***Original Research Article***

**Acute Assessment of Azorubine Exposure on Testosterone, Progesterone, Estradiol, Follicle Stimulating Hormone, Luteinizing Hormone, and Prolactin in Male and Female Albino Rats**

**ABSTRACT**

A study of azorubine toxicity on reproductive hormones and gonads of albino rats was carried out. A total of 80 albino rats weighing approximately 0.15kg were used, of which a total of 40 rats were used for the intraperitoneal study and another 40 were used for the oral study. The rats were divided randomly into 10 groups of 4 rats each for both the intraperitoneal and oral treatments. The groups were designed A, B, C, D, E, F, G, H, I, and J for the pilot study to determine the LD100 while the acute research only considered the groups A, B, C, D, E, and F following the determination of the LD100.The rats were treated with varying doses of azorubine for 48 hours and blood samples were collected using cardiac puncture. Hormonal parameters like testosterone, estradiol, progesterone, follicle stimulating hormone, luteinizing hormone, and prolactin were analysed from blood samples collected from the treated rats using the ELISA method. The H & E staining technique was used for histologically evaluating the ovaries and testes. Statistical analysis was done using GraphPad Prism, version 9.02, and results obtained were expressed as Mean±SD. In terms of pilotstudy, doses used for the intraperitoneal administration were 0.0g/kg, 0.17g/kg, 0.50g/kg, 1.0g/kg, 1.53g/kg, 2.0g/kg, 2.5g/kg, 3.33g/kg, 4.17g/kg, and 5.0g/kg while doses used orally were 0.0g/kg, 5.0g/kg, 10.0g/kg, 12.5g/kg, 17.5g/kg, 22.5g/kg, 25.0g/kg, 32.5g/kg, 37.5g/kg, and 40.0g/kg. In terms of acute study, the intraperitoneally treated groups were designated ACIP, BCIP, CCIP, DCIP, ECIP, and FCIP with doses of 0.0g/kg, 0.17g/kg, 0.50g/kg, 1.0g/kg, 1.53g/kg and 2.0g/kg respectively. In contrast, that of the oral were designated ACO, BCO, CCO, DCO, ECO, and FCO were given doses of 0.0g/kg, 5.0g/kg, 10.0g/kg, 12.5g/kg, 17.5g/kg, and 22.5g/kg respectively after LD100 determination. The results of the acute study showed significant dose-dependent decreases in testosterone in male rats while in the female rats, significantly reduced values of follicle stimulating hormone were observed. However, luteinizing hormone, progesterone, estrogen, and prolactin indicated significantly higher values in rats treated with high doses of azorubine. In addition, estrogen, progesterone, and luteinizing hormones indicated dose-dependent increased values in the azorubine treated rats. Non-significant decreases were observed in the absolute weight of the testes and ovaries. Histological evaluation of the testes indicated vacuolated portions of spermatogonia layer, pycnosis, scanty, and distorted Leydig cells, distorted flagella, and basement membrane at a dose of 22.5g/kg given orally. The ovaries showed intense lutein cells with yellowish colouration, follicular cells, and antrum of ovarian follicle surrounded by granulosa cells with vacuolations. Conclusively, azorubine could be described as an endocrine disruptor and a precocious agent especially when consumed in high doses even for a very short period of 48 hours as indicated by increased presence of lutein cells, Graafian follicles, estrogen, progesterone, prolactin, and luteinizing hormone in female rats. Spermatogenesis could also be impeded in male rats due to an induced significant fall in testosterone concentration alongside testicular damage. Therefore, azorubine should be used with caution and high doses should be avoided.

keywords: Azorubine; Ovary, Testis; Hormones; Follicle Stimulating Hormones; Luteinizing Hormone

