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Research Article

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Protective and Curative Roles of Vitamin C Against Formaldehyde Toxicity in Female Albino Mice Reproductive System

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ABSTRACT

Infertility refer to the biological inability of an individual to contribute to conception, or to a female who cannot carry a pregnancy to full term. Vitamin C is a water-soluble vitamin that is naturally present in some foods; it is a highly effective antioxidant and used in preventing and treating many diseases. Formaldehyde is an organic compound, and it is used as a chemical intermediate, disinfectants, fumigants, photography, and wood preservation.

The aim of this study is to investigate the protective and curative roles of antioxidants as Vitamin C toward formaldehyde induce damage in female reproductive system.

Female mice (n=50, 25-30 gm) were divided into 5 groups: group I (control) administered normal saline (5 ml/kg) for 5 days, group II received formaldehyde (30 mg/kg) for 5 days, group III received Vitamin C (250 mg/kg) for 5 days, group IV administered formaldehyde and Vitamin C (prophylactic) for 5 days, group V received formaldehyde for 5 days followed by Vitamin C for another 5 days (curative). At the end of 21 days, animals were sacrificed, ovaries and uterus were removed and fixed in 10% formalin solution for routine histological techniques. Intraperitoneal administration was used in this study.

It was found that formaldehyde cause reduction in ovulation process and damage in lining endometrial epithelial. Administration of Vitamin C only increase in ovulation process and showed normal tunica albuginea and produce hyper stimulation for blood vessels and uterine epithelial. Vitamin C administration for treatment or prophylaxis repair the damage induced by formaldehyde and improve the ovary and uterus to nearly healthy condition. The treated group with Vitamin C showed more repair and improvement compared to the prophylaxis group.

Formaldehyde induce histological alteration in ovary and uterus of female mice and in turn, Vitamin C has a prophylaxis and treatment role against the damage induced in female mice. It is recommended for the future work, to find the protective role of Vitamin C against formaldehyde induce damage in fetus and offspring.

Key words- *Female infertility; Formaldehyde; Vitamin C; Mice; Histopathology*

INTRODUCTION

Female infertility refers to an inability to conceive after having regular unprotected sex, to the biological inability of an individual to contribute to conception, or to a female who cannot carry a pregnancy to full term.[1,2] Female infertility may be due to hormonal imbalance,[3,4]thyroid disease, polycystic ovaries syndrome (PCOS), hyperprolactinemia,[3]endometrio sis,[5] sexually transmitted disease[6] and pelvic inflammatory disease (PID).[7,8]

Medication may cause female infertility,as chemotherapy, can result in ovarian failure.[1,9] Women who take non-steroidal anti-inflammatory drug (NSAIDs) for long-term may find it harder to conceive.[1]

Risk factor of female infertility are age, smoking, alcohol consumption, obesity, or over-exercising, not exercising, exposure to some chemicals, mental stress,[1]caffeine or recreational drugs (such as marijuana).[10]

Vitamin C is water-soluble vitamin that is naturally present in some foods. Humans, unlike most animals, are unable to synthesize vitamin C endogenously.[11]Vitamin C is essential in healthy diet as being a highly effective antioxidant[12] and an enzyme cofactor for the biosynthesis of many important biochemical as collagen, carnitine, and neurotransmitters.[13] Vitamin C act as electron donor for important enzyme;[14] it used to prevent and treat many of disease as heart disease, arthritis, acute respiratory infection, high blood pressure, scurvy, cataracts, and help in prevent inflammation by lowering blood level of histamine,[15] it reduce the severity of cold symptoms, also is used as antiaging, and help body absorption of iron.[16]

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Formaldehyde is an important precursor to many other materials and chemical compounds,[17,18]highest levels of airborne formaldehyde have been detected in indoor air, it is released from various consumer products such as building materials and home furnishings, smoking, also present in food, either naturally or as a result of contamination.[19] Formaldehyde increased incidence of menstrual disorders, anemia, toxemia and low birth weight of offspring in female workers exposed to formaldehyde.[20]

The present study was designed to investigate the protective and curative roles of antioxidants as vitamin C toward formaldehyde induce damage in female reproductive system.

MATERIALS AND METHODS

Materials

Vitamin C supplement was obtained from PIS, Tunisia; Formaldehyde, NaCl and Eosin Y were purchased from Red El-De Haen AG Seelze-Hannover- Germany; Tween 80 obtained from Leicestershire, UK.; Formalin prepared by Al Hashan pharmacy, Ain Zara-Tripoli-Libya; Calcium chloride dehydrate obtained from BDH limited Poole England.

Methods

Female mice (n=50, 25-30 gm) were divided into 5 groups: group I (control) administered normal saline of 5 ml/kg[21] for 5 days; group II received formaldehyde of 30 mg/kg[22] for 5 days; group III received vitamin C of 250 mg/kg[23] for 5 days; group IV administered formaldehyde and vitamin C (prophylactic) for 5 days; group V received formaldehyde for 5 days followed by vitamin C for another 5 days (curative). At the end of 21 days, animals were sacrificed, ovaries and uterus were removed and fixed in 10% of formalin solution for routine histological techniques. Intraperitoneal administration was used in this study.

