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The protective role of ascorbic acid in the reproductive functions of female laboratory mice treated with methomyl pesticide

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Abstract:

The present study aimed to evaluate the preventive role of ascorbic acid in female laboratory mice treated with the methomyl Carbamate insecticide, The research included a study of physiological changes in the level of the sex hormone estrogen and the Phase Oogenesis and measuring of the antioxidant glutathione and catalase enzymes level . In the current study, 40 females mice of age (10-12 weeks) and weight (23-25 g) were used with sexual experience and were divided into five groups. Each group contained eight mice. The first group was injected with the physiological solution, The second group treated with low dose of the pesticide 1 mg / kg methomyl pesticide, the third group (with high dose of the pesticide) was injected with a concentration of 2mg / kg of methomyl pesticide, the fourth group was injected with a concentration of 200 mg/kg of ascorbic acid After being injected with the 1 mg / kg dose of the pesticide and the fifth group was injected with a concentration of 200 mg/kg of ascorbic acid after being injected with the 2 mg / kg dose of the pesticide. The results showed a significant decrease in the level of estrogen hormone in the serum of female laboratory mice with both doses treated with the pesticide. The injection of mice treated with ascorbic acid also improved the level of the hormone, which led to an increase in its level in the serum compared to its level in the two groups treated with the pesticide only (methomyl), as the statistical analysis showed that a reduction occurred significant number of Ovarian vesicles and the two doses treated with the pesticide, while treatment the mice with ascorbic acid recorded a distinct protective role in the process of Oogenesis. In addition, ascorbic acid preserved the levels of antioxidants in the serum of mice after it increased significantly due to treatment with methomyl only.

Key words: Methomyl pesticide, Ascorbic acid, Estrogen, Oogenesis, antioxidants enzyme.

Introduction:

Methomyl is an insecticide from the group of Carbamate pesticides, whose chemical formula is ($C_5H_{10}N_2O_2S$), and it has been classified as a toxic and harmful chemical to humans and to non-target organisms. The two groups of oxime (H3-NH-O) and Dimethyl sulfide (CH_3-S-CH_3) are toxic the pesticide is due to less stability (Tamimi *et al*., 2008).

Methomyl pesticide was used to control and eliminate many agricultural pests and insects of all kinds, as well as to get rid of worms, snails and others due to its wide and influential effects, which were detected through the pesticide residues in soil, agricultural products and natural waters (EPA, 1998).

Ascorbic acid is also known as Vitamin C or Ascorbate and its chemical formula ($C_6H_8O_6$), the oxidized form of it is known as Dehydro ascorbic acid (DHAA), which together with ascorbic acid represents the physiologically active forms of the vitamin C (Choi *et al*., 2009) and it is non-toxic as the value of LD50 is estimated. It has 11.9 g / kg orally and is a water-soluble vitamin naturally available in various amounts in fruits, liver, kidneys and vegetables (Nishikimi et al., 1994).

Ascorbic acid acts as a free radical scavenger and draws electrons to reduce reactive oxygen species (ROS) and prevent damage to fats, proteins and DNA during cell metabolism or during exposure to toxins and pollutants (Carr, 2017; Lykkesfeldt *et al*., 2014). Ascorbic acid is beneficial for Mammalia reproduction through enhances the meiotic maturation of oocytes in pigs , and improves embryo growth efficiency (Gomes *et al*., 2015; Tao *et al*., 2010; Yu *et al*., 2018; Kere *et al*., 2013), moreover. Ascorbic acid can reduce the defects in eggs and embryos caused by environmental pollutants such as heavy metals and pesticides of all kinds (Zhou *et al*., 2019).

Insecticides, despite their presence in small quantities, if compared to other pollutants such as industrial waste and fertilizers, constitute public concern and the scientific community as they have high biological activity and tend to persist and have the ability to accumulate in the body of the organism, which increased concern about the reproductive system as they cause Changes in reproductive behavior that contribute to decreased fertility, infertility, pregnancy loss, growth retardation, intrauterine deaths, birth defects, and ovarian failure (Fleming *et al*., 1999).