Progesterone; Testosterone; Estradiol, Prolactin; High Doses; Carmoisine

1. **Introduction**

Azorubine is one of the synthetic dyes that colour food and food dyes red in appearance and the use of these food dyes is not a recent development but rather an ancient practice [1, 2]. Azorubine is a nitrous derivative synthetic dye belonging to the azo class of food dyes widely used in food, pharmaceutical, and cosmetic industries that produce red appearance. It is also present in edibles such as soft drinks, energy drinks, cereals, ice creams, some coloured rice, snack food, biscuits, chocolates, yoghurts, and so on [1, 3]. Azorubine toxicity is derived mainly from the biotransformation process in the liver and by the actions of intestinal micro-organisms to aromatic amines, aryl amines, and free radicals [2, 4]. It is also known as carmoisine, acid red 14 0r C.I. 14720. Structurally, a molecule of azorubine has two pairs of benzene rings linked by an azo bond (N = N), with one pair consisting of sodium sulphate, nitrogen, and hydroxyl group while the other pair consists sodium sulphate and nitrogen as shown in figure 1[5].

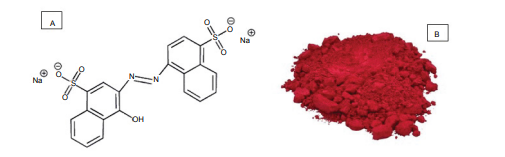


Figure 1: Chemical Structure of Azorubine (A) and the powered form of Carmoisine dye (B) – [5]

Consumption of azorubine in food products has been reported to have induced some toxicity and anaphylactic reactions such as asphyxia, insomnia, depression, anxiety, weakness, and blurred vision when consumed especially in high doses [6, 7]. This dye has also been reported to react with proteins covalently which leads to distortion of the proteins’ active site and configuration [8, 9]. Therefore, the purpose of this study is to assess the 48-hours toxicity of azorubine on the gonadal-endocrine tissues, particularly, the testes and ovaries, and their hormones in albino rats using varying doses. Blood samples were collected for biochemical analysis while testes and ovaries were harvested for the histological investigations.

Testosterone, progesterone, estradiol, luteinizing hormone (LH), follicle stimulating hormone (FSH), and prolactin are major hormones or endocrine chemicals associated with reproduction. Several scientific reports on the influence of azorubine on reproductive derangements have been documented. However, most of these reports are contradictory probably due to the dose of azorubine or the type of experimental animal used. Montaser et al.,[10], reported that azorubine at 5xADI and 10xADI induced significant dose-and-time-dependent down regulation of testin gene which induced lowered values of testosterone and derangements of sperm production and sperm motility in treated rats for 15, 30, and 45 days. The histological reports of their studies further revealed focal mild testicular degeneration of single or several layers of vacuolated spermatocytes, congested interstitial blood vessels, desquamated spermatocytes in the lumen of some seminiferous tubules as well as shrunken, disorganized tubules especially in the 5xADI and 10xADI. More so, Arjwam & Hussain, [11], also reported in male rats a significantly lower values of FSH and LH in rats treated with azorubine for 60 days at 250mg/kg. In another related work, Shok et al, [12], in a separate study, reported that azorubine consumption for 28 days at a dose of 250mg/kg induced significantly higher and lower values of prolactin and LH respectively in male rats compared to control rats. In addition, Sattir & Amin, [5], revealed a significant fall in FSH and LH levels in female rats treated with 5, 10, and 20mgkg of azorubine daily for 30 days. It was further revealed that estradiol and progesterone indicated significantly lowered values in the 5, 10, and 20mg/kg except in progesterone where there was a significantly higher value at the dose of 20mg/kg compared to 5 and 10 mg/kg treated rats. It has been observed that in most of these studies the exposure period to azorubine is usually above 20 days. Therefore, our study is designed to investigate the 48-hours acute exposure of high doses of azorubine on testosterone, estradiol, progesterone, LH, FSH, and prolactin in albino rats and its effect on the histology of the testes and ovaries of these rats.

1. **Materials and Methods**

**2.1 Materials**

The materials used include azorubine from Gessete, Italy**,** Polypropylene gavage tubes, MPW bucket centrifuge, Olympus Microscope with a digital camera, Shandon AS 325 Rotary Microtome, Haematoxylin& Eosin stain, Leica automatic tissue processor, Ohaus Scout-Pro Electronic weigh balance, 10% formal-saline, Albino rats, Stat Fax 4200 microplate Reader, progesterone, Estradiol, rProlactin, rFSH, rLH, and Testosterone Enzyme Linked Immunosorbent Assay (ELISA) kits were purchased from Bioassays, China.

