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Assessment of Nephro-, Hepato-, and Sex-Dependent Toxicity of Carmoisine Exposure in Albino Rats

Ibioku Elekima[1,2*], Uchechukwu Achor Obisike[1], Holy Brown[1], Helen Anthony Waribo[1], Ngozi Brisibe[1], Ibitoroko George-Opuda[1], Adline Ben-Chioma[1], Donatus Onwuli[1]

[1]Chemical Pathology Unit, Department of Medical Laboratory Science, Rivers State University, Port Harcourt, Nigeria
[2]Medical Diagnostics, Cranfield School of Health, Cranfield University, Bedford, UK
Email: *ibioku.elekima@ust.edu.ng

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Abstract

Aim: To evaluate chronic exposure of carmoisine at ADI doses on some hepatocellular and renal parameters of male and female albino rats as well as to determine sex-dependent toxicity. **Study Design:** The study involves treatment for 30, 60, and 90 days. Each phase consists of 40 rats, divided into treatment and control groups. The treated groups were orally administered with 4.0 mg/kg of carmoisine daily for the periods of 30, 60, and 90 days. **Methodology:** At the end of the treatment, the rats were allowed to fast for 18 hours followed by the collection of 5 ml of whole blood specimens by means of cardiac puncture into Lithium Heparin bottles and fluoride oxalate bottles (for glucose only). Plasma obtained was analyzed for glucose (GLU), AST, ALT, ALP, creatinine (CRT), and urea. Hepatic and Renal tissues collected were fixed in 10% formol saline and later examined histologically using H&E stain. Statistical data analysis was done using GraphPad Prism version 9.02. **Results:** Glucose indicated significant increases after 30, 60, and 90 days of chronic treatment at ADI doses. Urea, Creatinine, AST, ALT and ALP showed significantly higher values after 60 and 90 days of treatment (except creatinine in male rats and ALP in female rats after 60 and 90 days respectively). Hepatic distortions, vacuolation, compression of central vein were seen in the liver section while distortion of proximal and distal tubules, and inflammation of the glomerulus were observed in the renal tissue of the treated rats. **Conclusion:** The administration of camoisine over a period of 30 days at ADI dose did not indicate hepatocellullar and renal derangements as well histological distortions in liver, and kidneys. However, after 60 and 90 days, mild hepatocellular, and renal derangements were seen. No sex-dependent toxicity was observed.

Keywords

Carmoisine, Toxicity, Liver Enzymes, Renal Tissue, Sex-Dependent, Azo Dye, Creatinine, Urea

1. Introduction

The use of colours for the beautification of edible food and food products has played a significant role in consumers' satisfaction and acceptance [1] [2]. Food dyes have been known to maintain or improve on the colour of the foods or food products by covalently binding to the food particles [3] [4]. Carmoisine, tartrazine, erythrosine, fast green, and so on are synthetic azo dyes derived mainly from benzene [5]. The use of azo dyes is also seen in textile, leather, paper, rubber, cosmetics and pharmaceutical industries [6]. Carmoisine is a nitrous derivative synthetic dye belonging to the azo class of food dyes that gives food products a reddish appearance with an ADI of 0 - 4.0 mg/kg [7]. Carmoisine is also reduced through the biotransformation process in the liver and by the actions of intestinal microorganism to aromatic amines, aryl amines and free radicals [7] [8]. It is also known as Azorubine, food red 3, azorubine S, Brilliant carmoisine 0, acid red 14 or C.I. 14720 [7]. Structurally, a molecule of carmoisine 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 consist sodium sulphate and nitrogen as shown in **Figure 1**. It is seen in food products such as jams, swiss roll, yoghurts, breadcrumbs, cheesecake mixes, jellies, etc. It is soluble in water and tends to react with food particles covalently [2] [7].

The Acceptable Daily Intake (ADI) of carmoisine is 0 - 4.0 mg/kg [5] [7]. The Food and Agricultural Organization (FAO) and World Health Organization (WHO), have put in place laws and regulations towards the use of food dyes especially synthetic ones [8] [9]. Reviews of literature reveals that carmoisine as a synthetic food dyes originate from coal tar which is toxic and carcinogenic [9] [10]. The toxicity of carmoisine is connected to the reductive biotransformation of the azo bond in the intestine and hepatocellular tissue releasing reactive amines, aryl amines and other free radicals [10]. However, the degree of toxicity

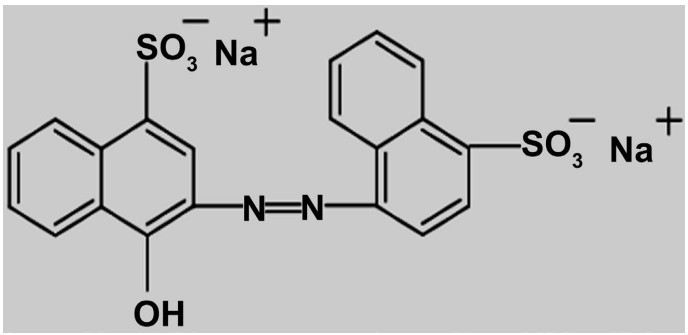

Figure 1. Structure of carmoisine dye [8].

of carmoisine is also associated with the dose, duration of exposure, age, sex, and body weight as well as interaction with other product [11]. Therefore, the purpose of this study is to assess the chronic effect of carmoisine dye on glucose, the hepatocellular and renal integrity, and sex-dependent toxicity at recommended ADI doses.

