Original Article

The effectiveness of resveratrol in protection against histological alterations induced by hyperprolactinemia in reproductive organs of female albino rats

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ABSTRACT

Introduction: Hyperprolactinemia (HPRL) commonly causes primary amenorrhea and reproductive disorders. Resveratrol (RES), a natural phenol, possesses antioxidant, anti-inflammatory and anti-apoptotic effects. However, its protective role on the histological structure of female reproductive organs in case of HPRL remains unclear.

Aim of the work: This study aimed at evaluating the protective effect of RES against the HPRL induced by metoclopramide in female albino rats.

Materials and Methods: Female rats that showed three regular estrus cycles (determined by vaginal smears) were divided into three groups. Group I was the control group. Group II rats were given oral metoclopramide (2 mg/Kg/day) for 28 consecutive days. Group III rats were given oral RES (20 mg/kg/day) for 28 days concurrently with metoclopramide. Serum superoxide anion (O_2^-), prolactin (PRL), estradiol (E2), progesterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) were assessed, in addition to measurement of tissues tumor necrosis factor-alpha (TNF-α). Sections from uteri, oviducts and ovaries were stained with hematoxylin and eosin and caspase-3 immunohistochemical stain and were subjected to morphometric and statistical studies.

Results: Group II showed significant increase in O_2^-, TNF-α and PRL, with significant decrease in the sex hormones. There were histological alterations in the uteri, oviducts and ovaries, with significant increased caspase-3 immunoexpression compared to the control. While, in group III, there was significant decrease in O_2^-, TNF-α and PRL, with significant increase in the sex hormones. The uteri, oviducts and ovaries showed minimal changes, with significant reduction in caspase-3 immunoreactivity compared to group II.

Conclusion: Administration of resveratrol ameliorates the oxidative stress, inflammation, hormonal assay alterations, histological changes and apoptosis resulted from induced HPRL in female rats.

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Key Words: Apoptosis, female reproductive organs, hyperprolactinemia, inflammation, oxidative stress, resveratrol.

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INTRODUCTION

Hyperprolactinemia (HPRL), a common endocrine disorder in hypothalamus-pituitary-gonadal axis, can be caused by either endogenous or exogenous factors. It occurs in more than 0.4% incidence among the whole population predominantly in young women and leads to menstrual cycle disorders, amenorrhea, and infertility. It thus affects people's health and life quality, which makes it a major health concern worldwide[1]. The HPRL state can be caused by diseases affecting the hypothalamus and pituitary gland, or diseases of other organs as the liver, kidneys, ovaries and thyroid, also it may result from disruption of the normal regulation of prolactin (PRL) levels by drugs or heavy metals[2].

Being one of the most usual disorders in gynecology, there are several queries regarding the effects of HPRL on the histology of female reproductive organs, particularly on the endometrium, oviducts and ovaries, because of their direct impact on female fertility. The development of experimental model by administering metoclopramide, an antiemetic agent, is able to simulate such disorder. This drug acts on a specific dopamine receptor (D2) antagonist in the pituitary; this binding inhibits the apoptosis in lactotrophs, so increasing PRL production[3].

Treating HPRL is done primarily with dopamine agonist drugs that have a lot of side effects occurring in 20-78% of the patients. These effects include dizziness, nausea, vomiting, postural and orthostatic hypotension, vasospasms, myocardial infarction, stroke and acute psychosis. In addition, these drugs are expensive with the need for long-term therapy. That is why natural products have remained the cornerstone of health care[4].

Resveratrol (3, 5, 4′-trihydroxystilbene) is a phytoalexin molecule that is produced by a variety of plants especially peanuts and grapes. Studies have mentioned its wide variety

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of bio-properties; including its anti-inflammatory, anti-oxidant, anti-microbial, anti-aging and cardio-protective effects. The RES also functions as a phytoestrogen regulating the reproductive system by changing the level of estrogen. More notably, it has a powerful anti-apoptotic effect through its action on several pathways including p53 signal, interleukin family and reactive oxygen species (ROS) dependent pathways[5].

The present work was carried out to investigate if resveratrol supplementation could protect against hyperprolactinemia-induced alterations in sex hormones and histopathological changes in female reproductive organs in albino rats; this was achieved using serological and histological methods.

MATERIALS AND METHODS

Chemicals

• Metoclopramide hydrochloride (Primperan) was purchased from Sanofi Aventis, Al Amireyah, Cairo, Egypt. It was in the form of tablets (each 10 mg) that were dissolved in distilled water.

• Resveratrol (RES) was purchased from Sigma-Aldich, Saint Louis, MO, USA (CAS No. 501-36-0) and was supplied as a bottle of 100 mg white powder. It was prepared by dissolving it in 20% dimethyl sulfoxide (DMSO).

Animals

Forty-two female albino rats weighing 150-170 gm and about 2 months old were obtained from the laboratory animal house unit of Kasr Al-Ainy Faculty of Medicine, Cairo University. They were housed in standard stainless-steel cages at a 12 h cycle of light and dark, room temperature was kept at 24±1°C and humidity maintained at 50%. Rats received standard rat chow diet and water ad-libitum. All animal experiments were carried out in strict compliance with the guidelines of the Cairo University Animal Ethics Committee on the care and use of laboratory animals.

Establishment of estrous cycle

This was determined by examining the cytology of vaginal smears obtained daily between 8.00 a.m. and 9.00 a.m. for 15 days. Normal saline was drawn into the tip of the pipette that was pushed gently 2mm deep into the vagina and 2 drops flushed into the vaginal canal. The mixture of vaginal fluid and normal saline was then sucked into the tip of the pipette. The smear was expelled on glass slide labeled with female identification number and date, then stained with crystal violet[6] and examined under the light microscope. Rats have very short cycle (4-5 days) consisting of four phases. The metestrus phase showed leucocytes, cornified, and nucleated epithelial cells in the same proportion, lasts for 21 hours and ovulation occurs at its end (Fig. 1A).

The diestrus phase lasts up to 57 hours and showed predominance of leucocytes (Fig. 1B). The third phase showed clusters of large nucleated epithelial cells and called the proestrus and lasts for 3-12 hours (Fig. 1C). The estrous phase showed predominant anucleated cornified cells, present in densely packed clusters and lasts for 12 hours (Fig. 1D)[7]. Rats showed three regular estrus cycles (15 days) were chosen for the study and subsequently the start of experimental design (day one) was considered.

Experimental design

The thirty-five rats with established estrus cycle were randomly divided into three groups as follows:

• Group I (Control group): fifteen rats divided equally into 3 subgroups: Subgroup IA received no treatment. Subgroup IB received 0.5ml distilled water / day orally through a gastric tube for 28 consecutive days. Subgroup IC: prepared as subgroup IB with concomitant oral administration of 0.5 ml of 20% DMSO/day for 28 consecutive days.

• Group II (Hyperprolactinemia; HPRL group) included ten rats. Each rat received metoclopramide (2 mg/kg/day) dissolved in 0.5 ml distilled water orally for 28 consecutive days[4].

• Group III (Resveratrol; RES group): included ten rats. Each rat received 20 mg/kg/day of RES dissolved in 0.5 ml of 20% DMSO/day orally for 28 days concomitantly with metoclopramide (1; 8).

To avoid fluctuation in prolactin level during the estrous cycle and because HPRL results in prolonged diestrus phase that may reach more than 14 days[4]; only animals in the diestrus phase were included in the current study.

Biochemical investigations:

At the end of the experiment, the following investigations were done in Biochemistry Department, Faculty of Medicine, Cairo University:

Before scarification, blood samples were drawn from tail veins of all rats in collecting heparinized capillary tubes. Samples were assessed for serum superoxide anion (O_2^-), prolactin (PRL), estradiol (E2), progesterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH). Superoxide anion was measured using LumiMax superoxide anion detection kit supplied by Agilent Technologies, Canada[9]. Detection of PRL, E2, progesterone, FSH, and LH was done using ELISA kits according to the manufacturer's instructions (DRG International Inc, NJ 07081, USA).

Then animals were sacrificed by decapitation; to obtain unstressed levels of prolactin, which is a stress hormone[10]. Uteri, ovaries and oviducts were dissected, washed with

saline. A part of each right uterine horns, right ovaries and right oviducts were homogenized for measurement of TNF-α[11] using commercial ELISA rat kits supplied by R and D systems (USA).

Light microscopic studies

Specimens from the middle segments of left uterine horns, ampulla of left oviducts and left ovaries were fixed in 10% buffered formalin. Paraffin-embedded serial sections were cut at 5-7 µm thickness and were subjected to:

1-Hematoxylin and Eosin stain (H&E)[12].

2-Immunohistochemical stain using streptavidine-biotin peroxidase technique[12] for caspase-3 (a rabbit polyclonal antibody, ab2302). This was purchased from Abcam, Massachusetts, USA. Tissue sections were counterstained with Mayer's hematoxylin.

