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RESEARCH ARTICLE

Effect of Copper Sulphate Pollution and its Antidote Penicillamine on Liver and Serum Markers of Albino Rats

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Abstract

Copper is an essential trace element and is required for many metabolic functions. The present study was designed to study the negative impact of excess copper sulphate ($Cuso_4$) on liver of albino rats and studying how its antidote d-penicillamine play role in the development of its side effects. Seventy albino rats were divided into seven equal groups each containing 10 rats (G_1:control group received distilled water) ; (G_2: 0.1 LD_{50} of $CuSO_4$) ; (G_3: 0.2 LD_{50} of $CuSO_4$) ; (G_4: 0.4 LD_{50} of $CuSO_4$) ; (G_5: 0.1 LD_{50} of $CuSO_4$ +100 mg/kg/day of penicillamine) ; (G_6: LD_{50} of $CuSO_4$.+100 mg/kg/day of penicillamine) and (G_7: LD_{50} of $CuSO_4$+ 100 mg/kg/day of penicillamine) for 30 days. At the end of the experiment all rats were sacrificed, and blood samples and liver tissues were collected for biochemical and molecular assaying. The result showed that administration of copper sulphate with different levels induced a significant increase in fasting blood glucose level (FBG), lipid peroxidation marker (MDA), serum copper level, white blood cells (WBCs) and serum tyrosinase activity, but a significant decrease in total antioxidant activity (TAC), Hb and platelet count. Moreover, copper sulphate administration elicited a significant ($P < 0.05$) downregulation of cytochrome c oxidase (Cyto co) and glucose -6- phosphate dehydrogenase (G6PD). It could be approved that d-penicillamine (DPA) can decrease the negative impact of copper sulphate on hepatic tissues and serum enzymes. DPA can reduce hepatotoxicity and oxidative stress caused by copper pollution.

Keywords: D-Penicillamine, Copper sulphate pollution, Liver, Gene expression.

Introduction:

Heavy metals are metallic intrinsic components of the environment. Their presence is considered unique in the sense that it is difficult to remove them completely from the environment once they enter in it. Metal constitutes an important class of toxic substance which are encountered in numerous occupational and environmental circumstances [1].

Copper is not lethal in its metallic state, but several of its salts are poisonous, particularly the sulphate and sub-acetate salts. High exposure to copper, it induces

toxicity [2]. The major sources of copper are electroplating, fertilizer, pesticide, and iron and steel industries [3]. Copper's function as a cofactor in numerous copper proteins contributes to its role in various physiological processes and as a cofactor in oxidoreductase enzymes. It participates in the production of melanin by oxidizing tyrosine and dopamine to aldehydes and tyrosinase [4]. Mitochondrial cytochrome c oxidase is a copper-containing enzyme complex that plays a role in mitochondrial respiration [5]. Ceruloplasmin (Cp), a copper-dependent oxidase, transforms Fe2+ divalent iron to Fe3+, and this mechanism is critical for transferrin-mediated iron transport in the plasma [6]. The liver, which stores most of the copper, is the principal organ responsible for its processing. In hepatocytes, ATP7B goes into vesicles at the apical membrane in response to elevated copper levels, transferring excess copper to the bile [7]. Excess copper can cause cell necrosis, which can be followed by an inflammatory response. ROS, which is created by copper in the Fenton reaction, can trigger and/or amplify the inflammatory process [8]. ROS are signal transduction pathway mediators that can trigger the production of pro-inflammatory cytokines in cells that cause damage to protein, lipid, and nucleic acid structures [9].

DPA is a byproduct of penicillin without any antibiotic properties. It is a chelator for heavy metals, such as copper and lead. Penicillamine is absorbed from the gastrointestinal tract (GIT), it’s an oral bioavailability between 40 and 70%. In humans, DPA is used to treat several diseases, including Wilson’s disease, cystinuria, rheumatoid arthritis, and it is used in liver disease treatment caused by the accumulation of excess copper. Adverse reactions for DPA are rash, nausea and thrombocytopenia, proteinuria and have been associated with nephrotoxicity, bone marrow toxicity, and skin elastosis [10].

