Egy. J. Pure & Appl. Sci. 2022; 60(2):36-50

Egyptian Journal of Pure and Applied Science

# Exposure to copper nanoparticles induces oxidative stress and alters *Hsp70* and *Sod2* gene expression in *Drosophila melanogaster*

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## ARTICLE INFO

Received 25 April 2022
Accepted 23 June 2022

**Keywords**

*Drosophila,*
Copper nanoparticles,
Oxidative stress,
Nanotoxicology

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## ABSTRACT

Copper nanoparticles (CuNPs) are used in a range of industries such as semiconductors, catalysts, sensors, and antimicrobial agents. While there are already studies on its possible genotoxicity, few of these reports evidence *in vivo*. Copper nanoparticles (CuNPs) were prepared via chemical reduction and characterized by electronic transmission microscopy (TEM) and X-ray diffraction. *Drosophila melanogaster* (*D. melanogaster*) were reared on CuNPs, and $Cu^{+2}$ (as $CuSO_4$) treated food from egg to egg stage. The total number of progeny, percentage of aberrant phenotypes, oxidative stress, and gene expression of heat shock protein-70 (Hsp70) and superoxide dismutase 2 (*Sod2*) were investigated. Results showed that the acute exposure of CuNPs did not affect the fly's survivorship, unlike $Cu^{+2,}$ which showed higher toxicity. Chronic exposure of *D. melanogaster* to CuNPs (100 ppm) and $Cu^{+2}$ (50 and 100 ppm) resulted in a delay in the development of three consecutive generations. Furthermore, the ingestion of $Cu^{+2}$ and CuNPs during early developmental stages caused a dose-dependent reduction in the number of emerged flies. CuNPs and $Cu^{+2}$ treatments resulted in distinctive phenotypic aberrations, such as deformed wings transmitted to the offspring in subsequent generations. Finally, CuNPs and $Cu^{+2}$ treatments caused downregulation of the *Sod2* gene and upregulation of the *Hsp70* gene in the second and third generations. This study indicated that CuNPs are mutagenic for *D. melanogaster*. So, it is necessary to evaluate CuNPs toxicity to reduce human health-related issues.

## 1. Introduction

Nanoparticles (NPs) (particle diameter <100 nm) show exceptional physical properties (optical, electrical, and magnetic), making them more attractive for commercial and medical applications [1, 2]. On the other hand, NPs have great potential to migrate through living cells. Therefore, the widespread and diverse use of nanomaterials will pose high risks to ecosystems and humans if not properly managed [3, 4].

Therefore, nanotoxicology has been defined to bridge the knowledge gap and study the mechanisms and properties of toxicity of nanomaterials in biological systems [5].

Copper nanoparticles (CuNPs) have received much interest due to their unique physicochemical properties and low fabrication cost [6]. CuNPs are extensively used in electronics and metallic inks due to their optical, electrical, catalytic, and antimicrobial properties [7-9]. CuNPs have various applications, which increase, in turn, their environmental exposure [10, 11]. Therefore, the bioaccumulation and toxicity of CuNPs have been reported in plants [12] and animals such as rainbow trout (*Oncorhynchus mykiss*), zebrafish (*Danio rerio*) [13], nematode (*Caenorhabditis elegans*), algae, daphnia [14], and *Drosophila melanogaster* [15].

*D. melanogaster* offers a vital choice organism in toxicology studies because of the reasonable understanding of its genetics, short lifespan, and low maintenance cost. In addition, the molecular pathways and behavioral and developmental parameters can be assessed using this model in different assays, allowing a logical classification of the toxicity levels of different nanoparticles [16].

Despite the extensive study of the toxicity of NPs, there are only limited studies about CuNPs. In the current study, The effect of CuNPs on phenotype, oxidative stress, and gene expression heat shock protein-70 *(Hsp70)* and superoxide dismutase 2 *(Sod2)* in *D. melanogaster* were investigated.

Oxidative stress was considered one of the primary causes of nanotoxicity and was stated to be used as a biomarker for evaluating the toxic effects of NPs [17, 18]. CuNPs are thought to induce oxidative stress by disrupting enzyme activity such as catalase (CAT), superoxide dismutase (SOD), and glutathione-S-transferase [19, 20].

The expression of heat shock proteins correlates with the general response to shock in the animal kingdom. Induction measurement of the heat shock protein, *Hsp70*, has been suggested as a valuable technique for toxicology assessment and environmental monitoring for several stress factors, including anoxia, heavy metals, teratogens, and heat, have been demonstrated [21]. These environmental issues necessitate a rigorous assessment of the toxic effects of CuNPs, which negatively affect human and environmental health [22].

## 2. Materials and Methods

### 2.1 Synthesis of copper nanoparticles

The CuNPs were synthesized by the chemical reduction method [23]. $CuSO_4.5H_2O$ was used as precursor salt and ascorbic acid as a reducing agent. ten mL of 0.1 M $CuSO_4$ solution was added to 500 mL of 0.2 M ascorbic acid solution. Subsequently, 30 mL of 1 M NaOH solution was added dropwise with constant stirring and heating at 80 °C for two hours. The latter obtained ocher solution was then allowed to settle overnight. CuNPs were collected via centrifugation at 16000 rpm for five minutes, washed with ethanol three times, then dried.

### 2.2 X-Ray Diffraction analysis

X-Ray Diffraction (XRD) for the CuNPs was performed in the National Research Centre, Giza, Egypt, using RIGAKU Japan/ULTIMA-IV diffractometer. The powdered sample was subjected to X-ray with CuKα radiation, $\lambda = 0.154$ nm, with $2\theta$ in the range of 10°–80° and a scanning rate of 2° per min.

### 2.3 Transmission Electron Microscopy (TEM)

The particle size and morphology of CuNPs were studied by TEM imaging using a Hitachi H-7600 tungsten-tip instrument at an accelerating voltage of 100 kV. In brief, about100 µg CuNPs were suspended in one mL of water. Then 5µL of CuNPs suspension was deposited onto formvar/carbon-coated copper TEM grids. The AMT software for the digital TEM camera was calibrated for the size measurement of the nanoparticles.

### 2.4 Drosophila Culture and Procedures

The flies and larvae of wild-type *D. melanogaster* (strain Oregon-R) were cultured at 24 °C ± 1 on standard *Drosophila* sterile food containing per one liter (8.9 g agar, 76.5g cornmeal, 24g glucose, 77.5g sucrose, 27g yeast, and 5 mL propanoic acid). Fifty adult flies (20 males and 30 females) were added to glass vials containing *Drosophila's* prepared media for multiplication. After two weeks, adults were transferred to new vials to keep the cycle going.

