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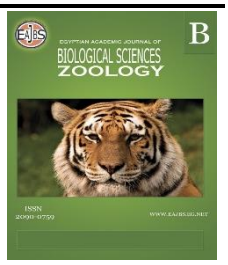

Alleviative Role of Hemin on Reproductive Functions of Adult Male Rats Treated with Copper Oxide Nanoparticles

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ABSTRACT

Background: The potential toxicity of copper oxide nanoparticles (CuO NPs), which are frequently employed in a variety of commercial and industrial uses, remains unknown. Hemin is the inducer of heme oxygenase 1, which exerts cytoprotective actions.**Objectives**: We aimed to assess the potential protective impact of hemin on the sperm of adult rats treated with CuO NPs. **Material and methods:** 50 adult rats were split into five groups: Group I: control, group II: animals received 40 μmol/kg b.w./day of solvent, group III: rats received 40 μmol/kg b.w./day of hemin, group IV: animals received 250 mg/kg b.w./day of CuO NPs and group V: rats received 40 μmol/kg b.w./day of hemin followed by CuO NPs at a dose of 250 mg /kg b.w./day. Each rat was administered orally as a single dose day after day for 2 weeks. At the completion of the investigation, all rats were sacrificed. Serum sex hormones were quantified and testes homogenized for biochemical analysis. Sperm analysis was processed for light microscopic examination.**Results:** Nanocopper reduces fertility hormone levels leading to sperm toxicity. CuO NPs also significantly decreased the activities of antioxidant enzymes accompanied by significant elevation in the MDA levels in testes in contrast to the control group. Hemin treatment reduced the majority of the toxic impacts of CuO NPs *via* their antioxidative capacity. **Conclusion:** Administration of hemin ameliorated sex hormones, oxidative stress, lipid peroxidation and sperm characteristics in CuO NPs intoxicated rats.

INTRODUCTION

We have shown a rapid advancement of nanotechnology in many domains of human existence, including biomedicine, agriculture, manufacturing, nutrition, cosmetics, and more (Kalpana and Devi Rajeswari, 2018; El-Shorbagy *et al*., 2019). Molecules or particles of a size between one and one hundred nanometers are known as nanoparticles (NPs) (Singh, 2022). According to Rothen-Rutishauser *et al*. (2007), the primary characteristics of NPs are their electrical configurations linked to their shape and size, their nanoscale structure and their very high surface-to-volume ratio in comparison to bulk substances. These distinctive

qualities give rise to worries about possible health risks to animals, people and the environment (Hendren *et al*., 2011).

According to Handy and Shaw (2007), individuals are already subjected to a wide range of natural and artificial nanomaterials in the air *via* the food chain, supply of water, and medicinal applications. Since their minuscule size, nanomaterials may either penetrate cells and go to other regions of the body or penetrate cell membranes to reach cells, with the respiratory tract being the primary route of entry for them (De Braganca *et al*., 2021). Nanoparticles (NPs) generate a high amount of reactive oxygen species (ROS) when they invade the cell, and this can result in oxidative stress (OS). The NP-induced cytotoxicity, genotoxicity, cellular harm and death provide a foundation for the OS process (Mishra and Panda, 2021).

Male reproductive issues brought on by work-related exposures in humans have received greater interest (Phillips and Tanphaichitr, 2008). According to earlier research, a variety of nanomaterials, including carbon and metal-based particles in addition to diesel exhaust containing nanoparticles, can cross the blood-testis barrier (BTB) and have a negative impact on the reproductive and endocrine systems (Liu *et al*., 2016). Changes in the way the male reproductive system functions can be a particularly sensitive indicator of environmental dangers and have a direct impact on reproduction. The most often mentioned threats to male reproductive function have been chemical and physical exposure. The effects of hazardous components at low exposure levels on the structure and function of the male reproductive system are rather well-demonstrated by the dosages and exposures (Massányi *et al*., 2020). Following exposure, NPs go through a variety of pathways to enter the reproductive system. In men, the epididymis and testis are the primary NP target sites (Zhao *et al*., 2014).

Male infertility is reported to have OS as a causal component (Agarwal and Bui, 2017). When the male reproductive tract's natural antioxidant defense is exceeded by the creation of ROS or free radicals, OS results (Bisht *et al*., 2017). According to Bisht *et al*. (2017), ROS interferes with sperm nucleus DNA integrity and ATP synthesis, which has detrimental impacts on the quality of semen. Polyunsaturated fatty acids are abundant in the spermatozoa cell membrane, making them the most susceptible to peroxidation of lipids and free radical destruction. As a result, they affect the motility, count, viability, and morphology of the sperm (Agarwal *et al*., 2014). According to Agarwal and Sengupta (2020), an excess of ROS produces OS, which lowers sperm fertility.

In several fields of technology and science, such as electronics (Jana *et al*., 2018), agriculture (Pelegrino et al., 2020), medicine (Selvaraj, 2020), and solar energy (Amalraj and Michael, 2019; Abdullah *et al*., 2020), CuO NPs, are now in broad application. Owing to their numerous uses, CuO NPs are ingested orally, inhaled, and administered by injection. Humans are exposed to them on a regular basis. Organ deposits might possibly be harmful to people's health (Pohanka, 2019; Areecheewakul *et al*., 2022).

Hemin (H) is the byproduct of heme oxidation and has a wide range of biological roles. It is also referred to as porphyrin iron chloride, hematin chloride, and ferriheme (Song *et al*., 2014). Heme oxygenase 1 (HO-1) is a stress-inducible protein that is abundantly expressed in tissues. Hemin is both the substrate and inducer of HO-1, which catalyzes the breakdown of heme into biliverdin, free divalent iron, and carbon oxidant (Even *et al*., 2018). According to reports, HO-1 possesses a variety of cytoprotective properties, such as anti-oxidative stress, anti-inflammatory, and anti-apoptotic impacts (Gozzelino *et al*., 2010). Hemin can lessen the toxic effects of excitatory amino acids, shield vital organs from hypoxic-ischemic injury, and stop the damage from free radicals (Martín *et al*., 2019).

The cauda epididymis is the final portion of the epididymis when spermatozoa become motile and have the capability to fertilize oocytes, marking the completion of sperm maturity. Additionally, mature sperm are stored in the cauda epididymis until ejaculation.

The intact, normal epididymal environment that the absorptive and secretory characteristics of the epididymal lining epithelium offer is necessary for these sperm changes (Hinton and Cooper, 2010). There is insufficient information from animal experiments to support the possibility that the unique features of nanomaterials might be damaging to the testicles, despite prior *in vitro* and *in vivo* experiments showing this (Hong *et al*., 2022). Hence, utilizing biochemical methods and sperm analysis is required to assess the potential protective function of hemin against the damaging impacts of CuO NPs on the testis and epididymal sperm of adult albino rats.

MATERIALS AND METHODS

1. Animals:

Fifty mature male albino rats in excellent physical condition, weighing between 150 and 200 grams, were acquired from Hellwan Farm, the Egyptian Holding Company for Biological Products and Vaccines. They were housed in hygienic cages with enough ventilation, free access to water, and well-balanced lab food. Prior to the experiment and for the whole research duration, the rats were maintained on a 12-hour light/12-hour dark cycle. Prior to the trial, they were housed for two weeks to allow them to acclimate. The Al-Azhar University's Local Ethics Committee for the Faculty of Science (For Boys) in Cairo, Egypt, authorized the study's concept.