DPA effects on hepatic copper content by the metal chelating mechanism. Copper levels are reduced by chelating systems that cause increased copper excretion in urine by forming soluble complexes with copper [11].

The current work aims to study the negative impact of copper sulphate pollution on enzymes in serums and organs especially liver tissues and how decreasing its side effects by using its antidote d-penicillamine.

Materials and methods

Experimental design

The experiment was done according to the general rules of the National Institutes of Health (NIH) for the Care and Use of Laboratory Animals in scientific investigations and affirmed by the Ethics of Animal Use in Research Committee (EAURE). The protocol of this study has been reviewed and approved by ZU-IACUC/2/F/62/2023 committee.

Seventy male adult Albino rats with mean body weight 200 ± 10 g were used in the experimental investigation of this study. Rats were purchased from the Central Animal House of Faculty of Veterinary Medicine, Zagazig University, Egypt. Rats were housed in the research building, Faculty of Veterinary Medicine, Zagazig University under conventional laboratory conditions throughout the period of the experiment. The animals were fed a standard rat pellet diet and allowed free access to water. Rats were kept at constant environmental and nutritional condition throughout the periods of the experiment and received a balanced ratio. All animals were acclimatized for a minimum period of three weeks prior to the beginning of the study. The rats were divided into seven equal groups each containing 10 rats (G_1:control received distilled water) ; (G_2:0.1 half of lethal dose (LD_{50} 472 mg/kg of BW) of Copper sulphate hydrate ($Cuso_4.5H_2O$; Sigma-Aldrich, Chemical Cp. St. Louis, Mo, USA); (G_3:0.2 LD_{50} of $CuSO_4$); (G_4:0.4 LD_{50} of $CuSO_4$); (G_5:0.1 LD_{50} of $CuSO_4$ +100 mg/kg of BW /day of d-penicillamine; Sigma-Aldrich, Chemical Cp. St. Louis, Mo, USA); (G_6: 0.2 LD_{50} of $CuSO_4$.+100 mg/kg of BW /day of penicillamine) and (G_7: 0.4 LD_{50} of $CuSO_4$+ 100 mg /kg of BW/day of penicillamine)

orally by gastric tube for 30 days [12, 13]. At the end of the experiment all rats were weighted and anesthetized by thiopental sodium (EIPICO, Chile) (500 mg) diluted in 12.5 mL distilled water, the injected dose is 0.1 ml for each 100 g of BW of rats intraperitoneally [14] then sacrificed and blood samples and brain tissues were collected for biochemical assaying and relative gene expression of Cytochrome c Oxidase, and Glucose-6- Phosphate Dehydrogenase.

Sampling

Blood samples were collected from the orbital venous plexus. The capillary tube was inserted into the medial canthus of the eye (30-degree angle to the nose), and with slight thumb pressure, the blood came through the capillary tube, kept for a time, centrifuged at 3000 r.p.m for 15 min, the resulting supernatant was collected and used for the measurement of biochemical parameters [15]. Another sample was collected in 3 ml lavender-top (K2EDTA) tube for a complete blood count (CBC) test. All samples were kept cool (at refrigerated temperature, but not frozen) during storage and shipping to minimize changes in cells that can occur with storage.

Tissue samples

Immediately after scarifying, liver tissues were taken, weighted, and every sample was divided into 2 parts, the first part was wrapped in aluminum foil and put immediately in a liquid nitrogen container to make snap-freezing of tissue and minimize the action of endogenous RNases for molecular investigations. The second part was kept at -20 ºC to be homogenized for antioxidants measurements.