The liver is involved in the detoxification of harmful substances ingested into the body and the detoxification process may produce reactive intermediate metabolites that can attack macromolecules leading to direct insults and hypersensitivity [12]. Hepatocellular damages and alteration of the liver architecture occur when the mechanism of conjugation by glutathione is saturated or where the rate of toxic metabolites produced exceeds the bioavailability of glutathione [12]. Liver enzymes such as alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are sensitive markers of hepatocellular disturbances. ALT and AST play important function in metabolism of amino acid precisely, in their synthesis and degradation in a reversible reaction called transamination [7] [12]. AST catalysis the transfer of amino group from glutamate to oxoglutarate to from oxaloacetate and aspartate while ALT catalyses the transfer of amino acid from glutamate to oxoglutarate to form pyruvate and alanine [13]. ALP is a hydrolase enzyme that catalyses the release of inorganic phosphates from phosphateester substrates and it is present in all body tissues mostly in bones, liver, placenta, erythrocytes and renal tubules [13]. In a study carried out by Hussain *et al.* [13], they stated that carmoisine orally administered in rats caused a significant increase in hepatic AST, ALT, and ALP in the plasma when rats were fed with 30 mg/kg and 300 mg/kg for 12 weeks. The kidneys play crucial roles in maintaining of body homeostasis, fluid and electrolyte balance, buffering, removal of metabolic wastes, as well as the synthesis of erythropoeitic hormones [14]. Creatinine and urea are markers routinely used in evaluating renal function [14]. Amin *et al.* [7] revealed in their studies that carmoisine when fed to rats induced increased level of serum creatinine and urea. Increase in plasma creatinine and urea are indicators of renal disturbances [14]. The use of urea alone to assess renal integrity is not sufficient due to several factors such as high protein diet, hydration, intestinal obstruction, and so on affecting their plasma level [15]. Glucose is the simplest form of carbohydrate that acts as a major source of energy to cells and tissues through the Kreb's cycle [16]. Maintenance of plasma glucose concentration within a relatively narrow interval is essential to avoid metabolic disorders such as hyperglycaemia or hypoglycaemia [16]. As reported by Amin *et al.* [7], a significant increase in glucose concentration was observed when carmoisine was administered at low and high doses in male albino rats for 30 days.

2. Materials and Methods

2.1. Materials

Materials used in this study include, Polypropylene gavage tubes (Intech Labor-

atory Incorporated, Plymouth Meeting, USA), Haier thermocool refrigerator (China), MPW bucket centrifuge Model 351 (MPW Medical Instruments, Warsaw, Poland), Ohaus Scout-Pro Electronic weigh balance (Ohaus Corporation, New Jersey, USA), Albino rats, Vis spectrophotometer (Axiom Medical Limited, United Kingdom), Carmoisine dyes (E122) with serial no of FI18784 (Colori Spa, Gessete, Italy), with purity of 86.7%. Glucose, Urea, Creatinine, ALT, ALP and AST kits were purchased from Atlas Medicals (Cowley Road, Cambridge, United Kingdom) except ALP reagent that was purchased from Teco Diagnostics. Other materials used include automatic pipettes and glass test tubes.

2.2. Experimental Animals

Male and female albino rats used for the study weighed 150 gm approximately. 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.

2.3. Preparation of Carmoisine Food Dye

In the chronic study, 0.60 grams of carmoisine was weighed and dissolved in 1.0 litre of distilled water. This means that, 1.0 ml of the carmoisine solution contains 0.006 grams, which is equivalent to 4.0 mg/kg when given to 0.15 kg rat.

2.4. Experimental Design and Administration of Food Dyes

The method of treatment involved oral techniques. In the oral method, the food dyes were administered using orogastric tube to ensure complete delivery of carmoisine. The experiment was divided into phase 1, 2 and 3 which had duration of 30, 60 and 90 days respectively. Forty (40) experimental rats were used in each phase of the study. In each phase of the experiment, the rats were divided into two groups designated CT (Carmoisine 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 CT1, CT2…C10. In the treatment, 4.0 mg/kg of carmoisine was administered orally. The control group, were not treated with carmoisine. At the end of each phase, the animals were anaesthetized with chloroform before blood samples were collected by means of cardiac puncture for biochemical investigations while kidney and liver organs were harvested for histologic examination.

