EFFECT OF COPPER SULPHATE POLLUTION AND ITS ANTIDOTE PENICILLAMINE ON SERUM AND BRAIN TISSUES MARKERS OF ALBINO RATS

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Background: Copper is an essential trace element and is required for many metabolic functions. Aim of work: The present study was designed to study the negative impact of excess copper sulphate on the central neural function of albino rats and studying how its antidote d-penicillamine play role in improving its side effects.

Material and methods: Seventy albino rats were divided into seven equal groups each containing 10 rats (G1: control received distilled water); (G2: 0.1 LD₅₀ of CuSO₄); (G3: 0.2 LD₅₀ of CuSO₄); (G4: 0.4 LD₅₀ of CuSO₄); (G5: 0.1 LD₅₀ of CuSO₄ + 100mg/kg/day of penicillamine); (G6: LD₅₀ of CuSO₄ +100 mg/kg/day of penicillamine) and (G7: LD₅₀ of CuSO₄+100 mg/kg/day of penicillamine) for 30 days and at the end of the experiment all rats were sacrificed and blood samples and brain tissues were collected for biochemical assaying of fasting blood glucose (FBG), serum Cu level, serum tyrosinase activity, oxidative stress marker malondialdehyde (MDA) and total antioxidant activity (TAC). also, DNA determination of relative gene expression of cerebral adenosine monophosphate-activated protein kinase (AmpK), protein kinase (AKT), phosphatidylinositol-3-kinase (PI3K), cytochrome c oxidase (Cyto co ), and glucose-6-phosphate dehydrogenase (G6PD).

Results: The results showed that administration of copper sulphate with different levels induced a significant increase in fasting blood glucose level, lipid peroxidation marker MDA, serum copper level, and serum tyrosinase activity, and a significant decrease in TAC. Moreover, copper sulphate administration elicited a significant downregulation (AmpK, AKT, PI3K, Cytochrome c oxidase, G6PD). it could be approved that penicillamine could abolish the negative impact of copper sulphate on neural tissues and serum enzymes.

Conclusion: D-penicillamine can reduce neurotoxicity and oxidative stress caused by copper pollution. Recommendations: Exposure to the pollution of copper must be controlled. Search for sensitive blood and neural markers for early detection of neurological disorders and further studies are needed to use its antidote d-penicillamine in the treatment of copper-induced pollution.

Keywords: D- Penicillamine; Copper sulphate pollution; Albino rats; Gene Expression.

INTRODUCTION

Copper is an essential trace element and is capable of being toxic to humans and animals if copper is ingested in excess amounts. copper could cause vomiting as it has an irritating effect on the gastrointestinal tract and any disturbance in its excretion leading to
accumulation of it in organs and tissues. The major sources of copper are electroplating, fertilizer, pesticide, and iron and steel industries. Copper is required for many metabolic functions. It acts as a cofactor in various copper proteins and in oxidoreductase compounds. It participates in the arrangement of melanin. Mitochondrial cytochrome c oxidase is a copper-containing complex that assumes a part in mitochondrial respiration. Ceruloplasmin (Cp), a copper-subordinate oxidase, changes Fe2+ divalent iron to Fe3+ for transferrin-intervened iron vehicle in the plasma. The liver, which stores most of copper, is the principal organ responsible for its processing and transferring excess copper to be excreted in bile. The brain is the second important organ to accumulate the most copper that can get copper from blood or CSF.

Excess copper is the result of genetic mutation that is leading to neurodegeneration diseases including Parkinson's disease, Alzheimer’s disease, ROS, which is produced by copper can lead to the inflammatory process. It also causes conditions of high oxidative stress and damage to protein, lipid, and nucleic acid structures and leading to translocation of nuclear and conformational changes and decrease these toxic effects, we can use chelators agents as D-penicillamine that acts on copper content by the chelating system and forming soluble complexes with copper ions so it is easily excreted in urine.

The current work aims to study the negative impact of copper sulphate pollution on enzymes in serums and organs especially brain tissues and how decreasing its side effects by using its antidote D-penicillamine. D-penicillamine was obtained from (Sigma-Aldrich, Chemical Cp. St. Louis, Mo, USA) and also dissolved in distilled water given orally in one therapeutic dose (100 mg/kg/day) for 30 days to (5, 6, 7) groups with copper sulphate in doses (0.1, 0.2, 0.4 LD$_{50}$ of CuSO$_{4}$) respectively.