Preparation of formalin solution

10% Formalin solution was prepared by adding 10 ml formalin and 10 ml of 10% calcium chloride to 80 ml distilled water. 10% Calcium chloride was prepared by dissolving 10 gm anhydrous $CaCl_2$ in 100 ml distilled water.[24,25]

Histological study

At the end of the administration, mice were sacrificed, uterus and ovary of treated mice were removed and then fixed in 10% formalin for 24 hours. The specimens were washed twice with 70% alcohol. The fixed tissues were dehydrated in an ascending series of alcohol ranging from 70% to 100% (absolute). The dehydrated tissues were cleared in xylene (twice), infiltrated, and then were embedded in paraffin wax. The uterus or ovary were sectioned on a rotary microtome, sections were 5μm in thickness. The prepared sections were stained by routine methods using hematoxylin and eosin (H&E) method. The stained sections were examined under the microscope and the different cell types were carefully studied and photographed.[26,27]Uterus and ovary sections from each study group were evaluated for structural changes, blind by a histologist. Light microscopy (Leica, Germany) was used for the evaluations.

RESULTS

Control group

***Ovary control*:**

Examination of H&E- stained sections of control mice's ovary revealed that the ovary was covered by a single layer of cuboidal epithelium (Figure 1a). The epithelium was separated from the underlying ovarian tissue by a layer of collagenous fibers. The ovarian parenchyma formed of cortex and medulla, but there was no sharp demarcation between these two components.

In the ovarian cortex, the primordial follicles were seen underneath the tunica albuginea (Figure 1a,b).

Different forms of growing follicles were seen in the ovarian cortex, they comprised of primary and secondary follicles (Figure 1a). The secondary follicle was formed of large primary oocyte in the center and surrounded by clear material (zona pellucida) and multilayers of follicular granulosa cells.

Tertiary follicle was larger follicle, located near the surface. It was formed of primary oocyte surrounded by clear zona pellucida and few follicular cells called corona radiate. The whole follicle was surrounded by theca interna, and theca externa (Figure 1b). The corpus luteum was formed of both granulosa and theca lutein cells which were polyhedral cells containing large spherical nuclei and large amount of vacuolated cytoplasm. The luteal cells were separated by spindle shaped fibroblasts and blood capillaries. The stroma of the ovary was formed of connective tissue showing collagen fibers and blood vessels (Figure 1b).

***Uterus control*:**

Examinations of H&E- stained sections of control mice's uterus revealed that the mucosal lining of the uterus, is composed of a simple columnar epithelium and a lamina propria. The epithelium is composed of nonciliated secretory columnar cells and ciliated cells, whereas the lamina propria houses simple tubular glands that extend as far as the myometrium (Figure 1c, d). The glandular cells resemble those of the surface epithelium, there are no ciliated cells in the glands. The dense, irregular collagenous connective tissue of the lamina propria is highly cellular and contains star-shaped cells, mononuclear phagocytic cells, and an abundance of fibers, the superficial layer is vascularized by numerous arteries; the myometrium is composed of inner longitudinal, middle circular, and outer longitudinal layers of smooth muscle (Figure 1d).

Formaldehyde exposure group

Formaldehyde exposure ovaries:

Sections of the ovary mice injected with formaldehyde showed: Proliferation of the interstitial cells among growing follicles in concomitant with the increase of the thickness of tunica albuginea covering the ovary than those of the control group (Figure 2a,b). There was a great number of growing follicles and few numbers of primordial follicles (Figure 2a), however numerous dilated blood vessels in interstitial tissue among atretic follicles and corpora lutea were observed (Figure 2c).

The degeneration appeared in different forms, some follicles showed lysis of the primary oocyte and appearance of acidophilic material filling the cavity of follicular antrum (Figure 2d). The surrounding granulosa cells showed pyknosis of their nuclei and of their cytoplasm (Figure 2e,f). Other follicles were undergone atresia (Figure 2). Normal growing follicle was also seen (Figure 2a). The most striking feature of this group was the presence of engorged blood vessels occupying the ovarian stromare in concomitant with the vacuolation of the interstitial cells (Figure 2 c).

Formaldehyde exposure uterus:

Alterations in cellular architecture of uterus of formaldehyde treatedgroups are shown in Figure (3). Compared to the uteri of control group, uterus of mice ischaracterized by decreased prominence of endometrial glands, less and few uterine glands. Degeneration of luminal epithelium and decrease in its thickness. Cellular vacuolar degeneration (Figure 3a,b). Most nuclei of glandular and luminal epithelium have heterochromatin. Note congested blood vessel (Figure 3b). Distortion and degeneration of luminal epithelium. Necrosis of the endometrial glands. Stromal mitotic figures (Figure 3c,d).

Proliferative endometrium with a small focus to the gland, some cells within the gland (abortive cells), vacuolar degeneration in endometrial epithelial cells, damage to endometrial glands. In addition, there was sloughing off from the lining endometrial epithelial cells (Figure 3b).

Vitamin C group

Vitamin C exposure ovaries:

Sections of the ovary mice injected with Vitamin C showed normal appearance of tunica albuginea covering the ovary (Figure 4a,b). Numerous blood vessels in interstitial tissue among atretic follicles and corpora lutea were observed (Figure 4b).

Vitamin C exposure uterus:

Sections of the uterus mice injected with vitamin C showed marked thickness of endometrium with hyperplasia and metaplasia of the uterine epithelial and glandular epithelial, dilated and congested blood vessels well seen (Figure 4c,d).

