



# Novel insights into acute/chronic genotoxic impact of exposure to tungsten oxide nanoparticles on *Drosophila melanogaster*

Fatma Turna Demir · Esref Demir

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**Abstract** Tungsten oxide nanoparticles (WO<sub>3</sub> NPs) have now been employed by various products including electronics, smart screens, gas-biosensors, water purifiers, disinfectants, and biomedical applications. Despite this wide-ranging adoption, little research has investigated their potential endpoint biomarkers in different *in vivo* models. We therefore propose the use of *Drosophila melanogaster* as a testing model in assessing genotoxic risks of exposure to WO<sub>3</sub> NPs. Our study examined toxicity, phenotypic alterations, locomotor behavior (climbing assay), intracellular oxidative stress (ROS), DNA damage (Comet assay), and somatic recombination (wing spot assay) in *Drosophila melanogaster* after exposure to WO<sub>3</sub> NPs (43.71 ± 1.59 nm) and microparticulated (MPs) of WO<sub>3</sub>. *Drosophila* larvae were exposed to the test materials via ingestion at doses ranging between 1 and 10 mM, and two greatest doses of NPs (5 and 10 mM) were found to cause mutagenic/recombinogenic effects, while the MPs caused no effects. Wing-spot assay detected genotoxic activity of NPs mostly through somatic recombination, and Comet assay showed DNA damage after exposure to NPs at certain

doses (1, 2.5, 5, and 10 mM). Other observations included ROS generation in hemocytes, phenotypic alterations in the mouths and wings of adult flies, and impaired locomotor behavior. This is the first research to report genotoxic evidence on the impact of WO<sub>3</sub> exposure in *Drosophila* larvae, highlighting the significance of this model organism in exploring the potential biological impact of nanoparticles and MPs of WO<sub>3</sub>. The results of our *in vivo* testing should make a vital contribution to the existing database on the genotoxicity of WO<sub>3</sub> NPs.

**Keywords** Tungsten oxide nanoparticles · *Drosophila melanogaster* · Genotoxicity · Comet assay · Somatic mutation and recombination test · Oxidative stress · DNA damage · Phenotypic alterations · Locomotor ability · Hemocytes · Somatic recombination · Risk assessment

## Introduction

Lately, huge strides in the sphere of nanotechnology have culminated in more than 20-fold increase in the amount of nanomaterial production. Nanoparticles (NPs), ranging in size between 1 and 100 nm, are key components of nano-based products [1]. Certain characteristics of NPs, including surface area, diameter, and shape, give them some properties considerably different from their microparticulated (MPs) (> 100 nm), which are prized across a wide range of

F. Turna Demir (✉) · E. Demir (✉)  
Department of Medical Services and Techniques, Medical Laboratory Techniques Programme, Vocational School of Health Services, Antalya Bilim University, 07190, Dosemealti, Antalya, Turkey  
e-mail: fatma.demir@antalya.edu.tr

E. Demir  
e-mail: esref.demir@antalya.edu.tr

fields [2]. Such broad-spectrum of NP utilization is predicted to cause a significant increase in their leakage or escape into diverse environments [3]. They can find their way into the organisms dwelling in these environments, raising concerns over their hazardous health impact on animal and plant life. Human populations could be exposed to NPs through natural routes like ingestion, inhalation, and dermal contact [4].

There is still limited research into the toxic and genotoxic impacts of NPs despite the potential risks associated with their acute and chronic exposure [5, 6]. Earlier research in the relevant literature employing various in vivo testing models to explore the toxic and genotoxic effects of several NPs showed that some could induce significant genotoxicity, along with oxidative stress [5, 7, 8]. Tungsten trioxide (or tungsten (VI) oxide ( $\text{WO}_3$ ) is a thermally stable and valuable semiconductor compound with a wide band-gap (2.5–2.8 eV). Industrial applications of  $\text{WO}_3$  include utilization as pigment for tint and ceramics on account of its yellowish color [9]. Its nanoparticle form,  $\text{WO}_3$  NPs, attracts considerable attention thanks to the large surface area of particles and their stability at extreme temperatures [10]. Different nano forms of  $\text{WO}_3$ , such as nanocrystals and nanosheets, have been widely used in applications like memory devices, micro/optoelectronics [11], smart screens [12] gas-sensing devices [13], polluted water purifiers [14], disinfectants [15], and photoelectrocatalysis [16]. On top of that, current efforts are geared towards using  $\text{WO}_3$  in biomedical applications, since they offer several advantages in this field such as improved visibility of target tissues in CT scans. Besides,  $\text{WO}_3$  nanorods have been employed as both therapeutic and diagnostic agents in tumor CT imaging and photothermal therapy [8].

Although we still have no reliable figure as to the total global production of  $\text{WO}_3$ , the US market is forecast at 9.9 thousand metric tons in 2021, while China is estimated to reach 67.5 thousand metric tons by 2026 ([https://www.reportlinker.com/p04159772/?utm\\_source=GNW](https://www.reportlinker.com/p04159772/?utm_source=GNW)).

In spite of its heavy use across various industries and prevalence of its compounds in the nature, potential biological endpoints of  $\text{WO}_3$  NPs have yet to be fully illuminated by using reliable in vivo models. Limited number of in vitro studies, on the other hand, report that  $\text{WO}_3$  NPs may have some toxic potential. There is also evidence that  $\text{WO}_3$  NPs may

cause mutagenicity in a bacteria species called *Salmonella typhimurium* [17]. Also, cytotoxic effects of  $\text{WO}_3$  NPs have been determined in different cell lines (Caco-2, 3T3, A549 cell, AGS cell line of human stomach cancer, and cultured primary rat hepatocytes) [18–21]. In addition, the genotoxic and mutagenic effects caused by  $\text{WO}_3$  NPs were explored by in vitro studies employing micronucleus (MN) tests and Comet assays in cultured human lymphocytes [22]. So far, very little in vivo research has been carried out to explore the toxicity of  $\text{WO}_3$  NPs to living organism, and yet they revealed that chronic or acute exposure to  $\text{WO}_3$  NPs could exert toxic effects on hamsters and rats [8, 23–26]. Exposure via oral route is the most common entrance way of the NPs into the human body, which is well reflected in in vivo model organisms that are often exposed to such materials via ingestion [20].

Over the recent decades, researchers have been striving to find a way to avoid the high costs and prevailing ethical issues related to the handling of mammalian testing models in experiments, trying alternative in vivo model organisms with a capacity to act as near-perfect counterparts of higher vertebrates [27–31]. To that end, a number of non-mammalian organisms including *Daphnia magna* [32, 33], *Caenorhabditis elegans* [29, 30], and *Drosophila melanogaster* [34–36] have been used in search of an alternative in vivo model organism.

Research previously established that certain engineered NPs might escape or leak into the nature during their fabrication, transfiguration, and utilization, causing significant pollution and endangering plant and animal life. Despite their common usage, there still exists rather little scientific evidence as to the cytotoxic and genotoxic capacity of  $\text{WO}_3$  NPs across different fauna and flora. A nanomaterial's capacity to produce toxicity is principally regulated by the particle size, shape, surface area, form, and surface coating or modification [37]. More recently, toxicity research into NPs has reported that several types of NPs could bring about oxidative stress, ROS generation, and detrimental consequences to genetic makeup and essential components of cell membranes mainly including lipids and proteins [38, 39].

Around 75% of human genes linked to diseases have been discovered to exhibit matches or homologs in the genes of *Drosophila* fruit flies [40], and since then this species has been adopted as a popular

genetic model by researchers to study various diseases including gastrointestinal infections [41], heart and circulatory problems [42], oxidative stress [43], carcinoma [44], genetic disorders [45], and neurodegenerative disorders [46, 47]. Furthermore, several biological commonalities are conserved between humans and fruit flies, which include gene expression, cell proliferation, cell signaling, differentiation, homeostatic cellular balance, and cell death [48]. Best of all, *Drosophila* enables bypassing frustrating ethical rules in place to restrict testing on mammals or higher vertebrates [49, 50]. A new field commonly referred to as Drosophotoxology [51, 52] involves the development of biological data from toxicological research using *D. melanogaster* [53]. Also, this organism is among those recommended by the researchers [54, 55] including the European Center for the Validation of Alternative Methods (ECVAM) [56] to minimize experiments on higher vertebrates like mice.