**2.2 Experimental Animals**

Male and female albino rats (80) used for the study weighed 0.15kg approximately. All the rats used for the experiment were obtained by breeding. The rats were fed with rat pre-mix rat feed and water *ad libitum*. The animals were placed in a well-ventilated rat cage with water cans and feed containers in place.

**2.3 Preparation of Azorubine Treatment**

For the intraperitoneal study, 2.0 grams of azorubine was weighed and dissolved in sterile containers containing 8.0 ml of distilled water. This implies that 1.0 ml of this solution contains 0.25 grams while for the oral treatment, 3.0 grams of the azorubine was weighed and dissolved in sterile containers containing 8.0 ml of distilled water. This implies that 1.0 ml of this solution contains 0.375 grams.

**2.4 Experimental Design and Administration of Food Dyes**

The method of administration of treatment in the acute studies involved both intraperitoneal and oral techniques. In the intraperitoneal method, the dyes were injected into the intraperitoneal space of the rats using 2 ml and 5 ml hypodermic syringes. In contrast, in the oral method, the food dyes were administered using an orogastric tube to ensure complete delivery of the dye. The corresponding volume and doses of azorubine given per group are indicated in Table 1 and Table 2 for intraperitoneal and oral treatments respectively. The LD100 of the dye was established following the arithmetic method of Karber as described by Dede *et al*., [13] (Table 1 and Table 2).

**Table 1: Intraperitoneal Treatment: Volume and Corresponding Dose of Azorubine Administered per Group**

|  |  |  |  |
| --- | --- | --- | --- |
| Groups | No of rat | Volume (ml) | Dose (g/kg) |
| 1 | 4 | 0.0 | 0.00 |
| 2 | 4 | 0.10 | 0.17 |
| 3 | 4 | 0.30 | 0.50 |
| 4 | 4 | 0.60 | 1.00 |
| 5 | 4 | 0.90 | 1.53 |
| \*6 | 4 | \*1.20 | \*2.00 |
| 7 | 4 | 1.50 | 2.50 |
| 8 | 4 | 2.00 | 3.33 |
| 9 | 4 | 2.50 | 4.17 |
| 10 | 4 | 3.00 | 5.00 |

\*Intraperitoneal LD100

**Table 2: Oral Treatment: Volume and Corresponding Dose of Azorubine Administered per Group**

|  |  |  |  |
| --- | --- | --- | --- |
| Groups | No of rat | Volume (ml) | Dose (g/kg) |
| 1 | 4 | 0.0 | 0.0 |
| 2 | 4 | 2.0 | 5.0 |
| 3 | 4 | 4.0 | 10.0 |
| 4 | 4 | 5. 0 | 12.5 |
| 5 | 4 | 7.0 | 17.5 |
| \*6 | 4 | \*9.0 | \*22.5 |
| 7 | 4 | 10.0 | 25.0 |
| 8 | 4 | 13.0 | 32.5 |
| 9 | 4 | 15.0 | 37.5 |
| 10 | 4 | 16.0 | 40.0 |

\*Oral LD100

**2.5 Specimen Collection, Preparation, and Analysis**

At the end of the study, the animals were anaesthetized with chloroform, and 5 ml of blood samples were collected by cardiac puncture into plain bottles for hormonal assay. More so, ovarian and testicular tissues were also collected for histological examinations. These tissues were washed with normal saline to remove blood stains before being fixed in 10% formol-saline before tissue processing. The blood specimens were spun at 4500 rpm for 10 minutes to obtain serum which was transferred into other sets of labelled plain bottles and stored at -4°C. The laboratory analysis of the hormonal parameters was based on Enzyme Linked Immunosorbent Assay (ELISA) Technique based on the method described by Engvall & Perlmann, [14]

**2.6 Statistical Analysis**

Statistical analysis was performed using GraphPad Prism version 9.02 (San Diego, California, USA). Results were presented as Mean ± Standard deviation (SD). Inferential statistics using the One-Way ANOVA (Post Hoc: Tukey’s multiple comparative test) was used. Statistical significance was set at P<0.05.