Insecticides tend to interfere with the female hormonal functions, which negatively affects the reproductive system through disturbance and hormonal inhibition, which affects the proper functioning of the reproductive system, as insecticides affect one of the necessary mechanisms in the aesthetic fertilization process, which includes synthesis and secretion of hormone (Kara and Oztas ,2020). a mechanism of their storage , hormones metabolism and transporting them, identifying hormone receptors, the action mechanism of hormonal activation in the

receptors , the mechanism of action of the nervous system, and which is related to the function of the thyroid gland (Bretveld *et al*., 2006).

Exposure to carbamate insecticides leads to insufficiency of the female reproductive system through disturbances in ovarian function, which include changes in hormone secretion, follicle maturation, ovulation process and ovarian cycle, as these changes ultimately cause fertility decline, and the induced ovarian toxicity is affected by insecticides by endocrine disruption and Free radical formation (Rajnesh *et a*l., 2020). In addition, the carbamate carbaryl pesticide causes inhibition of estrogen synthesis by affecting granule cells (Cheng *et at*. 2006). The present study aimed to investigate the protective role of ascorbic acid in physiological changes of ovaries of female laboratory mice treated with methomyl pesticide.

Materials and methods:

Experiment animals:

Female mice have prepared the type experiment of *Mus musculus* L. belonging to the Balb / C strain, from the animal house belonging to the College of Education for Pure Sciences - University of Basra, and were bred and mated under controlled conditions of temperature (23 ± 1 C^{o}) and a periodic lighting system (12 hr. light and 12 hr. dark) and standard feeding. Laboratory mice were injected daily for a period of (16) days in the intraperitoneal (I.P) (Shimizu ,2004). Female adult mice (10-12 weeks) with weights ranging from (23 -25 g) were divided into five groups, as each group included 8 mice as follows:

First: the control group: It was injected with normal Saline at a dose of 0.1 ml.

Second: The treatment group with low concentration was injected with a concentration of 1 mg / kg and a volume of 0.1 ml of pesticide solution.

Third: The high concentration treatment group was injected with a concentration of 2 mg / kg and a volume of 0.1 ml of pesticide solution.

Fourth: The first treatment group was injected with a concentration of 200 mg/kg of ascorbic acid after 30 minutes of injection with a low concentration of pesticide solution at a concentration of 1 mg / kg.

Fifth: The second treatment group was injected with a concentration of 200 mg/kg of ascorbic acid and after 30 minutes of injection with a high concentration of pesticide solution at a concentration of 2 mg / kg.

Measurement of serum estrogen level:

Estrogen level was measured using the method (Gore - Langton and Armstrong, 1988), and using an American instrument from CALBIOTECH.

Measuring the level of glutathione in the blood serum:

Bioassay Technology Laboratory test kit of Chinese origin, using the method, was used. (Kickligher *et al*.,1989).

Measuring the level of catalase in the blood serum:

Kohl and Ascoil (2017) is approved using Sandwich Kit ELISA technology and a China-produced Bioassay Technology Laboratory measuring kit.

The Histological Study:

The laboratory mice were dissected on the seventeenth day of treatment after being anesthetized with chloroform, then the ovaries were isolated and kept in screw tubes containing formalin at a concentration of 10%, the tubes containing the samples were passed with increasing concentrations of ethanol alcohol (100%, 95%, 70%), the alcohol was removed from the tissues by washing with xylenes for two hours, then placed with molds of molten paraffin wax, then the molds were cut into strips 6 µm thick and fixed on glass slides by gluing them and stained with hematoxylin and eosin dyes and examined under the Optical microscope (Humason, 1972).

Statistical Analysis:

The statistical program (SPSS ver.20) used the Statistical Package for the Social Sciences to statistically analyze the results of the current study and by using the Analysis of Variance (ANOVA) test, and also used the Least Significant Difference (LSD) test at a significant level of $P < 0.05$.

