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Toxicity Assessment of Zinc, Copper and Chromium on Various Organs in Adult Male Rats

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ABSTRACT

This study was designed to investigate the mechanism of zinc, copper and chromium toxicity after exposure for two different durations (4 weeks and 8 weeks). Ninety six adult male albino rats were divided into 8 groups 12 rats each. G1 (C1): served as healthy control; G2 (Zn1), G3 (Cu1) and G4 (Cr1) were received 500, 200 or 8 mg/day of zinc, copper and chromium, respectively for 4 weeks. While G5 (C2) served as healthy control; G6 (Zn2), G7 (Cu2) and G8 (Cr2) were received the same doses of metals for 8 weeks. The results of this study revealed that exposure to the three metals have detrimental effects in a time dependent manner. A significant reduction ($P \leq 0.05$) in liver and kidney functions; also hemotoxic effect was confirmed by reduction in Hb concentration and deteriorations in blood cells count and shapes; in addition, results showed a significant increase in serum MDA, NO and OSI and a significant reduction ($P \leq 0.05$) in TAC and GSH levels and SOD activity; marked elevations in TNF-α, IL-6, IL-1β, CRP, MPO and EMAP-II. Moreover, tested metals caused disturbances in immune system, negative effect on brain neurotransmitters, sever elevation in DNA fragmentation percentage and PCG level in spleen and brain tissues and serum LDH activity, cytosolic and mitochondrial dysfunction in spleen and brain tissues appeared as significant elevation in IDH activity and CCO. The microscopic examination for spleen and brain confirmed damage to these tissues.

1. INTRODUCTION

Industrial and anthropogenic activities have increased the exposure of humans and animals to metals, leading to food, water, and air contamination, which is a major environmental concern [22], Luo et al., 2020). Metals can exert toxic effects on biological systems by forming metal cations that bind to vital macromolecules, leading to dysfunction of the gastrointestinal, immune and kidney systems [12]. Furthermore, simultaneous exposure to multiple metals can have cumulative effects on health [13], [21].

Zinc (Zn) is a vital trace element commonly used as supplement and food additive [15]. It is the second essential after iron and plays a crucial role as a structural, catalytic, and regulatory component [41]. However, exposure to Zn and its toxicity is not uncommon due to its presence in various environmental sources. There are several cases of toxicity resulting from either inhalation of Zn from occupational sources or overuse of Zn in dietary supplements and denture cream, also incorrectly prepared parenteral nutrition is a source for Zn toxicity. Unfortunately, these cases may lead to fatal outcomes [23]. Zinc toxicity varies in severity according to the involved compound and exposure duration. For instance, zinc chloride in smoke bombs is a main cause for chest pain and airway irritation [45]. While inhalation of zinc oxide can lead to "metal fume fever," commonly seen with occupational exposure [23]. Furthermore, overuse of makeup, sunscreen, and ointments can lead to dermal toxicity from zinc oxide exposure [66]. Excessive use of denture cream can cause zinc overdose and secondary copper deficiency [35].

Copper (Cu) is an essential nutrient has a vital role in the whole living organisms biochemistry. It acts as a cofactor of several proteins and enzymes named cuproenzymes which involved in basic mechanisms as oxygen carrying, energy production, cell metabolism and signaling, and hematopoiesis [65]. Copper toxicity due to excess Cu in the body is referred to as copperiedus [56], [68] which is rare in healthy individuals but can occur in those with genetic disorders of copper metabolism or in individuals exposed to high levels of copper through environmental or occupational sources [39]. Eating acidic foods cooked in uncoated copper utensils, use of dietary supplements or drinking water with excess Cu are among other environmental origins which can cause Cu toxicity [52], [9]. Cu is also extensively used in manufacturing industries, electronic products, and agrochemicals [47], [3]. Toxicity has also been reported in individuals using copper-containing intrauterine devices for contraception [10].

Chromium (Cr) is a common element in nature and a transition metal that plays a crucial role in various physiological processes in the human body, including glucose metabolism, lipid metabolism, and insulin signaling [74]. However, exposure to high levels of Cr can lead to respiratory, renal, and gastrointestinal disorders, among others [78]. It has been found to exist in several oxidative states from divalent to hexavalent [57]. In soil and groundwater, the common forms of Cr are the hexavalent state or the trivalent state. These states exhibit different chemical activities and toxicities [80]. It has been implicated as one of metals tend to accumulate into the body tissues up to very toxic levels that cause substantial harm to the health of an organism [64]. Cells can accumulate a high concentration through an anionic transport system and reduction reactions, which accompany the transport system [11]. The main sources of Cr are river drainage, dredging sludge, and dumping of industrial wastes. The concentration of Cr varied in seawater, further, in fish organs, gills showed a higher value whereas it was least in the muscle [55]. Compounds derived from Cr have been used widely as dyes and mordant in industries dealing in textiles, manufacturing industries dealing in saccharin production, purification process of fats and oils. Such a wide industrial use of it has adversely led to the detriment of the environment [34].

Various mechanisms perceived as the way metal toxicity occurs has been proposed ranging from free radical production, formation of DNA adducts and apoptosis [32]. Studies have shown that most of the damage has

been due to an upsurge in ROS production. To find out more about the toxic effects of zinc, copper, and chromium on different tissues systematically, the present study was designed to investigate their negative impact on the hematopoietic system, nervous system, cytogenetics and biochemistry in rats.

2. Materials and Methods

2.1 Chemicals

Zinc acetate dihydrate ($Zn(CH_3COO)_2 .2H_2O$), copper sulphate pentahydrate ($CuSO_4.5 H_2O$) and potassium dichromate ($K_2 Cr_2 O_7$) were purchased from Sigma Aldrich Chemical Co. (St. Louis, Missouri, USA).

2.2 Animals and experimental design

Ninety-six adult male Wistar rats weighing (160±10 g) were obtained from National Research Center (Dokki, Giza, Egypt) and acclimatized to the laboratory conditions for 1 week. They were kept individually in standard laboratory conditions. The food and water were introduced *ad libitum* in special cups and bottles, respectively. All rats were maintained on a standard diet prepared according to the American Institute of Nutrition (AIN-93M) and adjusted by [54]. Rats randomly divided into eight groups 12 rats each as follow: - Groups 1 and 5: Healthy control groups (C1 and C2) were received distilled water (5ml /kg body weight /day) for 4 and 8 weeks respectively. The set of groups were received a daily oral doses of Zn, Cu or Cr salts (500, 200 or 0.8 mg /kg/day) dissolved in distilled water by gastric tube according to [38], [1], [58], respectively, as follow: Groups 2 (Zn1), 3 (Cu1) and 4 (Cr1) were received the doses for 4 weeks, while Groups 6 (Zn2), 7 (Cu2) and 8 (Cr2) were received the doses for 8 weeks.

At the end of the experimental period (4 or 8 weeks) all rats were fasted for 12 hours with water *ad libtium*, then weighed and sacrificed under sodium barbiturate anesthesia. Blood samples were collected directly from hepatic portal vein in two tubes. The first tube was contains ethylene diamine tetra acetic acid (EDTA) as an anticoagulant for separation of whole blood and the second tube used for separation of serum by allowing blood samples left for 15 min at room temperature then were centrifuged at 4000 rpm for 20 min and were kept in plastic vials at -20°C until analysis. Spleen and brain tissues were separated immediately and washed by saline solution (0.9 % NaCl) then either preserved in 10% neutral formalin for microscopic examination or stored at -20ºC until used for the tissue biochemical analyses.

2.3 Biochemical analyses

2.3.1 Determination of Tested Heavy Metals Concentrations

The concentration of Zn, Cu and Cr were determined in spleen and brain tissues according to (APHA, 2017).