1. **RESULTS**

**3.1 Results of Reproductive Hormonal Parameters**

The results obtained indicated significantly lower values or decline that is dose-dependent in the testosterone values as seen in the intraperitoneal group treated with 1.0g/kg, 1.5g/kg, and 2.0g/kg. Progesterone and estradiol indicated significantly higher values in treated groups at doses of 1.0g/kg – 2.0g/kg and 1.5g/kg – 2.0g/kg respectively. Likewise, prolactin also indicated a similar significant increase in treated groups at doses of 1.5g/kg – 2.0g/kg. FSH indicated significantly lower values from the dose, 1.0g/kg – 2.0g/kg while LH showed significantly higher values in a dose-dependent pattern from the dose of 0.5g/kg to 2.0kg (Table 3).In orally treated rats, testosterone and prolactin indicated significantly lower and higher values in a dose-dependent pattern from the dose of 17.5g/kg – 22.5g/kg. Progesterone and LH also indicated higher values from dose 10.0g/kg – 22.5g/kg while estradiol had higher values at the doses of 12.5g/kg – 22.5g/kg. Finally, FSH showed significantly lower values from dose 5.0g/kg – 22.5g/kg (Table 4). The weights of testes and ovaries indicated a non-significant reduction in the treated male and female rats compared to control rats (Table 5 and Table 6).

**Table 3. Hormonal Parameters of Rats Administered with Azorubine Intraperitoneally**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Parameters | 0.0g/kg  (ACIP) | 0.17g/kg  (BCIP) | 0.5g/kg  (CCIP) | 1.0g/kg  (DCIP) | 1.5g/kg  (ECIP) | 2.0g/kg  (FCIP) | P value | F value |
| \*TESTO(ng/ml) | 4.40±0.57a | 3.60±0.57a | 4.35±0.50a | 3.0±1.27b | 1.90±0.28c | 1.25±0.35d | 0.0150 | 7.415 |
| \*\*PROG(ng/ml) | 4.25±3.61a | 5.35±2.05a | 3.20±1.56a | 8.50±0.85b | 8.40±1.56b | 8.45±0.78b | 0.1113 | 2.933 |
| \*\*E2(ng/ml) | 17.90±1.41a | 18.80±1.70a | 20.75±2.09a | 19.15±1.46a | 32.50±6.79b | 34.10±1.13b | 0.0891 | 3.301 |
| \*\*FSH(mlu/ml) | 0.73±0.43a | 0.55±0.39a | 0.30±0.12a | 0.18±0.10b | 0.15±0.10b | 0.18±0.10b | 0.0193 | 3.621 |
| \*\*LH(mlu/ml) | 0.63±0.61a | 0.83±0.47a | 1.0±0.44b | 1.18±0.05a | 1.43±0.05c | 1.43±0.10c | 0.1680 | 1.779 |
| \*\*PRL(ng/ml) | 0.35±0.13a | 0.38±0.10a | 0.38±0.10a | 0.38±0.05a | 0.65±0.21b | 0.60±0.12b | 0.0829 | 2.349 |

PostHoc: Values in the same row with different superscripts differ significantly at p<0.05. \*Male rats \*\*Female rats.

**Table 4. Hormonal Parameters of Rats Administered with Azorubine Orally**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Parameters | 0.0g/kg  (ACIP; Control) | 5.0g/kg  (BCO) | 10.0g/kg  (CCO) | 12.5g/kg  (DCO) | 17.5g/kg  (ECO) | 22.5g/kg  (FCO) | P value | F value |
| \*TESTO (ng/ml) | 4.40±0.57a | 4.30±0.42a | 4.20±0.99a | 3.30±1.0a | 2.65±0.78b | 1.35±0.50b | 0.0340 | 5.234 |
| \*\*PROG (ng/ml) | 4.25±3.61a | 5.40±1.77a | 9.30±0.14b | 7.15±4.17b | 8.90±0.42b | 9.60±0.71b | 0.0316 | 4.497 |
| \*\*E2(ng/ml) | 17.90±1.41a | 22.80±3.11a | 22.5±3.54a | 29.50±5.37b | 30.15±0.50b | 36.10±3.11b | 0.0114 | 8.312 |
| \*\*FSH(mlu/ml) | 0.73±0.43a | 0.30±0.08b | 0.23±0.15b | 0.45±0.13b | 0.35±0.13b | 0.18±0.10b | 0.0193 | 3.621 |
| \*\*LH(mlu/ml) | 0.63±0.61a | 0.74 ±0.56a | 0.92±0.60b | 1.03±0.20b | 1.31±0.05b | 1.60±0.36c | 0.0168 | 4.779 |
| \*\*PRL(ng/ml) | 0.35±0.13a | 0.48±0.19a | 0.43±0.21a | 0.40±0.16a | 0.65±0.17b | 0.68±0.19b | 0.0429 | 4.349 |

