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Effects of anabolic androgenic steroids on mice ovarian and the possible protective role of ascorbic acid

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ABSTRACT

Testone – E (TE) is an anabolic androgenic steroids (AAS) and widely used as a medical drug which possesses multiple clinical therapeutic benefits. The aim of this study was to determine the effects of TE on the ovarian tissue of adult female mice and the possible protective role of ascorbic acid (AA).

Materials and Methods: Thirty adolescent (21- to 28-day-old) female Swiss albino mice (17-20g) were equally divided into 3 groups. The first group (GI) served as control. The second group (GII), was injected intraperitoneally (IP) with TE at a dose (25mg/kg) daily for four weeks. The third group (GIII) was injected (IP) by TE (25mg/kg) concurrently with AA at a dose (100mg/kg) daily for four weeks.

Results: Remarkable histopathological changes occurred in the ovaries of TE-treated group, including degenerative changes in the ovary with many atretic follicles and vacuolated stroma. Ultrastructural examination showed the follicles in TE-treated group appeared to have vacuolated oocyte with nuclear shrinkage and the granulosa cells demonstrated some signs of degeneration. Also, estrogen (ER) and progesteron (PR) showed weak immunoreactivity within the cells in TE-treated group. The above mentioned toxic effects in TE-treated group were markedly improved by treatment with AA.

Conclusion: TE caused detrimental effects on the ovarian tissue; However, AA prevents the harmful effects of TE on the ovarian follicles.

Key words: Anabolic androgenic steroids; Testosterone; Ovary; Ovarian follicle; Polycystic ovary; Ascorbic acid.

INTRODUCTION

Anabolic–androgenic steroids (AAS) are a group of hormones including testosterone and its synthetic derivatives [1]. They consists of four different testosterone esters (testosterone propionate, testosterone phenylprpropionate, testosterone isocaproate and testosterone decanoate), which provides a continuous release of testosterone into the blood and producing a stable testosterone level for a long period of time extending from 3-4 weeks [2]. These hormones are used clinically to treat conditions such as reproductive system dysfunction, hypogonadism [3, 4], senile osteoporosis [5] and some types of anemia [6].

In recent years, the intentional abuse of anabolic androgenic drugs especially the testosterone derivatives by athletes have increased rapidly in many countries to become a serious negative phenomenon [7]. Moreover, some healthy individuals have been using AAS for non-medical purposes [8] to enhance performance or body image [9]. However, the elevation in testosterone concentration stimulate protein synthesis resulting in improvements in muscle size, body mass and strength [10]. Abusers and many athletes, especially in the power sports like body building and weight lifting, administer illegally high doses of these drugs during sport competitions [11]. The adverse effects caused by abusing anabolic androgenic drugs included, liver dysfunction [12], kidney disease [13] and testicular problems [14].

Anabolic, androgenic steroid abuse by women and adolescent females has been associated with a number of adverse effects, including menstrual abnormalities and reproductive dysfunction [15]. However, elevated androgen testosterone levels associated with polycystic ovary syndrome (PCOS), a condition caused by an imbalance of sex hormones that can lead to menstrual cycle changes, ovarian cysts, difficulty conceiving, and another health change where, women with Polycystic ovary syndrome (PCOS), are hyper- androgenic [16]. Several studies have been performed on the effects of AAS on ovarian follicles. It has been shown that AAS induce atresia of developing follicles and typical PCOS features in rodents [17-21].

Ascorbic acid (AA) is non-enzymatic antioxidant form in plants, animals and humans and exists within cells to protect them against oxidative damage by neutralization excess reactive oxygen species (ROS). AA plays crucial roles in many biological process, electron transport, hydroxylation reactions, oxidation catabolism of aromatic compound in animal metabolism [22], biosynthesis of collagen and other components of the extracellular matrix [23]. AA Ascorbic acid has long been associated with fertility [24]. However, a large amount of AA was contained in the preovulatory follicles in rat ovaries [25]. Moreover, the addition of AA to media could prevent follicular apoptosis in cultured rat [26] and mouse [27] follicles.

