Histological and Immunohistochemical study on the possible protective effect of hesperidin on the ovaries of adult female albino rats treated with cyclophosphamide

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Abstract: Cyclophosphamide (CP) is an alkylating agent widely used in the treatment of many types of malignant tumors and autoimmune disorders. Although, CP treatment is important for survival of the patient, might have negative side effects, including detrimental effects on the reproductive system. It is usually associated with a high risk of female infertility resulting from premature ovarian insufficiency. Hesperidin (HSP) is a plant chemical that is classified as bioflavonoid, it is found in citrus fruits, vegetables, in food products and beverages derived from plant, as tea and olive oil. It has been reported to exert a wide range of pharmacological effects, which include, antioxidants, antitumor, anti- allergic, hypolipidemic, anti-inflammatory and vasoprotective effects. It has strong cellular antioxidant protection against the damaging effects induced by cyclophosphamide treatment. The target of the present study is to estimate the possible protective effect of hesperidin against cyclophosphamide induced ovarian toxicity. The animals were randomly divided into four groups. Group I (Control group), the animals were given phosphate buffered saline for eight days. Group II (Hesperidin treated group), the animals were given HSP 100 mg/kg/d orally for eight days. Group III (Cyclophosphamide treated group), the animals were given CP 150 mg/kg single intraperitoneal injection on the 8th day of the experiment. Group IV (Cyclophosphamide and hesperidin treated group), the animals were given HSP 100 mg/kg/d orally for eight days and CP 150 mg/kg single intraperitoneal injection on 8th day of the experiment. Animals were then sacrificed at the end of experiment and ovaries were used for histological, histochemical and immunohistochemical study. CP-treated group showed degenerative changes of the ovary with highly significant reduction of primordial, primary, secondary and graafian follicles when compared with the control group. However, combined treatment of HSP and CP showed amelioration of the histological changes in the ovary. **Conclusion**: It has been concluded that hesperidin improves the histological changes caused by cyclophosphamide in the ovary.
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Keywords: Ovary, Cyclophosphamide, Hesperidin, Caspase-3

1. Introduction

Cyclophosphamide (CP) is an alkylating agent widely used in the treatment of many types of malignant tumors such as Hodgkin's disease and breast cancer (Tomao et al., 2010). Breast cancer is the second most frequent malignancy, affecting around 11,000 women per year, early diagnosis and treatment with CP improve prognosis of malignancy but can have damaging effect on the gonadal function (De Santis et al., 2011).

Cyclophosphamide is transformed by the liver into two chemicals, acrolein and phosphoramide which are the active compounds and they slow the growth of cancer cells by interfering with deoxyribonucleic acid (DNA) actions within the cells. Normal cells are also affected and this results in serious side effects and suppression of the immune system and is referred to as immunosuppressive (Tomao et al., 2010).

CP treatment could cause several side effects as acute cardiotoxicity, acute damage to kidneys, bone marrow suppression, opportunistic infections, hemorrhagic cystitis, nausea, vomiting and hair loss. It is associated with a high risk of female infertility resulting from premature ovarian insufficiency (Ezoe et al., 2014). It causes progressive and irreversible damage to oocytes and is thought to be related to duration of therapy and the state of gonadal function at the time of treatment (Yener et al., 2013).

Cyclophosphamide deteriorates ovarian functions by rapid depletion of the oocyte reserve which was mediated by prevention of cell division and inhibition of DNA function with disappearance of resting primordial follicles and growing follicles (Salama et al., 2013).

The alkylating agents could cause damage of blood vessels, cortical fibrosis, reduction of ovarian follicles and apoptosis in granulosa cells (Meirow et al., 2007)].

Cyclophosphamide and its toxic metabolites run against intracellular antioxidation systems which play an important role in detoxifying the reactive oxygen species (Yener et al., 2013).

Young patients treated with chemotherapy might suffer from permanent ovarian failure and infertility (Jeruss et al., 2009), so, there is an urgent need for a method for preserving ovarian function.

Hesperidin (HSP) is a plant chemical that is classified as bioflavonoid, it is found in citrus fruits (such as oranges, lemons or pummelo fruits), vegetables, in food products and beverages derived from plant, as tea and olive oil. It is a sugar bound form of the flavonoid hesperitin. Hesperitin is known to mediate the hesperidin actions in the body (Hwang et al., 2012).

HSP is most often used for blood vessel conditions such as hemorrhoids, varicose veins, chronic venous insufficiency and poor circulation, it is also used to treat lymphedema after breast cancer surgery (Hwang et al., 2010), arthritis and venous leg ulcers. It has been reported to exert a wide range of pharmacological effects, which include, antioxidants (Sun et al., 2013), antitumor (Coelho et al., 2013), antiallergic, hypolipidemic, vasoprotective and anti-inflammatory effects (Saiprasad et al., 2013).

It has strong cellular antioxidant protection against the damaging effects induced by cyclophosphamide treatment (Wilmsen et al., 2005). As, a citrus bioflavonoid, hesperidin facilitates the formation of vitamin C complex, which supports healthy immune system functions (Peterson et al., 2006).

The purpose of the present study is to estimate the possible protective effect of hesperidin against cyclophosphamide induced ovarian damage.

2. Material and Methods

Animals

Forty adult female rats of average weight 150-200 grams were used in this study. The animals were get from the animal house of faculty of medicine, Menoufia University. The rats were put in a healthy standard environmentalconditions and fed with basal diet and tap water. The rats were adapted to laboratory conditions a week before start of the study.

Chemicals and Drugs

Cyclophosphamide (CP): (cycram) 500 mg vial was purchased from EIMC Pharmaceuticals CO.

Hesperidin (HSP): HSP powder was dissolved in phosphate buffered saline (PBS). HSP and PBS were obtained from Sigma chemical company (St. Louis, MO, USA).

