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Pathophysiological Effects of Azorubine on Female Reproductive Organs and Hormones in Sprague Dawley® Rat

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ABSTRACT

Monitoring of negative effects of the chemical additives in food and environment is an important function of quality control laboratories around the world. Therefore, the aim of this study is to evaluate the effect of various doses of azorubine (AZ) on ovary histopathology in female Sprague Dawley® rats, in addition to testing some of the hematological parameters and hormones of the experimental groups. We found that there were various moderate to severe changes in the ovary histopathology at different doses of treatment. As well as, the hormone levels are affected drastically by the effect of different doses of AZ. On the other hand, hematological outcomes are not so significantly affected by this synthetic dye. Finally, we conclude that AZ can be considered as one of the most important causes of infertility, hormonal disturbances and irregular menstruation in the female that should be banned from the foodstuff that are imported to our country from other countries.

Keywords: Chemical additives, Histopathological study, Sprague Dawley® rat model, Sex hormones

INTRODUCTION

Addition of a specific, globally allowed safe substances or chemicals to eligible products such as food, beverages, pharmaceutical drugs and medicines, paints, biological samples, cosmetics, perfumery, wood, and many other products are termed as a preservative [1]. A preservative food additive reduces the risk of foodborne infections, decrease microbial spoilage and decomposition, and preserve fresh attributes and nutritional quality [2].

In general, preservation is implemented in two modes, chemical and physical.

Chemical: In the chemical, preservation entails chemical compounds are added to the product.

Physical: In the physical preservation entails processes such as refrigeration or drying, dehydration, UV-C radiation, and freeze-drying are involved.

In most of the cases, chemical and physical preservation techniques are combined [3,4].

In recent years, food additives in general, and colors, in particular, have increasingly come under investigation for the evaluation of their safety in use [5]. One of the mostly used preservatives nowadays worldwide is azorubine (AZ), which is also termed as carmoisine, food red 3, azorubine S, brilliant carmoisine O, acid red 14, E122 or C.I. 14720 [6,7]. This preservative is a red to maroon synthetic organic dye powder that is derived from the azo dye group (coal tar), usually in the form of a disodium salt (Figure 1) [3,8].

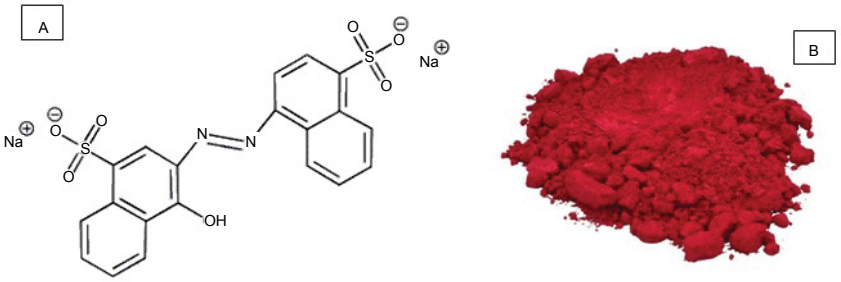

Figure 1 Chemical structure (A) and powder form (B) of Azorubine (AZ)

This powder is used mostly in the food and confectionery industry for making jellies, sweet candies, cheesecakes, and marzipan especially where the food is heat-treated after fermentation [9,10]. More worryingly AZ is commonly used in most of the pharmaceutical syrups and coating of pills and capsules, particularly in the children medicines [5].

Previously, AZ has shown no evidence of mutagenic or carcinogenic properties and an acceptable daily intake (ADI) of 0.0-4.0 mg/kg was established in 1983 by the WHO [11]. In oppose to that, some researches indicated that possible effects on human health are produced after consumption of this powder such as allergic reactions, rashes, skin swelling and hyperactivity [12,13].

Salam, et al., in their investigation addressed the potential of AZ to induce mutagenicity and hepatic carcinogenicity in male mice via protein expression profiles in native and SDS-electrophoresis, and seven isozymes analysis, besides the molecular parameters. They concluded that AZ affects adversely and alters the biochemical markers in the liver not only at a higher dose but also at low doses [5].