Biochemical determinations

Tyrosinase concentrations were assayed using (Tyrosinase Activity Assay Kit, Abcam, USA, Cat. No. ab252899) following colorimetric method Qu *et al*. [16], Serum copper was determined using (Copper Assay Kit, Sigma-Aldrich , USA, Cat. No. MAK127) following the method of Changfeng *et al*. [17]. Serum fasting glucose levels were determined using (Glucose Assay Kit, Abcam, USA, Cat. No. ab65333) as described by Saw *et al*. [18], Total Antioxidant (TAC) in liver tissue was determined using (Total Antioxidant Assay Kit, Elabscience Biochemistry Inc, USA, Cat. No. E-BC-K136-S) following colorimetric assay of Marziyeh *et al.* [19]. Malondialdehyde (MDA) in liver tissue was assayed using (Lipid Peroxidation (MDA) Assay Kit, Abcam, USA, Cat. No. ab118970) using a method adapted to Wang *et al.* [20]. Hemoglobin, White Blood Cells, and Platelet counts were determined by the automatic cell counter as previously described [21].

Molecular determinations of hepatic Cytochrome C Oxidase and Glucose-6-Phosphate Dehydrogenase (CYTO C and G6PD)

The real-time polymerase chain reaction procedure was carried out for determination of hepatic Cytochrome C Oxidase and Glucose-6- Phosphate Dehydrogenase as described before [22]. Total RNA was isolated from 50 mg of liver tissues using Trizol (Invitrogen; Thermo Fisher Scientific, Ink.). The concentrations of extracted RNA were determined using Nanodrop® system spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, Delaware USA). A Rotor-Gene Q 2 Plex (Qiagen, Germany) real-time PCR system was used to perform real-time RT-PCR with a total reaction volume of 20 µL containing 10 µL of top real syber Green master mix (Enzynomics, Korea), 1 µL of cDNA templet, 1 µL of both forward and reverse oligonucleotide-specific primers of cytochrome c oxidase and Glucose-6- Phosphate Dehydrogenase) (Beijing, China) **(Table 1)**, and nuclease-free water up to 20 µL. The cycling condition of initial denaturation at 95°C for 12 min was followed by 40 cycles of denaturation at 95 °C for 20 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 30 sec. A melting curve analysis was performed following PCR amplification. The expression level of the target genes was normalized using the mRNA expression of a known housekeeping gene (Gapdh). Results were expressed as fold-changes compared to the control group

following the $2^{-\Delta\Delta Ct}$ method [23]. Online Primer3 software (https:// bioinfo. ut.ee/ primer3-0.4.0/) was used to design gene-specific primers, which supported the cDNA sequences in GenBank, and the NCBI BLAST (http://blast.ncbi.nlm.nih.gov/) software was used to confirm all sequences.

Table 1: Primers used in real-time RT-PCR

Primer	Primer (5-3) sequence	Product length (bp)	Gene Accession No.
Gapdh		91	NM-017008.4
F	GCATCTTCTTGTGCAGTGCC		
R	GGTAACCAGGCGTCCGATAC		
G6pd		177	NM-017006.2
F	TGAGGACCAGATCTACCGCA		
R	TCAAAATAGCCCCCACGACC		
CYTO C		71	NM 012812.3
F	GGAACCACACGCTTTTCCAC		
R	GAGTCTTCAAGGCTGCTCGT		

Statistical analysis

To assess the influence of excess copper and penicillamine on different enzymes, one-way ANOVA was followed by least Significant Difference (LSD) test as a post hoc test**.** Homogeneity of variance of sample groups was checked using Levene's test.

All Analysis was done using Statistical Package for Social Sciences version 24.0 (SPSS, IBM Corp., Armonk, NY), and charts were done by Graph Pad prism 8.0.2 (GraphPad Software, Inc).

Results were reported in means ± Standard Error of Mean (SEM) and probability value. P – value < 0.05 was considered statistically significant.