PostHoc: Values in the same row with different superscripts differ significantly at p<0.05. \*Male rats \*\*Female rats.

**Table 5: Weights of Organ Extracted from Rats Administered with Azorubine Intraperitoneally**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Parameters | 0.0g/kg  (ACIP) | 0.17g/kg  (BCIP) | 0.5g/kg  (CCIP) | 1.0g/kg  (DCIP) | 1.5g/kg  (ECIP) | 2.0g/kg  (FCIP) | P value | F value |
| Ovaries (g) | 1.22±0.02 | 1.17±0.27 | 1.02±0.11 | 1.04±0.05 | 1.01±0.08 | 0.96±0.18 | 0.3999 | 1.225 |
| Testes (g) | 3.25±0.04 | 3.21±0.11 | 3.09±0.11 | 3.00±0.13 | 2.92±0.18 | 3.03±0.03 | 0.1455 | 2.523 |

No of Rats/group = 4 Rats

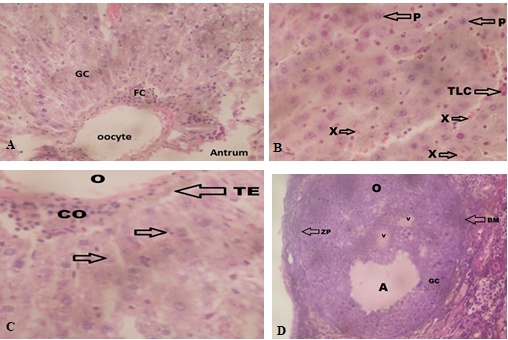
**Table 6: Weights of Organs Extracted from Rats Administered with Azorubine Orally**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Parameters | 0.0g/kg  (ACIP; Control) | 5.0g/kg  (BCO) | 10.0g/kg  (CCO) | 12.5g/kg  (DCO) | 17.5g/kg  (ECO) | 22.5g/kg  (FCO) | P value | F value |
| Ovary (g) | 1.22±0.02 | 1.11±0.18 | 1.19±0.21 | 1.15±0.18 | 0.87±0.13 | 0.89±0.13 | 0.2255 | 1.917 |
| Testis (g) | 3.25±0.04 | 3.24±0.31 | 3.04±0.19 | 3.28±0.28 | 2.79±0.37 | 2.72±0.23 | 0.2461 | 1.805 |

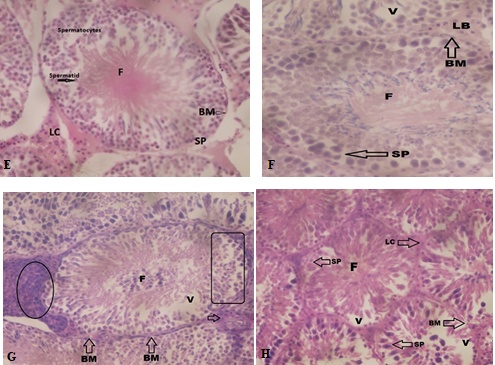
No of Rats/group = 4 Rats

**3.2 Histopathological Investigations of the Acute Toxicity Study**

Histopathology of examination of the ovaries and testes of the acute toxicity of azorubine were performed as seen in Plate A– H