2.3.2 Evaluation of Liver Function

The level of ALT, AST, ALP, γ-GT, total protein, albumin, globulin and total bilirubin were determined according to methods previously reported by [70], [77], [69], [43], [14], [75], using biomed kit, Egypt for ALT and AST; vitro scient Egypt for ALP and γ-GT; bio-diagnostic kit, Egypt for total protein, albumin and total bilirubin.

2.3.3 Evaluation of Kidney Function

Urea, creatinine and uric acid were determined according to methods reported by [19], [63], [61], respectively using bio-diagnostic kit, Egypt.

2.3.4 Hematologic Function and Blood Picture

All samples were brought to room temperature for 30 min before analysis and the following variables were

assessed: RBC count, hemoglobin (Hb) concentration, HCT (instrument-derived), MCV, MCHC, MCH, total WBC count and platelet count. Five high-power (100× oil objective) fields in the monolayer of the blood smear were examined for morphologic changes in erythrocytes; changes were categorized as previously described [18].

2.3.5 Evaluation of Brain Function

The serum dopamine, nor-epinephrine, serotonin and γ-amino butyric acid levels were measured by ELISA Kits (CA NO CSB-E08660r, CUSABIO China, CAT.NO MBS725497, CAT.NO MBS269152) using the manufacturer's protocol.

2.3.6 Measurement of Inflammatory Cytokines and Serum Immunoglobulins

The serum tumor necrosis factor-α (TNF-α), C- reactive protein (CRP), interleukin 6(IL-6), interleukin 1-beta (IL-1β), endothelial-monocyte activating polypeptide II (EMAP-II) levels and the activity of myeloperoxidase (MPO) were analyzed by ELISA Kits (cusabio, China CA NO SL0202Ra, cusabio, China CA.NO SL0402Ra, CAT NO MBS260410, CA NO MBS704859) following the manufacturer's protocol. Serum IgG and IgM were determined by the Sandwich-ELISA principle using SunLong Biotech kit CA.No (SL0362Ra) and CA.No (SL0363Ra).

2.3.7 Measurement of Antioxidant and Oxidative Stress Markers

Superoxide dismutase (SOD), reduced glutathione (GSH), catalase, total antioxidant capacity (TAC), malondialdehyde (MDA) and nitric oxide (NO) were analyzed by Biodagnostic Kits, Egypt (CAT NO SD2521, CAT.NO E-BC-K030M, CAT.No.(CA2517),CAT.NO TA 2513, CAT.NO MD2529,CAT.NO2533) according to the manufacturer's protocol. Also oxidative stress index (OSI) was calculated.

2.3.8 Determination of DNA Fragmentation Percentage and Tissue Damage

Spleen and brain tissues were used to determine the percentage of DNA fragmentation and Protein carbonyl group (PC) concentration using methods previously reported by [8], [76]. Also the serum activity of lactate dehydrogenase (LDH) was measured according to [73] using biomed kit Egypt.

2.3.9 Assessment of Cytosolic and Mitochondrial Dysfunction

Spleen and brain tissues were used to determine serum Iso-citrate dehydrogenase (IDH) and cytochrome C oxidase (CCO) activities as described by [7], [53].

2.3.10 Histopathological Examination

The histopathological examination of spleen and brain tissues was performed according to the method of [6]. Tissues were fixed in neutral buffered formalin. Following fixation, tissues were sectioned within paraffin-embedded blocks and stained with Hematoxylin and Eeosin (H&E) then examined microscopically by alight microscope (Olympus Bx 50,Japan).

2.3.11 Statistical Analysis:

The statistical analysis was done using the SPSS program for Windows, (version 16)(SPSS Inc., Chicago, IL, USA). The data was presented as mean ±standard deviation (SD). The One Way Analysis of Variance (ANOVA) LSD was used to illustrate differences between the groups. P-value <0.05 was recognized as statistically significant [37].

3. Results

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3.1 Tested Metals Concentration in Spleen and Brain Tissues

In all the samples, all the metals studied were present at levels above their detection limits (Tables 1a-1c). The highest concentration was estimated in the eight weeks tested groups, compared with the four weeks treated groups and the control groups.

Tables (1): Concentration of zinc, copper and chromium in spleen and brain tissues

a. Zinc concentration (µg/g Wet Tissue)

Groups	Spleen	Brain
G1: C1	23.1 ± 0.7[a]	14.9 ± 0.8 [a]
G2: Zn1	40.2 ± 3.7 [b]	27.9 ± 2.6 [b]
G5:C2	27.3±0.6 [a]	18.2 ±0.9 [a]
G6: Zn2	56.1 ± 1.4[c]	40.2 ± 4.12 [c]
LSD(P≤0.05)	10.4	11.8

b. Copper concentration (µg/g Wet Tissue)

Groups	Spleen	Brain
G1: C1	16.2 ± 0.5 [a]	2.5 ± 0.6 [a]
G3 :Cu1	40.8 ± 2.3 [b]	7.3 ± 0.3 [b]
G5: C2	19.4± 0.7 [a]	3.2 ± 0.5[a]
G7 :Cu2	56.0 ± 3.4 [c]	16.2 ± 0.25[c]
LSD(P≤0.05)	10.6	2.0

c. Chromium concentration (µg/g Wet Tissue)

Groups	Spleen	Brain
G1: C1	2.03 ± 0.38 [a]	2.9 ± 0.9 [a]
G4: Cr1	8.78 ± 0.14 [b]	10.8 ± 0.28[b]
G5: C2	2.6 ± 0.4 [a]	3.6 ± 0.8 [a]
G8: Cr2	11.57 ± 0.21[c]	15.6 ± 0.2 [c]
LSD(P≤0.05)	1.18	2.5

P≤0.05, there are no significant difference between means have the same letters in the same column

3.2 Hepatotoxic Effect of Zinc, Copper and Chromium Salts

The results of biochemical serum parameters in the control and experimental groups described in Tables (2.a and 2.b) showed that administration of Zn, Cu and Cr in rats initiated liver damage, as revealed by appreciable surge in activity of ALT, AST, ALP, γ-GT, enzymes and total bilirubin as compared with control rats while there was sever reduction in total protein, albumin and globulin concentrations. The harmful effect of metals increases over time as it is clear that the groups treated for a period of 8 weeks are more affected compared with the groups treated for a period of 4 weeks.

Table (2.a): Effect of zinc, copper and chromium on liver enzymes activities

Groups	ALT (IU/L)	AST (IU/L)	ALP (U/L)	γ-GT (U/L)
G1 :(C1)	5.2±1.5 [a]	6.4±1.0 [a]	70 ±2.2 [a]	2.5±0.9 [a]
G2 :(Zn1)	22.9±0.3 [b]	24.1±0.8 [b]	112±2.4 [b]	11.7±0.7 [b]
G3 : (Cu1)	43.6±2.0 [c]	47±1.6 [ce]	157±1.7 [ce]	31.4±0.29 [c]
G4 :(Cr1)	70.9±1.4 [d]	67.8±1.0 [de]	178±1.2 [d]	47.9 ±0.2 [d]
G5: (C2)	7.3±1.0 [a]	8.1±0.8 [a]	78.6 ±1.8 [a]	3.8±0.7 [a]
G6 :(Zn2)	42.7±0.8 [c]	41.4±2.5 [c]	145±2.7 [c]	23.5±1.0 [e]
G7 :(Cu2)	63.3±2.8 [d]	60.4±3.8 [e]	166±2.1 [e]	40.5 ±0.4 [f]

G8 :(Cr2)	88.6±0.9 [e]	82.6±2.0 [f]	197.7±1.1 [f]	57±1.1 [g]
LSD (P ≤0.05)	**10.8**	**14.0**	**13.8**	**5.0**

Table (2.b): Effect of zinc, copper and chromium on blood proteins and total bilirubin concentration