### 2.5 Acute toxicity of CuNPs and $Cu^{+2}$

Standard *Drosophila's* media containing CuNPs or $Cu^{+2}$ with different concentrations (50, 100, 250, 500, 1000, 2000, and 4000 ppm) were prepared by mixing the culture medium with the proper amount of either CuNPs or $CuSO_4$ solution. Acute toxicity of CuNPs and $Cu^{+2}$ on *D. melanogaster* was evaluated by incubation of 15 male wild-type flies (to avoid laying eggs and hatching of new flies) on a medium with normal, CuNPs, and $Cu^{+2}$ containing media; then dead adults were scored daily for eight days. All treatments were done in three replicates.

### 2.6 Chronic toxicity of CuNPs and $Cu^{+2}$

Chronic toxicity of CuNPs and $Cu^{+2}$ was assessed by treating the flies through the entire life cycle (eggs-to-eggs). Briefly, a group of 20 flies (10 males and 10 females) was transferred to vials containing normal, CuNPs, and $Cu^{+2}$ treated food with known concentrations (50 and 100 ppm). After five days of laying eggs, the parent flies were removed. Then the life cycle development, number of successfully emerged adults, percentage, and types of abnormal phenotypic changes were assessed in three subsequent generations (F1, F2, and F3) [24].

### 2.7 Chronic effects of CuNPs and $Cu^{+2}$ exposure

As summarized in Fig. 1, the adult flies of F1, F2, and F3 of control and treated groups were examined for any phenotypic changes under a stereomicroscope (OLYMPUS Co., Japan). For next-generation, flies with normal phenotypes (10 male and 10 female) were randomly selected, transferred to a new media (containing the respective food), and cross-mated. Each treatment was carried out in triplicates.

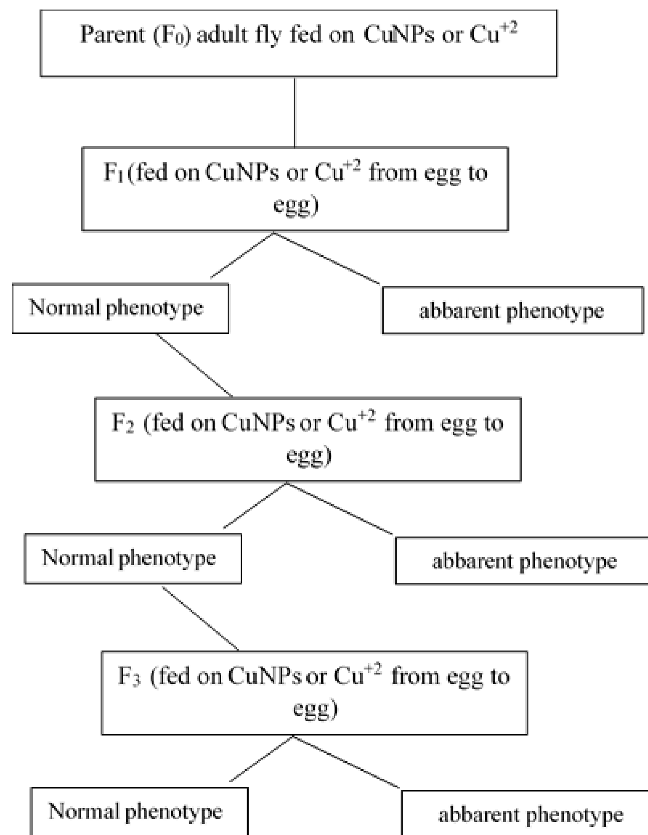


**Fig.1** Exprerimental design: chronic exposure of *D. melanogaster* to CuNPs or $Cu^{+2}$ (50 and 100 ppm)

### 2.8 Enzyme Activity Assay

#### 2.8.1 Sample preparation

Biochemical assays were performed to determine SOD and CAT activity levels in addition to lipid peroxidation assay in treated versus untreated third instar D. melanogaster larvae. Briefly, 10 mg of the larvae were homogenized in 5 mL cold 100 mM potassium phosphate buffer, pH 7.0, containing 2 Mm EDTA/ gram tissue. The solution was centrifuged at 4000 rpm for 15 min at 4 °C. The aqueous upper layer was collected and kept at a temperature from 0 to 4 °C for immediate assay [25]. Experiments were run in triplicate; the mean was used for statistical analyses.

#### 2.8.2 Superoxide dismutase (SOD) activity assay

Superoxide dismutase activity was determined by recording the inhibition of phenazine methosulphate-mediated reduction of nitro blue tetrazolium dye [26]. Phosphate buffer (50 mM, pH 8.5), nitro-blue tetrazolium (1 mM), and NADH (1 mM) were mixed in a ratio (8:1:1) to form a mixture solution. one mL of the

mixture solution was mixed with 0.1 mL of either deionized water or sample extract. Adding 0.1 mL (0.1 mM) phenazine methosulphate initiate the reaction. The nitroblue tetrazolium dye reduction rate was monitored for 5 min at 560 nm. Following the addition of the sample, the decrease in absorbance was monitored. The SOD activity was determined according to the following equations:

$$Inhibition\ \% = \frac{\Delta\ control - \Delta\ sample}{\Delta\ control}\ x\ 100$$

$$Enzyme\ activity\ U/gm\ tissue = (inhibition\ \%\ x\ 3.75)\ x\ (\frac{1}{tissue\ weight})$$

Δ control and Δ sample are the change in absorbance of control and sample, respectively.

#### 2.8.3 Catalase (CAT) activity assay

The CAT activity was determined by measuring $H_2O_2$ (5 mM) decomposition by the enzyme [27]. It was monitored by adding an aliquot ( 50μL) to the sample, followed by the addition of chromogen inhibitor, peroxidase, and 4-aminoantipyrine. The solutions were incubated for 10 min at 37°C and read at 510 nm. The CAT activity was determined according to the following equation:

$$Enzyme\ activity\ U/gm\ tissue = \left[\frac{A_{standard} - A_{sample}}{A_{standard}}\right]\ x\ \frac{1}{tissue\ weight}$$

$A_{standard}$ and $A_{sample}$ are the absorbances of the standard and sample, respectively.