Vitamin C (prophylaxis) exposure ovaries:

The ovaries of this group showed the presence of growing follicles but very little of primordial follicle, proliferation of the interstitial cells among growing follicles, tunica albuginea covering the ovary thicker than the control group, numerous dilated blood vessels in interstitial tissue

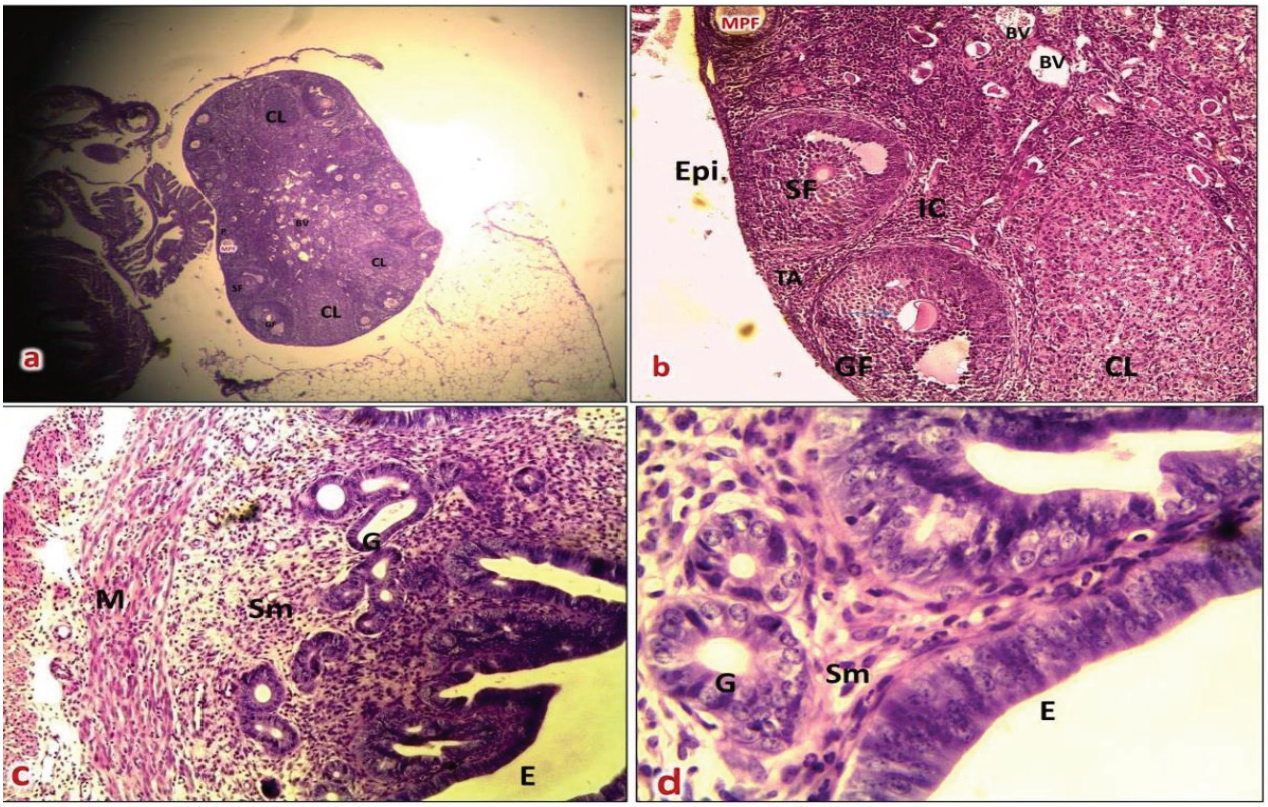

Figure 1. Photomicrographs of control mice ovary and uterus showing;

a) Overview of control ovary showing primordial follicle (P), corpus luteum (CL) (H&E, 2.5X).

b) A single layer of cuboidal surface epithelium (E), underneath it an aggregates of interstitial cells (IC) are found. Note the presence of Graffian follicle (GF), well defined corona radiate (H&E, 10X).

c) Normal histological features of endometrium containing lining epithelium of simple columnar (E), uterine glands (G) in lamina properia and Myometrium (M), (H&E, 10X).

d) Endometrium contain smooth muscle (Sm) in interstitium and uterine glands (G) (H&E, 40X).

