COPPER NANOPARTICLES AFFECTING APOPTOSIS RELATED GENE EXPRESSION AND THE PROTECTIVE EFFECT OF ALPHA-LIPOIC ACID IN ADULT MALE ALBINO RATS: A CHRONIC STUDY

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ABSTRACT

Background: Copper nanoparticles (CuNPs) attract considerable interest because of their specific chemical and pahysical characteristics. In nanotoxicology, reactive oxygen species are considered the primary underlying chemical process that sets off subsequent processes that can eventually result in cell damage. The antioxidant and metal/toxin chelating properties of alpha-lipoic acid have been shown to have beneficial effects on a broad range of clinical conditions.

Aim of the study: This study aimed to evaluate the effect of copper nanoparticles on cytoplasmic/mitochondrial apoptosis through apoptosis-related genes and to study the potential protective effect of alpha-lipoic acid against apoptosis induced by copper nanoparticles.

Methods: The toxicity was investigated by evaluating the levels of BCL-2 and BAX genes in the liver and kidney tissues of rats using quantitative real-time polymerase chain reaction analysis and evaluating hepato-nephrotoxicity and, oxidative stress markers as well.

Results: CuNPs caused increased expression of BAX gene (cytoplasmic/mitochondrial pro-apoptotic gene) and decreased expression of BCL-2 gene (anti-apoptotic gene) in rats receiving CuNPs compared to the control groups. The use of alpha-lipoic acid had a protective effect when given with copper nanoparticles. However, it had no significant effect when given alone compared to the control groups.

Keywords: Copper nanoparticles, Nanotoxicity, Apoptotic genes, Oxidative stress, Alpha-lipoic acid.

INTRODUCTION

Nanotechnology is a field that deals with the application and characterization of nanoparticles in biology, physics, and science. Nanomaterials have different chemical reactivity and surface chemistry from their bulk counterparts **(Naz et al., 2020).**

Developments in the field of nanotechnology, as well as their increased application in the last few decades, have increased environmental and human exposure to nanoparticles in general **(Elsaesser and Howard, 2012).**

Nanotoxicity is primarily caused by the presence of a large number of reactive sites on nanoparticles because of the large surface area to volume ratio of nanoparticles **(Naz et al., 2020).**

Copper in the human body is found in many proteins; it also plays a crucial role in many metabolic processes. Different body functions are controlled by copper-containing enzymes such as iron homeostasis and oxygen transport **(Vimbela et al., 2017; Al-Hakkani, 2020).**

Copper nanoparticles (CuNPs) possess thermal, magnetic, and electrical properties, and they are used in heat transfer systems, antimicrobial coating for surgical tools and water treatment **(Chandraker, 2020;**

Mohamed, 2020).

Exposure to copper nanoparticles is mainly through ingestion, inhalation, and skin contact **(Docter et al., 2015; Caracciolo et al., 2017).** The CuNPs are transported throughout the body through the circulatory and lymphatic systems **(Pattan and Kaul, 2014).** The liver reticuloendothelial system is exposed to nanoparticles absorbed from the gastrointestinal tract **(Khalaf et al., 2017).** One of the strong antioxidants that exhibits its properties in water and lipid-soluble media is alpha-lipoic acid (ALA) **(Rochette et al., 2013).**

On exposure of cells to copper nanoparticles, DNA damage occurs. This damage is dependent on copper nanoparticle concentration. This damage is induced via oxidative stress and lipid peroxidation causing apoptotic cell death **(Naz et al., 2023).**

Cytoplasmic/mitochondrial apoptosis is a form of cell death that is genetically regulated. Pro-apoptotic and anti-apoptotic activities are regulated by the protein family BCL-2 and BAX genes **(Qian et al., 2022).** The determination of a cell's fate in response to an apoptotic stimulus is based on the BAX/BCL-2 ratio. A rise in this ratio induces apoptosis by reducing the cell's resistance to apoptotic stimuli **(Chougule et al., 2011).**

This study therefore sought to evaluate the hepato-nephrotoxicity of copper nanoparticles, clarify the mechanism of hepato-nephrotoxicity, study the effect of copper nanoparticle exposure on oxidative stress enzymes and cytoplasmic/mitochondrial apoptosis-related genes, and examine the effect of cessation of exposure on hepato-nephrotoxicity, as well as evaluate the possible protective role of ALA against copper nanoparticle-induced apoptosis.