Among many commercial versions of AAS, TE which is known as a useful medical drug and possesses multiple clinical therapeutic benefits. It comprises testosterone propionate plus vitamin E. The aim of the present work to study the effect of TE, 25 mg on the histological, ultrastructural and immunohistochemical changes in the ovary of female Swiss albino mice and the possible protective role of ascorbic acid as an antioxidant.

2. MATERIALS AND METHODS

2.1 Chemicals:

Testone – E (TE) is the commercially name for Testosterone propionates, each one ml contain Testosterone propionate 25 mg + Vitamin E 50 mg was obtained from MISR CO. for Pharmaceutical industry, Cairo, Egypt. Ascorbic acid was obtained from ALAMIA Company for chemicals Cairo, Egypt.

2.2 Animals and treatment:

Thirty adolescent (21- to 28-day-old) female Swiss albino mice (17-20g) were used in this study. All animal experimental protocols were approved by Committee of Scientific Ethics at Universities of Ain Shams and Sohag, and were carried out in accordance with the guidelines for animal use. These mice were kept under good hygienic conditions and allowed free water and fed adlibitum. The animals were divided into three main groups: A group of 10 mice per experiment were taken for the study.

Group 1(GI): Mice served as control.

Group 2 (GII): Mice were injected (IP) TE at a dose (25mg/kg) for four weeks.

Group 2(GIII): Mice were injected (IP) by both TE (25mg/kg) and AA (100mg/kg) for four weeks.

At the assigned times of the experiment, mice from each group were sacrificed under anaesthesia with diethyl ether. Sections of ovary were cut for routine histological examination, ultrastructural study and immunohistochemical study.

.2.3 Histological Study:

The ovary samples were fixed in 10% neutral formalin for 24 hours. The specimens were then dehydrated in ascending grades of alcohol, cleared and embedded in paraffin. Sections of 3-5 microns thick were cut by microtome and stained with haematoxylin and eosin for general histological structure according to [28]**.**

2.4 Electron microscopic study:

ovaries were randomly collected (n = 10 from each groups) after equilibration in medium for 30 minutes and fixed in 2.5% glutaraldehyde in phosphate-buffered saline (pH 7.4) for 2h, and post fixed with 1% osmium tetroxide in the same buffer for 2h. After dehydration in an ascending series of ethanol, specimens were placed in propylene oxide and embedded in Epon 812. Semi thin sections (0.5 µm) were stained with toluidine blue for light microscopy. Ultrathin sections (60-80 nm) were contrasted with uranyl acetate and lead citrate and examined by electron microscopy (Cell Biology Unit, Cancer Institute, Giza, Egypt).

2.5 Immunostaining procedure:

According to [29] with modifications: Serial sections from the paraffin embedded blocks were cut at 5µm thickness and mounted on slides coated with poly-l-lysine (Sigma). Slides were deparaffinized, rehydrated through graded concentrations of alcohol to distilled water, transferred to sodium citrate buffer (pH 6.0), and heated two times for 10 min in a microwave oven, set at 800 W. Slides were cooled between microwave irradiations for 5min. After this procedure, slides were washed twice with 10mM PBS, pH 7.4, and incubated successively in: 3% hydrogen peroxide in PBS for 30 min (at room temperature), 1% normal goat serum plus 1% hydrogen peroxide in PBS for 30 min; 0.5% Triton X-100 in PBS for 30 min; estrogen (ER) or progesterone (PR) mouse monoclonal antibody, ready to use (DAKO, USA) for 12 hours at 4℃ in a humid chamber. Slides were incubated with a biotinylated secondary antibody for 2 hours at room temperature and later with conjugated streptavidin-peroxidase for 1 hour. Sections were washed twice with PBS among incubations. Peroxidase activity was evidenced by using 3, 3_-diaminobenzidine chromogen solution in the presence of hydrogen peroxide for 10 min. After washing, sections were counterstained with Mayer`s hematoxylin, then sections were dehydrated and a cover slip was applied with mounting medium (DPX).