2 .Chemicals:

The powder of CuO-NPs was obtained and prepared at the Nano Gate Center for Nanotechnology and its Application (Cairo, Egypt). Characterization of CuO NPs was done by X-ray diffraction (XRD) analysis and transmission electron microscopy (TEM). Hemin powder was obtained from Sigma-Aldrich, Inc. St. Louis, United States;1001146792. The oral dose of 250 mg/kg b.w./ day of CuO NPs was chosen based on the previous work done by Ouni *et al*. (2020). CuO NPs suspensions were made with a concentration of 10 gm of powdered CuO NPs, diluted with 100 ml of 9% Na Cl, and stirred with a magnetic stirrer for a whole night before being dispersed using ultrasonic vibration for one hour. Initially, hemin was dissolved in 0.1 M NaOH, titrated with 0.1 M HCl to achieve a pH of 7.4, and then diluted with normal saline (1: 10 v/v). The suspension was given to rats by oral gavage. Every rat has received hemin in a concentration of 40 μmol/kg b. w. according to the previous study (Al-Kahtani *et al*., 2014). Kits for physiological analysis were obtained from the Bio-diagnostic Company for Diagnostic and Research Reagents, Dokki, Giza, Egypt.

3. Experimental Design:

Rats were split randomly into five groups (each containing 10 animals) as follows:

- **Group I (negative control):** untreated control in which the rats were fed on a normal diet.
- **Group II (positive control) (S):** rats were treated with 40 μmol/kg b. w./day of solvent (0.1 M NaOH, titrated to pH 7.4 with 0.1 M HCl).
- **Group III (H):** rats were treated with 40 μmol/kg b. w./day of hemin.
- **Group IV (CuO NPs):** animals received 250 mg/kg b. w./ day of CuO NPs.
- **Group V (H + CuO NPs):** rats were treated with 40 μmol/kg b. w./day of hemin followed by CuO NPs at a dose of 250 mg /kg b. w./ day.

All doses were given orally by gavage as a single dose day after day for 14 days. Each of the rats was starved for an entire night, weighed and slaughtered under ether anesthesia at the conclusion of the experiment, which occurred after 28 days and 24 hours following the last dose.

Blood Sampling and Biochemical Analysis:

Heparinized capillary tubes were used to draw blood samples *via* the retro-orbital venous plexus and into dry, clean test tubes. To separate the serum, the blood was

centrifuged for ten minutes at 3000 r.p.m. To estimate the levels of male sex hormones, semen was separated, obtained, and frozen at -20°C using a Pasteur pipette and dry clean tubes. The levels of free sera sex hormones were measured for testosterone (T) (Rosner *et al*., 2007), luteinizing hormone (LH), follicle-stimulating hormone (FSH), (Bablok *et al*., 1988) and estradiol (E2) (Lichtenberg *et al*., 1992) using the Electrochemiluminescence Immunoassay (ELISA) commercial Kits.

Tissue Preparation, Testis Index Determination and Homogenate:

Immediately after dissection, both testes and epididymidis were removed, examined grossly and photographed. The testes were weighed to calculate the organ indexes according to the method of Laurent *et al*. (2008). The width and length of the testes were measured and the size was done based on the previous method by Shoemaker and Heideman (2002). The right testes of rats were homogenized to produce a 10% homogenate utilizing cold ice and 1.15% KCl. Following testicular supernatant preparation, OS markers were assessed. The antioxidant parameters such as SOD activity (Marklund and Marklund, 1974) and CAT (Aebi, 1984) activity, reduced GSH content (Beutler *et al*., 1963) as well as MDA content (Ruiz-Larrea *et al*., 1994) were estimated in testicular tissue homogenates.

Epididymal Sperm Analysis:

Each rat's caudal region of the right epididymis was removed, cut carefully with scissors in a petri dish, and placed in 15 mL of a Biggers-Whitten-Whittingham (BWW) medium. The sperm were then allowed to swim up from the epididymal ductules at 37°C for 30 minutes in an incubator with 5% CO_2 (Jaâ, 2015).

The measurements of sperm motility, count, and morphology are outlined here.

Sperm Count And Motility:

Sperm counts were determined in accordance with Nak-ung *et al*. (2018). A Neubauer hemocytometer was used for the counting, which was carried out under an optical microscope. The sperm counts were reported as follows: total number of spermatozoa in four tiny corner squares measuring × 125 × 102 = number of spermatozoa/ml of sperm solution (Saber *et al*., 2016). After combining sperm samples with sodium citrate dehydrate, the quantity of motile and immotile sperm was counted. The values for rapid, progressive, nonprogressive, sluggish and immotile sperm were represented as (%) (WHO, 2010).

Sperm Morphology:

According to Agarwal *et al*. (2016), the eosin–nigrosin stain was used to evaluate sperm morphology. The sperm specimens were spread out on a microscope slide and combined with an eosin-nigrosin stain (kept for incubation). To determine alterations in morphology, 200 spermatozoa from each rat were seen under a light microscope (400x magnification) (Moridi *et al*., 2018).

4. Statistical Analysis:

The data were statistically analyzed using the SPSS/PC computer software, version 20, which is a statistical package for social sciences. One-way analysis of variance (ANOVA) was applied to analyze the data. The data was presented as mean ± S.E. When $P \leq 0.05$, differences were deemed statistically significant (Turner and Thayer, 2001).

RESULTS

1. Body Weight And Body Weight Change:

The body weight and alterations of the body weight of control and all treated rats are presented in Table 1. There was a great significant reduction ($P \leq 0.01$) in the mean body weight of rats treated with CuO NPs (159.7 ± 1.2 gm) compared with both controls (184.2 ± 1.5 gm and 183.2 ± 1.7gm respectively) and H (184.7 ± 2.5 gm)-treated groups. However, H+ CuO NPs (178.5 ± 1.4 gm) treated rats showed significant elevation ($P \leq 0.05$) in the

mean body weight in comparison to the CuO NPs group at the completion of the experimental duration.

Moreover, the mean body weight change of rats treated with CuO NPs (6.5 ± 0.99 gm) or H+ CuO NPs (8.8 ± 1.1 gm) recorded highly significant reductions (P≤0.01) in comparison to the positive, negative (20.7 ± 0.8 gm and 18.5 ± 1.8gm respectively) controls and H (18.5 ± 1.4 gm)-treated groups. Although the present result recorded a gain, the group treated with H + CuO NPs did not reveal a significant difference from the CuO NPs group. In this regard, H did not have a noticeable impact on the animals' changes in body weight.

2. Absolute and Relative Testis Weight:

The absolute and relative weights of the testes of control and all treated rats are presented in Table I. Absolute right (0.67 ± 0.046 gm) and left (0.62 ± 0.04 gm) testicular weights of CuO NPs and H + CuO NPs (right, 0.7 ± 0.04 gm and left, 0.66± 0.04 gm) treated rats were significantly (P ≤ 0.05) declined in contrast to the control (right, 0.94± 0.033 gm and left, 0.97 ± 0.05 gm). In the same trend, their index weights in Cu ONPs (right, 0.41% and left, 0.38 %) and H+ CuO NPs (right, 0.39% and left, 0.36%)- treated were significantly declined in contrast to those of control rats.

At the completion of the investigation, there were no significant alterations in the testes' absolute and index weights between H + CuO NPs and CuO NPs groups. Moreover, no significant change was recorded between the absolute and relative testes weight of H-treated rats and both control groups. In this regard, H did not reveal a marked impact on the absolute and relative testes weight of the animals.

Table 1: Alterations in male rat body weight, absolute and index weights of testes in control and different treated groups.