Groups	Total Protein (g/dl)	Alb (g/dl)	Glob (g/dl)	Alb/Glob Ratio	Total Bilirubin (mg/dl)
G1 : (C1)	8.9±0.1 [a]	6.0±0.1 [a]	2.9 ±0.2 [a]	2.0±0.1 [a]	0.2 ±0.06 [a]
G2 : (Zn1)	6.0±0.1 [b]	3.8±0.2 [b]	2.3±0.2 [ab]	1.7±0.2 [ab]	1.34±0.1 [ab]
G3 : (Cu1)	3.6±0.06 [c]	2.2±0.2 [c]	1.5±0.1 [b]	1.4±0.1 [ab]	2.5±0.1 [b]
G4 : (Cr1)	3.1±0.1 [c]	2.0±0.1 [cde]	1.1±0.5 [b]	1.8±0.2 [a]	3.9±0.2 [c]
G5: (C2)	9.2±0.2 [a]	6.8±0.1 [a]	3.2 ±0.1 [a]	2.1±0.2 [a]	0.6 ±0.1 [a]
G6 : (Zn2)	3.3±0.3 [c]	2.1±0.2 [cd]	1.2±0.1 [b]	1.9±0.2 [a]	2.1±0.1 [b]
G7 :(Cu2)	3.0±0.1 [c]	0.96±0.1 [de]	2.1 ±0.2 [a]	0.47±0.1 [b]	4.5±0.2 [cd]
G8 : (Cr2)	2.7±0.2 [c]	0.85±0.1 [e]	1.8 ±0.2 [a]	0.46±0.1 [b]	5.3±0.1 [d]
LSD (P ≤0.05)	**1.3**	**1.2**	**1.4**	**1.4**	**1.2**

P≤0.05, there are no significant difference between means have the same letters in the same column

3.3 Nephrotoxic Effect of Zinc, Copper and Chromium Salts

The current results confirm renal toxicity of Zn, Cu and Cr exposure. Metals caused a significant rise (*P≤0.05*) in the serum urea, creatinine and uric acid levels when compared to the control rats. Figure (1) shows the detrimental effect of tested metals on kidney.

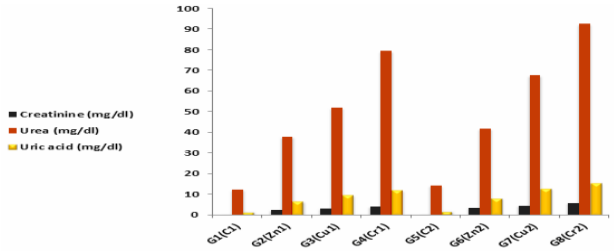

Figure (1): The renal function index of rats treated with metals

3.4 Hemotoxic Effect of Zinc, Copper and Chromium Salts

According to the results illustrated in tables (3-a) and (3-b), we can conclude that the three tested metals have the potential to cause hemotoxic effect in experimental rats. Also, this effect is time-dependent, as the most affected groups were those that were tested for a period of 8 weeks, compared with those tested for a period of 4 weeks. In addition, the most affected groups are those tested with chromium, followed by copper and then zinc.

Table (3-a): Effect of zinc, copper and chromium on Erythrogram

Groups	RBCS (×10^{6}cu.mm)	HB (g/dl)	HCT (%)	MCV (fl)	MCH (Pg)	MCHC (g/dl)
G1: (C1)	4.6 ± 0.2[a]	16.59 ± 0.3[a]	51 ± 1.12[a]	110 ± 5.98 [a]	35.9 ± 2.1 [a]	32.5 ± 1.0 [a]
G2 :(Zn1)	4.0 ± 0.1 [ab]	13.3 ± 0.2[b]	41.3 ± 1.1 [b]	103 ± 5.3 [ab]	33.4 ± 1.1 [ab]	32.4 ± 1.1 [a]
G3: (Cu1)	3.5 ± 0.1[b c]	11.06 ± 0.3[c]	33.9 ± 0.6 [bc]	96.9 ± 3.4 [bc]	31.6 ± 0.7 [be]	32.6 ± 0.63 [a]
G4 :(Cr1)	3.1 ± 0.1[cd]	8.4 ± 0.3[f]	28.5± 0.9 [cd]	92.3 ± 3.5 [bc]	27.4 ± 1.3 [c]	30.3 ± 2.5 [b]
G5: (C2)	4.8 ± 0.1[a]	17.0 ± 0.2[a]	43.2 ± 1.0 [a]	114± 3.6 [a]	36.2 ± 1.5 [a]	32.7 ± 1.0 [a]
G6: (Zn2)	3.3 ± 0.1[cbd]	10.4 ± 0.1[ce]	33.0 ± 0.6 [c]	98.9 ± 3.4 [b]	31.2 ± 1.1 [be]	31.6 ± 0.7 [a]

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G7 :(Cu2)	3.0 ± 0.08^{cd}	8.8± 0.1 ^{e}	27.8± 1.7 ^{cd}	89.9 ± 7.4 ^{c}	28.4 ± 0.9 ^{ce}	31.7 ± 1.9 ^{a}
G8 :(Cr2)	2.51 ± 0.1^{d}	6.9 ± 0.4 ^{f}	22.4 ± 1.27 ^{d}	89 ± 7.4 ^{c}	27.1 ± 1.9 ^{c}	29.7 ± 0.9 ^{b}
LSD (P ≤0.05)	**0.9**	**1.9**	**7.5**	**9.0**	**3.6**	**2.5**

Table (3-b): Effect of zinc, copper and chromium intoxication on WBCs count, WBCs differential, platelets count and Howell jolly Bodies

Groups	WBCs (×10^{3}cu.mm)	Neutrophil (%)	Lymphocyte (%)	Basophil (%)	Eosinophil (%)	Monocyte (%)	PLT (x 10^3/cmm)	Howell Jolly Bodies
G1 :(C1)	5.9 ± 0.2^{a}	53 ± 1.0 ^{a}	37.1 ±1.0 ^{e}	0 ± 0^{a}	8 ± 0.1^{a}	1.9 ± 1.6 ^{a}	268±1.5^{a}	...
G2 :(Zn1)	7.2 ± 0.1^{b}	46 ± 0.9^{ad}	43 ± 1.0 ^{b}	0 ± 0 ^{a}	6.0 ± 0.1^{b}	5.0± 1.0 ^{a}	244 ±2.5^{b}	...
G3:(Cu1)	7.7 ± 0.2 ^{bc}	36 ± 0.1 ^{b}	51.7± 0.8 ^{c}	0 ± 0^{a}	4 ± 0.1 ^{ce}	8.2 ± 0.6 ^{a}	171±1.0 ^{c}	(+)
G4 :(Cr1)	8.7 ± 0.1 ^{c,d}	26 ± 0.1^{c}	63.7±0.8 ^{d}	1 ± 0^{a}	2 ± 0.1 ^{df}	7.2±0.6 ^{a}	162±0.9^{c}	(+)
G5 : (C2)	6.1 ± 0.1^{a}	55 ± 1.0 ^{a}	35.0 ±1.0 ^{a}	0 ± 0^{a}	9 ± 0.1^{a}	1.0 ± 0.9 ^{a}	276±1.2^{a}	…
G6 :(Zn2)	8.1 ± 0.14^{cd}	40 ± 0.9^{bd}	48 ± 0.6 ^{b}	1 ± 0 ^{a}	5 ± 1.1^{bc}	6.0±0.9 ^{a}	202±1.7^{d}	(+)
G7: (Cu2)	9.2 ± 0.2^{d,e}	30.6±0.9^{bc}	60 ± 0.1 ^{d}	1 ± 0 ^{a}	3 ± 0.8^{de}	5.4±0.9 ^{a}	151±1.0^{e}	(++)
G8 :(Cr2)	10.4 ± 0.2^{e}	21.2 ±0.9 ^{c}	70.9 ± 1 ^{e}	2 ± 0 ^{a}	1.0±0.1 ^{f}	4.7±1.4 ^{a}	142±1.1^{e}	(++)
LSD(P≤0.05)	**1.2**	**9.9**	**5.1**	**1.0**	**1.7**	**7.4**	**9.9**	

P≤0.05, there are no significant difference between means have the same letters in the same column

3.5 Neurotoxic Effect of Zinc, Copper and Chromium Salts

The Neurotransmitters dopamine, nor-epinephrine, serotonin and γ-amino butyric acid levels in serum, as determined by ELISA and showed in figure (2), nor epinephrine level significantly elevated in all intoxicated groups as compared with control groups; while there was a significant (P<0.05) reduction in the serotonin, dopamine and γ-amino butyric acid concentration as compared with control groups. The more pronounced reduction was found in groups Cr2, Cu2 and Zn2 respectively which are treated with Cr, Cu and Zn salts for 8 weeks.