2.5. Specimen Collection, Preparation, and Laboratory Analysis

Blood specimens were collected by means of cardiac puncture after the rats were anaesthetized into lithium heparin bottle for all biochemical parameters except glucose sample that was collected into fluoride oxalate bottle. The blood specimens were spun at 4500 rpm for 10 minutes to obtain plasma which was transferred into other sets of labeled plain bottles and stored at −4˚C. The laboratory analysis of ALP as well as ALT and AST were determined using spectrophotometer as described by Kind and King [17] and Reitman and Frankel [18] respec-

tively. Plasma Urea was estimated using Berthelot's enzymatic method as described by Patton and Crounch [19] while creatinine was determined by kinetic colorimetric method as described by Vaishya *et al.* [20]. Plasma glucose was estimated using oxidase enzymatic method as described by Trinder [21].

2.6. Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 8.02 (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 tests) was also used to test treatment duration on toxicity. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Biochemical Parameters of Rats Chronically Treated with Carmoisine over a Period of 30 Days

The result indicated significant higher values of glucose in the male (Table 1(a)) and female rats (Table 1(b)) after 30 days treatment. Other parameters considered did not indicate any significant difference at $P < 0.05$. The comparison between male and female values also indicated no significant difference (Table 1(c)).

3.2. Biochemical Parameters of Rats Chronically Treated with Carmoisine over a Period of 60 Days Units

Results of the 60 days treatment in the male rats indicated significantly higher values in glucose, Urea, AST, ALT, and ALP except in creatinine (Table 2(a)). In similar manner significantly higher values were observed in glucose, Urea, AST, ALT, and creatinine except ALP in the female rats (Table 2(b)). Finally, when treated male and female treated rats were compared, no significant differences were seen in the biochemical parameters considered at $P < 0.05$ (Table 2(c)).

Table 1. (a) Results of male rats treated with carmoisine for 30 days type styles; (b) Results of female rats treated with carmoisine for 30 days; (c) Results of male and female rats treated with carmoisine for 30 days.

(a)

Parameters	Control Rats (Males) n = 18	Treated Rats (Males) n = 16	P value	T value	Remark
GLU (mmol/l)	2.18 ± 0.90	3.91 ± 2.27	0.0057	2.969	S
UREA (mmol/l)	4.09 ± 0.23	4.24 ± 0.35	0.1393	1.518	NS
CRT (μmol/l)	145.6 ± 45.76	176.8 ± 62.74	0.1052	1.667	NS
AST (U/L)	50.33 ± 32.51	54.56 ± 27.19	0.6857	0.408	NS
ALT (U/L)	25.41 ± 13.43	24.96 ± 8.84	0.9104	0.113	NS
ALP (U/L)	27.36 ± 14.92	25.81 ± 17.13	0.7795	0.282	NS

(b)

Parameters	Control Rats (Females) n = 22	Treated Rats (Females) n = 22	P value	T value	Remark
GLU (mmol/l)	2.11 ± 0.85	3.71 ± 1.75	0.0004	3.848	S
UREA (mmol/l)	4.12 ± 0.25	4.24 ± 0.21	0.0727	1.841	NS
CRT (µmol/l)	159.4 ± 38.21	168.8 ± 52.38	0.5014	0.678	NS
AST (U/L)	47.19 ± 32.88	60.50 ± 22.07	0.1224	1.576	NS
ALT (U/L)	20.24 ± 14.31	27.25 ± 11.05	0.0763	1.818	NS
ALP (U/L)	23.79 ± 14.84	27.95 ± 18.21	0.4111	0.830	NS

(c)

Parameters	Treated Rats (Males) n = 16	Treated Rats (Females) n = 22	P value	T value
GLU (mmol/l)	3.91 ± 2.27	4.40 ± 2.62	0.7512	0.319
UREA (mmol/l)	4.24 ± 0.35	4.24 ± 0.21	0.9534	0.058
CRT (µmol/l)	176.8 ± 62.74	168.8 ± 52.38	0.6700	0.429
AST (U/L)	54.56 ± 27.19	60.50 ± 22.07	0.4625	0.742
ALT (U/L)	24.96 ± 8.84	27.25 ± 11.05	0.4994	0.682
ALP (U/L)	25.81 ± 17.13	27.95 ± 18.21	0.7163	0.366

n = no of Rats, NS = Not Significant, S = Significant.

Table 2. (a) Results of male rats treated with carmoisine for 60 days; (b) Results of female rats treated with carmoisine for 60 days; (c) Results of male and female rats treated with carmoisine over a period of 90 days.

