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Consequences of a short-term exposure to a sub lethal concentration of CdO nanoparticles on key life history traits in the fruit fly (*Drosophila melanogaster*)

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Abstract

Nanoparticles of cadmium oxide (CdO NPs) are among the most common industrial metal oxide nanoparticles. Early adulthood (P1) fruit flies (*D. melanogaster*) were

exposed for 7 days to a sub lethal concentration (0.03 mg CdO NPs/ml, which was 20% of the LC_{50}), spiked into food media to test whether short episodes of CdO NPs exposures early in adult life have long-lasting effects on life history traits such as fecundity well beyond exposure times. All studied life history traits, as well as climbing behavior were adversely affected by exposure to CdO NPs. A blistered wing phenotype was also observed in the non-exposed progeny (F1) of adult flies (P1) and their fecundity was significantly decreased (-50 percent) compared to the fecundity of non-exposed (control) F1 flies. Expressions of antioxidant enzymes encoding genes; catalase and superoxide dismutase (SOD2) were significantly up regulated in P1 flies compared to control. Expression of metallothionein encoding genes (MTn A-D) were significantly up-regulated in both parent flies (P1) and their progeny (F1) after exposure of P1 flies to CdO NPs compared to non-exposed control flies, suggesting long-term potential effects. Taken together, these findings indicate that short-term exposure to a sub-lethal CdO NP concentration is sufficient to have long-lasting, adverse effects on fruit flies.

Keywords: Nano-sized Cadmium oxide, Phenotype effects, Metallothionein, Fruit fly, oxidative stress, Bioaccumulation.

1. Introduction

Among several human activities, including engineering, agriculture, manufacturing, medicine and public health, nanotechnology has gained considerable public attention. Since nanomaterials are part of our everyday lives, exposure of humans and wildlife to nanomaterials is inevitable and as a result, research into possible effects of nanotoxicity is increasing [1,2]. The specific properties of nanoparticles (NPs) allow them to enter organisms and to be transported to tissues, cells, and even organelles in such a way that larger particles may not [3]. This has raised possible threats to human health and the environment [1,4,5].

Cadmium (Cd) is a non-essential transition metal that poses a health risk for both humans and animals [6]. It has significant toxic potency of ongoing concern because its concentrations in the environment have increased due to continued mobilization and release by activities of humans. Cadmium ions exhibit strong affinities for biological structures that include – SH groups (cysteine and glutathione GSH) as well as disulfide – S – S-groups (cystine and reduced GS-SG glutathione) that may interfere with their function. Cadmium causes oxidative stress [7] by production of ROS that are normally balanced by the enzymatic (superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and non-enzymatic (glutathione (GSH), vitamin C, vitamin E) antioxidative barriers [8,9]. Xenobiotic-induced oxidative stress results in the oxidation and destruction to biologically important macromolecules, such as proteins, DNA, lipids, and cellular membrane phospholipids [10].

Nano-Cd oxide (CdO) is the initial substrate for the manufacturing of quantum dots (QDs*;* semiconductor particles a few nanometres in size, having optical and electronic properties that differ from larger particles due to quantum mechanics) in both medical diagnostic imaging and controlled therapy [11] and in solar cells [12]. It is present in the air in industrialized countries, including cigarette smoke [13], sediments of lakes and streams [14], fertilizers and waste sludge [15]. Releases of Cd NPs into the environment could result in their accumulation in the food chain and increased human exposure that could potentially affect health of humans, biodiversity, and the environment. Several studies have demonstrated toxic potencies of cadmium-containing nanoparticles (NPs) *in vitro* and *in vivo* [16–19] and on human health [20,21]. While

these studies provide independent empirical evidence for the toxic potency of these Cd-containing NPs, further information is needed on the potential risks of these metallic nanoparticles to living organisms, such as their effects on life history traits, potential short or long-term effects on genomic and proteomic physiology, during and after exposure. Such studies are required to assess both the potential for life history toxicity in mammalian models and the potential for environmental harm through their comprehensive commercial production and consumer use.

Although much remains unknown about the toxic effects of nanoparticles, it is clear that there is no single underlying mechanism found in all types of nanoparticles [2]. In biological environments, metallic nanoparticles appear to disassociate and release ions that can contribute to the formation of reactive oxygen species (ROS) and subsequent activation of the oxidative stress response system [22–25]. Ingestion of nano-Ag by *Drosophila* larvae, for example, has been demonstrated to trigger oxidative stress pathways, including SOD, catalase, caspase-3 and caspase-9, GSH and malondialdehyde (MDA), a lipid peroxidation agent [26]. Exposure to heavy metals also triggers a variety of adaptive responses, such as induction of metallothionein (MT) [27]. MTs are proteins that have been shown to be involved in the defense against heavy metals by binding free metal ions and thereby minimizing reactive oxygen formation [28]. Upon heavy metals stimuli, metallothionein genes are rapidly transcriptionally activated and function in protecting cells from damage [28,29]. MTs also exhibit antioxidant activity and involved in zinc homeostasis (Zn)[30]. MTs obviously can be a powerful biomarker for predicting toxicity of heavy metals and adverse biological effects [31,32]. Yet to be determined is

whether oxidative stress and induction of MTs by metallic nanoparticles have effects on the entire organism, in terms of development, reproduction, and survival.

Here, the fruit fly (*Drosophila melanogaster*) was used as an *in vivo* model organism. Around 50 percent of proteins and about 75 percent of genes of human disease exhibit associated sequences in *D. Melanogaster*, meaning that findings obtained for the fly are important for predicting possible effects in other species [33–35]. In a series of experiments, adult *D. melanogaster* flies were exposed during early adulthood to a sub-lethal and field relevant concentration of CdO NPs for a short-term (7 days). We then quantified long lasting effects on life history traits such as survival, developmental time, fecundity, food intake, motor ability, antioxidative response and molecular mechanisms involved in metal detoxification through expression of metallothioneins (MTs) genes. Phenotypic and ultrastructural effects have also been investigated. We therefore quantified whether short episodes of CdO NPs exposures early in adult life have long lasting effects on life history traits well beyond exposure times.