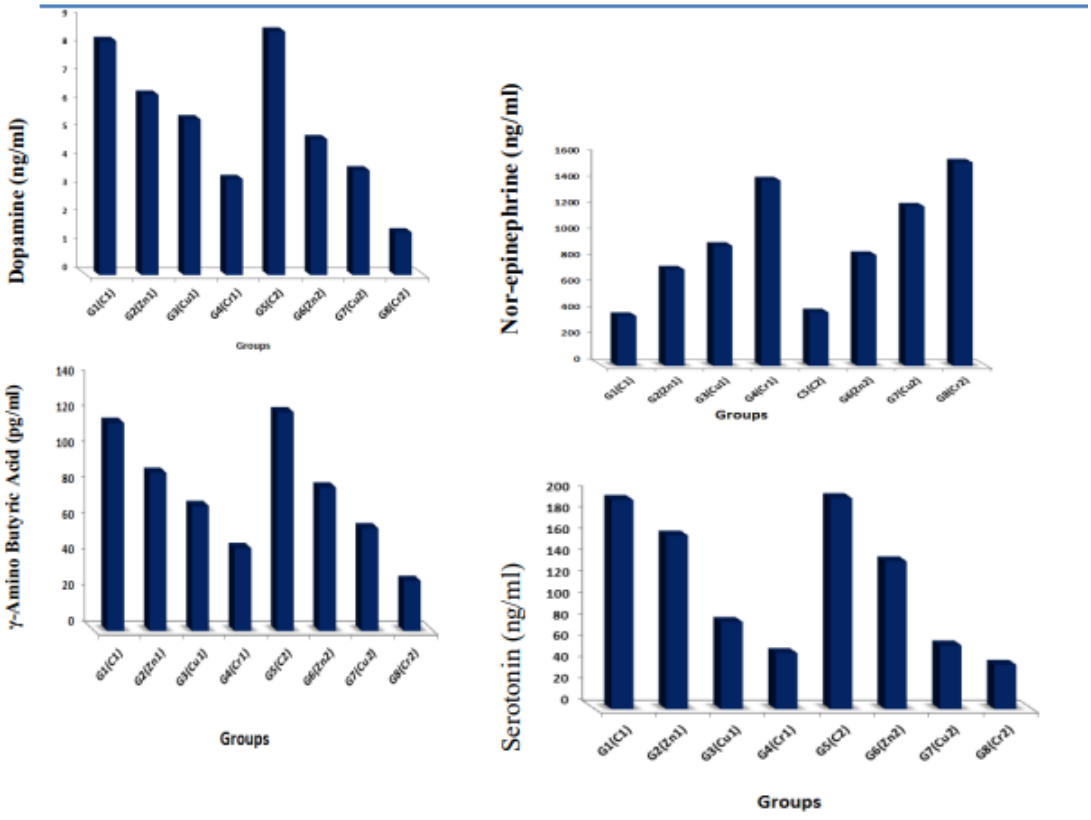

Figure (2): Effect of zinc, cooper and chromium intoxication on serum dopamine concentarion (ng/ml); nor-epinephrine concentration (ng/ml); γ-amino butyric acid (pg/ml) and serotonin concentration (ng/ml)

3.6 Adverse Effect on Serum Inflammatory Markers and Immunoglobulins Concentration

The mean of the serum levels of inflammatory markers and immunoglobulins at the end of the study are presented in tables (4-a) and (4-b). The results showed that the intoxicated groups had significantly rise (*P≤0.05*) in all inflammatory markers TNF-α, CRP, IL-6, IL-1β, EMAP-II levels, MPO activity and significantly decrease (*P≤0.05*) in immunoglobulins IgG and IgM concentrations as compared with control groups.

Table (4-a): Effect of zinc, copper and chromium on the inflammatory markers in experimental rats

Groups	TNF-α (Pg/ml)	IL-6 (P g/ml)	IL-1β (pg/ ml)	CRP (mg/L)	EMAP-II (Pg/ml)	MPO (ng/ml)
G1: (C)	5.9±0.4 ^{a}	5.4±1.6 ^{a}	78.9±2.0 ^{a}	0.93±0.22 ^{a}	150±6.0 ^{a}	8.2±0.1 ^{a}
G2 :(Zn1)	27.1±1 ^{b}	25.1±1.5 ^{b}	128±3.3 ^{b}	7.5 ±0.28 ^{b}	263±2.9 ^{b}	26.9±1.1 ^{b}
G3: (Cu1)	37.6±1.9 ^{bd}	54.9±2.2 ^{c}	170±2.7 ^{c}	13.9 ±0.6 ^{e}	525±3.7 ^{c}	55.4±0.95 ^{c}
G4 :(Cr1)	66.2±2.2 ^{c}	81.8±1.1 ^{d}	191±0.9 ^{d}	19.8±0.9 ^{d}	724±1.95 ^{d}	86.6±2.0 ^{d}
G5 : (C2)	7.2±0.3 ^{a}	8.3±0.2 ^{a}	81±1.0 ^{a}	1.4±0.3 ^{a}	161±3.0 ^{a}	9.3±0.1 ^{a}
G6 :(Zn2)	36.2±1.6 ^{bd}	40.6± 2.1 ^{e}	144±1.4 ^{e}	11.2±0.2 ^{c}	479±3.7^{e}	42±1.0 ^{e}
G7: (Cu2)	49.3±2.6 ^{d}	76.3±1.8 ^{f}	185±1.3 ^{d}	16.7±0.3 ^{e}	687±5.9 ^{f}	79 ±1.0 ^{d}
G8: (Cr2)	87.1± 4.0 ^{e}	93.2±0.6 ^{g}	213±1.4 ^{f}	25.0±0.5 ^{f}	865±1.7 ^{g}	98.9±1.0 ^{f}
LSD (P≤0.05)	**15.2**	**11.3**	**14.1**	**3.5**	**25.6**	**8.2**

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Table (4-b): Effect of zinc, copper and chromium on Immunoglobulins in experimental rats

Groups	IgG (mg/dl)	IgM (mg/dl)
G1 (C1)	1187±0.4 [a]	211±1.6 [a]
G2 (Zn1)	729 ±1.1 [b]	183±3.3 [b]
G3 (Cu1)	405±0.6 [c]	122±1.6 [c]
G4 (Cr1)	224±0.7 [d]	82.3±2.0 [df]
G5 (C2)	1190±0.4 [a]	210±1.2[a]
G6 (Zn2)	458 ±0.9 [e]	151±2.7 [e]
G7 (Cu2)	280±0.7 [f]	93.1±1.5 [d]
G8 (Cr2)	121±0.2 [g]	72.0±1.6 [f]
LSD (P ≤ 0.05)	**5.1**	**14.5**

P≤0.05, there are no significant difference between means have the same letters in the same column

3.7 Antioxidant and Oxidative Stress Parameters

In the current study, the effect of the three tested salts on free radicals-induced lipid peroxidation, as well as the antioxidant status was evaluated. The results in tables 5-a and 5-b revealed a significant decrease (*P≤0.05*) in the activity and level of antioxidant enzymes and parameters SOD, GSH, catalase, TAC and significant elevation (*P≤0.05*) in parameters of oxidative stress MDA and NO as compared to the control groups.