Our study aimed to characterize as many as endpoint biomarkers for MPs and nano forms of  $\text{WO}_3$  through a series of experiments on *Drosophila*. For an in-depth analysis and risk assessment, we adopted a combined approach, and as far as we are aware, our study is to become the first in vivo study to investigate this compound's cytotoxicity, genotoxicity, and morphologic alterations in fruit flies. To that end, we conducted several tests and microscopy imaging techniques, which included quantification of egg-to-adult viability, climbing assays to measure overall locomotion in exposed adult flies (direct cytotoxicity), observation of morphological alterations, Comet assays to detect the genotoxic capacity of  $\text{WO}_3$  compounds (as revealed by DNA strand breaks), SMART assays to analyze activities like genetic mutations and recombinations, and finally, determination of ROS generation. We firmly believe that our study findings will contribute significantly to our present understanding of risks pertaining to  $\text{WO}_3$  exposure.

## Experimental details

### Chemicals

Tungsten oxide (VI) NPs (mean diameter: 30 nm, nanopowder with 99.99% purity, CAS No. 1314–35–8) and its MPs (tungsten oxide (VI)) (99% purity;

CAS No. 1314–35–8, amorphous powder) were obtained from Acros Organics (Belgium). We procured hydrogen peroxide ( $\text{H}_2\text{O}_2$ , CAS No. 7722–84–1) and ethyl methanesulfonate (EMS, CAS No. 62–50–0) from Sigma Chemical (USA).  $\text{WO}_3$  NPs and MPs were dispersed in deionized water, a common solvent in previous research [57], and as for positive control substance in SMART and Comet testing, we used EMS at 1 mM [57, 58], EMS at 4 mM [57–60], respectively. In the tests conducted for the quantification of ROS levels, we employed 0.5 mM of hydrogen peroxide solution ( $\text{H}_2\text{O}_2$ ) [57, 58, 60].

### Characterization and dispersion of tungsten oxide (IV) nanoparticles

For the characterization of  $\text{WO}_3$  NPs, we utilized the following instruments: Environmental Scanning Electron Microscopy (ESEM, FEI QUANTA 260F) (Hillsboro, Oregon, USA), TEM, (Tecnai G2 F30) (Austin, Texas, USA) and Malvern Zetasizer Nano-ZS zen3600 laser Doppler velocimetry (LDV) (Worcestershire, UK), and Malvern Zetasizer Nano-ZS zen3600 dynamic light scattering (DLS) (Worcestershire, UK). In elemental analyses of test materials, we utilized energy-dispersive X-ray spectroscopy (EDX) (Malvern, UK), which is generally considered a well-established technique for the chemical evaluation of nanomaterials. We prewetted  $\text{WO}_3$  NPs in 0.5 vol% ethanol, and then dispersed the NPs in a stabilizing agent (BSA 0.05%) in Milli-Q water, purified by a Milli-Q lab water system. After that stage, once in the dispersion medium, we sonicated the NPs at 20 kHz for 30 min in a Branson digital sonifier (S-250D, Cambridge, MA, USA) to create a 2.56 mg/mL stock dispersion, as per the Nanogenotox protocol [61].

### Toxicity (viability) of $\text{WO}_3$ NPs and MPs

In our experiments, we used a wild-type strain of *Drosophila* commonly referred to as Oregon-R<sup>+</sup> to determine the toxic potential of  $\text{WO}_3$  NPs and MPs. Toxic doses of these materials were previously specified in detail by earlier research (genotoxicity, ROS production in cells, locomotor behavior, and phenotypic changes). Following exposure to study compounds, egg-to-adult viability was measured in percentages. The steps in this procedure were as follows:

50 eggs for each dose of WO<sub>3</sub> NPs and its MPs (1, 2.5, 5, and 10 mM) were placed into 5 plastic test vials containing food media (4 g). Agar-based food media was initially treated with different doses of WO<sub>3</sub> NPs (10 mL) and its MPs (0, 1, 2.5, 5, and 10 mM), which accounted for nominal doses of 231.84, 579.6, 1159.2, and 2318.4 µg/mL, and following dispersion in the media they accounted for 0.5796, 1.449, 2.898, and 5.796 mg/g food. We decided on the ideal doses of study compounds in reference to findings of earlier studies using in vivo and in vitro models [8, 20, 21, 24, 26]. The upper threshold for WO<sub>3</sub> NP and MPs doses was 10 mM, and for each test dose we performed 5 replications in total. After the eggs had been exposed to the study compounds throughout the stages of development, we noted the number of adult flies that successfully survived in order to quantify the survival rate. Throughout the paper, we presented the doses of WO<sub>3</sub> NPs and MPs in mM to maintain coherence and unity.

#### Intracellular oxidative stress (ROS) detection in *Drosophila* hemocytes

Oxidative stress, often caused by disturbances in the balance between free radicals and antioxidants, is a crucial phenomenon governing the extent of the toxic and genotoxic capacity of fabricated NPs. In detecting any possible elevation in ROS production, we utilized 2',7' dichlorodihydrofluorescein diacetate (DCFH-DA), a useful cell-permeable fluorogenic probe commonly used to quantify ROS levels in the cells of living organisms. Accordingly, our tests detected increased ROS in the hemocytes of 96 h-old 3rd instar *Drosophila* larvae upon exposure to both WO<sub>3</sub> NPs and MPs for 24 h. During these experiments, the steps we followed, in line with our previous studies [57, 58, 60], were as follows: First, we collected samples of *Drosophila* hemocytes, then incubated the samples in 5 µM DCFH-DA at 24 °C for 30 min. Second, the cells were examined under fluorescence microscopy equipped with a green filter (485-nm excitation and 528-nm emission). As a negative control, we used sterile distilled water, while 0.5 mM of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was employed as positive control. In the final step, the images created through microscopy studies were transferred to a software package called ImageJ for further analysis [62].

#### Phenotypic alterations

In an attempt to probe whether exposure to WO<sub>3</sub> NPs and MPs would induce any alterations in the phenotype, we examined a total of 50 Oregon R<sup>+</sup> adult flies through a stereomicroscope [34, 58], performing assessments in all body parts of flies (including eyes, legs, wings, and abdomen) and observed alterations and abnormalities mainly in the mouth and wings.

#### Climbing assay

Locomotor behavior of the flies was measured through climbing assays in accordance with the procedures proposed by previous studies [58, 63–68]. For this experiment, 10 flies randomly selected from control and study groups were transferred into separate vials, and before assessing the parameters of climbing behavior, they were kept there for 15 min at room temperature to allow acclimatization. The flies were moved to the bottom of the vial by a gentle tap on the vial, and the number of flies that managed to climb above the 7 cm mark in 10 s was recorded to assess their climbing ability. For each group, we repeated this climbing assay ten times after exposure to different doses of test materials.