Parameter / Groups	Body weight before treatment (gm)	Body weight after reatment (gm)	Body weight change (gm)	Right testis weight (gm)	Right testis index (%)	Left testis Weight (gm)	Left testis index (%)
	Mean ± SE						
Control	163.5 ± 1.3^{a}	184.2 ± 1.5^{a}	20.7 ± 0.8^{a}	0.94± .033^{a}	0.50 ±.01^{a}	0.97±.05^{a}	0.49±.006^{a,c}
S	164.7 ± 1.7^{a}	183.2 ± 1.7^{a}	18.5 ± 1.8^{a}	0.9 ± .026^{a}	0.51±.023^{a}	0.85±.02^{b}	0.46 ± .011^{c}
H	166.2 ± 1.9^{a}	184.7 ± 2.5^{a}	18.5 ±1.4^{a}	0.98 ± .035^{a}	0.53 ± .01^{a}	1.00 ± .039^{a}	0.51 ± .009^{a}
CuO NPs	165.2 ± 1.4^{a}	159.7 ± 1.2^{b**}	- 6.5 ± 0.99^{b**}	0.67 ± .046^{b}	0.36 ±.02^{b}	0.62±.04^{c}	0.36 ± .014^{b}
H+ CuO NPs	169.7 ± 1.2^{b}	178.5 ± 1.4^{c}	8.8 ± 1.1^{b**}	0.7 ± .04^{b}	0.37±.017^{b}	0.66± .04^{b,c}	0.37 ± .010^{b}

All data represent mean ± SE of 10 animals.
Within a column, values with identical letters do not differ significantly.
A one-way ANOVA was performed to determine the significant difference between the groups, and values with different letters within the column indicated a significant difference, P ≤ 0.05.
**: Highly significant different P ≤ 0.01 vs. control group.

3.Testis size:

When comparing the testicular size of CuO NPs-treated groups to both the negative and positive control groups, there is a significant reduction in relation to the absolute weight of the testis. The right testicular size in CuO NPs-treated group (0.42 ± 0.005 cm^3) is significantly (P ≤ 0.05) smaller than that of the control (0.92 ± 0.074 cm^3). In addition, the same trend was observed in the left testicular size of the experimental rats treated with CuO NPs alone. Moreover, there was no significant alteration in right testicular size between H + CuO NPs and CuO NPs as well as the S (0.71 ± 0.063 cm^3) group, but the mean of left testicular size of H + CuO NPs showed a significant increase compared to CuO NPs alone. Nonetheless, treating rats with H displayed improvement in left testes size with a significant increase (P ≤ 0.05) compared to the positive control, CuO NPs and H + CuO NPs treated groups. (Table 2 and Plate 1).

Table 2: Average testicular size (cm^3) in control and different treated groups.

Parameters / Groups	Testicular size (cm^3) Mean ± SE	
	Right	Left
Control	0.92 ± 0.074[a]	0.81 ± 0.045[a]
S	0.71 ± 0.063[b]	0.94 ± 0 .086[b]
H	1.10 ± 0.095[a]	1.0 ± 0.057[b]
CuO NPs	0.42 ± 0.005[c**]	0.41 ± 0.054[c**]
H + CuO NPs	0.61 ± 0. 057 [b, c]	0.58 ± 0.07[d]

All data represent mean ± SE of 10 animals.
Within a column, values with identical letters do not differ significantly.
A one-way ANOVA was performed to determine the significant difference between the groups, and values with different letters within the column indicated a significant difference, $P \leq 0.05$.
**: Highly significant different $P \leq 0.01$ vs. control group.

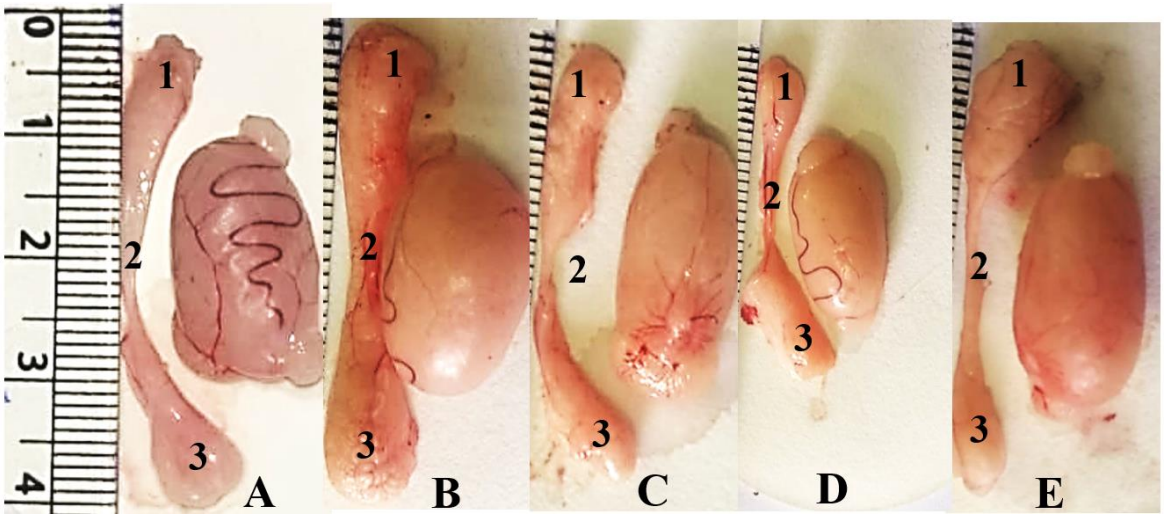

Plate 1: Gross anatomy of the rat testis and epididymis. 1) caput, 2) corpus, 3) cauda epididymidis of control (A), S (B), H (C), CuO NPs (D) and H+ CuO NPs (E) groups.

4. Antioxidant Enzyme Activities and MDA Content In Testicular tissue:

Table 3, displays the MDA content and antioxidant enzyme activities in the rat testes. Rats who received CuO NPs in addition to H + CuO NPs had considerably lower CAT and SOD activities ($P \leq 0.05$) when contrasted with both the H group and the controls, GSH content exhibited a significantly greater ($P < 0.01$) decline. Additionally, CuO NPs found that the experimental rats' testes had significantly higher MDA levels ($P \leq 0.01$) than the control group. However, there was no apparent alteration in the lipid peroxidation parameter between the H group and the control group. In contrast to the CuO NPs group, delivery of H with CuO NPs led to a substantial ($P \leq 0.05$) drop in MDA content and a rise in CAT, SOD activities and GSH content. Thereby, H-treatment had an ameliorated effect on antioxidants and OS in CuO NP-treated rats.

Table 3: CAT, SOD, GSH activities and MDA contents of testicular tissue in control and different treated groups.

Parameters / Groups	CAT (U/mg)	SOD (U/mg)	GSH (mmol/g)	MDA (nmol/g)
	Mean ± SE			
Control	20.9 ± 0.5$^{a, c}$	7.2 ± 0.12^{a}	0.75 ± .02^{a}	51.3 ± 1.9^{a}
S	19.7 ± 2.9$^{a, c}$	7.0 ± 0.16^{a}	0.71 ± .03^{a}	67.5 ± 1.4^{b}
H	22.6 ± 0.3^{a}	7.5 ± 0.13^{a}	0.77 ± .04^{a}	51.8 ± 1.6^{a}
CuO NPs	13.9 ± 0.6^{b}	4.1 ± 0.11^{c}	0.20 ± .02^{b**}	110.1 ± 3^{c**}
H + CuO NPs	17.2 ± 0.7^{c}	5.6 ± 0.14^{a}	0.50 ± .04^{c}	88.3 ± 3.2^{d**}

All data represent mean ± SE of 10 animals.
Within a column, values with identical letters do not differ significantly.
A one-way ANOVA was performed to determine the significant difference between the groups, and values with different letters within the column indicated a significant difference, $P \leq 0.05$.
**: Highly significant different $P \leq 0.01$ vs. control group.