Table (5-a): The Effect of zinc, copper and chromium on antioxidant markers in experimental rats

Groups	Blood SOD (U/mL)	Serum Catalase (U/L)	Blood GSH (mg/dL)	Serum TAC (mM/L)
G1:(C1)	71.0± 2.9 [a]	112±2.0 [a]	21.5±1.3 [a]	1.8±0.05 [a]
G2:(Zn1)	53.9 ±3.1 [b]	85±2.6 [b]	14.0±1.5 [b]	0.94±0.02 [b]
G3: (Cu1)	34.6 ±1.3 [cd]	66.7±3.2 [c]	9.3±0.97 [bc]	0.56±0.06 [c]
G4:(Cr1)	23.3±0.8 [ce]	30.3±3.0 [d]	5.6±0.7 [cd]	0.28±0.04 [d]
G5: (C2)	74.0± 0.8 [a]	116±1.0 [a]	23.5±1.0 [a]	1.9±0.5 [a]
G6:(Zn2)	43.4±1.7 [db]	76.2±1.8 [cb]	10.9±0.97 [bc]	0.82±0.07 [e]
G7:(Cu2)	26.2±0.8 [ce]	47.1±2.8 [e]	7.5±0.7 [bdc]	0.4±0.04 [f]
G8: (Cr2)	15.5±1.0 [e]	21±1.7 [d]	4.0±0.61 [d]	0.15±0.06 [g]
LSD (P ≤0.05)	**12.3**	**16.8**	**6.7**	**0.1**

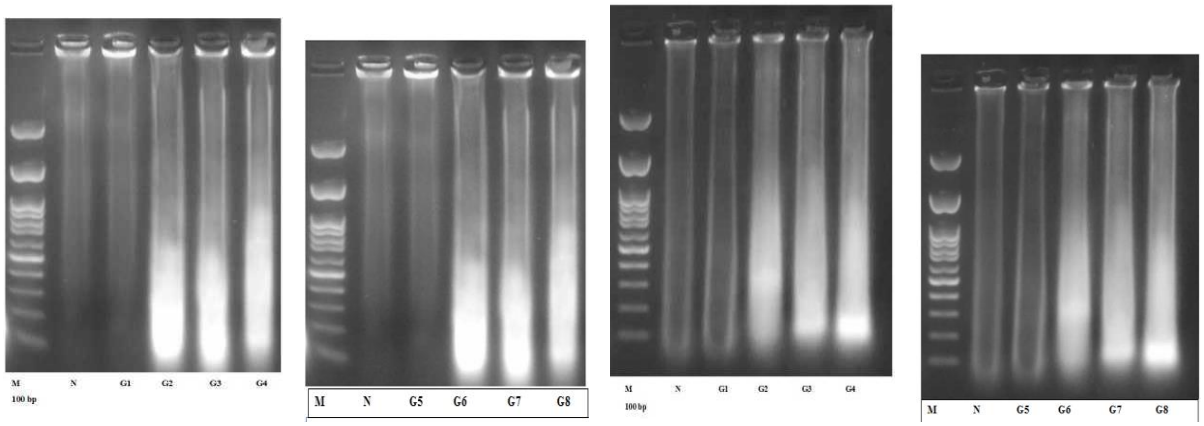
Table (5-b) The Effect of zinc, copper and chromium on oxidative stress markers in experimental rats

Groups	Serum MDA (nmol/mL)	Serum NO (µmol/L)	OSI (Index) MDA/TAC
G1 : (C1)	1.4 ±0.1 [a]	23.8±1.3 [a]	0.7±0.08 [a]
G2: (Zn1)	8.9 ±0.3 [b]	53.9±1.2 [b]	9.4±0.5 [a]
G3 : (Cu1)	18.5 ±1.1[c]	72.4±1.5 [c]	32.9±1.9 [ac]
G4 : (Cr1)	21.3±1.0 [cd]	106±3.1 [d]	76.4±4.2 [b]
G5: (C2)	2.1 ±0.1 [a]	26.1±0.6 [a]	1.7±0.8 [a]
G5: (Zn2)	16.6 ±0.4[c]	86.6 ±1.7 [e]	20.2 ±0.5 [a]
G6 : (Cu2)	27±1.2 [d]	98.1±1.5 [ed]	65.8±3.0 [bc]
G7: (Cr2)	36.6±1.0 [e]	141±0.8 [f]	237±14.0 [d]
LSD (P ≤ 0.05)	**5.8**	**13.2**	**39.1**

P≤0.05, there are no significant difference between means have the same letters in the same column

3.8 DNA Fragmentation Percentage and Tissue Damage

Results of the percentage of DNA fragmentation and PC concentration in spleen and brain tissues also the serum LDH activity confirm the damage induced by Zn, Cu and Cr toxicity as presented in figures (3) and (4).



a b c d

Figure (3): the percentage of DNA fragmentation (a) spleen tissue after 4 weeks, (b) spleen tissue after 8 weeks, (c) brain tissue after 4 weeks and (d) brain tissue after 8 weeks for all intoxicated rats.

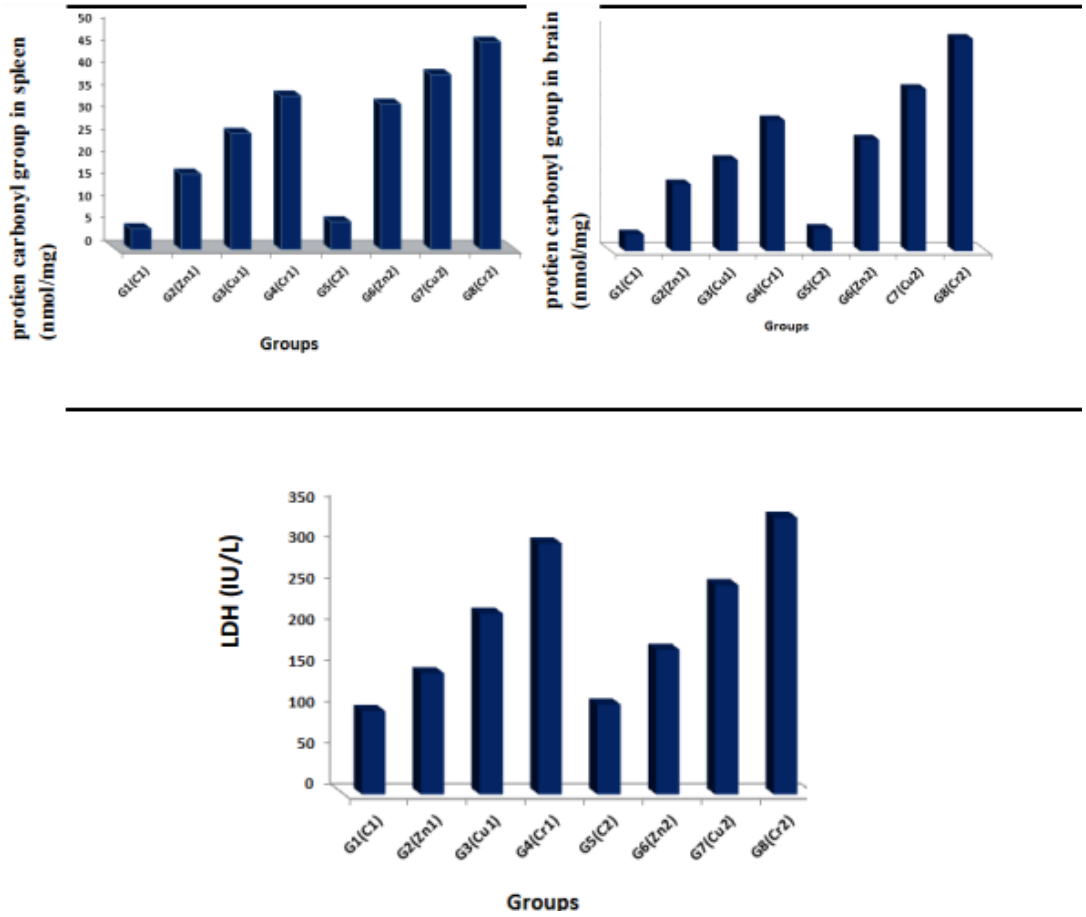


Figure (4): Effect of Zn, Cu and Cr on protien carbonyl group (PC) in spleen and brain (nmol/mg) and