#### Genotoxicity detection with *Drosophila* wing spot test and Comet assay

##### *The wing-spot assay (somatic mutations and recombination test, SMART)*

SMART assays, which yield results in a short period of time at significantly low costs, rapidly identify allelic imbalance and chromosome instability in larval somatic cells [69]. They have been used by many studies into genotoxicity and antigenotoxicity of environmental contaminants like plastics and nanomaterials [58, 70, 71]. In SMART assay, we employed two distinct strains of fruit flies: flare-3 (*flr<sup>3</sup>/In (3LR) TM3, Bd<sup>8</sup>*) and multiple wing hairs (*mwh/mwh*). More detailed information on these strains was given in early research [70]. Prior to this research protocol, we first crossbred virgin female flare-3 and male mwh flies to obtain trans-heterozygous flies for two recessive mutations, and then we cultured such trans-heterozygous eggs in test vials that were added food media (containing agar, sugar, corn in powder form)

for 8 h. Once the eggs had turned into  $72 \pm 4$  h-old larvae, we placed them into plastic vials for exposure to the study compounds. Before this exposure, we carried out a viability test in the larvae, and the final test doses for  $\text{WO}_3$  NPs and MPs were determined as follows: 1, 2.5, 5, and 10 mM. At all tested doses, the viability rate was calculated to be above 70%. Exposure vials contained 4 g instant *Drosophila* food media including 10 mL of  $\text{WO}_3$  NPs and its MPs and also negative (sterile distilled water) as well as positive control solutions (EMS, 1 mM). *Drosophila* larvae ( $72 \pm 4$  h-old) were exposed to  $\text{WO}_3$  NPs and MPs via ingestion throughout the larval-pupal transition. In line with the standards elaborated in great length in earlier research, the experiments were carried out under normal conditions ( $25 \pm 1$  °C, humidity at around 65%). Once the pupation was completed, adult flies were immersed in ethanol (70%) at 4 °C until they were ready for preparation of wing slides. Next, their wings were dissected from the body and fixed on the microscopy slides to allow detection of any possible clone formations through a light microscope (400 $\times$ ). Presence of *mwh* single spots is considered to indicate recombination and substitution of certain genes, or deletion of wild-type segments, whereas presence of *flr*<sup>3</sup> single spots could indicate deletion of wild-type alleles [69]. On the other hand, both *mwh* and *flr*<sup>3</sup> twin spots observed on the fly's wing blades usually result from gene recombination during mitosis [69]. In our study, we examined a total of 40 flies at each series by following step-by-step commonly adopted procedures for the wing-spot assay and scoring described by previous reports [6, 57, 58].

#### Comet assay

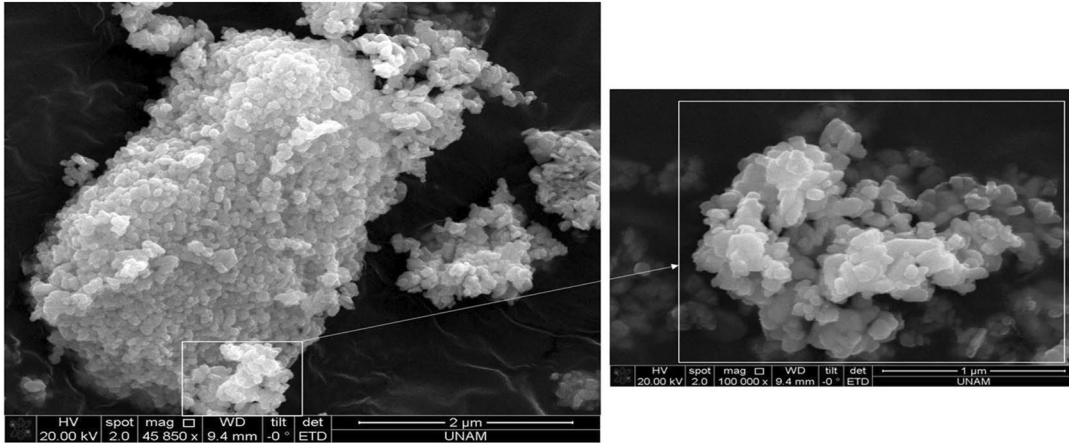
Third instar ( $72 \pm 4$  h-old) *Drosophila* larvae belonging to the Oregon R<sup>+</sup> strain were exposed to  $\text{WO}_3$  NPs, its MPs, as well as positive control substance (4 mM EMS) and negative control (sterile distilled water). The exposure to such compounds was achieved as follows: instant *Drosophila* food media (4 g) was mixed with 10 mL of varying doses of study compounds (0.1, 1, 2.5, and 5 mM). The larvae were exposed (through ingestion) to the mixture for 1 day ( $24 \pm 2$  h). Then, hemocyte cells were collected following the steps in the research protocol proposed by Irving et al. [72]. Comet assays were carried out

on isolated hemocytes by following the procedure specified by Singh et al. [73] along with some minor changes. Trypan blue exclusion assay was used to assess the viability of the hemocytes [74]. As well as low melting point agarose (75% LMA, 120  $\mu\text{L}$ ), slides coated with normal melting point (1% NMP) agarose were employed to spread the hemocytes (20  $\mu\text{L}$ ). Then, coverslips were placed on the slides to allow keeping them on ice (for 5 min) and to embed the isolated hemocytes into the slides. After the removal of these coverslips, we applied a second LMA (80  $\mu\text{L}$ ) spreading and kept them on ice for 5 min once again [73, 75]. In a dark chamber, the slides were saturated in lysing solution (10 mM Trizma base, 100 mM EDTA, 2.5 M NaCl, 1% Triton X-100, and 1% N lauroyl sarcosinate pH 10) at 4 °C for 2 h. We did not use dimethyl sulfoxide during the preparation of the lysing solution, as it could give rise to some extra damage in the fly tissues [76, 77]. Besides, all such procedures were carried out under dim light conditions to avoid any potential DNA breakage owing to strong light. The slides were transferred into a running buffer for electrophoresis (1 mM EDTA and 300 mM NaOH, pH  $\sim 12.8$ ) and kept there for as long as 20 min (300 mA and 1 V/cm). Tris buffer solution (pH 7.5) was used to wash the slides, which was repeated 3 times for 5 min. After that, they were fixed with cold 70% ethanol for 5 min and dried prior to EtBr (20 mg/ml) staining. To acquire images with fluorescence microscopy (filter 515–560 nm), the slides were stained for 20 min, after which Comet assays were performed on CaspLab (version 1.2.3b2) [78]. Such experiment protocols are realized by strictly following procedures described in detail by earlier research [79]. For each exposure series (300 cells in total), 100 cells randomly chosen in triplicate were analyzed. Damage in DNA was measured as the percentage of DNA in the tail (% DNA tail), so mean and standard error values were calculated accordingly.

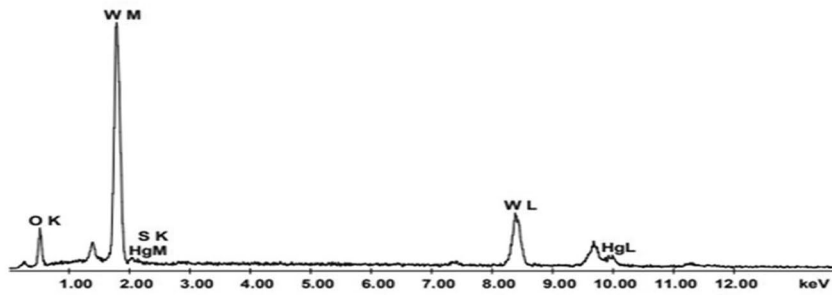
#### Statistical analysis

The conditional binomial test, tabulated in detail by Kastenbaum and Bowman [80], was used to assess the differences in the frequency of each type of wing spot in exposed flies and negative controls, and  $\alpha = \beta = 0.05$  was considered statistically significant. Mann–Whitney–Wilcoxon nonparametric U-test [81] and the multiple decision method [82] were employed

**A**



**B**



Weight % by Element				
Filename	O K	S K	W L	Hg L
genspc.spc	32.16	0.00	67.84	0.00

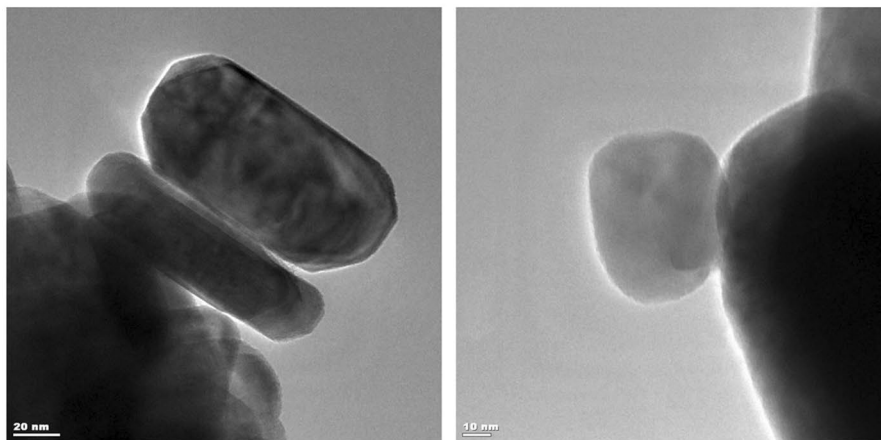
  

Atomic % by Element				
Filename	O K	S K	W L	Hg L
genspc.spc	84.49	0.00	15.51	0.00

