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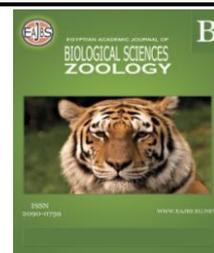


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Therapeutic Effects of Ascorbic Acid on Hormonal and Histological Alteration Produced in The Reproductive System of Albino Rats Intoxicated by Herbicide Atrazine

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ABSTRACT

The aim of this study was to examine the cytotoxicity of the herbicide atrazine on the reproductive system. 48 male and 48 female albino rats were treated with atrazine daily for two different durations (15 and 30 days). Reproductive system toxicity was monitored by quantitative analysis of the serum Follicle-stimulating hormone (FSH), Luteinizing hormone (LH), Prolactin (PRL), Estrogen (E2), Progesterone (Prog) and Testosterone (Testo). On the other hand, the reproductive organs were collected for histopathology study. The study showed a significant elevation of estrogen, progesterone hormones with a significant decrease in testosterone hormone in male groups while in female groups there was a significant decrease in estrogen, progesterone & hormones with a significant increase in testosterone hormone. But there was no effect on PRL and LH hormones in both male and female groups toxified by ATZ, in comparison to the control groups. In addition to that, the Light microscopic examination of the seminiferous tubule cells (st) showed vacuolations within seminiferous tubules (V), degeneration of spermatozoa formation and hemorrhage (hg) in the interstitial tissue. These effects were increased by increasing the dose or the time of exposure. By using ascorbic acid in the treatment of those effects, we find a significant improvement and detoxification of the atrazine effects on both hormonal tests and histological sections. From our study results, we concluded that there is a potential contribution of herbicide mixtures in the etiology of somebody's diseases, while ascorbic acid has beneficial effects as it tends to dampen atrazine toxicity, in albino rats.

INTRODUCTION

Atrazine exposure interferes with normal meiosis, which affects spermatozoa production in male mice. Oxidative stress and disruptions in calcium homeostasis play an important role in the induction of immune-toxicity in mice by atrazine as depicted by (Gao S, 2016) The prime target of chlorinated atrazine on humans and mammals is the disruption of the endocrine system (Jin Y., 2014); (Kroon F.J., 2014); (Weber G.J., 2013). Secondly, it also induces oxidative stress by the formation of reactive oxygen species resulting in the reduced semen quality, sperm dysfunction and infertility of amphibians, rats and pigs (Gely-Pernot A, 2015); (Jestadi D.B., 2014); (Kniewald J. J. M., 2000). Atrazine is known to cause hepatic

damage, as the liver is the primary organ for atrazine metabolism in mammals (Campos-Pereira F.D., 2012); (Gojmerac T., 1989). Atrazine is also responsible for cardiotoxicity in mice by disruption of ionic balance (Lin J. L. H., 2016a) (Lin J. L. H., 2016b). A considerable number of antioxidants as ascorbic acid and vitamin E have been tested in lab animals to minimize the clastogenicity induced by drugs (Antunes L.M., 1998), (Siddique YH, 2005). Antioxidant vitamins are able to inactivate highly reactive molecules, such as free radicals, which are generated during various biochemical processes in the cells (Costa W.F., 2006).

Atrazine (6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine is a synthetic triazine herbicide used to control grassy and broadleaf weeds in sugarcane, wheat, conifers, sorghum, nuts and corn crops (Iriel A., 2014); (Kumar A., 2016); (Zhao X., 2017). It is the second most widely consumed pesticide in the world with annual consumption of about 70,000–90,000 tons (Kumar V., 2013); (Cheng M., 2016). Because of its long half-life of 41–231 days (Karlsson A.S. W. L., 2016), low adsorption in soils and moderate aqueous solubility, it has a tremendous potential to contaminate not only agricultural areas but also ground and surface water with the highest concentration up to 30 µg/L (Cerejeira M.J., 2003); (Schwab A.P., 2006); (Kumar V., 2013). It was banned in several countries like Italy (Huang H., 2009), Denmark (Glæsner N., 2014), Finland and Germany (Vonberg D., 2014). Atrazine has been classified as an endocrine-disrupting pesticide by the US Environmental Protection Agency (Morales-Pérez A.A., 2016). The International Agency for Research on Cancer (IARC) has categorized atrazine in the list of carcinogenic pesticides (Mahler B.J., 2017)