MATERIALS & METHODS

2.1 Ethical consideration

The ethical committee of the Forensic Medicine and Clinical Toxicology Department at Kasr Alainy Faculty of Medicine as well as the Institutional Animal Care and Use Committee Cairo University (IACUC - CU) approved the study design (Code: CU III F 1 18).

2.2 Chemicals

- **Copper nanoparticles and alfa lipoic acid**

Copper nanoparticles (CuNPs) and alpha-lipoic acid (ALA) were obtained from Sigma Aldrich Co. (St. Louis, MO, USA) as shown in **Table 1.**

Table (1): Properties of copper nanoparticles and alpha-lipoic acid

Product name	**Copper nanoparticles**	**Alpha-lipoic acid**
Product brand	Sigma Aldrich	Sigma Aldrich
Product number	774081	T5625
CAS number	7440-50-8	1077-28-7
Molecular weight	63.55 g/mol	206.33 g/mol
Color	Brown or black	Light yellow to dark yellow and faint yellow-brown to yellow-brown
Form	Powder	Powder
Particle size	Average 25 nm	-
Purity/Assay	99.5% based on trace metal analysis	≥ 99% (titration with NAOH)

2.3 Study design and animal grouping

The animal house located inside the Research Institute of Ophthalmology (RIO) provided 100 adult male albino rats with an average body weight (bw) of 150-170 grams. Standard polypropylene cages with a stainless steel top grill were used to house the animals. All animals were exposed to a 12-hour cycle of light and dark and could freely obtain water and food. The rats were categorized into 4 groups after 2 weeks of acclimatization, and they received the following:

Group 1 controls (30 rats):

1. Group **1-A** (10 rats): negative control group.
2. Group **1-B** (10 rats): 2 ml of deionized water daily.
3. Group **1-C** (10 rats): 2 ml of oral olive oil daily.

Group 2 (30 rats):

1. Group **2-A** (20 rats): 40 mg/kg bw oral copper nanoparticles for 90 days.
2. Group **2-B** (10 rats (follow-up group)): 40 mg/kg bw oral copper nanoparticles for 90 days, after which they were fed normally for 4 weeks.

Group 3 (20 rats): 100 mg/kg bw oral ALA

daily.

Group 4 (20 rats): 40 mg/kg bw oral copper nanoparticles and a prophylactic dose of 100 mg/kg bw oral ALA.

Intraperitoneal injection of Ketamine hydrochloride (50 mg/kg) was used for the anesthesia of rats and then sacrificed by decapitation after 90 days. The follow-up group was preserved with no treatment for 30 days before being sacrificed as above. Blood samples (5 ml of blood from each rat) were collected through cardiac puncture. The abdominal cavity was opened, the kidneys and liver were removed, then frozen and stored at -80°C to be analyzed biochemically.

2.4 Dose selection and oral suspension preparation

- **Copper nanoparticles:**

The current study's application of the CuNPs dose (1/10 LD50, 40 mg/kg bw) was based on prior work in which rats experienced biochemical alterations at 1/10 LD50 without experiencing any morbidity **(Khalaf et al., 2017).** CuNPs powder was dissolved in deionized water and then subjected to vigorous vertexing and sonication to prepare the stock suspension.

- **Alpha-lipoic acid:**

The dose applied in the current study for ALA (100 mg/kg bw) was documented to have hepatoprotective effects in rats **(Pari and Murugavel, 2004).** ALA was dissolved in olive oil and then subjected to vigorous vertexing and sonication to prepare the stock suspension.

2.5 Biochemical assay of serum

The whole blood was centrifuged for 15 minutes at 3000 revolutions per minute (rpm) to obtain the serum (Sigma Aldrich, St. Louis, MO, USA). The biochemical levels include:

- Markers of hepatotoxicity: albumin (ALB), alanine transaminase (ALT), aspartate transaminase (AST), and total bilirubin concentration (TBC).
- Markers of nephrotoxicity**:** serum creatinine and blood urea nitrogen (BUN)
- Markers of oxidative stress: superoxide dismutase (SOD), glutathione (GSH), malondialdehyde (MDA), and catalase.
- Nitrative stress: expression of intracellular nitric oxide (NO) synthesis in the liver and kidney tissues.

2.6 Analysis of apoptosis-related genes

Quantitative real-time polymerase chain reaction (RT-PCR)

1. **RNA extraction:**

The tissue extraction kit (Qiagen, USA) was used to extract total RNA following the manufacturer's guidelines. Utilizing spectrophotometry, the purity as well as concentration (A260/A280 ratio) of RNA were defined (Spectrophotometer, dual-wavelength Beckman, USA).

