Effects of Azorubine food additive on female reproductive organs and hormones in Sprague Dawley rat

Faraidoon Abdul Sattar Muhamad Amin
Department of surgery and Theriogenology , College of Veterinary Medicine, University of Sulaimani, Sulaimani, Kurdistan Region, Iraq.
E-mail: faraidoon.muhamad@univsul.edu.iq

Received: 16/4/2018
Accepted: 30/5/2018
Publishing:31/1/2019

Summary

The objective of the present study was to assess the effects of various doses of Azorubine which is a food additive on the female reproductive organs in Sprague Dawley rats. Twenty four female Sprgue Dawley rats were divided randomly into 4 equal groups. Group 1(control group), group, 2, 3 and 4 were received Azorubine (5, 10 and 20 mg/kg) orally, daily for 30 days respectively. Blood samples were taken for estimation of white blood cells, red blood cells, hemoglobin and platelets, in addition Luteinizing, follicular stimulation, estrogen and progesterone hormones from the sera. The reproductive hormones levels affected drastically under the effects of different doses of treatment like Luteinizing hormone (0.69±0.25, 0.60±0.75 and 0.55±0.63), Follicular Stimulation hormone (0.17±0.11, 0.13±0.33 and 0.3±0.45), Progesterone hormone (0.50±0.77, 0.14±0.56 and 0.10±0.85), and estrogen hormone (0.45+0.43, 0.30±0.29 and 0.14±0.27) hormones were decreased significantly ($P<0.05$) in groups of rats treated with each 5, 10 and 20 mg/kg doses of Azorubine respectively. Histopathologically, the ovaries treated with 5 mg/kg doses of Azorubine showing follicles at the beginning stages of growth with no Graffian follicle while the ovaries with 10 mg/kg doses of Azorubine contain fully grown Graffian follicles with no follicles at various stages as well as those with 20 mg/kg doses of treatment displaying no mature Graffian follicle with many atretic and shrunk follicles. The hematological outcomes are significantly affected by this food additive. The results of this work is concluded that Azorubine can be considered as one of the most important causes of infertility, hormonal disturbances and irregular estrus cycle in the rat female.

Keywords: Food additive, Histopathology, Sexes hormones, Rats.

Introduction

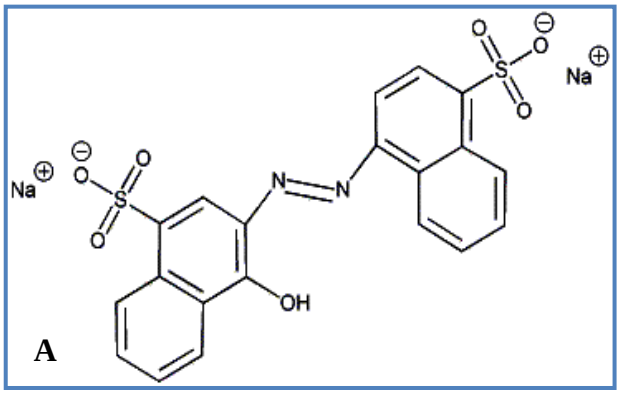
Adding of a specific, globally allowed safe substances or chemicals to eligible consumable products such as foods, beverages, medicines, and dyes, or other items such as cosmetics, perfumery, and wood are termed preservative (1). Adding of preservative food additives in a limited quantity lower the chance of food-borne diseases, bacterial spoilage, food decomposition as well as preserve the food freshness, taste, smell and quality (2). Generally, there are 2 modes of preservation implementation which are chemical and physical methods. The chemical mode means adding chemical compounds to the product in a limited quantity, while physical way means refrigeration, drying, dehydration, salting, sweetening, UV radiation, and freezing. In most cases, both techniques are combined and used together at the same time for the same product (3 and 4).

Recently, intensive and particular investigations are conducting on internationally used more common and popular food additives in general, and colors in particular for estimation of their dangerous effects (5). One of the mostly used preservative nowadays worldwide is Azorubine (AZ) (Fig. 1A), which is also termed carmoisine, Food Red 3, Brillant carmoisine O, Acid Red 14, E122 or C.I. 14720 (6 and 7). This preservative is a red (Fig. 1B) to maroon synthetic organic dye powder that derived from the azo dye group (coal tar), usually in the form of a disodium salt (3 and 8). This powder is commonly used in the food and confectionery manufacture for preparing jellies, sweets, cakes, creams, and muffins especially in the case of heat-treated products (9 and 10). More worryingly is the commonly used AZ in most pharmaceutical syrups and coating of pills and capsules, particularly in children medicines (5).

