Original Research Article

EVALUATION OF ACUTE AND CHRONIC TOXICITY OF TARTRAZINE (E102) ON STERIOD REPRODUCTIVE HORMONES OF ALBINO RATS

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ABSTRACT

Aim: To determine the acute and chronic effect of tartrazine on reproductive steroid hormones of albino rats.

Study design: The design involved acute and chronic study. The acute study investigated intraperitoneal and oral route of administration while the chronic study used oral route only. The rats used for the study weighed 150gm approximately. In the acute study, 48 rats (24 female and 24 male) were used for intraperitoneal treatment and were randomly selected into six groups treated with 0.0g/kg, 1.67g/kg, 3.33g/kg, 5.0g/kg, 6.67g/kg and 8.33g/kg of tartrazine. In orally treated rats, 48 rats (24 female and 24 male) were also used and were treated with 0.0g/kg, 2.5g/kg, 5.0g/kg, 10.0g/kg, 15.0g/kg and 20.0g/kg of tartrazine. In the chronic study, the experiment was divided into phase 1, 2 and 3 which lasted for 30, 60 and 90 days respectively. In each phase, 80 rats were used and were divided into treatment and control groups. The treatment groups were given 7.5mg/kg of tartrazine orally on daily basis over a period of 30, 60 and 90 days while the control groups were not treated with tartrazine.

Place and Duration of Study: The study was carried out in the Department of Medical Laboratory Science, Rivers State University, Port Harcourt, Nigeria over a period of 12 months (December, 2017 – December, 2018).

Methodology: At the end of the acute and chronic study, 5mls of whole blood specimens was collected by means of cardiac puncture into plain bottles. The specimens were spun at 4500 rpm for 10 minutes to obtain serum. The laboratory analysis of the hormonal parameters was based on Enzyme Linked Immunosorbent Assay (ELISA) Technique. Statistical analysis was performed using GraphPad Prism version 5.03. More so, ovarian and testicular tissues were also collected for histological examinations. These tissues were fixed in 10% formol-saline prior to tissue processing. Staining was done using Haematoxylin and Eosin stain.

Results: In acute study, female treated rats (intraperitoneally and orally) showed significantly higher values in Progesterone (PROG) and Estradiol (E_2) concentrations while male treated rats (intraperitoneally and orally) indicated significantly lower values in testosterone (TESTO) concentration compared with control rats. Histopathologic examination showed flagella distortion in the seminiferous lumen, vacuolation, pycnosis, distortion of basement membrane and loss of leydig cells of the testis. More so, mild vacuolation of follicular ovarian cells were also seen. In chronic treatments, hormonal parameters after 30 days, 60 days and 90 days showed no significant differences in testosterone (TESTO), Progesterone (PROG) and Estradiol (E_2) concentrations in tartrazine treated rats compared with their respective control rats. When the comparative analyses of treated groups after 30, 60 and 90 days using One-Way ANOVA were considered, testosterone (TESTO) concentration indicated significantly lower levels in treated male rats while Progesterone (PROG) showed significantly higher values over 30, 60 and 90 days in treated female rats. Histopathologic examination indicated mild changes such as flagella distortion, pycnosis and vacuolation in testicular tissues especially after 90 days of chronic treatment likewise mild vacuolation of ovarian cells.

Conclusion: In the acute study, reduction in testosterone (TESTO) concentration while increase in PROG and E_2 concentrations were seen. However, in the chronic study, significant differences were not seen in testosterone (TESTO), Progesterone (PROG) and Estradiol (E_2) concentrations. Finally, when the influence on duration of exposure at ADI doses (7.5mg/kg) were considered after 30, 60 and 90 days, reduction in testosterone (TESTO) and increase in Progesterone (PROG) concentrations were seen.

Keywords: Tartrazine, reproductive hormones, Progesterone, Testosterone, Estradiol.

1. INTRODUCTION

Colours are important components of food and food products which gives the first impression on the psyche of the consumer [1, 2]. Though food dyes occur in natural and synthetic forms, lately synthetic food dyes are commonly used in food industries because of their availability, cost effectiveness and stability. They are mainly organic compound (Azo dyes) with the capacity to reflect light. Examples include tartrazine, erythrosine, fast green, carmoisine and so on [3, 4]. The application of dyes is also seen in textile, leather, paper, rubber, cosmetics and even in pharmaceutical industries [4].

The use of synthetic food dyes has been reported to cause renal derangements, hepato-toxicity, anaemia, leucopenia and interference with enzymes activities resulting in reduced enzymes functions when studied in rats [2, 4, 5, 6]. However, according to the Australian Government through her Department of Health [7] in a scientific review report in 2014 stated that synthetic food dyes possesses no harmful effect within the acceptable daily intake (ADI) doses.

A review of literature reveals that almost all synthetic food dyes originate from coal tar which is toxic and carcinogenic [6]. The toxicity of synthetic dyes such as tartrazine has been linked to the reductive biotransformation of the azo bond during their metabolism in the intestine and liver producing reactive amines, aryl amines and free radicals [8]. These dyes have also been reported to react with proteins (enzymes) covalently which leads to distortion of the protein active site and configuration [5]. Though the effects of synthetic dyes on reproductive parameters and organs remains controversial, very few studies have reported reproductive derangements in rats [9, 10, 11], while other studies reported no reproductive derangements [12, 13, 14]. Reproductive parameters (Hormones) considered in this study include, testosterone, estrogen and progesterone.

Testosterone is the main androgen hormones secreted by the mature testes [15, 16]. It is an important steroid hormone that play vital role in the production and maturation of spermatozoa, development, growth and differentiation of male sex organs, sexual drive and secondary sexual characteristics that make sexual reproduction feasible in males [17]. In the absence of injury or toxicity of the testicular cells, there is no sharp reduction or fall in testosterone production [17]. Several chemicals and drugs have been reported to induce adverse effects on the reproductive organs [17]. Sies et al. [18], Ashida et al. [19], reported that tartrazine stimulates mutagenic processes and decreases cell viability. However, Mehedi et al. [9], Gautam et al. [20], reported that sperm production and sperm motility were decreased when xenobiobitics such as azo dyes were fed to 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 [21, 22]. The major role of progesterone involves the transformation of the proliferative endometrium in the secretary phase, which is necessary for implantation of fertilized egg [22]. Progesterone also enhances the viscosity of the cervical mucus making it more viscous and less permeable

and therefore play vital role in establishing pregnancy after implantation of fertilized egg(s) [22]. Estrogen is produced mainly by the granulosa cells of the developing ovarian follicle in the early part of the ovarian cycle and from the luteinized granulosa cells in the corpus luteum after ovulation [21]. The principal biological active form of estrogen is the 17β-estradiol [21]. The major function of estrogen includes promotion of growth and development of secondary sexual characteristics in the female such as growth and development of the oviducts, uterus, vagina, external genitals, among others making sexual reproduction feasible [21, 22]. According to Foster & Gray Jr., [17], exposure to xenobiotics has been implicated in the decline of normal fertility and reproduction. Takana [12], reported that 773mg/kg of tartrazine dyes fed to rats in diet had no adverse effect on reproductive parameters such as steroid hormones. However, Mehedi et al. [10] and Sharma et al. [23], reported in their separate studies that 2.5% of tartrazine induced significant weight reduction of the ovaries as well as significantly lowered concentration of steroid hormones in rats.

