RESEARCH PAPER

Adaptive and Adverse Multigenerational Responses to Zinc Oxide Nanoparticle Exposure in *Drosophila melanogaster*

Sampangiramaiah Jalajakshi*[1], Bangalore Satishbabu Manushree[1], Nageshwar Subramanyam Srividya[1]

[1]Department of Genetics, Vijaya College, R.V. Road, Basavanagudi, Bangalore-560004
***Corresponding Author**: Jalajakshi S, Department of Genetics Vijaya College, Bangalore-560004, Karnataka, India. e*mail- jalajasrinivasa09@gmail.com*

ABSTRACT

Introduction- Engineered nanomaterials are widely being developed and therefore their long-term effects on the biological system needs to be assessed. This present study focused on assessing the dose dependent and multi-generational toxicity of Zinc Oxide (ZnO) nanoparticles using *Drosophila melanogaster*.

Methods- Exposure was done with three doses (0.1, 0.01, 0.001mg ml^{-1}) in 4 generations. Development and cytogenetic alteration (polytene chromosome) were studied. Characterization of nanoparticles was performed using scanning electron microscopy (SEM) and X-ray diffraction (XRD). In the experiment, the development of flies exposed to the three concentrations for four generations revealed concentration and generation dependent effect. At high concentration (0.1 mg ml^{-1}), it showed acute toxicity and resulted in complete death by generation two.

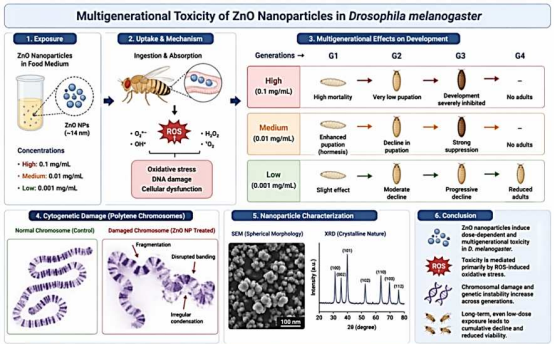
Results- At medium concentration (0.01 mg ml^{-1}) initially pupa production increased followed by complete reduction in later generation demonstrating a hormetic response. At the low concentration (0.001mg/ml^{-1}) flies exhibited tolerance at initial but declined with longer exposures. Significant differences were observed between treatment groups and generations using ANOVA ($p < 0.05$, $p < 0.01$). A significant interaction between dose and generation was noted. High Coefficient of variation up to 100% showed the developmental instability of treated group.

Conclusion- Cytogenetic analysis showed fragmentation, disruption of chromosome banding pattern and abnormality in chromosome condensation; indicating their genotoxic effects through the generation of reactive oxygen species (ROS). The SEM and XRD images show the nanosized (<14nm) spherical crystalline structure of ZnO, confirming high reactivity of these nanoparticles.

Keywords: *Drosophila melanogaster,* Genotoxicity, Oxidative stress, ZnO Nanoparticles

How to cite this article: Sampangiramaiah Jalajakshi*1 Bangalore Satishbabu Manushree1 Nageshwar Subramanyam Srividya1. Adaptive and Adverse Multigenerational Responses to Zinc Oxide Nanoparticle Exposure in Drosophila melanogaster. Int J Drug Deliv Technol. 2026;16(87s):1833-1839. DOI: 10.25258/ijddt.16.87s.196

.GRAPHICAL ABSTRACT



Introduction

The science of nanomaterials and nanotechnology has evolved to be a major multidisciplinary area with widespread impact on medicine, agriculture, environment, and biotechnology. The Engineered nano sized (1-100nm) materials known as nanoparticles have several peculiar physico-chemical properties such as high surface area to volume ratio, higher surface reactivity and ability of enhanced permeability into biological environments (Nel et al., 2006; Zhang et al., 2021). This may result in unique applications in various industries and in health care (Liu et al., 2020). It has observed that the nanoparticles may interacts with biological targets like protein, lipid, nucleic acids that affects cellular functionality. Among the most frequently used nanoparticles, zinc oxide (ZnO) nanoparticles have well known for its excellent antibacterial activities, UV absorption properties and affordability (Sirelkhatim et al., 2015; Agarwal et al., 2023). ZnO is the element zinc which is an essential trace mineral for enzyme activities, DNA repair and regulation, but nanoparticles of ZnO show different biological interactions than other ZnO. The most common cause of ZnO nanoparticles toxicity involves the

induction of reactive oxygen species (ROS) and consequent oxidative stress that disturbs the cellular equilibrium, leading to injury to cellular biomolecules such as DNA, proteins and lipids (Xia et al., 2008; Srivastava et al., 2022). This induced oxidative stress leads to various negative effects such as developmental malformations, reduced survival rates, and genotoxicity (Sharma et al., 2021).