2. **cDNA production:**

To convert total RNA (0.5–2 µg) to cDNA, a cDNA reverse transcription kit (Fermentas, USA) was utilized as follows:

- 3 µl of random primers were mixed with the 10 µl of RNA, which was denatured in the thermal cycler at 65°C for 5 minutes. The combination was then cooled to 4°C.
- For each sample: 19 µl of the cDNA master mix, after preparation, were added to 31 µl of RNA-primer mixture, producing 50 µl of cDNA.
- The last mix was placed in the thermal cycler and incubated for 60 minutes at 37°C, after which inactivation of the enzymes was done at 95°C for 10 minutes and cooled to 4°C. Then we converted RNA to cDNA, which was stored at -20°C.

3. **Quantitative RT-PCR using SYBR Green I:**

- Quantitative RT-PCR amplification was done via an Applied Biosystem software 3.1 version (StepOneTM, USA). The primer sequence is shown in **Table 2.**

Table (2): Primer sequence of the studied genes

Gene	Sequence
BCL-2 gene	Forward: TACAGGCTGGCTCAGGACTAT Reverse: CGCAACATTTTGTAGCACTCTG
BAX gene	Forward: CCCGAGAGGTCTTTTTCCGAG Reverse: CCAGCCCATGATGGTTCTGAT

Relative quantification (RQ) calculation:

The relative quantification was calculated with the Applied Biosystem software:

Δ Ct = Ct gene test – Ct endogenous control

$\Delta\Delta$Ct = ΔCt sample1 – ΔCt calibrator

RQ = Relative quantification = $2^{-\Delta\Delta Ct}$

The fold change compared to the calibrator is represented by the RQ **(Livak et al., 2001).**

2.7 Statistical analysis

Statistical Package for the Social Sciences (SPSS) version 25 was used for data coding. Mean and standard deviation were used to summarize quantitative variables. One-way Analysis of Variance (ANOVA) and post-hoc tests were used to compare quantitative variables **(Chan, 2003a).** Pearson correlation coefficient was used for analyzing correlations between quantitative variables **(Chan, 2003b).** Statistical significance was considered when p-values were less than 0.05.

RESULTS

Regarding hepatotoxicity markers, group 2 had higher mean ± standard deviation (SD) values of ALT, AST, ALB, and TBC than the 3 control groups and group 3 (ALA alone); group 4 demonstrated a significant improvement with a difference that is highly significant **(Table 3).**

Table (3): Hepatotoxicity markers level within the studied groups (mean ± SD) using one-way ANOVA

		Group 1 N = 30 Control			Group 2 N = 30 CuNPs		Group 3 N = 20 ALA	Group 4 N = 20 CuNPs + ALA	p-value
		1A N = 10	1B N = 10	1C N = 10	2A N = 20	2B N = 10			
Alanine transaminase	**Mean ± SD**	14.30 ± 3.65	16.50 ± 3.03	16.10 ± 1.91	61.50 ± 15.15	62.00 ± 10.88	15.10 ± 2.10	30.75 ± 6.27	< 0.001*
Aspartate transaminase	**Mean ± SD**	13.30 ± 2.11	15.00 ± 1.49	11.50 ± 1.35	50.95 ± 5.55	51.60 ± 10.19	10.90 ± 1.80	28.10 ± 4.75	< 0.001*
Albumin	**Mean ± SD**	4.99 ± 0.43	4.68 ± 0.33	5.00 ± 0.32	3.99 ± 0.47	3.79 ± 0.60	5.24 ± 0.21	4.81 ± 0.32	< 0.001*
Total bilirubin concentration	**Mean ± SD**	0.89 ± 0.07	0.96 ± 0.07	0.92 ± 0.07	2.30 ± 0.47	2.22 ± 0.40	0.88 ± 0.04	1.46 ± 0.37	< 0.001*

*p-value < 0.001 is statistically highly significant SD: Standard deviation
CuNPs: Copper nanoparticles ALA: Alpha-lipoic acid

Regarding nephrotoxicity markers, group 2 had higher mean ± SD of BUN and serum creatinine than the 3 control groups and group 3, while group 4 had a slightly above-normal mean ± SD of BUN and within-normal range mean serum creatinine, with a difference that is highly statistically significant **(Table 4).**