Experimental protocol

Animal experiments were carried out in an ethical manner following the guidelines set by the Ethical committee of Menoufia University. The animals were divided into four groups randomly (10 animals each).

Group I (control group): The animals of this group were given phosphate buffered saline for eight days.

Group II (Hesperidin treated group): The animals were given HSP 100 mg/kg/d orally for eight days (Hozayen, 2012).

Group III (Cyclophosphamide treated group): The animals were given CP 150.

mg/kg single intraperitoneal injection on the 8th day of the experiment (Yener et al., 2013).

Group IV (Cyclophosphamide and hesperidin treated group): The animals were given HSP 100 mg/kg/d orally for eight days and CP 150 mg/kg single intraperitoneal injection on 8th day of the experiment.

The animals were scarified by cervical decapitation, 24h after single i.p injection of CP. Both ovaries of each animal were removed and cleaned by normal saline. The weight of ovaries was measured and then fixed in 10 % formal saline. The ovaries were underwent to the following studies.

I. Histological study:

The ovaries were fixed in formal saline and processed for Paraffin sections of about 5-6 µm thickness. Sectioned were obtained and stained with hematoxylin and eosin (Hx & E) to show the histological details & Mallory's trichrome stain to detect the collagen fibers (Bancroft and Layton, 2010):

II. Histochemical study:

Periodic acid-Schiff (PAS) stain is specific for detection of glycogen (Bancroft and Gamble, 2013).

III-Immunohistochemical study:

Caspase3: Sections were subjected to staining with the primary rabbit polyclonal anti-caspase-3 antibody (Thermo Scientific, Lab Vision, USA **) (Sani *et al.*, 2012*).***

Quantitative Morphometric study

Haematoxylin and eosin stained sections of the ovaries from each experimental group were examined under microscopy at high power field.. Various fields were chosen and ten readings were obtained from each group. The number of different types of ovarian follicles was counted at a total magnification of 400 and the mean values were obtained.

Statistical analysis

The data (ovarian weight and the number of each type of ovarian follicles) were expressed as mean ±SD. The student t-test was used to evaluate the significant change in each parameter in the experimental groups when compared with the control group. The statistical analysis of data was carried out using Excel and statistical package for the social science software, version 11. The significance was set a P- value less than 0.05 (Peat and Barton, 2005):

3.Results

Histological results

H & E

Examination of sections of an adult ovary from the control group showed an intact germinal epithelium and wide cortex containing numerous primordial follicles, primary follicle, and a secondary follicle (Fig. 1). The primordial follicles were seen under tunica albuginea consisting of an oocyte with prominent nucleus and nucleolus surrounded by a single layer of squamous follicular cells (Fig. 2). The primary follicle consisted of an oocyte with prominent nucleus and surrounded by well-defined zonapellucida and follicular granulosa cells (Fig. 3).

The ovary also contained secondary follicles with multiple cavities surrounded by follicular granulosa cells and peripheral fusiform theca folliculi cells (Fig. 4)), clusters of stroma cells were seen (Figs. 1, 2, 3 & 4). Graafian follicles were noticed consisting of an oocyte with a well-defined zonapellucida and corona radiata and connected to the wall of the follicle by cumulus oophorus. Wide cavity full of liquor folliculi appeared within the follicle surrounded by multilayer of granulosa cells and fusiform theca cells (Fig. 5).

Sections from hesperidin treated group revealed a picture more or less similar to the control group.

Sections from cyclophosphamide treated rats showed multiple atretic follicles with loss of oocytes. The blood vessels (BV) are dilated and congested. The wall of some blood vessels is thickened (Fig. 6). Primary follicle consisted of an oocyte, darkly stained follicular granulosa cells, the blood vessel contained eosinophilic hyaline material (Fig. 7). Collagen fibers deposition with cellular infilteration were seen (Figs. 6, 7, 8 & 9). The tunicaalbuginea was thickened, secondary follicle consisted of a degenerated oocyte, small cavities, and disorganized darkly stained follicular granulosa cells (Fig. 8). The stroma cells appeared vacuolated and arranged in clusters (Figs.6,7 & 8). The germinal epithelium was darkly stained (Fig. 9 & 10). Degenerated follicle with degenerated oocyte was observed, the cells of the zonagranulosa were degenerated, some with dark pyknotic nuclei and others with faint nuclei (Fig. 9). The Graafian follicle consisted of a degenerated oocyte, surrounded by disorganized corona radiata cells, follicular granulosa cells with darkly stained nuclei and antrum containing exfoliated cells, the capsule of the ovary was corrugated (Fig. 10). The follicular granulosa cells appeared vacuolated (Figs. 9 & 10).

Examination of sections from cyclophosphamide and hesperidin treated group showed apparently healthy primordial, primary, and secondary follicles. Also, atretic follicles appeared (Fig. 11). The follicles were almost identical to those of the control group. The primary follicle was composed of anoocyte surrounded by clear zonapellucida, and granulosa cells (Fig. 12). The secondary follicle consisted of an oocyte with well-defined zonapellucida, and multiple cavities surrounded by follicular granulosa cells and peripheral fusiform theca folliculi cells (Fig. 13).

Graafian follicle appeared more or less similar to control, consisting of an oocyte with a well definedzonapellucida, corona radiata, multilayers of granulosa cells and theca folliculi cells with appearance of some haemorrhagic spots (Fig. 14).

Mallory's trichrome stain

Examination of an adult ovary from the control group exhibited minimal collagen fibers deposition around mature graafian follicle (Fig. 15). While massive collagen fibers deposition around ovarian follicles and blood vessels were observed in the ovary of cyclophosphamide treated group (Fig. 16). Sections from CP and HSP treated group showed minimal collagen fibers depositon around ovarian follicles (Fig. 17).