Morphometric study

Using "Leica Qwin 500" software image analyzer computer system (Leica image system Ltd; Cambridge, England) present in the Histology Department, Faculty of Medicine, Cairo University, ten non-overlapping randomly chosen fields from different sections of each group were used to measure the following parameters:

1. Uterine wall thickness, measured from the surface endometrium to the perimetrium in H&E stained sections at a magnification of x40.

2. The number of corpora lutea (CL) in H&E stained sections at a magnification of x40.

3. The number of normal and atretic follicles in H&E stained sections at a magnification of x40.

4. The area percent of caspase-3 immunoexpression in the uteri, oviducts and ovaries at a magnification of x100.

Statistical analysis

It was done for serum O_2^-, hormones, tissue TNF-α, and the morphometric results. Data were expressed as mean and standard deviation for the quantitative variable. Data were statistically analyzed using statistical package SPSS version 16 (SPSS Inc., Chicago, USA). Comparisons between groups were done using ANOVA (analysis of variance) followed by post hoc test for multiple comparisons. The results were considered significant when $p<0.05$[13].

RESULTS

General observations

No deaths were noticed among rats during the experiment.

All rats of the control subgroups (IA, IB and IC) showed the same biochemical, histological and immunohistochemical results. Therefore, they were represented as the control group (group I).

Biochemical investigations

Biochemical investigation results are illustrated in Table 1.

Histological examinations
Uterine sections
Hematoxylin & Eosin results

Group I (control group) displayed uteri with slit-like lumina and their walls formed of inner endometrium, middle myometrium, and outer perimetrium. The endometrial lining was simple columnar epithelial cells, the underlying lamina propria contained tall endometrial glands lined with simple columnar epithelial cells with vesicular nuclei. Few apoptotic cells with pyknotic nuclei and vacuolated cytoplasm were detected in the surface epithelium, the endometrial glands and the endometrial lamina propria. Some mitotic figures in the epithelium were noted. The smooth muscles of myometrium were organized into inner circular and outer longitudinal layers with a layer of blood vessels in between. The myocytes possessed acidophilic cytoplasm and pale nuclei (Fig. 2A).

Group II (HPRL group) revealed histopathological alterations and degeneration in the form of apoptosis in the surface and glandular epithelial cells. Apoptotic cells appeared with deep acidophilic cytoplasm and pyknotic nuclei. Most of epithelial cells lining the endometrial surface and the glands exhibited cytoplasmic vacuolation. Additionally, the lamina propria and the myometrium showed vacuolated cells with darkly stained nuclei, widening of intercellular spaces and minimal inflammatory cells infiltration. The sections also demonstrated congested blood vessels within the myometrial layer (Figs. 2B, 2C).

Group III (RES group) regained nearly normal appearance of the cells lining the endometrial surface and glands, in addition to almost normal myometrial layer. While few epithelial and stromal cells still showed dark nuclei and cytoplasmic vacuolation. Mitotic figures in the epithelium could be noted (Fig. 2D, 2E).

Immunohistochemical stain results

Sections of control group revealed cytoplasmic and nuclear caspase-3 immunoreactivity that was detected in few surface epithelial cells, glandular epithelial cells, endometrial stromal cells, myometrial cells and endothelial cells lining blood vessels (Fig. 3A). Regarding group II, it displayed wide distribution of caspase-3 immunoexpression in the endometrium and the myometrium, as well as some immunoreactive cells were noted lining the blood vessels (Fig. 3B, 3C). However, group III showed less caspase-3 immunoreaction especially in the stromal and myometrial cells (Fig. 3D).

Oviduct sections
Hematoxylin & Eosin results

The oviducts from group I was composed of three layers; inner mucosa, middle musculosa and outer

serosa. The mucosa presented elongated folds lined with a single layer of columnar epithelial cells, ciliated and non-ciliated, and an underlying lamina propria. These epithelial cells had eosinophilic cytoplasm and oval vesicular nuclei (Fig. 4A). As regards sections from group II, there were numerous apoptotic cells with pyknotic nuclei and vacuolated cytoplasm within the mucosa and the musculosa. Additionally fragmented nuclei of the surface epithelium especially within the ciliated cells and darkly stained nuclei of non ciliated epithelial cells were noticed (Fig. 4B). On the other hand, RES group showed morphological improvement regarding the epithelial lining and the musculosa. However, few epithelial cells with pyknotic nuclei and cytoplasmic vacuolation could be seen (Fig. 4C).

Immunohistochemical stain results

A positive cytoplasmic and nuclear reaction for caspase-3 was observed in mucosa and musculosa cells of the oviducts from the control rats (Fig. 5A). Samples from group II displayed abundant positive immunoprecipitation in cells of mucosa, along with the presence of positive reaction in the musculosa (Fig. 5B). As for group III, some immunoreaction for caspase-3 antibody was detected in the mucosa and musculosa (Fig. 5C).

Ovarian sections
Hematoxylin & Eosin results

Ovaries taken from the control covered by a thin layer of dense fibrous connective tissue called tunica albuginea, which was surrounded by a single layer of cuboidal or flat cells (germinal epithelium). Many follicles in various stages of development were observed. Primordial follicles consisted of single layer of squamous cells surrounding an oocyte. Also, unilaminar primary follicles with a single layer of cuboidal cells were detected. Secondary follicles were formed of an oocyte surrounded by several layers of granulosa cells with small fluid spaces. Tertiary follicles with single antral cavity were seen. These follicles were formed of oocyte surrounded by zona pellucida (acidophilic line), corona radiate (granulosa cells immediately surrounding the oocyte) and cumulus oophorus (granulosa cells that protrudes into the antrum). A third part of granulosa cells lining the follicular cavity was also present. Then the follicle was surrounded by theca folliculi. Large corpora lutea (CL) were composed of moderately eosinophilic cells with large vesicular nuclei and foamy cytoplasm. There were few atretic follicles (Figs. 6A, 6B, 6C).

Group II sections displayed few normal ovarian follicles, with the presence of many atretic follicles. Their cells appeared apoptotic with deep acidophilic cytoplasm and pyknotic nuclei, in addition to fragmentation of nuclei of some of them. Moreover, some degenerated oocytes were surrounded by irregular or disrupted zona pellucida. There was vacuolation in the granulosa cells of different follicles. As most follicles were extensively degenerated, their types were hardly identified. Some sections revealed tertiary follicle with almost lost oocyte, zona pellucida and granulosa cells. Furthermore, degenerated corpora lutea showed vacuolated or deeply eosinophilic cells with pyknotic nuclei. Dilated congested blood vessels were also seen (Figs. 7A, 7B, 7C).

Regarding ovarian sections from group III, there were apparently near normal ovarian follicles and CL, apart from the presence of few atretic follicles and apoptotic cells with darkly stained nuclei lining different types of follicles (Fig. 7D).

Immunohistochemical results

Examination of ovarian sections from the control stained with anti caspase-3 antibody displayed positive cytoplasmic and nuclear reaction in few cells (granulosa, theca, luteal and interstitial cells) (Fig. 8A). However, the reaction was markedly evident in group II in many granulosa, theca and interstitial cells (Fig. 8B). While in group III, obvious reduction of caspase-3 immunoreactivity in the ovarian sections was recorded (Fig. 8C).

Morphometric and statistical results

Morphometric results are illustrated in Table 2.

Table 1: Mean values (± SD) of serum PRL, sex hormone and O_2^- levels and tissue TNF-α level in the studied groups.

Parameters		Control	HPRL group	RES group
PRL (ng/ml)		36.2±3.69	66.9±5.43*	47.4±5.5*#
E2 (pg/ml)		18.3±1.6	8.7±1.01*	13.0±1.9*#
Progesterone (ng/ml)		47.2±2.03	26.9±1.58*	39.6±4.0*#
FSH (μ IU/ml)		3.45±0.4	1.23±0.38*	2.5±0.4*#
LH (μ IU/ml)		2.7±0.43	0.92±0.25*	1.9±0.44*#
O2− (FU)		1149.8±84.7	1850.3±68.8*	1224.4±22.5*#
	Uterine	33.8±3.42	70.9± 8.42*	43.2± 10.03*#
TNF-α (pg/g tissue)	Oviductal	24.9±3.34	78.57± 6.54*	45.7± 7.76*#
	Ovarian	29.6±4.9	62.2± 9.6*	49.8±11.26 *#

* significant compared to control group, # significant compared to HPRL group.

Table 2: Mean values (±SD) of morphometric parameters in the studied groups.