Table (4): Nephrotoxicity markers level within the studied groups (mean ± SD) using one-way ANOVA

		Group 1 N = 30 Control			Group 2 N=30 CuNPs		Group 3 N = 20 ALA	Group 4 N = 20 CuNPs + ALA	p-value
		1A N = 10	1B N = 10	1C N = 10	2A N = 20	2B N =10			
Blood urea nitrogen	**Mean ± SD**	42.90 ± 7.61	35.80 ± 3.05	44.20 ± 5.22	87.70 ± 11.12	86.60 ± 12.66	34.85 ± 4.40	53.20 ± 9.39	< 0.001*
Serum creatinine	**Mean ± SD**	0.13 ± 0.05	0.08 ± 0.02	0.10 ± 0.05	1.44 ± 0.48	1.60 ± 0.43	0.08 ± 0.02	0.32 ± 0.11	< 0.001*

*p-value < 0.001 is statistically highly significant SD: Standard deviation
CuNPs: Copper nanoparticles ALA: Alpha-lipoic acid

The mean ± SD of GSH, SOD, and catalase levels were decreased in group 2 in comparison with the 3 control groups and group 3; group 4 showed improvement with a difference that is highly statistically significant. Group 2 had higher mean ± SD values of MDA than the 3 control groups and group 3; group 4 demonstrated improvement with a difference that is highly statistically significant **(Table 5).**

Table (5): Oxidative stress markers level within the studied groups (mean ± SD) using one-way ANOVA

		Group 1 N = 30 Control			Group 2 N = 30 CuNPs		Group 3 N = 20 ALA	Group 4 N = 20 CuNPs + ALA	p-value
		1A N = 10	1B N = 10	1C N = 10	2A N = 20	2B N = 10			
Glutathione	**Mean**	60.67	64.89	57.34	25.68	30.98	65.27	52.88	< 0.001*
	± SD	± 4.82	± 4.28	± 1.84	± 6.22	± 9.74	± 2.83	±4.84	
Superoxide dismutase	**Mean**	2.76	2.84	2.41	0.71	0.71	2.72	1.78	< 0.001*
	± SD	± 0.36	± 0.24	± 0.22	± 0.24	± 0.33	± 0.33	± 0.38	
Catalase	**Mean**	137.48	124.38	128.43	59.14	65.47	150.13	116.01	< 0.001*
	± SD	± 12.11	± 2.68	± 16.73	± 9.34	± 13.42	± 8.10	± 7.37	
Malondialdehyde	**Mean**	5.37	4.66	5.34	44.29	41.30	4.82	17.16	< 0.001*
	± SD	± 1.32	± 0.27	± 0.82	± 10.93	± 15.81	± 1.14	± 6.30	

*p-value < 0.001 is statistically highly significant SD: Standard deviation
CuNPs: Copper nanoparticles ALA: Alpha-lipoic acid

Table 6 shows that the mean ± SD of NO level in liver and kidney tissues in group 2 increased compared to the 3 control groups and group 3, while group 4 showed improvement with a difference that is highly statistically significant.

Table (6): Nitric oxide expression in liver and kidney tissues within the studied groups (mean ± SD) using one-way ANOVA

		Group 1 N = 30 Control			Group 2 N = 30 CuNPs		Group 3 N = 20 ALA	Group 4 N = 20 CuNPs + ALA	p-value
		1A N = 10	1B N = 10	1C N = 10	2A N = 20	2B N = 10			
Nitric oxide liver	**Mean**	1.77	2.06	2.57	11.86	6.66	2.08	4.96	< 0.001*
	± SD	± 0.54	± 0.04	± 0.18	± 3.61	± 1.28	± 0.43	± 1.42	
Nitric oxide kidney	**Mean**	1.17	1.19	1.02	14.77	13.18	1.43	6.41	< 0.001*
	± SD	± 0.18	± 0.16	± 0.06	± 5.65	± 4.72	± 0.30	± 1.79	

*p-value < 0.001 is statistically highly significant SD: Standard deviation
CuNPs: Copper nanoparticles ALA: Alpha-lipoic acid

The mean ± SD of BCL-2 gene expression in liver and kidney tissues of group 2 was reduced compared to the 3 control groups and group 3, while group 4 showed significant recovery. On the other hand, the mean ± SD of BAX gene expression in liver and kidney tissues showed an increase in group 2 in comparison with the 3 control groups and group 3, while group 4 showed regression with a difference that is highly statistically significant **(Tables 7 and 8).**