#### 2.8.4 Lipid Peroxidation

Lipid peroxidation assay was done by [Thiobarbeturic acid (TBA) reactive substances] TBARS assay method [28]. TBA reagent (containing 0.037g TBA, 15% TCA, and 0.24N HCl per 10ml) was mixed with 100 µL of the larvae extracts or Malondialdehyde (MDA) standard. The reaction mixture was boiled for 15 minutes, and the absorbance was determined at 532nm. The concentration of MDA in the samples was calculated using the standard MDA curve.

### 2.9 Gene expression analysis by real-time PCR

Changes in the expression profiles of heat shock protein-70 and superoxide dismutase-2 were determined using Real-time quantitative PCR (RT-qPCR). Total RNA was extracted from a group of 10 third instar treated and untreated larvae of *D. melanogaster* using miRNeasy Mini Kit (Qiagen, Valencia, CA). RNA quality and quantity were verified using Thermo Scientific™ NanoDrop 2000.

cDNA was synthesized using one µg of total RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche, US) and stored at -20 ºC till further use. The resulting cDNA was amplified using Real-Time PCR analysis. SYBR green technology was used to assess the relative expression of the selected genes using the PowerUp SYBR green master mix. 60S ribosomal protein L32 (RPL32) was used as a housekeeping gene.

For each gene, one µL (20 ng) of cDNA, one µL forward primer, one µL reverse primer, 10 µL SYBR green master mix were mixed and diluted to 20 µL using deionized water. Reaction conditions for all genes were: pre-incubation for 5 min at 95 °C, one cycle, and the amplification was set for 45 cycles (10 s at 95 °C, 15 s at 61 °C, 72 °C for 25 s). primer sequences used in the present study are presented in Table 1.

### 2.10 Statistical analysis

The experiment was designed with three replicates for each test. One-way ANOVA followed by post-hoc multiple comparisons. Data were calculated as mean ±standard error. Significance was ascribed at $P \leq 0.05$.

**Table 1** Sequences of the primers used in the present study. Primers were designed using NCBI primer design tool.

| Gene name | Forward (5- > 3) | Reverse (5- > 3) |
|---|---|---|
| *RPL32* | GCCCAAGGGTATCGACAACA | GCGCTTGTTCGA TCCGTAAC |
| *Sod2* | GAGGGACGCACGTTCTTGTA | ATCTAAATGCCGCCGAGGAG |
| *Hsp70* | GAGCACGATGTCGTGGATCT | AGAGGACATGAAGCACTGGC |

## 3. Results

### 3.1 Characterization of CuNPs

X-Ray Diffraction was performed to confirm the crystallinity of the nanoparticles. XRD diffractogram (Fig.2A) reveals typical Bragg diffraction peaks of cubic Cu crystals. The absence of impurity peaks and high peak intensity indicate that these CuNPs are pure and crystalline

The diffraction pattern represents specific peaks for pure Cu at 2θ values of 43.29°, 50.41°, and 74.07° (reference code. 01-071-4610). Also, TEM was used to identify the sizes and shape of CuNPs. The TEM image (Fig. 2B) reveals the prism shape of CuNPs, whose size range from 13 to 20 nm.

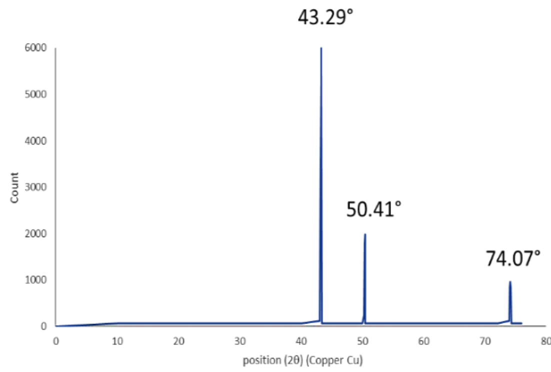


**Fig. 2A** X-ray diffraction pattern of synthesized CuNPs. The diffraction pattern represents specific peaks for pure Cu at 2θ values of 43.29°, 50.41°, and 74.07

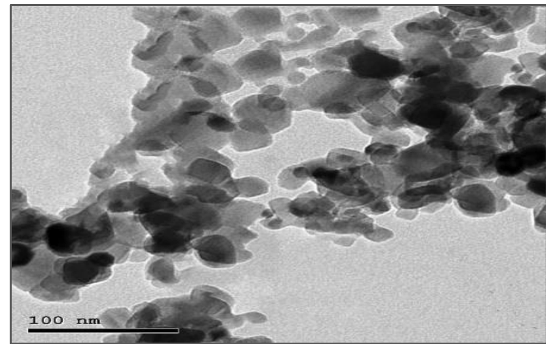


**Fig. 2B** TEM image of synthesized CuNPs reveals the spherical shape of CuNPs with a particle size range from 13 to 20 nm

### 3.2 Acute toxicity

The copper ion $Cu^{+2}$ caused acute toxicity at 1000, 2000, and 4000 ppm, while the concentrations of 50, 100, 250, and 500 ppm did not show acute toxicity. As shown in (Fig. 3), the number of surviving adults decreased significantly to 46.6% after exposure to a concentration of 1000 ppm for eight days. Concentrations of 2000 and 4000 ppm resulted in the death of all flies after 7 and 6 days, respectively. In contrast, CuNPs did not affect the fly's survivorship and showed no apparent negative impact on adult physical activity up to 4000 ppm for eight days. So, CuNPs showed no acute toxicity on *D. melanogaster* within the used concentrations.