This study is particularly relevant in our society because the exposure to food dyes cuts across almost everyone due to their diverse applications in the various industries and especially in the food industries (restaurants, fast food, and domestic use, among others). Secondly, studies on toxic effect of synthetic dyes on reproductive hormones even at the recommended acceptable daily intake (ADI) are still controversial, quite minimal and obscure. Sreenivasa et al. [24], reported that infertility and hormonal imbalances are on the increase with global record of 75 million couples suffering infertility annually of which 15% are idiopathic. Therefore, this research is aimed at using acute and chronic toxicity studies to perform toxicological evaluation of tartrazine toxicity on steroid reproductive hormones using albino rats.

2. MATERIALS AND METHODS

2.1 Materials

Materials used in this research include Polypropylene gavage tubes (Intech Laboratory Incorporated, Plymouth Meeting, USA), Haier thermocool refrigerator (China), MPW bucket centrifuge Model 351 (MPW Medical Instruments, Warsaw, Poland), Olympus Microscope (with digital microscopic camera for taking photomicrographs) Shandon AS 325 Rotary Microtome, Haematoxylin & Eosin stain, Leica automatic tissue processor (Leica Biosystems, USA), Ohaus Scout-Pro Electronic weigh balance (Ohaus Corporation, New Jersey, USA), 10% formal-saline, Albino rats, Stat Fax 4200 Microplate Reader (awareness, USA), Tartrazine dyes (CI. 19140, CAS No 1934-21-0, MW 534,37, E102, FD& C NO 5) with serial no of FI19371 purchased in a granular form from Fiorio Colori Spa, Gessete, Italy, with purity of 86.7% guaranteed by the manufacturer. Progesterone, Estradiol and Testosterone Enzyme Linked Immunosorbent Assay (ELISA) kits were purchased from BioCheck diagnostics (San Francisco,USA). Other materials used include automatic pipettes and glass test-tubes and glass slides.

2.2 Experimental Animals

Male and female albino rats used for the study weighed 150gm approximately. The reason for selecting male and female rats for the study was based on the fact that male predominate hormone is testosterone while female predominate hormones are estradiol and progesterone. All the rats used for the experiment were obtained by breeding. However, the parent rats used for the breeding were purchased from the University of Port Harcourt, River State, Nigeria. The rats were fed with rat pre-mix rat feed and water *ad libitum*. The animals were placed in a well-ventilated rat cages with water cans and feed containers in place.

2.3 Preparation of Tartrazine Food Dye

In the acute study, for intraperitoneal treatment, 250 grams of the tartrazine was weighed and dissolved in a sterile container containing 1 litre of distilled water. This implies that 1.0ml of this solution contains 0.25 grams. In terms of oral treatment (acute study), 375grams of the tartrazine dyes was also dissolved in sterile containers containing 1 litre of distilled water. This implies that 1.0ml of this solution contains 0.375grams of tartrazine. Finally, in the chronic study, 1.13 grams of tartrazine was weighed and dissolved in a sterile container containing 1.0 litre of distilled water. This implies that, 1.0ml of the tartrazine solution contains 0.00113grams and which is equivalent to 7.5mg/kg when administered into a 0.15kg rat. The contents of the containers were properly mixed to ensure complete mixture before administration.

2.4 Experimental Design and Administration of Food Dyes

The method of treatment in the acute studies involved both intraperitoneal and oral techniques while in the chronic study, treatment was strictly orally. In the intraperitoneal method, the dyes were injected into the intraperitoneal space of the rats using 2 ml and 5 ml hypodermic syringes while in the oral method, the food dyes were administered using orogastric tube to ensure complete delivery of the dye.

2.4.1 Acute treatment and Toxicity Study

Dose range of the tartrazine dye were determined after the obtaining the value of LD_{50} using the arithmetic method of Karber as described by Dede et al. [25], in both oral and intraperitoneal treated rats. The LD_{50} was calculated to be 5.83g/kg and 11.25g/kg for intraperitoneal and orally treated rats respectively. In the intraperitoneal treatment, 48 rats (24 male & 24 female rats) were used. The male and female rats were randomly selected into six different groups separately designated as A_{TIP} (control), B_{TIP}, C_{TIP}, D_{TIP}, E_{TIP} and F_{TIP} and were treated with 0.0g/kg, 1.67g/kg, 3.33g/kg, 5.0g/kg, 6.67g/kg and 8.33g/kg of tartrazine respectively. In terms of orally treated rats, 48 rats (24 males; 24 females) were also used. The male and female rats were randomly selected into six different groups separately. The groups were designated as A_{TO} (control), B_{TO}, C_{TO}, D_{TO}, E_{TO} and F_{TO} and were orally treated with 0.0g/kg, 2.5g/kg, 5.0g/kg, 10.0g/kg, 15.0g/kg and 20.0g/kg of tartrazine respectively. At the end of the 24 hours acute toxicity testing, blood samples as well as ovarian and testicular tissues were collected after the animals were sacrificed.

2.4.2 Chronic Treatment and Toxicity Study

In the chronic study, the experiment was divided into three phases depending on the duration of exposure of the rats to tartrazine dyes. The phase 1, 2 and 3 of the chronic toxicity studies lasted for a duration of 30, 60 and 90 days respectively. Eighty (80) experimental rats weighing approximately 150gm were used in each phase of the study (with a total of 119 females and 116 male rats of which 5 died in the course of the experiment). In each phase of the experiment, the rats were divided into two groups designated T_T (tartrazine treated group), and C (control, untreated group). Rats in each of these groups were further distributed randomly into ten cages with four rats per cage, designated $T_{T1,}$ $T_{T2...}T_{10}$. In the treatment pattern, the acceptable daily intake (ADI) of 7.5mg/kg of tartrazine was administered orally. The control group, were not treated with tartrazine. At the end of the chronic study, the animals were anaesthesized with chloroform and blood samples, ovarian and testicular tissues were collected investigations.

2.5 Study Area

The study was carried out in the Department of Medical Laboratory Science, Rivers State University, Port Harcourt. However, samples were transported in frozen form in a thermo-regulatory container to the University of Port Harcourt Teaching Hospital. However, prior to the actual assay, the serum samples were allowed to defrost at temperature. All of the hormonal parameters considered were analysed at the Chemical pathology Unit of the University Teaching Hospital while the histological examinations were carried out in the anatomical laboratory, College of Medical Science, University of Port Harcourt.