Parameters		Control	HPRL group	RES group
Uterine wall thickness (µm)		178.9±8.14	259.5±43.3*	209.8±11.5*#
Number of ovarian follicles	Normal	17.9±3.6	6.4±1.4 *	13.1±2.2 *#
	Atretic	4.2±0.9	13± 2.4 *	7.0± 1.5 *#
Number of CL		4.6± 0.5	2.4± 0.61 *	3.6±0.67* #
Area percent of caspase-3	Uteri	1.46±0.24	14.7± 1.015*	8.04±1.4 *#
	Oviducts	0.89±0.23	8.6±0.9 *	1.5±0.36 *#
	Ovaries	1.62±0.4	8.7± 1.8*	3.13± 0.34 *#

* significant compared to control group, # significant compared to HPRL group.

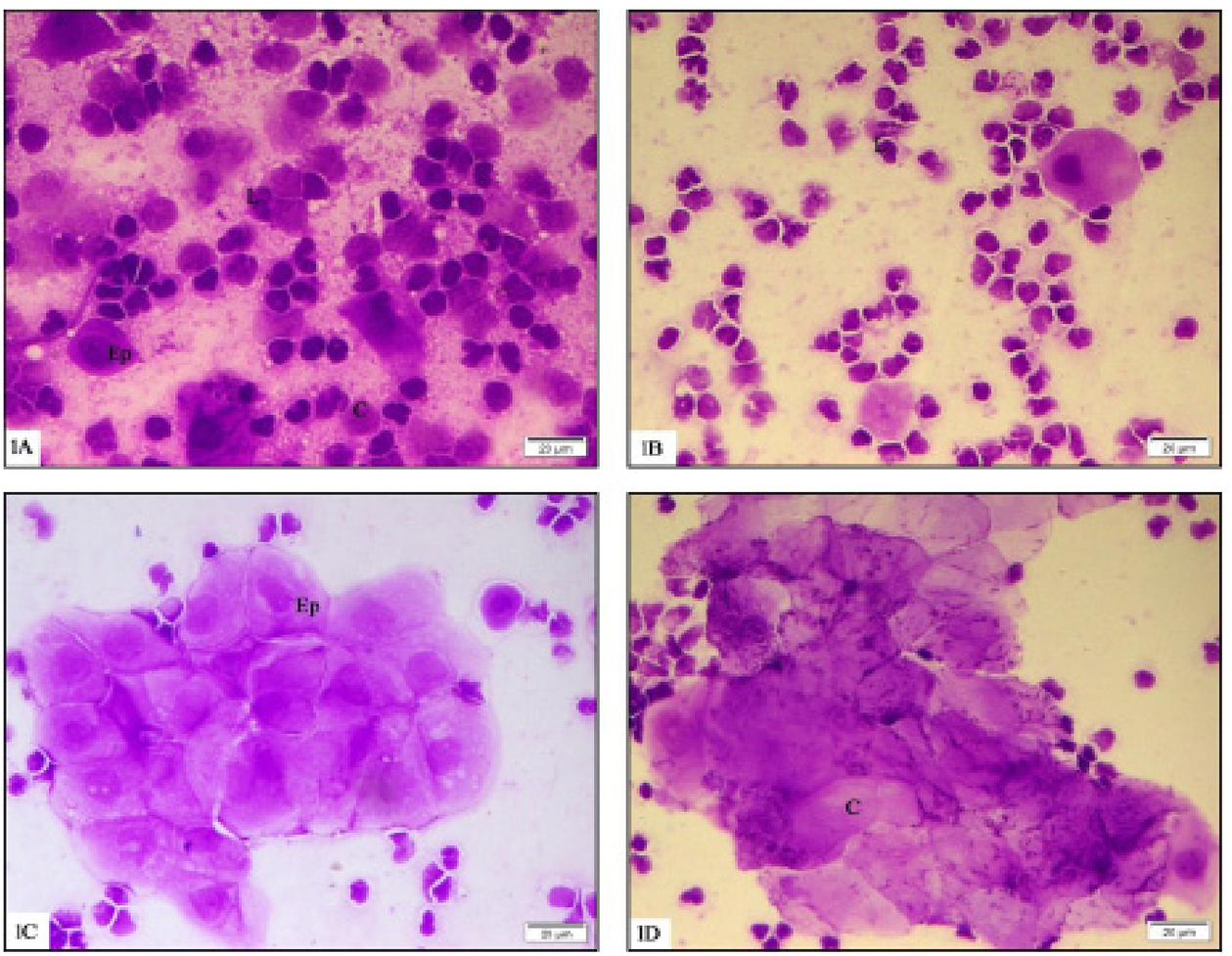

Fig. 1: Photomicrographs of vaginal smears stained with crystal violet: A: Metestrus phase shows leucocytes (L), cornified cells (C) and nucleated epithelial cells (Ep) in the same proportion. B: Diestrus phase shows predominance of leucocytes (L). C: Proestrus illustrates cluster of large nucleated epithelial cells (Ep). D: Estrous phase demonstrates predominant anucleated cornified cells (C) present in densely packed cluster [x400].