Previously, there were not enough data about the mutagenic and carcinogenic evidence of AZ even in developed countries that own the most reputable food industries and factories. Highlighting this issue, an acceptable daily intake (ADI) of 0.0 – 4.0 mg/kg was decided by World Health Organization (WHO) in 1983 (11). In opposite, some researchers indicated that possible effects on human health are produced after consumption of this powder such as allergic reactions, rashes, skin swelling and hyperactivity (12). Other researchers (5) in their intensive research study investigated the potential negative effects of AZ in male mice. They confirmed that this dye has the capacity to produce mutagenicity and hepatic carcinogenicity using protein expression profiles, SDS-electrophoresis, and molecular analysis. So, they suggested contraindicating this chemical as it adversely affects and alters biochemical markers in the liver even when used in a very low dose (5).

More seriously, the Hyperactive Children's Support (HACS) agency announced that AZ can intoxicate nervous system especially in children that results in the formation of Psychological diseases (13 and 14). More additionally, the carcinogenic properties of this dye are connected to urinary bladder cancer (15 and 16). Thus, on April 10th, 2008, UK Food Standards Agency (FSA) called for the voluntary removal of E122 (17 and 18). Presently, AZ is prohibited in Sweden, Canada, USA, Norway, and Japan and some other countries, since the dye is considered as the carcinogenic substance that leads to cancer development (5 and 14).

Nowadays, the majority of the processed, frozen, canned or even dried foods that are imported to Iraq from different countries especially China, Turkey, and Iran are treated with AZ as indicated on the outer cover of the stuff and at the same time too many various cancers, infertility, poor growth in infants were observed in the region that thought to be related to this dye (2). Therefore, the aim of this current work is to address the effect of various doses of AZ on the ovary function in female Sprague Dawley rats, in addition to testing some of the hematological parameters of the experimental groups.



Figure, 1A and B: Chemical structure (A) and powder (B) of Azorubine (3 and 8).

Materials and Methods

Twenty four female Sprague Dawley rats aged 6 to 8 weeks and weighed about 180 to 220 g was provided by the Animal House/ College of Veterinary Medicine/ University of Sulaimani after obtaining an official approval from the Ethical Committee of the College. The rats were kept in polypropylene plastic cages in which wood chips were used for bedding, as well as pellet and water *ad libitum* during the period of study. They were acclimatized to the laboratory temperature at 24 ± 1˚C under a 12-hour dark-light cycle for at least 5 days before starting the experiment.

Rats were divided randomly into 4 equal groups. Group 1 (control group) received tap water only. Other nominated groups (G2, G3 and G4) received 5, 10 and 20 mg/kg AZ(Fig.1A and B) (Sigma Aldrich, USA) orally, daily for 30 days respectively, following the standard method described earlier (19). Before daily treatment, the animals were deprived of food for at least 10 hours. Oral medication was performed using disposable syringe with a ball-tipped stainless steel gavage needle.

The rats in all groups were checked for clinical and behavioral abnormalities, toxicological signs, feed intake and gross appearance twice each day over a period of 30 days post-treatment. At the end of the experiment, deep anesthesia using a mixture of Xylazine and Ketamine were used to sacrifice the animals in all groups. Heart puncture technique was done to obtain blood samples, and divided into two part, the first one put in vacuumed collection tubes containing ethylene diamine tetra acetic acid (EDTA) mixed thoroughly, and analyzed promptly. The total and differential white blood cell (WBC), total red blood cell (RBC), hemoglobin (Hb), packed cell volume (PCV) and platelets were measured by automatic hematology analyzer (Cell Dyn, 3700, Abbot, USA). The second part of the blood samples was put in tubes without anticoagulant. Then, the tubes were centrifuged (Hettich zent-EBA20, Germany) at 4000 rpm for 8 minutes, serum separated and analyzed immediately. The concentrations of FSH, LH, estrogen, and progesterone were estimated using standard diagnostic kits (Roche) in an automatic biochemistry analyzer (Hitachi 902, Japan).

For histopathology study, ovary tissue samples were collected directly and immediately after sacrificing and were washed properly using distilled water. Then, pieces of 0.5 cm^2 sizes were prepared from the ovaries and put on 10% formalin for at least 2 days for fixation.Then prepared sections routinely and stained with Harris's hematoxylin and eosin, according to (20), viewed under light microscopy (Leica, Japan) at 400X magnification and photos were captured. The result is exhibited as mean ± SD and analyzed statistically using SPSS version 21.0 (SPSS Inc., Chicago, USA). Probability values of less than 0.05 ($P<0.05$) is considered statistically significant using Post hoc comparison test-one way ANOVA - Tukey's b-test.