Experimental animal and Ethical statement

seventy rats aged 8 weeks and weighing 100-200 gm, were used in the experimental investigation of this study. The animals were obtained from the Central Animal House of Faculty of Veterinary Medicine, Zagazig University, Egypt. Rats were acclimatized for 2 weeks under conventional laboratory conditions prior to any treatments. The animals were fed a standard rat pellet diet and allowed free access to water. Rats were kept at constant environmental and nutritional conditions throughout the periods of the experiment. This investigation has been audited and supported by ZU-IACUC panel, approval number ZU-IACUC/2/F/62/2023. After 2 weeks of adaptation, rats were divided into seven groups. The first group served as a normal control group (G1, 10 rats).

Experimental design

Seventy albino rats were divided into 7 equal groups each contains 10 rats. Copper sulphate and penicillamine were given orally for 30 days then the rats were sacrificed, and blood samples were collected for biochemical determination and brain tissues were collected for antioxidant investigation and gene expression.

Sampling

Rats were sacrificed via cervical decapitation, blood was collected, serum and plasma were separated by centrifugation at 3000 rpm for 20 mins and sent for the different biochemical determination. A 50 mg of brain tissue was collected and used for gene expression analysis and antioxidant determination.

Biochemical determinations

The following parameters were assayed serum tyrosinase was determined using (ab185435 kit) colorimetric assay method. Serum Copper ions were determined using
Serum Fasting glucose levels were determined using (ab65333 kit) according to \(^{16}\), TAC in brain tissue was determined using (Cat. No- E-BC-K136-S) according to colorimetric assay adapted to \(^{18}\) and MDA in brain tissue assayed using (ab118970) according to a method adapted to \(^{19}\).

**Molecular determinations**

The real-time Polymerase Chain Reaction procedure was carried out as described before\(^{20}\). Total RNA was isolated from 50mg of brain tissues using Trizol (Invitrogen; Thermo Fisher Scientific, Ink.), and quality and concentration were determined using Nanodrop\textsuperscript{®} system spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, Delaware USA). cDNA was synthesis Kit (iNtRON Biotechnology Co., South Korea ), 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 (1 µL of both forward and reverse primer) and nuclease-free water up to 20 µL with a cycling condition of initial denaturation at 95 °C for 12 minutes was followed by 40 cycles of denaturation at 95 °C for 20 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 30 seconds. the PCR product was amplified with oligonucleotide-specific primers listed in Table 1.(Beijing, China).

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 are expressed as fold-changes compared to the control group following the \(2^{-\Delta\Delta Ct}\) method\(^{21}\).

**Statistical analysis**

In order to assess the influence of excess cu and penicillamine on different enzymes, one-way ANOVA was followed by LSD least Significant Difference test as 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 are reported in means ± SEM (Standard Error of Mean), and probability value. The value of \(P< 0.05\) was used to indicate statistical significance.

| Primer name | Forward primer | Reverse primer | Product length | Accession No. |
|-------------|----------------|----------------|----------------|---------------|
| AMPK | GGCCTGTGAAGATCCGGACA | GGCCTGCAATTGATTTCTCC | 93 | NM-023991.1 |
| PI3K | CGAGAGTACGCTGTAGGCTG | AGAAACTGGCCAATCCCGTG | 164 | NM-053481.2 |
| AKT | GAAGGAGAAGGCAACAGGTC | TTCTGCAGGACACGTCTC | 111 | NM-033230.3 |
| Gapdh | GCATCTTCTTGTGCAATGCG | GGTAACCAGGCTCGATAC | 91 | NM-017008.4 |
| G6pd | TGGAGGACACAGCTACCCGCA | TCAATAGCCCCACGACC | 177 | NM-017006.2 |
| CYTO C | GGAACCACACGCTTTCAC | GAGTCTTCAAGGCTGCAGTG | 71 | NM 012812.3 |
RESULTS AND DISCUSSION

Results

Effect of penicillamine on (tyrosinase, FBG, and Cu level) in copper sulphate toxicity induced in rats

we reported in our results when compared with the control groups, that rat groups induced cytotoxicity with CuSO₄, highly observed significance increase with \( p < 0.05 \), in serum tyrosinase, FBG, and Cu level, and these results were proportional with the dosage amount (0.1, 0.2, 0.4 LD₅₀ of CuSO₄) respectively.