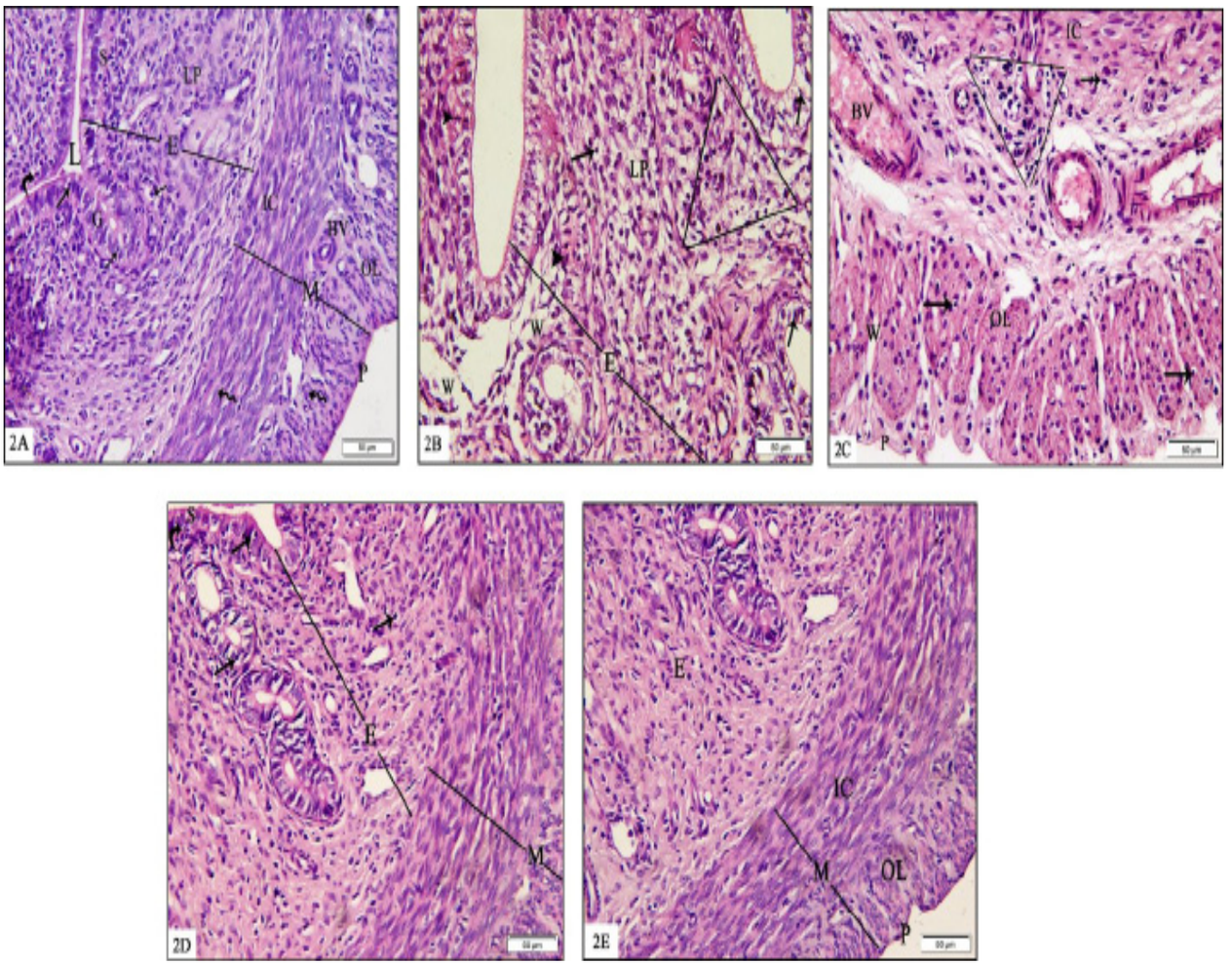

Fig. 2: Photomicrographs of uterine H&E stained sections: A: Control group shows uterus with slit-like lumen (L) and its wall consists of endometrium (E), myometrium (M), and perimetrium (P). The endometrium is composed of surface (S) simple columnar epithelial cells and underlying lamina propria (LP) containing endometrial glands (G) lined with simple columnar epithelial cells with vesicular nuclei. Few apoptotic cells (arrows) show cytoplasmic vacuolation and pyknotic nuclei in the endometrial surface (S), glands (G) and lamina propria (LP). Notice the presence of some mitotic figures (curved arrow) in the epithelium. Myometrium (M) shows myocytes having pale nuclei and acidophilic cytoplasm (wavy arrows) which are arranged into inner circular (IC) and outer longitudinal layers (OL) with a layer of blood vessels (BV) in between. B: HPRL group illustrates part of endometrium (E) with apoptotic cells having pyknotic nuclei and either deep acidophilic cytoplasm (arrowheads) or vacuolated cytoplasm (arrows). Additionally the lamina propria (LP) shows widening of the intercellular spaces (W) & minimal inflammatory cell infiltration (triangle). C: HPRL group shows the inner circular (IC) and outer longitudinal (OL) layers of the myometrium having some vacuolated myocytes with darkly stained nuclei (arrows) and widening of intercellular spaces (W). In addition to the presence of minimal inflammatory cells infiltration (triangle) and congested blood vessels (BV). Perimetrium (P) can be seen. D: RES group demonstrating nearly normal appearance of endometrium (E), however, few vacuolated cells with darkly stained nuclei (arrows). Note the presence of mitotic figures (curved arrow) in the surface (S) epithelium. Part of myometrium (M) can be observed. E: Part of endometrium (E) and myometrium (M) of group III appear almost normal. The myometrium (M) is formed of inner circular (IC) and outer longitudinal layers (OL). The perimetrium (P) is also noted [x200].

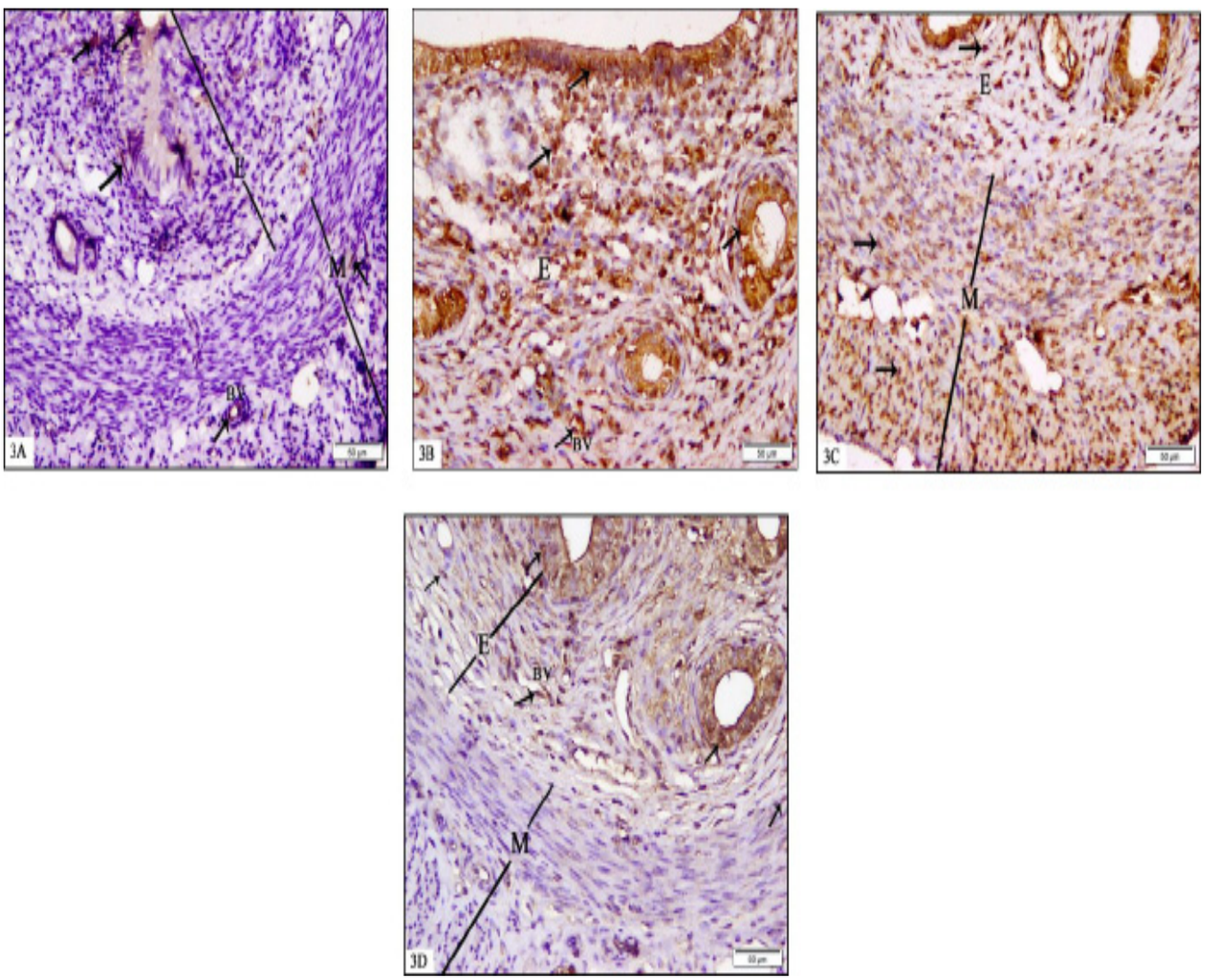

Fig. 3: Photomicrographs of caspase-3 immunostained uterine sections: A: Control group shows cytoplasmic and nuclear immunoreactivity (arrows) localized in few cells of the endometrium (E) (surface epithelial cells, glandular epithelial cells and stromal cells) & myometrium (M). Additionally, positive immunoreaction is noted in endothelial cells (arrow) lining blood vessel (BV). B: Group II; illustrating wide distribution of caspase-3 immunoexpression (arrows) in the endometrium (E) that is expressed in surface epithelial cells, glandular epithelial cells and stromal cells, in addition to some endothelial cells (arrow) lining blood vessels (BV). C: Group II demonstrating many immunoreactive cells (arrows) in the myometrium (M) and endometrium (E). D: Group III shows less caspase-3 immunoreaction (arrows) especially in stroma of the endometrium (E) and myometrium (M). Note that there are some positive immunoreactive cells (arrows) in the surface and glandular epithelium, as well as lining the blood vessels (BV) [x200].