2. Material and methods

2.1 Preparation and Characterization of CdO NPs

Cadmium oxide nanoparticles (CdO) were prepared according to methods previously published [36]. Then, the crystalline phase of synthetized CdO NPs was analyzed by Xray diffraction (XRD), size and morphology were also characterized using HR-TEM (high-resolution transmission electron microscopy) **(Fig.S1)**. The mean

crystalline size estimated for CdO NPs was 69.84 nm **(Table S1)** (for more details see [16] **)**

2.2. Fly strain and culture

Wild type *D. melanogaster* flies (Canton-s), obtained from Bloomington Drosophila stock center (#64349), were used in all experiments. This wild line strain gave consistent results under our laboratory conditions as reported earlier [37], compared to different wild strains (Oregon-R and Zimbabwe-S). Flies were grown on regular *Drosophila* food media containing cornmeal-agar (14–15 g agar, 18.5 g yeast, 61 g glucose, 30.5 g sucrose, 101 g corn meal/L, then kept at 25 °C, 50-60 % relative humidity (RH) with an 18/6-h light/dark cycle) [38]. We regularly change food media in vials and use few numbers of adults (~ 5 pairs) to avoid competition on food and any factors affecting population size such as bottleneck effects.

2.3. Determination of LC_{50}

Cadmium oxide NPs stock suspension (20 mg/mL) was prepared by placing 200 mg of CdO NPs in 10 mL of 10% sucrose solution (20 mg mL^{-1}). CdO NPs suspension was then sonicated for 30 min using an ultrasonic system (Powersonic 405) before use. Five serially diluted concentrations of synthetized CdO NPs (0.02, 0.06, 0.18, 0.54, 1.62 mg mL^{-1}) in standard medium were prepared for bioassays with adult *D. melanogaster*. For each concentration, 10 mL of CdO NPs spiked medium were poured into glass Petri dishes (7 cm in diameter). Then 3-day old male and females *Drosophila* adults (n=5) were transferred to each Petri dish containing medium spiked with CdO NPs. Exposures

to each concentration were performed in triplicate. Preliminary findings of the LC50 bioassay indicated that the data obtained after 24 hours and 48 hours of treatment did not meet the requirements for LC50 determination. Mortality was then recorded daily for 4 days, and the total mortality for each concentration measured was then determined. LC_{50} for CdO NPs was then calculated using the LdP Line^{R} program using the log-probit model (Ehabsoft (http://www.ehabsoft.com/ldpline). The LC_{50} of CdO NPs against 3rd larval instar of *D. Melanogaster* was also calculated **(see supplementary information for more details).**

2.4. Effects of a sub-lethal concentration of CdO NPs on *D. melanogaster*

2.4.1 Effects on life history traits

The LC_{50s} of CdO NPs against larvae and adult of *D. melanogaster* were respectively 0.027 mg mL^{-1} and 0.17 mg mL^{-1} **(Table S2).** In order to determine potential adverse effects of exposure to lesser concentrations of CdO NPs on survival, food intake, developmental time, fecundity, cellular structure and on detoxification related genes of *D. melanogaster*, newly emerged P1 adult flies (males and females) were chronically exposed for 7 days to a non-treated (control) or a sub-lethal concentration of CdO NPs (0.03 mg/ml: 20% of LC50) in supplemented media. We tested only one sub-lethal concentration of CdO NPs because we were more conservative to test a concentration that might represent a worst-case exposure scenario and based on our previous studies in which this sub-lethal level of CdO (20% of LC50) resulted in detrimental effects on honeybee workers [16,18]. Additionally, in a recent study in China, mean content of Cd

in atmospheric deposition was 2.99 mg kg^{-1} and ranged from 0.47 to 7.87 mg kg^{-1} [39] and ranged from 4 to 14.1 mg kg^{-1} DM in bottom sediments of water reservoir located in the key anthropogenic "hot spot" area in Poland [40], therefore the tested concentration of CdO NPs was environmentally-relevant and an order in magnitude lower than the levels of Cd detected in different environmental samples.

For potential effects on survival, ten pairs of newly hatched adults (P1) were placed into vials contained a non-treated (control) or a sublethal concentration of CdO NPs-supplemented media. Then, to avoid both the dryness of the media the mixing of generations (offspring–parent), both the control and the treated food media were changed every two days until the experiment ended [28,29 with few modifications]. Mortality was recorded daily. Dead flies were removed from both control and treated vials and sexes of dead flies determined. Three replicates per treatment were tested.

The developmental time and eclosion rate of progeny (F_1) generation (egg-adults) of P1 flies that were exposed to a sublethal concentration of CdO NPs in supplemented media were recorded. To do that, we started with vials contain laid eggs where we allowed 8-10 days old females to lay eggs for 8 hours, then removed the females, laid eggs were ~ 30-35 eggs in control and 25-30 in CdO NPs-treated vials and then we recorded the developmental time for every life stage (larvae, pupae and adults) in days. For eclosion assay, total eggs laid by 20 females were allowed to develop then after 13 days, the total number of pupae were counted including early stage pupae (light-colored pupae), late stage pupae (dark pupae) and hatched pupae (empty puparium) [43,44]. Three replicates per treatment were tested. To check for any long-term phenotypic

malformations, adults (3-5 days old) of F_1 flies (n=30) were checked relative to F_1 of non-exposed (control) flies by use of an Olympus BX61 microscope.

Potential effects on fecundity were assessed by mating newly emerged, virgin females and males of P1 flies that were exposed for 7 days to a non-treated (control) or a sub-lethal concentration of CdO NPs in supplemented media. To do that, three females and 5 males were placed in one vial containing food without CdO NPs but supplemented with blue dye. Flies were transferred every day to a new vial to count laid eggs. Fecundity was calculated as number of eggs laid per female per day. Potential long-term effects of CdO NPs on fecundity of F_1 flies was also investigated. To do that, virgin females (n=3) and males (n=5) F_1 flies were also mated and allowed to lay eggs on normal cultural media and the number of eggs laid per female per day have been counted for 2 days compared to control F_1 females. Three replicates per treatment were tested [45].

To quantify possible effects of short-term exposure to CdO NPs on both gene expression and mid gut cell structure of *D. melanogaster*, subsamples of P1 flies that had been exposed for 7 days to a non-treated (control) or a sub-lethal concentration of CdO NPs in supplemented media and their progeny F_1 flies were collected. Ten individuals (five males and five females) per treatment frozen at -80 °C. For ultrastructural investigations, only P1 flies were investigated as described below (section 2.4.4). In all experiments only P1 flies were exposed for 7 days to a non-treated (control) or a sub-lethal concentration of CdO NPs in supplemented media.

