytochrome P450 enzymes resulting in the formation of 4-hydroxycyclo-phosphamide, which rapidly interconverts to acyclic aldehydes, aldophosphamide. These aldehydes spontaneously decompose to acrolein, chloroacetaldehyde (CAA), and PM. This pathway accounts for less than 10% of the metabolism of CP. The therapeutic effects of CP are attributed to PM, while acrolein and CAA are associated with cellular toxicities. PM produces interstrand cross-links between the N7-positions of guanines on opposite DNA strands; these cross-links are believed to be the major cytotoxic lesion.[17]

Both acrolein and CAA alter Ca^{2+} homeostasis and disruption of mitochondria oxidative phosphorylation. At little concentrations, acrolein increases intracellular Ca^{2+} resulting in increased NO levels, which may explain the mechanism of how acrolein exposure results in apoptosis in human endothelial cells. Acrolein and CAA can alkylate DNA at guanine bases.[17]

In the present study, the observed decrease in ovary weight in CP-treated group was in accordance with previous studies,[18] which found that mice treated with CP exhibited a reduction in uterine weight concurrent with a decrease in plasma estradiol concentrations, and ovarian atrophy. Thereby indicates toxicity to the ovary.

In addition, histopathology and histomorphometry of rat ovaries showed that the number of layers of granulosa cells and follicle's count in CP treated rats did not increase as it should under physiological conditions. These results were

in agreements with previous reports which demonstrated that a single injection of 200 mg/kg CP, in adult rats, destroyed all types of follicles. This destruction was caused by induction of apoptosis in granulosa cells of these follicles and was associated with activation of caspase-9 and caspase-3.[19]

Our results showed that the toxicity of CP increased lipid peroxidation in ovarian mucosal tissue and level of MDA. These results may be explained on the basis that, ROS and reactive nitrogen species (RNS) include superoxide anion radicals, hydroxyl radicals, hydrogen peroxide (H_2O_2), peroxynitrite, and other peroxides and NO are formed through leakage of electrons from the inner mitochondrial membrane during oxidative phosphorylation and ATP generation in steroidogenic tissues such as the ovary.[20]

Oxidative stress disrupts cellular redox circuits, resulting in disturbances of redox-regulated cellular processes. ROS and RNS react with cellular lipids, proteins, and nucleic acids. Moreover, it has been reported that H_2O_2 is a potent oxidant that inhibits steroidogenesis in ovarian cells.[21]

The detoxification of ROS is important for the oocyte maturation and embryo development. If ROS and RNS are not neutralized by endogenous or exogenous antioxidant molecules, lipid peroxidation would occur and unsaturated lipids were converted to peroxides. The degradation products with toxic aldehyde moieties such as MDA interfere with the ovarian reproductive functions.[3] It was reported that lipid peroxidation within plasma membrane of luteal cells was associated with loss of gonadotrophin receptors and decreased steroidogenic ability of CL.[22]

In the present study NO level was elevated after single dose of CP. NO is a highly RNS that plays an important role in the pathogenesis of CP-induced toxicity. NO is produced from the amino acid L-arginine by the enzyme NO synthase (NOS), and it regulates a number of important physiological and pathophysiological processes including vascular tone, polymorphonuclear leukocytes adhesion and inflammation.[20]

NOS isozyme II is localized in granulosa cells of CL and produces large amounts of NO. NOS is activated by cytokines such as, interleukin-1, tumor necrosis factor α (TNF-α), and lipopolysaccharides. Overproduction of NO is a characteristic of inflammatory process. Excess NO inhibits progesterone production and causes apoptotic cell death in rat granulose cells. Moreover, generated NO in human granulosa-luteal cells appears to inhibit estradiol secretion by inhibiting aromatase.[23] These reports suggested the role of NO overproduction in CP-induced ovarian toxicity besides the role of ROS.

In the present study, antioxidant enzymes (SOD and GPx) were reduced by single dose of CP. CP induces oxidative stress due to over-production of ROS and RNS. Together, SOD and GPx are synergistically scavenging ROS. These interacting defense mechanisms permit cells to live in an oxidative environment, perform its biochemical processes, and even use these ROS as signaling molecules.[24]

SOD reacts with superoxide anion radicals to form oxygen and H_2O_2. SOD plays a role in regulating follicular development, ovulation, and luteal functions. It was reported that ovaries of adult female *Sod1* null mice had reduced numbers of preovulatory follicles and corpora lutea. They concluded that these mice were subfertile because of a defect in late follicular development or ovulation. There is an evidence that copper chaperone for SOD null mice, which have decreased ability to incorporate copper into Cu/Zn SOD (in cytoplasm) have abnormal development of antral follicles and no corpora lutea.[20]

GPx is a well-known first line of defense against oxidative stress; it catalyzes the transformation of H_2O_2 to harmless byproducts. In addition, GPx catalyzes the degradation of lipid peroxides and can metabolize lipid hydroperoxides to less reactive hydroxy fatty acids.[20]