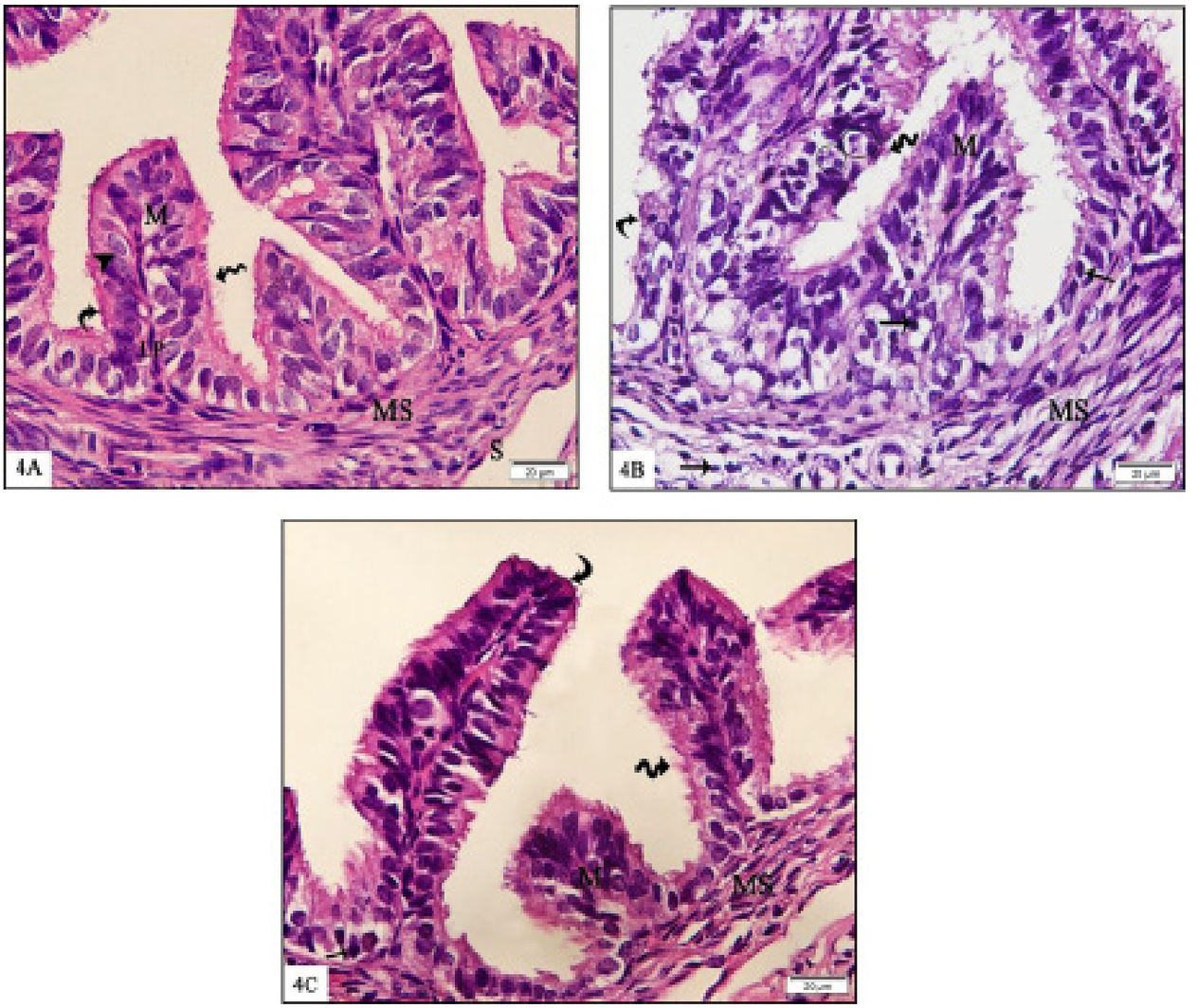

Fig. 4: Photomicrographs of oviduct sections stained with H&E: A: Control group shows mucosa (M), musculosa (MS) and serosa (S). Elongated folds of the mucosa are formed of single layer of columnar cells, mostly ciliated (wavy arrow) and others non-ciliated (curved arrow), and an underlying lamina propria (LP). These epithelial cells appear with eosinophilic cytoplasm and oval vesicular nuclei (arrowhead). B: Group II demonstrating many apoptotic cells (arrows) with pyknotic nuclei and vacuolated cytoplasm within the muocsa (M) and musculosa (MS). Fragmented nuclei (circles) of the surface epithelium especially the ciliated cells (wavy arrow) are also noted. Non ciliated surface epithelial cells (curved arrow) show darkly stained nuclei. C: Group III illustrating morphological improvement of the mucosa (M) & musculosa (MS). The mucosa is lined with ciliated (wavy arrow) and non ciliated (curved arrow) columnar epithelial cells. Few epithelial cells having pyknotic nuclei and cytoplasmic vacuolation (arrow) can be seen [x400].

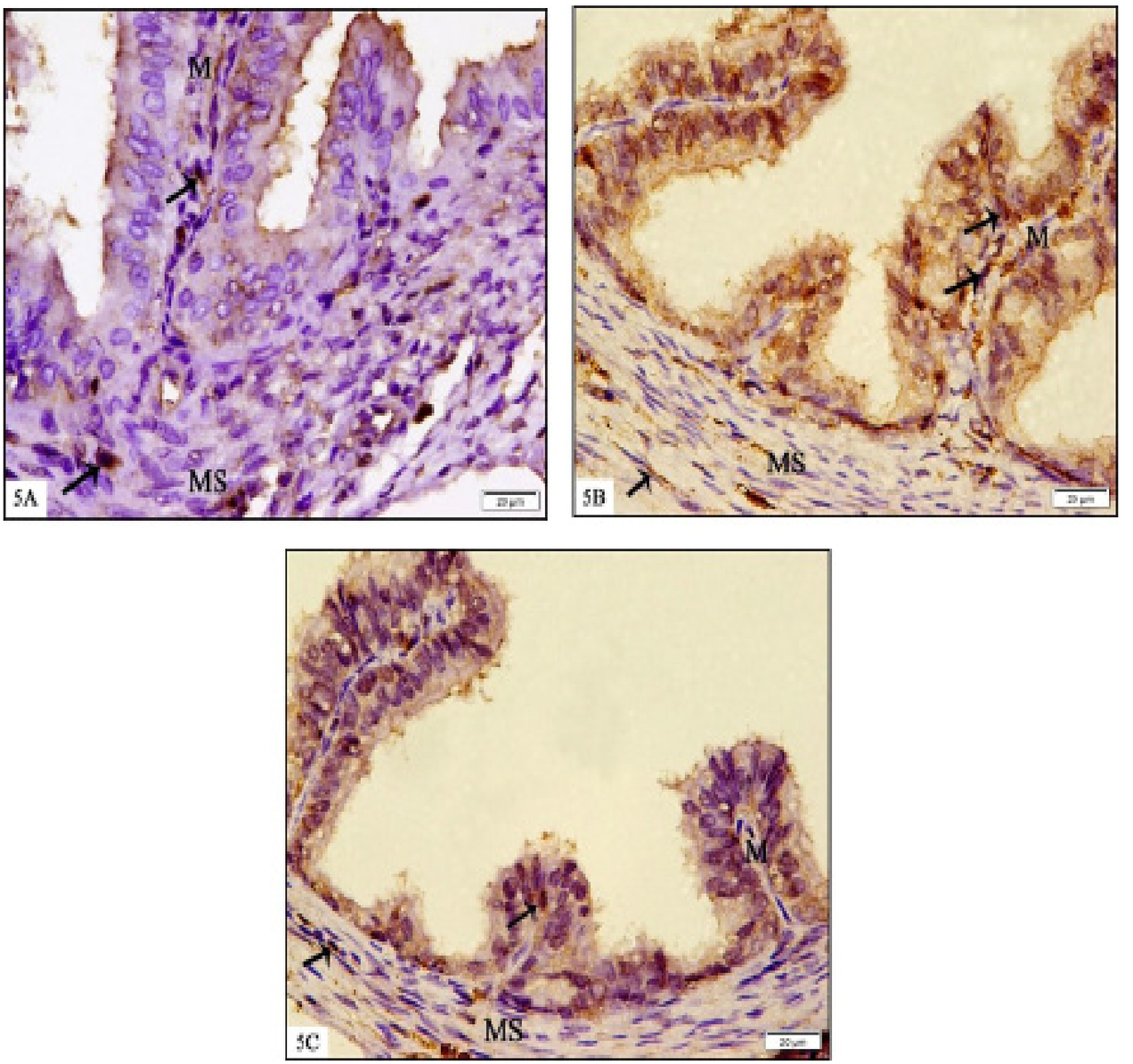

Fig. 5: Photomicrographs of caspase-3 immunostained oviduct sections: A: Control group shows cytoplasmic and nuclear immunoreaction for caspase-3 (arrows) in the cells of mucosa (M) and musculosa (MS). B: Group II illustrating abundant positive immunoprecipitation (arrows) within the mucosa (M), as well as the presence of positive immunoreactive cells (arrow) in the musculosa (MS). C: Group III demonstrating some caspase-3 immunoreaction (arrows) in the mucosa (M) and musculosa (MS) [x400].

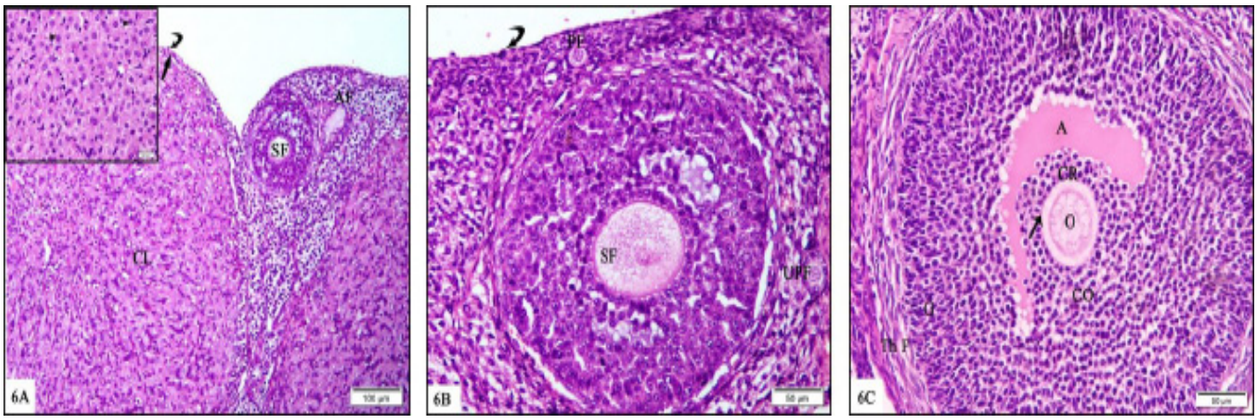

Fig. 6: Photomicrographs of ovarian H&E stained sections of control group: A: Part of ovary covered by tunica albuginea (thick arrow), a thin layer of connective tissue, is enclosed by germinal epithelium (curved arrow). Large corpora lutea (CL), secondary follicle (SF) and atretic follicle (AF) can be seen [x100]. An inset shows moderately eosinophilic luteal cells (arrowheads) with large vesicular nuclei and foamy cytoplasm [x400]. B: Germinal epithelium (curved arrow) consisting of cuboidal or flat cells surrounds the ovary containing primordial follicles (PF), unilaminar primary follicles (UPF) and secondary follicle (SF) [x200]. C: Tertiary follicle showing a single antral cavity (A). It is composed of oocyte (O) surrounded by zona pellucida (thin arrow), corona radiate (CR), cumulus oophorus (CO) and part of granulosa cells (G) lining the follicular cavity. A layer of theca folliculi (Th F) encloses the follicle [x200].