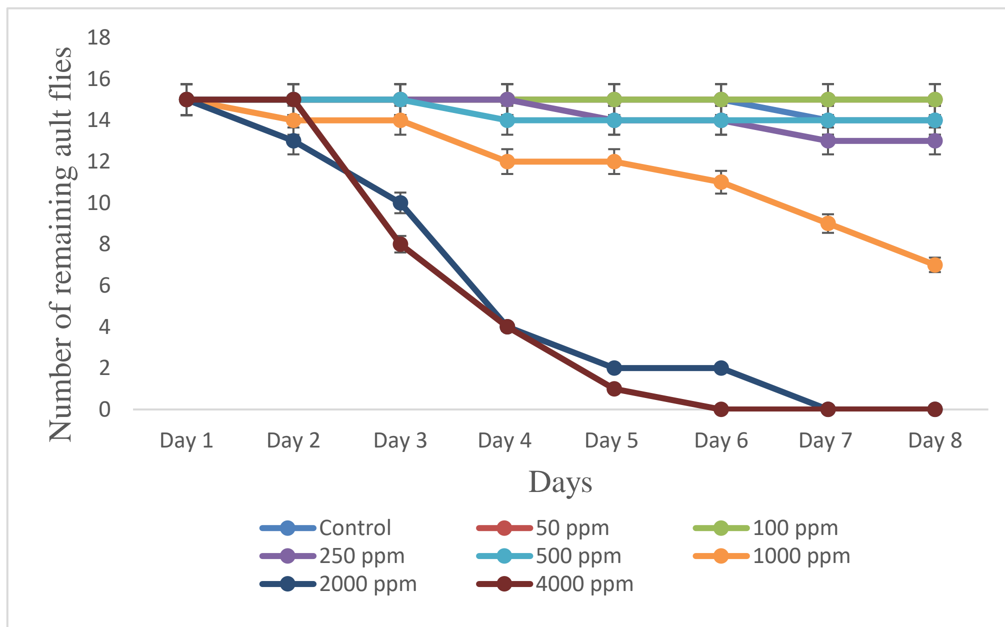


**Fig. 3.** The number of flies survived after oral administration of different concentrations of $Cu^{+2}$ compared to control. The 50, 100, 250, and 500 ppm concentrations showed no acute toxicity. At 1000 ppm $Cu^{+2}$ concentration, the number of surviving adults significantly decreased to 7 (46.6%). The 2000 and 4000 ppm concentrations resulted in the death of all flies after 7 and 6 days, respectively. Data analyzed represent mean ± standard deviation and error bar

### 3.3 Chronic toxicity

D. melanogaster adults were exposed to CuNPs and $Cu^{+2}$ for three generations to evaluate the chronic effects of CuNPs and $Cu^{+2}$ on the life cycle, the total number of emerging adult flies, morphological changes, levels of oxidative stress markers, and the expression of stress-associated genes.

Viability (egg-to-adult) was first determined to choose suitable doses to further experiments. The 500 ppm and 250 ppm concentrations of $Cu^{+2}$ and 500 ppm of CuNPs caused the death of a large percentage of larvae. The surviving larvae were not developed into the adult fly. So, the experiment was continued with the lower concentration for both $Cu^{+2}$ and CuNPs, *i.e.,* 50 and 100 ppm.

### *3.4 Chronic effects on the life cycle of flies*

After the flies laid their eggs in each vial, the development time was monitored in each vial compared to normal development in control. In all generations, there was a significant delay in development found at 100 ppm CuNPs and both concentrations in $Cu^{+2}$ treated flies (Fig. 4A & B). The first, second, and third instar larvae were observed in the untreated flies after 48±4, 72±2, and 96±5 hours. The pupa appeared within 144±4 hrs, and the adult flies started to hatch after 168±8 hrs.

After treatment, the developmental delay started to appear during the third larval instar in the first generation and the first larval instar in the second and third generations and continued until adulthood. Besides, $Cu^{+2}$ causes more developmental delay than CuNPs.

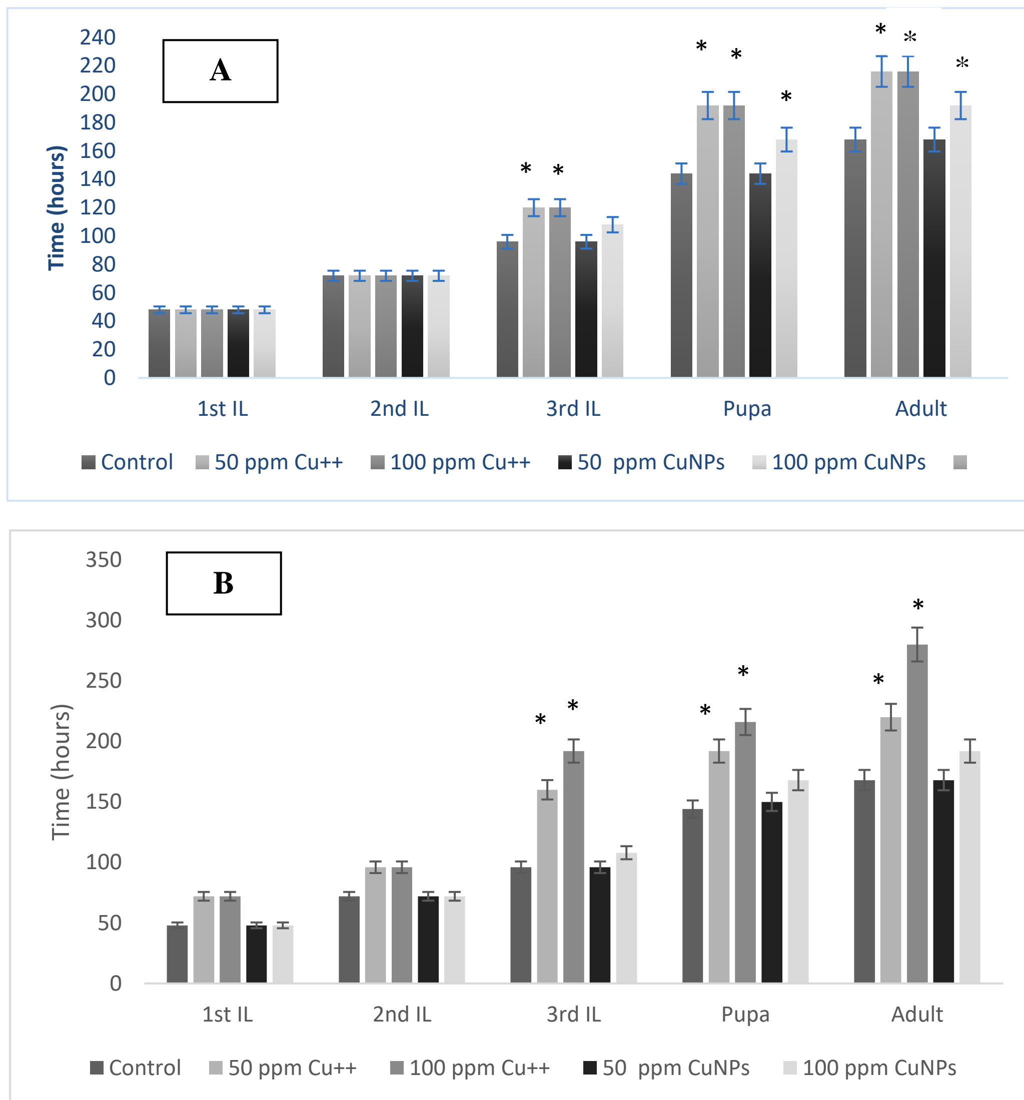


**Fig. 4A** The life cycle of first-generation (F1) *D. melanogaster* treated with different concentrations of CuNPs and Cu+2, IL: Instar Larvae. **B** The life cycle of the second generation (F2) of *D. melanogaster* treated with different concentrations of CuNPs and Cu+2. IL: Instar Larvae. (*) means Significantly different from the values of the control group at $p \leq 0.05$