Table (7): The expression of apoptosis genes in liver tissues within the studied groups (mean ± SD) using one-way ANOVA

		Group 1 N = 30 Control			Group 2 N = 30 CuNPs		Group 3 N = 20 ALA	Group 4 N = 20 CuNPs + ALA	p-value
		1A N = 10	1B N = 10	1C N = 10	2A N = 20	2B N = 10			
BCL-2 liver	**Mean**	1.00	1.00	1.01	0.17	0.20	1.01	0.71	< 0.001*
	± SD	± 0.01	± 0.00	± 0.01	± 0.08	± 0.05	± 0.01	± 0.14	
BAX liver	**Mean**	1.01	1.01	1.03	7.39	6.66	1.00	3.15	< 0.001*
	± SD	± 0.01	± 0.01	± 0.02	± 1.52	± 1.28	± 0.01	± 0.50	

*p-value < 0.001 is statistically highly significant SD: Standard deviation
CuNPs: Copper nanoparticles ALA: Alpha-lipoic acid

Table (8): The expression of apoptosis genes in kidney tissues within the studied groups (mean ± SD) using one-way ANOVA

		Group 1 N = 30 Control			Group 2 N = 30 CuNPs		Group 3 N = 20 ALA	Group 4 N = 20 CuNPs + ALA	p-value
		1A N = 10	1B N = 10	1C N = 10	2A N = 20	2B N = 10			
BCL-2 kidney	**Mean ± SD**	1.04 ± 0.03	1.01 ± 0.01	1.00 ± 0.01	0.25 ± 0.11	0.30 ± 0.07	1.03 ± 0.03	0.71 ± 0.18	< 0.001*
BAX kidney	**Mean ± SD**	1.01 ± 0.01	1.04 ± 0.03	1.04 ± 0.01	6.12 ± 1.20	4.77 ± 1.50	1.01 ± 0.01	2.19 ± 0.49	< 0.001*

*p-value < 0.001 is statistically highly significant
SD: Standard deviation
CuNPs: Copper nanoparticles
ALA: Alpha-lipoic acid

Tables 9 and 10 show that when post-hoc pairwise comparisons between the studied groups were performed, a highly statistically significant difference was detected between group 2 with groups 1, 3 and 4 as well as group 4 with groups 1, 2 and 3.

Table (9): Post-hoc pairwise comparisons between the studied groups concerning BAX gene expression in liver tissues

Main group (I)	Other group (J)	Mean Difference (I-J)	Standard error	p-value	95% Confidence Interval	
					Lower Bound	Upper Bound
Group 1	**Group 2A**	-6.37533-	0.23545	< **0.001***	-7.0520	-5.6986
	Group 2B	-5.64533-	0.29783	< **0.001***	-6.5013	-4.7894
	Group 3	0.01017	0.23545	1.000	-0.6665	0.6869
	Group 4	-2.13533-	0.23545	< **0.001***	-2.8120	-1.4586
Group 2A	**Group 1**	6.37533	0.23545	< **0.001***	5.6986	7.0520
	Group 2B	0.73000	0.31590	0.230	-0.1779	1.6379
	Group 3	6.38550	0.25793	< **0.001***	5.6442	7.1268
	Group 4	4.24000	0.25793	< **0.001***	3.4987	4.9813
Group 2B	**Group 1**	5.64533	0.29783	< **0.001***	4.7894	6.5013
	Group 2A	-0.73000	0.31590	0.230	-1.6379	0.1779
	Group 3	5.65550	0.31590	< **0.001***	4.7476	6.5634
	Group 4	3.51000	0.31590	< **0.001***	2.6021	4.4179
Group 3	**Group 1**	-0.01017	0.23545	1.000	-0.6869	0.6665
	Group 2A	-6.38550-	0.25793	< **0.001***	-7.1268	-5.6442
	Group 2B	-5.65550-	0.31590	< **0.001***	-6.5634	-4.7476
	Group 4	-2.14550-	0.25793	< **0.001***	-2.8868	-1.4042
Group 4	**Group 1**	2.13533	0.23545	< **0.001***	1.4586	2.8120
	Group 2A	-4.24000-	0.25793	< **0.001***	-4.9813	-3.4987
	Group 2B	-3.51000-	0.31590	< **0.001***	-4.4179	-2.6021
	Group 3	2.14550	0.25793	< **0.001***	1.4042	2.8868