Results

Effect of penicillamine on the mean value of tyrosinase, FBG, and Cu levels in copper sulphate toxicity induced in rats

The outcomes of the present investigation showed a significant ($P < 0.05$) increase in serum tyrosinase, FBG, and serum Cu^{+} level in $CuSO_4$ toxicity induced in groups 2, 3, and 4 compared with the control group (G1). On the other hand, G5, G6, and G7 with CuSO4 cytotoxicity and treated with penicillamine showed a significant mean value ±SE ($P <$ 0.05) decrease in serum tyrosinase, FBG, and Cu level **(Figure 1).**

Effect of penicillamine on the mean values of Hb, WBCs, and platelet counts in copper sulphate toxicity induced in rats

The results showed a significant ($p < 0.05$) decrease in Hb and platelet count but an increase in WBCs in $CuSO_4$ treated groups (G2, G3, and G4) compared with a control group (G1). However, d-penicillamine administration induced a significant (p <0.05) increase in Hb and platelet count but a significant ($p < 0.05$) decreasing in WBCs compared to groups with induced copper cytotoxicity (G 5, G6, and G7) **(Figure 2).**

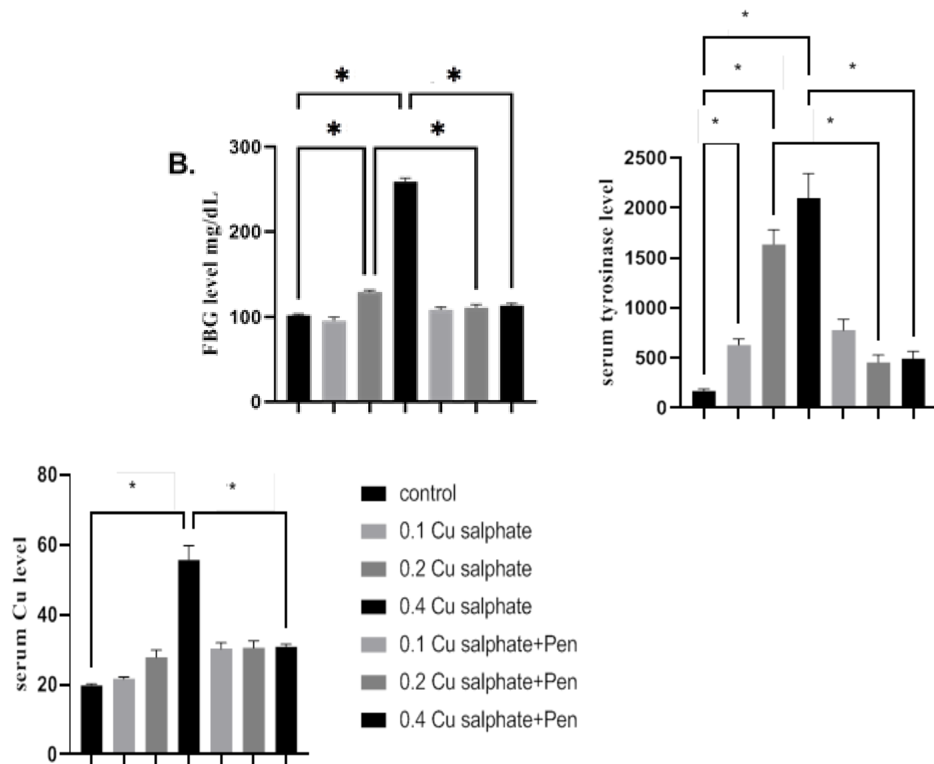

Fig. 1: Effect of penicillamine on the mean values of (tyrosinase, Fasting Blood Glucose, and Copper ions level) in copper sulphate toxicity induced in rats. (*) indicate a significant mean value ±SE ($P < 0.05$)