Exposure to atrazine affects both germ cells as reduced motility and sperm counts in male rats (Victor-Costa A.B., 2010), (Pogrmic K., 2009) Atrazine supposedly increases aromatase enzyme activity via inhibition of phosphor-di-esterase, which increases the aromatization of testosterone to estrogen (Hayes T.B., 2006); (Cooper R.L., 2007). According to (Gely-Pernot A., 2015), atrazine exposure interferes with normal meiosis, which affects spermatozoa production in male mice. ATZ also induces oxidative stress by the formation of reactive oxygen species resulting in the reduced semen quality, sperm dysfunction and infertility of amphibians, rats and pigs (Kumar V., 2013); (Huang H., 2009); (Glæsner N., 2014). Atrazine is known to cause hepatic damage, as the liver is the primary organ for atrazine metabolism in mammals (Vonberg D., 2014); (Morales-Pérez A.A., 2016). Atrazine is also responsible for cardiotoxicity in mice by disruption of ionic balance (Mahler B.J., 2017) (Victor-Costa A.B., 2010). A considerable number of antioxidants as ascorbic acid and vitamin E have been tested in lab animals to minimize the clastogenicity induced by drugs (Pogrmic K., 2009), (Hayes T.B., 2006). Antioxidant vitamins are able to inactivate highly reactive molecules, such as free radicals, which are generated during various biochemical processes in the cells (Cooper R.L., 2007).

MATERIALS AND METHODS

Animals:

All animals in this study were conducted in accordance with the criteria of the investigations and Ethics Committee of the Community Laws governing the use of experimental animals. The animals used in this study were 48 male and 48 female Albino rats, each weighing about 90 ± 10 g and 9 ± 1 Weeks old (obtained from Faculty farming and housed in the animal house in a room maintained at $22 \pm 2^\circ\text{C}$ with a 14: 10 hours light: dark respectively schedule (lights on at 0500 h: lights out at 1900 h). Food and water were provided ad libitum. In animal house; faculty of Science Al Azhar University. In accordance

with the guidelines of the ethical committee for research on laboratory animals (National Research Council., 1996)

Chemicals and Reagent:

Atrazine (organo-chlorine herbicide) with 80% purity is the chemical material used for the toxicity tests. Atrazine (C₈H₁₄ClN₅; 6-chloro-4-N-ethyl-2-N-propan-2-yl-1, 3, 5-triazine-2,4-diamine) is a commonly used chemical in farms in Egypt and worldwide for controlling weeds. The toxicity was induced by oral gavage tube (150 & 300 mg/kg) daily for (15 & 30) respectively consecutive days. Ascorbic acid (Vit C) from the International Company for Scientific and Medical Supplies at Cairo, Egypt, was evaluated for its antioxidant effect in a dose of (200 mg /Kg) for 15 and 30 days.

Experimental Design: -

The animals, male and female albino rats, were randomly divided into 6 equal groups and labeled as groups 1, 2, 3, 4, 5 & 6 and each group contains 8 rats. Rats received all treatments daily via oral gavage tube along the period of the experiment. Atrazine was given in two doses: low dose (L) = 150 mg/kg and high dose (H) = 300 mg/kg, while ascorbic acid was given in a dose of (200 mg/Kg).

Group (1a) contains the control male rats, Group (1b) contains the control female rats, Group (2a) contains male rats were given Vit-C (200 mg/Kg) only, Group (2b) contains female rats given Vit-C (200 mg/Kg) only, Group (3a) contains male rats given atrazine in low dose (L), Group (3b) contains female rats given atrazine in low dose (L), Group (4a) contains male rats given atrazine in high dose (H), Group (4b) contains female rats given atrazine in high dose (H), Group (5a) contains male rats given atrazine in low dose (L) and Vit-C, Group (5b) contains female rats given atrazine in low dose (L) and Vit-C, Group (6a) contains male rats were given atrazine in high dose (H) and Vit-C, Group (6b) contains female rats given atrazine in high dose (H) and Vit-C. The animals were observed daily for signs of atrazine toxicity during the period of the experiment.