### *3.5 Chronic effects on the emerged adult flies*

This assay determines the percentage of flies that successfully developed from the pupal stages. The number of emerged adults was calculated as a percentage of the control group of the same generation. Results showed that the ingestion of $Cu^{+2}$ and CuNPs during the egg-to-adult development caused a dose-dependent reduction in the number of emerging adults. As shown in Fig. 5, the percentage of emerging adult flies in the first generation was reduced to 87%, 58%, 97%, and 90% for 50 and 100 ppm $Cu^{+2}$ and 50, 100 ppm CuNPs, respectively. In the second generation, the percentage of emerging adult flies was reduced to 82%, 36%, 88%, and 77% for 50 and 100 ppm $Cu^{+2}$ and 50, 100 ppm CuNPs, respectively. In the third generation, the percentage of emerging adult flies was reduced to 71%, 30%, 80%, and 38% for 50 and 100 ppm $Cu^{+2}$ and 50, 100 ppm CuNPs, respectively.

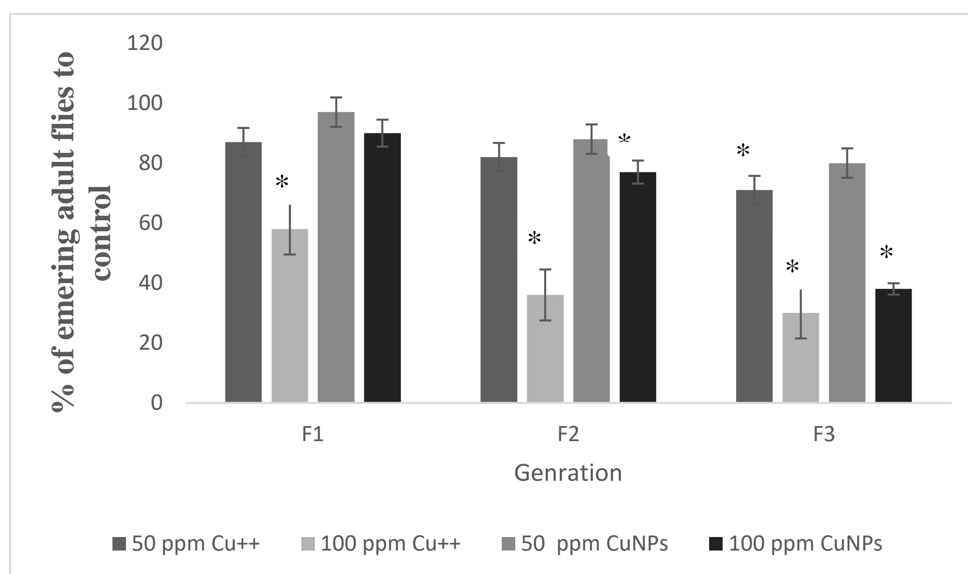


**Fig. 5** Effect of chronic CuNPs and $Cu^{+2}$ administration on the percentage of emerging adult flies to control. The percentage of emerging adult flies in the first generation was reduced to 87%, 58%, 97%, and 90% for 50 and 100 ppm $Cu^{+2}$ and 50, 100 ppm CuNPs, respectively. In the second generation, the percentage of emerging adult flies was reduced to 82%, 36%, 88%, and 77% for 50 and 100 ppm $Cu^{+2}$ and 50, 100 ppm CuNPs, respectively. In the third generation, the percentage of emerging adult flies was reduced to 71%, 30%, 80%, and 38% for 50 and 100 ppm $Cu^{+2}$ and 50, 100 ppm CuNPs, respectively. (∗) means Significantly different from the values of the control group at $p \leq 0.05$

### *3.6 Chronic effects on adult fly morphology*

The morphological analysis of the different adult structures revealed some morphological alterations in the fly's wings because of CuNPs and $Cu^{+2}$ exposure (Figs. 6, 7 & 8). For further evaluation of the chronic effects of CuNPs and $Cu^{+2}$ administration in the subsequent generations (F1, F2, and F3), flies were screened for phenotypic changes. As shown in (Fig. 6), in the 1st generation, the percentage of mutated flies was mild as 2.4%, 0.4%, 1.3%, and 0.9% in 50, 100 ppm CuNPs, and 50, 100 ppm $Cu^{+2,}$ respectively,compared with the control, which showed no mutations. In the second generation, the mutations were 0.4%, 4.8%, 3.3%, 3.2%, and 1.3% in the control, 50, 100 ppm CuNPs, 50 and 100 ppm $Cu^{+2}$ respectively. In the third generation, the mutations were raised to 13.12%, 8.14%, 3.2%, and 2.4% in 50, 100 ppm CuNPs, 50 and 100 ppm $Cu^{+2}$, respectively, while the control group showed only 0.6 % mutations.