More seriously, the Hyperactive Children's Support Group (HACSG) issued an alert that E122 can stimulate a child's nervous system and can lead to the development of psychological diseases [14,15]. More recently, the carcinogenic properties of this dye are connected to urinary bladder cancer [16,17]. In April 2008 UK Food Standards Agency (FSA) called for the voluntary removal of E122 [18,19].

Presently, it is banned in Sweden, USA, Norway, Canada and Japan and some other countries, as the substance is classified under the group of carcinogenic agents that can cause cancer [5,15].

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 azorubine 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.

Therefore, the aim of this study is to evaluate the effect of various doses of E122 on the ovary histopathology in female Sprague Dawley® rats, in addition to testing some of the hematological parameters and hormones of the experimental groups.

MATERIALS AND METHODS

Azorubine (AZ)

It is purchased from the Sigma Aldrich, the USA through a local supplier. The dry powder was stored in a tightly sealed amber colored glass container at 4°C until used in the experiment.

Animal Groups

Female Sprague Dawley® rat aged 6 to 8 weeks and weighed about 180 to 220 g were provided by the animal house of College of Veterinary Medicine, University of Sulaimani, Iraq after obtaining an official approval from the ethical committee of the college. The rats were housed in polypropylene plastic cages with wood chips as bedding. The rats were acclimatized to the laboratory condition at 24 ± 1ºC under a 12-hour dark-light cycle for at least 5 days before commencement of the experiment. The rats were provided pellet and water ad libitum during the period of study.

Evaluation of the Test Experiments

Around 24 female Sprague Dawley® rats were divided randomly into 4 groups of 6 animals each. Group 1 served as control negative and received tap water only. Groups 2, 3 and 4 received 5.0, 10.0 and 20.0 mg/kg AZ orally, daily and exactly for 30 days respectively, following the method described earlier [20]. The animals were deprived of feed 12 hours prior to the treatment. Oral intubation was done using a ball-tipped stainless steel gavage needle which was attached to a disposable syringe.

Clinical Observations

The animals were observed for clinical and behavioral abnormalities, toxicological symptoms, feed consumption and gross appearance twice each day over a period of 30 days post-treatment.

Histopathological Study

Histopathology is an important and rapid way to perceive effects of irritants and toxic chemicals and compounds in

various tissues and organs of the body systems. Thus, ovary tissue samples were collected directly and immediately after sacrificing and were washed properly using distilled water. Then, the organs were cut into pieces of about 0.5 cm^2 sizes and fixed in 10% formalin for at least 2 days. Later on, the samples were dehydrated using an automated tissue processor (Leica ASP300, Germany) and then embedded in paraffin wax (Leica EG1160, Germany). The blocks were sectioned to about 4.5×4.5×4.0 µm size using a microtome (Leica RM2155) and the sections were mounted on glass slides using a hot plate (Leica HI1220, Germany). The slides were subsequently treated in order with 100%, 90% and 70% ethanol for 2 minutes each and then rinsed in tap water. Finally, the sections were stained with Harris's hematoxylin and eosin for light microscopy [21].

Serum Biochemistry

Blood samples were collected from tail veins, centrifuged (Hettich zent-EBA20, Germany) at 3500 rpm for 10 minutes and serum were obtained and stored at -20ºC until analyzed. The concentrations of serum hormones such as FSH, LH, estrogen, and progesterone were determined using standard diagnostic kits (Roche) in an automatic biochemistry analyzer (Hitachi 902, Japan).

Hematological Study

On an experimental day, 30 animals were sacrificed under deep anesthesia using a mixture of xylazine and ketamine. Peripheral blood was collected from tail veins with an ethylenediamine tetraacetic acid (EDTA) vacuumed blood collection tubes, shaken immediately to mix well, and were analyzed directly without delay. The total and differential white blood cell (WBC), total red blood cell (RBC), hemoglobin (Hb), packed cell volume (PCV) and platelets in each sample were measured by automatic hematology analyzer (Cell Dyn, 3700, Abbot, USA).