Results:

The results showed a significant decrease in the level of estrogen in the female laboratory mice treated with two doses of methomyl pesticide (1,2 mg / Kg) at a probability level $P \leq 0.05$ compared to the control group, while treatment of mice with ascorbic acid showed a protective role at 200 mg / Kg. It caused a significant increase in the level of estrogen concentration for both groups treated with ascorbic acid compared with the two groups treated with the pesticide only and at the same level of significant difference, while no significant difference was observed between the control group and the two treatment groups with ascorbic acid (Table 1).

Table 1 The effect of Methomyl on the level of Estrogen in the serum of laboratory mice and the protective role of ascorbic acid. (mean ± SD)(n = 8)

Groups	Estrogen Pg/ml
Control group (physiological solution) 0.1ml N.S	**$20.27^{a} \pm 3.81$**

Second group Methomyl low dose group (1mg/kg)	**6.05 ^{c} ± 1.166**
Third group Methomyl high dose group (2mg/kg)	**2. 00^{d} ± 0.57**
Fourth group treatment group (Ascorbic acid 200mg/kg and 1mg/kg)	**18.19^{a} ± 3.05**
Fifth group treatment group (Ascorbic acid 200 mg/kg and 2mg/kg)	**15.31^{b} ± 1.33**

$^{a, b, c, d}$ different letters indicate significant differences ($p < 0.05$) among groups

Statistical analysis showed a significant increase in glutathione and catalase enzymes in the second treatment group with the pesticide at a concentration of 2 mg / kg compared with the control group and groups treated with ascorbic acid, in addition, the results did not record a difference significant between the two treatment groups with ascorbic acid and the control group (Table 2).

Table 2: Effect of methomyl fungicide on glutathione and catalase levels and the protective role of ascorbic acid.(mean ± SD)(n = 8)

Group	Catalase mg/l	Glutathione mg/l
Control group (physiological solution)0.1ml N.S	**12.12^{bc} ± 1.45**	**84.5^{c}±12.24**
Second group Methomyl low dose group (1mg/kg)	**14.25 ^{ac}± 2.65**	**93.87^{bd} ±12.62**
Third group Methomyl high dose group (2mg/kg)	**17.12 ^{a}± 1.8**	**110.87^{a} ±38.49**
Fourth group treatment group (Ascorbic acid 200mg/kg and1mg/kg)	**12.5^{bc} ± 2.00**	**78.00^{cd}±15.49**
Fifth group treatment group (Ascorbic acid 200 mg/kg and 2mg/kg)	**12.00^{bc}± 1.60**	**85.57^{c}±12.17**

$^{a, b, c, d}$ different letters indicate significant differences ($p < 0.05$) among groups

The Histological Study:

The results of the histological study showed a significant decrease in the number of primary, secondary and Graafian follicles of treated groups with the pesticide when compared to the control group at a statistical significance level (P <0.05). The treatment groups with ascorbic acid also recorded a significant increase in the number of primary, secondary and graafain follicles when compared to treated groups with the methomyl pesticide and with both doses. In addition, the pesticide caused a significant decrease in the number of the corpus luteum in the two treatment groups with pesticide , while the both treatment groups with ascorbic acid recorded a significant increase in the number of the corpus luteum when compared to the control group, as shown in Table 3, The histological study also showed the occurrence of histopathological changes in the ovaries in the treated groups with both doses of methomyl , as it occurred with the presence of areas of bleeding and congestion in the blood vessels, and the decrease in the numbers of ovarian follicles was evident, and in some ovaries a lack of Graafian follicle and a severe decrease in the number of corpus luteum , but in the two treatment groups with ascorbic acid , an improvement in the number of all ovarian follicles and corpus luteum was observed. The ovarian tissue was not affected, and no observations were recorded about the occurrence of any hemorrhage and tissue hyperplasia .