Histochemical results

PAS stain

PAS stained sections of the control group showed strong reaction in the germinal epithelium, growing follicles, developing ova, zonapellucida, granulosa and theca cells (Fig. 18). Sections from CP treated group showed weak reaction (Fig. 19). While sections from CP and HSP treated group showed strong PAS reaction in mature Graafianfollicle, developing ovum, zonapellucida, corona radiata, granulosa and theca cells (Fig. 20).

Immunohistochemical results

Caspase-3immunostaining

Section from the control group exhibited weak caspase-3 immunoreaction (Fig. 21). Strong cytoplasmic immunoreaction to caspase-3 was seen in the granulosa cells and stroma cells in the ovary of cyclophosphamide treated group (Fig. 22). While, section from cyclophosphamide and hesperidin treated group, showed weak immunoreaction in the granulosa cells and stroma cells (Fig. 23).

Morphometric and statistical results

The mean ovarian weight of an adult rats treated with CP (group III) showed significant decrease when compared with the control group ($P<0.05$). The mean ovarian weight of CP and HSP treated group and that of adult rats treated with HSP showed a non-significant decrease as compared with control group (Table 1). Rats treated with CP showed highly significant decrease in the number of primordial, primary, secondary and mature graafian follicles and corpus luteum ($P<0.001$) but a highly significant increase in the number of atretic follicles as compared with the control group ($P<0.001$). Rats treated with CP and HSP and rats treated with HSP only showed a non-significant decrease in the number of primordial, primary, seconday, and mature graafian follicles but a

nonsignificant increase in the number of atreticfollicles compared with control group (Table 1).

Table. 1

	group I (control group	group II (HSP treated group)	group III (CP treated group)	group IV (CP and HSP treated group)	F. test	P. value
Ovarian weight	0.04±0.01	0.04±0.02	0.03±0.01	0.04±0.01	3.277	0.028 P1 =0.881>0.05 P2 =0.020<0.05 P3 =0.765>0.05
Atretic. follicles	1.37±0.18	1.17±0.13	17.51±9.07	1.59±0.13	47.406	0.000 P1 = 0.904>0.05 P2 = 0.000<0.001 P3 = 0.892>0.05
corpus. luteum	1.35±0.14	1.31±0.13	0.19±0.14	1.27±0.17	228.752	0.000 P1 = 0.451>0.05 P2 = 0.000<0.001 P3 =0.106>.0.05
Graafian follicles	1.89±0.14	1.83±0.23	0.45±0.16	1.80±0.18	214.562	0.000 P1 = 0.23>0.05 P2 = 0.000<.001 P3 =0.192>0.05
secondary. follicles	4.23±0.14	4.21±0.14	1.30±0.16	4.13±0.21	1161.449	0.000 P1 = 0.740>0.05 P2 = 0.000<0.001 P3 =0.101>0.05
primary. follicles	5.05±0.16	4.99±0.16	2.00±0.16	4.94±0.30	789.475	0.000 P1 = 0.425>0.05 P2 = 0.000<0.001 P3 =0.46>0.05
Primordial follicles	23.13±0.34	23.14±0.24	3.44±0.16	23.12±0.32	19337.889	0.000 P1 = 0.895>0.05 P2 = 0.000<0.001 P3 =1.000>0.05

P1 Comparison was done between control group and HSP treated group (group II).
P2 Comparison was done between control group and CP treated group (group III)
P3 Comparison was done between control group and CP and HSP treated group (group IV). P>0.05 means NS. P<0.05 means significant.. P<0.001 means highly significant.

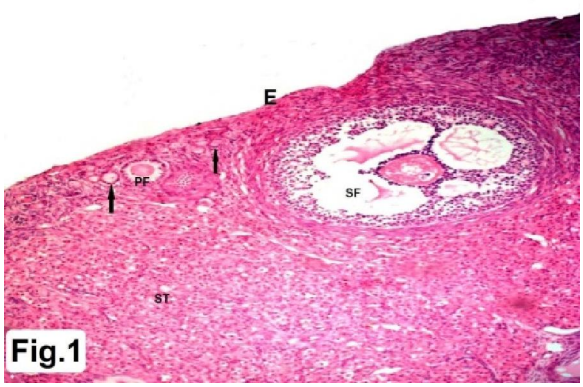

Fig. (1): Aphotomicrograph of an adult rat ovary from the control group showing wide cortex containing primordial follicles (arrows), a primary follicle (PF) and a secondary follicle (SF). Notice, ovarian stroma (ST) and germinal epithelium (E). (H & E x 200).

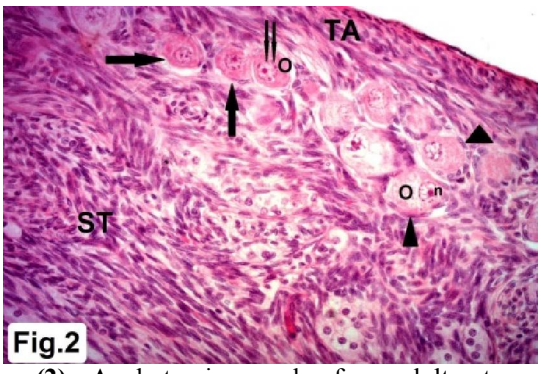

Fig. (2): A photomicrograph of an adult rat ovary from the control group showing multiple primordial follicles (arrows) under tunica albuginea (TA). Each consists of an oocyte (O) with prominent nucleus (double arrow) and nucleolus (n) surrounded by a single layer of squamous follicular cells (arrowheads). Clusters of stroma cells (ST) can be seen. (H & E x 400).