Results and Discussion

Although too many proposed method was suggested to determine AZ as a model substance in food samples such as a green, convenient, and fast methods (21), but until this moment there is no study showing the effects of AZ on fertility in the female. Thus, this current study considered to be the 1st report in this respect. Generally, no clinical signs of typical toxicity have been found such as anxiety, rough coat, and depression, in appetite, emaciation or mortality. But simple degrees of inactivity, drowsiness and slow growth were observed in few numbers of the animals. These observation were coincided with (2), found the clinical signs of toxicity related to AZ in white Sprague Dawley mice were the loss of appetite, drowsiness, tachycardia, the decrease in locomotion, and anorexia but without mortality when the LD50 value of the carmoisine was 4166.66 mg/kg.

The levels of each LH, FSH, estrogen and progesterone hormones of control group rats were of the normal range. While, the levels of LH, FSH, progesterone, and estrogen hormones were decreased significantly ($P<0.05$) in groups of rats treated with 5, 10 and 20 mg/ kg of AZ respectively as compared with control group as shown in (Table, 1). The findings of other workers (22-24) which were believed to be attributed to the ability of food additive Azorubine to damage the nerve cells of the hypothalamus and may alter the neural control of reproductive hormone secretion via the hypothalamic pituitary-ovaries that regulate and control the production of FSH, LH, Estrogen and Progesterone hormones of pituitary glands and ovaries, so the fertility rate has been reported to be reduced in female rats.

Table, 1: Mean values of FSH, LH, estrogen and progesterone levels following administration of Azorubine on day 30.

Hormone	G1 (Control)	G2 (5mg/k)	G3 (10mg/kg)	G4 (20mg/kg)
LH (mIU/ml)	0.78 ± 0.21	0.69 ± 0.25	0.60 ± 0.75*	0.55 ± 0.63*
FSH (mIU/ml)	0.90 ± 0.34	0.17 ± 0.11*	0.13 ± 0.33*	0.3 ± 0.45*
Estrogen (pg/ml)	53.07 ± 0.55	0.45 ± 0.43*	0.30 ± 0.29*	0.14 ± 0.27*
Progesterone (pg/ml)	19.22 ± 0.11	0.50 ± 0.77*	0.14 ± 0.56*	0.10 ± 0.85*

Values are given as mean ± SEM for each group. * indicates a significant difference ($p<0.05$) between treated groups compared to control group. Statistical level of significance was determined by one-way ANOVA.

As a result of obtained data from Hemogram, there is significant increased in

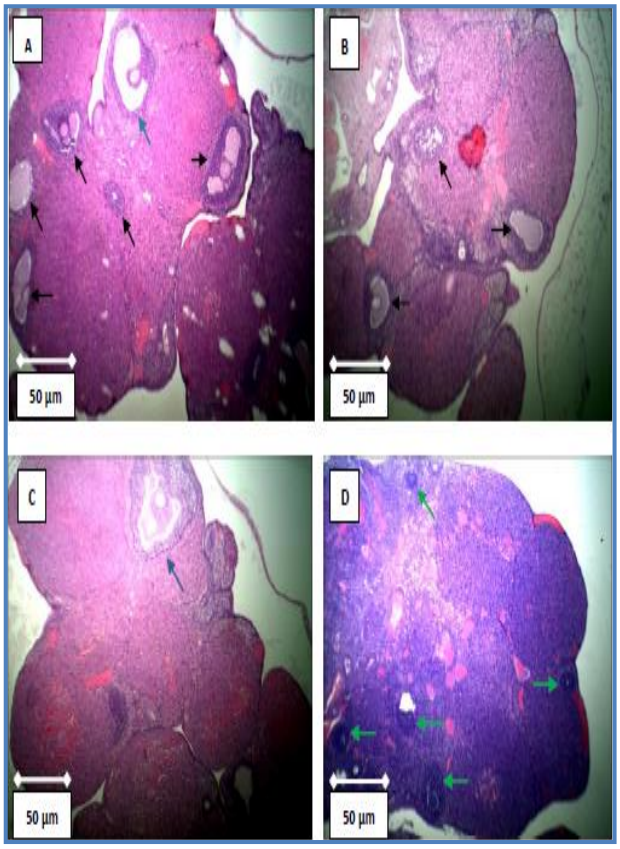
the counts of WBC levels were found in all treated groups of AZ (Table, 2), these observations suggest physiological inflammatory response as a result of tissue damage and inflammation causing acceleration of immune system to increase production of white blood cells this agreed with (23). On the other hand, significant decreasing in the levels of Hb,RBC and PCV counts were found in all treated groups with AZ. In the same manner, a significant ($P<0.05$) decreasing in the level of platelet counts was found in 10 and 20 mg/kg of AZ treatment (Table, 2), these findings suggests possible inhibitory effect of Azorubine on heme synthesis, this results coincided with (25-27) which were assumed to be associated with retarded haemopoeisis, destruction and shrinkage of RBC.