The increasing production and environmental release of ZnO nanoparticles have heightened concerns regarding their long-term ecological and biological consequences (Zhao et al., 2024). The continued discharge of nanoparticles into our surroundings makes it important to perform toxicity assessment, especially because of their cumulative effects upon continuous exposure and their potential to persist across generations (Fadeel et al., 2018; Wang et al., 2023). *Drosophila melanogaster*, being a readily available organism with a short life cycle and exhibiting similar characteristics to other higher eukaryotes in its genetics has been frequently employed in the evaluation of environmental toxicants. Studies have demonstrated that exposure to various nanoparticles negatively impacts development, reproduction, and genetic stability in this model (Pandey and Nichols, 2011; Alaraby et al., 2016; Demir et al., 2020). In particular, the giant polytene chromosomes of larval salivary glands provide an excellent cytogenetic model for directly detecting chromosomal abnormalities and genotoxic effects induced by environmental contaminants.

The present study aims to assess dose dependent as well as multi-generational toxicity of ZnO nanoparticles in *Drosophila melanogaster*. Life and developmental parameters were studied for four consecutive generation whereas cytogenetic changes in terms of polytene chromosome changes were analyzed. Also, physicochemical nature of ZnO particles using Scanning Electron Microscopy (SEM), X Ray Diffraction (XRD)and developmental, cytogenetic studies revealed the overall impact of long-term presence of ZnO nanoparticles in organisms.

Materials and methods

ZnO Nanoparticle synthesis and characterization

ZnO nanoparticles were synthesized by zinc oxide by following established protocols earlier (Sood et al., 2019). SEM was employed to determine particle morphology, whereas XRD was used to identify the crystalline phase and estimate the average crystallite size using the Debye–Scherrer equation (Nath et al., 2024).

Experimental design

Drosophila culture

Standard wild-type Drosophila melanogaster was kept at 25 ± 1°C on a standard media formula using: 5 g of jaggery, 9 g of wheat flour, 1.2 g of agar, 3 g of yeast powder prepared in 250 mL distilled water, then boiled, homogenized, bottled, solidified, and nine-drops of propionic acid solution per bottle was introduced to prevent any fungal growth (Anand et al., 2023).

ZnO nanoparticle exposure

The ZnO nanoparticles were dispersed in distilled water to prepare three exposure concentrations: 0.1 mg/mL (high), 0.01 mg/mL (medium), and 0.001 mg/mL (low). Four experimental groups were established, consisting of one untreated control and three ZnO NP-treated groups. Each treatment was performed in triplicate (n =3). Adult flies were introduced into the respective culture bottles, allowed to mate, and maintained under identical environmental conditions for four consecutive generations.

Developmental assessment

Larva and pupa development was monitored by recording the total number of larvae and pupae daily for ten days in each treatment group across four successive generations. Developmental performance was compared among treatment concentrations and generations to determine dose-dependent and cumulative toxic effects of ZnO NP exposure.

Lipid peroxidation assay

Thiobarbituric Acid Reactive Substances (TBARS) assay is the most widely used method. It measures Malondialdehyde (MDA), a primary secondary byproduct of lipid degradation. The larvae or pupa were taken in 1.5 ml Tris HCl buffer [pH 7.4] and the homogenate was prepared. The homogenate was centrifuged at 3000 g for 20 min. 100 µL of the supernatant was used for assay as described previously.