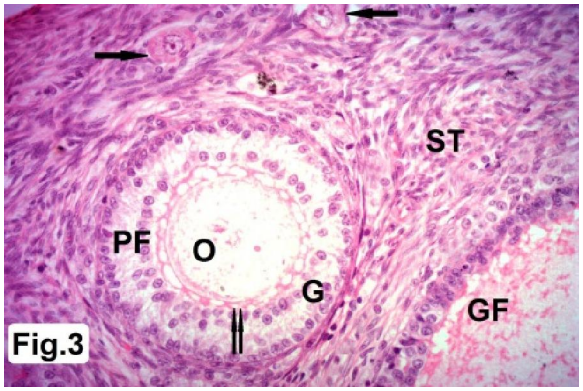

Fig. (3): Aphotomicrograph of an adult rat ovary from the control group showing primordial follicles (arrows), a primary follicle (PF) and a part of Graafian follicle (GF). The primary follicle consists of an oocyte (O) with prominent nucleus surrounded by well definedzonapellucida (double arrow) and follicular granulosa cells (G). Clusters of stroma cells (ST) can be seen. (H & E X400)

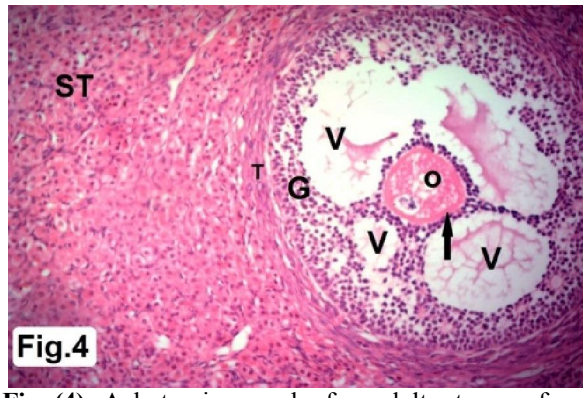

Fig. (4): Aphotomicrograph of an adult rat ovary from control group showing a secondary follicle. It consists of an oocyte (O) with well definedzonapellucida (arrow), and multiple cavities (V) surrounded by follicular granulosa cells (G) and peripheral fusiform theca folliculi cells (T). Clusters of stroma cells (ST) can be seen. (H & E x 400)

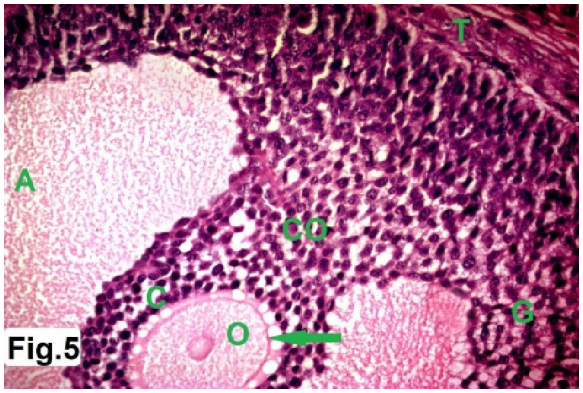

Fig. (5): A photomicrograph of an adult rat ovary from the control group showing a part of Graafian follicle. It consists of an oocyte (O) with a well definedzonapellucida (arrow) and corona radiata (C). The oocyte is attached to the wall of the follicle by cumulus oophorus (CO). A wide cavity (A) full of liquor folliculi appears within the follicle surrounded by multilayer of granulosa cells (G) and fusiform theca cells (T). (H & E x 400)

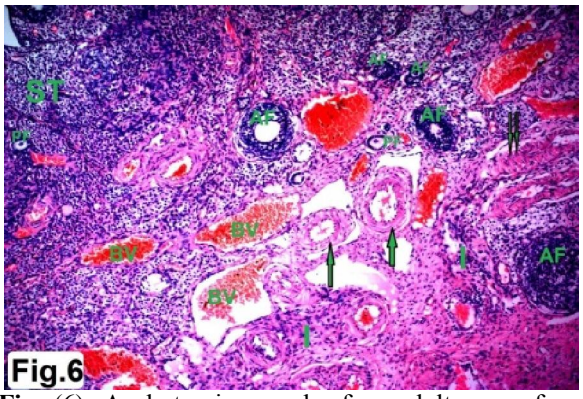

Fig. (6): A photomicrograph of an adult ovary from CP treated group showingprimary follicles (PF) and multipleatretic follicles (AF) with absence of oocytes. The blood vessels (BV) are dilated and congested. The wall of some blood vessels is thickened (arrows). Collage fibers deposition (double arrow) with cellular infilteration (I) can be seen. Notice, vacuolated stroma cells (ST) are arranged in clusters. H & E x 100

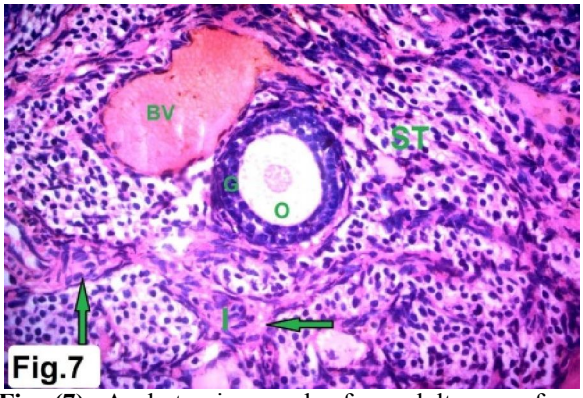

Fig. (7): A photomicrograph of an adult ovary from CP treated group showing a primary follicle consisting of an oocyte (O) and darkly stained follicular granulose cells (G). The blood vessel (BV) containseosinophilic hyaline material. Collagen fibers deposition (arrows) with cellular infilteration (I) are seen. Notice, vacuolatedstroma cells are arranged in clusters (ST). **H & E x400**