Table 3: The effect of methomyl on oogenesis and the protective role of ascorbic acid in female laboratory mice.

(mean ± SD)(n = 8)

Groups	primary follicles	secondary follicles	Graafian follicles	corpus luteum
Control group (physiological solution)0.1ml N.S	11.1^{a}±2.287	7.1^{a}±1.791	3.7^{a} ±0.948	1.9^{a} ±0.567
Second group Methomyl (low dose group (1mg/kg	5.5^{c}±1.269	4.5^{c} ±1.354	1.6^{b}±0.699	$1.2b^{d}$ ±0.632
Third group Methomyl (high dose group (2mg/kg	3.5^{d}±0.849	4.2^{c} ±0.918	0.7^{c} ±0.483	0.7^{cd} ±0.674
Fourth group treatment group (Ascorbic acid (200mg/kg and1mg/kg	7.5^{b}±1.715	4.7^{c} ±0.948	1.8^{b} ±0.788	1.5^{b} ±0.849
Fifth group treatment group (Ascorbic acid 200 (mg/kg and 2mg/kg	8^{b}±1.885	5.8^{b} ±1.588	2^{b} ±0.258	2.2^{a} ±0.249

$^{a, b, c, d}$ different letters indicate significant differences (p <0.05) among groups

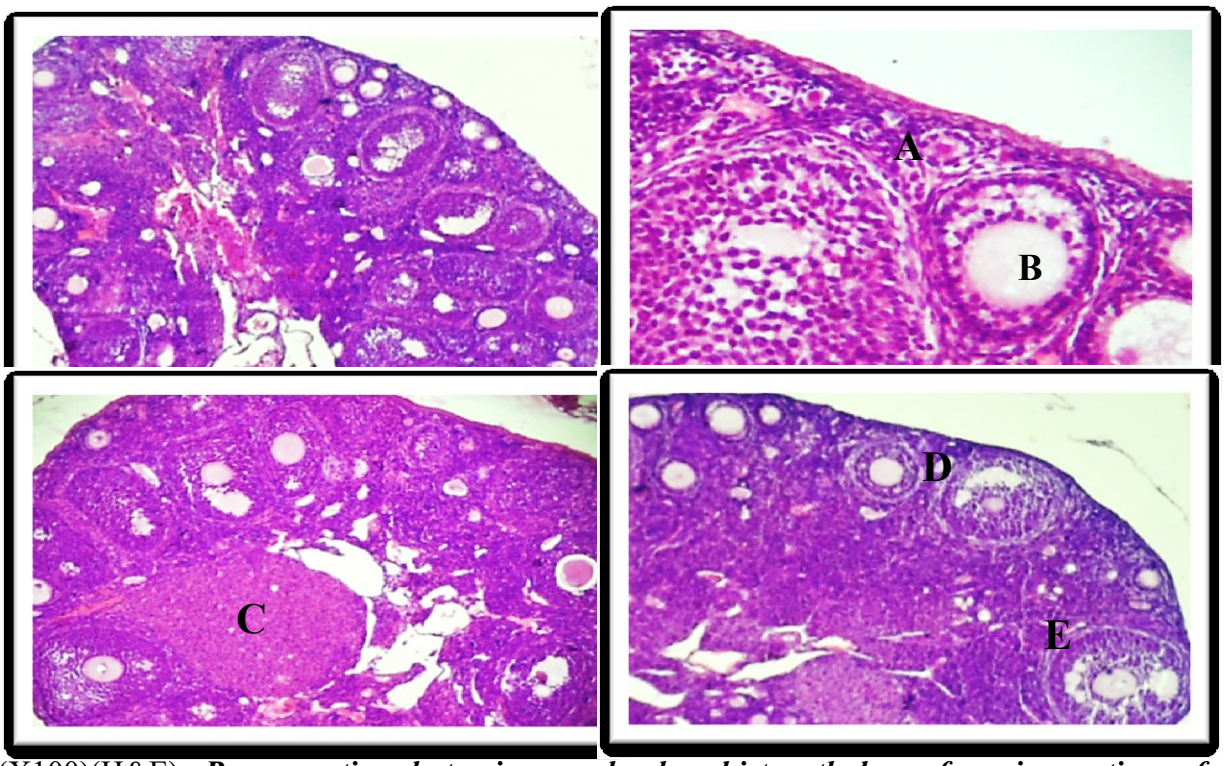

Fig 1(X100)(H&E) : ***Reprsesentive photomicrographs show histopathology of ovarian sections of mature mice ,show* A *primary,* B *secondary ,* D,E *Graafian follicles and* C *corpus luteum in the Control group.***

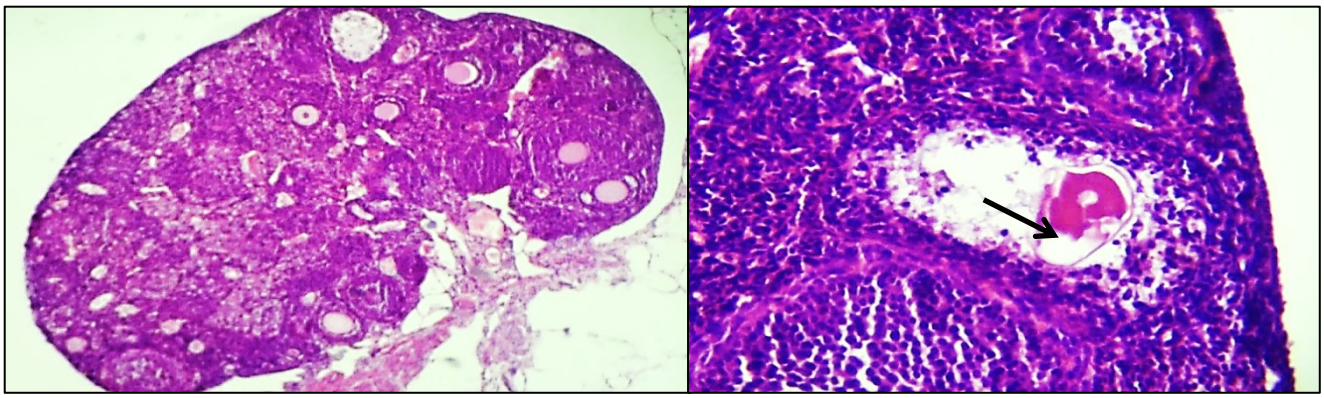

Fig 2 (X40)(H&E) ***: Cross-section in the ovarian from the methomyl-pesticide low-dose group show Reduction in the number of ovarian follicles and Oocyte lysis (arrow) .***