Sample Collection:

The animals were sacrificed after the treatments. Whole blood was taken on a vacutainer to separate the serum two separate times; firstly after two weeks from half animals and finally after four weeks from the rest of the animals and the following tests was performed: (E2, PRG, Testosterone, PRL, FSH and TSH). Then a section was performed in gonads to examine the changes in their structure.

Biochemical Assay:

Estimation of serum hormone level: coagulated blood samples were centrifuged for 15 min at 4000 rpm. Sera were separated for assessment of testosterone concentration by using ELISA kits. TSH was determined according to the method described by (Beck, 1986) and (Caldwell, 1985). Testosterone level was determined according to the method described by (Kim, 2012). PRL was investigated according to the method described by (Cooper R. e., 2000). LH was determined according to the method described by (Cooper R. J., 1999). Prog was determined according to the method described by (Cooper R.L., 1996). E2 was investigated according to the method described by (Connor, 1996).

Histological Examination:

H&E staining (Bancroft JD, 2008) and Immune-histo-chemical staining for detection of Bcl-2: the primary antibody used was mouse monoclonal Bcl-2 oncoprotein (N1587; Dako Corporation, Glostrup, Denmark). Bcl-2 was indicated by brown cytoplasmic staining. An immunohistochemical study was conducted using the avidin-biotin-peroxidase method. Paraffin sections were deparaffinized, rehydrated in descending grades of alcohol, and incubated overnight with the primary monoclonal antibody. Sections were rinsed three times with PBS, then incubated for 1 h with peroxidase-conjugated secondary antibody, and washed three times with PBS. The reaction was developed with 0.05% di-amino-benzidine

(Dakopatts, Glostrup, Denmark) as the substrate for peroxidase, and finally, the slides were counterstained with Mayer's hematoxylin. Negative control slides were prepared by replacing the primary antiserum with PBS. A tonsil slide was used as a positive control for Bcl-2 (Kiernan, 2008).

Statistical Analysis:

The statistical package for social sciences SPSS/PC computer program (version 19) was used for statistical analysis of the results. Data were analyzed using one-way analysis of variance (ANOVA). The data were expressed as mean \pm S.E. Differences were considered statistically significant at ($P < 0.05$).

RESULTS

Biochemical Examination: data found in (Table 1)

A non-significant effect of different doses (low and high) of atrazine on PRL hormone in male and female rats

A non-significant effect of different doses (low and high) of atrazine on LH hormone in male and female rats while there is a significant decrease in FSH level in male and female rats exposed to a low dose of atrazine on both 15 & 30 days periods and showed more decrease in female rats exposed to a high dose of atrazine.

A significant decrease ($p < 0.05$) in both estrogen and progesterone hormones in female rats exposed to a low dose of atrazine on both 15 & 30 days periods and showed more decrease in female rats exposed to a high dose of atrazine, while in male groups (table3) showed a significant increase ($p > 0.05$) in both estrogen and progesterone hormones when exposed to a low dose of atrazine on both 15 & 30 days periods and more increase in hormones level in males exposed to a high dose of atrazine.

On the other hand, data found in Table 1 showed a significant increase in testosterone hormone level in female groups which exposed to a low dose of atrazine on both 15 & 30 days periods and more increase in hormone level in female groups which exposed to a high dose of atrazine on both 15 & 30 days periods. While data showed a significant decrease in hormone level in male groups exposed to a low dose of atrazine on both 15 & 30 days periods and more decrease in groups exposed to the high dose on both 15 & 30 days periods. As regards, ascorbic acid effect, used as an antidote to atrazine in this study, (Tables 1, 2, 3 & 4) showed a significant decrease of atrazine toxicity in female & male rates but it doesn't eliminate the effect of atrazine completely. Yet with prolonged administration of ascorbic acid, the antidote effect became more prominent and atrazine toxicity became less obvious.

Histological Examination:

It showed gonads of males (Plate 1) (400x) in their normal structure in control groups (Fig. A) and groups exposed to ascorbic acid only (Fig. B), so we considered them as control groups. In male rats treated with a low dose of atrazine the photomicrograph of testicular tissue. Examination showed vacuolations within seminiferous tubules (V), degeneration of spermatozoa and hemorrhage in interstitial tissue (hg) in the group which treated for 15 days (Fig C.). While in the group treated for 30 days, the hemorrhage increased and atrophies appeared. (Fig. D).