2.4.2 Effects on food intake

To test for potential effects of exposure to a sub-lethal concentration of CdO NPs on feeding intake of adult flies (P1), the capillary feeder (CAFE) assay was conducted [46]. Briefly, CAFE chambers were made from standard fly vials (25 × 95 mm) cut to a height of 6 cm and filled with 5 ml of 1% agar which serves as a water source and maintains internal chamber moisture and closed with a sponge bung. Food capillaries containing 10 µl of untreated liquid food or a liquid food spiked with a sublethal concentration of CdO NPs (0.03 mg / ml: 20% LC50) were inserted into the sponge holes. Four hours starved P1 adults (n=3) were then transferred to either CAFE champers. After 24 h, the volume of liquid food taken was determined using ImageJ software. Identical flyless chambers were maintained as evaporative controls (typically <10% of ingested volumes) and these measurements have been subtracted from data on use. The quantity of food consumed per fly during 24 h was calculated. Five replicates per treatment were tested.

2.4.3 Effects on motor ability

To test possible adverse effects of short-term exposure to CdO NPs on P1 adult *D. melanogaster* locomotion, an assay based on negative geotaxis was used as described previously [47]. Briefly, newly emerged male flies (n=10) were exposed for 7 days to a non-treated (control) or a sublethal concentration (20% of LC_{50}) of CdO NPs-supplemented media, then transferred into an empty 100 mL glass cylinder, gently tapped to the bottom. After 10 min acclimation at room temperature, upward movement of

controls and treated flies to the top of the cylinder was videotaped for 30 sec. Speed of climbing (cm sec^{-1}) for each individual was then calculated from recorded videos using ImageJ software (version 1.2). We only used male flies to avoid the potential variation in locomotion ability between female flies due to the effect of oogenesis and/or ovulation on body mass that have been shown to affect the musculoskeletal function in *Drosophila* [48,49]

Potential long-lasting effects of CdO NPs on locomotion of progeny F_1 larva were also assessed compared to F_1 larva of control flies using the same assay. To do that, third larval instar were transferred by a paintbrush soaked in 1% Phosphate buffered saline (PBS) from food media into 9-cm empty glass petri dish which then used as a study arena. Larvae were placed on one side of the petri dish and a few uL of standard food media were placed on the opposite side in order to bustle directional movement. The petri dish was placed horizontally, and the camera was held over the experimental arena. Videos of crawling movement were recorded for 10 min and then videos were cut into 0.1 sec frames and frame –by-frame analysis of movement speed was done using image J software. Three replicates per treatment were tested.

2.4.4 Effects on gene expression

Real-time, quantitative polymerase chain reactions (RT-qPCR) were used to quantify the potential effects of CdO NPs on expressions of four genes encoding for metallothioneins (MTn A-D) and four antioxidant enzymes encoding genes, including glutathione S-transferase (GSTD2), catalase, superoxide dismutase (SOD1) and SOD2

that have been investigated in previous studies [50–52]. Primers for all genes were given previously [53,54] **(Table S2).** Total RNA was isolated from a composite sample of 10 individual P1 or F_1 flies (five males and five females) by use of an RNA extraction kit (Thermo scientific), then cDNA was synthesized from RNA extracts according to the manufacturer's protocol [16,55] using SensiFAST cDNA synthesis kit. Overall, qPCR was performed on RNA isolated from three composite samples (n=10) per treatment. Two step cycling SensiFAST SYPR Lo-ROX kit (Bioline) was used for RT PCR. RpL32 reference gene was used as recommended [56]. For each target gene, abundance of transcripts was quantified using $2^{-\Delta\Delta CT}$ method [57].

2.4.5 Effects on cellular structure (TEM)

Midguts from three composite samples (n= 10) of CdO NPs-treated and untreated P1 flies were used and processed according to the method of [58], then studied and photographed using an electron transmission microscope JEM-1200EX (JEOL, Japan) at an accelerating voltage of 60 kV (for more details, see [18]).

2.3.Statistical analysis

Data were analyzed using GraphPad Prism version 8.00 for Windows (www.graphpad.com). To better estimate the normality and homogeneity of the variance, the data were converted to log10 when necessary. The impact of treatments on survival of P1 flies has been assessed by log-rank (Mantel cox) paired test, $p < 0.05$ after Bonferroni correction (i..e a conservative test that protects from Type 1 Error to counteract the problem of multiple comparisons). Differences in food intake, developmental time and

eclosion rate were assessed by Student's *t*-test. Differences in motor ability of P1 and F_1 larva were assessed by Mann Whitney test. Effect of treatment on fecundity of P1 and F1 flies were assessed by Two-way RM ANOVA. Effects on gene expression between treatments were analyzed by one-way ANOVA followed by Tukey's post hoc test. An alpha level of 0.05 was used to define significance for all tests.

3. Results

3.1. Effects on life history traits

Chronic exposure of P1 *D. melanogaster* flies to food media spiked with a sub-lethal concentration of CdO NPs for 7 days, significantly reduced survival of both male and female flies as compared to controls (log-rank (Mantel cox) paired test, $X^2 = 164.6$, $df = 3$, $p < 0.008$, after Bonferroni correction). All treated flies died after 17 days. Survival of males did not differ significantly compared to females regardless of whether they were exposed to CdO NPs or not (log-rank (Mantel cox) paired test, $X^2 = 3.17$, $df = 1$, $p = 0.07$) **(Fig.1a).**

Developmental time and rates of eclosion of *D. melanogaster* F_1 generation (egg-adults) of P1 flies that were exposed to lesser concentrations of CdO NPs resulted in adverse effects compared to controls **(Table 1)**. Eggs hatched after one day exposure in both exposed and non-exposed control groups and the time required for larval and pupal development was slightly longer in CdO NPs-treated flies, however this difference was not statistically significant compared to the control (Student's *t*-test, $p > 0.05$). Developmental time (egg-adult) was significantly longer in flies exposed to CdO NPs (11.72 ± 0.60 days) compared with the control (8 ± 0.00 days) (Student's *t*-test: $df = 4$, p

< 0.001). Eclosion rate (%) was also significantly less in treated flies (12.38 % ± 2.94) compared to control (69.50 % ± 0.60) (Student's *t*-test: *df* = 2, $p < 0.001$) **(Table 1)**.