3. Results

3.1 Histological results

Microscopic structure of the ovary in adult mice reveals its organization in two areas, fully separated, that is cortical and medullary area. Cortical area covered by the germinal epithelium and composed of a large number of different stages of ovarian follicles (primordial, primary and secondary) and corpora lutea separated by loose fibrous stroma and groups of ovarian interstitial cells [Fig.1a]. The medulla comprises vascular, dense fibrous connective tissue stroma [Fig.1a& b]. Sections from adult mice ovary of (GII) showed enlarged ovary size, multiple degenerated follicles with many atretic follicles within the cortex. The degenerated follicles had degenerated oocytes with pyknotic nuclei and vacuolated cytoplasm, the zona granulosa showed degenerated cells in follicles. The medulla appeared degenerated, having multiple vacuoles with congested blood vessels [Fig.1c&d]. The ovary of (GIII) showed amelioration of the ovary tissue. The histological findings of this group were more or less similar to those of the control group (Fig. 1e &f).

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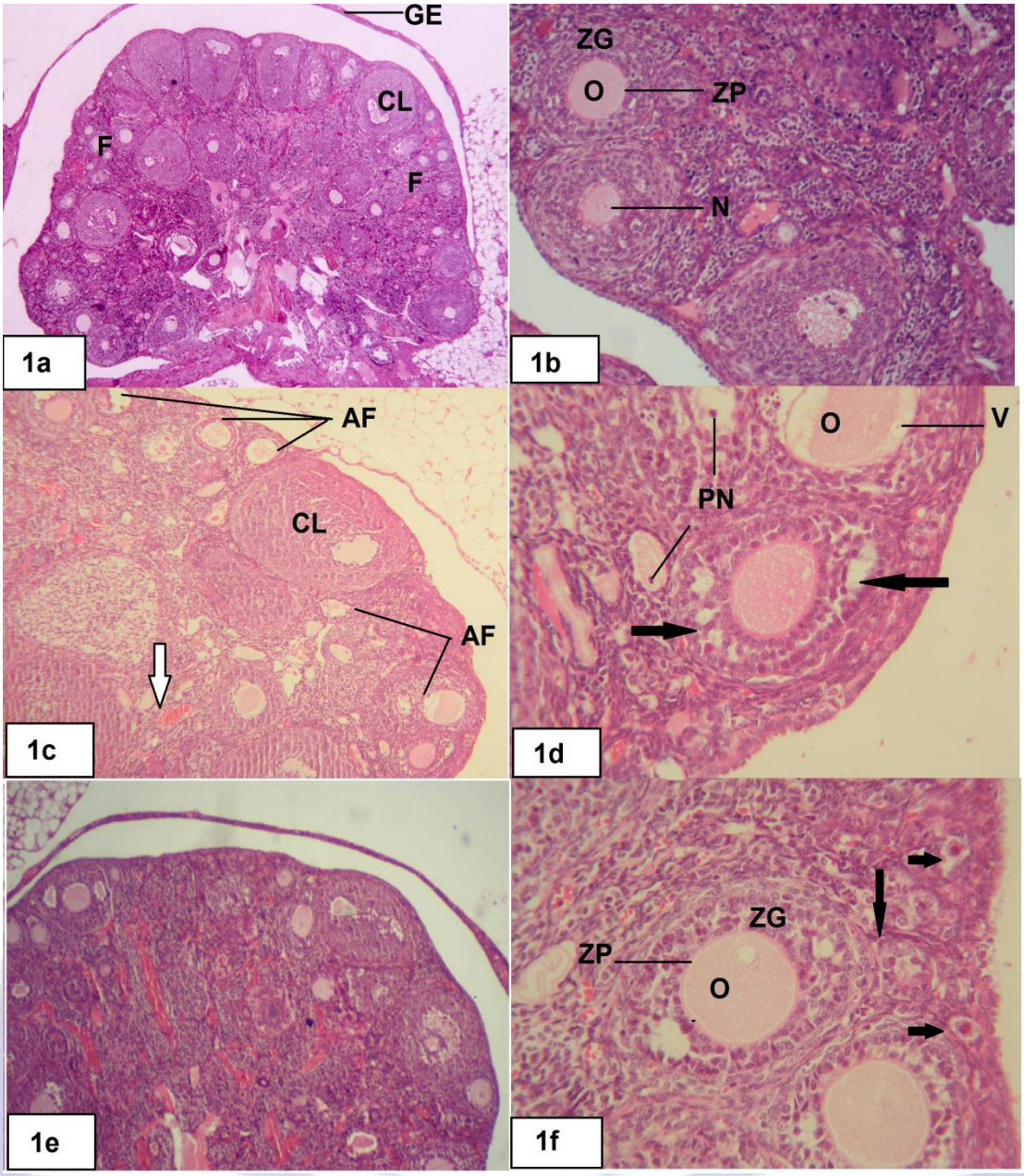