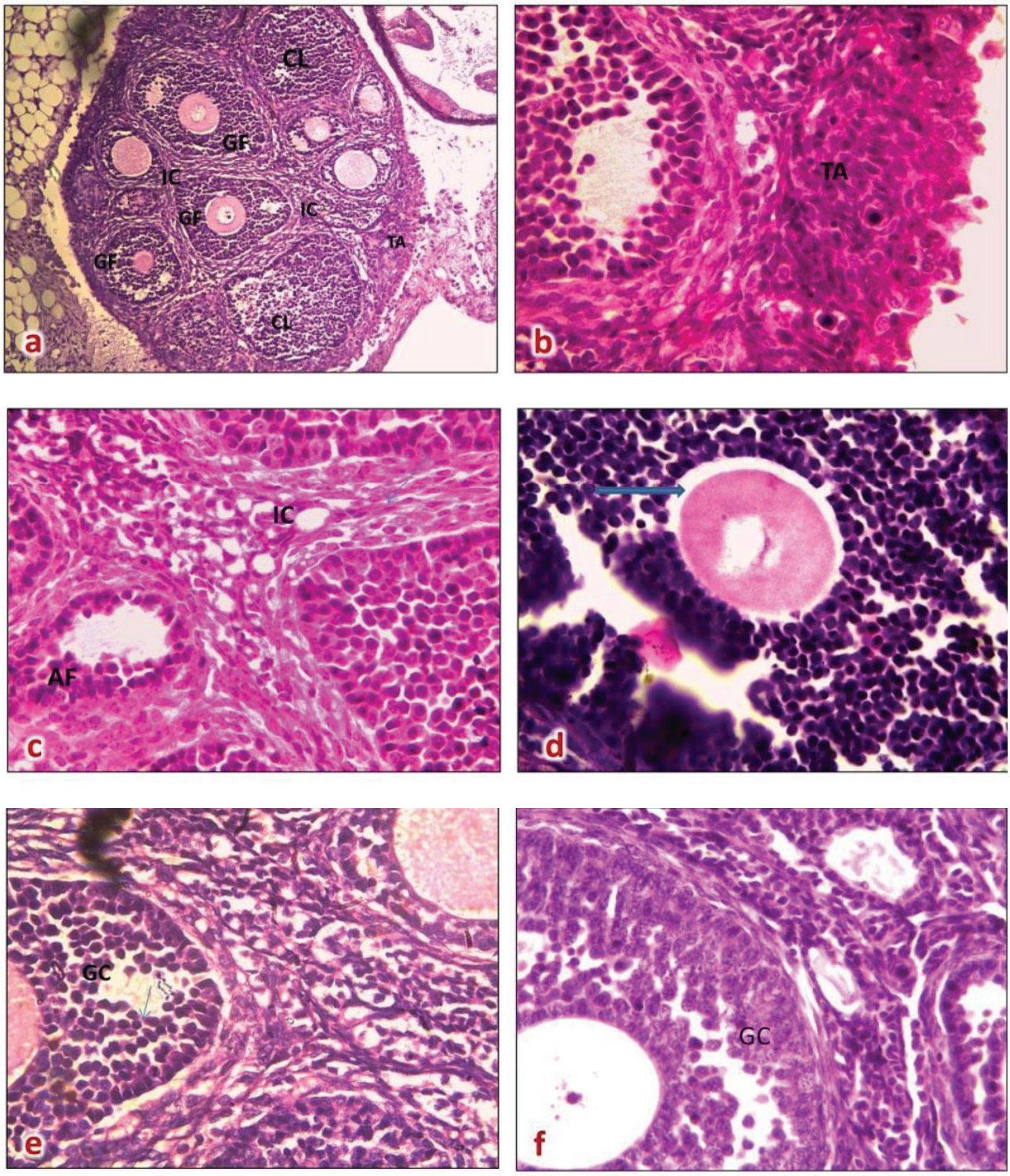

Figure 2: Photomicrographs of formaldehyde treated mice ovary showing;

a) Denoting proliferation of the interstitial cells among growing follicles (GF) (H&E, 10X).

b) A thickening of Tunica albuginea (TA), (H&E, 10X).

c) Numerous blood vessels in interstitial tissue (IC) among atretic follicles (AF) (H&E, 40X).

d) Lysis of the primary oocyte and appearance of acidophilic material filling the cavity of follicular antrum (H&E, 40X).

e) & f) Pyknosis of nuclei and cytoplasm of granulosa cells (H&E, 40X)