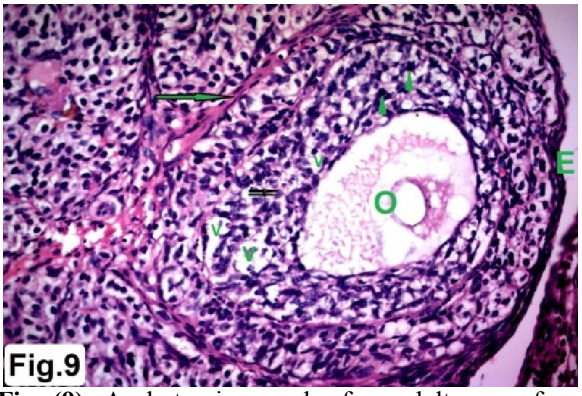

Fig. (9): A photomicrograph of an adult ovary from CP treated group showing a follicle with degenerated oocyte (O) and disorganization of the granulosa cells, some with dark nuclei (arrowheads) and others with faint nuclei (double arrows) with multiple vacuoles (V). Notice, collagen fibers deposition (arrow) and daklystained germinal epithelium (E). H & E x400

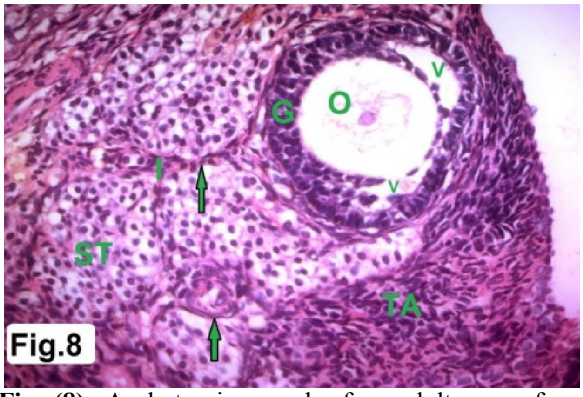

Fig. (8): A photomicrograph of an adult ovary from CP treated group showing thickened tunica albuginea (TA), a secondary follicle consisting of a degenerated oocyte (O), small cavities (V), and disorganized darkly stained follicular granulosa cells (G). Collagen fibers deposition (arrows) with cellular infilteration (I) are seen. Notice, vacuolated stroma cells (ST) are arranged in clusters. H & E x400

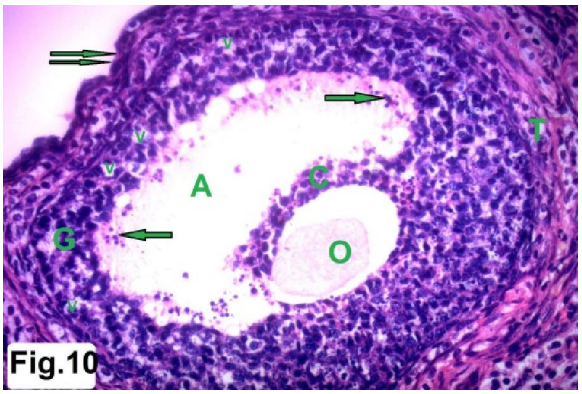

Fig. (10): A photomicrograph of an adult ovary from CP treated group showing Graafian follicle consisting of degenerated oocyte (O) surrounded by disorganized corona radiata cells (C) follicular granulose cells (G) with darkly stained nuclei and vacuoles (V), theca folliculi cells (T) and antrum (A) containing exfoliated cells (arrows). Notice, corrugated capsule and darkly stained germinal epithelium (double arrows). H & E x 400

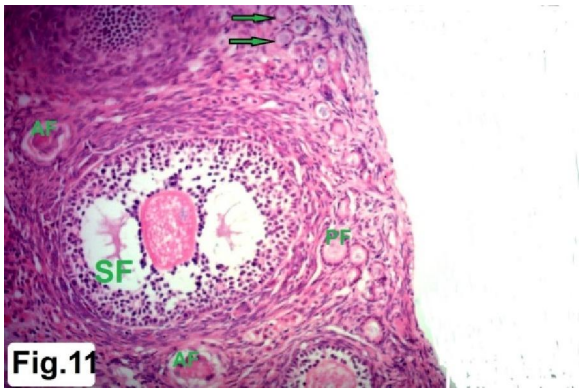

Fig. (11): **A**photomicrograph of an adult rat ovary from CP and HSP treated group showing primordial follicles (arrows), a primary follicle (PF), a secondary follicle (SF) and atretic follicles (AF). H & E. x 400

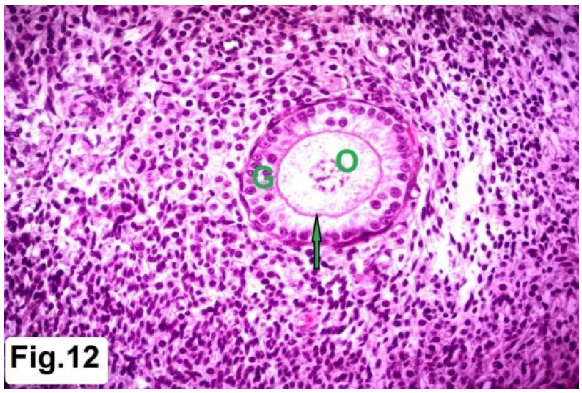

Fig. (12): **A**photomicrograph of an adult rat ovary from CP and HSP treated group showing a primary follicle. It consists of anoocyte (O) surrounded by clear zonapellucida (arrow), andgranulosa cells (G). (H & E. x 400)