*p-value < 0.001 is statistically highly significant

Table (10): Post-hoc pairwise comparisons between the studied groups concerning BCL-2 gene expression in renal tissues

Main group (I)	Other group (J)	Mean difference (I-J)	Standard error	p-value	95% Confidence interval	
					Lower bound	Upper bound
Group 1	Group 2A	0.76917	0.02776	< **0.001***	0.6894	0.8490
	Group 2B	0.71367	0.03512	< **0.001***	0.6127	0.8146
	Group 3	-0.01333	0.02776	1.000	-0.0931	0.0665
	Group 4	0.30467	0.02776	< **0.001***	0.2249	0.3845
Group 2A	Group 1	-0.76917-	0.02776	< **0.001***	-0.8490	-0.6894
	Group 2B	-0.05550	0.03725	1.000	-0.1626	0.0516
	Group 3	-0.78250-	0.03041	< **0.001***	-0.8699	-0.6951
	Group 4	-0.46450-	0.03041	< **0.001***	-0.5519	-0.3771
Group 2B	Group 1	-0.71367-	0.03512	< **0.001***	-0.8146	-0.6127
	Group 2A	0.05550	0.03725	1.000	-0.0516	0.1626
	Group 3	-0.72700-	0.03725	< **0.001***	-0.8341	-0.6199
	Group 4	-0.40900-	0.03725	< **0.001***	-0.5161	-0.3019
Group 3	Group 1	0.01333	0.02776	1.000	-0.0665	0.0931
	Group 2A	0.78250	0.03041	< **0.001***	0.6951	0.8699
	Group 2B	0.72700	0.03725	< **0.001***	0.6199	0.8341
	Group 4	0.31800	0.03041	< **0.001***	0.2306	0.4054
Group 4	Group 1	-0.30467-	0.02776	< **0.001***	-0.3845	-0.2249
	Group 2A	0.46450	0.03041	< **0.001***	0.3771	0.5519
	Group 2B	0.40900	0.03725	< **0.001***	0.3019	0.5161
	Group 3	-0.31800-	0.03041	< **0.001***	-0.4054	-0.2306

*p-value < 0.001 is statistically highly significant

Regarding Pearson correlation between hepato-nephrotoxicity markers and apoptosis gene expression in liver and kidney tissues within the studied groups, BAX gene expression showed a strong negative correlation with ALB level and a strong positive correlation with AST, ALT, TBC, BUN, and serum creatinine levels. On the other hand, BCL-2 gene expression showed a strong positive correlation with ALB level and a strong negative correlation with AST, ALT, TBC BUN, and serum creatinine levels **(Tables 11 and 12).**

Table (11): Pearson correlation between hepatotoxicity markers and apoptosis gene expression in liver tissues within the studied groups

Pearson correlation between hepatotoxicity markers and apoptosis gene expression in liver tissues N = 100		BAX liver	BCL-2 liver
Alanine transaminase	Correlation coefficient (r)	0.868	-0.899-
	p-value	< **0.001***	< **0.001***
Aspartate transaminase	Correlation coefficient (r)	0.912	-0.956-
	p-value	< **0.001***	< **0.001***
Albumin	Correlation coefficient (r)	-0.703-	0.722
	p-value	< **0.001***	< **0.001***
Total bilirubin concentration	Correlation coefficient (r)	0.884	-0.918-
	p-value	< **0.001***	< **0.001***

*p-value < 0.001 is statistically highly significant

Table (12): Pearson correlation between nephrotoxicity markers and apoptosis gene expression in kidney tissues within the studied groups

Pearson correlation between nephrotoxicity markers and apoptosis gene expression in kidney tissues N = 100		BAX kidney	BCL-2 kidney
Blood urea nitrogen	**Correlation coefficient (r)**	0.873	-0.893-
	p-value	**< 0.001***	**< 0.001***
Serum creatinine	**Correlation coefficient (r)**	0.834	-0.860-
	p-value	**< 0.001***	**< 0.001***

*p-value < 0.001 is statistically highly significant

DISCUSSION

It has been demonstrated that copper nanoparticles, in comparison to either copper-based microsize particles or other metal-based nanoparticles, exhibit heightened cytotoxicity and genotoxicity **(Karlssonet al., 2009).** Furthermore, chromosomal damage was increased as a result of incubation with CuNPs. This damage was determined by the formation of micronuclei **(Aliko et al., 2024).**