Fecundity of P1 females of *D. melanogaster* that exposed for 7 days to a control or food media spiked with a sub-lethal concentration of CdO NPs and their F_1 progeny is shown in **Figure 1 (b)**. Two-way RM ANOVA results showed that the number of eggs laid per female per day varied significantly between treated and untreated flies (*df* = 1, $p < 0.001$) and also between P1 and F_1 flies *(df* = 1, $p < 0.05$).

3.2 Effects on food intake

Flies fed on a liquid food spiked with sub-lethal concentration of CdO NPs significantly consumed less food (< 50 %) compared to flies fed on non-treated (control) food (Student's *t*-test, *df = 7.51* $p < 0.001$) **(Fig.1c)**.

3.3 Effects on motor ability

Short-term exposure of male P1 flies for 7 days to a sub-lethal concentration of CdO NPs affected their instinctive negative geotaxis behavior. Exposed flies were excessively disturbed, hyperactive preferred to jump or use their wings, therefore, climbed in short paths. Only data of flies walked up vertically against the gravity are considered. Their climbing speed significantly impaired compared to control (Student's *t*-test, *df* = 35.37, $p < 0.0001$) **(Fig. 2a).** There was no significant difference in crawling speed of F_1 larva of treated flies compared to F_1 larva of controls (Student's *t*-test, *df* = 18, $p = 0.70$) **(Fig. 2b).**

3.4 Effects on gene expression

Genes encoding for metallothionein (MTn A-D) were significantly up-regulated in CdO NPs- exposed P1 flies and their progeny F_1 flies compared to P1 of controls (Mtn A: $F = 7.84$, $df = 2$, $p < 0.05$; MTn B: $F = 38.54$, $df = 2$ $p < 0.001$; MTn C: $F = 68.30$, $df = 2$, $p < 0.001$; MTn D: $F = 17.88$, $df = 2$, $p < 0.01$). Additionally, expressions of MTn B-C encoding genes were significantly greater in F_1 flies compared to P1 flies **(Fig. 4)**. Expressions of antioxidant enzymes encoding genes; catalase and SOD2 were significantly up-regulated in P1 flies compared to control (Catalase*:* $F = 27.23$, $df = 2$, $p < 0.05$*;* SOD2*:* $F = 16.33$, $df = 2$, $p < 0.05$). Although expression of GSTD2 and SOD1 were significantly upregulated in P1 flies compared to F_1 flies ($p < 0.05$), this difference between flies exposed to CdO NPs and controls was not statistically significant ($p > 0.05$) **(Fig. 4).**

3.5 Phenotypic effects and ultrastructure observations by TEM

F_1 flies (n=30) of P1 that exposed for a short-term to a sub-lethal concentration of CdO NPs were carefully checked for any physical abnormalities in body size and appendages. Malformations of wings of 83.3 % (n=25) were observed and flies exhibited blisters and bubbles at the apical area of the wings and reduction of the axillary cord at the jugal area of the wings compared to control **(Fig. 4.)**.

Bioaccumulation of CdO NPs in mid-gut cells of P1 flies that were exposed for 7 days to food media spiked with a sub-lethal concentration of CdO NPs is shown in **Figure S2**. Cellular alterations in mid gut cells of P1 treated flies as compared to control flies are shown in **Figure 5**. Midgut cells of non-treated (control) P1 flies exhibited

typical columnar cell morphology with the apical border, which was straight bearing multiple, long filaments like microvilli. Large and dense mitochondria, rER and an oval nucleus were also found **(Fig. 5 A, C, E)**. Ultrastructure's of midgut epithelial cells in flies exposed to CdO NPs were adversely affected. Lysis of the smooth endoplasmic reticulum(sER), microvilli were found to be fragmented and large lytic region observed. Some mitochondria have been found swollen, had matrix lysis, mitochondrial crystal breakage and auto-phagosomal appearance. Moreover, we also observed lysis of the nuclear membrane and in rER membranes, its layered structure was thus lost **(Fig. 5 B, D, F)**.

4 Discussion

Exposure of aquatic and terrestrial organisms to potentially toxic metals and metalloids can have adverse effects [59]. Even at sublethal concentrations, metals and metalloids can cause toxic effects [16,18]. Given a substantial amount of existing literature on potential risks associated with exposure to emerging nanomaterials [60], to our knowledge, this is the first study to investigate and report adverse long lasting effects on key life history traits of *D. melanogaster* well beyond exposure times to CdO NPs even at a lesser concentration.

Early stages of life-history seem more vulnerable to environmental stress than stages of adult life-history [61]. Here, CdO NPs exerted greater toxic potencies "exert adverse effects" to 3rd larval instar *of D. melanogaster* than they did to adult flies. Where, the calculated median lethal concentration (LC_{50}) of CdO NPs against adult flies (0.17

mg mL^{-1}) was 6.5-fold greater than the calculated LC_{50} against larvae (0.027 mg mL^{-1}), findings that match previous research showing that early life stages such as first-instance daphnia, juvenile mysids, juvenile fish and embryos are more sensitive to toxicants such as metals than adults. [62–65]. Early life stages might lack fully-expressed enzymes for efficient detoxification and removal of toxicants and therefore using the most vulnerable life stage would offer protection to all life stages in the natural environment [66].

In the current study, short-term exposure of adult *D. melanogaster* to a sub-lethal concentration of CdO NPs adversely affected all studied life history traits of the fly including survival, developmental time and reproductive fitness. Such results are in line with previous studies revealing that exposure to Cd, either as salts ($CdCl_2$) or as nano-sized (CdO) can cause toxic effects in *D. melanogaster* as well as other organisms [16,18,20,67–69]. Inhalation of CdO NPs during pregnancy negatively affects fecundity and inhibits the development of fetal and postnatal offspring of mice [20]. Here, the flies exposed to CdO NPs by oral intake and long-lasting effects beyond direct exposure times were further investigated by assessing the fecundity of both parents (P1) flies and its non-treated progeny (F_1) flies where, the fecundity of F_1 flies were significantly reduced (-50%) compared with fecundity of unexposed (control) F_1 flies. These findings also match the results of previous studies, which indicated that the presence of Cd in the environment may impair the fitness of *D. melanogaster* adulthood, even at lesser concentrations for short durations [67,69], because it might negatively affect expressions of genes associated with reproduction of *D. melanogaster* and trigger the transcription of defense-related genes [68]. Flies fed on a liquid food spiked with sub-lethal concentration

of CdO NPs significantly consumed less food (< 50%) compared to flies fed on non-treated (control) food. This result also indicated the CdO NPs antifeeding effect that may explain the adverse effects recorded on *D. Melanogaster* growth and reproduction, as previously reported [70–73].