On the other hand, rat groups induced cytotoxicity with CuSO₄ and treated with penicillamine, showed a significant decrease in serum tyrosinase, FBG, and Cu level, levels with \( p < 0.05 \) (Fig.1).

Effect of penicillamine on antioxidant enzymes (brain TAC and MDA) in copper sulphate toxicity induced in rats

we reported in our results when compared with the control groups, that rat groups induced cytotoxicity with CuSO₄, highly observed significance with \( p < 0.05 \), hence it affected brain-specific enzymes (TAC and MDA) where CuSO₄ lead to increase of MDA enzyme, while we noticed a significant decrease of TAC enzyme in blood serum, and these results were proportional with the dosage amount (0.1, 0.2, 0.4 LD₅₀ of CuSO₄) respectively.

On the other hand, rat groups induced cytotoxicity with CuSO₄ and treated with penicillamine, showed a significant decrease in MDA enzyme, while TAC enzyme levels significantly increased \( p < 0.05 \) (Fig.2).

Fig. 1: (A) Effect of penicillamine (100 mg/kg/day) on the mean value of (serum tyrosinase) in induced copper sulphate toxicity (0.1, 0.2, 0.4 LD₅₀) in rat,(B) Effect of penicillamine (100 mg/kg/day) on the mean value of (serum FBG level) in induced copper sulphate toxicity (0.1, 0.2, 0.4 LD₅₀) in rat. (C) Effect of penicillamine (100 mg/kg/day) on the mean value of (serum Cu) in induced copper sulphate toxicity (0.1, 0.2, 0.4 LD₅₀) in rat. the values are expressed as mean + SEM.

Fig. 2 : (A) Effect of penicillamine (100 mg/kg/day) on the mean value of (brain tissues TAC) in induced copper sulphate toxicity (0.1, 0.2, 0.4 LD₅₀) in rat,(B) Effect of penicillamine (100 mg/kg/day) on the mean value of (brain tissues MDA) in induced copper sulphate toxicity (0.1, 0.2, 0.4 LD₅₀) in rat the values are expressed as mean + SEM.
Effect of penicillamine on (Brain PI3K, AKT, and AMPK mRNA relative expression) in copper sulphate toxicity induced in rats

We noticed a significance $p<0.05$ downregulation of PI3K, AKT, and AMPK mRNA expression in cerebral cortex tissue, in CuSO4 cytotoxicity induced group, where the downregulation was proportional with the dosage groups of (0.1, 0.2, 0.4 LD$_{50}$ of CuSO$_4$) respectively, while when CuSO4 cytotoxicity induced groups were treated with penicillamine, it showed a significance $p<0.05$ upregulation of expression levels when compared with control groups (Fig.3).

Fig. 3: (A) Effect of penicillamine (100 mg/kg/day) on the mean value of (Brain PI3K mRNA relative expression) in induced copper sulphate toxicity (0.1, 0.2, 0.4 LD$_{50}$) in rat, (B) Effect of penicillamine (100 mg/kg/day) on the mean value of (Brain AKT mRNA relative expression) in induced copper sulphate toxicity (0.1, 0.2, 0.4 LD$_{50}$) in rat. (C) Effect of penicillamine (100 mg/kg/day) on the mean value of (Brain AMPK mRNA relative expression) in induced copper sulphate toxicity (0.1, 0.2, 0.4 LD$_{50}$) in rat. The values are expressed as mean + SEM.

Effect of penicillamine on the mean value of (neuro G6PD and Cyto c-o mRNA relative expression) in copper sulphate toxicity induced in rats

The outcomes of the present investigation showed a significant mean value of $p<0.05$ downregulation in mRNA expression of the cerebral cortex 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 CuSO$_4$) respectively, while when CuSO4 cytotoxicity induced groups were treated with penicillamine, it showed a significance $p<0.05$ upregulation of expression levels when compared with control groups (Fig.4).