CuNPs' high cytotoxicity may be caused by the direct interaction of undissolved particles with cellular components and cell membrane. This interaction is facilitated by the increased surface area of CuNPs 23-fold. The reduction of [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] (WST-8) to the corresponding formazan was found to be impeded by CuNPs **(Semisch et al., 2014).**

The Trojan-horse mechanism proposes that nanoparticles reach intracellular compartments and release copper ions. CuNPs enter the cells through endocytic vesicles. On entering the lysosomal compartment, CuNPs dissolve in an acidic medium affecting the endo-lysosomal pathway and engulfing secondary lysosomes. This leads to leakage of the cell membrane, glutathione depletion, DNA damage, and cell death **(Karlsson et al., 2013).**

The accumulation, dissolution, and interaction of CuNPs in the cell membrane alter its permeability, thus contributing to CuNPs toxicity. Alteration of cell membrane permeability leads to a decrease in proton motive force across the membrane **(Chatterjee et al., 2014).** Reactive oxygen species (ROS) formation by ions liberated from the nanoparticles causes DNA degeneration and protein oxidation **(Raffi et al., 2010).** Changes in ATP production and DNA damage are caused by metal ions within the cell. Denaturation and other structural alterations can result from interactions between copper ions with the -SH and DNA phosphate groups and proteins **(Ingle et al., 2014; Wang et al., 2017).** This suggests that when metal nanoparticles are oxidized, Cu^{+2} ions are released, which mediates the toxicity of CuNPs.

Regarding hepatotoxicity markers, our study showed that CuNPs caused elevation in AST, ALT, and TBC levels, and this was in accordance with **Ghonimi et al. (2022),** whose results revealed hepatic parenchymal degenerative changes as well as hemosiderosis, congestion of blood vessels, glycogen depletion, and steatosis following the injection of CuNPs in Westar rats. Also, it was in accordance with **Doudi and Setorki (2014)** study, which proved that CuNPs caused elevation in AST and ALT levels.

Liver toxicity can be explained as follows: since the liver tissue serves as the main site for detoxification, exposure to toxins from both intrinsic and extrinsic sources exposes the liver to various disorders **(Khalaf et al., 2017).**

This work also showed that the rats receiving CuNPs combined with ALA as prophylaxis showed significant improvement in liver biomarkers. This result was consistent with **Tanaka et al. (2015) and El-Feki et al. (2016)** who proved that ALA has a hepatoprotective effect.

Regarding nephrotoxicity markers, the current study showed that rats receiving CuNPs had significant elevation in BUN and serum creatinine levels. This was in accordance with the work of **Chen et al. (2006).** Also **Ghonimi et al. (2022)** found degenerations of the renal corpuscles and renal tubules with congestions of the glomerulus.

The nephrotoxicity of copper nanoparticles could be explained by **Chen et al. (2007),** who proved that the kidney and spleen were the primary target organs of

nanocopper. After reacting with gastric juice, nanocopper particles can partially change into copper ions that are absorbed into the bloodstream. The pH of the renal tissues, where the cupric salt is deposited, decreases as a result of HCO_3 production.

Regarding the analysis of apoptosis-related genes in liver and kidney tissues, the present study found that in the group of rats treated with CuNPs, BAX gene expression increased while that of BCL-2 decreased (p-value < 0.001).

Through direct contact with undissolved particles after endocytotic uptake, nanoparticles can cause damage to mitochondria. Furthermore, it has been documented that nanoparticles affect intracellular calcium concentrations, which trigger the expression of genes linked to apoptosis **(Manke et al., 2013).**

Our results were in accordance with **Mukhopadhyay et al. (2018),** who found that the apoptotic marker genes BAX, caspase 9 and 3 were increased, while on exposure to CuNPs, there was a decrease in the gene BCL-2, the anti-apoptotic gene. **Abudayyak et al. (2016)** showed that copper oxide nanoparticles significantly induced DNA damage, depending on the concentration.

Our study found that in the group of rats receiving CuNPs combined with ALA, there was an improvement in BAX and BCL-2 gene expression in liver and kidney tissues compared to the group receiving only CuNPs.

In accordance with our study, **Dixit et al. (2015)** proved that ALA causes BCL-2 to be upregulated and BAX genes to be downregulated. On the other hand, **Wenzel et al. (2005)** found that exposure of cells to ALA can cause a decrease in BCL-2.