2.6 Specimen Collection, Preparation and Analysis

At the end of the study, the animals were anaesthetized with chloroform and 5mls of blood samples was collected by means of 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 prior to 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 -4^0C. The laboratory analysis of the hormonal parameters was based on Enzyme Linked Immunosorbent Assay (ELISA) Technique. The ELISA procedure (outlined by BioCheck Diagnostics, San Francisco, USA) for the determination of Progesterone, Estradiol and Testosterone concentrations were based on method described by Engvall and Perlmann [26]. The concentration of the analytes in the samples viz-a-viz the intensity of colour change in the microplate wells was determined using Stat Fax 4200 microplate reader.

2.7 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 5.03 (San Diego, California, USA). Results were presented as Mean ± Standard deviation (SD). Inferential statistics using Students' statistical t-test was employed to compare values of the treated rats and control rats. In addition, the One-Way ANOVA (Post Hoc: Tukey's multiple comparative test) was also used to analyse the influence of treatment duration. Statistical significance was set at P=0.05.

3. RESULTS

3.1 Results of Acute Treatment on Reproductive Hormonal Profile in Rats Administered with Tartrazine

Table 1 and table 2 showed hormonal indices in Rats administered with tartrazine intraperitoneally and orally respectively. In intraperitoneally treated rats, testosterone (TESTO) in treated male rats showed a significant decrease compared to control from dose 3.33g/kg while Progesterone (PROG) and Estradiol (E_2) in female rats showed significant increases when compared to control from dose 1.67g/kg at P=0.05. More so, in orally treated rats, testosterone (TESTO) showed a significantly lower value in tartrazine treated male rats compared with control male rats from 2.5g/kg dosage while Progesterone (PROG) and Estradiol (E_2) in treated female rats indicated a significantly higher value in tartrazine treated female rats compared with control female rats at P=0.05.

Table 1. Reproductive Hormonal Profile in Rats Acutely Treated with Tartrazine Intraperitoneally

Parameters	*TESTO (ng/ml)	**PROG (ng/ml)	**E2 (ng/ml)
0.0g/kg (A_{TIP})	5.75±0.21[a]	1.10±0.70[a]	11.95±2.62[a]
1.67g/kg (B_{TIP})	3.75±1.49[a,c]	4.20±0.28[b,c]	15.0±2.83[a,c]
3.33g/kg (C_{TIP})	4.0±0.28[b,c,e]	4.35±0.07[b,c,e]	12.65±1.06[a,c]
5.0g/kg (D_{TIP})	3.10±0.14[b,c,e]	7.90±0.99[b,d,f,g]	26.10±11.88[b,c,e]
6.67g/kg (E_{TIP})	2.90±0.42[b,d,f,g]	7.90±0.99[b,d,f,g,h]	56.25±1.91[b,d,f,g]
8.33g/kg (F_{TIP})	2.15±0.78[b,d,f,g]	7.40±0.28[b,d,f,g,h]	37.65±1.49[a,d,f,g]
P value	0.03	0.0003	0.0008
F value	5.85	33.37	22.76
Remark	S	S	S

Values in each column with different superscript letter (a, b) differ significantly (P=0.05) when comparing the control with other groups. Values in the same column with different superscript letter (c, d) differ significantly (P=0.05) when comparing the group B_{TIP} with other groups. Values with different superscript letters (e, f) in the same column are significantly different (P=0.05) when comparing group C_{TIP} with other groups. *Male rats, ** female rats. No of female Rats/group = 4 Rats, No of male Rats/group = 4 Rats.

Table 2. Reproductive Hormonal Profile in Rats Acutely Treated with Tartrazine Orally

Parameters	*TESTO (ng/ml)	**PROG (ng/ml)	**E2 (ng/ml)
0.0g/kg (A_{TO})	5.75±0.21[a]	1.10±0.70[a]	11.95±2.62[a]
2.5g/kg (B_{TO})	3.25±0.07[b,c]	7.05±6.45[a,c]	37.25±18.6[a,c]
5.0g/kg (C_{TO})	3.35±0.35[b,c,e]	2.45±1.85[a,d,e]	14.85±3.75[a,d,e]
10.0g/kg (D_{TO})	3.10±0.71[b,c,e,g]	6.90±0.14[b,c,f,g]	48.95±0.35[b,c,e,f]
15.0g/kg (E_{TO})	1.85±0.21[b,d,f,h,i]	8.10±2.12[b,c,f,g,h]	29.25±1.20[b,c,e,g,h]

20.0g/kg (F_{TO})	2.40±0.28[b,c,e,h,i]	8.30±0.42[b,c,f,g,h]	28.60±0.99[b,c,e,g,h]
P value	0.0005	0.4101	0.020
F value	26.88	1.197	6.210
Remark	S	NS	S

Values in each column with different superscript letter (a, b) differ significantly (*P*=0.05) when comparing the control group (A_{TO}) with other groups. Values in each column with different superscript letter (c, d) differ significantly (*P*=0.05) when comparing the B_{TO} with other groups. Values in each column with different superscript letter (e, f) differ significantly (*P*=0.05) when comparing the C_{TO} with other groups. Values in the same column with same superscript letter (i) do not differ significantly (*P*=0.05) when comparing the groups E_{TO} and other. *Male rats **Female rats. No of female Rats/group = 4 Rats, No of male Rats/group = 4 Rats

3.2 Results on Reproductive Hormonal Profile in Rats Chronically Treated with Tartrazine Over a Period of 30 Days

The comparison of tartrazine male treated Rats and male control Rats showed no significant difference in Testosterone (TESTO) (Table 3). When tartrazine female rats and female control were considered, the comparison showed non-significant differences Progesterone (PROG) and Estradiol (E_2) at *P*=0.05 (table 3).

Table 3. Reproductive Hormonal Profile in Rats Chronically Treated with Tartrazine Over a Period of 30 Days

Parameters	Control Rats	Treated Rats	*P* value	T value	Remark
*TESTO (ng/ml)	4.24±2.21	3.94±1.99	0.6729	0.4259	NS
**PROG (ng/ml)	6.26±1.96	6.25±0.87	0.9950	0.0064	NS
**E2 (ng/ml)	33.30±11.84	37.97±11.07	0.7754	0.2872	NS

NS= Not Significant. No of control rats: Male=18, Female=22. No of treated rats: Male = 17, Female=22. *Male rats, **Female rats.

3.3 Results on Reproductive Hormonal Profile in Rats Chronically Treated with Tartrazine Over a Period of 60 Days

When tartrazine treated male rats were considered, no significant differences were seen in Testosterone (TESTO) in tartrazine treated male rats compared with control male rats at *P*=0.05 (table 4). In addition, when tartrazine treated female rats were considered, no significant differences were also seen in Progesterone (PROG) and Estradiol (E_2) of tartrazine treated female rats compared with control female rats at *P*=0.05 (table 4).