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Serum Lactate dehydogenase activity LDH (IU/L(of intoxicated rats

3.9 Cytosolic and Mitochondrial Dysfunction Induced by Metals in Spleen and Brain Tissues

The results illustrated in figure (5) indicated cytosolic and mitochondrial dysfunction which manifested by sever reduction in IDH activity and significant elevation in CCO activity in spleen and brain in all intoxicated rats as compared with control groups.

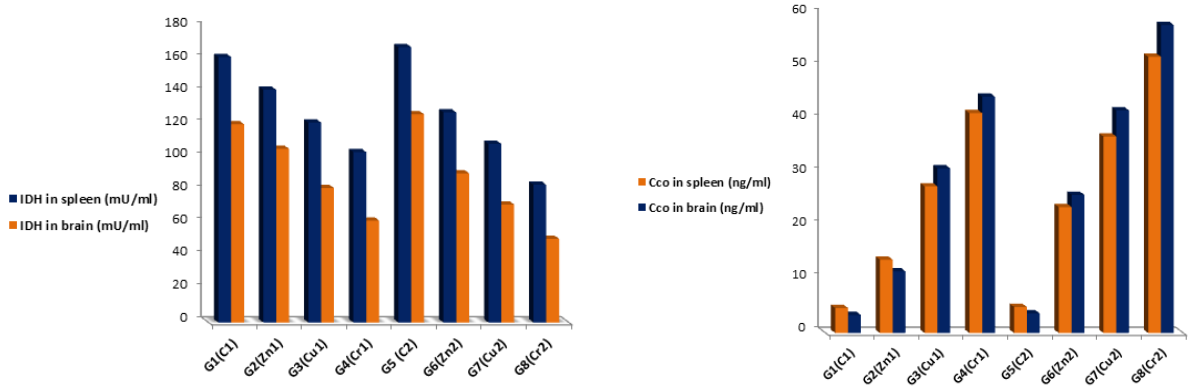
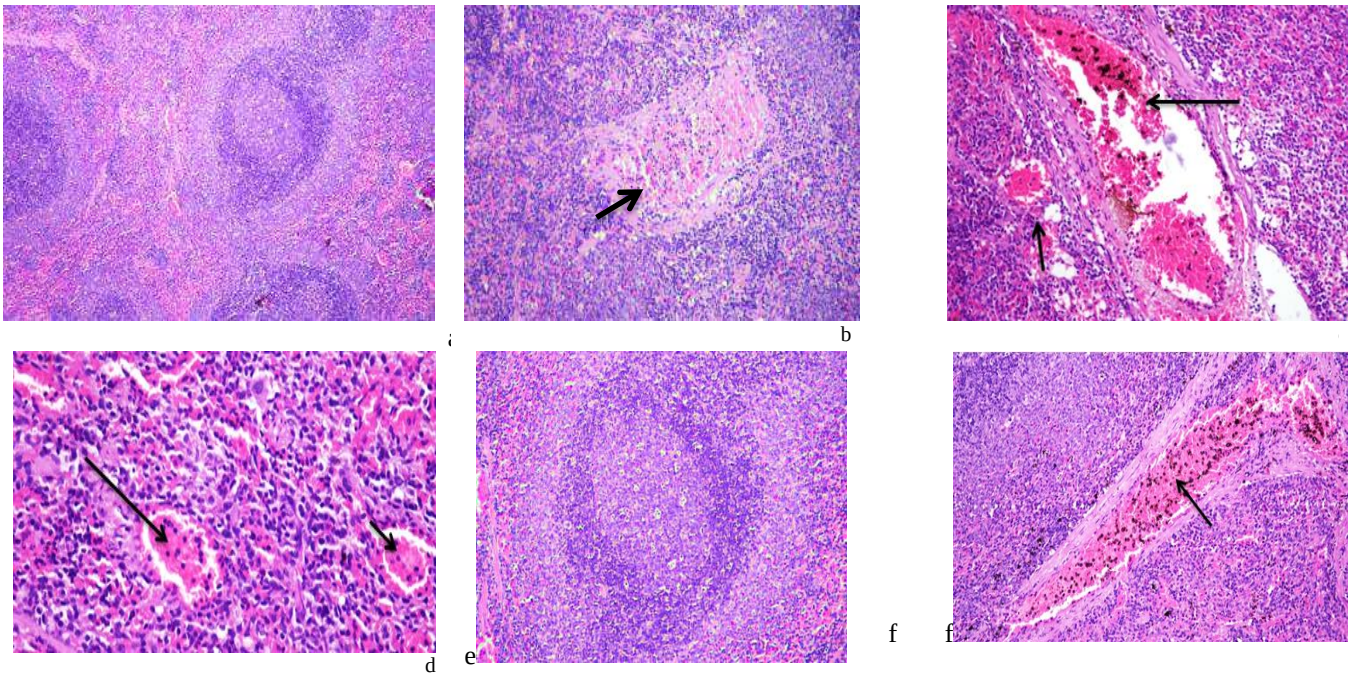


Figure (5): Effect of Zn, Cu and Cr on IDH Cco activities in spleen and brain (mU/ml) in all intoxicated rats

3.10 Histopathological Changes of Spleen and Brain Tissues

Microscopic examination of spleen and brain tissues from the control groups revealed normal histological structure. Adversely, the intoxicated groups treated with the three metals for 8 weeks showed severe pathological features. While intoxicated groups treated with three metals for 4 weeks showed moderate pathological features. The results of microscopic examination of spleen and brain tissues are illustrated in figures (6and7).



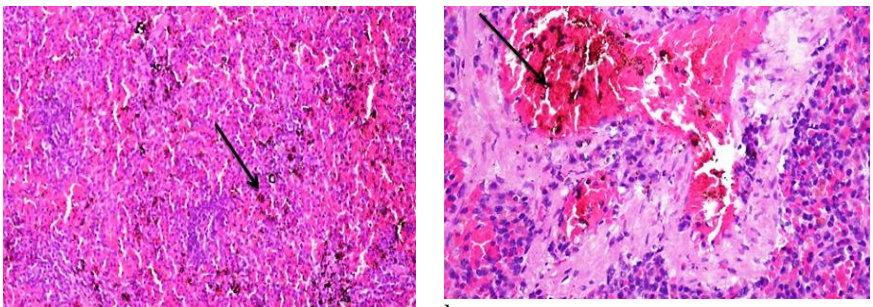


Figure (6): *Photomicrograph of rat brain with hematoxylin and eosin (H & E) following metal exposure*. a- G1(C1) showing normal histological structure, b-G2 (Zn1) showing congestion and thickening of central arteriole (arrow), c- G3(Cu1) showing congestion of central arteriole (long arrow) and sinusoids (short arrow), d-G4(Cr1) showing congestion of sinusoids (long arrow) and hemorrhage (short arrow): e- G5(C2) showing normal histological structure, f- G6(Zn2) showing congestion and thickening of central arteriole (arrow), g- G7 (Cu2) showing hemosiderosis and lymphoid depletion in white pulp, h- G8 (Cr2) showing thickening and congestion of central arteriole (arrow).