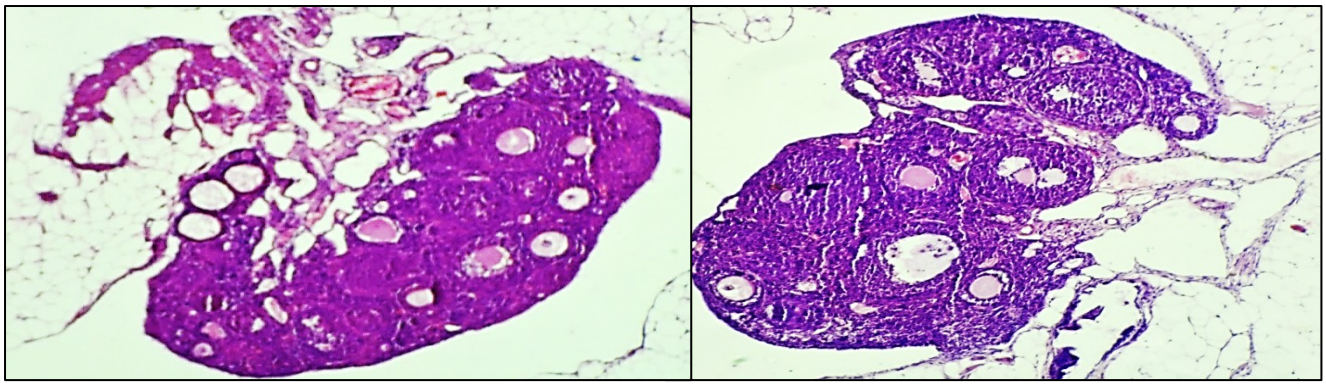

Fig 3 (X40)(H&E) : ***Ovarian cross-section from the methomyl pesticide high-dose group show demonstrating reduction in ovarian follicle numbers and ovarian small size.***

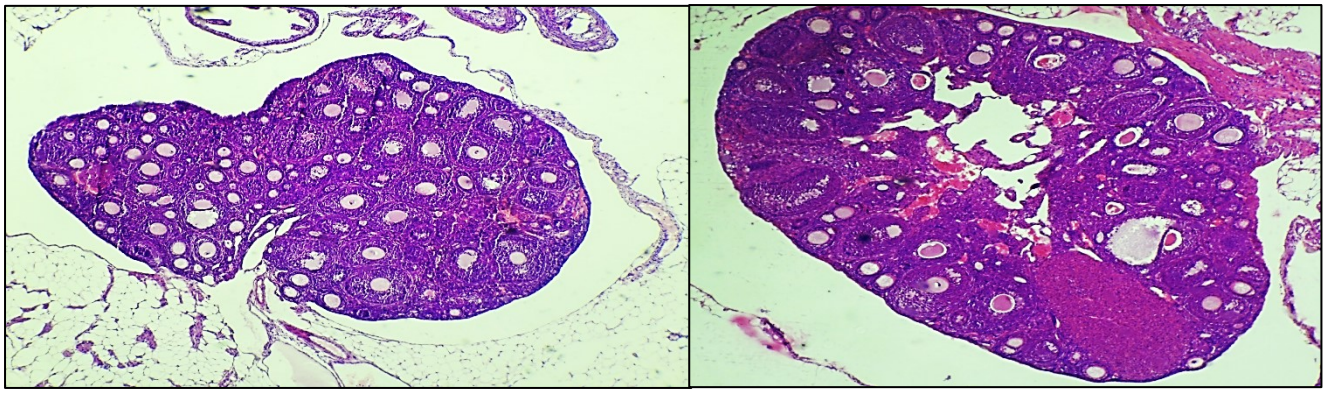

Fig 3 (X40)(H&E) : ***Ovarian cross-section from the low dose group of methomyl pesticide with ascorbic acid showing increased numbers of ovarian follicles and continuation of the Oogenesis.***

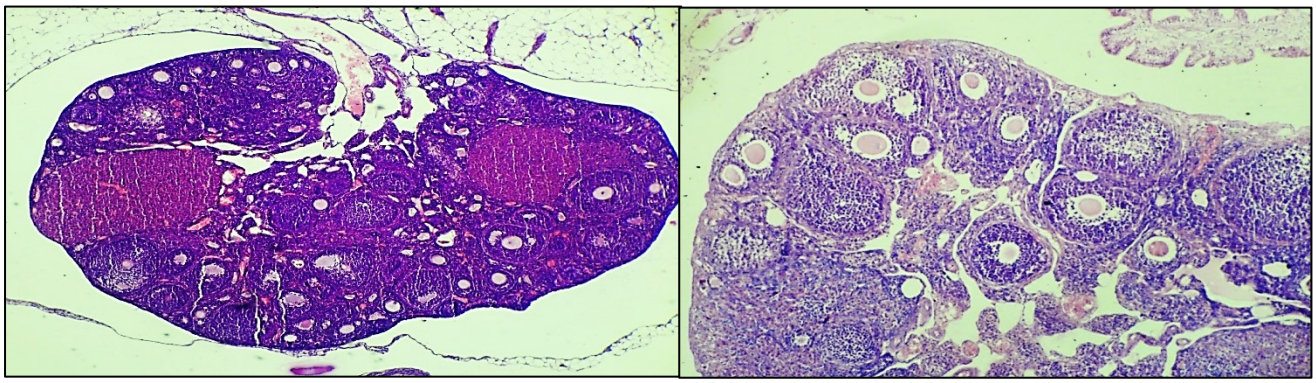

Fig 4(X40)(H&E) : ***Bleaching cross-section from the methomyl pesticide high dose group with ascorbic acid showing normal ovarian follicle numbers , Oogenesis and It shows normal ovarian size.***