In male rats treated with a high dose of atrazine the photomicrograph of testicular tissue: Examination showed degeneration of seminiferous tubule cells, wide gaps between neighboring cells and loss of spermatozoa & spermatids (arrowhead). Note the degeneration of sertoli cells (arrow) in the group treated for 15 days (Fig. E), while the group treated for 30 days showed degeneration of seminiferous tubule cells, abnormal spermatogenesis, loss of seminiferous tubule sheath, wide gaps between neighboring cells and interstitial tissues showed edema, hemorrhage and vacuolations (IT), (Fig. F).

Table 1: Serum E2, PROG, and Testo, level (U/L) in adult female albino rats subjected to different treatment conditions for 15 and 30 days

	Female	Control	Vit C	ATZ (L)	ATZ (H)	ATZ (L) + VitC	ATZ (H) + VitC
E2	15 days	6.0±0.4a	6.01±0.2a	5.57±1.3a, b	4.54±0.5a, b	5.95±0.7a	5.08±0.2a, b
	30 days	5.95±0.4a	5.90±0.3a	5.06±0.6a, b	4.05±0.2b	5.46±0.6a, b	4.55±0.4a, b
Prog.	15 days	17.8±2.2a	15.9±0.9a, b	11.2±0.6b, c, e	6.50±0.4c, d	8.25±2.2c, d	16.9±4.0a
	30 days	16.8±0.4a	18.3±1.3a	9.43±0.9c	3.85±0.6d	9.80±2.7c	15.4±0.5a, e
Testo	15 days	6.20±0.6a	5.93±0.7a, b	3.68±0.3c, e	3.17±0.4e	4.00±0.6c, e	3.10±0.5e
	30 days	4.65±0.1b, c	5.60±0.4a, b, d	4.05±0.4c, e	5.23±0.2a, b	4.35±0.4c, d	5.48±0.5a, b

Each value represented means of 4 records ± S.E.

a,b,c,d,e means comparison between all groups which the groups have the same letter mean there is no significance ± difference and which have different letter mean there is a significance ± change

Table 2: Serum FSH, LH and PRL level (U/L) in adult female albino rats subjected to different treatment conditions for 15 and 30 days.

	Female	Control	Vit C	ATZ (L)	ATZ (H)	ATZ (L) + VitC	ATZ (H) + VitC
FSH	15 days	20.6±1.8a, d	26.6±0.9bce	20.6±0.2a, f	19.0±0.7d, f	23.7±0.5a, e	23.3±0.5a, e
	30 days	23.0±0.3a, b	28.2±3.1c	19.3±1.0d, f	20.5±1.2a, f	23.9±1.0a, e	22.0±1.0a, f
LH	15 days	7.05±0.7a, c	9.00±0.5abc	8.70±0.1abc	8.00±0.1abc	6.80±0.6a, c	9.20±0.5a, b
	30 days	7.10±0.3a, c	9.20±0.1a, b	7.88±0.6abc	8.58±0.1abc	9.80±2.5b	6.53±0.9c
PRL	15 days	316.0±39.1abc	389.3±15.6b	356.0±14.1abe	272.0±4.3cdg	216.5±69.1d	338.5±15.0abcg
	30 days	302.0±9.9a, c	390.0±2.2b	368.0±3.4abe	335.0±3.8abg	344.5±32.5abcg	288.0±45.3adeg

Each value represented means of 4 records ± S.E.

a,b,c,d,e means comparison between all groups which the groups have the same letter mean there is no significance ± difference and which have different letter mean there is a significance ± change.

Table 3: Serum E2, PROG, and Testo, level (U/L) in adult male albino rats subjected to different treatment conditions for 15 and 30 days.