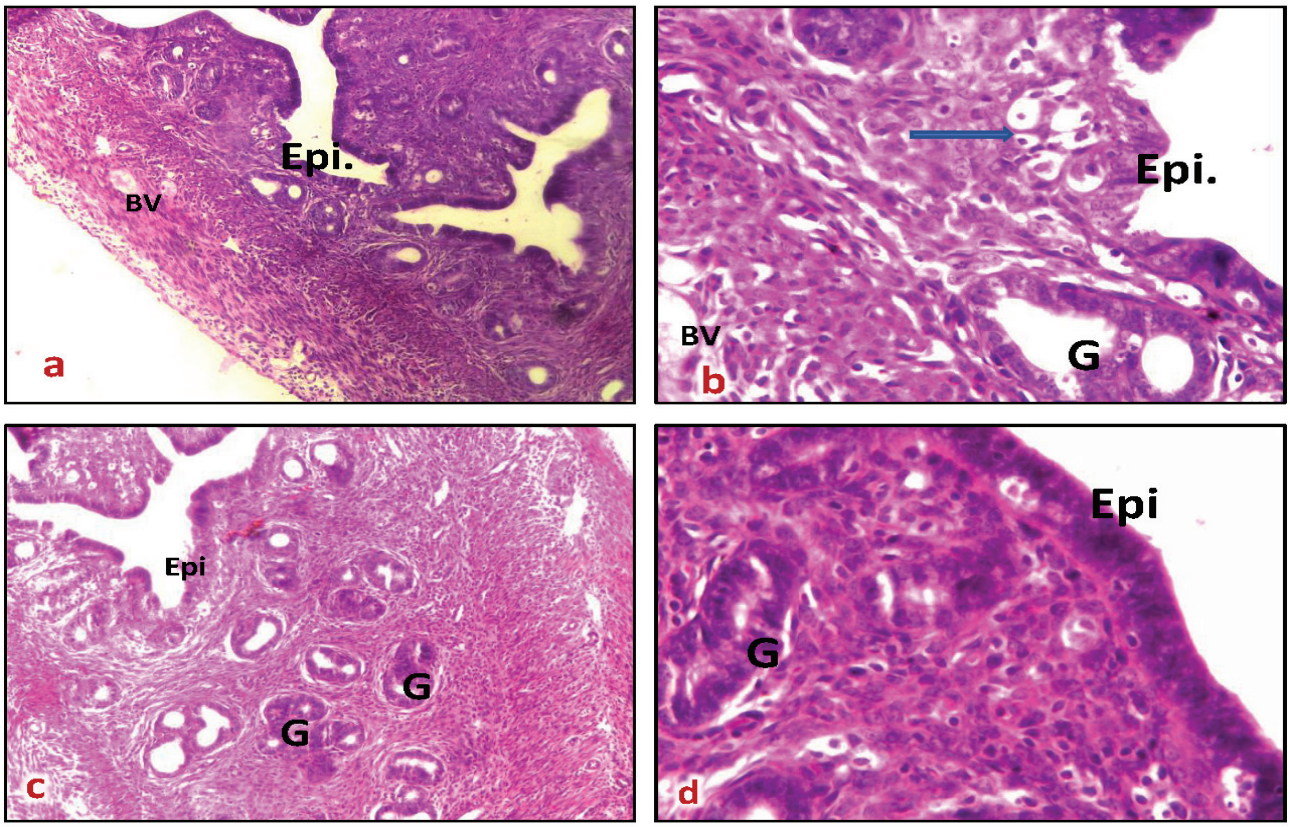

Figure 3: Photomicrographs of mice uterus treated with formaldehyde showing;
a) Endometrium showing decreased prominence of endometrial glands,congested blood vessels (BV) (H&E, 10X).
b) Vacuolar degeneration in endometrial epithelial cells (arrow),damage of endometrial glands (G), congested blood vessels (BV) (H&E, 40x).
c) Sloughing off of the lining endometrial epithelial (Epi.) (H&E, 10x).
d) Higher magnification of previous figure showing degeneration of glandular epithelium (G) &vacuolar degeneration in endometrial epithelial cells (Epi.) (H&E, 40X).

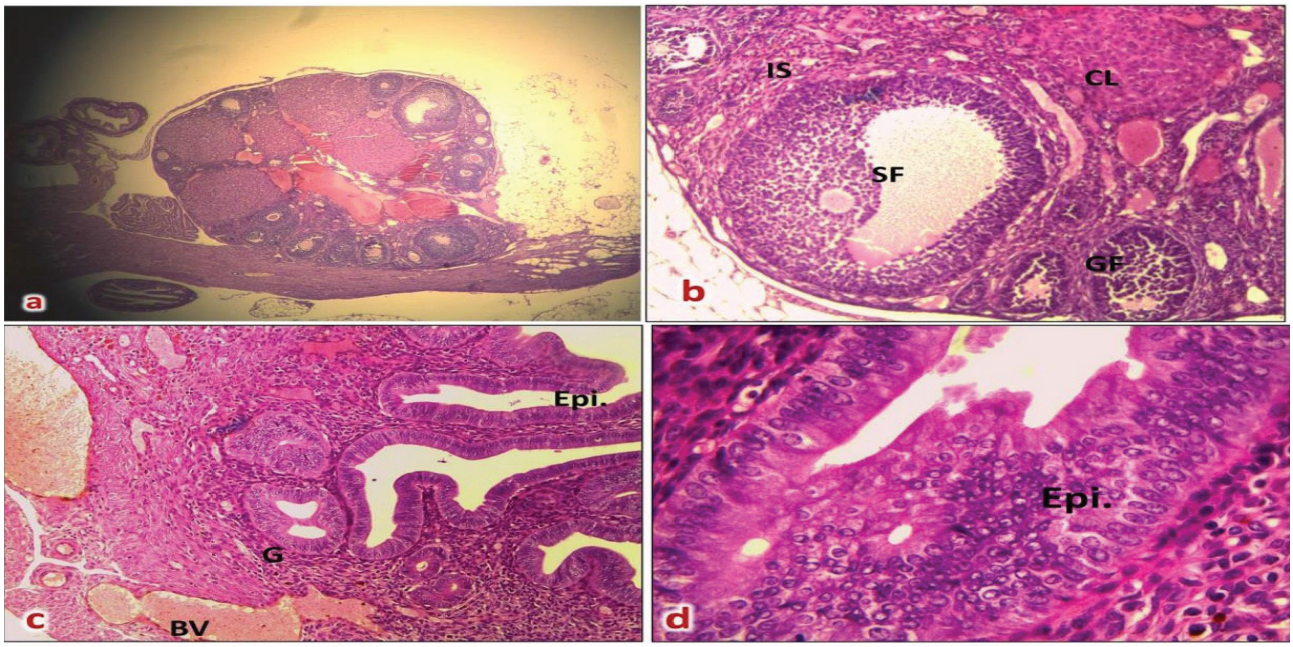

Figure 4: Photomicrograph of mice ovary and uterus treated with vitamin C showing.
a) A photomicrograph of mice ovary showing corpora lutei and growing follicles, (H&E, 2.5X).
b) Normal tunica albuginea, primordial follicles, Secondary follicles, corpous letium, blood vessels (H&E, 10X).
c) Proliferation of uterine and glandular epithelium (Epi.), (G), dilated and congested blood vessels (BV) (H&E, 10X).
d) Higher magnification of previous figure, hyperplasia of uterine epithelial (Epi.) (H&E, 40X).