Table, 2: Mean values of hematological tests following administration of Azorubine on day 30.

Parameter	G1 (Control)	G2 (5 mg/kg)	G3 (10mg/kg)	G4 (20mg/kg)
WBC (10^9/L)	5.3 ± 0.22	6.5 ± 0.12*	7.5 ± 0.33*	10.1 ± 0.17*
RBC (10^{12}/L)	6.4 ± 0.19	6.3 ± 0.25	6.0 ± 0.44	4.9 ± 0.69
Hb (g/dl)	12.9 ± 0.47	12.7 ± 0.41	12.5 ± 0.77	9.9 ± 0.35*
PCV (%)	38.7 ± 0.65	35.7 ± 0.95	33.11 ± 0.65	25.7 ± 0.83*
Platelet (10^9/L)	5.4 ±1.0	5.0 ±1.1	4.4 ±1.6*	3.5 ±1.5*

Values are given as mean ± SEM for each group. * indicates a significant difference ($P<0.05$) between treated groups compared to control group. Statistical level of significance was determined by one-way ANOVA.

Histopathological changes of the ovaries in treated groups showed various degrees of lesions. In ovaries of control group showing normal texture that contains follicles at different stages of growth with fully matured graffian follicle (Fig. 2A). While ovaries treated with 5 gm/kg of AZ, showing follicles at the beginning stage of growth with no graffian follicle (Fig. 2B), whereas ovaries treated with 10 mg/kg of AZ contains fully grown graffian follicle with no follicles at various stages (Fig. 2C). Ovaries treated with 20 mg/kg of AZ displaying no mature graffian follicle with too many atretic and shrunk ovarian follicles (Fig. 2D). Macroscopically the size of the ovaries of this group (20 mg/kg) appeared smaller in size compared to other groups. The results of the present study showed that Azorubine causes many pathologies in the ovaries, it is believed that they may be arisen through disruption of the hypothalamic pituitary –ovaries axis regulation as well as suppressing release of gonadotropin hormones to cause follicullogenesis and crpora lutea formation, therefore the ovaries of rats appeared smaller in size, these findings were also observed by other researchers (23 and 24). It concluded that Azorubine can be considered as one of the most important causes of infertility, hormonal disturbances and irregular estrus cycle in the female that should be banned from the foodstuff that will be imported to our country from other countries.



Figure, 2: Histopathological appearance of ovaries from Sprague Dawley rat after sacrificing and stained with Hematoxylin and Eosin double staining (H & E). (A) Control group showing normal ovarian texture that contains 5 follicles at different stages of growth (black arrows) with 1 fully matured graffian follicle (blue arrow), (B) Ovaries treated with 5 mg/kg of azorubine (AZ), showing 3 follicles at beginning stage of growth (black arrows) with no graffian follicle, (C) Ovaries treated with 10 mg/kg of AZ showing 1 fully grown graffian follicle (blue arrow) with no follicles at various stages, and (D) Ovaries treated with 20 mg/kg doses of AZ displaying no mature graffian follicle with 5 atretic ovarian follicles (green arrows). Scale bars showing magnification at 50 µm.