(a)

Parameters	Control Rats (Males) n = 20	Treated Rats (Males) n = 15	P value	T value	Remark
GLU (mmol/l)	2.62 ± 0.77	4.69 ± 1.59	<0.0001	5.305	S
UREA (mmol/l)	4.0 ± 0.41	4.88 ± 0.69	<0.0001	4.586	S
CRT (µmol/l)	174.6 ± 152.1	218.0 ± 88.33	0.3318	0.985	NS
AST (U/L)	33.22 ± 19.47	50.67 ± 23.73	0.0227	2.390	S
ALT (U/L)	17.26 ± 5.78	31.87 ± 10.9	<0.0001	5.125	S
ALP (U/L)	22.48 ± 7.71	28.53 ± 9.08	0.0407	2.131	S

(b)

Parameters	Control Rats (Females) n = 20	Treated Rats (Females) n = 24	P value	T value	Remark
GLU (mmol/l)	3.06 ± 1.66	4.01 ± 1.08	0.0270	2.291	S
UREA (mmol/l)	4.25 ± 0.42	4.84 ± 0.54	0.0002	4.007	S

Continued

CRT (μmol/l)	126.0 ± 61.30	173.1 ± 63.45	0.0167	2.492	S
AST (U/L)	38.9 ± 12.98	65.67 ± 28.17	0.0003	3.912	S
ALT (U/L)	19.22 ± 7.86	30.76 ± 12.17	0.0007	3.655	S
ALP (U/L)	25.75 ± 12.91	26.27 ± 7.84	0.8710	0.163	NS

(c)

Parameters	Treated Rats (Males) n = 15	Treated Rats (Females) n = 24	P value	T value	Remark
GLU (mmol/l)	4.69 ± 1.59	4.01 ± 1.08	0.1145	1.617	NS
UREA (mmol/l)	4.88 ± 0.69	4.84 ± 0.54	0.8385	0.205	NS
CRT (μmol/l)	218.0 ± 88.33	173.1 ± 63.45	0.0728	1.847	NS
AST (U/L)	50.67 ± 23.73	65.67 ± 28.17	0.0948	1.715	NS
ALT (U/L)	31.87 ± 10.9	30.76 ± 12.17	0.7746	0.288	NS
ALP (U/L)	28.53 ± 9.08	26.27 ± 7.84	0.4136	0.826	NS

n = no of Rats, NS = Not Significant, S = Significant.

3.3. Biochemical Parameters of Rats Chronically Treated with Carmoisine over a Period of 90 Days

When carmoisine treated male rats were considered, all the parameters indicated significantly higher values in the treated rats compared to the controls except in the female rats were ALP indicated no significant difference at $P < 0.05$ (Table 3(a), Table 3(b)). The comparison between male and female rats all indicated no significant differences at $P < 0.05$ (Table 3(c)).

3.4. Biochemical Parameters of Duration on Chronically Treated Rats with Carmoisine over a Period of 30, 60 and 90 Days

Table 4(a) shows the biochemical parameters for 30 days (phase 1), 60 days (phase 2) and 90 days (phase 3) of carmoisine treated male rats. The ANOVA results obtained indicated significantly higher values in GLU (Glucose), UREA and ALT of carmoisine treated rats over 30, 60 and 90 days at $P < 0.05$. Glucose showed a significantly higher value between 30, 60 and 90 days. More so, significantly higher values were also seen between 60 and 90 days. Urea and ALT also showed a significantly higher value from 69 and 90 days compared to 30 days treatment.

Table 4(b) showed biochemical parameters for 30 (phase 1), 60 (phase 2) and 90 days (phase 3) carmoisine treated female rats. The ANOVA results obtained indicated significantly higher values in UREA and ALT as the duration was prolonged at $P < 0.05$. UREA and ALT showed significantly highest values in 90 days treatment compared to 30 and 60 days. More so, significantly higher values were seen in 60 days treatment compared to 30 days treatment (Table 4(b)).

Table 3. (a) Results of male rats treated with carmoisine for 90 days; (b) Results of male rats treated with carmoisine for 90 days; (c) Results of male and female rats treated with carmoisine over a period of 90 days.

(a)

Parameters	Control Rats (Males) n = 19	Treated Rats (Males) n = 18	P value	T value	Remark
GLU (mmol/l)	2.94 ± 1.39	5.02 ± 2.57	0.0039	3.095	S
UREA (mmol/l)	4.0 ± 0.51	5.10 ± 0.83	<0.0001	4.889	S
CRT (µmol/l)	137.2 ± 100.9	220.5 ± 67.03	0.0058	2.940	S
AST (U/L	32.0 ± 15.03	63.02 ± 26.04	<0.0001	4.467	S
ALT (U/L)	16.58 ± 6.25	31.98 ± 15.59	0.0003	3.982	S
ALP (U/L)	21.04 ± 8.48	34.16 ± 12.41	0.0006	3.771	S