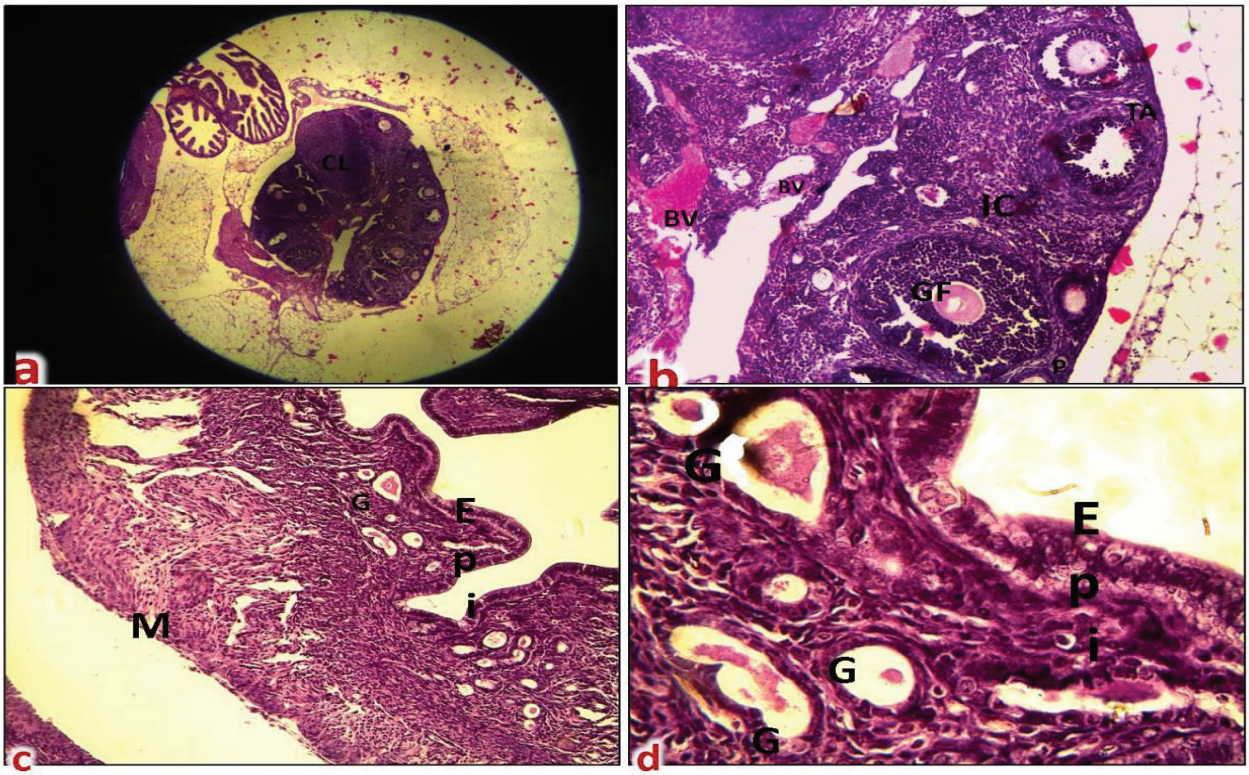

Figure 5: Photomicrographs of mice ovary and uterus treated with formaldehyde and vitamin C (prophylaxis) showing;
a) A Photomicrographs of mice ovary contain growing follicles (GF) and primordial follicles (P), (H&E, 2.5X).
b) Slightly thickness of tunica albuginea (TA), growing follicles (GF), interstitial cells (IS), dilated blood vessels in interstitial tissue (BV), (H&E, 10X).
c) The endometrium containing little uterine glands contain secretion in their lumen (H&E, 10X).
d) Higher magnification of previous figure showing proliferation of uterine epithelium, pseudo stratification (Epi.) (H&E, 40X).