Cytogenetic analysis

Cytogenetic analysis was performed on third instar larvae. The salivary glands were isolated dissected with a scalpel and fixed in acetic acid with a subsequent staining with aceto orcein method reported earlier (Kolesnikova et al., 2022). Polytene chromosome squash was obtained applying light pressure with the coverslip above the squashed chromosomes. Preparations were observed at the light microscope (40 objective lens); observed structural abnormalities were characterized by chromosomal fragmentation, abnormal banding, disrupted chromosome condensation, etc. and a photograph of the respective chromosomes was taken.

Statistical analysis

Data are expressed as mean ± standard deviation (SD), obtained from three independent experiments. The effect of the different treatments was determined by one-way ANOVA and interaction effect between nanoparticle generation and concentration on a specific parameter by two-way ANOVA. The coefficient of variation (CV%) was

calculated to assess the degree of variability within each treatment group, using the formula:

CV (%) = (Standard Deviation / Mean) × 100

A significance level of $p < 0.05$ and $p < 0.01$was considered statistically significant for all analyses. Statistical calculations were performed using standard software packages such as SPSS.

Results and discussion

Drosophila melanogaster individuals were exposed to ZnO nanoparticles, which demonstrated both dose-dependent and multi-generational effect in its development and thus similar to the results demonstrated previously indicating a concentration-and exposure-time dependency to nanoparticle-induced toxic effects. In the case of 0.1 mg ml^{-1} of ZnO nanoparticles, being the maximum concentration used, resulted in complete mortality by generation 2 which is consistent with acute toxicity induced by high doses of nanoparticles in *Drosophila* causing oxidative stress and death (Sharma et al., 2021; Khan et al., 2022). The medium concentration (0.01 mg ml^{-1}) induced a biphasic response (hormesis). Generation 1-3 showed an increase in pupal output, but this was followed by complete suppression in Generation 4. Such responses, where low or moderate stress can enhance biological activity while chronic high levels lead to toxicity have been observed in other nanoparticle exposure studies (Motulsky, 2014; Roco, 2019). A similar biphasic effect has been observed in studies with high particle concentration exposure, suggesting initial stimulation before damage. The lowest concentration (0.001 mg ml^{-1}) led to a general pattern of consistent development in the initial generations, which gradually declined by Generation 4. Such chronic, low-dose exposure leading to delayed toxicity has been seen in multiple nanoparticle exposure studies (Fadeel et al., 2018). On other side control flies showed stable developmental patterns throughout the study.

The progressive declines in the development of both larva and pupa after repeated exposure demonstrates a cumulative effect in larva at multigeneration exposure of ZnO NPs probably due to accumulative oxidative stress. NPs accumulated inside cells, and a decrease in cellular self-repaired function of larva. Multi-generation negative effects on organism have also been observed on animal model exposed to nanomaterials in the last decade (Wang et al., 2023). SEM of ZnO NPs showed that the morphology of the NPs used were mainly spherical nanosized and mildly aggregated. This small size of nanoparticles with larger surface area-to-volume ratio will strengthen their binding ability on the cell surface and result in more pronounced intracellular absorption as well as potent induce ROS level (Adamcakova-Dodd et al., 2021).

The particle agglomeration can affect the bioavailability, but the nanoparticle still retains highly reactive. The XRD data indicates highly crystalline hexagonal wurtzite ZnO structure and characteristic diffraction peaks for planes (100) (002) (101) (102) (110) (103) (112) (Figure 2a and b). There is no impurity peaks existed in the spectra which means good purity phase. The average crystallite size (around 13.97nm) and particle size (ranging from 11.5-18 nm) demonstrate that the particle size is at nanoscale. It was observed that the lipid peroxidation levels increased for all generation (Fig 4). The high crystallinity of the ZnO NPs and large surface-to-volume ratio of the ZnO NPs are well known to generate ROS and the ROS will lead to high toxic nature (Kumari et al., 2020).

The effects of ZnO NP exposure on the genomic content are reflected through the cytogenetic examination performed on salivary gland polytene chromosomes, indicating severe genotoxicity of ZnO NP upon administration (Liao et al., 2017). As revealed from the results presented in Fig.3, control larva showed the defined structure of chromosome, composed of banding pattern and the normal morphology of chromosomes indicating the genomic integrity of the control larvae, while, treated larvae have chromosome breakage and altered banded patterns with abnormal condensation of chromosomes and sometimes there are puffs, that increased depending on the concentration of the treatments. The changes above suggested that ZnO NPs may induced Oxidative DNA damage, may interfere DNA replication and repair processes in chromosomes, and may decrease genomic integrity through the generation of reactive oxygen species ROS. Our data have coincided with earlier studies that demonstrate Oxidative DNA damage induced by ZnO NP (Valdiglesias et al., 2013).