Discussion:

In the current study , the reason for the decreased in the estrogen hormone level was due to the toxic and oxidative effects of methomyl pesticide through the accumulation of reactive oxygen species ROS resulted from oxidative stress, which cause the oxidation of lipids , proteins, and genetic materials such as DNA and RNA (Singh *et al*., 2006; Zama *et al*, 2007). The destruction of antioxidant defense systems and increased ROS accumulation cause inhibiting RNA synthesis in follicular vesicles, as well as work on DNA damage through the attack of the hydroxyl radical on guanine nucleic acid at the site of the OHdG-8, which is one of the most important bases studied and is considered an acceptable biological evidence on DNA damage in biological systems (Orrenius *et al*., 2007). Also, the oxidative stress generated by the pesticide affects the oxidative enzymes in the mitochondria of ovarian cells, which induces cell apoptosis (Harvey, 2019). The above is consistent with Rattan *et al*. (2017) which indicated in their study the effect of chemicals including, pesticides, on disruption endocrine.

Another factors that reduce estrogen hormones , under the toxic action of methomyl pesticide , are due to the disturbance of the hypothalamic-pituitary-genital axis that responsible on regulation and secretion of reproductive hormones (Sakr *et*

al., 2017; Bretveld *et al*., 2006).Also the reduction of estrogen hormone is due to decrease in its receptors which include ERα and ERβ, and these receptors are playing a main role in physiological development and function of the ovaries, and the disorder in them lead to decrease in the FSH and LH hormone , infertility , a failure in the ovulation process (Tang *et al*. , 2019). And it has been demonstrated that exposure to low dose of methoxychlor pesticide can lead to defect of hypothalamic and pituitary function in rodents . It is assumed that any environmental compounds that mimics or antagonizes the action of the steroid hormone can, alter the process of Glycosylation in LH and FSH, and thus reducing their biological activity (Palanza *et al*, 1999; Cooke and Eroschenko, 1998).

Carbamate pesticides also impede the synthesis of estrogen through the pathological and histological damage of granular cells in the ovaries , which most important functions of their production and secretion of sex hormones such as estrogen and progesterone (Cheng *et al*., 2006). The reason for the diminishing in estrogen level may be due to the damage in ovarian cells and the reduction of LH and FSH receptors for hormone in granular cells that cause decreasing in estrogen production and secretion (Holesh *et al*., 2003)

The results of the current histological study also indicated that the pesticide caused failure in ovarian follicles development , which was observed through the decreasing in the number of primary, secondary, graafain follicles and corpus luteum with a marked increased in the number of atretic follicles. The pesticide also caused a smallness size of the ovaries when comparing with control group, and these results are in agreed with the study of Baligar *et al*. (2002). They showed that the administration of Swiss mice with the Carbofuran insecticide caused histopathological effects through a decreasing in the number and duration of estrous cycles in mice, a decreasing in mature ovarian follicles, an increasing in the atresia follicles, as well as a decrease in ovarian weight. The total tissue changes that occurred in the ovaries are clear evidence of physiologically disruption in ovarian action , as these changes caused a decreasing in the secretion of estrogen hormone, which plays a main role in the formation of ovarian follicles. Therefore any decrease in the number of these follicles are resulted from a low level of estrogen hormone (Al-Hamdani and Yajurvedi, 2017).