(b)

Parameters	Control Rats (Females) n = 20	Treated Rats (Females) n = 24	P value	T value	Remark
GLU (mmol/l)	2.94 ± 1.66	4.21 ± 1.56	0.0236	2.370	S
UREA (mmol/l)	4.24 ± 0.35	5.15 ± 0.92	0.0004	3.932	S
CRT (µmol/l)	140.0 ± 62.97	188.6 ± 71.76	0.0379	2.161	S
AST (U/L	36.17 ± 13.13	64.88 ± 21.47	<0.0001	4.840	S
ALT (U/L)	19.35 ± 7.79	39.11 ± 19.97	0.0004	3.911	S
ALP (U/L)	24.76 ± 12.19	27.77 ± 13.44	0.4862	0.704	NS

(c)

Parameters	Treated Rats (Males) n = 15	Treated Rats (Females) n = 24	P value	T value	Remark
GLU (mmol/l)	5.02 ± 2.57	4.21 ± 1.56	0.2578	1.151	NS
UREA (mmol/l)	5.10 ± 0.83	5.15 ± 0.92	0.8649	0.171	NS
CRT (µmol/l)	220.5 ± 67.03	188.6 ± 71.76	0.1767	1.380	NS
AST (U/L	63.02 ± 26.04	64.88 ± 21.47	0.8164	0.234	NS
ALT (U/L)	31.98 ± 15.59	39.11 ± 19.97	0.2405	1.195	NS
ALP (U/L)	34.16 ± 12.41	27.77 ± 13.44	0.1474	1.483	NS

n = no of Rats, NS = Not Significant, S = Significant.

3.5. Weight of Organs Extracted from Rats Chronically Treated with Carmoisine over a Period of 30, 60 and 90 Days

Table 5(a) and Table 5(b) show the results of the weights of organs (in grams) in male and female rats respectively. The results obtained indicated no significant difference in both sexes in the 30, 60, and 90 days treatment when treated rats were compared to their respective control at $P < 0.05$.

Table 4. (a) Results of varying duration on carmoisine treated male rats over a period of 30, 60 and 90 days; (b) Results of varying duration on carmoisine treated female rats over a period of 30, 60 and 90 days.

(a)

Parameters	Phase 1 (Male) n = 16	Phase 2 (Male) n = 15	Phase 3 (Male) n = 18	P value	F value	Remark
GLU (mmol/l)	3.91 ± 2.27[a]	4.69 ± 1.59[b]	5.02 ± 2.57[c]	0.0209	4.206	S
UREA (mmol/l)	4.24 ± 0.35[a]	4.88 ± 0.69[b]	5.10 ± 0.83[b]	0.0017	7.373	S
CRT (µmol/l)	176.8 ± 62.74	218.0 ± 88.33	220.5 ± 67.03	0.1701	1.841	NS
AST (U/L)	54.56 ± 27.19	50.67 ± 23.73	63.02 ± 26.04	0.3734	1.006	NS
ALT (U/L)	24.96 ± 8.84[a]	31.87 ± 10.9[b]	31.98 ± 15.59[b]	<0.0001	13.20	S
ALP (U/L)	25.81 ± 17.13	28.53 ± 9.08	34.16 ± 12.41	0.1948	1.694	NS

(b)

Parameters	Phase 1 (Female) n = 22	Phase 2 (Female) n = 24	Phase 3 (Female) n = 18	P value	F value	Remark
GLU (mmol/l)	3.71 ± 1.75	4.01 ± 1.08	4.21 ± 1.56	0.5535	0.597	NS
UREA (mmol/l)	4.24 ± 0.21[a]	4.84 ± 0.54[b]	5.15 ± 0.92[c]	<0.0001	17.02	S
CRT (µmol/l)	168.8 ± 52.38	173.1 ± 63.45	188.6 ± 71.76	0.1772	1.781	NS
AST (U/L)	60.50 ± 22.07	65.67 ± 28.17	64.88 ± 21.47	0.7507	0.288	NS
ALT (U/L)	27.25 ± 11.05[a]	30.76 ± 12.17[b]	39.11 ± 19.97[c]	0.0043	5.962	S
ALP (U/L)	27.95 ± 18.21	26.27 ± 7.84	27.77 ± 13.44	0.7529	0.285	NS

Values in the same column with different superscript letter (a, b) or (a, b, c) differ significantly ($P < 0.05$) when compared against each others. NS = Not Significant, S = Significant, n = No of Rats.

3.6. Histopathological Investigations

Histopathological examination of the liver and kidneys were performed to correlate the findings of hepatic and renal parameters of the chronic toxicity of carmoisine as seen in **Figure 2** and **Figure 3**.