Statistical Analysis

The result is expressed as mean ± SD and was analyzed statistically using SPSS version 20.0 (SPSS Inc., Chicago, USA). Post-hoc comparison test-one way ANOVA was done using the Tukey's b-test. Probability values of less than 0.05 ($p<0.05$) were considered statistically significant.

RESULTS AND DISCUSSION

Although too many proposed methods were suggested to determine the AZ as a model compound in food samples such as a green, convenient, and fast method but until this moment there is no study showing the effects of AZ on fertility in female [22]. Thus, this current study is considered to be the 1st report in this respect.

Clinical Observation

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.

On the other hand, Ai-Mashhedy, et al., found that the clinical signs of toxicity related to AZ in white Sprague Dawley® mice were loss of appetite, drowsiness, tachycardia, and decrease in locomotion, and anorexia but without mortality when the LD50 value of the carmoisine was 4166.66 mg/kg [2].

Histopathology

The histopathological changes in the ovaries of different treated groups showed various degrees of lesions using various doses of AZ. Ovaries of the control negative group showed normal texture that contains follicles at different stages of growth with the fully matured Graafian follicle. On the other hand, ovaries treated with low doses of AZ, showed follicles at the beginning stage of growth with no Graafian follicle, whereas ovaries treated with mild doses of AZ contains fully grown Graafian follicle with no follicles at various stages. Ovaries treated with high doses of treatment displayed no mature Graafian follicle with too many atretic and shrank ovarian follicles. Macroscopically the size of the ovaries of this group appeared smaller as compared to the other groups (Figure 2).

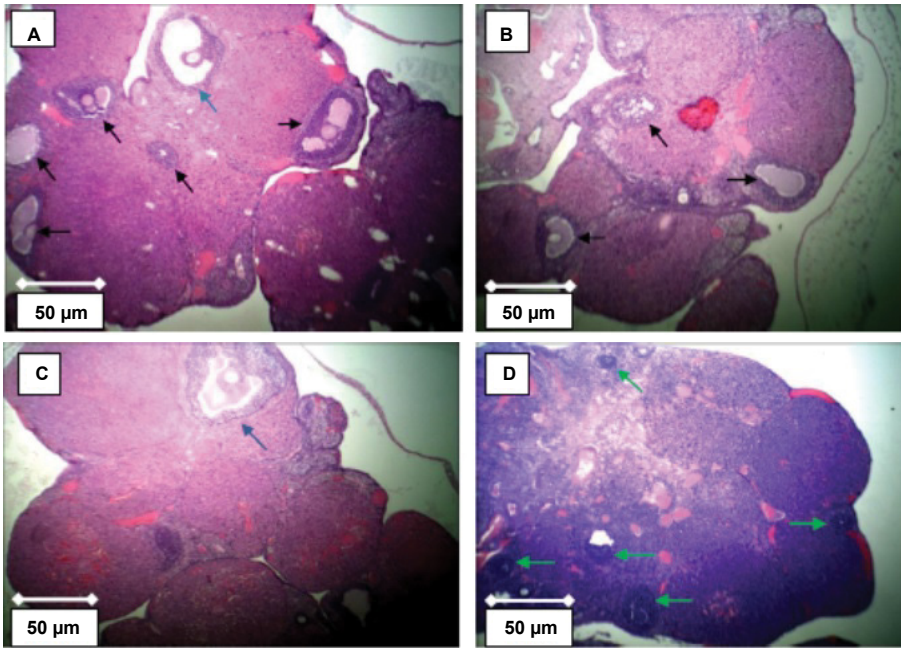

Figure 2 Histopathological appearances of ovaries from Sprague Dawley® rat after sacrificing and stained with Hematoxylin and Eosin double staining (H & E). (A) Control negative showing normal ovarian texture that contains 5 follicles at different stages of growth (black arrows) with 1 fully matured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

Recently, histological examination of the renal and the cardiac tissues correlated with the biochemical results marked distortions in the kidney and heart of rats administered AZ. These results suggest that AZ have the tendency of inducing nephrotoxicity and cardiac dysfunction in Wister albino rats [16].