The central nervous system (CNS) is very important in the integration of hormonal and behavioral activity, and disturbances in these activities, which operate in accurate coordination, can impair the reproductive behavior . Because of many pesticides are known to be neurotoxic, they can disrupt the coordination activity of the nervous system by damaging brain cell functions and thus reflect their negative affect on reproductive function in females (Crisp *et al*., 1998).

The results of the current study showed that the insecticide methomyl caused a significant increase in the level of the enzyme glutathione in the serum of female mice in the treatment with high concentration only when compared to the control group, and the study was compatible with the study (Nur *et al*. 2011). While the

methomyl pesticide caused an increase significant of glutathione level in the two groups treated with the pesticide in the serum of laboratory females compared to the control group, and the present study agreed with several studies, including Naz *et al*. (2019) and (Friscic *et al*. 2014) as the results of these studies showed the occurrence of an increase of the antioxidant catalase enzyme level after exposure to insecticides.

The increase in the level of the glutathione enzyme may be attributed to a natural reaction to a free radicals scavenger which are induced by the methomyl pesticide . The enzyme glutathione participates in many detoxification reactions, due to its high ability to protect cells from the damage induced by oxidative stress. As a result of the oxidation and reduction cellular state characterized by the levels and ratio of oxidized Glutathione disulfide (GSSG) and reduced (GSH) glutathione, which is an important indicator of cell integrity, and due to the high toxicity of oxidized glutathione (GSSG) it converts very quickly and returns to the reduced form by the enzyme glutathione reductase (GSR). In this way, the level of the enzyme glutathione increases (Deleve and kaplowitz 1991).

The significant increase in the level of the catalase enzyme is also due to the fact that the methomyl pesticide causes the formation of the H2O2 peroxide radical rather than the other types of the free radicals (Tamimi *et al*., 2008). And due to the fact that the catalase enzyme catalyzes the decomposition of the hydrogen peroxide radical into water and oxygen, as result, the increasing in hydrogen peroxide concentration raises the level of catalase enzyme as a normal reaction (Kaushal *et al*., 2018).

On the other hand, the results of the current study showed the protective role of ascorbic acid in maintaining the estrogen hormone in the low and high doses treated groups .This is due to the role of ascorbic acid in suppression of ROS formed by the insecticide methomyl, and this was declared in the results of the current study . As well as there was no significant difference in the level of the estrogen hormone in the group treated with ascorbic acid comparing with control group, and this agreed with the study conducted by Cemek *et al*. (2010). The difference between the ovarian tissues in high and low concentration of pesticide treating groups and treatment group with ascorbic acid, was cleared through remaining the number of graafain vesicles, corpus luteum and ovarian vesicles within the normal range , when comparing with control group. And This result was agreed with study of Jaiswal *et al*. (2013), whose indicated the role of ascorbic acid in preserving of ovarian from the damage caused by the pesticide Carbofuran in rats.

The results showed the role of ascorbic acid in production of glutathione and catalase enzymes in female laboratory mice treated with methomyl pesticide and ascorbic acid at a dose 200 mg / kg with no significant difference when comparing with control group, and this results agreed with Milosevic *et al*. (2018). The results of their study showed the protective role of vitamin C and selenium Se against the various toxic compounds due to their antioxidant properties, after exposure to the insecticide fenitrothion and the maintaining of the catalase enzyme level within the normal ranges. Ascorbic acid also improves antioxidant enzymes activity, reduces

lipid peroxidation and prevents oxidative stress caused by methomyl. The antioxidant mechanisms of ascorbic acid depend on donating of a hydrogen atom to the free radicals, suppressing the reactive oxygen and renewal of α-tocopherol (vitamin E). (Milosevic *et al*., 2018).

Conclusion:

Methomyl pesticide inhibits the number of proliferating and evolving follicles in ovaries . Ascorbic acid by its antioxidant activity was able to ameliorate the toxicity of methomyl . For that reason , ascorbic acid can protect ovarian follicles against this toxicity.

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