**Plate A:**Dose: 0.0g/kg, Treatment Substance: Nil. Observation: Antrum of the ovarian follicle surrounded by granulosa cells (GC). The oocyte is surrounded by follicular cells (FC). Normal histology. **Plate B:** Dose:2.5g/kg and 5.0g/kg. Observation: P= Primordial Follicles, TLC= Lutein cells with yellowish colouration X. **Plate C:**Dose: 0.17g/kg and 0.5g/kg.Azorubine (I.P). Observation: The ovarian follicle is surrounded by granulosa cells (Arrow). The oocyte (O) is surrounded by follicular cells. TE= theca externa. **Plate D:** Dose: 1.0g/kg, 1.5g/kg and 2.0g/kg.Azorubine (I.P), Observation: A= Antrum of ovarian follicle surrounded by granulosa cells (GC) with vacuolations (V). Oocyte (O) surrounded by GC. ZP= Zona pellucida and BM= Normal basement membrane. H&E, Mag: ×400, Ovary.



**Plate E:** Dose: 0.0mg/kg, Treatment: Nil.F=flagella of spermatogonia in the lumen of seminiferous tubule of the testis, BM=basement membrane, SP=spermatogonia, LC=leydig cells. Inference = normal histology of the testis. **Plate F:** Dose: 0.17g/kg and 0.5g/kg, 1.0mg/kg; Azorubine (I.P). F = Flagella within the lumen of the seminiferous tubule. Spermatogonia (SP) layer with vacuolated portions (V), basement membrane (BM) appears normal. **Plate G:** Dose: 10mg/kg, 12.5g/kg, and 17.5g/kg, Azorubine (Oral): F=distorted flagella with nuclear materials in the lumen of seminiferous tubule. vacuolation of spermatid and spermatogonia layer (v). The spermatogonia layer is filled with degenerative scattered nuclear materials (pycnosis) and vacuolated portions (rectangular shape). The basement membrane (BM) appears distorted at some point. Scanty and distorted Leydig cells (arrow). Clusters of degenerating nuclear materials (circle shape). **Plate H:** Dose: 22.5g/kg, azorubine (oral): F=distorted flagella within the lumen of the seminiferous tubule. Vacuolation (V) of spermatid, spermatocyte, and spermaatogonia (SP) layers (V), distorted basement membrane (BM). H&E, Mag: ×400, Testis.

1. **DISCUSSION**

Our results indicated that the intraperitoneal treatment had more severity than the oral administration. The severity of the different routes of administration could be associated with the role of hepatic first-pass, gastrointestinal and microbial interaction with the dye, and intestinal secretions. These interactions as seen in the oral route could account for the lesser severity compared to the intraperitoneal route of administration.

The dose-dependent significantly lower values of testosterone observed in the azorubine treated male rats concur with the reports of Shok et al., [12]. They reported significantly reduced values of testosterone in rats fed with 250mgkg of azorubine for 28 days. Our findings are also collaborative with the reports of Wopara et al., [15] and Montaser et al., [10]. Wopara et al., [15], reported a significant fall in testosterone concentration in the rats treated with a combination of azo dyes tartrazine and erythrosine at higher doses of 10 and 20mgkg for 23 days. Montaser et al., [10], documented down regulation of the testosterone gene and a decline in testosterone concentration at 5xADI and 10xADI doses over 45 days. They further documented distortion of seminal vesicles, epididymis, testicular basement membrane, exfoliation of testicular cells into the lumen, vacuolization, and pyknosis when rats were treated with azorubine. Alabi et al., [16], also documented a significant reduction in testosterone in mice treated with three different food additives at higher doses. The fall in testosterone could be associated with oxidative damages induced by metabolic by-products including reactive oxygen (ROS) and nitrogen species (RNS) of azorubine breakdown. The fall in testosterone observed a dose-dependent pattern indicating that the higher the dose of the dye, the more the fall in the testosterone concentration in the plasma. Our testosterone results also aligned with the histological reports on the testicular tissues of the treated rats. The histology indicated vacuolation within the lumen of the seminiferous tubule in smaller doses of 0.17g/kg and 0.5g/kg. However, as the doses were increased, distortion of flagellation of spermatocytes, basement membrane, degeneration, and scattered nuclear materials (pycnosis) were also observed. Higher doses indicated distorted Leydig cells, clusters of degenerating nuclear materials as well as major distortion of the basement membrane at 22.5g/kg dose of azorubine administered orally. The distortion of the basement membrane (associated with Sertoli cells) and Leydig cells could further explain the significant fall in testosterone concentration in the azorubine-treated rats. The Leydig cells are responsible for the production of luteinizing hormones that subsequently stimulate the testes for the production of testosterone. More so, the observed vacuolation of spermatogonia layer in the azorubine treated male rats suggest loss of spermatogenic precursors necessary for the formation of mature and active spermatocytes. These deficits could also be tied altered Leydig and Sertoli cells activities. Therefore, a significant loss of Leydig and Sertoli cells in the azorubine-treated male rats could also account for the significant fall in the testosterone concentration in the rats.