	Male	Control	Vit C	ATZ (L)	ATZ (H)	ATZ (L) + VitC	ATZ (H) + VitC
E2	15 days	4.10±0.5a	4.18±1.1a	8.05±0.3b, e	10.0±0.7cdf	7.02±0.3e	9.55±0.5d, g
	30 days	4.13±0.4a	4.13±0.5a	9.00±0.1b, d	11.2±0.2cf	8.50±0.3bg	10.5±0.2f, g
Prog.	15 days	0.47±0.1a	0.78±0.1a	1.50±0.2b	5.95±0.4d	0.68±0.1a	0.17±0.1a
	30 days	0.39±0.1a	0.71±0.1a	2.70±0.4c	6.03±0.4d	0.70±0.1a	0.33±0.1a
Testo.	15 days	42.8±1.7a	43.8±1.0a	19.5±3.4b	5.4±0.6d, e	26.8±3.6f	9.1±1.0g, e
	30 days	39.3±2.6a	41.8±2.6a	13.0±1.6c, g	4.1±0.6d, e	15.1±1.5b, c	5.9±0.6e

Each value represented means of 4 records ± S.E.

a,b,c,d,e means comparison between all groups which the groups have the same letter mean there is no significance ± difference and which have different letter mean there is a significance ± change.

Table 4: Serum FSH, LH and PRL, level (U/L) in adult male albino rats subjected to different treatment conditions for 15 and 30 days.

	Male	Control	Vit C	ATZ (L)	ATZ (H)	ATZ (L) + VitC	ATZ (H) + VitC
FSH	15 days	25.3±0.5abe	27.9±1.0b, e	26.6±3.5b, e	20.6±1.0acd	24.3±0.2abc	24.5±0.1bde
	30 days	27.4±2.1b, e	28.5±0.7b, e	20.9±0.9acd	19.0±1.9c	29.0±4.5e	23.1±0.8a, b
LH	15 days	5.75±0.7a, b	7.10±0.6b, c	8.13±0.1c, d	7.63±0.2c	8.18±0.4c, d	8.23±1.0c, d
	30 days	5.85±1.0a, b	7.10±0.5abc	8.40±0.2c, d	7.13±0.5abc	9.25±0.3d	7.85±0.1c, d
PRL	15 days	328.0±10.7a	347.0±14.3a	356.0±4.1a	364.0±24.3a	315.0±46.1a	362.0±20.0a
	30 days	326.0±22.3a	359.0±18.9a	366.3±5.5a	372.0±42.9a	325.3±21.7a	344.0±2.1a

Each value represented means of 4 records ± S.E.

a,b,c,d,e means comparison between all groups which the groups have the same letter mean there is no significance ± difference and which have different letter mean there is a significance ± change.

Ascorbic acid as an antidote to atrazine effect in male groups was examined by (400x) in this study:

In male rats treated with a low dose of Atrazine in combination with ascorbic acid: showed necrosis (N) in the seminiferous tubules, deformation in the spermatocytes (arrowhead), with vacuolation (V) and hemorrhage (hg) in interstitial cells in the group

treated for 15 days (Fig. G). While the group treated for 30 days showed normal seminiferous tubule architecture with normal appearance of seminiferous tubule sheath (arrowhead), spermatogonia (SG), spermatocytes (SC), spermatids (ST) and spermatozoa (SP) (Fig. H).

In male rats treated with a high dose of Atrazine in combination with ascorbic acid: showed congestion of blood in the entire seminiferous tubule (arrowhead) and increase in vacuolation (V) and deformation of spermatogenesis. Note atrophy and hemorrhage in interstitial tissue in the group treated for 15 days (Fig. I). While the group treated for 30 days showed recovery of spermatogenesis and well-developed seminiferous tubule sheath (Fig. J). Histological examination of female gonads (Plate 2) showed normal structure in control groups by using (400x) showed pre-ovulatory follicle with mature oocyte with normal nucleus (N) surrounded by granulosa cells (GC) with normal zona pellucida (zp). (Fig. A). And groups exposed to ascorbic acid only showed normal ovarian structure like that of the control group (Fig. B), so we considered them as control groups.

In female rats treated with a low dose of atrazine the photomicrograph of the ovarian section: showed reduced Grafian follicle (GF) number and size, deformed oocyte (Oo) with degenerated nucleus (N) and vacuolated (V) zona granuloza in the group treated for 15 days (Fig. C). While the group treated for 30 days showed atretic follicles (AF) with vacuolated (V) zona granuloza and blood congestion (cg) (Fig. D).