3. 5. Serum fertility hormone levels:

As shown in **Table 4**, CuO NPs caused a highly significant decrease ($P \leq 0.01$) in serum testosterone, LH and FSH (0.7 ± 0.1 ng/ml, 0.08 ± 0.01 ng/ml and 0.06 ± 0.002 ng/ml respectively) in contrast to the control group (3.7 ± 0.1 ng/ml, 0.5 ± 0.03 ng/ml and 0.3 ± 0.02 ng/ml respectively). Additionally, the level of E2 in CuO NPs (32.6 ± 0.6 ng/ml), caused a high significant increase ($P \leq 0.01$) followed by significant elevation ($P \leq 0.05$) in H+ CuO NPs group (23.4 ±0.7 ng/ml) when compared with that in the control (17.0 ± 0.5 ng/ml) and H-groups (4.3 ± 0.2 ng/ml). However, there was no E2 apparent difference between the H (17.3 ± 0.6 ng/ml) and control groups. Nonetheless, treating rats with H concurrently with CuO NPs significantly ($P \leq 0.05$) improved the levels of testosterone and E2 concentration in contrast to the CuO NPs-exposed group. Moreover, the level of both LH and FSH hormones is still low compared to the control group.

Table 4: Serum fertility hormones of adult male rats in control and different treated groups.

Parameters / Groups	Testosterone (ng/ml)	E2 (ng/ml)	LH (ng/ml)	FSH (ng/ml)
	Mean ± SE			
Control	3.7 ± 0.1^{a}	17.0 ± 0.5^{a}	0.50 ± .03^{a}	0.30 ± .02^{a}
S	3.3 ± 0.17 $^{a, d}$	21.1 ±0.4^{b}	0.20 ± .03^{c}	0.20 ± .04^{a,c}
H	4.3 ± 0.2^{b}	17.3 ± 0.6^{a}	0.80 ± .04^{b}	0.50 ± .03^{b}
CuO NPs	0.7 ± 0.1^{c**}	32.6 ± 0.6^{c**}	0.08±.01^{d**}	0.06 ±.002^{d**}
H + CuO NPs	3.0 ± .07^{d}	23.4 ±0.7^{d}	0.24±.04^{e}	0.18 ± 0.03^{c}

All data represent mean ± SE of 10 animals.
Within a column, values with identical letters do not differ significantly.
A one-way ANOVA was performed to determine the significant difference between the groups, and values with different letters within the column indicated a significant difference, $P \leq 0.05$.
**: Highly significant different $P \leq 0.01$ vs. control group.

3. 6. Epididymal sperm parameters:

Table 5 summarizes the quantitative and qualitative sperm parameters for each group. Administration of CuO NPs at a dose of 250 mg/kg b. w./ day for 14 days day after day led to a highly significant ($P \leq 0.01$) decrease of epididymal sperm count (6.1 ± 0.5) in contrast to the negative, positive control and H-treated groups (24± 2.1, 22.7 ± 0.5 and 24.5 ± 1.8 respectively). However, the H + CuO NPs treated group had significantly ($P \leq 0.05$) enhanced sperm motility (rapid and progressive) and count compared to the CuO NPs group. Comparing these alterations to the CuO NPs (65.7) group, there was a notable drop in spermatozoa with nonprogressive motility of 16.4% ($P \leq 0.05$) and spermatozoa with immotility of 31.4%. Additionally, there was a substantial rise in the count of rapid motility sperm (17.6%; $P \leq 0.05$).

Table 5: Epididymal sperm parameters of adult male rats in control and different treated groups.

Epididymal sperm parameters							
	Motility and count			Types of sperm motility			
Parameters / Groups	Motile sperm (%)	Non motile (%)	Sperm count (10^6/ml)	Rapid (%)	Progressive (%)	non-Progressive (%)	Sluggish (%)
	Mean ± SE						
Control	77.1± 2.4^{a}	22.9±2.4^{a}	24 ± 2.1^{a}	23.6 ± 1.4 ^{a,b}	20.0 ± 1.1^{a}	18.6 ± 0.9^{a}	15.0 ±1.1^{a}
S	75.3± 4^{a,d}	24.7 ± 4^{a,d}	22.7 ± 0.5^{b}	20.0 ± 1.5^{b}	20.7 ± 0.7^{a}	19.3 ± 1.3^{a}	14.6±1.1^{a}
H	85.7 ± 1.7^{b}	14.3 ± 1.7^{b}	24.5 ± 1.8^{a}	27.1 ± 2.1^{a}	29.0 ± 1.7^{b}	17.4 ± 0.8^{a}	12.0 ± 1.3^{a}
CuO NPs	34.3 ±2.5^{c**}	65.7 ± 2.5^{c**}	6.1 ± 0.5^{c**}	6.5 ± 0.2^{c**}	8.3 ± 0.9^{c**}	12.6 ± 1.6^{b}	8.7 ±1.2^{b}
H + CuO NPs	68.6 ± 1.4^{d}	31.4 ± 1.4^{d}	19.7 ± 0.6^{b}	17.6 ± 1^{b}	21.4 ± 0.9^{a}	16.4 ± 1^{a}	12.9±1.5^{a}

All data represent mean ± SE of 10 animals.
Within a column, values with identical letters do not differ significantly.
A one-way ANOVA was performed to determine the significant difference between the groups, and values with different letters within the column indicated a significant difference, $P \leq 0.05$.
**: Highly significant different $P \leq 0.01$ vs. control group.

Exposure to CuO NPs caused a significant ($P \leq 0.05$) elevation in sperm general morphological abnormalities of the head and flagellum (15.1 % ± 0.9) compared to control. Table 6, indicates that the proportion of spermatozoa with general abnormalities considerably dropped only after treatment with H and CuO NPs (10.4 % ± 0.6) in contrast to rats treated with CuO NPs, whose values were closer to those of the control group (10 % ± 0.7). In addition, the percentages of an abnormal flagellum were 6.20 ± 0.4 in rats of the CuO NPs group and parallel to spermatozoa head abnormalities (6.2 ± 0.4). Many abnormalities included a flexed head, amorphous head, hookless head, hairpin loop, coiling tail, bent tail, fused tail, headless sperm, and zigzag tail as well as double head and double tail in comparison to the normal structure of the sperm. This consistency with representative photos using light microscopy is shown in Plate 2, 3 and 4 (a-f), which explain the morphologically normal and malformed sperm.

Table 6: Epididymal sperm morphology of adult male rats in control and different treated groups.

Parameters / Groups	General abnormality %	Abnormal head %	Abnormal tail %
	Mean ± SE		
Control	10 ± 0.7^{a}	3.9 ± 0.3^{a}	4 ± 0.3^{a}
S	6.7 ± 0.4^{b}	2.3 ± 0.1^{b}	2.8 ± 0.2^{b}
H	6.6 ± 0.5^{b}	2.4 ± 0.2^{b}	2.7 ± 0.3^{b}
CuO NPs	15.1 ± 0.9^{c}	6 ± 0.4^{c}	6.2 ± 0.4^{c}
H+ CuO NPs	10.4 ± 0.6^{a}	3.8 ± 0.3^{a}	4.1± 0.35^{a}

All data represent mean ± SE of 10 animals.
Within a column, values with identical letters do not differ significantly.
A one-way ANOVA was performed to determine the significant difference between the groups, and values with different letters within the column indicated a significant difference, $P \leq 0.05$.
**: Highly significant different $P \leq 0.01$ vs. control group.