4. Discussion

The significant higher values seen in GLU over a period of 30, 60 and 90 days concurs with the findings of Amin *et al.*, [7] when carmoisine were fed to rats but contrast the findings of Mehedi *et al.* [22], who reported no significant difference in GLU levels when azo dyes were fed to rats. The significant higher values seen in GLU in the treated rats could be due to the negative pharmacological interaction between these azo dyes and insulin. Azo dyes tend to interfere with proteins (enzymes) three dimensional configuration reducing their functionality. Also, the increase of GLU levels could be probably due to direct oxidative insult on the pancreas leading to reduction in insulin production.

Table 5. (a) Weights of organs treated with carmoisine over a period of 30, 60, and 90 days in male rats; (b) Weights of organs treated with carmoisine over a period of 30, 60, and 90 days in female rats.

(a)

Days	**30 Days Treatment**				
Parameters	Control Rats (Males) n = 18	Treated Rats (Males) n = 16	P value	T value	Remark
Kidney (g)	1.25 ± 0.31	1.21 ± 0.33	0.7206	0.360	NS
Liver (g)	5.99 ± 1.36	5.48 ± 1.38	0.2822	1.094	NS
Days	**60 Days Treatment**				
Parameters	Control Rats (Males) n = 20	Treated Rats (Males) n = 15	P value	T value	Remark
Kidneys (g)	1.19 ± 0.22	1.18 ± 0.21	0.3528	0.942	NS
Liver (g)	5.13 ± 1.00	5.39 ± 0.80	0.6197	0.520	NS
Days	**90 Days Treatment**				
Parameters	Control Rats (Males) n = 19	Treated Rats (Males) n = 18	P value	T value	Remark
Kidneys (g)	1.21 ± 0.28	1.19 ± 0.30	0.8182	0.231	NS
Liver (g)	5.41 ± 0.83	5.71 ± 0.95	0.3127	1.024	NS

(b)

Days	**30 Days Treatment**				
Parameters	Control Rats (Females) n = 22	Treated Rats (Females) n = 22	P value	T value	Remark
Kidney (g)	1.34 ± 0.27	1.17 ± 0.34	0.0731	1.838	NS
Liver (g)	5.29 ± 1.37	5.12 ± 1.85	0.7254	0.353	NS
Days	**60 Days Treatment**				
Parameters	Control Rats (Females) n = 20	Treated Rats (Females) n = 24	P value	T value	Remark
Kidneys (g)	1.22 ± 0.47	1.07 ± 0.34	0.0293	2.257	S
Liver (g)	5.13 ± 1.00	4.77 ± 1.05	0.2442	1.181	NS
Days	**90 Days Treatment**				
Parameters	Control Rats (Females) n = 18	Treated Rats (Females) n = 18	P value	T value	Remark
Kidneys (g)	1.20 ± 0.55	1.06 ± 0.27	0.3187	1.012	NS
Liver (g)	5.38 ± 0.93	5.21 ± 1.28	0.6381	0.474	NS

n = no of Rats, NS = Not Significant, S = Significant.

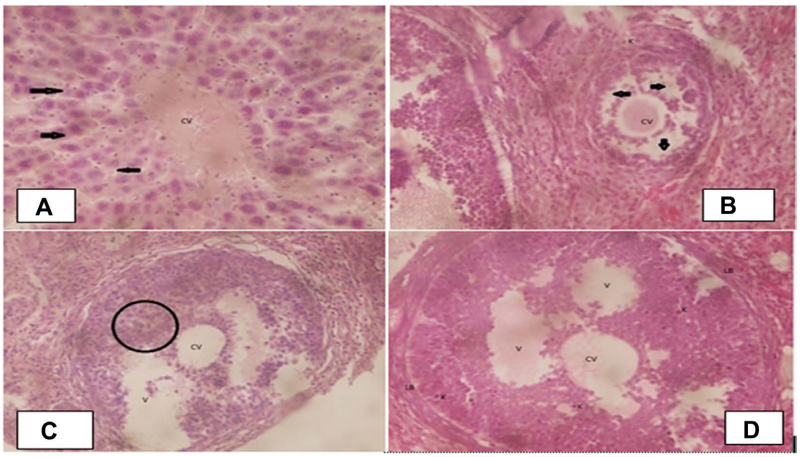

Figure 2. Histological examination of the liver. Plate A: H&E, Mag: ×400, Dose: 0.0 mg/kg, CV = Central Vein Appearing Normal, Hepatocyte Appears in Thick Plate Radiating (Arrow) between Thick Plates are Sinusoids. Inference: Liver Slide Appears Normal. Plate B: H&E, Mag: ×400, Dose: 4.0 mg/kg for 30 Days, CV = Central Vein Surrounded by Radiating Hepatic Cells. Hepatic Cells are Destroyed Leaving Vacuoles (arrows), K = Kupffer Cells. Plate C: H&E, Mag: ×400, Dose: 4.0 mg/kg for 60 Days, CV = Central Vein, V = Vacuolated Portion of Lobule, Circled Portion: Cluster of Inflamed Hepatic Cells. Plate D: H&E, Mag: ×400, Dose: 4.0 mg/kg for 90 Days, CV = Central Vein, V = Vacuolated Portions (Loss of Hepatic Plates), LB = Lobule Boundary, K = Pigmentation of Kupffer Cells in Sinusoid Areas. Inference: Injury within Hepatic Lobule.