K-Ratio				
Filename	O K	S K	W L	Hg L
genspc.spc	0.0724	0.0000	0.6312	0.0000

**C**



◀**Fig. 1** Characterization of WO<sub>3</sub> NPs (43.71 ± 1.59 nm). **A** Typical SEM images. **B** EDX spectroscopy illustrates the chemical structure of WO<sub>3</sub> NPs in the dispersion solution by SEM. **C** Typical TEM images

to decide whether there were positive, weakly positive, negative, or inconclusive responses to the test materials ( $P \leq 0.05$ ). While Kolmogorov–Smirnov and Shapiro–Wilk test assessed the normality of variance, Levene’s test analyzed the homogeneity of variance. Data displaying normal distribution and equal variance were analyzed by the Student’s *t*-test (Comet assay and ROS production) and one-way ANOVA (climbing assay) on SigmaPlot 11.0 (SPSS). Non-parametric Mann–Whitney U-test analyzed data exhibiting unequal variance or skewed distribution (toxicity/viability). Unless specified otherwise, all research data were presented as means of two independent experiments, as well as duplicates of each test, and the figures represent arithmetic means ± standard error values.

## Results

### Physicochemical characterization of WO<sub>3</sub> NPs

The physicochemical characterization of WO<sub>3</sub> NPs (mean diameter 30 nm) was achieved by means of TEM, ESEM, EDX, LDV, and DLS, whose results are presented in Figs. 1, 2. Figure 1A shows the examples of ESEM images, while Figs. 1C, 2B present relevant TEM images. Spectroscopy images obtained with EDX are shown in Fig. 1B. Details of WO<sub>3</sub> characterization are presented in Figs. 1, 2. The mean diameter of WO<sub>3</sub> NPs was 43.71 ± 1.59 nm, measured through respective TEM images. The histogram graph in Fig. 2A illustrates particle size distribution. Average size of WO<sub>3</sub> was 43.71 ± 1.59 nm, zeta potential  $-22 \pm 5.26$  mV (Fig. 2C), and the mean diameter calculated by DLS was 756.8 ± 161.9 nm (Fig. 2D). This zeta potential value indicates the good dispersion of WO<sub>3</sub> NPs. The Polydispersity index (PDI) determined by DLS was 0.483 for WO<sub>3</sub> NPs. PDI gives information about the size distribution in a sample or agglomeration, corresponding to the measurement of the heterogeneity of a chemical based upon size [83]. In order to avoid any potential aggregation, all experimental applications were carried out

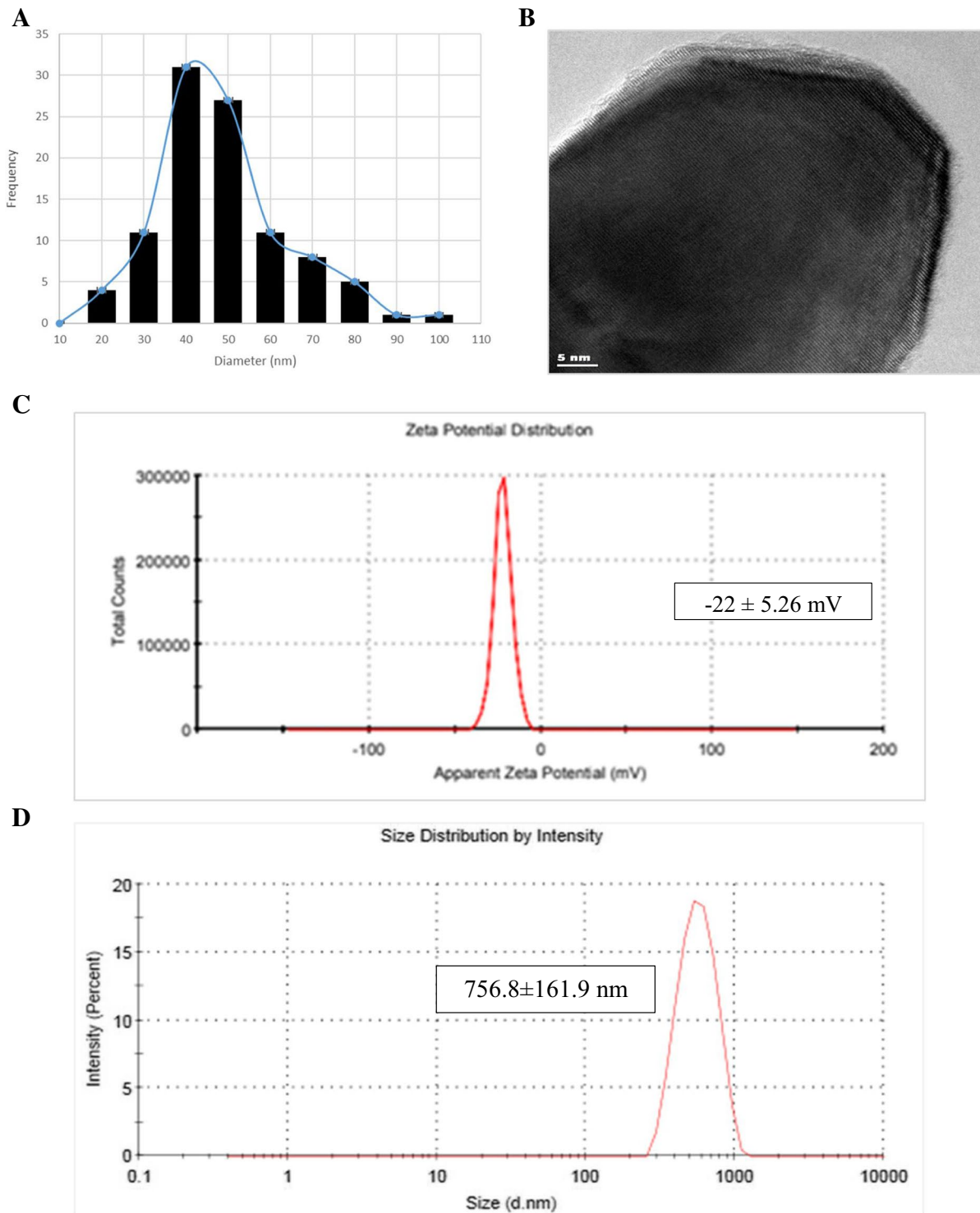
with freshly prepared NPs solutions. On the other hand, energy-dispersive X-ray (EDX) spectroscopy via ESEM was performed to assess the chemical composition of WO<sub>3</sub> and showed that oxygen (O) peak was 84.49% and tungsten (W) peak of 15.51% (atomic % by element) (Fig. 1B).

### Toxicity of WO<sub>3</sub> NPs and its MPs

Toxic capacity of study compounds was measured with viability assays by calculating the egg-to-adult survival rate. The results of these assays showed no significantly impaired survival at doses of 1, 2.5, 5, and 10 mM. On the other hand, *Drosophila* eggs exposed to our negative control substance (sterile distilled water) exhibited perfect viability at 100%. Following exposure to 1, 2.5, 5, and 10 mM (greatest dose) of WO<sub>3</sub> NPs, the eggs showed the following viability rates: 96, 95, 96, and 94%, respectively, while exposure to the MPs resulted in slightly higher rates at 98, 99, 97, and 96% (Fig. 8). *Drosophila* larvae were also exposed to non-toxic doses (0.1, 1, 2.5, and 5 mM) of WO<sub>3</sub> NPs and its MPs to conduct ROS assays, genotoxicity studies (SMART and Comet assay), climbing assays, and to assess alterations in phenotypes.