Fig. 1: Photomicrograph of control mice ovary (Fig.1a&1b) showing, germinal epithelium (GE), several corpora lutea (CL), different stages of follicles (F), oocyte (O) surrounded by clear zona pellucid (ZP). Zona granulosa (ZG) formed of multilayers of cubical cells surround the follicle. H&E (20 &40X). Fig. (1c &1d) for (GII) showing, multiple degenerated follicles, atretic follicles (AF) within the cortex, oocyte (O) with pyknotic nuclei (PN), vacuolated cytoplasm (V). Degenerated granulose cells (black arrow) and few corpora lutea (CL). The medulla appeared degenerated, having multiple vacuoles with congested blood vessels (white arrow). H&E (20 &40 X). Fig. (1e&1f) for (GIII) showing relatively normal primordial (short arrow), primary (long arrow) and secondary follicles with normal oocyte (O) surrounded by clear zona pellucid (ZP) and Zona granulosa cells (ZG). H&E (20 &40 X).

3.2 Ultrastructure results:

The ultrastructure of oocyte for control mice ovary (GI) showed normal nucleus with regular neuclear envelope (Fig. 2a). The uniform cytoplasm had rounded mitochondria with normal cristae and continuous mitochondrial membrane (Fig. 2a). Also, the cytoplasm contained Golgi complex, endoplasmic reticulum and intermediate filaments. However, the oocyte of (GII) showed irregular shape of the nucleus (Fig. 2b) and condensed heterochromatin. The nucleus of oocyte of (GIII) revealed normal shape but exhibit some vacuolation (Fig. 2c).

Theca layer, Granulosa cells (GC) in all groups arranged in one or two layers. The normal granulosa layer formed a compact layer around the oocyte which have normal large nucleus with numerous dense chromatin aggregates, these finding revealed in control group (GI) and (GIII) (Fig. 3a & 3c). In samples of (GII) a great number of Granulosa cells (GC) exhibited clear signs of cell damage such as abnormal nuclear karyolysis, presence of cytoplasmic large vacuoles and lipid droplets (Fig.3b).

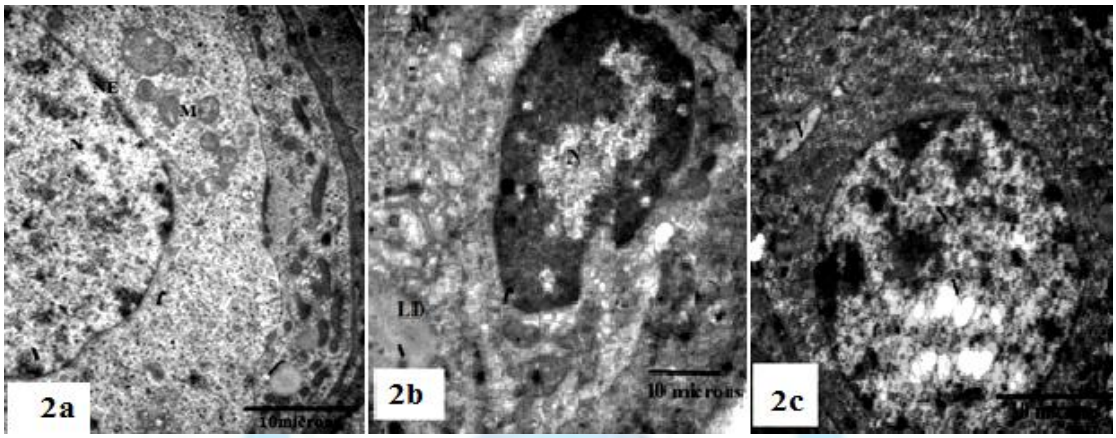


Fig. 2: TEM photos showing oocytes of different groups, (Fig.2a) showing normal oocyte of (GI) with nucleus (N), mitochondria (M) and nuclear envelop (NE). (Fig. 2b) of (GII) with abnormal nucleus (N) and lipid droplets (LD). The nucleus of (GIII) appear normal with mild vacuolation (V) (Fig.2c).