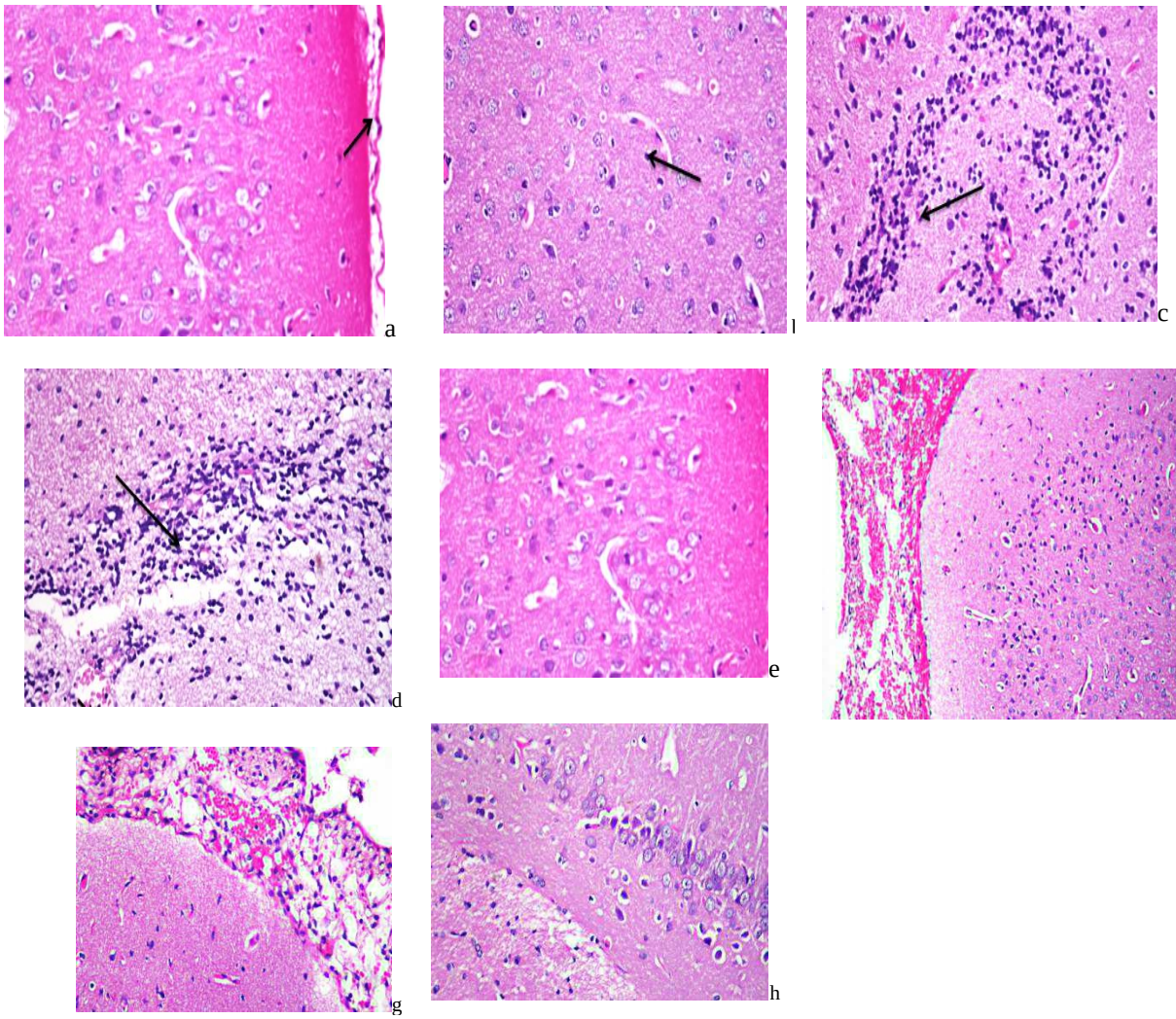


Figure (7): *Photomicrograph of rat brain with hematoxylin and eosin (H & E) following metal exposure*. a- G1(C1) normal histological structure of cerebral cortex and meninges (arrow), b-G2 (Zn1) showing hypertrophy of tunica intima of cerebral blood vessels (arrow), c- G3(Cu1) showing neuronal degeneration

with focal gliosis (arrow), d-G4(Cr1) showing gliosis (long arrow) and capillary congestion (short arrow): e-G5(C1) normal histological structure of cerebral cortex and meninges f- G6(Zn2) showing severe meningeal hemorrhage (arrow), g- G7 (Cu2) showing meningeal congestion and edema (arrow), h- G7 (Cr2) showing degeneration of pyramidal cells of hippocampus (arrow)

4. Discussion

Zinc, copper and chromium are a necessary trace elements for many of biological functions but prolonged exposure can cause side effects [20]. It is important to carefully monitor the levels of these elements in the environment and to minimize exposure to toxic concentrations [4]. The present work was designed to study the negative impact of zinc acetate dihydrate, copper sulfate pentahydrate and potassium dichromate chronic exposure on various organs such as spleen, brain, liver and kidney; Our experiment was carried out also to study how the exposure period aggravates the deleterious effects in intoxicated rats this was proved by measuring changes in the biological and biochemical parameters after 4 weeks and 8 weeks.

The current results showed that in a time dependent manner there were a significant accumulation of zinc, copper and chromium in spleen and brain tissues of experimental rats as compared with control. This is consistent with previous findings reported by [16], [29] who demonstrated an increase in copper and chromium levels in brain, blood, spleen, and liver tissues in rats supplemented with copper sulfate and potassium dichromate, respectively. However, it is worth noting that the accumulation of these elements can be detrimental to the body, leading to toxic effects.

Hepatotoxicity and degeneration of hepatocytes induced by Zn, Cu and Cr salts revealed by massive increase in activities of liver enzymes as serum ALT, AST, ALP and γ-GT in intoxicated groups when compared with a healthy control group. Release of these enzymes from cytoplasm is an indication of damage of liver tissue and oxidative stress process and consumption of the liver glutathione that is one of the main antioxidant defense elements [71]. In addition plasma concentration of ALT is higher than AST in the cytoplasm which indicate inflammations or infections. But, in infiltrative diseases that damage the cytoplasmic and mitochondrial membranes, the AST is higher than ALT [17].

Displayed results also showed marked reduction in total protein, globulin, albumin and significant elevation in total bilirubin as compared with control. The reduction in total protein explained by [30] who found a significant decrease in the protein levels in rats treated with potassium dichromate as compared with control group and explained these by impaired protein production. A decrease in protein level in groups exposed to Cr was also observed in studies by [31], [67]. Moreover, [42] assessed the toxicity of copper and found the liver toxicity is clearly evident with increased serum bilirubin and transaminases.

Regarding nephrotoxicity which manifested by significant ($p<0.05$) increase in serum creatinine, urea and uric acid levels. This was in accordance with the results obtained by [42] who observed a severe degenerative changes in kidney tissues of rats that orally received copper salt once daily for 90 days as compared with control; as well as those of [2] who concluded that chronic administration of copper sulfate orally to rats for 14 days induced nephrotoxicity. In line with our results [59] said that the peroxidative damage by Cr causes reduction in kidney function, which was reflected by significant increase in serum levels of blood urea nitrogen and creatinine suggesting nephrotoxicity.

Kidney is the site for synthesis of erythropoietin, the primary hormone that stimulates red blood cell generation in bone marrow [50]. The nephrotoxicity induced by metals led us to expect their effect on erythropoiesis through mechanisms include disruption of heme synthesis, iron accumulation and

erythropoietin production [50]. In accordance with our results [24] found microcytic hypochromic anemia in rats fed Zn excess diets as compared with control. Moreover [79] reported that in rats treated with $CuCl_2$ orally for 28 consecutive days; a significant increase in WBC, lymphocytes, RBC and significant decrease in HCT and PLT counts in copper treated group. While [20] found that large doses of copper induced decrease in Hb, the destruction of red blood cells, and as a consequence, it can lead to the development of anemia. [30] reported that oral administration of 5 mg/kg potassium dichromate was administered for seven days caused a significant decrease in hematological indices such as Hb, PCV, RBC, MCH, and MCHC.