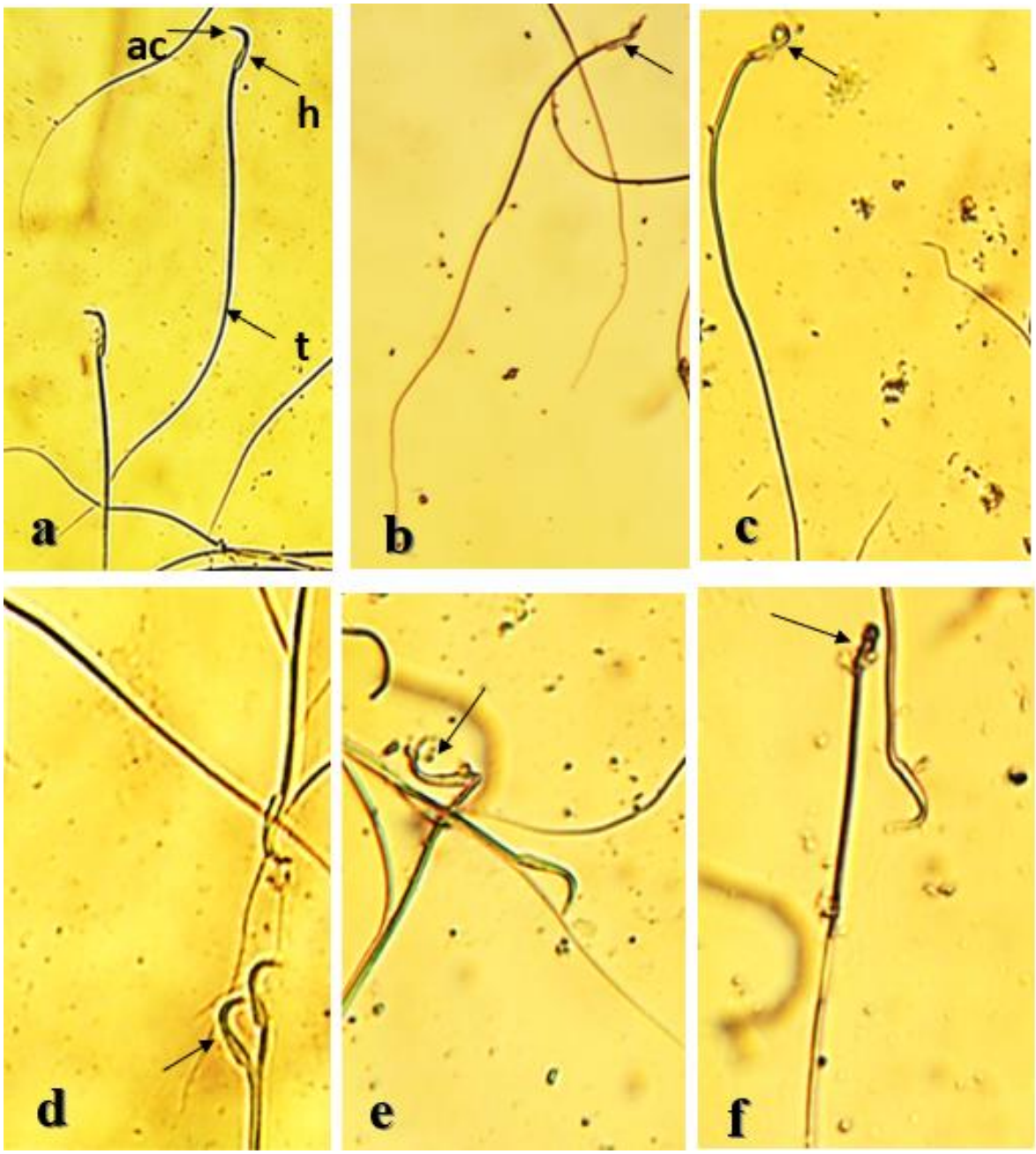

Plate 2: Photomicrographs showing normal sperm and sperm abnormalities of head in copper oxide nanoparticles treated rat. a) Normal spermatozoa with characteristic hook and a single tail (h: head; ac: acrosome; t: tail), b) Hookless head, c) Tip of the head is coiled upward, d) Double head, e) Flexed head, tip of the head facing outwards the flagellum and f) Amorphous head. Eosin-nigrosine stain, X 400.