Irritable and fast climbs point to a chronic neuronal motor defect in *D. melanogaster* flies [74]. Here, climbing behavior was impaired in only P1 flies exposed to CdO NPs. Cadmium may affect the degree and balance of excitation inhibition in synaptic neurotransmission and also the levels of antioxidants in the animal brain [75]. Cd inhibits the release of acetylcholine, probably by interfering with calcium metabolism [76]. In an earlier study, in which honeybees were exposed to a sub-lethal concentration of CdO NPs, AChE activity in heads of bees was inhibited by 3.8-fold relative to control and bees also showed malaise-like' behaviors [16]. These findings confirmed the neurotoxicity of CdO NPs and future studies are therefore required to explore underlying mechanisms of its neurotoxicity on living organisms. Possible translocation of CdO NPs through the alimentary canal to hemolymph and then to the brain of the treated flies has yet to be verified.

Normally, wings of insects are smooth, consisting of single dorsal and ventral epithelial layers kept together by cell adhesion and any interaction with cell adhesion leads to the apposition of wing epithelial sheets and thus to an aberrant 2D wing structure [77]. Here, chronic exposure of P1 flies led to blistered wings in their F_1 progeny flies compared to F_1 control flies. Similarly, adverse birth outcomes were also detected however, as a result of moderate prenatal Cd exposure of pregnant women [78]. Birth

defects detected due to either dietary CdO NPs exposure in the current study or due to inhaled CdO NPs [20] emphasize the great toxicity of nano-sized CdO even at sub-lethal level and give a clear public health warning message to women who are pregnant and those of childbearing age who are exposed to CdO NPs at work. Yet to be investigated is the potential genotoxic effects and the expression of proteins and genes which regulate the wing blister phenotype that have been observed in F1 progeny in the current study.

Metal oxide nanoparticles are known to generate oxidative stress and deregulate normal cellular activity, which in turn contributes to cellular toxicity and has been reported as a bioindicator for assessing the toxic effects of nanoparticles [79–81]. In the current study, antioxidant enzymes encoding genes; catalase and SOD2 were induced by CdO NPs in only P1 flies compared to control. While expression of GSTD2 and SOD1 were significantly up regulated in P1 flies however compared to F_1 flies and not with control. These results are in line with previous studies reporting similar findings using *D. melanogaster* as a model as well, however in response to other metal oxide nanoparticles [26,82]. Moreover, CuO NPs exposure was shown to cause a significant accumulation of intracellular reactive oxygen species (ROS) in various cultured cell models and marine model species, such as zebrafish or shrimp [83].

Metallothioneins (MTs) are central to intracellular metal regulation such as Cu, Zn, and Cd. Studies carried out in MT models of transgenic mice or MT-null mice show strong evidence that MTs play an essential role in protecting cells from acute metal exposure [84–86]. In the present study, MTn (A-D) encoding genes were significantly induced by CdO NPs in both P1 and F_1 flies compared to control. This is noteworthy

because there was a two-week time interval between the exposure of P1 parent flies to CdO NPs and the measures of gene expression in their F_1 progeny flies, which is almost spanning the developmental time (egg-adult) of the fly [87]. This was in addition to the phenotype effects observed in wings of F_1 flies indicates the long-lasting adverse effects of CdO NPs even at low exposure levels and for short periods during early adulthood. It also gives more evidence that MTs can be a valuable biomarker for predicting metal toxicity and adverse biological outcomes [31,32].

Cadmium is a non-metal that can be deposited in animal tissues, especially if it is found in nanosized materials capable of disrupting physiological functions that cause significant internal tissue damage [88]. Accumulation of CdO NPs in midgut cells of *D. Melanogaster* is more reflective of the pollutant's intrinsic toxicity [89]. Common cytological changes observed in epithelial cells of P1 flies in the current study were swelling and lysis of both mitochondria and sER and lysis of nuclear and rER membranes. Moreover, microvilli appeared fragmented and large lytic area were observed as well. Such ultrastructural changes reflect the key features of cell necrosis and apoptosis [90,91] and are comparable to those found in the midgut of the honey bees exposed to CdO NPs [18], HeLa cells treated with CdS NPs [92] and *in vitro* in cell lines— IMR-32, HEK-293 and MAEC treated with either CdCl2 or CdS NPs [93]. Moreover, it could explain the observed long lasting adverse effects on studied life history traits of *D. melanogaster*.

Conclusions and environmental implications

Extensive production and use of metal (oxide) nanoparticles increase potential for their release into the environment. Based on **cafe** assay in the current study, we estimated the daily CdO NPs intake per fly (2×10^{-4} µg fly^{-1} day^{-1}), which was 14-fold less than the calculated LC50. Consequently, the tested conc. was sub-lethal and environmentally relevant. Therefore, results of this study showed for the first time that short-term exposure to a sub-lethal concentration of CdO NPs is sufficient to cause long-lasting, harmful effects on life history traits of the fruit fly, which might also occur in other organisms. Induction of oxidative stress pathways and common cytological changes detected that were nearly identical to those seen in other insects or invertebrates exposed to these metals, might explain the observed long lasting adverse effects on studied life history traits of *D. melanogaster* and the observed deformities in wings of F_1 progeny flies. Taken together, the findings of this study provide insight into CdO NP's possible danger to living organisms using *D. melanogaster* as an *in vivo* model. CdO NPs nanotechnologies based on cadmium (Cd) may pose risks to humans and the environment and their use needs to be regulated and the environment monitored for potential exposure. Further information is therefore required to evaluate the potential risks of these metallic nanoparticles to living organisms, such as their potential short or long-term effects on genomic and proteomic physiology, during and after exposure.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Figure legends