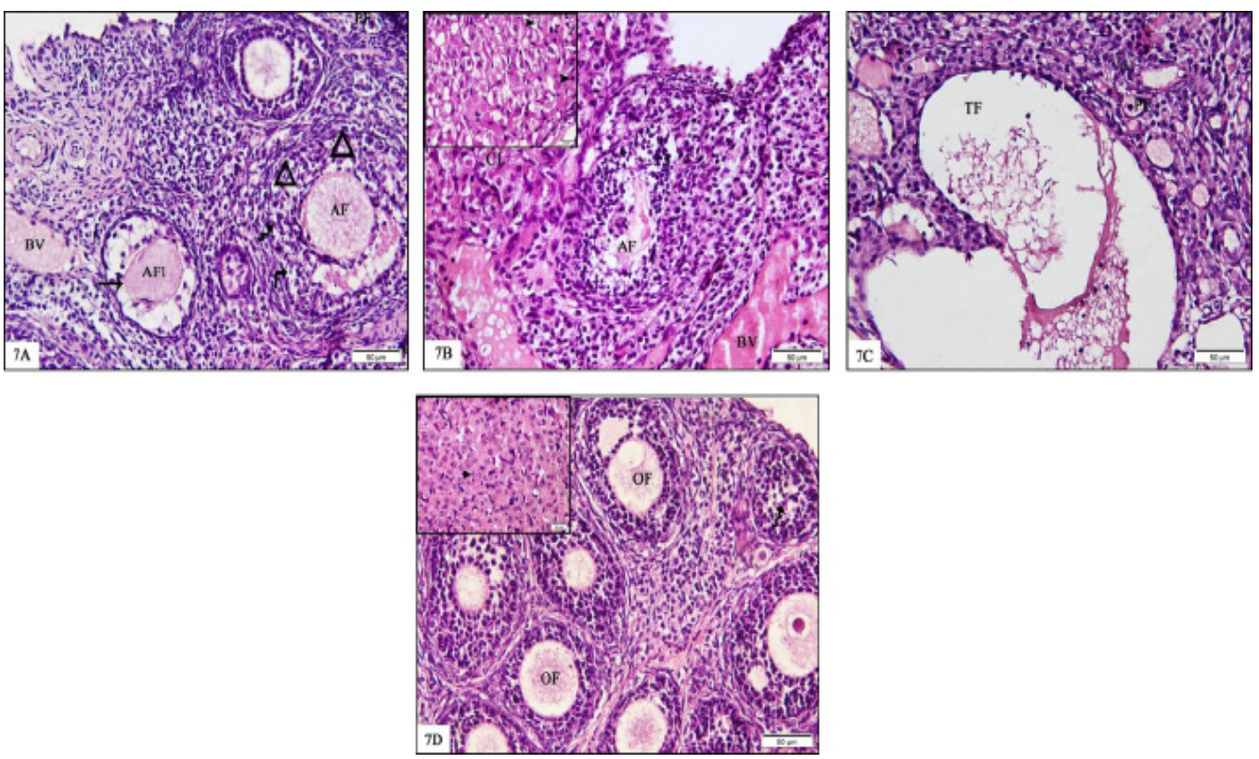

Fig. 7: Photomicrographs of ovarian H&E stained sections: A: Group II demonstrating atretic follicles (AF). These follicles show apoptotic cells having deep acidophilic cytoplasm with pyknotic nuclei (wavy arrow) or fragmented nuclei (triangles). Other granulosa cells appear vacuolated (right-angled arrow). Some degenerated oocytes are surrounded by irregular or disrupted zona pellucida (thin arrow). Note that the type of some atretic follicles (AF 1) is hardly identified. Degenerated primordial follicle (PF) and congested blood vessel (BV) are also seen [x200]. B: Group II shows atretic follicle (AF), corpus luteum (CL) and dilated congested blood vessels (BV) [x200]. An inset illustrating luteal cells (arrowheads) having vacuolated or deeply eosinophilic cytoplasm and pyknotic nuclei [x400]. C: Group II presents tertiary follicle (TF) with almost lost oocyte, zona pellucida and granulosa cells. Primordial follicle (PF) appears degenerated [x200]. D: Group III illustrating almost normal ovarian follicles (OF). Few apoptotic cells with darkly stained nuclei (wavy arrow) lining different types of follicles are present [x200]. An inset shows luteal cells (arrowhead) with moderately eosinophilic cytoplasm and vesicular nuclei [x400].

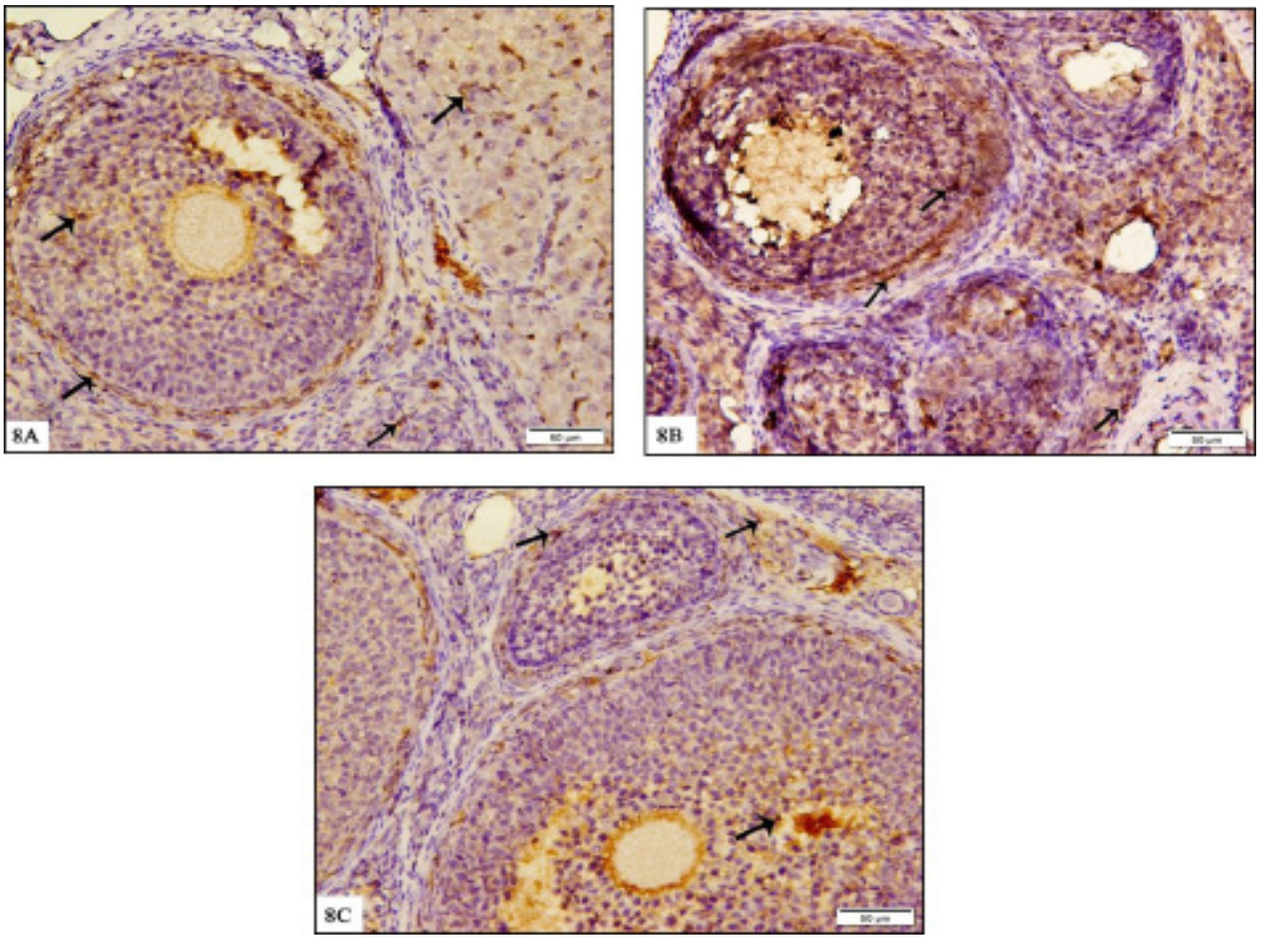

Fig. 8: Photomicrographs of caspase-3 immunostained ovarian sections: A: Control group showing few immunoreactive cells (granulosa, theca, luteal and interstitial cells) (arrows) having brown cytoplasmic and nuclear reaction. B: Group II exhibits markedly evident caspase-3 immunoexpression (arrows) in many granulosa, theca and interstitial cells. C: Group III illustrating obvious reduction of caspase-3 immunoreactive cells (granulosa, theca and interstitial cells) (arrows) [x200].