Hormonal Level Changes

The levels of each LH, FSH, estrogen, and progesterone hormones of the control negative group rats were of the normal range. While, the levels of LH, FSH, progesterone and estrogen hormones decreased significantly ($p<0.05$) in groups of rat treated with each low, mild and high doses of AZ respectively as shown in Table 1.

Table 1 Mean value of FSH, LH, estrogen and progesterone levels following administration of azorubine on day 30

Hormone	G1 (Control negative)	G2 (Low dose)	G3 (Mid dose)	G4 (High dose)
LH (mIU/ml)	0.78 ± 0.21	0.69 ± 0.25	0.60 ± 0.75*	0.55 ± 0.63*
FSH (mIU/ml)	0.17 ± 0.34	0.13 ± 0.11*	0.90± 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	0.14 ± 0.77*	0.10 ± 0.56*	0.50 ± 0.85*
Values are given as mean ± SEM for each group; *indicates a significant difference ($p<0.05$) in treated groups compared to group A (control negative group); Statistical level of significance was determined by one-way ANOVA				

Additionally, Elekima, et al., in their study suggested that oral administration of AZ dye induced a dose-dependent increase in the plasma levels of tri-iodo-tyrosine (T3) and tetra-iodo-tyrosine (T4) as well as the reduced level of thyroid stimulating hormone (TSH) of albino rats [12].

Hematology

As a result of the obtained data from hemogram, we realized that no changes in RBC counts were seen in all treated groups with AZ, whereas a significant increase in the counts of WBC levels was found in all treated groups of AZ. On the other hand, a significant decrease in the levels of each Hb and PCV counts was only observed in the groups treated

with high doses of AZ. In the same manner, a significant ($p<0.05$) decrease in the level of platelet counts was found in mild and high doses of AZ treatment (Table 2).

Table 2 Mean values of hematological tests following administration of azorubine on day 30

Parameter	G1 (Control negative)	G2 (Low dose)	G3 (Mid dose)	G4 (High dose)
WBC (10^9/L)	7.5 ± 0.22	6.5 ± 0.12*	5.3 ± 0.33*	3.7 ± 0.17*
RBC (10^{12}/L)	6.3 ± 0.19	6.0 ± 0.25	6.4 ± 0.44	6.6 ± 0.69
Hb (g/dl)	12.9 ± 0.47	12.5 ± 0.41	12.7 ± 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 ($p<0.05$) in treated groups compared to group A (control group); Statistical level of significance was determined by one-way ANOVA				

El-Wahab, et al., in their research indicated that carmoisine (70 mg/kg diet) leads to a significant increase in the serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) activities, bilirubin, urea, creatinine, total protein and albumin in all the groups of treated Sprague Dawely® rats with AZ when compared to the control negative group [23]. Moreover, the observed significant increase in the activities of enzymes ALP, AST, Creatinine Kinase (CK) and lactic acid dehydrogenase (LDH) in the serum was also found in Wister albino rats, which was considered as cardiac tissue damage [16].

CONCLUSION

In conclusion, the overall results of this study are clearly demonstrated that oral administration of azorubine at various doses will not induce severe behavioral alterations, toxicological sign or any other adverse effect on the experimental animals during the 30 day test period. On the other hand, AZ caused clear changes in the histology of the ovaries and reduced the numbers of follicles in the ovaries of treated animals. Simultaneously, hormonal changes also have been seen. Thus, AZ is considered as a toxic material for human consumption and it is advisable to limit the uses of this food colorants additive especially those used by children.

DECLARATIONS

Conflict of Interest

The authors have disclosed no conflict of interest, financial or otherwise.

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