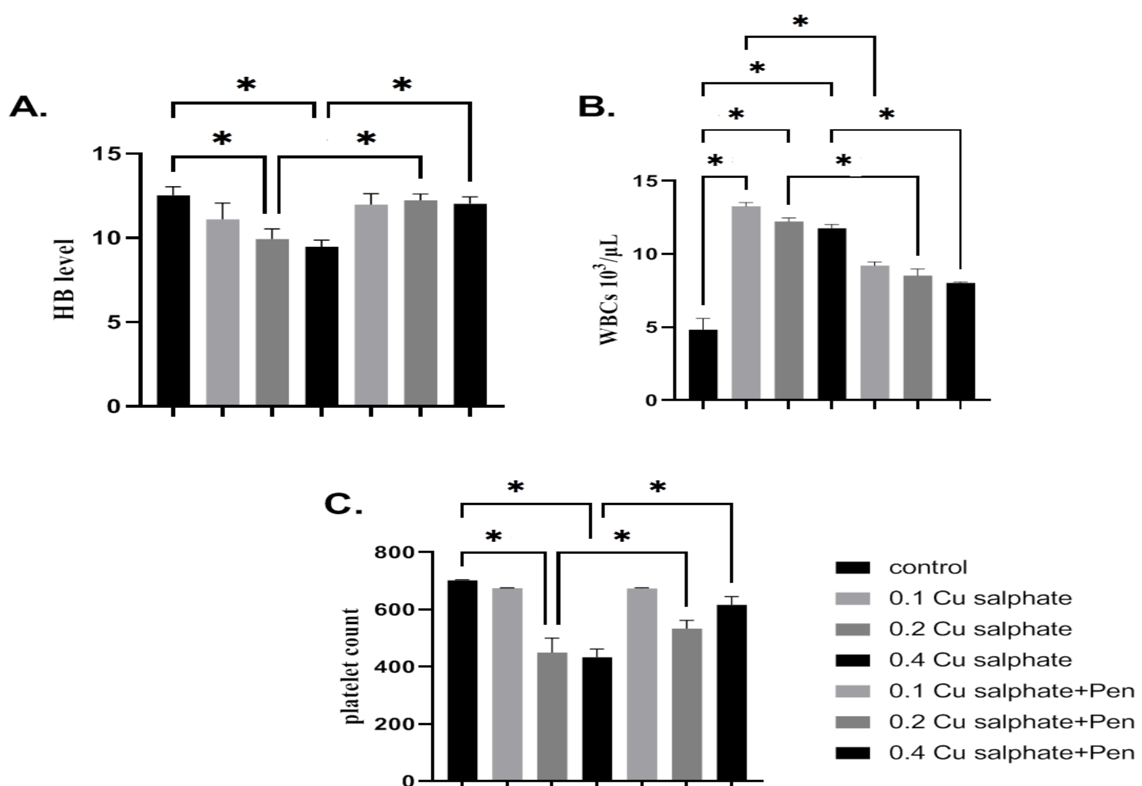

Fig. 2: Influence of penicillamine on the mean values of (hemoglobin, white blood cells and platelet counts) in copper sulphate toxicity induced in rats. (*) indicate a significant mean value ±SE ($P < 0.05$)

Effect of penicillamine and copper sulphate toxicity induced in rats on the antioxidant enzymes

In comparison with the control group, rat groups with induced CuSO4 cytotoxicity showed a significant mean value ±SE increase of MDA enzyme. However, we noticed a significant decrease of TAC enzyme in serum, and these results were proportional with the dosage amount (0.1, 0.2, 0.4 LD_{50} of CuSO4) respectively.

On the other hand, rat groups induced cytotoxicity with CuSO4 and treated with penicillamine, showed a significant mean value ±SE decrease in MDA enzyme, while TAC enzyme levels significantly increased mean values ±SE **(Figure 3).**

Effect of penicillamine and copper sulphate toxicity on the hepatic G6PD and Cyto c-o mRNA relative expression

We noticed a significant ($p < 0.05$) downregulation in mRNA expression of the hepatic G6PD and Cyto c-o in $CuSO_4$ toxicity induced in groups, where the downregulation was proportional with the dosage groups of 0.1, 0.2, 0.4 LD_{50} of CuSO4, respectively. While, when CuSO4 cytotoxicity induced groups (G5, G6, and G7) were treated with d-penicillamine it showed a significant ($p < 0.05$) upregulation of expression levels in comparison with the control group (G_1) **(Figure 4).**