### Oxidative stress in hemocytes after exposure to WO<sub>3</sub> NPs and its MPs

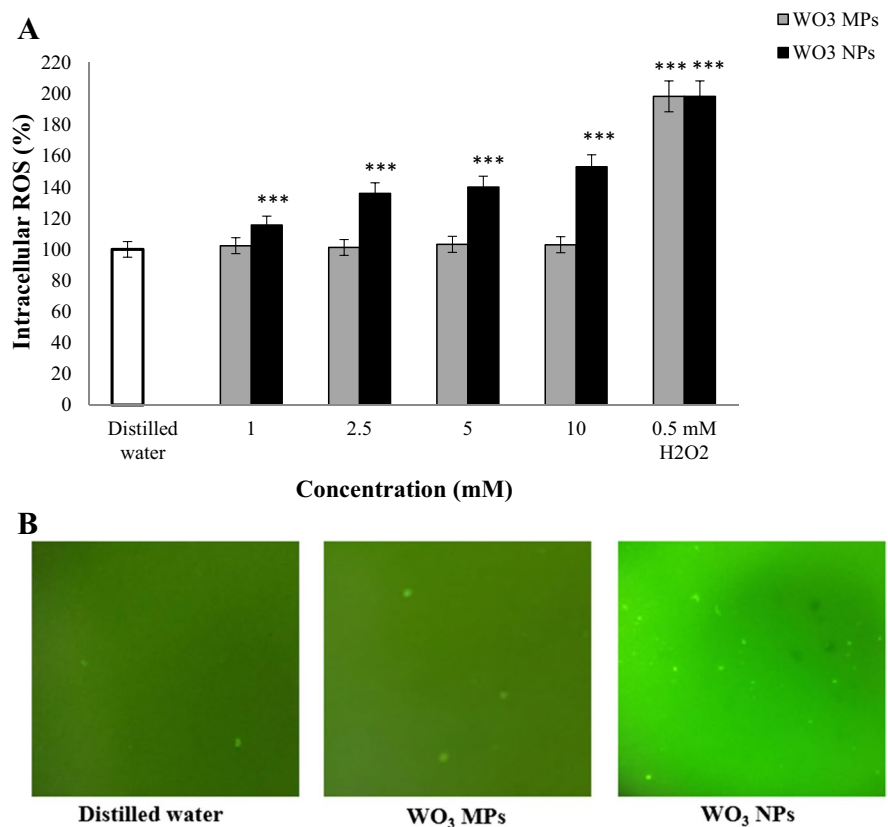
Closely associated with the accumulation of ROS in cells, oxidative stress can be accepted as an indicator of hazardous consequences of exposure to WO<sub>3</sub> NPs and MPs, as it induces the generation of ROS in several cells within the organism. Therefore, we explored ROS levels in the 3rd instar larvae hemocytes upon exposure to WO<sub>3</sub> NPs and its MPs by means of fluorescent dye, 6-carboxy 2,7'-dichlorodihydrofluorescein diacetate (DCFH-DA) under fluorescent microscopy. Exposure to WO<sub>3</sub> NPs at doses of 1, 2.5, 5, and 10 mM brought about statistically significant increases in ROS accumulation ( $P \leq 0.001$ ), with a direct concentration–response relationship (Fig. 3A). This is especially observed for WO<sub>3</sub> NPs (Fig. 3B). ROS generation at the highest dose (10 mM) was considerably greater following exposure to MPs (103%) as compared to WO<sub>3</sub> NPs (153.1%), suggesting that WO<sub>3</sub> NPs caused greater ROS induction than WO<sub>3</sub> MPs (Fig. 3A). WO<sub>3</sub> NPs exposure was found to



**Fig. 2** Characterization of  $\text{WO}_3$  NPs ( $43.71 \pm 1.59$  nm). **A, B** TEM image and size distribution histogram through images. **C, D** Zeta potential and size distribution, by LDV and DLS characterization, respectively



**Fig. 3** Intracellular ROS generation in hemocytes of unexposed 3rd instar larvae (distilled water only) and of those exposed to varying doses of WO<sub>3</sub> NPs and MPs (A). Hemocytes were incubated in 5 μM DCFH-DA for 30 min at 24 °C and then observed under fluorescence microscopy. ImageJ analysis quantified the fluorescence intensity of hemocytes in larvae exposed to WO<sub>3</sub> NPs and MPs (B). Positive control substance was 0.5 mM H<sub>2</sub>O<sub>2</sub>. \*\*\* $P \leq 0.001$  as compared to the negative control by Student's *t*-test



induce some ROS build-up in hemocytes. However, WO<sub>3</sub> MPs induced no ROS formation (Fig. 3A).

#### Phenotypic alterations

Experiments with non-toxic doses (1, 2.5, 5, and 10 mM) of WO<sub>3</sub> NPs and its MPs were carried out in 3rd instar larvae of *Drosophila* to probe whether any alterations specific to fly phenotypes would be observed in the wings, head, thorax, abdomen, or legs of the flies. All doses of WO<sub>3</sub> NPs caused such abnormalities in the mouth and wing parts (Figs. 4B, E and Figs. 5B, E). The mouth of the flies was enlarged, and the wings were defective in shape following exposure to WO<sub>3</sub> NPs (1, 2.5, 5, and 10 mM). On the other hand, no such abnormalities were detected after exposure to WO<sub>3</sub> MPs at doses of 1, 2.5, 5, and 10 mM.

#### Climbing behavior

Climbing test, conducted to quantify a *Drosophila* fly's ability to climb upwards, is a useful assay to

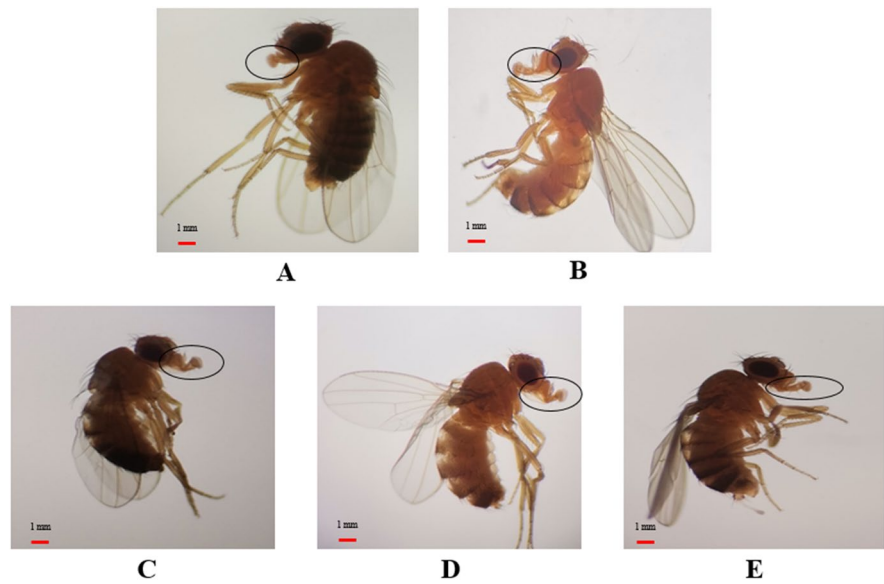
assess whether locomotor behavior is affected. Our climbing tests, after chronic exposure to the WO<sub>3</sub> NPs and its MPs (1, 2.5, 5, and 10 mM) for 7 days, detected significant differences between the climbing behavior of the study and control groups. All three doses of WO<sub>3</sub> NPs (1, 2.5, 5, and 10 mM) caused impairment in climbing performances ( $86 \pm 2.3$ ,  $80 \pm 3.1$ ,  $62 \pm 2.6$ , and  $57 \pm 3.3\%$ , respectively) as compared to the control group, while exposure to WO<sub>3</sub> MPs at doses of 1, 2.5, 5, and 10 mM produced milder effects ( $91 \pm 2.6$ ,  $86 \pm 3.4$ ,  $82 \pm 3.7$ , and  $77 \pm 3.2\%$ , respectively) (Fig. 6). Two highest doses of WO<sub>3</sub> NPs (5 and 10 mM) were found to impair locomotor activity at statistically significant levels.