Table 4. Reproductive Hormonal Profile in Rats Chronically Treated with Tartrazine Over a Period of 60 Days

Parameters	Control Rats	Treated Rats	*P* value	T value	Remark
*TESTO (ng/ml)	2.21±1.31	1.92±1.16	0.4361	0.7662	NS
**PROG (ng/ml)	16.32±11.76	13.86±6.45	0.4693	0.7321	NS

**E2 (ng/ml)	61.89±25.29	58.95±23.19	0.7266	0.3526	NS

NS= Not Significant. No of control rats: Male=20, Female=20. No of treated rats: Male = 25, Female=15. *Male rats, **Female rats.

3.4 Results on Reproductive Hormonal Profile in Rats Chronically Treated with Tartrazine Over a Period of 90 Days

The comparison of tartrazine treated male rats and control male rats indicated no significant difference in Testosterone (TESTO) concentration of tartrazine treated male rats compared with the control male rats (table 5). When tartrazine treated female rats were considered, no significant differences were seen in Progesterone (PROG) and Estradiol (E_2) concentrations in tartrazine treated female rats compared with control female rats at *P*=0.05 (table 5).

Table 5. Reproductive Hormonal Profile in Rats Chronically Treated with Tartrazine Over a Period of 90 Days

Parameters	Control Rats	Treated Rats	*P* value	T value	Remark
*TESTO (ng/ml)	3.14±0.98	2.58±1.21	0.1373	1.522	NS
**PROG (ng/ml)	7.47±3.53	10.80±6.25	0.0516	2.009	NS
**E2 (ng/ml)	31.09±19.31	44.94±23.40	0.0514	2.012	NS

NS= Not Significant. No of control rats: Male=19, Female=18. No of treated rats: Male = 17, Female=22. *Male rats, **Female rats.

3.5 One-Way ANOVA on Reproductive Hormonal Profile in Rats Chronically Treated with Tartrazine over a Period of 30, 60 and 90 Days

Table 6 showed hormonal parameters over a period of 30, 60 and 90 days of tartrazine male treated rats. The ANOVA results obtained showed a significantly lower value in TESTO of tartrazine treated male rats from phase 1 to phase 3. When Turkey's multiple comparison test was used, significant decreases were seen between phase 1 and phase 2 as well as phase 1 and 3. However, no significant differences were seen between phase 2 and 3. More so, Table 6 also showed hormonal parameters of female rats treated with tartrazine over a period of 30, 60 and 90 days. The ANOVA results obtained indicated a significantly higher value in PROG from phase 1 to phase 3. When Turkey's multiple comparison test was used, significant increase was seen between phase 1 and phase 2 as well as phase 1 and 3. However, no significant differences were seen between phase 2 and 3 at *P*=0.05.

Table 6. Effect of Duration on Reproductive Hormonal Profile in Rats Treated with Tartrazine Over a Period of 30, 60 and 90 Days

Parameters	Phase 1 (Rats)	Phase 2 (Rats)	Phase 3 (Rats)	*P* value	F value	Remark
*TESTO (ng/ml)	3.94±1.99^{a}	1.92±1.16^{bc}	2.58±1.21^{bc}	0.0002	9.786	S
**PROG (ng/ml)	6.25±0.87^{a}	13.86±6.45^{b,c}	10.80±6.25^{b,c}	0.0005	8.712	S
**E2 (ng/ml)	37.97±11.07^{a}	58.95±23.19^{a,b}	44.94±23.4^{a,b}	0.2397	1.466	NS

Values in the same row with different superscript letter (a, b) differ significantly (*P=0.05*) when comparing phase 1 with other phases. Values in the same row with same superscript letter (c) do not differ significantly (*P=0.05*) when comparing phase 2 with phase 3. S = Significant. NS=Not Significant.

No of Male rats for Phase 1, 2 & 3 were:17, 25 & 17 respectively. No of Female rats for Phase 1, 2 & 3 were: 22, 15 & 22 respectively. *Male rats, **Female rats.

3.6 Histological Examination of Reproductive Organs

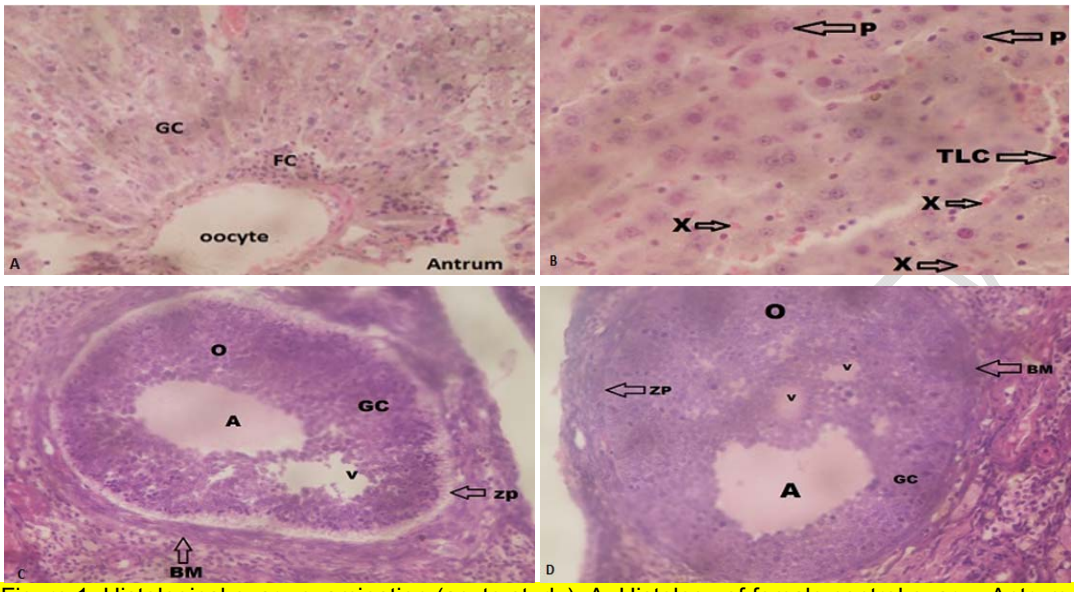

Figure 1: Histological ovary examination (acute study). A. Histology of female control ovary. Antrum of ovarian follicle surrounded by granulosa cells (GC). Oocyte is surrounded by follicular cells (FC). B. Dose: 2.5g/kg & 5.0g/kg (oral). P= primordial follicles, TLC= Lutein cells with yellowish colouration X. C. Dose: 15.0g/kg and 20.0g/kg (Oral) A=Antrum of the ovarian follicle surrounded by granulosa cells (GC) with mild vacuolation (V), O= Oocyte is surrounded GC. BM=Basement membrane is normal, ZP= Zona Pellucida appears normal. D. Dose: 1.67g/kg, 3.33g/kg (I.P). 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 stain. X400.