Fig 1. Kaplan–Meier plot showing effects of CdO NPs on survival of male and female P1 *D. melanogaster* flies (a), fecundity (b) and (c) food intake. **(a)** Adult flies were exposed for 7 days to a control or food media spiked with a sublethal concentration (0.03 mg mL^{-1}) of CdO NPs and then transferred to normal cultural media every two days. Different lowercase letters indicate statistical differences between treatments after Bonferroni correction (log-rank (Mantel cox) paired test, $p < 0.008$). Vertical dashed line indicates the end of CdO NPs exposure time. **(b)** Number of eggs laid per female per day (mean ± sem) of P1 flies (parents) that exposed to a control or food media spiked with a sublethal concentration (0.03 mg mL^{-1}) of CdO NPs for 7 days and their F1 progeny. Number of eggs laid were significantly different between both treated and non-treated flies and between P1 and F_1 (Two-way RM ANOVA, $p < 0.05$). **(c)** Food intake (µl. day^{-1}. Fly^{-1}) of adult P1 flies fed a liquid food spiked with sub-lethal concentrations of CdO NPs compared to flies fed non-treated (control) food. Different lowercase letters indicate statistical differences between treatments (Student's *t*-test, $p > 0.05$).

Fig.2 Climbing speed (cm. sec^{-1}) of **(a)** males (P1: parents) that were exposed to a control or food media spiked with sublethal concentrations of CdO NPs for 7 days and (b) crawling speed (cm. sec^{-1}) their progeny F_1 larva. Symbols on the box plot represent maximum and minimum values (n=32) (whiskers: ⊤ ⊥), mean values (-). Different lower-case letters denote significant difference from the control (Mann Whitney test, $P < 0.0001$).

Fig.3 Fold-change in abundances of transcripts of metallothionein (MTn A-D) and antioxidant enzymes encoding genes involved in detoxification of heavy metals in *Drosophila melanogaster* P1 and their F_1 progeny flies. Adult P1 flies (parents) have been exposed to a control or food media spiked with a sublethal concentration (0.03 mg mL^{-1}) of CdO NPs for 7 days. Bars represent the mean ± SEM concentration of three samples. Different lower-case letters denote significant differences among treatments (one-way analysis of variance with Tukey's post-hoc test, $p < 0.05$).

Fig.4 Phenotypic effects of CdO NPs on wings of 3-days old adults F_1 *Drosophila melanogaster* flies compared to F_1 control flies. Parents (P1) of these flies have been exposed for 7 days to a control or food media spiked with a sublethal concentration (0.03 mg mL^{-1}) of CdO NPs. A, C and E are F_1 of control flies showing normal wings. B, D, F are F_1 of treated flies showing malformation in wings. Note, the blisters & bubbles at the apical margin of the wings and reduction of the axillary cord at the jugal area of the wings (black arrows).

Fig 5. Transmission electron microscopy photomicrographs of midgut cells of P1 *Drosophila melanogaster* flies that exposed to a sublethal concentration (20 % of LC_{50}) of CdO NPs for 7 days compared to control. A, C and E Control group, exhibiting typical morphology of columnar cells with the apical border that was straight bearing numerous, long filament like microvilli. Note, columnar cells with oval nucleus, abundant and dense mitochondria and rough endoplasmic reticulum (rER). B, D and F flies exposed to a sublethal concentration of CdO NPs (0.03 mg ml^{-1}). Note, lysis of smooth endoplasmic reticulum (sER) (white stars), microvilli appeared fragmented (white arrowhead) and large lytic area (black star). Some mitochondria were found swollen and showed lysis of matrix and breakage of mitochondrial cristae and appeared autophagosome (burlywood arrows). Note, the lysis in the nuclear membrane (white arrow) and in rER. N Nuclei; sER smooth endoplasmic reticulum; rER rough endoplasmic reticulum MV microvilli; M mitochondria.

Tables

Table 1. Developmental time in days and percentage of pupa (Mean ± SD) of *Drosophila melanogaster* exposed to a control or food media spiked with a sublethal concentration (0.03 mg mL^{-1}) of CdO NPs for 7 days.

Treatment	Development time (days)				Eclosion assay (%)		
	Egg deposition	Larvae	Pupae	Adult	Early pupa stage	Late pupa stage	Hatched pupa
Control	1 ± 0.00 A	2 ± 0.00 A	5 ± 0.00 A	8 ± 0.00 A	17.78 ± 1.47 A	12.72 ± 1.10 A	69.50 ± 0.60 A
CdO NPs	1 ± 0.00 A	3 ± 0.00 A	7 ± 0.00 A	11.72 ± 0.60 B	72.74 ± 4.22 B	14.88 ± 1.30 B	12.38 ± 2.94 B

*Different uppercase letters denote significant differences in development time (days) for each stage and the different number of each pupa type compared with the control (Student's *t*-test, $p < 0.05$).

Fig.1

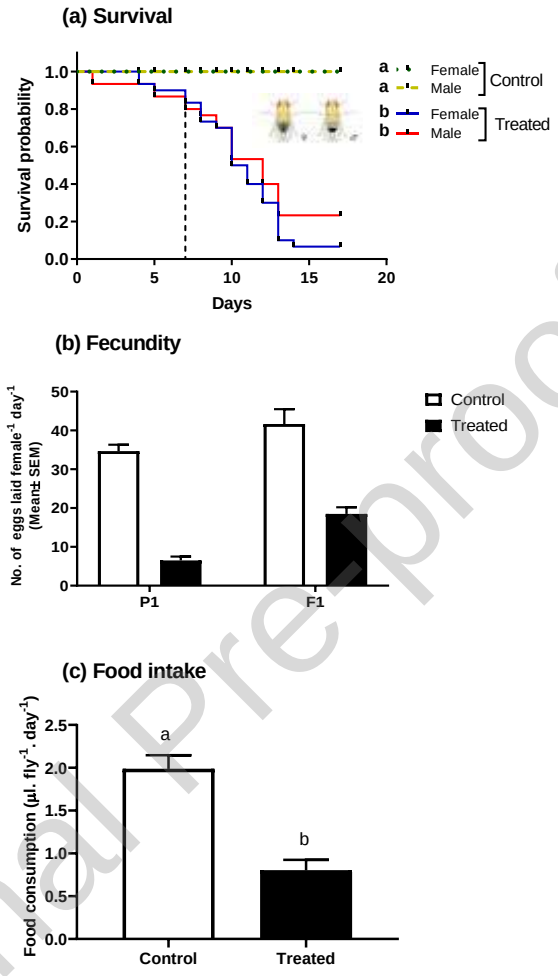


Fig.2.