Oocytes, granulosa cells, and lutein cells all express high levels of GPx. Because CL produces progesterone by consuming molecular oxygen with the reaction of cytochrome P450. ROS is produced as a byproduct, damage could be inflicted by ROS. The detoxification of the produced ROS by glutathione in conjunction with antioxidative enzymes is important for the CL and surrounding cells.[22]

Our results also showed that CP caused a significant increase in the MPO activity, a marker of inflammation and oxidative stress. MPO is a hemoprotein characterized by powerful pro-oxidative and pro-inflammatory properties. It is stored in azurophilic granules of polymorphonuclear neutrophils and macrophages and released into extracellular fluid during inflammation.[25]

MPO catalyzes the production of hypochlorous acid from H_2O_2 and chloride. H_2O_2 is formed from the dismutation of superoxide, which is generated by an NADPH oxidase in the plasma membrane of neutrophils. The hypochlorous acid is a sulfhydryl inhibitor that could inactivate proteins containing sulfhydryl groups and causes damage to vascular endothelium.[4]

In the present study, pre-treatment of rats with MTZ and HSP as antioxidant agents against ovarian oxidative damage induced by single dose of CP significantly ameliorated the histological deterioration and the biochemical changes in rat ovaries. NO and MDA levels and MPO activity were reduced by MTZ and HES administration. Scavengers of ROS (SOD and GPx) were increased in their activities by MTZ and HES treatment. These results were also confirmed by increased number of follicles and ovarian weights in both MTZ-CP and HSP-CP groups compared with CP-treated group.

The antidepressant MTZ restores the antioxidant status of the ovary. MTZ reduces the formation of potent oxidant peroxynitrite which is produced by the reaction of NO with superoxide anion by scavenging the superoxide anion with SOD activity. In addition, it was found that the incidence of depression was high among cancer patients because of the adverse effects of chemotherapy. The severity of depression and anxiety was found to be higher among infertile women.[4]

Previous studies demonstrated that MTZ (15, 30 and 60 mg/kg) displayed a significant reduction in mucosal damage induced by indomethacin through ROS mediated lipid peroxidation. Also, it was reported that MTZ (10 and 20 mg/kg) has anti-ulcer and anti-inflammatory effects in gastric ulcer models.[26]

In the present study, the citrus flavonoid HSP showed a protective effect against CP-induced ovarian toxicity. It has numerous biological activities such as immune stimulation and scavenging the ROS. HSP can utilize reactive oxygen metabolites, protecting biopolymers and reduce oxidative DNA damage.[27] Previous studies demonstrated that HSP administration to breast cancer bearing rats improved the macromolecular structure such as total protein content in breast, liver tissues, and serum suggesting the maintenance of cell structure and integrity and also modulation of nucleic acids.[28] Moreover, HSP attenuates the peroxidation reaction and membrane bound marker enzyme activities as well as upregulates adenosine triphosphatases, tricarboxylic acid cycle enzymes, and antioxidants.[29]

Conclusion

Based on biochemical and histomorphometrical analyses of rat ovaries, single dose (150 mg/kg) of CP resulted in ovarian toxicity, increased lipid peroxidation and oxidative stress in rat ovary, and decreased the ovarian follicle counts. Pre-treatment with HSP and MTZ protected rat ovaries from CP-induced toxicity and infertility. MTZ as antidepressant and antioxidant may be recommended as adjuvant therapy in patients receiving chemotherapy. Adding HES to CP therapy suggests its promising uses in the future treatment of oxidative stress-mediated toxicity.

Author contributions: The author participated in the design, interpretation of the study and analysis of the data, and review of the manuscript. All the experiments were conducted by the author except the histopathology part; it was conducted by an expert pathologist.

ACKNOWLEDGMENT

The authors thank Dr Karima Marea professor of pathology (Faculty of medicine, Tanta University, Egypt) for her excellent assistance with histopathology of ovary tissue and data analysis.
The study is financially supported by faculty of pharmacy, Tanta University Egypt.