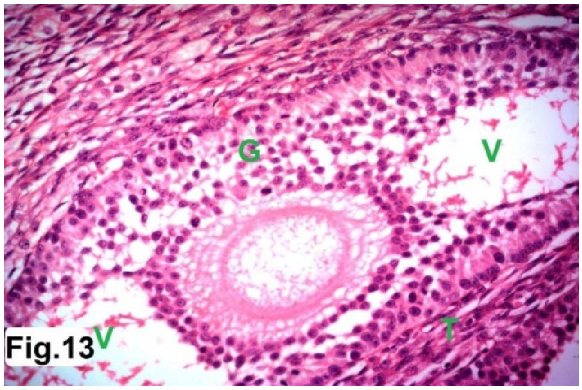

Fig. (13): **A**photomicrograph of an adult rat ovary from CP and HSP treated group showing a secondary follicle. It has multiple cavities (V) surrounded by follicular granulosa cells (G) and peripheral fusiform theca folliculi cells (T). (H & E x 400)

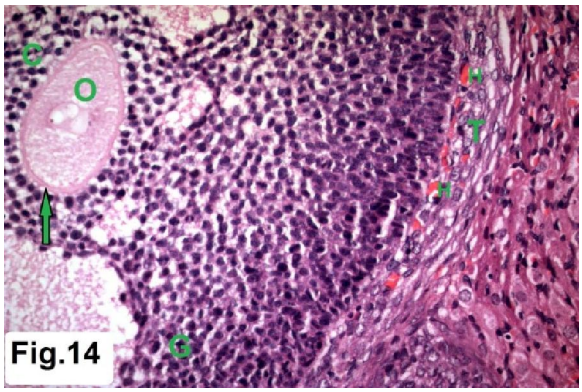

Fig. (14): A photomicrograph of an adult ovary from CP and HSP treated group showing a part of Graafian follicle appeared more or less similar to the control, consisting of an oocyte (O) with a well definedzonapellucida (arrow), corona radiata (C), multilayers ofgranulosa cells (G) and thecafolliculi cells (T). Notice, haemorrhagic spots (H). H & E x 400

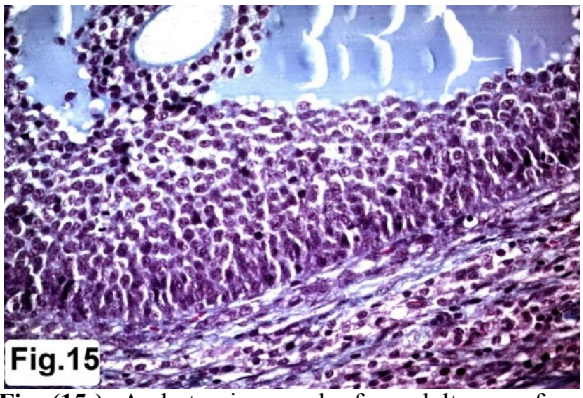

Fig. (15): A photomicrograph of an adult ovary from the control group showing minimal collagen fibers deposition around mature graafianfollicle. M.T x 400

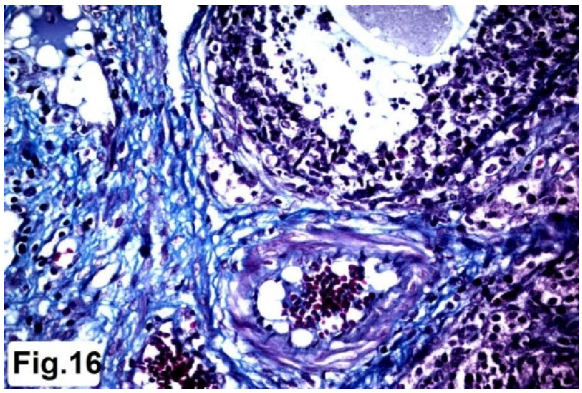

Fig. (16): A photomicrograph of an adult ovary from CP treated group showing massive collagen fibers deposition around ovarian follicles and blood vessels. M.T x 400

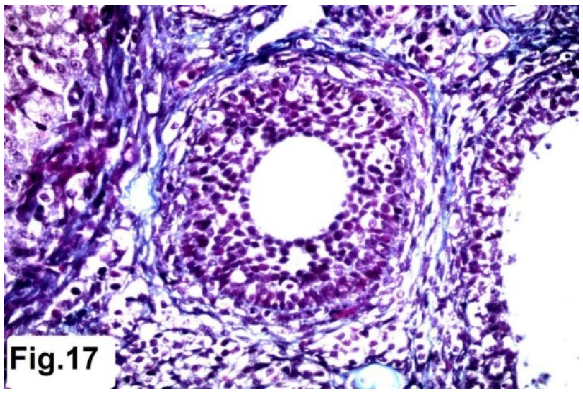

Fig. (17): A photomicrograph of an adult ovary from CP and HSP treated group showing minimal collagen fibers depositon around ovarian follicles. M.T x400

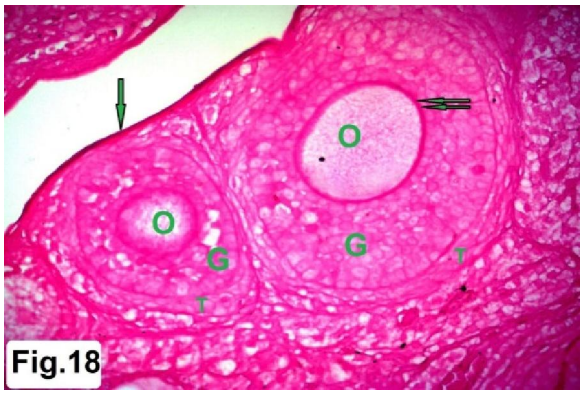

Fig. (18): A photomicrograph of an adult ovary from the control group showing strong PAS reaction in the germinal epithelium (arrow), growing follicles, developing ova (O), zona pellucid (double arrow), granulosa (G) and theca cells (T). PAS x 400