DISCUSSION

In the present work, the successful establishment of HPRL model was confirmed by the significantly elevated PRL in the blood samples from group II (HPRL group). Dopamine, a central regulator for PRL secretion, inhibits lactotroph proliferation and decreases the size of hypertrophied lactotrophs. So, treatment with metoclopramide (D2 antagonist) induces a consistent HPRL state via increasing the number and the volume of lactotrophs[14].

This HPRL condition was accompanied by significant decrease in E2, progesterone, LH, and FSH when compared to the control. These findings are in agreement with previous studies[1, 14, 15]. They mentioned that HPRL could decrease the secretion of sex hormones via short feedback loop. Prolactin suppresses gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus, which directly affects the pituitary physiologic functions and causes the decrease in gonadotropins (LH and FSH)[10]. Moreover, there is increased evidence that PRL exerts a direct inhibitory effect on gonadotropin actions in the ovary. Physiological levels of PRL suppress FSH-induced estradiol production by reducing aromatase expression and increases progesterone production by augmenting the steroidogenic acute regulatory protein (StAR), side-chain cleavage enzyme (P450scc), and 3β-hydroxysteroid dehydrogenase type 2 (3βHSD) in granulosa cells. However, HPRL may inhibit the release of GnRH leading to reduced ovulation and progesterone secretion[14].

Reproductive tissues remain stable when the scavenging antioxidants and free radical production remain in balance. Oxidative stress is a confusing factor in infertility as free oxygen radicals react with the cellular components of reproductive organs, producing destructive effects. PRL is a major stress-induced hormone, and its secretion follows physical, psychological or environmental stress. A positive correlation between serum PRL and high

lipid peroxidation suggested that HPRL could contribute to infertility by inducing oxidative damage[16]. In addition, high concentration of ROS affects folliculogenesis, oocyte maturation and uterine function, and initiates or develops pathological conditions that affect the reproductive processes[17]. Also the excessive generation of ROS stimulate the activation of mediator signaling molecules as the transcription factor, nuclear factor kappa-B (NF-κB), which up-regulates the production of inflammatory cytokines, such as interleukin-1β (IL-1β) or tumor necrosis factor-α (TNF-α) (18). Adding up, the association between high prolactin and an increase in inflammatory markers has been described[19].

In current work, there was significant increase in the levels of both superoxide anion (O_2^-) and TNF-α in HPRL group as compared to the control group. Superoxide anion is one of the reactive oxygen species that proved to have damaging effects on cells[20]. TNF- α is a cytokine synthesized in the endometrium, in the fallopian tubes and also secreted by mammalian pre-ovulatory follicles. This cytokine is released in relation to the menstrual cycle, which suggests a regulation by ovarian steroid hormones[21-23]. The expression of TNF- α and its receptors has been detected in glandular epithelial cells, oviducts and on oocytes, granulosa cells and interstitial cells suggesting autocrine as well as paracrine interactions[24].

Under physiological conditions, TNF- α is involved in numerous cellular processes; as survival, apoptosis inflammation, cell migration, proliferation and differentiation[25]. One particular function of TNF-α is the induction of apoptosis in fallopian tubes[22] and in endometrial epithelial and stromal cells associated with menstruation, as it provokes growth arrest as well as loss of epithelial cell-cell contact and vascular integrity. In humans, the increased levels of TNF-α occur when steroid hormones are decreased. Thus, TNF-α expression might be controlled by withdrawal of the hormones[24]. Another evidence for such control documented in another study, which clarified that depletion of estrogen in nude mice transplanted with human endometrial carcinoma cells could result in elevation of TNF-α concentrations[26]. These reports should not exclude that ovarian steroid hormones only regulate expression of TNF-α. As it was recorded that pathology of the uterus might also contribute to the unexpected high concentrations. In postmenopausal patients who had significant pathological variation of the whole uterus, high concentrations were found[21]. High levels of TNF-α appear to have negative effects on female reproductive physiology; it is a potential causative factor for follicular atresia, causing inhibition of GnRH and LH release, which leads to ovulatory dysfunction. Additionally, alterations in TNF-α level in follicular fluid lead to decreased oocyte quality[27]. It is also implicated in regression of the corpus luteum since luteal TNF was shown to increase upon the decrease in progesterone secretion[24].

Light microscopic examination of uterine and ovarian specimens from group II displayed marked degenerative changes that appeared as apoptosis (confirmed by the widespread caspase-3 immunoreactivity). This was accompanied by significant reduction in normal follicles and corpora lutea count within the ovaries as compared to the control. As well, there was marked increase in the number of atretic follicles. Along with vascular congestion, that points to the existence of inflammatory response in the uterus and ovary. That was associated with significant increase in the thickness of uterine wall. These findings are consistent with other researchers[1, 14, 15, 28, 29].

Concerning the remarkable increased number of atretic follicles and the marked apoptosis appeared in ovarian sections, this could be attributed to the decreased gonadotropins (FSH and LH) together with reduced E2. Though basal follicular growth until the secondary follicles does not depend mainly on gonadotropins, follicular growth from the antral follicles depends on them[30]. Estrogen is essential for follicular growth and differentiation, and for preventing apoptosis in the preantral and early antral follicles[15]. Histological examination of the ovaries from HPRL rats exhibited low numbers of CL. This could be explained by the increased levels of PRL which depresses gonadotrophic hormones and interfering with CL formation and function. That was confirmed by significantly decreased progesterone. This was supported in a previous study[31]. Further evidence came from the fact that PRL has a luteotrophic effect in rats. However, when the CL stop progesterone production, PRL stimulates luteolysis, which is responsible for the elimination of luteal cells in the nonfunctioning CL[32].

On other hand, several causes leading to sustained increase of PRL may result in pseudopregnancy and preserve functional corpora lutea. Consequently, elevated ovarian weights and hypertrophic CL are recoded[33]. These discrepancies could result from the use of different experimental HPRL models.

Several mechanisms by which PRL affects the endometrium were reported. It might be directly through the PRL receptor or indirectly through the decrease in the ovarian hormones production, which may hinder the embryo implantation. Besides, HPRL affects pinopodes, which are essential structures allowing embryo attachment to the endometrium. This occurs either by inhibiting their formation, or via reducing the progesterone needed for their development. Furthermore, PRL could act on the endometrium by modifying the immunologic activity or regulating the gland-secreted immune factors that could determine trophoblast proliferation and endometrial embryo invasion. Another potential effect of high serum PRL levels is the interference with the development of follicles and oocytes, leading to oocytes of poorer quality. All of the above mentioned negatively affects embryo implantation and fertility[29].

Minimal inflammatory cell infiltration was detected in the uterine wall of group II that most probably resulted from increased level of PRL. One of the most controversial aspects of PRL biology is related to its role in regulating inflammation and immune responses. In fact, several studies have documented the ability of PRL to stimulate the proliferation and the inflammatory activity of immune cells[34]. In agreement with the previously mentioned, some investigators observed that low-grade inflammation accompanies hyperprolactinemia[35].

Furthermore, the presence of widened intercellular spaces in the endometrial and myometrial layer might be a sign of inactivity and disintegration, which could be related to lack of estrogen and progesterone influence. Another explanation of such wide intercellular spaces might be the presence of interstitial edema accompanying the inflammatory process. This was furtherly supported by the significant increase in uterine thickness. However, previous authors attributed that to the increased cellularity in the endometrial stroma with more fibroblasts and abundant extra cellular matrix (ECM)[31]. The endometrial ECM has an important role in decidualization processes, implantation of embryo, invasion of trophoblast and maintenance of pregnancy. Dermatan sulfate (DS), chondroitin sulfate (CS), heparan sulfate (HS) and hyaluronic acid (HA) are the main glycosaminoglycans (GAGs) in the ECM of the uterine tissue (36). Changes in the composition of these GAGs, especially an increase in the synthesis of DS and CS with consequence increased uterine wall thickness is due to water retention associated with metoclopramide-induced HPRL[31].