REFERENCES

1. Mark-Kappeler CJ, Hoyer PB, Patrick J, Devine PJ. Xenobiotic effects on ovarian preantral follicles. *Biol Reprod* 2011;**85**:871–83
2. Lemos CN, Reis FM, Pena GN, Silveira LC, Camargos AF. Assessment of fertility protection and ovarian reserve with GnRH antagonist in rats undergoing chemotherapy with cyclophosphamide. *Reprod Biol Endocrinol* 2010;**18**:51–7
3. Yener NA, Sinanoglu O, Ilter E, Celik A, Sezgin G, Midi A, Deveci U, Aksungar F. Effects of spirulina on cyclophosphamide-induced ovarian toxicity in rats: biochemical and histomorphometric evaluation of the ovary. *Biochem Res Int* 2013;**2013**:764262
4. Altuner D, Gulaboglu M, Yapca OE, Cetin N. The effect of mirtazapine on cisplatin-induced oxidative damage and infertility in rat ovaries. *Sci World J* 2013;**22**:327240
5. Wilmsen PK, Spada DS, Salvador M. Antioxidant activity of the flavonoid hesperidin in chemical and biological systems. *J Agric Food Chem* 2005;**53**:4757–61
6. Cho Jungsook. Antioxidant and neuroprotective effects of hesperidin and its aglycone hesperetin. *Arch Pharm Res* 2006;**29**:699–706
7. Ataya KM, Valeriote FA, Ramahi-Ataya AJ. Effect of cyclophosphamide on the immature rat ovary. *Cancer Res* 1989;**49**:1660–4
8. Hozayen WG. Effect of hesperidin and rutin on doxorubicin induced testicular toxicity in male rats. *Int J Food Nutr Sci* 2012;**1**:31–42
9. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med* 1967;**70**:158–69
10. Nishikimi M, Roa NA, Yogi K. Measurement of superoxide dismutase. *Biochem Biophys Res Commun* 1972;**46**:849–54
11. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin-phenol reagent. *J Biol Chem* 1951;**193**:265–75
12. Ridnour LA, Sim JE, Hayward MA, Wink DA, Martin SM, Buettner GR, Spitz DR. A spectrophotometric method for the direct detection and quantitation of nitric oxide, nitrite, and nitrate in cell culture media. *Anal Biochem* 2000;**281**:223–9
13. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979;**95**:351–8
14. Westwood FR. The female rat reproductive cycle: a practical histological guide to staging. *Toxicol Pathol* 2008;**36**:375–84
15. Erickson GF. Follicle growth and development. In: Sciarra JJ (ed.). *Gynecology and obstetrics* (Vol. 5). Philadelphia: Lipincott-Raven, 2000
16. Landau S, Everitt BS. *Handbook of statistical analyses using SPSS*. USA: Chapman & Hall/CRC Press LLC, 2004
17. Hansen RJ, Susan M, Ludeman SM, Sari J. Paikoff SJ, Pegg AE, Eileen Dolan ME. Role of MGMT in protecting against cyclophosphamide-induced toxicity in cells and animals. *DNA Repair (Amst)* 2007;**6**:1145–54
18. Plowchalk DR, Meadows MJ, Mattison DR. Reproductive toxicity of cyclophosphamide in the C57BL/6N mouse: effects on uterine structure and function. *Reprod Toxicol* 1992;**6**:423–9
19. Sato MI, Shiozawa K, Uesugi T, Hiromatsu R, Fukuda M, Kitaura K, Minami T, Matsumoto S. Collaborative work on evaluation of ovarian toxicity. 7) Effects of 2- or 4- week repeated dose studies and fertility study of cyclophosphamide in female rats. *J Toxicol Sci* 2009;**34**:SP83–89
20. Devine PJ, Perreault SD, Luderer U. Roles of reactive oxygen species and antioxidants in ovarian toxicity. *Biol Reprod* 2012;**86**:1–10
21. Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. *Reprod Biol Endocrinol* 2005;**3**:28–39
22. Motta AB, Estevez A, Tognetti T, Gimeno MAF, Franchi AMF. Dual effects of nitric oxide in functional and regressing rat corpus leuteum. *Mol Hum Reprod* 2001;**7**:43–7
23. Fujii J, Iuchi Y, Okada F. Fundamental roles of reactive oxygen species and protective mechanisms in the female reproductive system. *Reprod Biol Endocrinol* 2005;**3**:43
24. Kamarzaman S, Shaban M, Abdul Rahman S. The prophylactic effect of *Nigella sativa* against cyclophosphamide in the ovarian follicles of matured adult mice: a preliminary study. *J Anim Plant Sci* 2014;**24**:81–8
25. Arnhold J. Free radicals. Friends or foes? Properties, Functions, And Secretion Of Human Myeloperoxidase. *Biochemistry (Moscow)* 2004;**69**:4–9
26. El-Awdan SA, Zaki HF. Gastroprotective activity of mirtazapine, escitalopram and venlafaxine in depressed rats. *Afr J Pharm Pharmacol* 2013;**7**:2701–9
27. Al-Jasabi S, Abdullah MS. The role of antioxidant hesperidin in the attenuation of lung cancer caused by benzo[a] pyrene in Balb/c mice. *World Appl Sci J* 2013;**22**:1106–10

28. Nandakumar N, Jayaprakash J, Rengarajan T, Ramesh V, Balasubramanian MP. Hesperidin, a natural citrus flavonoglycoside, normalizes lipid peroxidation and membrane bound marker enzymes in 7, 12-dimethylbenz (a) anthracene induced experimental breast cancer rats. *Biomed Prev Nutr* 2011;**1**:255–62
29. Nandakumar N, Balasubramanian MP. Hesperidin protects renal and hepatic tissues against free radical-mediated oxidative stress during DMBA-induced experimental breast cancer. *J Environ Pathol Toxicol Oncol* 2011;**30**:283–300

(Received September 25, 2014, Accepted January 27, 2015)