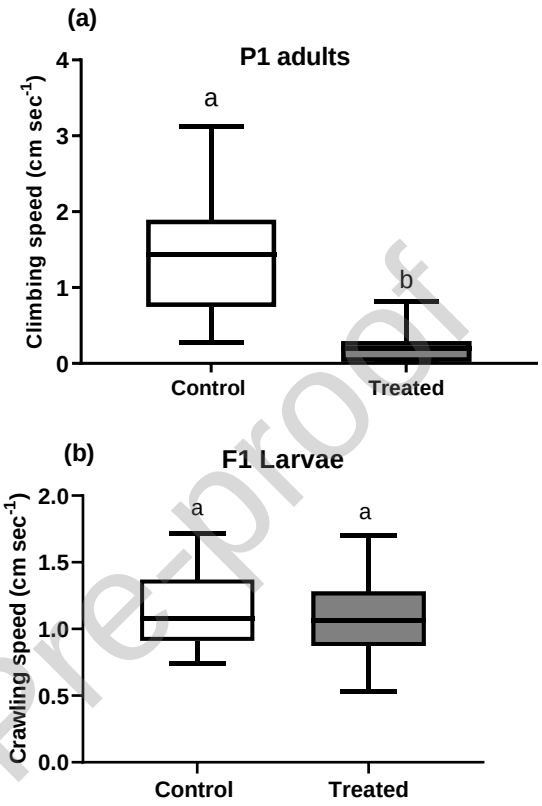


Fig.3

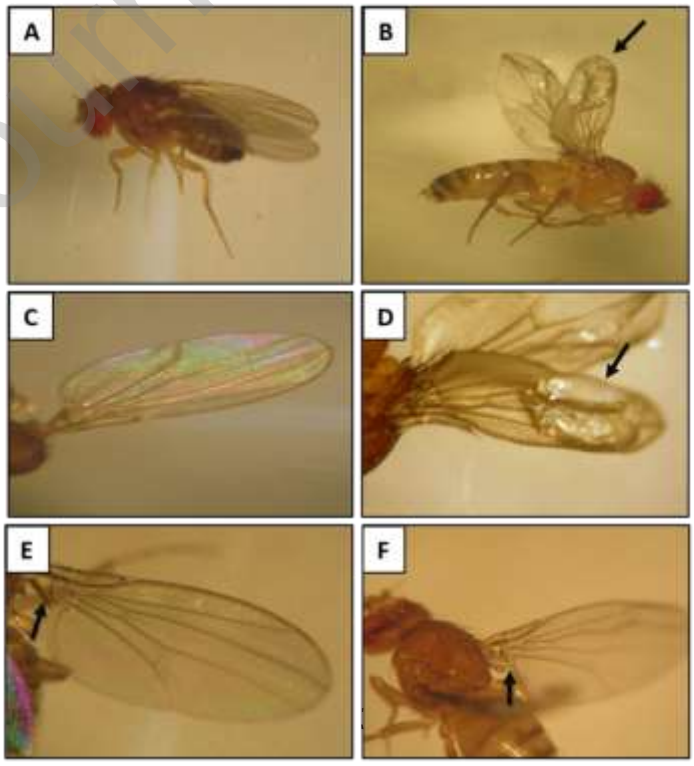


Fig.4

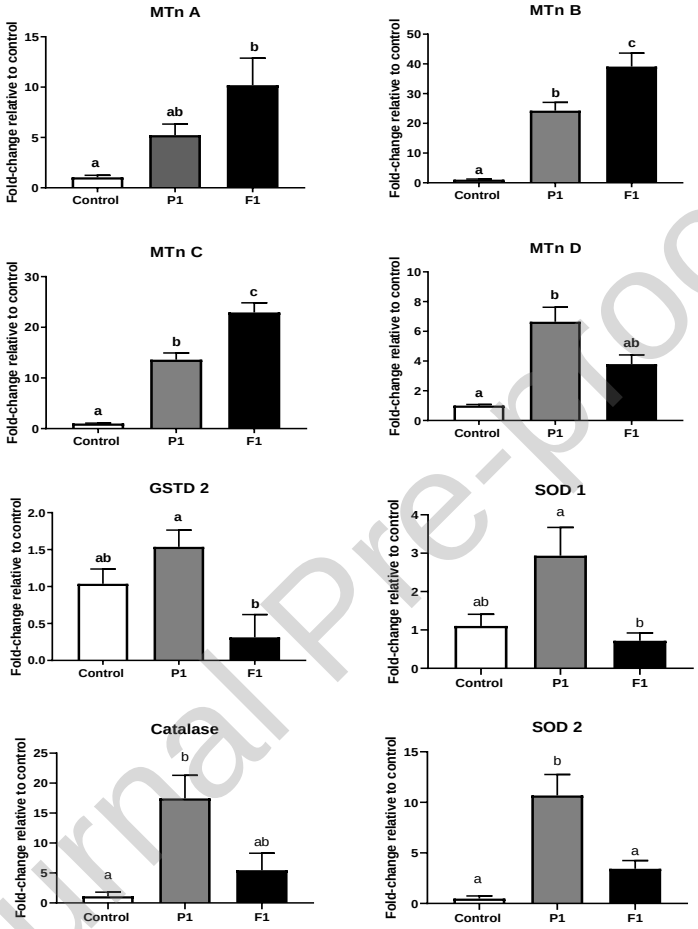
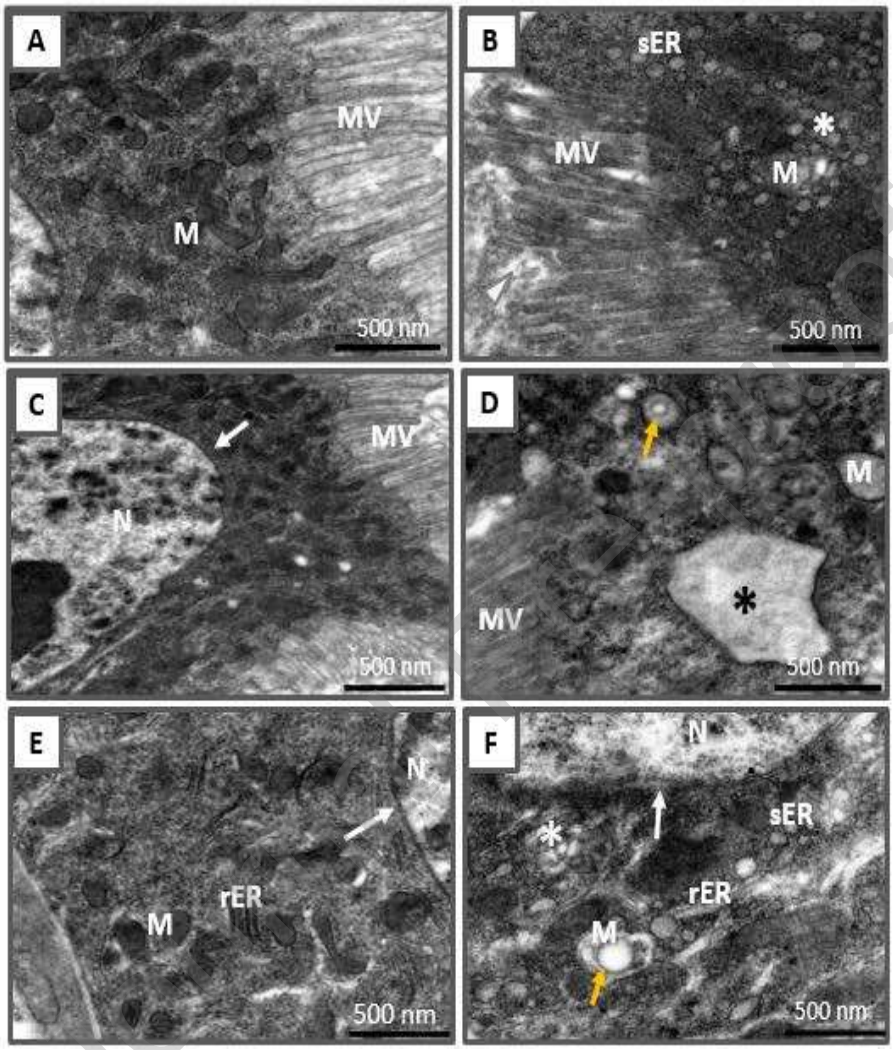