Genotoxicity tests (*Drosophila* wing spot test and Comet assay)

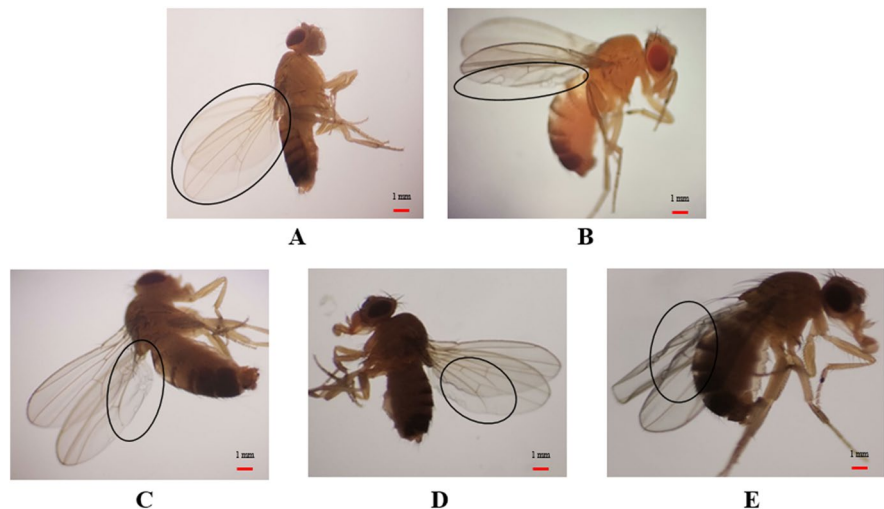
#### *Drosophila* SMART assay

We undertook a series of *Drosophila* SMART assays to determine WO<sub>3</sub> mutagenicity and recombinogenic

**Fig. 4** Phenotypic alterations in *D. melanogaster* after exposure to  $\text{WO}_3$  NPs and MPs. Normal mouth phenotype after exposure to  $\text{WO}_3$  MPs at all doses (A) and abnormal mouth phenotypes after exposure to  $\text{WO}_3$  NPs at doses 1 mM (B), 2.5 mM (C), 5 mM (D), and 10 mM (E). The dark-colored circles in the image show the defective areas



**Fig. 5** Phenotypic alterations in *D. melanogaster* after exposure to  $\text{WO}_3$  NPs and MPs. Normal wing phenotype after exposure to  $\text{WO}_3$  MPs at all doses (A) and abnormal wing phenotypes after exposure to  $\text{WO}_3$  NPs at doses 1 mM (B), 2.5 mM (C), 5 mM (D), and 10 mM (E). The dark-colored circles in the image show the defective areas

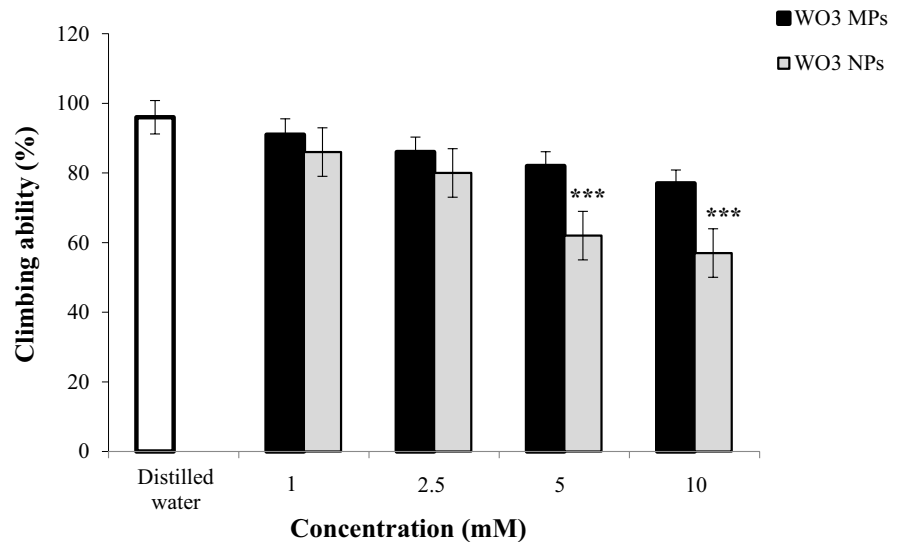


sequences, whose results are presented in Tables 1, 2. In Table 1, we present data relating to trans-heterozygous 3rd instar (3-day-old) larvae exposed to varying doses of  $\text{WO}_3$  NPs (1 to 10 mM) until they completed larval development. In such test conditions, single mutant spots on the wings are triggered by both somatic mutations and somatic recombination events, whereas twin spots on wing blades occur only because of somatic mutations.  $\text{WO}_3$  NPs were found to cause a significant rise in the frequency of small single *mwh* spots, large single spots, and in the total mutant spots in a dose-dependent manner. Genotoxic potential of an agent does not singly determine

the presence of small or large wing spots, rather the exposure time needed to allow the compound to reach the target cells appears to be the key factor, so we can assume that  $\text{WO}_3$  NPs caused genetic damage during the final stages of larval development.

We carried out one experiment on balanced heterozygous *Drosophila* larvae, a genotype suppressing gene recombination and allowing observation of clones induced by somatic mutation only, in an attempt to gain insight into possible mechanisms through which  $\text{WO}_3$  NPs could induce mutant wing spots, however, no significant single, large, or total mutant clones were observed; the findings of this

**Fig. 6** Climbing assay results following chronic exposure (for 7 days) to WO<sub>3</sub> NPs and MPs. Numerical values represent mean  $\pm$  standard error (SE) of the mean. \*\*\* $P \leq 0.001$  as compared to the negative control by one way ANOVA



experiment are summarized in Table 1. We can therefore suggest that WO<sub>3</sub> NPs caused genotoxicity mainly through somatic recombination. We should also note that exposure doses were found to increase incidence of mutant clones depending on doses, yet this was not statistically significant.

Finally, we assessed the genotoxic potential of WO<sub>3</sub> MPs, and doses varying between 1 and 10 mM caused no significant genotoxic effects (Table 2); however, high doses of WO<sub>3</sub> MPs could have greater genotoxicity than do WO<sub>3</sub> NPs; Fig. 7 presents a comparison of WO<sub>3</sub> NPs and MPs. Examination of results obtained in negative and positive groups reveals that our findings are mostly in parallel with those reported in our earlier papers [6, 58, 59, 84, 85].

#### Comet assay

Assessment of genotoxicity is vital in any risk assessment of physical or chemical compounds. Comet assay is a useful and practical technique to detect DNA strand breaks. Our Comet assays revealed that all doses of WO<sub>3</sub> NPs significantly elevated % DNA in the tail (1, 2.5, 5, and 10 mM), in a dose-dependent way, as compared to the controls exposed only to sterile distilled water (Fig. 8A). This is especially observed for WO<sub>3</sub> NPs (Fig. 8B). The greatest dose of WO<sub>3</sub> NPs (10 mM) resulted in more severe DNA damage in the hemocytes, by 8.67%, as compared with control. No DNA damage occurred in the group exposed to WO<sub>3</sub> MPs (Fig. 8A). Our findings seem to

confirm that the amount of WO<sub>3</sub> NPs in the concentration is a primary determinant of genotoxic effects.