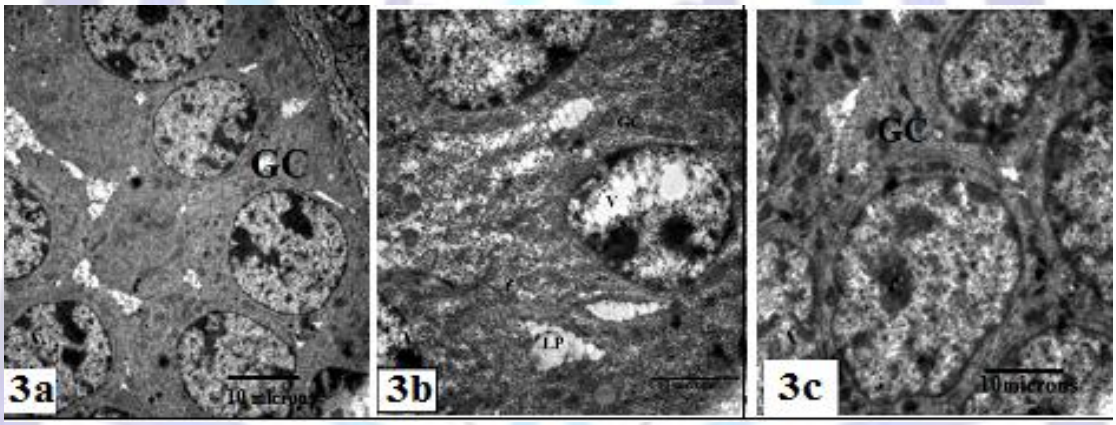


Fig. 3: TEM photos showing normal granuloza cells (GC) in (GI) (Fig.3 a) and (GIII) (Fig.3c). But, (Fig.3b) of (GII) showing sever vaculation in nucleus (V) and lipid droplets in cytoplasm (LP).

Immunohistochemistry Results

Estrogen (ER) is mainly synthesized in the ovary. The ER in ovaries of control mice (GI) highly expressed in granulosa cells, endothelial cells and interstitial cells (Fig. 4a), and weakly expressed in mice of (GII) (Fig.4b). The Moderate expression of ER was represented in (GIII) in the theca cells, luteal cells and endothelial cells. However, granulosa cells showed weakly expressed of ER (Fig. 4c)