References

1. Smith-Palmer, A.; Stewart, J. and Fyfe L. (2001). The potential application of plant essential oils as natural food preservatives in soft cheese. Food Microbiol., 18(4):463-470.
2. Ai-Mashhedy, L.A.M.; and Fijer, A.N. (2016). Acute toxicity of food additives tartrazine and carmoisine on white male mice. Int. J. Pharm. Tech. Res., 9:364-367.
3. Russell, N.J. and Gould, G.W. (2003). Food preservatives: Springer Science and Business Media. pp: 605–611.
4. Anchovy, P.; Kotolova, H. and Ruda-Kucerova, J. (2015). Health safety issues of synthetic food colorants. Regulatory Toxicol. Pharmacol., 73(3):914-922.
5. Salama, M.S.; Ismail, M.A.; Shahin, M.A. and Yassin, H.M. (2016). The use of GST-μ Gene and Isoenzymes as biomarkers to evaluate the mutagenicity and hepatic carcinogenicity in the mouse by carmoisine 'E122'. Merit Research J. Med. and Med.Sci., 4(6):294-316
6. Basu, A. and Kumar, G.S. (2014). Study on the interaction of the toxic food additive carmoisine with serum albumins: A microcalorimetric investigation. J. Hazardous Materials. 273:200-206.
7. Basu, A. and Kumar, G.S. (2015). Binding of carmoisine, a food colorant with hemoglobin: Spectroscopic and calorimetric studies. Food Res. Int., 72:54-61.
8. Amin, K.A.; Hamed, H.A. and Elsttar, A.H.A. (2010). Effect of food azo dyes tartrazine and carmoisine on biochemical parameters related to renal, hepatic function and oxidative stress biomarkers in young male rats. Food and Chem. Toxicol., 48(10):2994-2999.
9. Basu, A. and Kumar G.S. (2016). Spectroscopic and microcalorimetric studies on the molecular binding of food colorant acid red 27 with deoxyribonucleic acid. J. Molecular Recognition. 29(8):363-369.
10. Doganlar, Z.B.; Doganlar, O.; Ongoren, G.; Mimiroglu, P.A.; Kahraman, O. and Soykan, A. (2016). Single and combined toxicity of aluminum and azorubine: Physiological and genetic responses of Drosophila melanogaster. Toxicol. Letters. 258:184.
11. Shukla, S.K.; Singh, A.K.; Murulana, L.C.; Kabanda, M.M. and Ebenso, E.E. (2012). Inhibitive effect of Azorubine dye on the corrosion of mild steel in hydrochloric acid medium and synergistic iodide additive. Int. J. Electrochemical Sci., 7:5057-5068.
12. Elekima, I. and Ollor, A.O. (2016). Effect of Carmoisine Orally Administered to Thyroid Hormones and Thyroid Stimulating Hormone in Albino Rats. Int. J. Sci. Res., 5(10):29-32.
13. Himri, I., Bellahcen, S., Souna, F., Belmekki, F., Aziz, M. and Bnouham M. (2011). A 90-day oral toxicity study of tartrazine, a synthetic food dye, in Wistar rats. Int. J. Pharm. and Pharmaceutical Sci., 300(3):159-169.
14. Karatepe, A., Akalin, Ç. and Soylak, M. (2017). Spectrophotometric determination of carmoisine after cloud point extraction using Triton X-114. Turkish J. Chemistry., 41(2):256-262.
15. Oyewole, O.I. and Oladele, J.O. (2016). Assessment of Cardiac and Renal Functions in Wistar Albino Rats Administered Carmoisine and Tartrazine. Adv. Biochem., 4(3):21-25.
16. Peksa, V.; Jahn, M.; Štolcová, L.; Schulz, V.; Proška, J. and Procházka, M. (2015). Quantitative SERS analysis of azorubine (E122) in sweet drinks. Analyt. Chem., 87(5):2840-2844.
17. Mehedi, N.; Mokrane, N.; Alami, O.; Ainad-Tabet, S.; Zaoui, C. and Kheroua, O. (2013). A thirteen-week ad libitum administration toxicity study of tartrazine in Swiss mice. Afr. J. Biotechnol., 12(28):4519-4529
18. Montaser, M.M. and Alkafafy, M.E. (2013). Effects of Synthetic Food Color (Carmoisine) on Expression of Some Fuel Metabolism Genes in Liver of Male Albino Rats. Life Sci. J., 2:10.
19. Lorke, D. (1983). A new approach to practical acute toxicity testing. Arch Toxicol. 54(4):275-287.
20. Luna, L.G. (1968). Manual of histologic staining methods of the Armed Forces Institute of Pathology. 3rd ed. New York: Mc Graw-Hill.
21. Zargar, B.; Pourreza, N.; Bayat, E. and Hatamie, A. (2016). Zein bio-nanoparticles: a novel green nanopolymer as a dispersive solid-phase extraction adsorbent for separating and determining trace amounts of

azorubine in different foodstuffs. RSC Advances. 6(77):73096-73105.

22. Abdul- Hamid, M.; Galaly, S.R.; Ahmed, R.R. and Hamdalla, H.M. (2017). Monosodium glutamate as a food additive: Toxic implications and the protective role of quercetin. Merit Res. J. Med. Med. Sci., 5(8):384-402.