Fig. 4: (A) Effect of penicillamine (100 mg/kg/day) on the mean value of (Brain G6PD mRNA relative expression) in induced copper sulphate toxicity (0.1, 0.2, 0.4 LD$_{50}$) in rat, (B) Effect of penicillamine (100 mg/kg/day) on the mean value of (Brain Cyto c-o mRNA relative expression) in induced copper sulphate toxicity (0.1, 0.2, 0.4 LD$_{50}$) in rat. The values are expressed as mean + SEM.
Discussion

Copper is a necessary trace element for many biological functions and prolonged exposure to an excess concentration of copper ions can cause side effects. The present work was carried out to compare the efficacy of D-penicillamine in experimental copper intoxication and associated biochemical and molecular changes in rats and to investigate the effect of excess copper on brain tissues and serum markers of rats and using chelating agent d-penicillamine to improve the negative impact of copper sulphate pollution.

CuSO₄ toxicity is one a cause of serious neurodegenerative diseases in the world. A high level of copper harms lipids, proteins, and DNA. Increased production of Reactive Oxygen Species (ROS) through Fenton reaction has been the most important reason for copper toxicity side effects. D-penicillamine is the first-line approved copper ion chelator that scavenged excess copper in urine by forming a soluble complex with Cu⁺ ions to be easily excreted in urine. Thus, the present study was designed to investigate the possible improvement effect of d-penicillamine in CuSO₄ toxicity induced in rats.

Orally ingested copper gets absorbed through Gastrointestinal Tract and reaches the liver to be excreted through bile and undergoes redox cycles leading to the formation of ROS so copper pollution leads to the development of type-II diabetes. The result of the current study showed that CuSO₄ pollution induced a significant increased FBG that might to owed to heavy metals – induced hypoxia, glucose may be released into bloodstream and the release of catecholamine was taken place that stimulates gluconeogenesis by the action of cortisol. Interestingly, penicillamine abolished the CuSO₄ induced hyperglycemia that could be attributed to penicillamine induced hypoglycemia, or it increased beta-cell insulin secretion. The excess level of CuSO₄ increased the level of free copper ions in serum due to mutation in ATP7B transporter, which has a role in the excretion of excess copper ions through the bile, leading to an elevation in copper level and accumulation of excess copper in organs and tissues.

Copper acts as a cofactor factor for many enzymes such as tyrosinase and participates in the formation of melanin. The outcomes showed that CuSO₄ pollution elevated the level of tyrosinase in serum that could be attributed to that copper pollution induced mutated tyrosinase in albino rats leading to defect in melanocyte development (T. Kuramoto, et al. 2012). Also, ATP7B is translocated reversibly to facilitate the transportation of copper into copper containing enzymes like tyrosinase. Also, the result showed that penicillamine administration induced a significant reduction in serum tyrosinase and serum copper level as it acts as a chelating agent for copper.

G6PD is a key player in a cellular energy balance as a member of PPP pathway, also it regulates the oxidant and antioxidant state of the tissues. Excessive copper level induced mitochondrial stress and decreased mitochondrial membrane potential which activated the Endoplasmic reticulum (ER) stress. ER stress switch off protein biosynthesis many of these proteins is G6PD. Since, G6PD is responsible for the production of GSH, and its inhibition induced an oxidation stress condition the finding of the current study showed that excess CuSO₄ induced a significant downregulation of G6PD relative expression. So, results showed decreased in TAC and increased in MDA in CuSO₄ toxicity induced in rats. Copper is essential for many biological functions, such as the synthesis of phospholipids in cell membranes. Excess amount of Cu cause lipid peroxidation and damage of cell membrane as it led to the formation of hydroxyl radicals (•OH) that are involved in lipid peroxidation leading to a decrease in membrane integrity, and an increase in permeability of the membrane to H⁺, which leads to release of contents from cells.