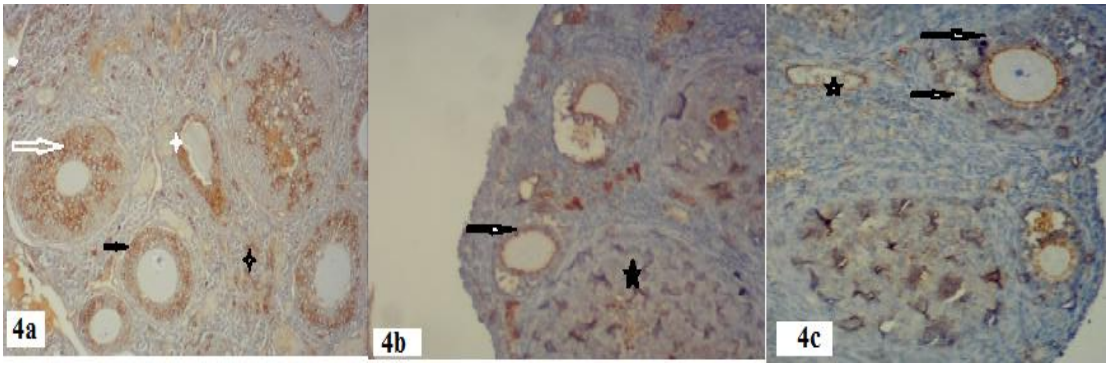
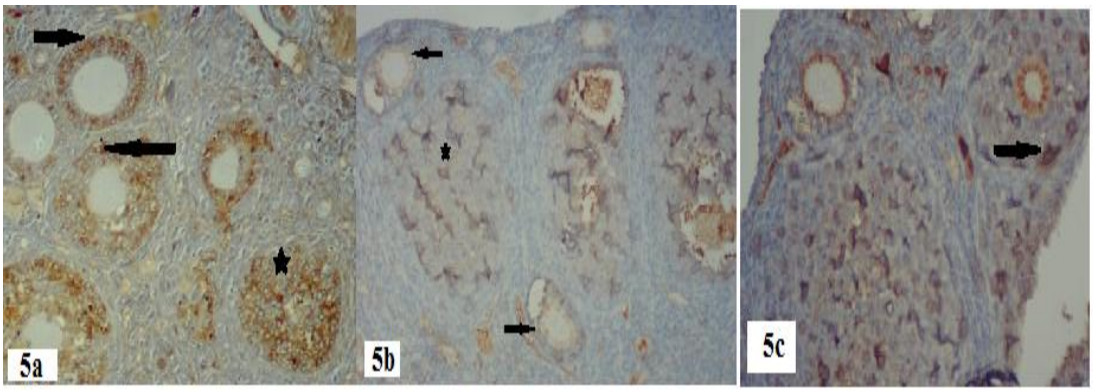


Fig 4: ER immunostaining photomicrograph (10X) showing, the high expression of ER in ovaries of control mice (Fig.4a) in granulosa cells (white arrow), theca cells (black arrow), interstitial cells (black asterisk), and endothelial cells (white

asterisk). However, in (Fig.4b) of (GII) the expression of ER is weak in granulosa cells (black arrow), There was no immunoreaction in theca cells and in luteal cells (black asterisk). (Fig.4c) showing moderate expression of ER in (GIII) in the theca cells (short black arrow), luteal cells and endothelial cells (black asterisk).

In the ovary, Progestron (PR) expression was present throughout the estrous cycle during follicle development and ovulation and in the corpus luteum. The PR expression was highest in the granulosa cells of preantral and antral follicles, also in the theca cells or in the granulosa cells and these highly positive expressions was observed in (GI) (Fig.5a) and to a moderate level in (GIII) (Fig. 5c). However, (GII) exhibit a much weak PR-positive expression (Fig.5b).



(Fig.5): PR immunostaining photomicrograph (10X) Showing highly positive PR in control mice (GI) in the granulose cells (arrows) and in the corpus leutum (asterisk) (Fig.5a). (Fig.5c) of (GIII) showing the moderate positive expression of PR in granulose cell (arrow). While, the (GII) showing faintly expression of PR in granulose cell (arrow) and corpous leutum (asterisk)(Fig.5b).

4. Discussion

Ovarian follicles have an important role in the female reproductive biology [30]. In the present study, detectable changes in the ovary induced by the effect of TE, these changes were rebalanced with the treatment of antioxidant vitamin, AA.

In light of our findings, the microscopical examination of the ovarian sections of adult mice treated with TE showed considerable structural changes, including multiple degenerated follicles with vacuolated zona granulosa cells and degenerated oocytes exhibiting some reproductive features of PCOs, including a greater number of atretic cystic-like follicles. These structural changes are also in confirmations with ultrastructural examination in which the oocyte and the granulosa cells demonstrated some signs of degeneration including vacuolated oocyte with nuclear shrinkage and vacuolated cytoplasm.

These results are in agreement with many authors using androgen-induced PCO. They reported that the excess of androgen can induce atresia of developing follicles as increase of the number of atretic follicles in female rats [18, 19, 31] and reduction in the number of ovarian oocytes [17]. Moreover, the same findings were seen in ovaries from letrozole-treated females where, the letrozole is an aromatase inhibitor which prevents the conversion of androgens to estrogens by reflecting the block in aromatase activity and accumulation of endogenous ovarian androgen secretion [20, 32]. In addition, the effects of AAS on ovarian morphology may vary depending on the period, duration and the amount of hormone administered [21].