23. Ali, A.A.; El-Seify, G.H.; El-Haroun, H.M.; Abd El Mawla, M. and Soliman, M. (2014). Effect of monosodium glutamate on the ovaries of adult female albino rats and the possible protective role of green tea. Menoufia Med. J., 27:793-800.

24. Bojanic, V.; Bojanic, Z.; Najman, S.; Savic, T.; Jakovi-jevic, V.; Najman, S. and Jancic, S. (2009). Diltiazem prevention of toxic effects of monosodium glutamate on ovaries in rats. Gen. Physiol. Biophys., 28:149-154.

25. Walton, K.; Walker, R.; Van de Sandt, J.J.; Castell, J.V. and Knapp, A.G.A.A. (1999). The application of in vitro data in the derivation of the acceptable daily intake of food additives. Food chem.Toxicol.37:1175-1197.

26. Onyema, O.; Farombi, E.; Emerde, G.; Ukoha, A. and Onyeze, G. (2006). Effect of vitamin E on monosodium glutamate induced hepatotoxicity and oxidative stress in rats. Indian J. Biochem. Biophys., 43:20-40.

27. Rim, K. (2017). Toxicology and environmental health sciences toxicological evaluation of MSG for the manufacturing workers health. A literature review. 9(1):1-11.

تأثير المضاف الغذائي الأزوربين في الأعضاء التكاثرية الأنثوية والهرمونات في فئران Sprague Dawley

فريدون عبد الستار محمد أمين

فرع الجراحة و الولادة، كلية الطب البيطري، جامعة السليمانية، مدينة السليمانية، إقليم كردستان، العراق.

E-mail: faraidoon.muhamad@univsul.edu.iq

الخلاصة

هدفت الدراسة إلى تقييم تأثير جرع مختلفة من الأزوربين وهو مضاف غذائي في الأعضاء التكاثرية الأنثوية في فئران سبراكو داولي. أربعة وعشرون من إناث فئران السبراكو داولي قسمت عشوائيا إلى أربع مجاميع متساوية. المجموعة الأولى (مجموعة سيطرة) و كلا من المجاميع الثانية والثالثة والرابعة أعطيت الأزوربين (٥ و١٠ و٢٠ ملغم/كغم) عن طريق الفم يوميا لمدة ٣٠ يوم بالترتيب. أخذت عينات الدم من أجل تقييم كريات الدم البيض وكريات الدم الحمر والهيموكلوبين والصفائح الدموية فضلا عن الهرمون اللوتيني والهرمون المحفز للجريبات وهرموني الأستروجين والبروجستيرون من المصل. تأثرت مستويات الهرمونات التكاثرية تأثيراً كبيراً بسب المعالجة بالجرع المختلفة للأزوربين، مثلا الهرمون اللوتيني (0.25±0.69، 0.75±0.60 و0.63±0.55) والهرمون المحفز للجريبات (0.11±0.17، 0.33±0.13 و 0.45±0.3) والبروجيستيرون (0.77±0.50، 0.56±0.14 و 0.85±0.10) والأستروجين (0.43+0.45، 0.29±0.30 و 0.27±0.14). أظهرت الهرمونات انخفاض مهم إحصائيا على مستوى ($P<0.05$) في مجموعة الفئران المعالجة بالجرع ٥ و١٠ و٢٠ ملغم/كغم من الأزوربين وبالترتيب. أظهرت نتائج الجانب النسجي المرضي في المبايض المعالجة بالجرعة ٥ ملغم/كغم جريبات في المرحلة الأولى من النمو ومن دون ظهور جريبات غراف بينما المبايض المعالجة بالجرعة ١٠ ملغم/كغم من الأزوربين أظهرت إحتوائها على جريبات غراف كاملة النمو بدون جريبات في مراحل النمو المختلفة في حال المبايض المعالجة بالجرعة ٢٠ ملغم/كغم لم تظهر جريبات غراف بالغة مع العديد من الجريبات المرتقة والمنكمشة. الاختبارات الدموية تظهر اختلافات مهمة إحصائيا بالتأثر بهذا المضاف الغائي. نستنتج بأن الأزوربين ممكن أن يعتبر مهم لأسباب العقم وإضطرابات الهرمونات وعدم انتظام دورة الشبق في إناث الفئران.

الكلمات المفتاحية: مضاف غذائي، نسجي مرضي، هرمونات جنسية، فئران.