Progesterone is one of the principal hormones secreted by the ovaries and produced mainly by the corpus luteum under the influence of luteinizing hormone. It plays a major role in the transformation of the proliferative endometrium in the secretory phase, which is necessary for the implantation of a fertilized egg, and establishing pregnancy Sharma, [17]. The dose-dependent significantly lower value of progesterone in the treated female rats concurs with the records of Sharma, [17], who also documented a fall in progesterone levels when azo dyes were fed to rats. Sattar, & Amin [5], also reported a significantly lower value of progesterone in rats treated with azorubine at 10mgkg and 20mgkg for 30 days. In a different work, Shosha et al., [18], reported a significant change in the progesterone concentration in rats administered with food additives; monosodium glutamate for 18 days at high dose of 6g/kg. The significant fall in Progesterone could also be attributed to ovarian cell secretions following their exposure to xenoestrogenic characteristics of azorubine. Again, the increases observed in progesterone concentration could also be attributed to enhanced activities of the corpus luteum of the ovarian cells initiated by the xenoestrogenic properties of azorubine. Progesterone is the primary hormone produced by the corpus luteum. The histological findings further explain the higher level of progesterone following the observation of the ovaries indicating intense lutein cells with yellowish colouration and follicular cells (plate B).

The dose-dependent higher values of E2 observed in our study also concur with the reports of Sattar, & Amin [5], who also reported significantly higher values of estrogen in rats treated with azorubine at 10mgkg and 20mgkg for 30 days. Elekima & Nwachuku 19], also reported similar findings in one of our works when tartrazine was administered to rats in high doses above the recommended ADI. In addition, the significantly higher values in E2 observed in our study is also in line with the report Mindang et al., (2022), who reported significantly higher values of E2 in female rats treated with tartrazine for 40 days at 47mgkg bodyweight. The significantly higher values in E2 could be as a result of the fact that azorubine are sources of xenoestrogen. Xenoestrogenic substances are known agents affecting the maturation of ovarian follicular cells through a false negative feedback mechanism in the ovarian-pituitary-Hypothalamus axis. The significantly higher values of E2 could also suggest early onset sexual maturation (precocious puberty) in the female rats due to the xenoestrogenous effect of azorubine.

In addition, the significantly lower values of FSH as observed in our study are contrary to the reports of Sattar, & Amin [5], who reported significantly higher values of FSH in rats treated with azorubine at 10mgkg and 20mgkg for 30 days. However, our study on the other hands, also agrees with the reports of Sattar, & Amin [5], when 5mg/kg of azorubine was administered to rats for 30 days. The fall in FSH (not dose-dependent) could also be attributed to structural changes associated higher doses of azurobine use such as follicular cells degeneration, distortion of the granulosa cells, and vacuolations seen in the histology. The histological findings are also in line with the reports of Sharma, [17], who stated that azorubine in combination with sunshine yellow (FD & C 6) on female albino rats induced degenerative features in the ovarian follicles such as shrunken oocytes, distorted basement membrane, absence of the zona pellicuda, loosing of theca layer, degenerative corpus luteum, vacuolization, and degenerative granulose cells. Though Sharma reported a significant reduction in ovarian weight, our results in the weight of the ovaries observed a non-significant difference. The fall in FSH as seen in our study could be associated with negative feedback limitation induced by increased E2 and progesterone. Gonadotropins such as FSH have been known to be limited by the increased presence of progesterone following enhanced activities of the corpus luteum of the ovum.