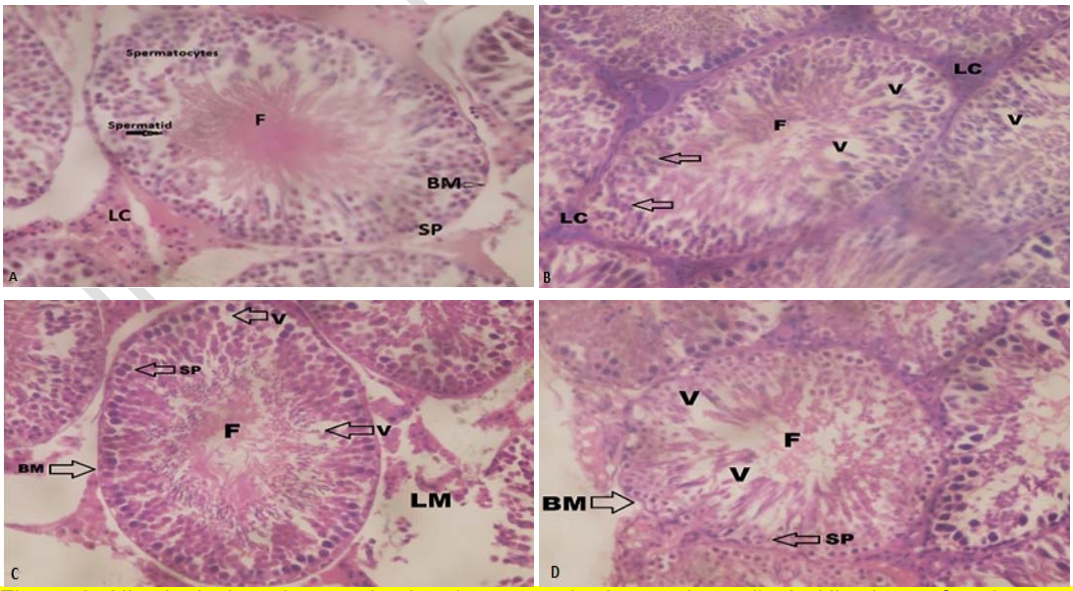

Figure 2: Histological testis examination (acute study: intraperitoneal). A. Histology of male control testis. F= Flagella of spermatogonia in lumen of seminiferous tubule, BM = Basement membrane, SP = Spermatogonia, LC = Leydig Cells. B. Dose: 3.33g/kg, F = Distorted flagella in lumen of seminiferous

tubule. Spermatocyte and spermatogonia (SP) layers indicate mild vacuolated (V) with clustered spermatids (Arrows). Normal basement membrane (BM) with leydig cells (LC). C. Dose: 5.0g/kg and 6.67g/kg. F = Distorted flagellated lumen. Vacuolation (V) of spermatid and spermatogonia portions (V), Basement membrane (BM) appears distorted with loss of interlobular materials (LM) and leydig cells due to vacuolation. D. Dose: 8.33g/kg, F = Distorted flagellated lumen. Spermatid, spermatocyte and spermatogonia (SP) layers have vacuolated portions (V), Basement membrane (BM) appears distorted with loss of leydig cells at interlobular junctions. H& E stain. X400.