The anti-apoptotic effect of ALA is due to the restoration of the level of BAX: BCL-2 to (1:2). Additionally, ALA scavenges superoxide and peroxide radicals, which are the primary causes of cell death, and lowers the amount of hydroxyl radicals. ALA can regenerate endogenous antioxidants, including intracellular glutathione (GSH), vitamin E, and vitamin C **(Na et al., 2009; Tanaka et al., 2015).**

The present work suggests that the effect of CuNPs on the expression of apoptosis genes in liver tissues may be reversible, as there is non-significant regression in BAX gene expression and increased expression of BCL-2 gene in liver tissues in the follow-up group after cessation of CuNPs for 30 days.

CONCLUSIONS

- Copper nanoparticles had hepato-nephrotoxic effects.
- The pro-apoptotic gene BAX showed increased expression on exposure to copper nanoparticles, while the anti-apoptotic gene BCL-2 showed decreased expression.
- Oxidative stress status and apoptosis gene expression improved in the follow-up group after exposure to copper nanoparticles was stopped.
- Alpha-lipoic acid can cause significant improvement in hepato-nephrotoxicity, DNA damage, and oxidative and nitrative stress caused by copper nanoparticles.

RECOMMENDATIONS

- The health hazards of nanoparticles should be further studied as there is increased use of nanoparticles in our daily environments.
- Applying international standards for the prevention of occupational exposure to nanoparticles in Egypt is of extreme importance to decrease the fatal health risks caused by exposure to nanomaterials.
- The findings of the current work suggest routine medical examinations and investigations when there is ongoing exposure to copper nanoparticles.
- It is recommended to stop exposure to copper nanoparticles for at least 4 weeks if hepatic or renal functions worsen as a result of ongoing exposure.
- Alpha-lipoic acid can be used as an antioxidant that protects against nanocopper exposure and toxicity.

CONFLICT OF INTEREST

The authors report there are no competing interests to declare.

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الملخص العربي

جسيمات النحاس النانوية التي تؤثر على التعبير الجيني المتعلق بموت الخلايا المبرمج والتأثير الوقائي لحمض ألفا ليبويك في ذكور الجرذان البيضاء البالغة: دراسة مزمنة

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الخلفية: تجتذب جسيمات النحاس النانوية (CuNPs) اهتمامًا كبيرًا بسبب خصائصها الكيميائية والفيزيائية المحددة. في علم السموم النانوية، تُعتبر أنواع الأكسجين التفاعلية هي العملية الكيميائية الأساسية التي تؤدي إلى عمليات لاحقة يمكن أن تؤدي في النهاية إلى تلف الخلايا. لقد ثبت أن خصائص حمض ألفا ليبويك المضادة للأكسدة لها تأثيرات مفيدة على مجموعة واسعة من الحالات الإكلينيكية.
هدف الدراسة: هدفت هذه الدراسة إلى تقييم تأثير جسيمات النحاس النانوية على موت الخلايا السيتوبلازمي/الميتوكوندريا من خلال الجينات المرتبطة بموت الخلايا المبرمج ودراسة التأثير الوقائي المحتمل لحمض ألفا ليبويك ضد موت الخلايا المبرمج الناجم عن جسيمات النحاس النانوية. **طريقة الدراسة:** تمت دراسة السمية من خلال تقييم مستويات جينات BCL-2 وBAX في أنسجة الكبد والكلى لدى الجرذان باستخدام تحليل تفاعل البوليميراز المتسلسل الكمي في الوقت الحقيقي وتقييم السمية الكبدية الكلوية وعلامات الإجهاد التأكسدي أيضًا. **النتائج:** تسببت CuNPs في زيادة التعبير عن جين BAX (الجين السيتوبلازمي/الميتوكوندريا المؤيد للاستماتة) وانخفاض التعبير عن الجين BCL-2 (الجين المضاد لموت الخلايا المبرمج) في الفئران التي تستقبل CuNPs مقارنة بمجموعات التحكم. كان لاستخدام حمض ألفا ليبويك تأثير وقائي عند إعطائه مع جزيئات النحاس النانوية. ومع ذلك، لم يكن له تأثير عند إعطائه بمفرده مقارنة بمجموعات التحكم.
الكلمات الدالة: جزيئات النحاس النانوية، السمية النانوية، جينات الاستماتة، الإجهاد التأكسدي، حمض ألفا ليبويك.