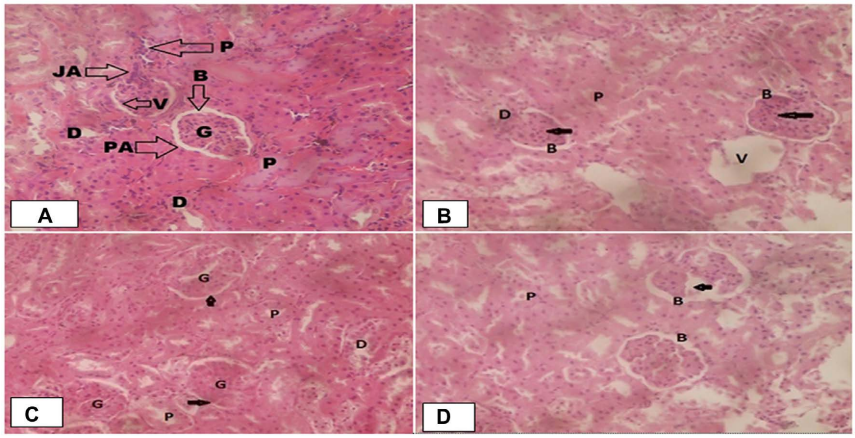

Figure 3. Histological examination of the kidney. Plate A: H&E, Mag: ×400, Dose: 0.0 mg/kg, G = Glomerulus (Normal), Visceral Layer (V), B = Bowman's Capsular Space (Normal), Parietal Layer (PA) with Juxtaglomerular Apparatus (JA), P&D = Proximal and Distal Convoluted Tubule (Normal) Inference: Kidney Slide Appears Normal. Plate B: H&E, Mag: ×400, Kidneys, Dose: 4.0 mg/kg for 30 Days, Glomerular Cells Appear Clustered (Arrow) with not Well-Defined Mesangial Areas. B = Bowman's Capsule and Space (Normal), P & D = Proximal and Distal Convoluted Tubule (Normal) V = Vacuolated Area Lined By Endothelial cells. Plate C: H&E, Mag: ×400, Kidneys, Dose: 4.0 mg/kg for 60 Days, G = Glomerulus (Normal). Bowman's Space (Arrow) almost Obscured. Mesangial Area Is Not Hypercellular, P and D Proximal and Distal Convoluted Tubules (Normal) with Pockets of Endothelial Distortion in the Distal Tubule. Plate D: H&E, Mag: ×400, Kidneys, Dose: 4.0 mg/kg for 90 Days, Distorted Glomerular Arrangement (Arrow) which is Vacuolated, B = Bowman's Capsule Appears Normal, P = Podocyte Inference: Possible Glomerulonephritis.

The significant higher values seen in creatinine and urea in our results over a period 60 and 90 days whether in carmoisine treated male or female rats is in line with the records of Ashour & Abdelaziz, [23]; Helal *et al.* [24] but contrast the findings of Aboel-Zahab *et al.* [25]. The significant higher values in CRT and Urea could also be as a result persistent oxidative stress on the nephrons of the kidneys. ROS tends to reduce cell viability by disrupting cell membrane integrity thereby inducing cell membrane leakage. The histology of the kidneys in the treated rats indicated vacuolation, distorted glomerular arrangements and clustered mesengials that were not well-defined within the glomerulus (Plate G, H). These changes suggest glomerular inflammation or an indication of inflammatory responses to nephritic damages. This present finding contradicts the reports of Reza *et al.* [26], stated that when carmoisine dyes fed to rats at ADI doses, no renal distortion or glomerular distortions and dilations were seen for over a period 120 days except when the doses were increased to 200 and 400 mg/kg.