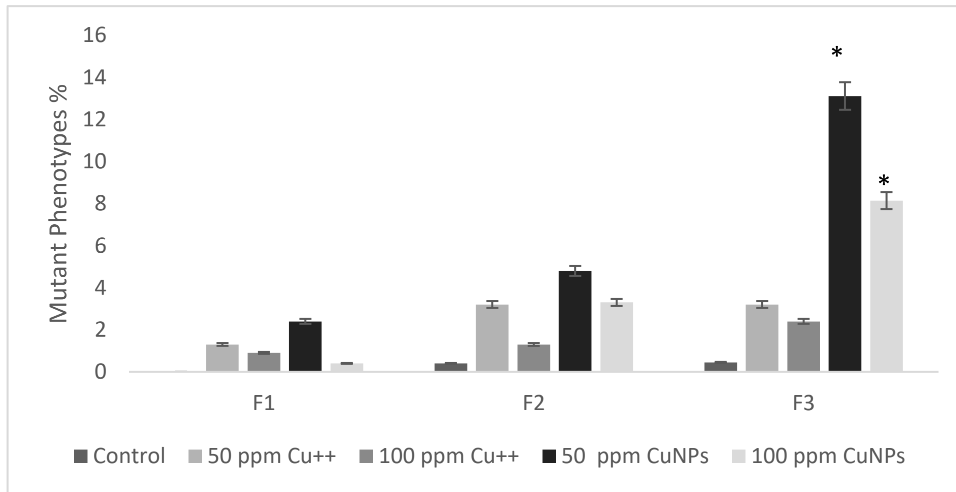


**Fig. 6** Percentage of mutant phenotypes observed in the progeny arising from *D. melanogaster* treated with $Cu^{+2}$ and CuNPs. (∗) means Significantly different from the values of the control group at $p \leq 0.05$

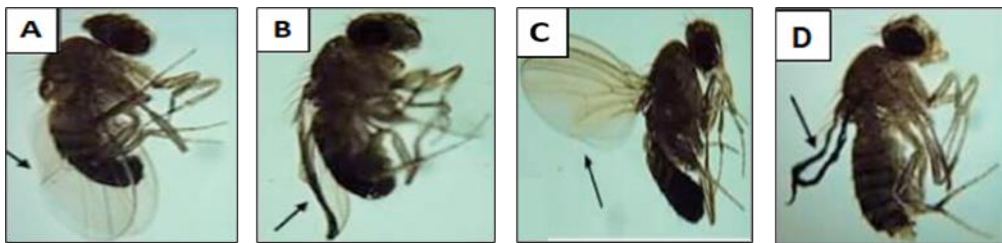


**Fig. 7** Representative images of mutant phenotypes observed in the 2nd generation progeny arising from flies treated with CuNPs and $Cu^{+2}$. Several wings' deformations were shown. (A) wing deformation in fly treated by 50 ppm CuNPs (B) lethal mutation (necrosis) in the fly's wings exposed to 100 ppm CuNPs (C) Drosophila wing venation in 50 ppm $Cu^{+2}$ treatment (D) lethal mutation (necrosis) in the fly's wings exposed to 100 ppm $Cu^{+2}$

**Fig. 8** Representative images of mutant phenotypes observed in the 3rd generation progeny arising from flies treated with CuNPs and $Cu^{+2}$. Several wings' deformations were shown. (A) lethal mutation (necrosis) in the fly's wings exposed to 50 ppm CuNPs (B) wing deformation in fly treated by 50 ppm CuNPs (C) Drosophila wing venation in 50 ppm $Cu^{+2}$ treatment (D) lethal mutation (necrosis) in the fly's wings exposed to 100 ppm $Cu^{+2}$

### *3.7 Antioxidant enzyme activity levels*

The CAT activity of treated and untreated flies is shown in Fig. 9. In the first generation, the mean CAT activity significantly decreased ($p < 0.05$) in concentrations of 100 ppm of $Cu^{+2}$ and CuNPs compared with the control group. The adverse effect of $Cu^{+2}$ and CuNPs on CAT activity became more severe in the second and third generations. Compared to the control group, the CAT activity significantly decreased in all treated groups. Furthermore, mean MDA levels significantly increased after treatment with $Cu^{+2}$ and CuNPs in a similar pattern of CAT enzyme (Fig. 10). In contrast, the SOD activity showed insignificant change in all treatment groups in all generations compared with the control group (Fig. 11).

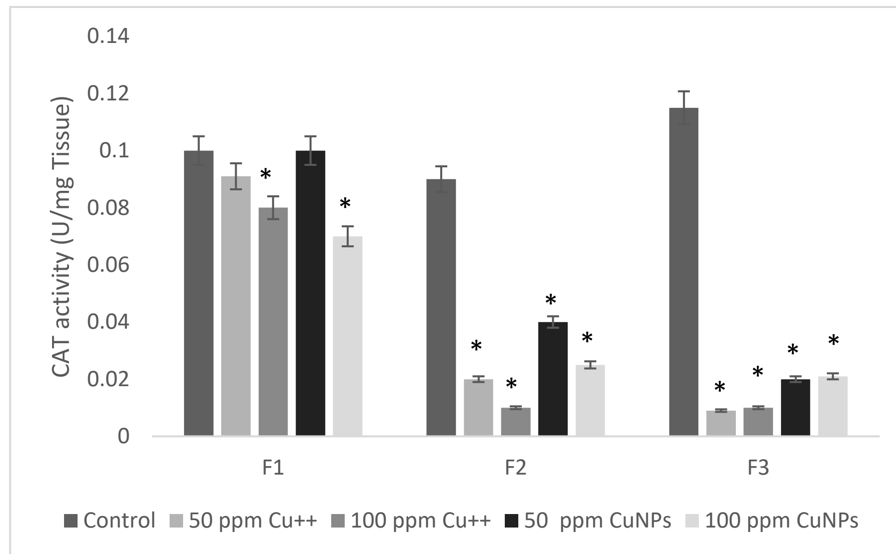


**Fig. 9** CAT enzyme activity in third instar larvae of *D. melanogaster* treated with CuNPs and $Cu^{+2}$ at 50 and 100 ppm concentrations. (*) means Significantly different from the values of the control group at $p \leq 0.05$

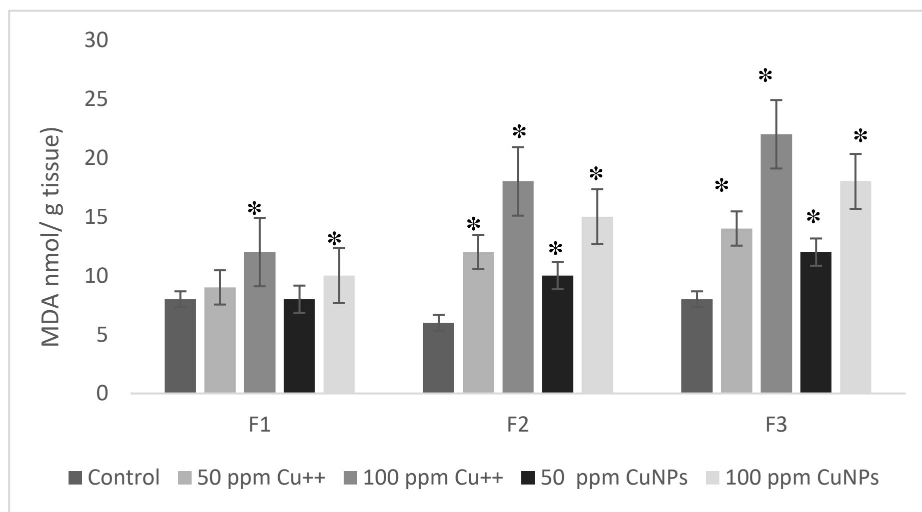


**Fig. 10** Lipid peroxidation in third instar larvae of *D. melanogaster* treated with CuNPs and $Cu^{+2}$ at 50 and 100 ppm concentrations. (*) means Significantly different from the values of the control group at $p \leq 0.05$