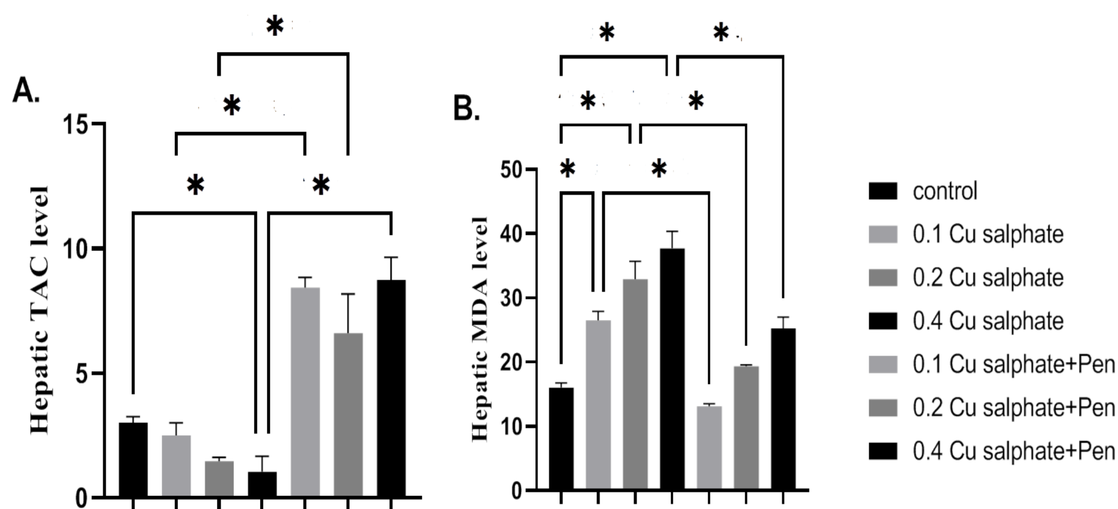

Fig. 3: Impact of penicillamine on the mean values of antioxidant enzymes (Hepatic Total Antioxidant and Malondialdehyde) in copper sulphate toxicity induced in rats. (*) indicate a significant mean value ±SE (*P* < 0.05)

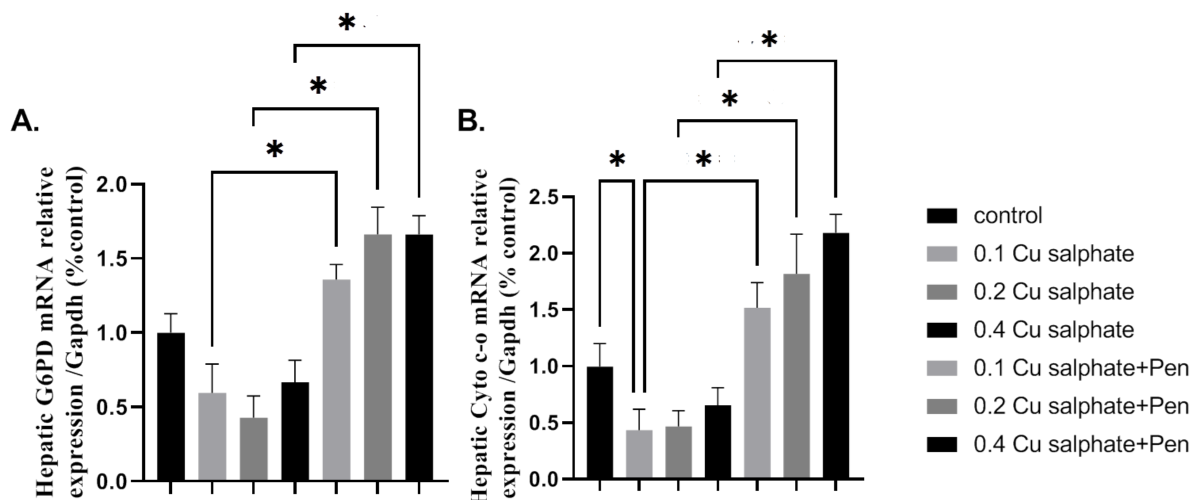

Fig. 4: Effect of penicillamine on the mean values of hepatic G6PD and Cyto c-o mRNA relative expression in copper sulphate toxicity induced in rats. (*) indicate a significant mean value ±SE (*P* < 0.05)

Discussion

Copper is a necessary trace element for many of biological functions and prolonged exposure to an excess concentration of copper ions can cause side effects [24]. Our experiment was carried out to study the efficacy of DPA in experimental copper intoxication induced in rats, also to study the negative impact of copper intoxication on liver tissues and serum markers and using its antidote DPA to decrease adverse effects of $CuSO_4$ pollution.