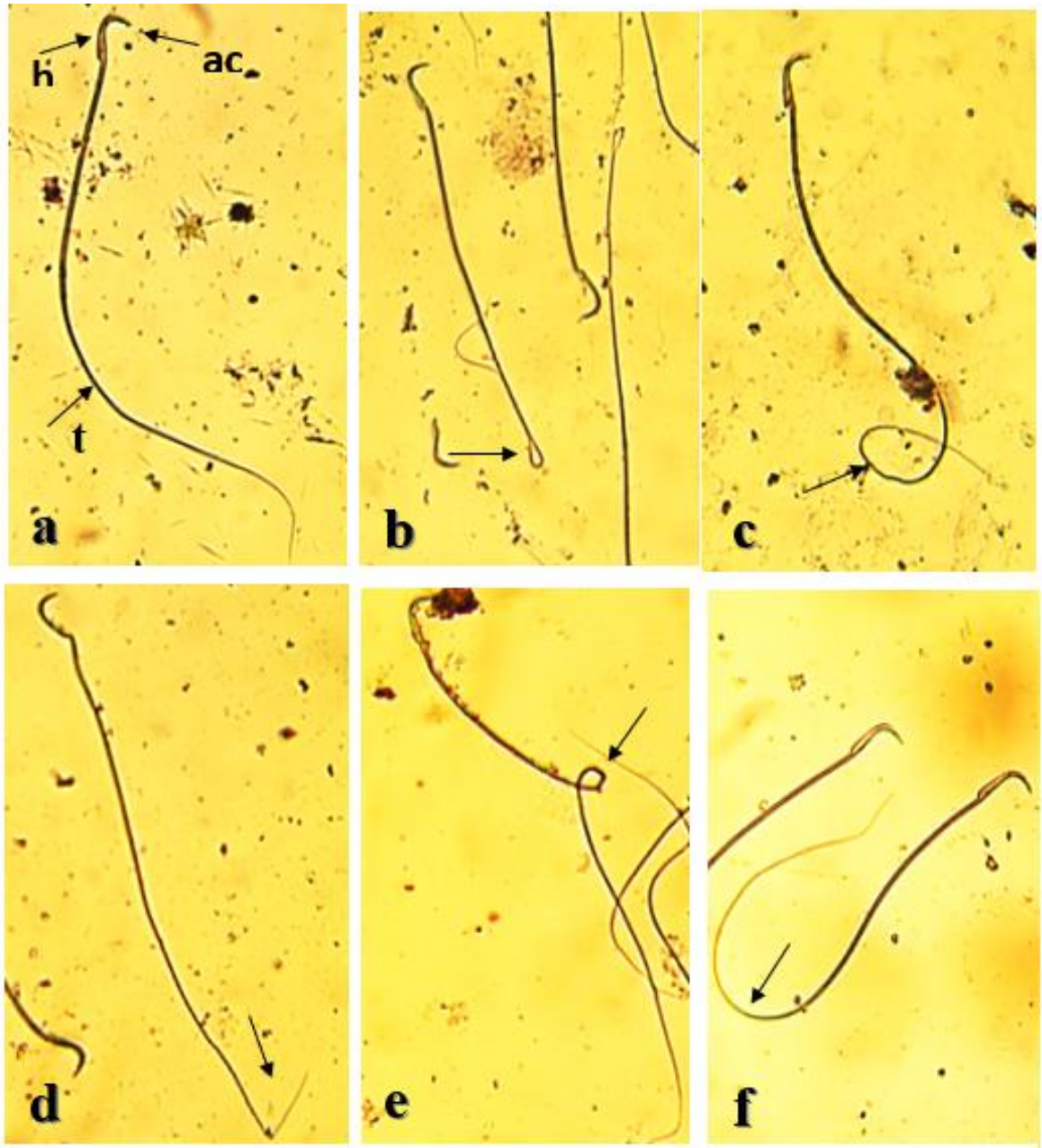

Plate 3: Photomicrographs showing normal sperm and sperm abnormalities of tail in copper oxide nanoparticles treated rat. a) Normal spermatozoa with characteristic hook and a single tail (h: head; ac: acrosome; t: tail), b) Hairpin loop, c) Coiling tail, tip of the tail facing outwards the head, d) Bent tail, e) Coiled tail and f) Coiling tail, tip of the tail facing towards the head. Eosin-nigrosine stain, X 400.

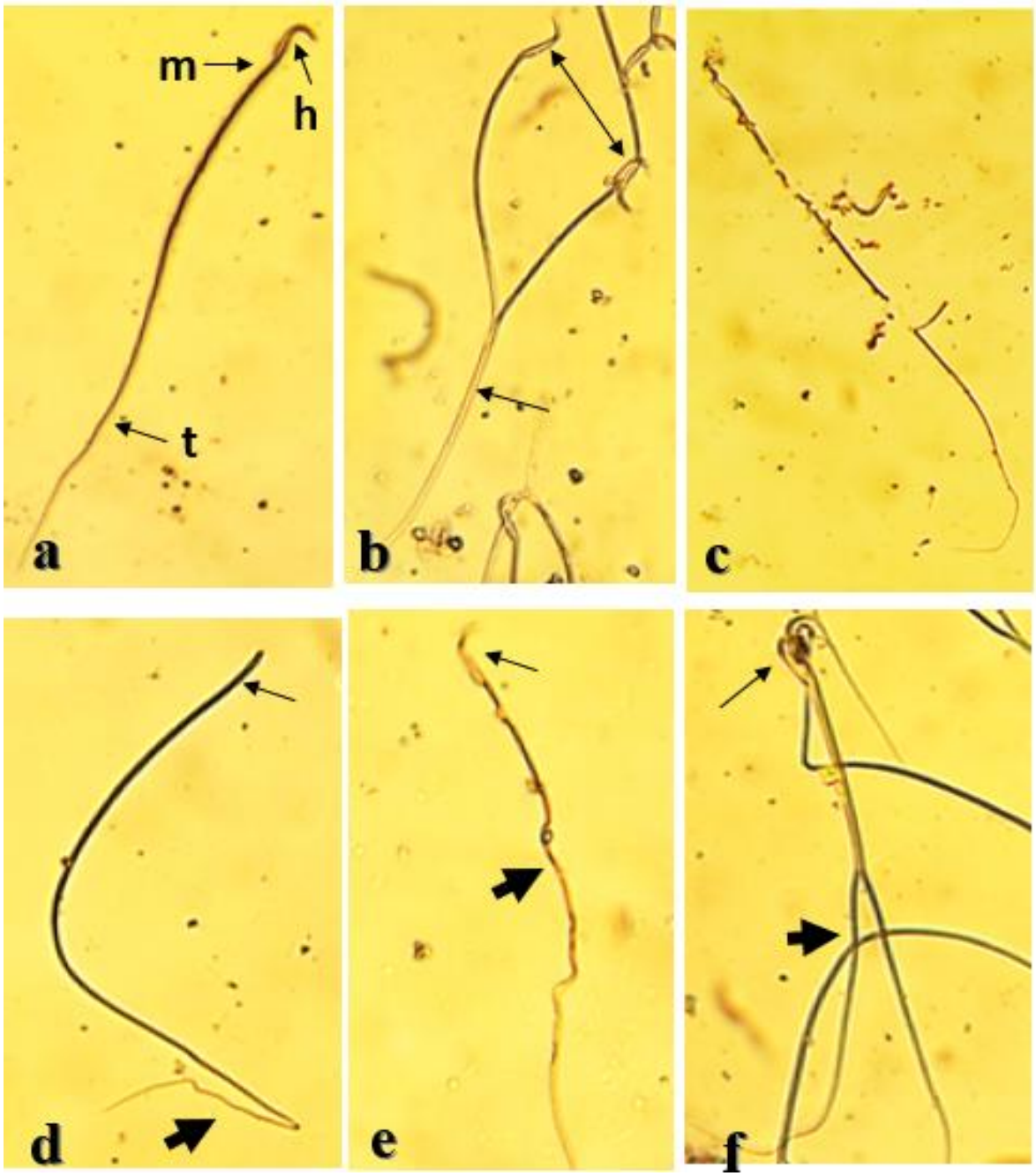

Plate 4: Photomicrographs of normal sperm and sperm abnormalities of head and tail in copper oxide nanoparticles treated rat. a) Normal spermatozoa with characteristic hook and a single tail (h: head; ac: acrosome; t: tail), b) Double head (double arrow) and fused tail (thin arrow), c) Damaged sperm, d) Headless sperm (thin arrow) and bent tail (thick arrow), e) Lack of hook (thin arrow) and zigzag tail (thick arrow) and f) Double head (thin arrow) and double tail (thick arrow). Eosin-nigrosine stain, X 400.

DISCUSSION

New developments in the field of nanotechnology have led to the use of NPs in the manufacturing processes of many consumer goods, in addition to a range of industrial uses and several novel medical procedures (Arato *et al*., 2023). According to Baranowska-Wojcik *et al*. (2020), NPs accumulate in the respiratory tract, alimentary tract, heart, liver, spleen and kidneys following inhalation or oral exposure. Infertility rates have increased over the past few decades from 6.7% to 25% due to advances in social modernization and prevalent

pollution of the environment (Zhou *et al*., 2018). According to estimates, males only may be responsible for up to 30% of infertility, and poor sperm count, low sperm quality, or both, account for up to 90% of these cases (Leaver, 2016). There is a critical demand to investigate the system toxicity of nano-copper since it is extensively employed as surface coatings, feed additives, and in other industries (Chen *et al*., 2022). There have been several studies on nano-copper published, but few have discussed the toxicity of the material to the male reproductive organs. As environmental stressors such as xenobiotics, heavy metals, microwaves, and nanoparticles have garnered a lot of attention lately, the reproductive organs are extremely vulnerable to them (Wang *et al*., 2016).

According to Liu *et al*. (2016), the reproductive organs are a fragile biological system that is vital for passing the genome to the offspring and is extremely vulnerable to external threats. Given the special characteristics of the reproductive system, it is becoming more widely acknowledged that the biological impacts of NPs on this sensitive system play a significant role in total toxicity (Wang *et al*., 2018).