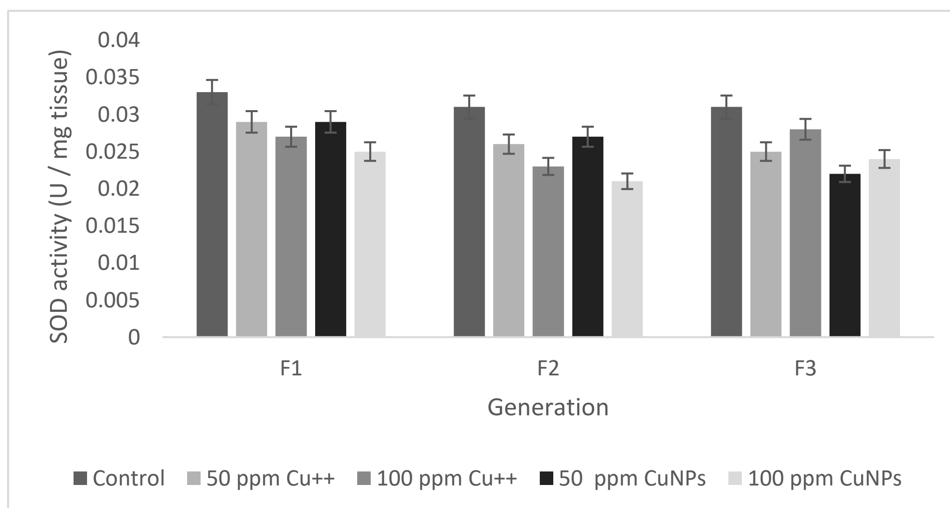


**Fig. 11** SOD enzyme activity in third instar larvae of *D. melanogaster* treated with CuNPs and $Cu^{+2}$ at 50 and 100 ppm concentrations. The results showed a nonsignificant change in treated and untreated flies

### 3.8 Gene expression analysis

Real-time quantitative PCR was used to determine the expression level of *Hsp70* and *Sod2* genes in the third instar larvae after exposure to 50 and 100 ppm of CuNPs and $Cu^{+2}$ in three generations. The obtained results showed changes in gene expression in third instar larvae after exposure to 50 and 100 ppm of CuNPs and $Cu^{+2}$. *Hsp70* did not change at all concentrations applied in the first generation, while it was significantly upregulated in the second and third generations in all treated flies by dose and generation independent manner. The fold change in the expression profile of *Hsp70* in all generations was calculated (Fig. 12). On the other hand, *Sod2* was upregulated in the first generation and downregulated in the second and third. The fold change in the expression profile of *Sod2* in all generations was calculated (Fig. 13).

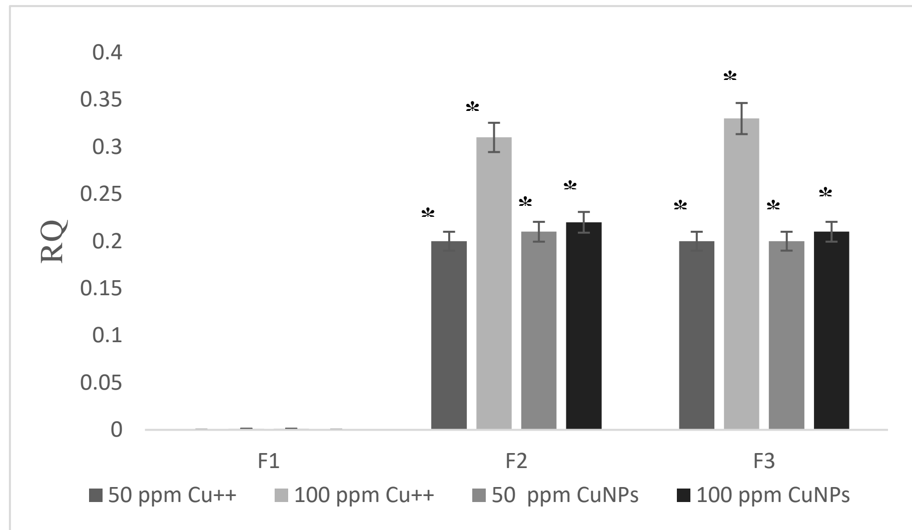


**Fig. 12** The expression of *Hsp70* in third-instar larvae of *D. melanogaster* treated with different doses of CuNPs and $Cu^{+2}$ in different generations. (∗) means Significantly different from the values of the control group at $p \leq 0.05$

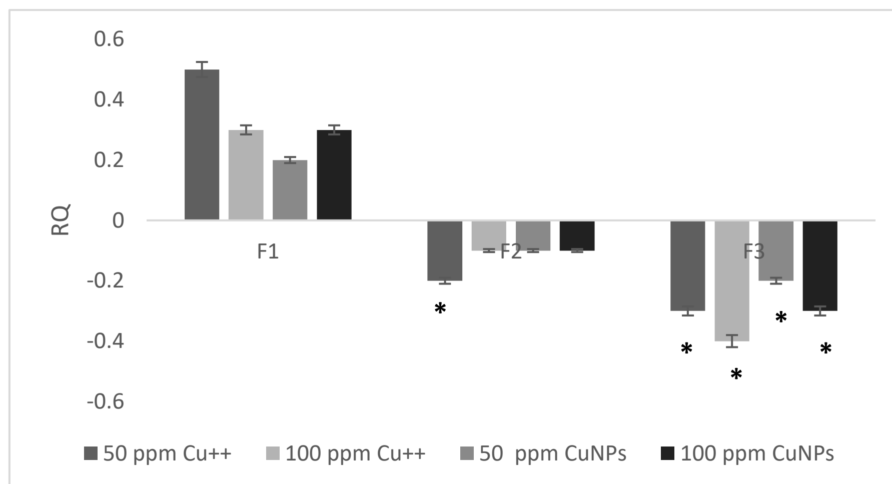


**Fig. 13** The expression levels of SOD in third-instar larvae of *D. melanogaster* treated with different doses of CuNPs and $Cu^{+2}$ in different generations