Tyrosinase belongs to a type 3 copper enzyme family with a binuclear copper center. The results showed that $cuso_4$ pollution increased the level of tyrosinase in serum. Intracellular copper levels are tightly controlled by transmembrane transporters including (ATP7A and ATP7B, respectively) and metallochaperones [25]. Copper is transported into the hepatocyte secretory route via ATP7B, as well as loading copper into a large number of other copper-dependent enzymes including tyrosinase [26].

Copper poisoning has long been thought to play a role in the development of type 2 diabetes. It is also known that the presence of copper ions facilitates the generation of reactive oxygen species (ROS). ROS are produced in diabetic patients and have been linked to the development of type 2 diabetes [27]. The obtained findings showed that $cuso_4$ pollution induced a significant increase in FBG due to disruption in carbohydrate metabolism, potentially due to increased breakdown of hepatic glycogen (glycogenesis) and extrahepatic tissue proteins and amino acids are used to synthesis glucose and in heavy metals – induced hypoxia [28]. Glucose may also be released into circulation, enhancing catecholamine mobilization, and the glycogenesis process by cortisol [27]. However, DPA induced hypoglycemia as it increased beta-cell insulin secretion [29].

Increasing serum copper levels may indicate excessive copper exposure or be linked to disorders that reduce copper excretion. Excess $cuso_4$ raises the level of free copper in serum in untreated albino rats. This is due to a mutation in ATP7B resulting in an increase in copper concentration and accumulation in organs and tissues [30]. Because penicillamine chelates copper, it allows excess copper to be eliminated in the urine as a soluble copper complex. The results showed that G5, G6, and G7 that received penicillamine had lower copper levels in their serum [10].

G6PD is a housekeeping enzyme which reduces NADP+ to NADPH in the first step

of the pentose shunt (PPP). In RBCs, the pentose shunt is the only source of NADPH. This is essential for maintaining high levels of Glutathione in the cell to protect it from oxidative stress-induced damage [31]. Excessive copper level induced mitochondrial stress and decreased mitochondrial membrane potential which activated the endoplasmic reticulum (ER) stress. Endoplasmic reticulum stress leads to a significant downregulation in biosynthesis of many proteins such as Glucose-6-Phosphate Dehydrogenase gene expression [32]. G6PD is a key player in a cellular energy balance as a member of pentose phosphate pathway; also it regulates the oxidant and antioxidant state of the tissues. Glucose-6-Phosphate dehydrogenase is responsible for the production of glutathione, and its inhibition induced an oxidation stress condition [33].

The finding of the current study showed that excess $CuSO_4$ induced in rats leading to a significant downregulation of G6PD relative expression so results showed decreased in TAC and increased in MDA.

The oxidation of RBCs is caused by free radicals produced by excess copper ions in body, resulting in decreasing hemoglobin solubility and irreversible precipitation of oxidized hemoglobin Copper penetrates erythrocytes and inhibits G6PD, which caused oxidative damage to the red cell membrane. Hemolytic anemia is caused by a mutation in the ATP7B gene on chromosome 13 and is linked to severe liver disorders [34].

We 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**.** Excess of copper leads to hemolytic anemia and methemoglobinemia as copper plays an important role in iron metabolism and heme synthesis in the body [35]. Also, oxidative stress leads to inflammatory reactions that causing a significant increasing in WBCs in blood [36]**.** A significant decrease in platelet counts were observed in groups 2, 3, and 4 excess copper causes hemolytic anemia that is caused either by direct cell membrane damage or indirectly as a result of the inactivation of enzymes that protect against oxidative stress [35].