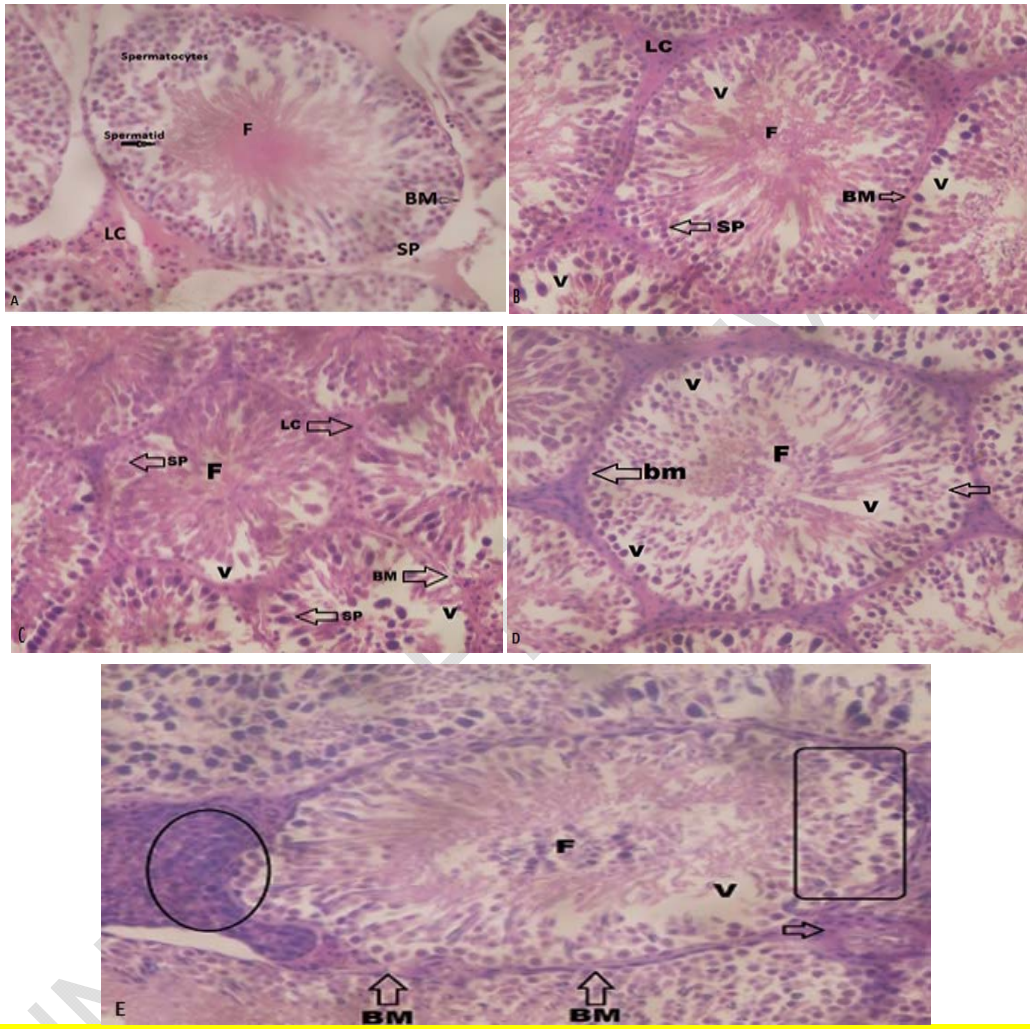

Figure 3: Histological testis examination (acute study: Oral). A. Histology of male control testis. F= Flagellated lumen of the testis, BM = Basement membrane, SP = Spermatogonia, LC = Leydig Cells. B. Dose: 2.5 and 5.0g/kg, F= Distorted flagellated lumen. Leydig Cells (LC) appears distorted at interlobular junctions. Spermatogonia layer (SP) appears distorted and vacuolated (V), Normal basement membrane (BM). C. Dose: 10.0g/kg, F= Distorted flagellated lumen. Vacuolation (V) of spermatid, spermatocyte and spermaatogonia (SP) Layers (V), distorted basement membrane (BM). D. Dose: 15.0g/Kg, F = Distorted flagellated lumen. The spermatid, spermatocyte & spermatogonia layer are filled with degenerative nuclear materials (pycnosis) and vacuolated portions (V), Basement Membrane (BM) appears normal. E. Dose: 20.0g/kg, F = Distorted flagella with nuclear materials in the lumen of seminiferous tubule. Vacuolation (V) and Spermatogonia layer filled with degenerative scattered nuclear materials (pycnosis) and vacuolated portions (Rectangular Shape). Basement membrane (BM) appears distorted at some points. Scanty and distorted Leydig Cells (Arrow). Clusters of degenerating nuclear materials (Circle Shape). H& E stain. X400.

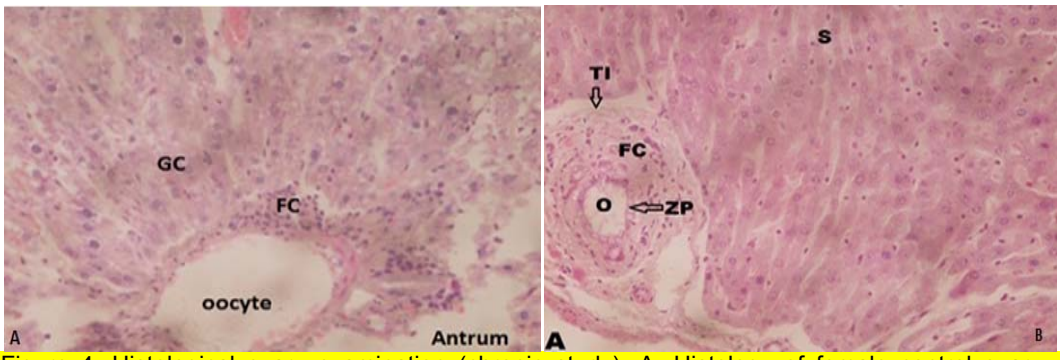

Figure 4: Histological ovary examination (chronic study). A. Histology of female control ovary. B. 7.5mg/kg for 90 Days. A=Antrum of the ovarian follicle surrounded by granulosa cells (GC), Oocyte (O) surrounded by zona pellucida (ZP) and follicular cell (FC) which appear unorganised, TI= Theca Interna (normal), S=Stroma well-organised with numerous primordial cells.

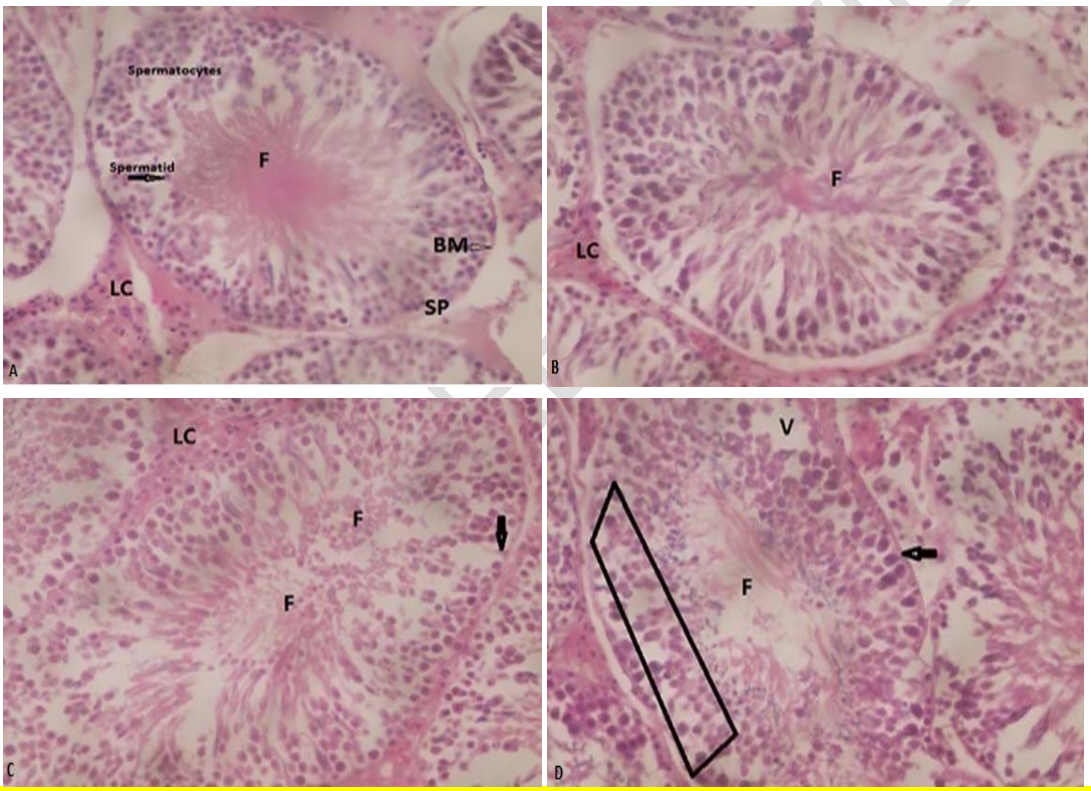

Figure 5: Histological testis examination (chronic study). A. Histology of male control testis. F= Flagella of spermatogonia in lumen of seminiferous tubule. BM = Basement Membrane, SP = Spermatogonia, LC = Leydig Cells. B. 7.5mg/kg for 30 Days. F = Flagella in lumen of seminiferous tubule, LC= Leydig cells (Normal). Maturing spermatocytes migrating towards basement membrane (Intact). C. 7.5mg/kg for 60 Days. LC= Leydig cells appears normal, F = Flagellated lumen distorted, portions of clustered spermatids and spermatocytes with vacuolation, basement membrane appear normal (Arrow) with sertoli cell attached. D. 7.5mg/kg for 90 Days. F = Mildly Distorted Flagellated Lumen. Spermatogonia layer is filled with degenerative scattered nuclear materials (Pycnosis) and some vacuolated portions (Rectangular Shape), Basement membrane (Arrow) is normal. H& E stain. X400.