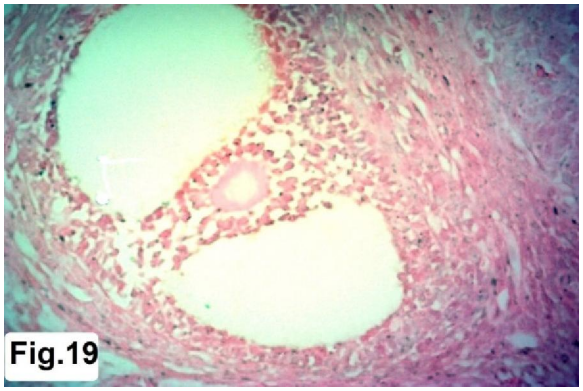

Fig. (19): A photomicrograph of an adult ovary from the CP treated group showing weak PAS reaction. PAS x 400

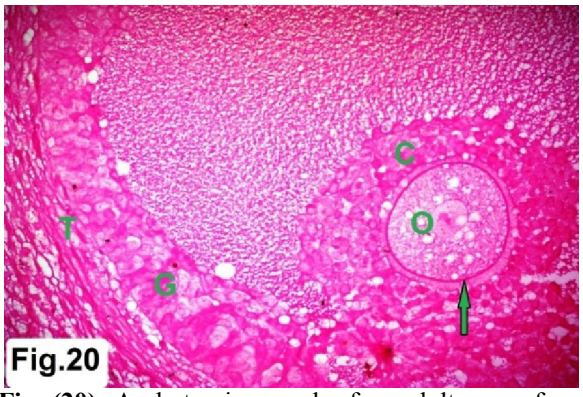

Fig. (20): A photomicrograph of an adult ovary from CP and HSP treated group showing strong PAS reaction in mature graafian follicle, developing ovum (O), zona pellucid (arrow), corona radiata (C), granulosa (G) and theca cells (T). PAS x 400

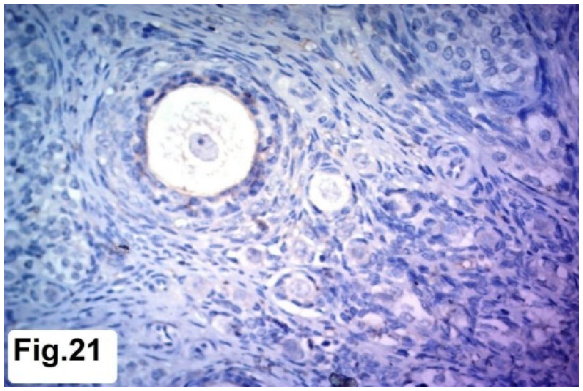

Fig. (21): A photomicrograph of an adult ovary from the control group showing weak cytoplasmic immunoreactivity for caspase-3. Caspase-3 X 400

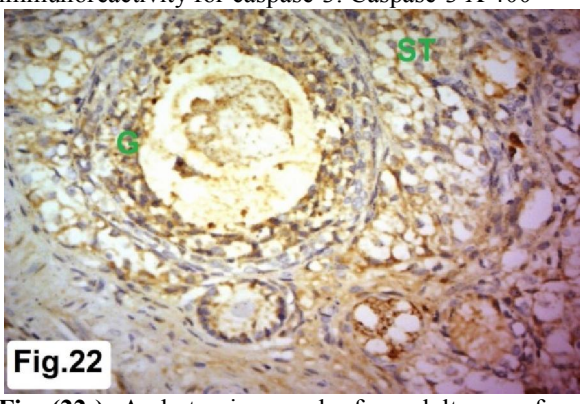

Fig. (22): A photomicrograph of an adult ovary from CP treated group showing strong positive cytoplasmic immunoreactivity for caspase-3 in the granulosa cells (G) and in the stroma cells (ST). Caspase-3 X 400

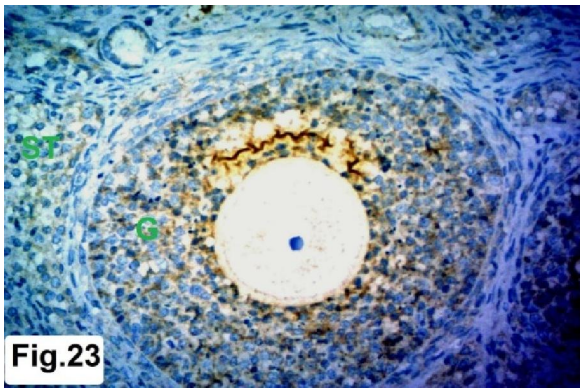

Fig. (23): A photomicrograph of an adult ovary from CP and HSP treated group showing weak cytoplasmic immunoreactivity for caspase-3 in the granulosa cells (G) and in the stroma cells (ST). Caspase-3 X400.

4. Discussion

Cyclophosphamide (CP) is an alkylating agent widely used in the treatment of many types of malignant tumors and autoimmune disorders. Although, CP treatment is important for survival of the patient, might have negative side effects, including detrimental effects on the reproductive system. It is associated with a high risk of female infertility resulting from premature ovarian insufficiency (Tomao et al., 2010).

The target of the present study is to estimate the possible protective effect of hesperidin against cyclophosphamide induced ovarian toxicity.

In the present study, cyclophosphamide (CP) treated group showed significant decrease in the ovarian weight and highly significant reduction in the number of primordial, primary, secondary and mature graafian follicles when compared to the control group. These results confirmed by histopathological changes and could be explained by damaging rapidly dividing granulosa cells in the developing follicles after CP therapy (Marcello et al., 2014). Destruction of follicles at all developmental stages has been found in humans and rodents (McLaren and Bates, 2012). The consequent decrease in gonadal steroid secretion stimulates primordial follicles into the pool of growing follicles which further destroyed by CP (Langan et al.,2011). The observed reduction in ovarian weight in cyclophosphamide treated group was in accordance with previous studies, which found that mice treated with CP exhibited reduction in uterine weight and ovarian damage (Plowchalk et al., 1992).