Conclusions

It can be concluded that excessive intake of copper may be associated with various disturbances including hepatic disturbance, hemolytic anemia, and imbalance in oxidative stress. The side effects of copper pollution can be decreased by using its antidote d-penicillamine that has a chelating effect on copper ions and increasing its excretion in urine. Further studies should be performed for histological assessment for the changes in rat liver and measuring copper level in liver tissues.

Conflicts of interest

The authors declare no conflicts of interest.

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الملخص العربي

تاثير التلوث بكبريتات النحاس وترياقه البنيسيلامين على انزيمات الدم وانسجه الكبد للجرذان البيضاء

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يعتبر النحاس من العناصر الهامة للعلميات الحيوية داخل جسم الانسان ولكن زيادة نسبته بالجسم تؤدى الى اضطرابات فى الوظائف الحيوية. تهدف هذه الدراسة الى تقصى تاثير التلوث بكبريتات النحاس على أنسجة الكبد وإنزيمات الدم وكيفية الكشف المبكر للتلوث بالنحاس وذلك بإجراء التحاليل الكيميائية وتقليل مخاطره باستخدام عقار البنيسيلامين. تم تقسيم الجرذان إلى سبع مجموعات متساوية كل مجموعة تحتوى على عشر جرذان وتم معاملتها كالأتى: المجموعة الأولى: المجموعة الضابطة تم إعطائها محلول ملحى عادى عن طريق الفم بإستخدام اللى المعدى لمدة 30 يوم يوميا. المجموعة الثانية: جرذان يتم إعطائها عن طريق الفم (0.1 LD_{50} من وزن الجسم من كبريتات النحاس) لمدة 30 يوم يوميا. المجموعة الثالثة: جرذان يتم إعطائها عن طريق الفم (0.2 LD_{50} من وزن الجسم من النحاس) لمدة 30 يوم يوميا. المجموعة الرابعة: جرذان تم إعطائها عن طريق الفم (0.4 LD_{50} من وزن الجسم من النحاس) لمدة 30 يوم يوميا. المجموعة الخامسة: جرذان تم إعطائها عن طريق الفم (0.1 LD_{50} من وزن الجسم من النحاس + 100 ملجم/ كجم من وزن الجسم) لمدة 30 يوم يوميا. المجموعة السادسة: جرذان تم إعطائها عن طريق الفم (0.2 LD_{50} من وزن الجسم من النحاس + 100 ملجم/ كجم من وزن الجسم) لمدة 30 يوم يوميا. المجموعة السابعة: جرذان تم إعطائها عن طريق الفم (0.4 LD_{50} من وزن الجسم من النحاس + 100 ملجم/ كجم من وزن الجسم) لمدة 30 يوم يوميا. وقد تم تجميع عينات الدم في نهاية التجربة من كل مجموعة لفصل المصل لقياس التحاليل البيوكيميائية.كما تم أخذ عينات الكبد سريعا وذلك لتقييم الإجهاد التأكسدى والتعبير الجينى.

وقد أوضحت النتائج أن التلوث بكبريتات النحاس قد أدى إلى زيادة ملحوظة فى نسبةالسكر بالدم , نسبة النحاس بالدم , MDA ,كرات الدم البيضاء ونسبة إنزيم tyrosinase بالدم ولكن وجد إنخفاض ملحوظ فى TAC , Hb و Platelet Count ; كما أظهرت النتائج أن كبريتات النحاس أدت إلى إنخفاض ($P < 0.05$) ملحوظ فى التعبير الجينى ل Cyto (co) و (G6PD) .وعليه يمكن استنتاج أن عقار البنيسيلامين يؤدى إلى تقليل التاثير السلبي لكبريتات النحاس على أنسجة الكبد وإنزيمات الدم كما له دور فى تقليل الإضطرابات الكبدية والإجهاد التأكسدى الذى حدث بسبب التلوث بالنحاس