Fig.5.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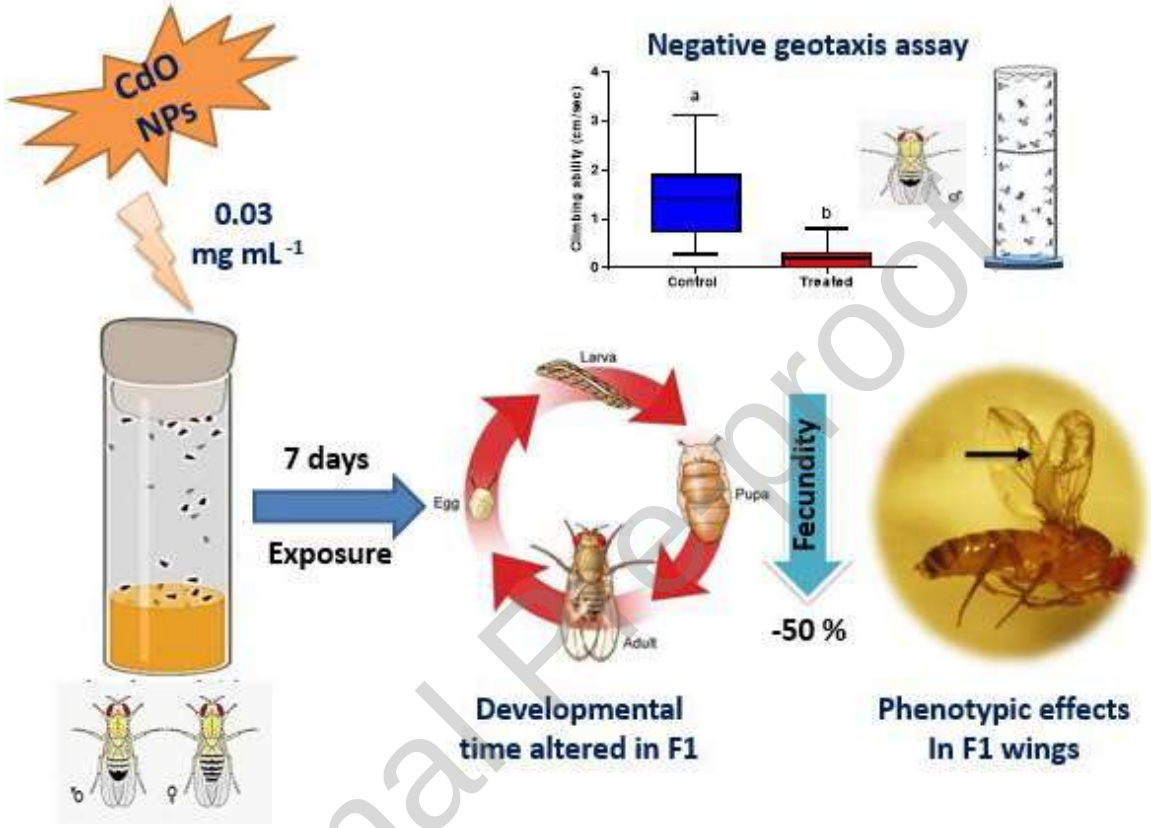
Graphical Abstract



Credit Author statement

Samar El Kholy (Investigation) (Methodology) (Writing- original draft, review and editing), John Giesy (review and editing) and Yahya Al Naggar (Conceptualization) (Data curation & analysis) (Writing original draft - review and editing).

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- CdO NPs adversely affected Life history traits of fruit flies.
- Fecundity and food intake significantly reduced (-50%) due to CdO NPs.
- Deformities were observed in wings of non-treated F1 progeny flies.
- CdO NPs affected fruit fly negative geotaxis behavior.
- Histopathological and cellular alterations have been observed in all CdO NPs treated groups.