In female rats treated with a high dose of atrazine the photomicrograph of ovarian section: showed atretic follicles (AF) with vacuolation (V) and blood vessel congestion (cg) in corpus luteum in the group treated for 15 days (Fig. E). While the group treated for 30 days showed Grafian follicle (GF) with mature oocyte (Oo) with a degenerative nucleus, well-formed zona pelucida (double arrowheads) and deformed zona granuloza (arrowhead). Note vacuolated corpus luteum (arrows) and hemorrhage in interstitial cells (hg) (Fig.F).

Ascorbic Acid as An Antidote to Atrazine Effect in Female Groups in This Study:

In female rats treated with a low dose of Atrazine in combination with ascorbic acid: showed some recovery of the follicular tissue; Oocyte (Oo) within Grafian follicle (GF) and recovery of zona granuloza in the group treated for 15 days (Fig. G). While the group treated for 30 days showed atretic oocytes within vacuolated follicles (V) and hemorrhage in the interstitial tissue (hg). (Fig.H).

In female rats treated with a high dose of Atrazine in combination with ascorbic acid: showed atretic follicles (AF) with vacuolated (V) zona granuloza, Corpus luteum (CL) showing degenerative luteal cells with vacuolations in the group treated for 15 days (Fig. I). While the group treated for 30 days showed atretic follicles (AF) with vacuolated (V) zona granuloza, lymphocytes infiltrations (LI) and blood vessel congestion (cg) (Fig. J).