Previous reports have claimed that some NPs may be able to cross the BTB and blood epididymal barrier (BEB), two physiological barriers in the system of reproduction. According to Liu *et al*. (2016), the chemical, physical, and administration modes of NPs have the potential to affect their capacity to penetrate. One explanation for the negative impacts of nanomaterials is said to be their tiny size (Strauch *et al*., 2017). According to Taran *et al*. (2017), NPs exhibit deep tissue penetration and enhanced cell absorption due to their minute size.

It is generally known that H can induce HO-1. According to Mateus *et al*. (2018), HO-1 is a rate-limiting enzyme of heme metabolism that generates carbon monoxide, free iron, and biliverdin, among other antioxidant and anti-inflammatory compounds. Heat stress, heavy metals, hyperoxemia, UV radiation, and other triggers can easily cause the HO-1 protein to be produced (Loboda *et al*., 2016).

According to Chen *et al*. (2020), among the most prevalent and sensitive markers of a drug's adverse impacts on animals are their weight and organ weight. Previous investigations have established characteristics related to toxicity generated by NPs, such as a reduction in weight in the body and associated organs (Radhi and Al-Bairuty, 2019; AL-Musawi *et al*., 2022).

In this work, oral administration of CuO NPs in a dose of 250 mg/kg b.w./ day for 2 weeks, day after day, caused a highly significant reduction of the final body weights of rats. These findings support the suggestions made by Al-Bairuty and Taha (2016) and De Jong *et al*. (2019) that this type of exposure may be actually harmful. The cause of the animal's declining average body weight might be a biological alteration that influences the animal's appetite and feed intake, or it could be the disruption of several metabolic activities brought on by oral gavage (Assar *et al*., 2023). However, in recent studies performed by Ouni *et al.* (2020) and Bugata *et al.* (2019), it was found that daily oral administration of CuO NPs did not impact the feed intake and the weight of the body. The size of the NPs may account for this discrepancy (De Jong *et al*., 2013).

Accordingly, the current research's outcomes also reported a substantial decline in the absolute and relative weight and size of the testis, along with a concurrent deterioration in sperm production as indicated by a substantial reduction in motility and count of sperm. Additionally, sperm abnormality was observed to be significantly higher in the CuO NPs group than in the control group. Comparing the CuO NPs group to the control, there was a considerable reduction in serum T, LH, and FSH following a similar pattern. The decrease of sex hormones and their possible significance in spermatogenesis might be the cause of CuO NPs' adverse impacts on spermatogenesis (Kerr *et al*., 2006). These findings correspond with those of Kalirawana *et al*. (2018), who discovered that mice given CuO NPs had lighter reproductive organs. Furthermore, rats treated with Ag NPs showed a substantial decrease in

testicular weight, as reported by Assar *et al*. in 2023. The study's reported reduction in relative testis weight might be attributed to many factors such as histopathological alterations (Kong *et al*., 2014), spermatogenesis defects and germ cell death (Karimi *et al*., 2019), or metal deposits in the testes. Furthermore, it's possible that the large drop in serum T levels or the generation of OS in the testicular tissue by NPs is what caused the testes' weight and size to significantly decrease in the current outcomes, which had a negative impact on the Leydig cells which discharge T hormone (Ahmed *et al*., 2017). The current results are consistent with those of Olugbodi *et al*. (2020), who found that rats given Ag NPs had significantly lower levels of T, LH, and FSH. In contrast, the results of Al-Bairuty and Taha (2016) showed a statistically significant increase in the relative testis weight with Cu NPs treatments compared to control group.

Regarding changes in body weight, H+ CuO NPs - treated rats revealed a substantial elevation in the mean final body weight in contrast to CuO NPs group. The improvement of H in the weight could be attributed to the decrement in MDA content which increased by CuO NPs or to the antioxidant property of H (Ali *et al*., 2019). Silva *et al*. (2022) disagreed with these findings and reported that H did not show any apparent impact on the animals' body weight.

Furthermore, despite a percentage acquire was observed, the groups were orally given H in a dose of 40 μmol/kg b. w./day followed by CuO NPs did not reveal a crucial impact on the testis weight and size contrasted with CuO NPs -treated group. On the contrary, the concentrations of serum T in rodents that were exposed to H+ CuO NPs matched those of the control group. Hence, the absence or minimal harm to the testis in the group treated with H+ CuO NPs was determined by the normal hormone levels. Furthermore, when H was administered simultaneously with CuO NPs to rats, LH and FSH levels increased substantially in comparison to the group exposed to CuO NPs but remained lower than in the control group. Hemin, an extremely potent inducer of HO-1, has demonstrated encouraging potential as a protective agent against inflammatory and oxidative damage to various types of tissue, involving testicular tissue. (Islam *et al*., 2019).

The results of the existing study revealed exhaustion of the antioxidant defense mechanism (GSH), accumulation of ROS, and MDA which in turn induces OS in the testicular tissue decreasing the vitality of the testes. This alteration was significantly observed in CuO-treated groups after 28 days of exposure. Notably, contrasted to control, CuO NPs were observed to drastically damage antioxidant defenses and raise levels of intracellular oxidized products, MDA, in addition to diminishing CAT activity, SOD, and GSH levels. These findings showed that OS could be the source of CuO NPs' cytotoxicity to epididymal sperm cells. CuO NPs' primary source of cytotoxicity is their capacity to produce ROS, which causes cell death (Denluck *et al*., 2018). Otherwise, the H administration retrieved CAT, SOD and GSH activities to approach the normal values. This amelioration in the oxidant/antioxidant status in the testis tissue can be attributed to the antioxidant activities of HO-1 induced by H. Furthermore, HO-1 is considered a possible antioxidant that could be useful in preventing OS (Chen *et al*., 2019).

The testis's seminiferous tubules create sperm, which are then moved and stored in the epididymis before exiting the male body (Yang *et al*., 2022). NPs mostly affect the epididymis and testis (Zhao *et al*., 2018). Consequently, the injury to the epididymis or/and testis may be a possible cause of the loss of sperm in both quality and quantity following oral gavage with CuO NPs. T, LH, and FSH are three hormones that are especially essential to the destiny of male germ cells since changes in them can result in abnormal sperm factors or even germ cell death (Shiraishi and Matsuyama, 2017).

The sperm functional indicators in the CuO NPs-treated rats were negatively impacted, with the exception of sperm viability. These measures include sperm abnormalities, sperm progressive motility, and epididymal sperm number. The current research suggests that ROS

may have caused a changed and hostile interior of the epididymis, leading to a substantial reduction in the number of epididymal sperm, motility (progressive and rapid), and raised abnormalities, along with a rise in the number of nonmotile and dead sperm in the CuO NPs treated group (Farombi *et al*., 2016). According to Al-Bairuty and Taha (2016), the impacts of NPs on germ cells disrupt the spermatogenic procedure, resulting in a decrease in the number of sperm. According to research by Gromadzka-Ostrowska *et al*. (2012), NPs can cross the BTB, damage male germinal cells, and lower the quality of sperm. Additionally, OS in the testis is caused by mitochondrial damage and a decrease in adenosine triphosphate (ATP) concentrations (Xu *et al*., 2014). Additionally, mice treated with 20 mg/kg of silica NPs for 15 days showed decreased amount and quality of epididymal sperm in addition to damage to DNA (Xu *et al*., 2014).

CuO NP's impact on Sertoli cell activity or OS might be the reason for a rise in the percentage of malformed epididymal sperm. The current research's findings support the hypothesis put out by Kruszewski *et al*. (2011), who claimed that Ag NPs might interact with DNA in the cell to cause oxidative injury, inflammatory processes, and cellular disorder, which in turn led to mutations in genes and aberrantly shaped sperm cells.