The oviduct is an essential organ in the female reproductive system. It is concerned with the uptake of the oocyte released from the ovaries and its transportation to the uterus. Thus, it is critical in providing the proper circumstances for fertilization and survival of the fertilized egg. For performing these functions, keeping the proper structure of all the elements forming the oviduct is fundamental to avoid infertility[37]. Moreover, there is lack in the histological studies that focus on the oviduct changes during hyperprolactinemia state. That is why this work paid an attention to the oviducts as influential parts in the female reproductive system. Examined specimens from group II revealed marked affection in the epithelial lining together with increased apoptosis in muocsa (mostly the ciliated epithelial cells). The presence of negative charges on the oviductal cilia glycocalyx is considered the principle of the electrostatic interaction between the cilia and oocyte–cumulus cell complexes as well as pickup and transport of oocyte[38]. The oviductal pathology could be attributed to the significant decrease in the circulating estrogen and progesterone, where the oviducts are dynamic tissues whose cellular components respond to hormonal changes.

Resveratrol (RES) is proved to be a potent antioxidant; by its known effect on low density lipoprotein oxidation, it scavenges cellular ROS and corrects the resultant DNA damage[39]. Moreover, numerous in vitro studies proved its strong anti-inflammatory effect on different cells as macrophages, endothelial cells, microglial cells, smooth muscle cells, chondrocytes and adipose tissue. Such effect seems to be unrelated to the inflammatory stimulus; as RES was found to suppress activated NF-κB in cells stimulated with TNF-α, IL-1 or other known activators of inflammation[40]. Also, researches involving RES treatment in animal models of inflammatory diseases have verified down regulation of proinflammatory mediators as IL-1/-6, TNF-α, inducible nitric oxide synthase (iNOS), NFκB and cyclooxygenase-2 (COX-2), and oxidative stress markers as malondialdehyde and nitric oxide. Simultaneously it resulted in up regulation of anti-oxidant proteins as superoxide dismutase and anti-inflammatory proteins as IL-10[41].

In addition, RES is known as a phytoestrogen; it was found to bind to estrogen receptors and compete with synthetic estrogens in vitro, also it was postulated to have an estrogenic activity because of the structural similarity to some synthetic estrogens. Others mentioned that it could regulate the reproductive system by changing the level of estrogen via its ability to bind to estrogen receptors, increasing progesterone secretion as well as the mRNA levels of LH receptor and steroidogenic regulatory protein in cells[5]. However, it is controversial whether RES is an estrogen agonist or antagonist, but it was established that it plays an imperative role through its estrogenic modulatory activity[42].

Upon the abovementioned, this work was conducted to clarify RES effects in case of HPRL, regarding the PRL and sex hormones level, together with the histology of the female reproductive organs. It resulted in significantly reduced PRL and significantly increased E2, progesterone, LH, and FSH when compared to group II, together with significant decrease in the levels of both $O2^{-}$ and TNF-α. As regards the histological examination, there was remarkable improvement in the uterine, oviduct and ovarian specimens, with significantly decreased apoptosis. This is consistent with Zhang *et al.*[1], who tested the effect of a RES derivative called pterostilbene in treating HPRL and its related mechanisms and reported similar results to the current study.

Cell death occurs in oocytes and granulosa through mitochondrion-mediated (intrinsic) pathway. In oocytes, the oxidative phosphorylation pathway in mitochondria is the only source of adenosine triphosphate (ATP) needed by all cellular activities. In addition, the distribution of mitochondria in the ooplasm is a marker of cytoplasm maturity and is strongly related to its developmental ability. Moreover, the impaired ROS balance is considered the main factor leading to apoptosis; as accumulation of ROS interferes with nuclear and cytoplasmic maturation, leading to cell death. Notably, RES results in a powerful

anti-apoptotic effect through its action on several different pathways including ROS-dependent pathways, maintaining mitochondrial homeostasis, acting on both their function and distribution and reduced the alterations of mitochondrial morphology. Furthermore, RES protective effect might be via preventing the methylation of H3 histone; which has been discovered to correlate with oocyte apoptosis[5]. Additionally, RES role in modulating mitochondrial function was furtherly documented, where disturbances in the mitochondrial function lead to numerous negative consequences, including impaired cellular osmolarity, impaired synthesis of biomolecules (as proteins, lipids, nucleic acids) and cell death. It was found that RES could attenuate mitochondrial impairment in other organ, together with upregulation of mitochondria-located antioxidant enzymes, thus decreasing the production of ROS and reducing apoptosis[43].

Recently, scientists have focused on the protective antioxidant effect of RES on the female reproductive organs because of its proven beneficial effects on porcine oocyte maturation and subsequent embryonic development[44]. Moreover, it protects against the reduction of fertility ability of mice with aging[45]. Likewise, the therapeutic capability of RES in treating polycystic ovarian syndrome (PCOS) has been postulated; as it improved the estrus cyclicity in acyclic rat model of PCOS, also it increased the ovarian follicular reserve and prolonged the ovarian life span in rats. Therefore, it seems to be a good candidate through its antioxidant capacity and its ability to return the ovarian morphology to normal limits[46].

CONCLUSION

Hyperprolactinemia obviously affects follicle development that might decrease the ova quality with the subsequent uteri and oviducts affection from this condition, causing infertility problems. Resveratrol treatment ameliorates such effects. The protective benefits of RES might be attributed to its anti-inflammatory, antioxidant and anti-apoptotic effects. In addition to its estrogenic activity which can be concluded from this study. Thus, this work may be a theoretical ground for optimizing approaches for treating patients with HPRL after additional clarification in future experimental studies and clinical trials.

CONFLICT OF INTEREST

There are no conflicts of interest.

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الملخص العربى

فعالية ريسفيراترول في الحماية ضد التغيرات النسيجية المستحثة بفرط بروﻻكتين الدم في الاعضاء التناسلية لإناث الجرذان البيضاء

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المقدمة: فرط بروﻻكتين الدم عادة ما يسبب انقطاع الطمث الأولى واضطرابات الإنجاب. يعد ريسفيراترول فينول طبيعي ويمتلك اثار مضادة للأكسدة، مضادة للالتهابات ومضادة لموت الخلايا المبرمج. ومع ذلك، فإن دوره في حماية التركيب النسيجي للجهاز التناسلي للإناث في حالة فرط بروﻻكتين الدم لا يزال غير واضح.

الهدف من البحث: هدفت هذه الدراسة إلى تقييم التأثير الوقائي للريسفيراترول ضد فرط بروﻻكتين الدم الذي يسببه ميتوكلوبراميد لإناث الجرذان البيضاء.

مواد وطرق البحث: تم تقسيم الجرذان التي أظهرت ثلاث دورات شبق منتظمة (حددت بواسطة المسحة المهبلية) إلى ثلاث مجموعات. المجموعة الأولى: الضابطة. المجموعة الثانية: أعطيت الجرذان ميتوكلوبراميد عن طريق الفم (2 ملغ / كغ / يوم) لمدة 28 يوما متتالية. المجموعة الثالثة: أعطيت الجرذان ريسفيراترول عن طريق الفم (20 ملغ / كغ / يوم) لمدة 28 يوما بالتزامن مع ميتوكلوبراميد. تم تقييم أنيون الاكسيد الفائق والبروﻻكتين والاستراديول والبروجسترون وهرمون التحوصل والهرمون اللوتيني في مصل الدم. بالإضافة الى قياس عامل التننخر الورمى-ألفا في الانسجة. كما صبغت شرائح الارحام، قنوات فالوب، والمبايض بالهيماتوكسلين والإيوسين والصبغة الهستوكيميائة المناعية للكاسباس3-. ثم أجريت القياسات المترية الشكلية وتم تحليل القياسات إحصائياً.

النتائج: أظهرت المجموعة الثانية زيادة ذات دلالة احصائية في أنيون الاكسيد الفائق وعامل التننخر الورمى-ألفا والبروﻻكتين، مع انخفاض ذو دلالة احصائية في هرمونات الجنس. وقد كانت هناك تغيرات نسيجية في الارحام، قنوات فالوب، والمبايض، مع زيادة ذات دلالة احصائية للظهور المناعي للكاسباس3- مقارنة بالمجموعة الضابطة. بينما أظهرت المجموعة الثالثة انخفاض ذو دلالة احصائية فى أنيون الاكسيد الفائق وعامل التننخر الورمى-ألفا والبروﻻكتين، مع زيادة ذات دلالة احصائية في هرمونات الجنس. وأظهرت الارحام، قنوات فالوب، والمبايض تغيرات طفيفة، مع انخفاض ذو دلالة احصائية في التفاعل المناعي للكاسباس3- مقارنة مع المجموعة الثانية.

الإستنتاج: يضعف تناول ريسفيراترول الإجهاد التأكسدي، الالتهاب والتغيرات الهرمونية القياسية، والتغيرات النسيجية وموت الخلايا المبرمج الناجم عن فرط بروﻻكتين الدم المستحث في إناث الجرذان.