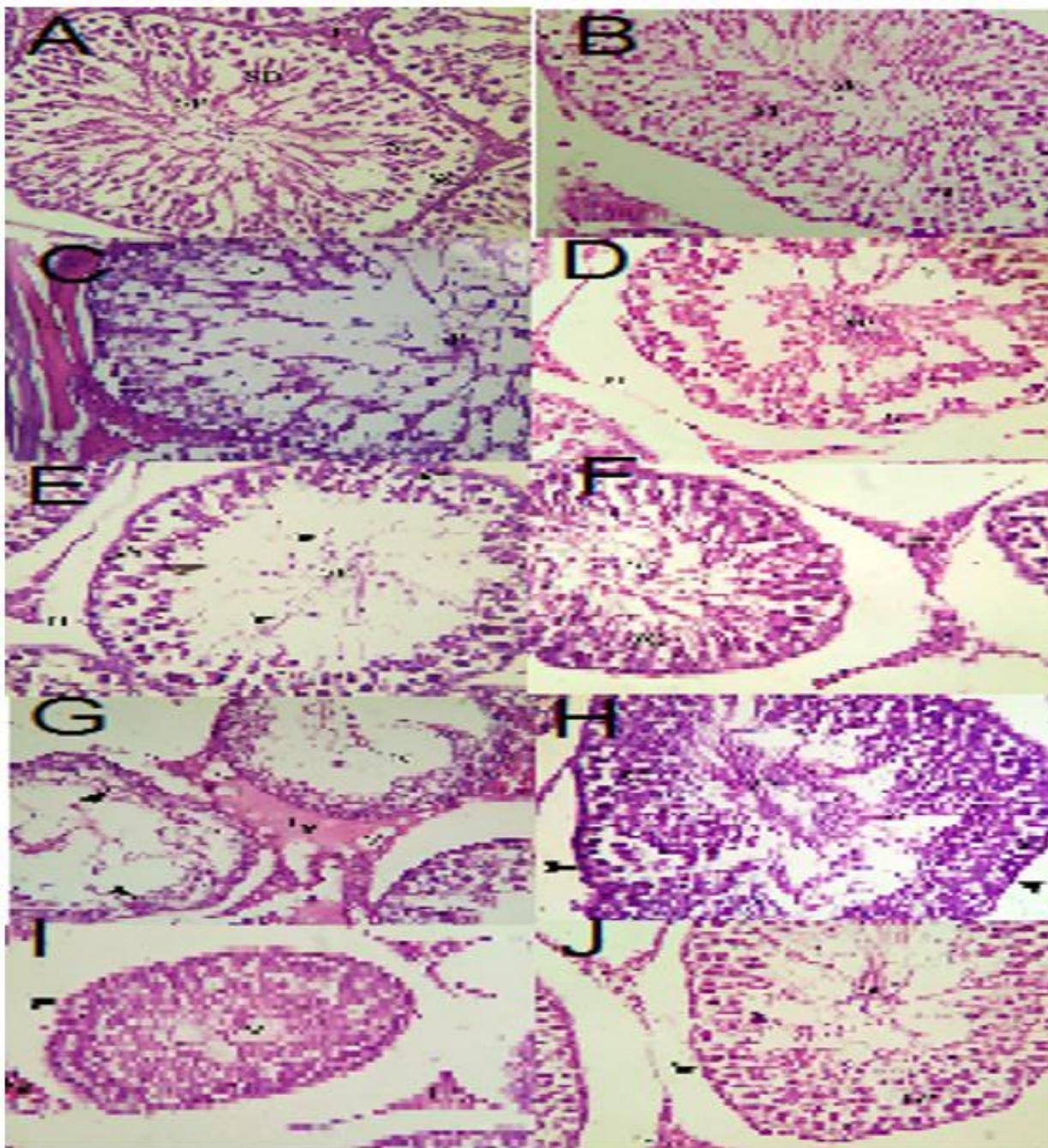


Plate 1: Photomicrograph of testicular tissue of male albino rats 400X subjected to different treatment condition.