#### Discussion

The field of nanotechnology has been expanding at an accelerating rate, bringing new synthesized NPs into our lives with everyday products. Over the recent years, WO<sub>3</sub> NPs have garnered significant attention in various fields owing to their superior properties like thermal stability and conductivity [10]. However, the potential hazards of WO<sub>3</sub> NPs to animal and plant life have not been explored in detail. The endpoint biomarkers that we used in this study not only revealed the toxic and genotoxic potential of WO<sub>3</sub> NPs and its MPs for the first time but also reinforced, once again, the practicality of *D. melanogaster* as an in vivo model organism. The current study was conducted to obtain the first report of the toxicity, genotoxicity, ROS induction, locomotor behavior, and phenotypic alterations caused by WO<sub>3</sub> NPs and MPs upon exposure via oral route (1, 2.5, 5, and 10 mM) in *D. melanogaster*. Accordingly, WO<sub>3</sub> NPs were found to cause genotoxic effects at all tested doses without any toxicity/mortality in the Comet assay. Our SMART showed induction of mutant clone formations at the two highest doses (5 and 10 mM), which could be associated with somatic mutations. In contrast, WO<sub>3</sub> MPs caused no genotoxicity in any doses, as revealed by SMART and Comet assays, and similar

**Table 1** Results of *Drosophila* somatic mutation and recombination tests on *mwht/ft<sup>r</sup>* and *mwht/TM3* wings upon exposure to WO<sub>3</sub> NPs

Test compound (mM)	Number of wings (N)	Small single spots (1–2 cells) (m=2)			Large single spots (> 2 cells) (m=5)			Twin spots (m=5)			Total <i>mw/h</i> spots (m=2)			Frequency of clone formation per 10 <sup>5</sup> cells			
		No	Fr	D	No	Fr	D	No	Fr	D	No	Fr	D				
<b>Marker heterozygous wings (<i>mwht/ft<sup>r</sup></i>)</b>																	
Distilled water	80	13	(0.16)		4	(0.05)		0	(0.00)		17	(0.21)		17	(0.21)	0.87	
1 mM EMS	80	76	(0.95)	+	51	(0.64)	+	7	(0.09)	+	121	(1.51)	+	134	(1.68)	+	6.87
1	80	16	(0.20)	i	1	(0.01)	-	0	(0.00)	i	17	(0.21)	i	17	(0.21)	i	0.87
2.5	80	28	(0.35)	+	3	(0.19)	-	0	(0.00)	i	31	(0.39)	i	31	(0.39)	i	1.59
5	80	40	(0.50)	+	9	(0.11)	+	0	(0.00)	i	48	(0.60)	+	49	(0.61)	+	2.51
10	80	48	(0.60)	+	15	(0.19)	+	0	(0.00)	i	62	(0.78)	+	63	(0.79)	+	3.23
<b>Balancer heterozygous wings (<i>mwht/TM3</i>)</b>																	
Distilled	80	12	(0.15)		2	(0.03)					14	(0.18)		14	(0.18)		0.72
1 mM EMS	80	49	(0.61)	+	56	(0.70)					105	(1.31)	+	105	(1.31)	+	5.38
1	80	13	(0.16)	i	2	(0.03)	-				15	(0.19)	i	15	(0.19)	i	0.77
2.5	80	13	(0.16)	i	0	(0.00)	-				13	(0.16)	-	13	(0.16)	-	0.67
5	80	11	(0.14)	-	0	(0.00)	-				11	(0.14)	-	11	(0.14)	-	0.56
10	80	14	(0.18)	i	0	(0.00)	-				14	(0.18)	i	14	(0.18)	i	0.72

No: number, *Fr* frequency, *D* statistical diagnosis according to Frei and Würgler [81, 82]; + positive, - negative, *i* inconclusive, *m* multiplication factor, probability levels  $\alpha = \beta = 0.05$ . A total of 40 flies were scored per concentration

**Table 2** Results of *Drosophila* somatic mutation and recombination tests on *mw/hflr<sup>3</sup>* wings upon exposure to MPs of WO<sub>3</sub>

Test compound (mM)	Number of wings (N)	Small single spots (1–2 cells) (m=2)			Large single spots (> 2 cells) (m=5)			Twin spots (m=5)			Total <i>mw/h</i> spots (m=2)			Frequency of clone formation per 10 <sup>5</sup> cells
		No	Fr	D	No	Fr	D	No	Fr	D	No	Fr	D	
Marker heterozygous wings ( <i>mw/hflr<sup>3</sup></i> )														
Distilled water	80	16	(0.20)		3	(0.04)		0	(0.00)		19	(0.24)		0.97
1 mM EMS	80	83	(1.04)	+	55	(0.69)	+	4	(0.05)	+	125	(1.56)	+	7.28
1	80	14	(0.18)	-	1	(0.01)	-	0	(0.00)	i	15	(0.19)	-	0.77
2.5	80	13	(0.16)	-	2	(0.03)	-	0	(0.00)	i	15	(0.19)	-	0.77
5	80	14	(0.18)	-	2	(0.03)	-	0	(0.00)	i	16	(0.20)	-	0.82
10	80	17	(0.21)	i	2	(0.03)	-	0	(0.00)	i	19	(0.24)	i	0.97

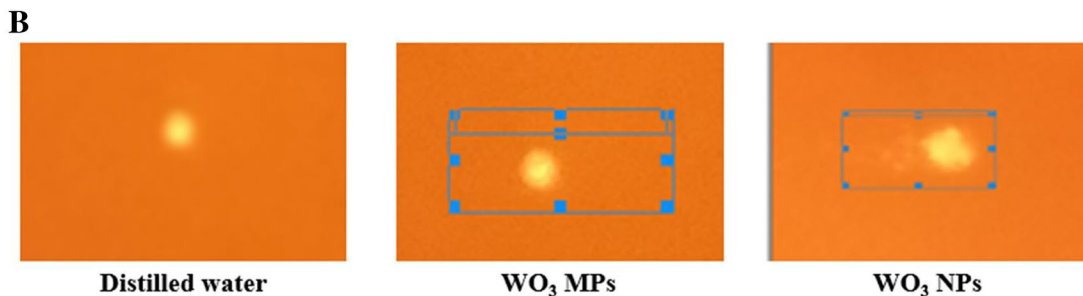
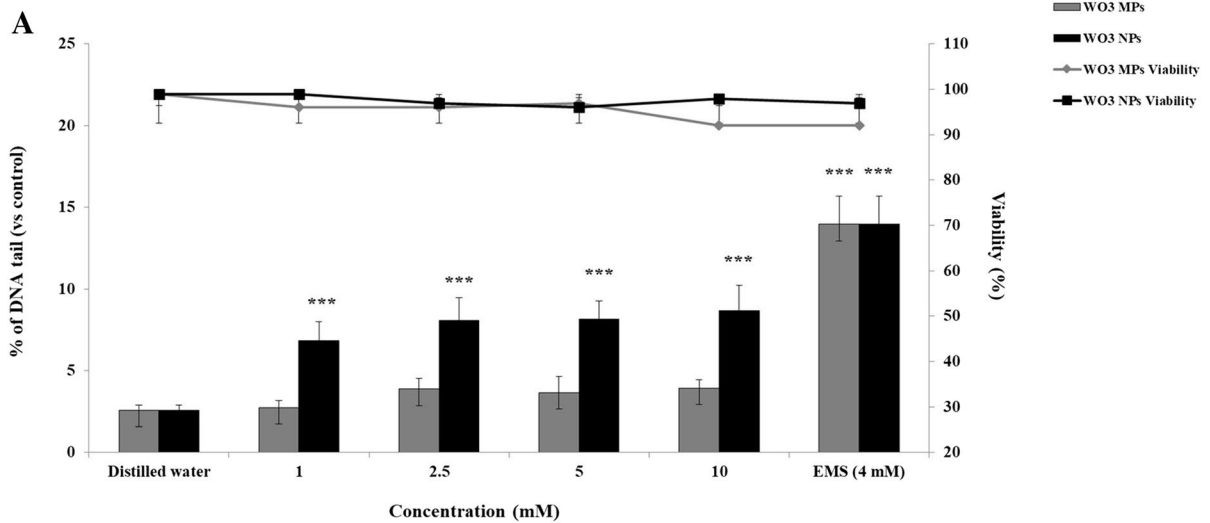
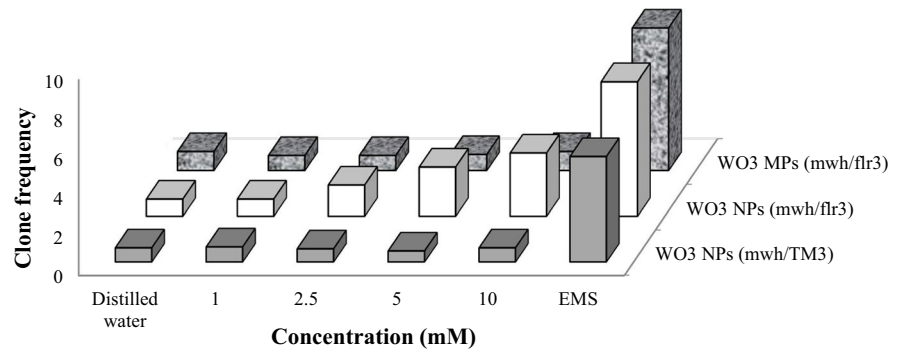
No: number, Fr frequency, D statistical diagnosis according to Frei and Würgler [81, 82]; + positive, - negative, i inconclusive, m multiplication factor, probability levels  $\alpha = \beta = 0.05$ . A total of 40 flies were scored per concentration

results were obtained from tests for ROS level determination and phenotypic alterations. Mouth and wing defects were detected in flies exposed to WO<sub>3</sub> NPs (1, 2.5, 5, and 10 mM), while intracellular ROS production elevation was detected in only hemocytes of WO<sub>3</sub>-exposed flies. We assume that the entrance of NPs into the cells was much easier than microparticles due to their smaller size, wide surface area, and crystal form. In this context, the nanoparticle form of WO<sub>3</sub> could be considered a toxic substance in cases of high doses of acute exposure, which appears to be in accordance with previous in vivo studies conducted with mammals [8]. In addition, biobehavior of NPs may be influenced by a wide range of physicochemical properties, which in turn increase their toxic/genotoxic effects as compared to their MPs, especially if exposed via oral route [8, 86–89].