4. DISCUSSION

When hormonal or reproductive hormones were considered in acute toxicity study at high doses, male rats treated (intraperitoneally and orally) indicated significantly lower values in testosterone (TESTO) concentration compared with control rats while female rats treated (intraperitoneally and orally) showed significantly higher values in Progesterone (PROG) and Estradiol (E_2) concentrations. The significantly lower value in TESTO observed in tartrazine treated rats supports several findings [9, 20, 23, 27]. Mehedi et al. [9], reported that 2.5% of tartrazine administered orally for 13 weeks in male rats induced decreased sperm count, sperm abnormalities viz-a-viz reduction in testosterone concentration compared to control rats. Gautem et al. [20], also reported reduction in sperm density, motility and presence of varying degree of abnormalities in the spermatozoa of rats treated with 0.2g/kg and 0.4g/kg bodyweight of tartrazine for 30 days. More so, Sharma et al. [23], reported low levels of steroid hormones including testosterone in rats treated with Kerisi powder (a dye mixture of tartrazine and sunset yellow 6). Khiralla et al. [27], reported that high dose (5 times ADI) of synthetic yellow dye (tartrazine) led to the reduction in testosterone concentration as well as LH concentration. In a related study, Dixit & Goyal [11], reported that the use of an azo dye; indigo Carmine at a dose 39mg/kg bodyweight for 6 weeks induced significant decrease in the concentration of testosterone and the weight of the testes as a result vacuolation of spermatogonia of the testis, flagella distortion of the seminiferous lumen, nuclear degeneration (pycnosis), distortion of basement membrane, distortion and loss of leydig cells in indigo Carmine treated male rats. Similarly, Ali et al. [28] reported that administration of 200mg/kg of tartrazine administered to rats induced decreased superoxide dismutase (SOD) and reduced glutathione (GSH) and increased malondialdehyde (MDA) suggesting oxidative stress induced by tartrazine dyes at high dose. More so, Bousssada et al. [29], reported that sub-chronic treatment of tartrazine (E102) for 30 days at 300mg/kg bodyweight induced altered sperm characteristics and quality accompanied with significantly lowered testosterone concentrations and increased MDA levels in the testicular tissue of tartrazine treated rats. However, our finding when tartrazine was administered at high doses contradicts the reports of [12]. Tanaka [12], reported that the administration of synthetic food dyes such as tartrazine at a high dose of 773mg/kg bodyweight in rats did not induce deleterious effect on reproductive hormonal parameters. The reduction in testosterone concentration observed in our study, could be as a result of disruption of the hypothalamus-pituitary-testes axis regulating testosterone production by the Leydig cells of the testes. The disruption might have resulted from the oxidative insults on the testes arising from azo dye metabolism which might have led to distortion or loss of spermatogenic precursors (spermatogonia) owing to pathologic alteration of the leydig (testosterone production) and sertoli cells (FSH and LH production) architecture. Our present findings further support the reports of [28, 29]. Ali et al., [28], reported that administration of 200mg/kg of tartrazine in rats induced decreased superoxide dismutase (SOD) and reduced glutathione (GSH) and increased malondialdehyde (MDA) suggesting oxidative stress induced by tartrazine dyes at high dose. More so, Boussada et al. [29], also reported increased MDA levels in the testicular tissue of tartrazine treated rats indicating increased oxidative stress in the testes of rats treated with 300mg/kg of tartrazine.

The significantly higher values seen in E_2 in our study when tartrazine was given in high doses collaborates with the report of [30, 31] but contradicts the findings of [23]. Akinloye et al.[30], reported in their work that azo dyes induced increase in E_2 when azo dyes were fed to rats. Zahra et al. [31], reported that the use of tartrazine and other food dyes such as sunset yellow 6 mimic estrogen in the body and thus stimulates increase in E_2 concentration and a reduction in testosterone concentration thereby affecting libido in men. However, Sharma et al. [23], reported reduced E_2 in rats treated with Kerisi powder (a dye mixture of tartrazine and sunset yellow 6). The increase observed in E_2 in the acute study could be due to xenoestrogenic attributes of tartrazine which is implicated in hormonal imbalance. More

so, the significant increase seen in PROG level in the acute treatment (intraperitoneal) also contradicts the finding of [23]. Sharma et al. [23], also reported reduced PROG in rats treated with Kerisi powder (a dye mixture of tartrazine and sunset yellow 6) as a result of apoptosis of ovarian cells being induced by the synthetic dye.

The histopathologic findings in testicular tissue in the acute study showed flagella distortion in the seminiferous lumen, nuclear degeneration (pycnosis), distortion of basement membrane and loss of leydig cells as well as vacuolation of spermatogonia layer (figure 2 and 3). More so, unlike Sharma et al. [23], who reported severe vacuolation of ovarian cells as result of cellular apoptosis when rats were treated with Kerisi powder (a dye mixture of tartrazine and sunset yellow 6), our finding showed mild vacuolation of the granulosa cells region (figure 1). The histologic changes seen in the testicular tissues (figure 2 and 3) suggest loss of spermatogenic precursors (spermatogonia) as well as altered leydig and sertoli cells functions. Our finding is in line with the records of [11]. Dixit & Goyal, [11], documented vacuolation of spermatogonia of the testis, flagella distortion of the seminiferous lumen, pycnosis and distortion of basement membrane when indigo Carmine at a dose 39mg/kg bodyweight was administered in male rats for 6 weeks. Our histopathologic findings support the significantly lowered testosterone concentration in tartrazine treated male rats observed in the acute study.

Furthermore, when chronic treatments were considered, hormonal parameters after 30 days, 60 days and 90 days chronic treatment showed non-significant reductions in testosterone (TESTO) concentration in the tartrazine treated male rats compared with their respective control male rats. The non-significant difference seen in testosterone when given ADI doses compared with control group is in line with the findings of [12, 13, 14]. Tanaka [12], Elhkim et al. [13], EFSA [14], reported in their separate work that tartrazine did not induce any deleterious effect on reproductive hormonal parameters in rats when given at ADI doses. More so, Gil [32], also reported that synthetic dye such as tartrazine in a concentration of 1mM in a cell culture medium did not induce a decrease in testosterone concentration compared to vehicle treated control. However, oxidative stress in the adrenal cortex affecting steroid hormones production when exposed to high concentration of azo dyes was reported. The non-significant decreases seen in testosterone concentration in the chronically treated rats compared with the control rats over a period of 30, 60 and 90 days could be as a result of the testes or the body system not been overwhelmed by the vehement effects of reactive oxygen species (ROS) produced by the tartrazine during metabolism.