(Fig. A) control

(Fig. C) low ATZ after 15 days

(Fig. E) high ATZ after 15 days

(Fig. G) low ATZ & Vit C after 15 days

(Fig. I) high ATZ & Vit C after 15 days

(Fig. B) Vit C

(Fig. D) low ATZ after 30 days

(Fig. F) high ATZ after 30 days

(Fig. H) low ATZ & Vit C after 30 days

(Fig. J) high ATZ & Vit C after 30 days

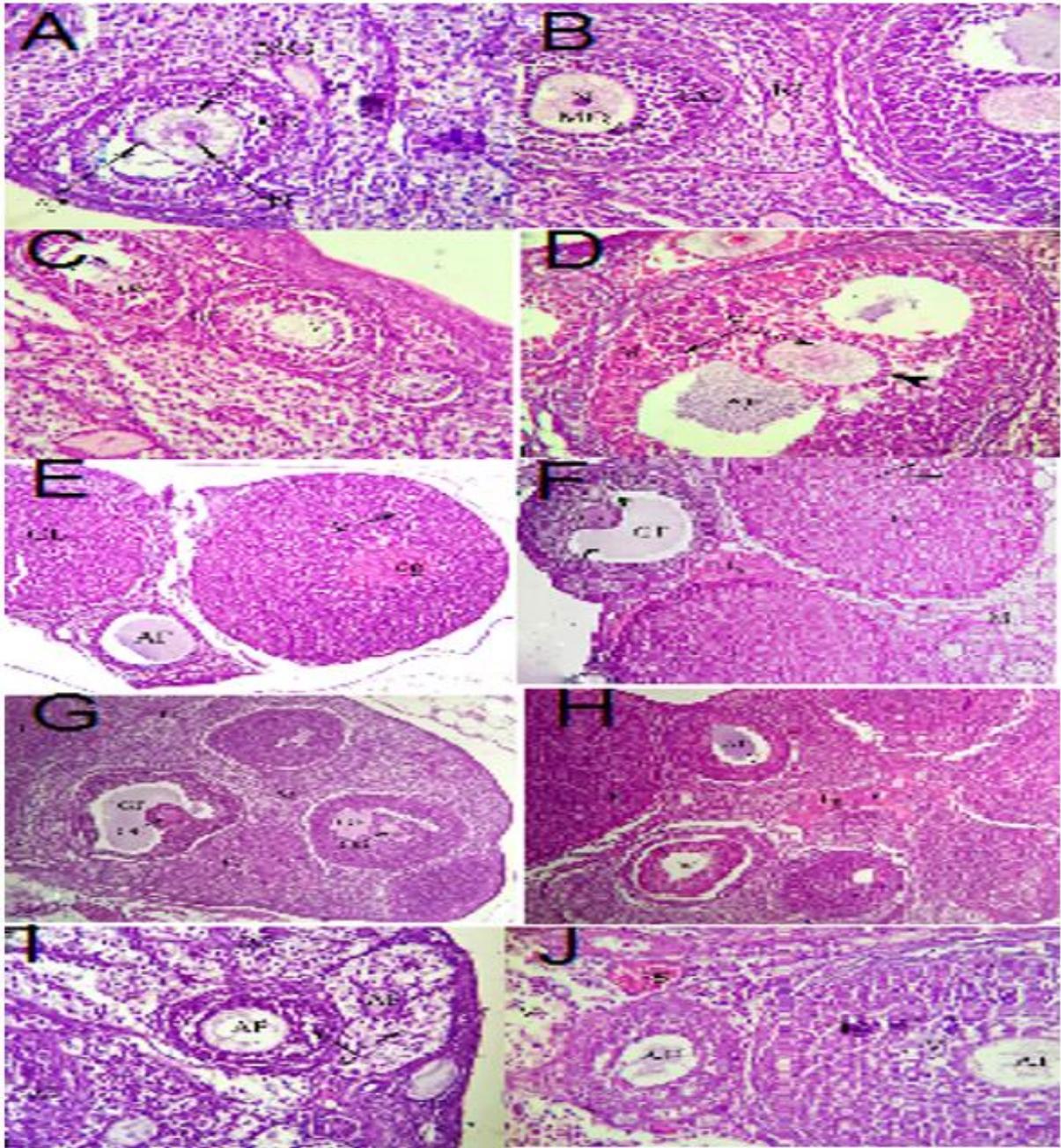


Plate 2: Photomicrograph of ovary of female albino rat 400X subjected to different treatment condition.

(Fig. A) control

(Fig. C) low ATZ after 15 days

(Fig. E) high ATZ after 15 days

(Fig. G) low ATZ & Vit C after 15 days

(Fig. I) high ATZ & Vit C after 15 days

(Fig. B) Vit C

(Fig. D) low ATZ after 30 days

(Fig. F) high ATZ after 30 days

(Fig. H) low ATZ & Vit C after 30 days

(Fig. J) high ATZ & Vit C after 30 days

DISCUSSION

The results showed that after treatment with both low and high doses of atrazine during 15 & 30 days periods, there is a significant decrease in FSH level in male rates, Similar to (Mokhtari, 2010) and inconsistent with (Yang, 2014), also showed a significant decrease in FSH level in female rates similar to (Bohn T., 2011) but our results are inconsistent with (Bohn T., 2011) in results of LH level where he found a decrease in LH level and our results didn't show any difference in the hormone level with a control group.

Results showed a significant decrease in both Estrogen and progesterone level on treatment with both low and high doses of atrazine in female groups during 15 & 30 days periods, although this result is inconsistent with (Taketa Y., 2011) who found that ATR induces luteal cell Hypertrophy and increases progesterone production, and this caused by newly formed corpora lutea in female rats, while in male groups Our results showed a significant increase in both Estrogen and progesterone level on treatment with both low and high doses of atrazine during 15 & 30 days periods as (Hayes T. B., 2011) who studied the evidence for atrazine as an endocrine disruptor that demasculinizes and feminizes the gonads of male vertebrates.

Results showed a significant decrease in testosterone level on both low and high doses of atrazine male groups during 15- & 30-days periods, similar to (Kniewald, 1979) (Babic-Gojmerac, 1989) & (Yang, 2014). And this is caused by the reduction of the number of testosterone receptors in the prostate gland by ATZ exposure (Kniewald *et al.*, 1995).

The study showed that no significant effect of ATR on the level of prolactin level in both male and female groups, and this is inconsistent with the results of previous studies (Stoker T. E., 1999). That may be explained that previous studies were done on the period of lactation when the physiological level of prolactin is normally high because of active mammary glands which became more active on ATR use.

Effect of Ascorbic Acid:

The study showed that using ascorbic acid caused improvement in the level of (E2, PROG, TESTO, FSH and LH), although didn't reach the normal value, in all groups treated with atrazine via capturing the free radicals released from herbicide reaction with these hormones, as similar to (Lin J L. H., 2016a), (Lin J L. H., 2016b), (Antunes LM, 1998), (Siddique YH, 2005) and (Costa WF, 2006).

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