Cytochrome c oxidase is the copper-containing respiratory complex's terminal electron acceptor. In our results, copper poisoning led to downregulation in the activity of cytochrome c oxidase (complex IV) that is associated with increased ROS production and cellular toxicity. Also, excess copper leading to inactivation of ATP7A or ATP7B results in disturbances in copper homeostasis and the inactivation of copper-containing enzymes. PI3K-AKT Pathway promotes cell metabolism, proliferation, survival of cells, apoptosis, and angiogenesis in response to extracellular signals. AMPK has a role in stimulating
In our results show that excess copper sulphate downregulated PI3K and Akt that was agreed with a study that showed increased excess of copper blocked expression of PI3K and AMPK as a result of greater production of ROS that led to dephosphorylations of AKT, condensation of DNA, swelling of mitochondrial and dysfunction and that leading to apoptosis and cell death. Also L. Chaiqin, et al., results showed downregulation of AMPK and mTOR that agreed with our result. Research has shown that the phosphorylation level of AMPK and mTOR will change in excess copper-induced-hypoxic conditions, leading to an imbalance of cell energy metabolism, and leading to apoptosis.

Conclusions
These findings recommended that penicillamine represented a valid candidate in abrogating the negative impact of CuSO4 toxicity via modulating PI3K, AKT, AmPK, G6PD, oxidative stress signaling pathway. Histological assessment for the changes in rat brains and measuring Cu levels in brain tissues will be considered in future work.

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تأثير التلوث بكبريتات النحاس وترياقه البنيسيلامين على إنزيمات الدم وانسجة المخ للجرذان البيضاء

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يعتبر النحاس من العناصر الهامة للعوامل الحيوية داخل جسم الإنسان ولكن زيادة نسبته بالجسم تؤدي إلى اضطرابات في الوظائف الحيوية. تهدف هذه الدراسة إلى تقصي تأثير التلوث بكبريتات النحاس على أنجاع المخ وإنزيمات الدم وكيفية الكشف المبكر للتلوث بالنحاس وذلك بإجراء التحليل الكميائي وتقليل مخاطره باستخدام عقار البنيسيلامين.

سيمون من الجرذان البيضاء تم تقسيمهم إلى سبع مجموعات متساوية كل مجموعة تحتوى على عشر جرذان وتم محايمتهم كالأتي: المجموعة الأولى: المجموعة الضابئة تم إعطائها مياه مقطرة 

\( LD_{50} \) عن طريق الفم لمدة 20 يوم يوميا. المجموعة الثانية: جرذان تم إعطائها عن طريق الفم (0.1 مليمول) من وزن الجسم من كبريتات النحاس لمدة 30 يوم يوميا. المجموعة الثالثة: جرذان تم إعطائها عن طريق الفم (0.2 مليمول) من وزن الجسم من النحاس لمدة 45 يوم يوميا. المجموعة الرابعة: جرذان تم إعطائها عن طريق الفم (0.4 مليمول) من وزن الجسم من النحاس لمدة 60 يوم يوميا. المجموعة الخامسة: جرذان تم إعطائها عن طريق الفم (0.1 مليمول) من وزن الجسم من النحاس + 100 مليمول/ل计量 من وزن الجسم لمدة 20 يوم يوميا. 

المجموعة السابعة: جرذان تم إعطائها عن طريق الفم (0.2 مليمول) من وزن الجسم من النحاس + 200 مليمول/ل计量 من وزن الجسم لمدة 30 يوم يوميا. وقد تم تجميع عينات الدم في نهاية التجربة من كل مجموعة لقياس التحليل البيوكييميا. كما تم أخذ عينات المخ سريعا وذلك لتقييم نسبة السكر بالدم.
نتيجة التحليلات التي أجريت، تمكنا من اكتساب المعلومات حول تأثير السكريات على النهايات، بالإضافة إلى تأثير تأكسيد tyrosinase وMDA في النهايات، بالإضافة إلى التأثير على التعبير الجيني AmpK، AKT، PI3K (G6PD) في النهايات. في النهايات، التأثير البوليتيكي لكريبتات النهايات على نسج المخ وانزيمات الدم، الخلاصة: عقار البنسيلامين له دور في تقليل اضطرابات الدم والإجهاد التأكسدي الذي يحدث بسبب التلوث بالنهايات.