The significant higher value in AST, ALT and (ALP in male rats only) seen in our results over a period of 60 and 90 days is in line with the records of Amin *et al.* [7]; Ai-Mashhedy *et al.* [27]. They reported higher values in AST, ALT and ALP when azo dyes were fed to rats for 60 days at ADI doses. However, contrary to our findings, Reza *et al.* [26], reported no significant differences in liver enzymes in carmoisine dye treated rats at ADI doses. The significant higher value seen in AST, ALT and ALP enzymes could indicate of hepatocellular distortion due to the presence of aryl amines, aromatic amines and other free radical derived from azo dye metabolism leading to the increase presence of these enzymes in the plasma. In our histopathological findings, the liver in treated rats showed the presence of inflamed hepatocytes, vacuolation, compression of the central vein, distorted lobular boundary (Plate C and Plate D), loss of hepatic plates and pigmented kupffer cells at the sinusoids (Plate D). These observations were also pointed out by Metwally & Mohamed [28]. They reported that ADI doses for over a period of 30 and 45 days resulted in hepatic degeneration, associated with swollen hepatocytes with fatty changes. It was further observed in their studies that 60 and 90 days treatment at higher dose also indicated severe hepatic degenerations. The presence of kupffer cells and vacuolation might indicate immunological response and hepatocellular damage as a result increased oxidative stress on the hepatocytes. Similar findings were documented by Adele *et al.* [29] were tartrazine azo dye at ADI doses over a period of 90 days was observed to induce hydropic dilation, vacuolation, and infiltration of parenchymal materials (including kupffer cells) of the central vein. It addition, significantly higher values of malondialdehyde (MDA) was also reported suggesting oxidative damages and lipid peroxidation. These changes indicate hepatocytic cell damage such that as the hepatic damage increases, the vacuole appears larger occupying the whole cytoplasm. The histopathological results obtained correlates with our biochemical findings were significant increases in liver: AST and ALT and ALP were ob-

served in both tartrazine and carmoisine treated rats.

The non-significant differences observed in the weight of the organs contrast the findings of Reza *et al.* [26]; Ai-Mashhedy *et al.* [27]. These also reported non-significant changes in weights of liver and renal tissues in rats fed with carmoisine dyes at ADI doses over a chronic period. The non-significant differences were further observed when different durations of 30, 60, and 90 days were compared. However, in the treated female rats, after 60 days treatment, significantly lower levels were seen in the weight of the kidney which could be due to loss of parenchymal materials due to vacuolation. Our findings are in line with the reports of Himri *et al.* [26], who reported no significant decrease after 90 days of azo dye administration in rats at ADI doses.

Our study also revealed that the sex did not play significant role in the severity of toxicity of carmoisine. All parameters considered indicated no significant difference between male and female rats compared. Gregson *et al.* [30], reported that testosterone tends to induce inhibitory mechanisms in biliary and urinary excretion of azo dyes via the hepatic and renal systems thereby extending the oxidative stress time frame cum toxicity in male rats. Gregson *et al.* [30] stated that due to testosterone, male rats excreted 22% of azo dyes while female excreted 63% of azo dyes within three hours of intraperitoneal or oral administration. However, this study did not find such difference in toxicity of azo dye in particular, carmoisine.

When the different duration of exposure were considered, the significantly higher values seen in GLU, UREA, and ALT over 30, 60 and 90 days could be as a result of cells inability to adapt to oxidative stress induced by the dyes over time. This finding also implies that prolonged duration of exposure tends to induce more adverse effects on the vital organs such as the liver as a result of oxidative damages. This report is in agreement with the findings of Mekkaway *et al.*, [31]. The higher values seen in UREA concentration without a corresponding increase in CRT might also suggest dehydration and increased protein degredation induced by the azo dyes.

The mechanism of toxicity of carmoisine could be connected to the reductive biotransformation of the azo bond (N=N) of the dye in the hepatocellular tissue and by the activities of intestinal microorganisms releasing reactive nitrogen species (RNS) and reactive oxygen species (ROS) such as reactive amines, aryl amines, superoxides, and other free radicals. Umbuzeiro *et al.* [10] and Adele *et al.* [29], reported that these reactive species could interacts with micro- and macro-biomolecules such as proteins, carbohydrates, and lipids, altering their physiological functions and most times causing pathological lesions on cells and eventually cell death, when the mechanism of conjugation by the system (especially glutathione) is saturated or where the rate of reactive species generated exceeds the bioavailability of glutathione. In addition, the degree or severity of carmoisine (azo dye) toxicity is also depended on the dose, duration of exposure, age, and body weight as evident in other of our studies [1] [6] [29].

5. Conclusion

Carmoisine administered orally at ADI doses in male and female induced deranged of renal and hepatic parameters as well as distortion of these tissues for over a period of 90 days. However, the weights of these tissues were not significantly affected. Our findings also indicated that there is no difference in the severity of carmoisine toxicity in male and female rats.

Authors' Contribution

Elekima, I. contributed to the study design, performing the study, analysis of the data, writing the manuscript, literature search, and guarantor of the manuscript. Obisike, U.A., Ben-Chioma, A.E. and Brown, H. contributed to the analysis of the data, and reviewing of the manuscript. Waribo, H.A., George-Opuda, I., Onwuli, D. and Brisibe N. contributed to the integrity of the manuscript, literature search, and reviewing of manuscripts.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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