The current findings indicate a possible cause of impaired sperm motility: a change in testicular SOD activities following the delivery of CuO NPs. Since the sperm tail contains the structural elements directly associated with motility, the mid-piece, containing the mitochondria for energy ATP synthesis, maybe to exhibit bent sperm tails in CuO NPs-treated rats (Paoli *et al*., 2011). The current research's reported sperm factor data are not compatible with those reported by Wang *et al*. (2018) and Olugbodi *et al*. (2020). The experimental methodology, mode of administration, and animal models all contribute to the discrepancies in the findings (Farombi *et al*., 2016). CuO NPs have a well-established cellular activity that involves the release of free radicals and the activation of OS (Chen *et al*., 2022). Sperm cell motility and genomic integrity have been shown to be affected by OS (Iommiello *et al*., 2015). CuO NPs have been noticed to penetrate the organelles of cells, specifically the mitochondria, causing impairment of membrane potential and induction of the formation of free radicals. This is indicated by the noticed rise in MDA levels and suppression of antioxidant enzyme activities in the testicular tissue of rats administered with CuO NPs. Following exposure to CuO NPs, there may be a decrease in CAT, SOD and GSH activities as a result of Cu NPs complexing with thiol groups (Sangodele *et al*., 2017) or a rise in the use of CAT, SOD, and GSH to counteract the impacts of free radicals (Lawal *et al*., 2015). According to Kim *et al*. (2014) and Olugbodi *et al*. (2020), the present findings are in line with the theory that Ag NPs toxicity is caused by oxidative damage and ROS generation. Yuan *et al*. (2020) have reported that OS-induced injury may additionally interfere with antioxidant enzymes and reduce endogenous non-enzymatic antioxidants.

The current findings demonstrated that giving H to rats treated with CuO NPs significantly improved sperm functional variables such as epididymal sperm number, motility, and deformities. According to earlier research, this enhancement is accompanied by an increase in the body's antioxidant abilities and a decrease in oxidative lipid injury (Chi *et al*., 2016; Martin *et al*., 2019). Furthermore, biliverdin/bilirubin, an anti-oxidative product of HO-1 that can scavenge peroxide radicals and reduce the peroxidation of lipids, may be responsible for some of the protective properties of the compound (Shan *et al*., 2019). The HO system's protective impacts on testicular tissue have been highlighted by earlier research (Heeba *et al*., 2016).

The hypothalamic-pituitary-gonadal axis damage seen in the current study in rats treated with CuO NPs could be the cause of the notable decrease in the blood levels of T, LH, and FSH. Particularly, co-treatment with H enhanced the levels of these sex hormones, a finding earlier established (Aziz *et al*., 2013). The observed enhancement in Leydig cell alterations caused by OS may be attributed to the antioxidant capability of HO-1, as reported

by Islam *et al*. (2019). This might enhance T secretion and, in turn, the reproductive function of rats treated with CuO NPs (Glade and Smith, 2015). According to Islam *et al*. (2019), H may have a direct stimulative impact on the pituitary gland, raising LH release, and on the Leydig cell, boosting the function of steroidogenic enzymes, which would account for its favorable effects on the blood level of T.

The noticed restoration of semen parameters to control values in rats treated with H + CuO NPs may be related to the antioxidant properties of H, its enhancement of sex hormones, as well as its defense against DNA damage and apoptosis (Heeba *et al*., 2016; Islam *et al*., 2019). According to Shiraishi and Naito (2005), who concurred with the current findings, spermatogenesis was protected by elevated Leydig cell expression and HO-1 was shown to be solely expressed in Sertoli cells, which is crucial for regular spermatogenesis (Middendorff *et al*., 2000).

Conclusion

The current findings showed that CuO NPs may be classified as a male reproductive toxicant because they reduced levels of fertility hormones, sperm quantity and quality, and body and testicular weight declines, all of which had an adverse impact on the male reproductive system's activity. Therefore, it might be concluded that these NPs are highly toxic to reproductive function and might alter the fertility of animals. In addition, H treatment significantly attenuated reproductive toxicity caused by CuO NP exposure in rats. The primary components of H's protective impact are its metabolic products' antioxidant, anti-inflammatory, and antiapoptotic properties, which are induced by HO-1.

Declarations:

Ethical Approval:Ethical Approval is not applicable.

Conflicts of Interest: The authors claim that there are no conflicts of interest.

Authors Contributions:I hereby verify that all authors mentioned on the title page have made substantial contributions to the conception and design of the study, have thoroughly reviewed the manuscript, confirm the accuracy and authenticity of the data and its interpretation, and consent to its submission.

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ARABIC SUMMARY

الدور الوقائي للهيمين على وظائف التكاثر لذكور الجرذان البالغة المعاملة بجسيمات أكسيد النحاس النانوية

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تُستخدم جسيمات أكسيد النحاس النانوية (CuO NPs) على نطاق واسع في العديد من التطبيقات الصناعية والتجارية ولا يُعرف سوى القليل عن سميتها المحتملة على الحيوانات المنوية. الهيمين هو محفز الهيم أوكسجيناز 1 والذي لديه دور وقائي في حماية الخلايا. في هذا البحث استخدمت جسيمات أكسيد النحاس النانوية لدراسة تأثيرها على أوزان الخصى وكذلك معايير النطف لذيل البربخ في الجرذ ان كنموذج للكائنات الثديية. الهدف الرئيسي لهذه الدراسة هو تأثير الهيمين على النطف بعد معاملة الجرذان بجسيمات أكسيد النحاس النانوية. استخدم في هذا البحث خمسون جرذ قسمت إلى خمس مجموعات (كل مجموعة عشر جرذان): **المجموعة الأولى:** مجموعة ضابطة، **المجموعة الثانية:** مجموعة تناولت 40 ميكرو مول/كجم من وزن الجسم/يوم من المذيب، **المجموعة الثالثة:** جرذان تلقت 40 ميكرو مول/كجم من وزن الجسم/يوم من الهيمين، **المجموعة الرابعة :** عوملت الحيوانات بجرعة 250 مجم/كجم من وزن الجسم/يوم من جسيمات أكسيد النحاس النانوية **والمجموعة الخامسة:** تلقت الجرذان 40 ميكرو مول/كجم من وزن الجسم/يوم من الهيمين متبوعة بجسيمات أكسيد النحاس النانوية بجرعة 250 مجم/كجم من وزن الجسم/يوم. أعطيت كل الجرعات عن طريق الفم يوم بعد يوم لمدة 14 يوم. في نهاية التجربة تم تشريح جميع الجرذان وأخذت عينة من الدم لقياس هرمونات الخصوبة في المصل وكذلك تم طحن الخصية لقياس مستوى أنزيمات مضادات الأكسدة. تم اجراء تحليل السائل المنوي من ذيول البرابخ لتقييم العدد والحركة وعمل سحبات منوية صبغت بالإيوسين والنجروسين لدراسة مورفولوجية النطف. أظهرت النتائج أن جسيمات النحاس النانوية خفضت مستويات كل من هرمونات الخصوبة ومضادات الأكسدة مصحوبة بارتفاع كبير في مستوي إنزيم محول الأكسيد الفوقي MDA مقارنة بالمجموعة الضابطة مما أدى ذلك لقلة عدد وكفاءة الحيوانات المنوية. وقد أظهرت النتائج أن للهيمين دور وقائي ملحوظ ضد التأثيرات السامة لـ جسيمات أكسيد النحاس النانوية من خلال قدرته كمادة مضادة للأكسدة.