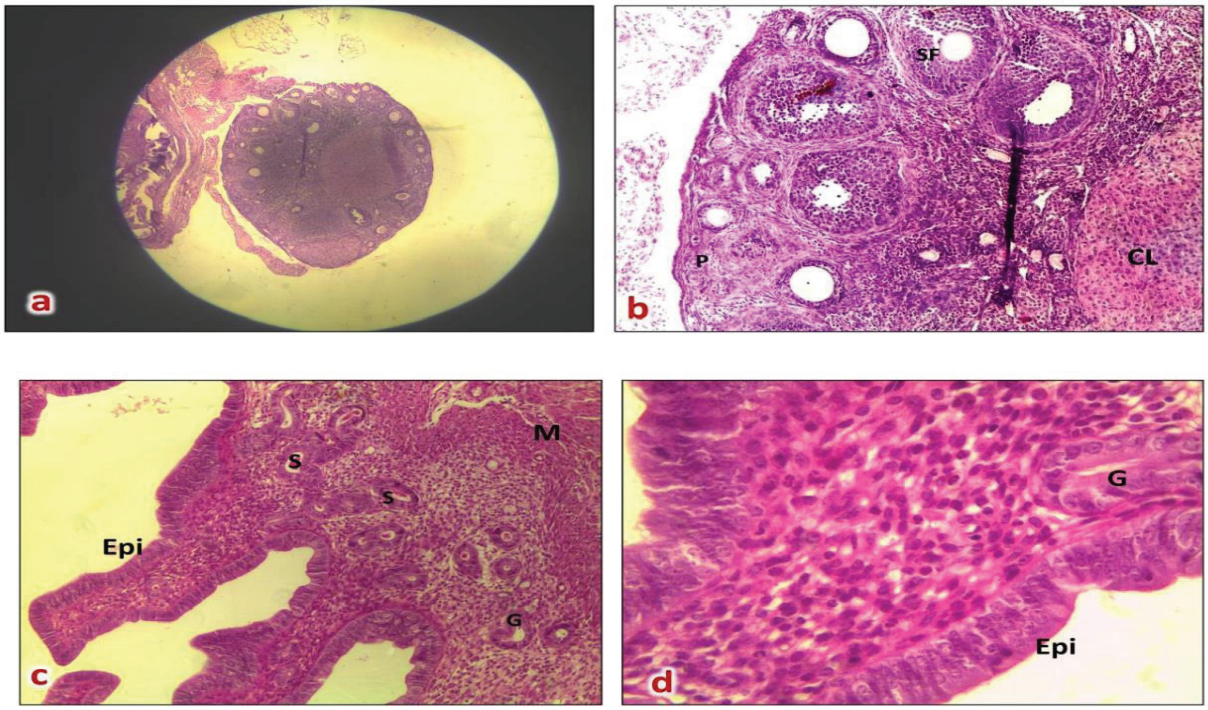

Figure 6: Photomicrographs of mice ovary and uterus treated with formaldehyde followed by vitamin C (treatment) showing,
a) Ovary appear nearly normal (H&E, 2.5X).
b) Presence of primordial follicles (P), secondary follicles (SF), corpus letum (CL), (H&E, 10X).
c) Normal uterine epithelial projections (Epi.), normal appearance of myometrium (M), the presence of glandular secretion (S) (H&E, 10X).
d) Higher magnification of previous figure showing normal uterine gland (G) and normal epithelium (Epi.) (H&E, 40X).

among atretic follicles and corpora lutea were observed (Figure 5a,b).

Vitamin C (prophylaxis) exposure uterus:

This group showing more increase in the thickness of luminal epithelium (pseudostratification). Endometrial glands showed branching and outpouching with appearance of secretion in their lumen (Figure 5c,d).

Vitamin C (treatment) exposure ovaries:

Sections of the ovaries mice injected with vitamin C (as treatment) showed nearly normal appearance of the ovaries; there are no marked changes compared to control group (Figure 6a,b).

Vitamin C (treatment) exposure uterus:

This group showed nearly normal appearance of glandular and luminal epithelium; the presence of glandular secretion is well seen in the lumen (Figure 6c,d).

DISCUSSION

The control group showed healthy ovary, the egg passed all stages, as well as normal uterus and normal number of uterus glands.

In formaldehyde treated group, ovary showed great number of mature follicles and few number of primoridial follicles. This means that formaldehyde cause damage that made primoridial follicles in unfinished stages, and low number of corpus luteum compared to the control group; this means that formaldehyde cause reduction in ovulation process, because the number of corpus luteum indicate on the amount of ovulation, and also showed proliferation for interstitial cell and increase thickness of tunica albugina and this considered abnormal.

The uterus that exposedto formaldehyde showing sloughing off from lining endometrial epithelial and low number of endometrial glands compared with the control group. The damage that caused by formaldehyde in ovary and uterus may indicate the potential mechanisms underlying formaldehyde-induced reproductive and developmental toxicities.

Formaldehyde was found to induce oxidative stress through increasing lipid peroxidation,[28] and formation of reactive oxygen species (ROS) and depletion of the antioxidant enzymes such as superoxide dismutase and glutathione peroxidase and caused destruction of mitochondria.[29] Formaldehyde induce chromosome and DNA damage (genotoxicity), oxidative stress, altered level and/or function of enzymes, hormones and proteins, apoptosis, toxicogenomic and epigenomic effects (such as DNA methylation),[22] where formaldehyde is known as genotoxic and mutagenic to mammalian cells and that it induces broad spectrum of genetic effects.[30-32]

The primary and direct genotoxic effect of formaldehyde seems to be the formation of DNA–protein crosslinks (DPC) in target tissues;[33] the exact mechanism of formaldehyde action toxicity is not clear, but it is known that it can interact with molecules on cell membranes (e.g., proteins and DNA) and disrupt cellular functions. High formaldehyde concentrations cause precipitation of proteins, which results in cell death.[34]

Formaldehyde is produced endogenously as an essential component of human cellular metabolism. External expose to formaldehyde cause genotoxicity, carcinogenicity, and teratogenicity.[35] Formaldehyde induced chromosome aberrations (CA).[36,37] It induced both base change and frame-shift mutations.[32]