Genotoxicity (DNA damage) not only causes carcinogenesis but also affects the fertility and health of offspring if a compound penetrates the reproductive cells. Genotoxicological research has been focusing on revealing the genetic damage potentials of different test compounds and risk assessment of potential carcinogens. Because of their small size, NPs can easily pass into the cells and affect the biomolecules including DNA [90, 91]. Both in vivo and in vitro studies have reported that various classes of NPs showed genotoxic effects [5, 36, 57, 89, 92–95].

We assessed the amount of damage in DNA on *Drosophila* hemocytes caused by NPs through genotoxicity assays, including Comet assays to detect single and double-strand breaks in DNA, often used in a number of cell targets in this species, for example, hemocytes responsible for immune response [96, 97]. Hemocytes in fruit flies have functions comparable to those of lymphocytes found in the bloodstream of mammals [72]. Therefore, they could suffer direct exposure to certain compounds and agents running through the hemolymph system, hence a valuable focus of research on the toxic and genotoxic capacity of various nanomaterials [57, 60]. Comet assays have been widely employed in ecotoxicology research efforts involving the testing of substances on various organisms like fruit flies [89]. In this study, Comet assay results revealed that WO<sub>3</sub> NPs induced % tail DNA significantly in *D. melanogaster* hemocytes at all tested doses as compared to the distilled water. Similarly, limited number of genotoxicity studies looking into WO<sub>3</sub> NPs with Comet assays

**Fig. 7** Frequency of clone formations after exposure to WO<sub>3</sub> NPs and MPs in *Drosophila* wing spot assay. Positive control was EMS (1 mM)



**Fig. 8** Comet assay shows genotoxic effects of WO<sub>3</sub> NPs and MPs and viability of *Drosophila* hemocytes (A). After 24-h exposure to WO<sub>3</sub> NPs and MPs, the % of DNA tail in larvae (three replicates were performed and 100 randomly selected cells were examined for each exposure dose). Numerical val-

ues represent the mean  $\pm$  standard error (SE) of the mean. Positive control was EMS (4 mM). \*\*\* $P < 0.001$  as compared to the negative control by Student's *t*-test. Representative comet images are shown (B)

have reported DNA damage induction after in vivo testing [8, 20]. The current literature contains rather limited research into in vivo toxicity of WO<sub>3</sub> NPs [24, 26]. Chinde and Grover [8] reported that WO<sub>3</sub> NPs induced DNA damage in white blood cells and

liver cells of Wistar rats upon chronic oral exposure for 28 days at higher doses (1000 mg/kg). Besides, Chinde et al. [20] also showed that oral single administration of WO<sub>3</sub> NP (1000 mg/kg) caused DNA damage in white blood cells (leukocytes) and liver cells

of Wistar rats. Another *in vivo* study on the toxic potential of WO<sub>3</sub> NPs explored the impacts of exposure via inhalation and its results showed that WO<sub>3</sub> NPs were toxic to the cells, caused morphological changes, and lung injury in tested rodents [24]. Most recently, research investigating *in vivo* effects upon intraperitoneal injection of WO<sub>3</sub> nanorods has reported that such exposure induced hepatotoxicity while melatonin administration diminished this toxicity in rats [26]. Researchers note that smaller WO<sub>3</sub> nanorods (125–200 nm) caused more severe toxic effects and oxidative stress than did micro-size particles (0.8–2 μm) [26].

Most of the research into WO<sub>3</sub> NPs toxicity was carried out as *in vitro* using different kinds of cell types such as Caco-2 and A549 [18–21]. Data from *in vitro* tests should be compared and reinforced with *in vivo* results to understand true effects within a living organism by using reliable *in vivo* model organisms in experiments [98]. As *Drosophila* has shown its potential as a reliable testing model in toxicity/genotoxicity assessment of several nanomaterials, we chose to use this organism in our study into WO<sub>3</sub> NPs and MPs.

Furthermore, intracellular ROS levels in hemocytes were accepted as an oxidative stress parameter in this current study. Although the genotoxic mechanism has yet to be fully illuminated, the most prominent source of NP-induced toxicity is thought to be oxidative stress caused by ROS production. Elevated levels of ROS could lead to DNA and cell membrane damage, disrupt the protein-lipid balance, and impair enzyme and hormone mechanisms [99]. A recent *in vivo* study by Mishra and Panda [39] proposed that ROS accumulation could be associated with the toxic impacts of nanomaterials in *D. melanogaster*. Ever since the first research to address the toxicity of NPs in *Drosophila* [100], substantial research efforts have been geared towards characterizing a through toxic profile of many nanomaterials [66, 101]. The toxic effects of certain compounds mediated by cytotoxicity, cellular internalization, and pigmentation may cause alterations in the genetic composition of fruit flies. Despite the knowledge gaps existing in the mechanism by which genotoxicity occurs, oxidative stress triggered by ROS accumulation is often considered as the culprit behind the nanoparticle toxicity [101, 102]. Increased ROS levels are

known to trigger inflammation response, as well as protein damage and DNA strand breaks [103, 104]. Our findings suggest that the injurious effects of WO<sub>3</sub> NPs responsible for modifications in normal functions of DNA could be attributed to ROS production in cells. However, WO<sub>3</sub> MPs did not cause significant elevation in ROS levels. Similar results obtained upon exposure to WO<sub>3</sub> NPs and MPs in mice hint that smaller WO<sub>3</sub> particles could induce greater hepatotoxicity [26]. Different properties of NPs such as particle size, shape, and surface area may determine their toxicity and genotoxicity.

## Conclusions

The findings of this study confirm that both nanoparticles and MPs of WO<sub>3</sub>, if exposed to high doses can cause some hazardous effects on *D. melanogaster*, underscoring the importance of a thorough risk assessment of WO<sub>3</sub> NPs for the first time. This is the first study to use *Drosophila* in testing *in vivo* effects including parameters like toxicity, genotoxicity, phenotypic alterations, locomotor behavior impairments, and ROS induction of exposure to WO<sub>3</sub> NPs (43.71 ± 1.59 nm) and MPs of WO<sub>3</sub> at varying doses. Non-toxic doses of WO<sub>3</sub> NPs caused significant % tail DNA damage, elevated ROS production in hemocytes, phenotypic alterations in wing shapes, and mouth parts as well as impaired locomotor behavior in adult flies. Most importantly, evidence from our assays establishes that a considerable portion of toxic effects is linked to somatic mutations (5 and 10 mM doses of WO<sub>3</sub> NPs), which may alter key functions in cells and play a role in carcinogenesis. In this context, the use of *Drosophila* could be further extended to research into cancer and nanoparticles. Our model organism, *Drosophila*, has shown its potential to reliably detect and quantify potential biological and genetic effects of WO<sub>3</sub> NPs and its MPs. Future studies should focus on the underlying mechanisms of various endpoint biomarkers of such widely adopted substances.

**Author contribution** FTD and ED contributed to the conception, experimental design, experimental performance, data analysis, and writing original manuscript.

## Compliance with Ethical standards

**Competing interests** The authors declare no competing interests.

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