The significantly higher values of LH seen in our results are also contrary to the findings of Sattar& Amin, 2018, who documented a significantly lower value of LH in rats treated with azorubine at 10mgkg and 20mgkg for 30 days. The significantly higher values of LH observed in our study is also agrees with the reports of Mindang et al., [20], who reported significantly higher values of LH in female rats treated with tartrazine for 40 days at 47mgkg bodyweight. More so, our result on LH also agrees with the findings of Abbas & AlHamadavi [21], who reported significantly higher values of LH in rats treated compared with control rats with chocolate brown (azorubine mixture) azo food dye at a high dose of 200 mg/kg and 400 mg/kg for 8 weeks. However, Khiralla et al. [22] observed a significant reduction in LH hormones when rats were treated with tartrazine azo dye at a dose 5 times that of the ADI dose. The higher values of LH in the treatment could be due to increased activities of pituitary gonadotropin cells due to reduced follicular response following the negative feedback mechanism. Also, the higher LH values could be due to enhanced folliculogenesis, stimulating estrogen increment in the treated rats. Another possibility concerning the increase in LH could be due to the stimulation of kisspeptin (Kp) from kiss1 neuron in the anteroventral periventricular nucleus (AVPV) of the hypothalamus by xenoestrogenic moieties of azorubine in a manner that could trigger increases in LH release.

The significantly higher values of prolactin contradicts the reports of Dixit & Goyal, [23], who documented significantly lowered values of prolactin when high doses of indigo carmine azo dyes were administered in rats at a dose of 39mg/kg bodyweight for 6 weeks. In addition, Khiralla et al. [22], documented non-significant differences in the level of prolactin, noraldrenaline hormone, and dopamine when rats were treated with tatrazine azo dye at 5xADI. However, Elekima et al., [24], also documented higher values of prolactin in tartrazine azo dye treated rats at ADI of 7.5gkg for 30 days.

The non-significant decreases observed in the testes and ovaries in the treated rats contradict the reports of Sattar & Amin [5] and Shok et al., [12]. Sattar & Amin [5] observed a significant reduction in the weight of the ovaries of rats treated with azorubine at 10mgkg and 20mgkg for 30 days while Shok et al., [12] documented a significant fall in the weight of the testes following exposure to 250mg/kg of azorubine for 28 days. Similarly, Dixit & Goyal, [23], also observed a significant reduction in the weight of the ovaries after 6 weeks exposure to indigo carmine azo dyes at a dose of 39mg/kg bodyweight. The non-significant reduction in the absolute weights of the testes and the ovaries in our work could be due to the short duration of the study, which is 48 hours compared the observed reductions in weight as seen in the above-mentioned studies. Furthermore, the observed non significant fall in the weights of the testes and ovaries could be tied to the loss of intrinsic parenchymal materials of these gonadal tissues as indicated by vacuolations in the histology examination. Therefore, by projections, we believe there could be a significant decline in the weight of these organs if the exposure periods were extended.

1. **Conclusion**

Azorubine could be described as an endocrine disruptor especially when consumed in high doses even for a very short period as indicated by the induced high levels of estrogen, progesterone, prolactin, and luteinizing hormone in female rats with a decreased follicle stimulating hormone level. Spermatogenesis could also be impeded in male rats due to an induced significant fall in testosterone concentration alongside testicular damage. The intense presence of lutein cells, graafian follicles, increased progesterone, and estrogen in female could also suggest early onset of puberty. Therefore, the consumption of high doses of azorubine even in cases of brief exposure (48 hours) could cause reproductive derangements in male and female rats.

**Ethical Approval**

Animal Ethic committee approval has been collected and preserved by the author(s)

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**Limitation**

The study did not consider the assay of luteinizing hormones (LH), prolactin, follicle stimulating hormones (FSH), estradiol, and progesterone in male rats. We believe the analysis of these hormonal parameters in the male rats would have provided additional insight into the disruptive capabilities of azorubine in male reproduction.

**Conflict of Interest**

We declare no conflict of interest.

Disclaimer (Artificial intelligence)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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