In addition, Progesterone (PROG) and Estradiol (E_2) concentrations after 30, 60 and 90 days chronic treatment showed non-significant increases in tartrazine treated female rats compared with their respective control female rats. The non-significant differences observed in PROG and E_2 concentration concurs with the findings of [12, 13, 14] but contrast the reports of [23, 30, 31], when the effects of azo dyes on reproductive parameters were evaluated. Tanaka [12], Elhkim et al. [13], EFSA [14], recorded in their separate work that ADI doses of tartrazine did not cause harmful effect on reproductive hormonal parameters. However, Sharma, [23], reported a fall in E_2 and PROG levels due to vacuolation or apoptosis of ovarian tissues when tartrazine mixed with sunset yellow 6 were fed to rats. In addition, Akinloye et al. [30] and Zehra et al. [31], reported in their separate studies that xenoestrogenic activities of tartrazine azo food dyes induced low levels of progesterone in plasma. The non-significant differences observed in E_2 and PROG in the chronic treatment could be as a result of complete removal of reactive oxygen species by the body anti-oxidative mechanism when these dyes are administered at ADI doses. It is quite possible that the derangements caused by synthetic food dyes are mainly due overwhelming tendencies of the anti-oxidative capacity of the body system when these days are consumed in high doses.

Finally, when the comparative analyses of hormonal parameters of tartrazine treated rats were considered using One-Way ANOVA over the period of 30, 60 and 90 days, TESTO concentration indicated significantly lower levels in tartrazine treated male rats. Significant decreases were seen between 30 days and 60 days as well as between 30 days and 90 days. However, no significant difference was seen between 60 days and 90 days. When treated female were considered, PROG showed significantly higher values over 30, 60 and 90 days in tartrazine treated female rats. Significant increase was seen between 30 days and 60 days as well as between 30 days and 90 days. However, no significant differences were seen between 60 days and 90 days.

The significantly lower value seen in TESTO over the period of 30, 60 and 90 days suggest gradual fall in the TESTO level over time and possibly alteration in membrane structure of parenchymal cells of the leydig and sertoli cells (of the testis) disrupting the hypothalamic-pituitary-testes axis. In a related study, Helal et al. [33], reported that administration of food additives such as sodium nitrate and monosodium glutamate at recommended dose induced reduction in testosterone concentration. Also, Mahmoud et al. [34], reported reduction in spermatogenesis when azo dye brilliant black was given to male rats orally at a dose of 0.08g/kg and 0.4g/kg over a period of 30 days. In addition, the significantly higher levels of PROG seen in our study is contrary to the reports of [23, 32].Sharma et al. [23], reported severe degeneration of corpus luteum of the ovaries in tartrazine treated female rats which was attributed to reduction in plasma PROG level. More so, Gil [32], also reported no significant difference in E_2 when tartrazine treated cells were compared with vehicle treated control in a cell culture medium at a concentration of 1mM. The significant increase seen in PROG could be related to hormonal imbalance induced by distortion of the follicular cells, theca interna and externa (of the ovaries) due to persistent oxidative stress induced by azo dyes. However, no significant increase was seen E_2 concentration over 30, 60 and 90 days. Our finding collaborates with the report of [12, 21] but contrary to the reports of [23, 30]. Tanaka [12], reported that tartrazine at a dose of 773mg/kg did not affect reproductive hormonal parameters when tested in rats. More so, Meyer et al. [21], recorded that administration of 0.5mg/kg and 50mg/kg of tartrazine did not induce significant change in the weight of the ovaries viz-a-viz estradiol concentration. However, Akinloye et al. [30], reported in their work that azo dyes such as tartrazine possesses xenoestrogenic attributes and are therefore stimulates increase in E_2 which is implicated in hormonal imbalance. Sharma *et al*, [23], further reported reduced E_2 levels in rats treated with tartrazine which they attributed to apoptosis of ovarian cells. The non-significant difference observed in E_2 over the period of 30, 60 and 90 days in the treated female rats could be as a result of intact membrane structure of parenchymal (follicular and luteal) cells of the ovaries maintaining the hypothalamic-pituitary-ovaries axis as well as poor xenoestrogenic activities of tartrazine on the ovarian cells associated with the ADI doses given over time.

Finally, the histologic examination of ovarian and testicular tissues in the chronic study did not indicate any obvious alteration especially after 30 and 60 days of treatment. However, mild histopathologic changes were very obvious after 90 days of treatment in the testis and ovary (figure 4 and 5). Though, there were mildly distorted flagella in the lumen of seminiferous tubules, nuclear clusters and spermatogonia layer filled with degenerative scattered nuclear materials and some vacuolated portions but the basement membrane appeared undisturbed (figure 5). More so, the histopathologic examination of the ovaries showed unorganised follicular cells, normal theca interna and corpus luteum (figure 4). The non-significant differences seen between the treated and control rats in the chronic study could be as a result of the intact structural arrangement of the testes and ovaries over the period of 30, and 60 days especially. However, the mild alterations in the testis and ovary probably explained the gradual fall in testosterone and the rise in PROG when the treated rats over a period of 30, 60 and 90 days were compared using ANOVA.

5. CONCLUSION

In the acute toxicity study, reduction in TESTO concentration as well as increase in PROG and E_2 was seen which suggest possible disturbance in the fertility profile or capacity when these dyes are consumed in high doses. Histopathologic alterations such as flagella distortion in the seminiferous lumen, pycnosis, distortion of basement membrane and loss of leydig cells as well as vacuolation of spermatogonia layer were seen in testicular tissues. More so, mild vacuolation of ovarian cells were also seen in the acute study. However, in chronic study, significant differences were not seen in TESTO concentration as well as increase in PROG and E_2 was seen which suggest possible disturbance in the fertility profile or capacity when these dyes are not consumed in high doses. Finally, when the influence of duration of exposure at ADI doses were considered over 30, 60 and 90 days, gradual reduction in TESTO concentration and increase in PROG concentration was seen. Mild histopathologic alterations such as flagella distortion, pycnosis and vacuolations were seen in testicular tissues especially after 90 days of chronic treatment likewise mild vacuolation of ovarian cells was also seen in the chronic study. This implies that there could be possibility of hormonal derangements when food dyes are consumed even at ADI doses on daily basis over prolonged period.

6. RECOMMENDATION

It is therefore recommended that high doses of tartrazine in foods or food products should be avoided. Also, the ADI dose of tartrazine should be reconsidered by international and national agencies on reducing the ADI dosage over a life time. More so, marketers/consumers should be sensitized/educated/re-educated on the use of food dyes and finally, governmental policies/consumer protection agency should regulate and ensure appropriate labelling of food dyes and food products with additives.

LIMITATION OF THE STUDY

The limitation of this study is that the status of enzymes related to synthesis of steroid reproductive hormones were not investigated. Therefore, our findings are subject further research and validation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

CONSENT

Not applicable

ETHICAL APPROVAL

We hereby declare that Principles of laboratory animal care (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the Rivers State University research/ethics committee.

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ABBREVIATIONS

TESTO	=	TESTOSTERONE
PROG	=	PROGESTERONE
E_2	=	ESTRADIOL
ADI	=	ACCEPTABLE DAILY INTAKE
PHASE 1	=	30 DAYS
PHASE 2	=	60 DAYS
PHASE 3	=	90 DAYS