In addition, our results agreed with [36], [67] who reported that Cr (VI) administration caused marked increment in WBC's as compared to control as well as they concluded that treatment by Cr (VI) lead to high production of immature WBC's and leukocytosis portraying a stimulation of the defense mechanism after 30 th day.

As copper plays an important role in iron metabolism and heme synthesis the excess of copper leads to hemolytic anemia and methemoglobinemia. Also, Cu toxicity induce oxidative stress leads to inflammatory reactions that causing a significant increasing in WBC's in blood as well as a significant decrease in platelet counts [20]. On the other hand, [33] explained the direct effect of heavy metals on the hematopoietic stem cells in the spleen and kidney with unusual membrane permeability, thereby leading to anemia by reducing the oxygen supply as a result of decreased red blood cell concentration and hemoglobin level.

The present results revealed that chronic exposure for zinc, copper and chromium increased lipid peroxidation and disturbed antioxidants status in all intoxicated groups as compared to healthy control groups. In the studdy of [39] high doses of Cu could induce oxidative stress, by increasing the levels of ROS and decreasing the activities of GSH, as well as activities and mRNA expression levels of antioxidant enzymes as SOD, CAT, and GSH-Px and increase the contents of MDA and hydroxyl radical.

Our observations was in agreements with the results by [72], [20] who reported that high exposure to copper sulfate and potassium dichromate cause severe alteration in antioxidant defense system and result in a significant increase in oxidative stress in albino rats that orally administrated copper sulfate and potassium dichromate. While [42] reported that copper sulfate induce oxidative stress and deplete the antioxidants defense system by decrease the activity of GSH and SOD as well as increase in lipid peroxidation levels as compared with the control. Also, our results were supported by [17] who reported that in rats that received daily dose of potassium dichromate; chromium motivated oxidative stress process, through producing significant down regulation of the gene expression levels of the anti-oxidant genes (CAT, SOD and GPx) as compared to control rat.

Concerning neurotoxicity, announced results clearly indicated that zinc acetate dihydrate, copper sulfate and potassium dichromate pentahydrate has significantly altered the levels of dopamine, serotonin, norepinephrine, and γ- amino butyric acid in all intoxicated rats; Maximum alteration was noticed in chromium intoxicated groups followed by copper intoxicated groups then zinc intoxicated groups. Our results confirmed the results of [60] who reported a significant reduction in serotonin and dopamine level in Cr-intoxicated group that received 10 mg/kg b.w. of potassium dichromate orally.

[28], [49] observed that copper exposure significantly increased the pro-inflammatory cytokine TNF- α, IL-6, IL-1β whereas an anti-inflammatory cytokines level such as IL-4 level remained inhibited. However, [40] stated that IL-1β and TNF –α was increased significantly in the potassium dichromate group as compared with control. While [26] indicated that a dose- time-dependent brain oxidative stress and inflammatory

reaction were induced in rats by intranasal instillation of potassium dichromate. These findings also showed that the effect of the highest dose of potassium dichromate and longer duration was more destructive than that conveyed either by the other doses of potassium dichromate or through shorter durations.

Recent studies clarified the mechanism of brain injury through the overproduction of TNF-α, IL-1β, and other inflammatory mediators and many other inflammatory factors, which increase the inflammatory immune response to damage, further altering the function of synapses and neurons [26], [28]. our study confirmed that exposure for zinc, copper and chromium produce negative impact on immune system which manifested by sever decline in IgG and IgM titre in all intoxicated rats these might be due to a significant depletion of spleen as observed histopathologically.

In accordance with our observations, results announced by [79] showed that copper induced a significant decrease in IgG and IgM levels as compared with control in rats that received $CuCl_2$ 200 mg/kg by intragastric administration every morning continuously for 28 days. Although, [27] suggested that chronic exposure to Cr is associated with impaired immune function in male tannery workers which manifested by significantly decrease of serum IgG as compared with unexposed control subjects.

Our results confirmed significant elevations in protein carbonyl groups and DNA fragmentation in spleen and brain tissues and serum LDH of all intoxicated rats as compared to control that indicated sever cell damage. [46] reported that bioaccumulation of high concentration of metals such as zinc, copper and cadmium have apoptotic and/or necrotic effects over cells of different organs and induced a significant increase in DNA fragmentation. As well as, [62] confirmed that Cu provoked DNA fragmentation, in brain of rats received 100 mg/kg $CuSO_4$ and suppose that generated ROS are the potent oxidizing agents that provoke the oxidative damage of lipids, proteins, and DNA, leading to lipid peroxidation, DNA breaks, and other deleterious effects.

Further, [11] displayed various mechanisms perceived as the way Cr toxicity occurs been proposed ranging from free radical production and formation of adducts of Cr-DNA, which are supposed to be stable to double as well as single-strand breaks in the DNA and cross-links between DNA and the proteins. Apoptosis has also been to occur due to Cr accumulation in cells, which is mediated by the production of ROS. Studies have shown that most of the damage due to Cr toxicity has been due to an upsurge in ROS production.

[25] also support the theory that Cr induces tissue damage via induction of oxidative stress. Due to structure similarity of chromate and sulfate, Cr can enter the cell and induces DNA damage not only by direct binding to DNA but also by reacting with H_2O_2 to produce ROS, which damage lipids, DNA, and other macromolecules through promote oxidative stress. On the other hand, [48] reported that LDH activity was significantly increased in the kidney tissue of Cr-treated mice compared with that of the control group. In agreement with our study [5] showed that metals such as chromium can disrupt DNA synthesis and repair. The toxicity and carcinogenicity of heavy metals are dose dependent as well as high- dose exposure leads to sever responses in animal and human which causes more DNA damage and neuropsychiatric disorders. [60] reported that ;Cr(VI) altered protein pattern and caused DNA damage in brain tissue in Cr- intoxicated group received10 mg/kg b.w. of potassium dichromate orally by gavage. Our results indicated cytosolic and mitochondrial dysfunction which manifested by significant elevation in Iso-citrate dehydrogenase (IDH) activity and sever reduction in cytochrome C oxidase (CCO) activity in spleen and brain in all intoxicated rats as compared with control.

The histopathological results indicate significant morphological changes in spleen of all intoxicated rats. The results of our study corroborate with [51] who evaluated the effects of single-dose intragastric administration

of 100 mg/kg zinc salt on the structure and function of organs in rats after treatment; they confirmed that the analysis of the brain structures in rats showed that the soft meninx could be traced in the form of small fragments, and the vessels were mostly full-blooded. While, [79] demonstrated that Cu induced obvious spleen damage in rats received $CuCl_2$ group, 200 mg/kg by intragastric administration for 28 days. In accordance with our study [59] reported that rats which received a single dose of potassium dichromate showed histopathological alterations in brain.

5. Conclusion

Collectively, our findings suggest that the oral administration of zinc acetate, copper sulfate, and potassium dichromate induces the accumulation of these heavy metals in the spleen and brain tissues of rats, leading to activation of free radical oxidation processes and to micronutrients' imbalance which manifested by increasing the levels of ROS and PC, suppressing the ability to scavenge free radical and reducing the activities of antioxidant enzymes, which can contribute to the development of histopathological and biochemical changes in spleen, brain, liver, kidney and inflammatory response in a time dependent manner. These results underscore the importance of monitoring heavy metal exposure and implementing measures to prevent or minimize its harmful effects.

Conflict of Interests

There are no conflicts of interest.

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