Formaldehyde acts as an electrophile and react mostly with guanine and adenine in DNA and forms various kinds of DNA lesions. The DNA lesions are classified into DNA adducts, DNA intra-strand crosslinks, DNA inter-strand crosslinks and DNA-protein crosslinks.[38-40] Formaldehyde mainly induces *N*-hydroxymethyl mono-adducts on guanine, adenine and cytosine, and *N*-methylene crosslinks between adjacent purines in DNA; these crosslinks DNA damage potentially fatal for cell survival.[41] Epigenetic changes include altered DNA methylation, histone methylation, and changes in microRNA (miRNA) expression.[42] Formaldehyde reacted predominantly with deoxyguanosine (dG). It also readily formed cross-links between lysine and deoxyguanosine.[43]

Once absorbed, formaldehyde is oxidized to formic acid and CO_2, which may cause acid-base imbalance.[34] Formaldehyde is metabolized to formic acid and CO_2 by formaldehyde hydrogenase (FDH). The toxicity of the metabolite and formaldehyde in humans as well as animals includes metabolic acidosis[34,44,45]Acidosis may exaggerate damage by accelerating free radical production via H+dependent reactions, by perturbing the intracellular signal transduction pathway, leading to changes in gene expression or protein synthesis, or by activating endonucleases which cause DNA fragmentation.[46] Reactive oxygen species target is the macromolecules in cells (e.g. lipids, proteins, and nucleic acids) and cause peroxidative damage.[47] Reactive oxygen species affect multiple physiological processes from oocyte maturation to fertilization. Ovulation induced oxidative base damage to DNA of the ovarian epithelium can be prevented by antioxidant.[48] Since vitamin C is considered as a strong antioxidant compound that can strongly scavenge free radicals especially ROS.[49,50]

Vitamin C was shown to act as a chain breaking scavenger for peroxy radicals,[51]Ascorbic acid may also prevent gametes from damage by free radicals during reproduction and fertilization.[52] Vitamin C is needed for the synthesis of collagen during tissue development and at sites of tissuedamage,[53] and collagen synthesis is required for follicle growth, for repair of the ovulated follicle,[54] and for corpus luteum development.[55]

Kim et al studied the ischemic tissue damage in ovarian cortex and evaluate the effectiveness of ascorbic acid and antioxidant to protect ovarian tissue from apoptosis. They found that ovarian cortex could tolerate ischemia at least for 3 hours and ascorbic acid treatment reduced apoptosis in ovarian cortex up to 24 hours of incubation in vitro.[56] Murry *et al*. 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.[57]

Free radical accumulation and the alterations in protein structure and function, may contribute to the development of these changes that occurred in ovary and uterus due to formaldehyde.

Ovarian function of female rats can be impaired after subchronically exposed to formaldehyde for 14 days, it produced hormonal disturbance as a decrease in serum estradiol (E2) and Inhibin B and increase the level of FSH, without changes in LH.[58] Formaldehyde has adverse effects on estrous cycle and ovary female mice; it produced atretic follicle, swelling of oocyte mitochondria and vacuolar degeneration; also, some oocytes disintegration.[59]

Additionally, it was suggested that the form of DNA damage induced by gaseous formaldehyde on reproductive cell of female mice was DNA-protein crosslinks; this damage increased with the increasing of formaldehyde concentrations. Formaldehyde has obvious genetic toxicity on the reproductive cells of female mice since DNA-protein crosslinks is a grievous damage of DNA.[60]

In this study, vitamin C administration showed healthy ovary with normal appearance of tunica albuginea covering the ovary, and high number of corpus luteumcompared to formaldehyde treated group. This mean that vitamin C stimulate ovulation and repair the damage induced by formaldehyde. Vitamin C dilate blood vessels in ovary and uterus and uterine epithelial compared to the control and formaldehyde group. This confirm that vitamin C is considered effective antioxidant in various biological systems.[61-64]Ascorbic acid has three biological actions of particular relevance to reproduction, each dependent on its role as a reducing agent: it is required for the biosynthesis of collagen, biosynthesis of steroid and peptide hormones, and prevent or reduce the oxidation of biomolecules. It is frequently involved in mixed-function oxidation, resulting in the incorporation of oxygen from molecular oxygen into a substrate.[65]

The role of ascorbic acid in maintaining the structure of collagenous tissues was confirmed.[66] It is needed for the synthesis of collagen during tissue development and at sites of tissuedamage, also for the maintenance of the slow collagen turnover which occurs in mature tissues.[53]More studies with luteinizing granulosa cells showed that ascorbate is stimulatory to progesterone and oxytocin secretion[67,68], consistent with its known roles in hormone biosynthesis, and synergizes with neurotransmitters in stimulating hormone secretion.[69]Collagen synthesis is required for follicle growth, for repair of the ovulated follicle,[54]and for corpus luteum development.[55]Ascorbate also needed for secretion of collagen and proteoglycans into follicular fluid.70[70-72]Vitamin C treatment and prophylaxis groups showed nearly healthy condition compared to the control group, where it showed primary and secondary and graffian follicles; but the treatment group show better repair and improvement than the prophylaxis group.

CONCLUSION

Based on the findings of this study, it can be concluded that formaldehyde induce histological alteration in ovary and uterus of female mice reproductive system and in turn, vitamin C has prophylaxis and treatment role against this damage induced by formaldehyde.

Future studies on animal models provide novel information on protective role of vitamin C against formaldehyde damage in fetus and offspring.

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