The degeneration of ovarian follicles and their oocytes detected in present study might be due to oxidative stress caused by the high testosterone rate which induces oxidative stress by alteration of the balance between ROS production and antioxidant defenses [33]. Since testosterone can increase the metabolic rate [34-36] where, high metabolic rate might lead to increased free radical production and oxidative damage [37]. Moreover, testosterone can also increase locomotor activity where, a high muscle activity might increase oxidative stress [38]. Furthermore, ovarian ROS overproduction can have deleterious effects on cellular function by inducing oxidative damage of intracellular components and inducing apoptosis [39, 40]. [18, 31] reported that AAS damage folicullogenesis, suppress corpora lutea maturation, inhibit ovulation and induce follicular atresia, due to disrupt of neuroendocrine functions, resulting in the decrease of circulating follicle-stimulating hormone (FSH) and luteinizing hormone (LH) [17, 41]. Moreover, the inhibitory effect of androgens related to apoptotic in granulosa cells of follicles is expected even at lower doses depending on AAS metabolism and oestrogen conversion rates [42]. In addition, the increase of circulating androgen levels inhibits the production and release of LH, FSH, estrogen and progesterone resulting in the inhibition of ovarian follicle formation, ovulation and sexual cycle irregularity [41, 43].

On the other hand, the present investigation reveled that the histological structure and ultrastructural changes in the ovaries tissues of mice treated with TE and AA (GIII) showed somewhat variable degrees of amelioration of the previous changes, this could be due to antioxidative stress effect of ascorbic acid. However, the antioxidants such as ascorbic acid can minimize the level of ROS and protect the growing follicles in normal ovarian tissue from the toxic effects of those radicals [44]. Also, our results are in agreement with the other authors who reported that ascorbic acid can prevent follicular apoptosis in ovarian follicular cells by suppressing oxidative stress [26, 27]. Ascorbic acid enhances porcine oocyte development competence [45]. However, the ovaries accumulate large amounts of ascorbic acid in the granulosa, thecal and luteal cells [46] and it has long been associated with fertility [24]. [47] reported that ascorbic acid is necessary for remodeling the basement membrane during follicular growth and that the ability of follicles to uptake ascorbic acid

confers an advantage in terms of granulosa cell survival. [48] found that the ascorbic acid treatment appeared to be effective in reducing apoptosis in ovarian tissue damage [49].

Immunohistochemical results of ovaries sections in mice treated with TE showed weak immunoreactivity of zona granulosa cells for ER and PR. This result could be attributed to degeneration of granulosa cells of degenerated ovarian follicles which due to oxidative stress caused by the high exogenous testosterone rate. The present findings come in accordance with the results of [50]. [17] reported that the exogenous steroids administration may increase the levels of circulating androgens, thus altering the function of the hypothalamus–pituitary–gonad axis which results in altered levels of hormones, such as FSH, LH, oestrogen and progesterone [41, 51, 52]. In addition, higher concentrations of androgens inhibit aromatase expression by granulosa cells. This lead to reduce production of oestrogen, which is necessary for follicle growth [53]. Anabolic androgenic steroids concentration induces a dose-dependent effect on the oestrogen synthesis to regulate ovarian functions, so that high doses can result in advanced stage of atresia and follicular apoptosis, while lower doses stimulate folliculogenesis [54-56]. Also, the present study showed that immunoreactivity of zona granulosa cells for ER and PR were improved to nearly normal content in the ovaries tissues of adult mice treated with TE and AA, this could be due to AA which had caused an increase in progesterone biosynthesis by activating luteinizing hormone [57]. Furthermore, ascorbic acid stimulates production of progesterone [58]. AA does have an important role as a modulator of aromatase activity in vivo in rats. This would in turn increase production of oestrogen [59].

In conclusion, our experiments showed that TE induced many histological, ultrastructural, and immunohistochemical changes in the ovary. The use of ascorbic acid as antioxidant can ameliorate these changes. Thus, AA can prevent free oxygen radicals that might occur during TE administration and protect the growing follicles from the toxic effects of those radicals. AA can be recommended as a protective agent against TE-induced ovarian damage.

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