In the present study, ovarian sections in rats treated with cyclophosphamide showed considerable structural changes including degenerated follicles with degenerated oocytes. The granuolsa cells appeared with darkly stained or faintnuclei and some of these cells were exfoliated within the cavities of the follicles. The stroma cells were vacuolated with appearance of congested blood vessels and cellular infilteration.

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 (Sato et al., 2009). Ovarian atrophy was associated with inactive interstitial glands, and interstitial stromal cell hypertrophy or hyperplasia (Long et al., 2001).

Previous study reported that animals treated with cyclophosphamide showed irreversible destruction and disintegration of the granulosa cells associated with vascular complication and endovascular damage (Meirow et al.,2004). Follicular damage is believed to be the main cause of ovarian failure and infertility induced by chemotherapy (Sonmezer and Oktay, 2004). CP has been reported to destroy ovarian follicles by targeting granulosa cells in rats and mice (Desmeules and Devine, 2006).

Oxidative stress has been reported in CP-induced toxicity to granulosa cells of antral follicles, but the mechanism underlying small ovarian follicle loss remains unknown (Petrillo et al., 2011).

Also, previous studies exhibited that the ovaries after CP treatment showed marked cortical fibrosis and reduced number of follicles especially primordial follicles. CP could seriously damage ovarian endocrine function and induce infertility due to its gonadal toxicity (Xiu-Ying, 2016),

The cyclophosphamide requires metabolic activation via oxidation by hepatic cytochrome p 450 enzymes to the reactive metabolite phosphoramide mustard (PM). PM is believed to be the active metabolite responsible for CP's anticancer activity as well as its ovarian toxicity (Tomao et al., 2010).

Nese et al., 2013 found that MDA (Malondialdehyde) was markedly increased and the activity of SOD and GPx (antioxidant enzymes) were markedly decreased in the ovary of CP treated rats suggesting that CP treatment caused oxidative damage to the proteins and lipids, induce lipid peroxidation and promote the apoptotic cell death.

Cyclophosphamide administration led to a significant increase in the MPO (myeloperoxidase) activity, a marker of inflammation and oxidative stress. MPO is a hemoprotein distinguished by great pro-inflammatory and pro-oxidative properties. It is stored in azurophilic granules of polymorphonuclear neutrophils and macrophages and released into extracellular fluid during inflammation (Arnhold, 2004).

The alkyl end groups of CP were coupling to DNA and the alkylated DNA decomposed rapidly. The DNA damage caused by CP may lead to DNA

mutations that result in cytotoxicity (Meirow et al., 2001).

It is found that the cytotoxic effects of CP occur by formation of DNA adducts, thus inhibiting cell division by inhibiting DNA strand separation, required for cancer cell damage, these DNA adducts are responsible for its cytotoxicity Previous studies found that a marker for DNA double- strand breaks was detected in oocytes of cultured ovaries (Petrill et al., 2011).

Sudharsan et al., 2006 recorded increased lysosomal enzymes activity after CP treatment. Once the lysosomal membranes are disrupted under any pathological conditions, such enzymes become free in the cytoplasm bringing about marked lyses and dissolution of the target materials, DNA and RNA.

In the present study, PAS stained section of rats treated with cyclophosphamide showed weak reaction in the oocyte with complete absence of zonapellucida. This result indicated decrease or depletion of carbohydrate within the oocytes and their surrounding zonapellucida. These results were in accordance with Wutzen, 1990 who reported that CP administration led to small amount of glycogen in the myocardial fibers. Also, electron microscopic examination of hair follicles after CP treatment showed increased density of the inner root sheath, loss of glycogen and intracellular edema of the outer root sheath (Kim et al.,1987).

In the present study, ovarian sections in rats treated with cyclophosphamide showedpositive cytoplasmic immunoreactivity for caspase-3 in the granulosa cells. Subsequent studies revealed that occurance of apoptosis by CP in granulosa cells of ovarian follicles was associated with activation of caspase-9 and caspase-3 (Hussein, 2005).

PM induced cell death was observed in granulosacells. PM binds to DNA to first form DA1 at a higher PM concentration after 24 h of exposure followed rapidly by the formation of DA2, observed in PM exposure after 48 h. These DNA adduct formation is involved in the ovotoxicity (Shanthi and Aileen, 2016).

Hesperidin (HSP) is a plant chemical that is classified as bioflavonoid, it is found in citrus fruits, vegetables and in food products [8]. It has strong cellular antioxidant protection against the damaging effects induced by cyclophosphamide treatment (Wilmsen et al., 2005).

In the present study, the hesperidin showed a protective effect against CP induced ovarian toxicity. HSP acts by scavenging free radicals and by maintaining intracellular superoxide dismutase (SOD) and glutathione levels, thereby preventing lipid peroxidation and tissue damage (Al-Jasabi and Abdullah, 2013).

HSP suppressed the level of TNF-α (tumor necrosis factor- alpha) which is considered as the master cytokine that is involved in a number of cytokine productions and therefore, it aggravates the severity of disease by producing and accumulating various types of ROS. Such accumulation of free radicals can damage a number of molecules present in the cells or cell membranes that may lead to lipid peroxidation and protein cross-linking resulting in the formation of malondialdehyde (MDA) (Ahmad et al., 2016).

Pre-administration of HSP is used to ameliorate oxidative stress, histological changes and subsequent cell death after radiation treatment (Rezaeyan et al., 2016). Some studies showed that HSP supplementation reduces oxidative and pathologic damages induced by irradiation in the liver, heart and kidney (Kalpana et al., 2011).

HSP has anti-inflammatory and specific protective effects against inflammatory disorders which are done through a mechanism involving the antioxidant activity of free radicals (Gholam et al., 2017). The anti-inflammatory an anti-oxidant actions of the naturally occurring compound, hesperidin, seem to be effective against cyclophosphamide induced ovarian toxicity.

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