## 4. Discussion

Metallic nanoparticles such as CuNPs tend to exhibit a wide range of applications due to their novel properties than their bulk counterparts[29]. Therefore, to take advantage of the unique properties of CuNPs, it is necessary to evaluate toxicity under various conditions to determine the safe limits of the use of these NPs. This study investigated the acute and chronic toxic effects of CuNPs administration on *D. melanogaster*. The X-ray diffractogram and transmission electron microscope confirmed that the synthesis methodology produced pure, homogeneous, and uncontaminated CuNPs, in a spherical shape, with an average size of 13 nm.

Generally, the ionic form of metal is more chemically reactive than its metallic form. In the biosystem, ions have a higher ability to bind with the vital biomolecule, which hinders their activity [30]. At physiological conditions, NPs release a small amount of metal ions that are proposed to cause toxicity [31]. So, $Cu^{+2}$ usually causes higher toxicity than CuNPs [28, 32]. In the current study, the survival assay is performed to evaluate the effect of $Cu^{+2}$ & CuNPs and screen their toxicity on *D. melanogaster*. In agreement with previous studies [12, 33-35], the ionic form of copper ($Cu^{+2}$) showed a higher harmful effect than CuNPs.

On the other hand, $Cu^{+2}$ and CuNPs retard the developmental stages and cause a reduction in the success of the development of larvae to the adult stage [15]. However, there was no apparent negative impact on larval fitness or food consumption due to exposing *D. melanogaster* to $Cu^{+2}$ and CuNPs [36, 37].

In the chronic exposure study, the number of successfully emerging flies was dose- and generation-dependent for $Cu^{+2}$ and CuNPs. The reduction in the total number of progenies may be due to reduced fecundity (fewer fertilized oviposit eggs) [38] or unsuccessful development from egg to larvae due to egg damage or retarded development [39]. Furthermore, the developed larvae have a higher chance of exposure to CuNPs due to the accumulation of the latter in the gut. The higher concentration of NPs in the gut is toxic for the larvae, resulting in early death and blackening in many larvae. The extensive egg and larval loss at different developmental stages led to fewer adult flies in high concentrations of CuNPs [40].

NPs are small enough to penetrate cellular membranes and interact with cell components to stimulate oxidative stress and inflammatory responses [41]. In the present study, some morphological alterations in the fly's wings were observed. In the third generation, the mutations were raised to 8.14% and 13.12 % in 100 ppm CuNPs and 50 ppm $Cu^{+2}$, respectively. These data were consistent with previous findings that showed the phenotypic defect in the flies' eye, wing, and bristle exposed to different types of metal nanoparticles [42-45].

Oxidative stress has been mentioned as one of the most effective mechanisms of toxicity related to nanoparticle exposure [46]. Oxidative stress is further associated with several aspects of delay in development in various species, including *D. melanogaster* [47].

The antioxidant enzyme can be induced under mild oxidative stress, although extreme oxidative stress can cause such enzymes to be suppressed [48]. The induction of the antioxidant defense systems of *Drosophila* exposed to lower concentrations of CuNPs indicates an apparent adaptive threshold. The over-accumulation of free radicals and the impact of CuNPs might disrupt the balance of the antioxidant defense system [49].

SOD and CAT activity and lipid peroxidation are used as oxidative stress markers. Decreasing CAT activity and increasing lipid peroxidation were previously reported [50, 51]. SOD enzyme was insignificantly decreased in the treated groups in agreement with previous studies [52, 53].

The primary function of heat shock proteins is to protect the cells from oxidative stress-promoting damage. Such proteins assist with the proper folding of nascent and misfolded proteins. Heat shock protein genes are known as "stress genes," which serve as indicators of the cell toxicity of various environmental stressors [54]. Stress inducible *Hsp70* protein is a potential first biomarker of cell change due to its conservation through evolution and its inducibility by various triggers [55]. Herein, the expression profile of *Hsp70* in treated and untreated flies was evaluated to elucidate the role of *Hsp70* in cell toxicity. Our study showed an increase in the expression of *Hsp70* in the treated flies of the second and third generations. Consistent with our findings, the induction of *Hsp70* in

*D. melanogaster* has been reported after exposure to silver NPs and graphene- zinc oxide nanocomposite [49, 56].

On the other hand, our results showed significant upregulation of the *Sod2* gene in the first generation in all studied doses of CuNPs and $Cu^{+2}$ compared with the untreated flies. Then a significant downregulation in the second and third generations. This irregular trend in *Sod2* expression was also observed in earthworms exposed to zinc oxide NPs [57]. Meanwhile, downregulation of *Sod2* was also previously reported in *D. melanogaster* [44, 58]. Moreover, the upregulation of the *Sod2* has been previously reported in *D. melanogaster* [59]. In addition, consistent with our findings, [60] reported that exposure to cobalt nanoparticles resulted in detectable deregulation of *Hsp70* and *Sod2* genes. One possible justification for the irregular pattern of *Sod2* gene expression is that mild oxidative stress (in the first generation) induces upregulation in the *Sod2* gene as a rapid response. In the second and third generations, *Hsp70* was upregulated to act as a primary inhibitor of ROS that causes the altering of *Sod2* expression [61].

## 5. Conclusion

Since CuNPs are commonly used nanoparticles in consumer products, safe doses need to be evaluated to minimize human health problems. Nonetheless, the understanding of the toxicity mechanisms associated with CuNPs is limited and needs to be further elucidated to provide nanosafety evaluation for the safe expansion of CuNPs use. The current report shows that CuNPs are mutagenic in *Drosophila,* and these effects may be mediated by oxidative stress. However, most of the effects seem to be associated with copper ions. This report confirmed the toxicity of nanomaterials and may help to understand their toxicity mechanism. Also, it showed the need to develop standardized and robust toxicological characterization protocols for testing nanomaterials to select those that comply with health and environmental safety standards.

## 6. References


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