However, the continued generation of chromosomal damage across the generations implies accumulating genetic damage under a prolonged period of exposure to nanoparticles (Cardozo et al., 2019). Moreover, these cytogenetic alterations were tightly coupled to the developmental defects in survival, from larva to adult and through pupation stage and also, it supports the existence of common pathway induced by the oxidative stress to achieve the developmental and genomic toxicity. While, two phase results at the intermediate level was probably associated to the initially acclimatizing the oxidative environment, afterwards irreparable damaged to the cellular system upon chronic exposure of ROS to the system.

The effect of ZnO NPs exposure resulted in a concentration-dependent decrease in developmental stability of *Drosophila melanogaster* larva and pupa (Anand et al, 2017). Data of the larval development as depicted in Tables 2-5 shows that the larval development was almost stable in the control experiment (Mean=25.75, CV=30.6%), whereas, variability was the increased among different ZnO NPs exposures concentration. Low concentration (0.001 mg mL^{-1}) treatment resulted in slightly increased number of larvae (Mean=33.75), showing transient hormetic effects but at the cost of higher coefficient variation (CV=68.2%) which indicates poor variable efficiency of larva with this treatment concentration as well.

Although, the larva number under medium concentration (0.01 mg mL^{-1}) of exposure was similar as control but the variation was relatively higher (CV = 81.2%) indicates higher stress in the physiological condition, whereas in high exposure (0.1 mg mL^{-1}), developmental instability was noted at highest value of CV=100% including complete developmental failure in some replicas, this depicts strong dependency of toxicity on the concentration of ZnO NPs.

In case of pupa development same was noted, although larva no of control group was showing quite a high variability (CV=64.1%), lower number of pupae at low dose treatment with similar variability was noted (CV=60.3%). In case of medium concentration, number of pupae was maximum (which is indicating temporary adaptation or hormesis) but had quite standard Deviation that means very high variability between replicas. In case of high doses treatments had complete failure of larva into pupae and many cases failed (CV=100%).

The significant effects of ZnO NPs concentration were additionally identified by two-way ANOVA in development of the larvae ($F = 4.87$, $p = 0.018$) and pupae ($F = 6.42$, $p = 0.006$) respectively (Table 4, 5). Coefficient of variation at successive concentrations in this variable was increasing as a reflection of enhanced developmental instability caused by oxidative stress and damage to physiological development. That means exposure to ZnO NPs impact the insect development at different dose-dependent patterns and confirmed the additive impact on developmental toxicity after chronic exposure (Ji et al., 2024). During nanoparticles induced stress, significant enhancement in developmental instability was found among the ZnO NPs treated groups of the examined insect compared to that of controls.

Therefore, the results revealed ZnO nanoparticles show the cumulative development toxicity and genotoxicity to *D. Melanogaster* and indicated to perform life cycle or multi-generation tests or longer term of animal studies or in environmental context the accumulation for the ZnO NPs could potentially lead to significant risk to ecological populations over a prolonged and even across generations.

Authors declaration

The authors declare that no part of this paper has been copied or reproduced from any external source. All figures, tables, and graphical representations are generated from our own experimental data.

Authors' contributions

Dr.Jalajakshi S. conceptualized and designed the study, supervised experimental work, and prepared the manuscript. Manushree B.F. and Srividya N.S. conducted laboratory experiments, collected data, and assisted in statistical analysis. All authors contributed to data interpretation, manuscript drafting, and approved the final version for submission.

Acknowledgement

The authors gratefully acknowledge the Department of Genetics, Vijaya College Bangalore, for providing laboratory facilities and technical support throughout the study.

Conflict of interest

The authors declare that they have no competing interests.

References

Adamcakova-Dodd, A., Stebounova, L. V., Kim, J. S., Vorrink, S. U., Ault, A. P., O'Shaughnessy, P. T., Grassian, V. H., & Thorne, P. S. (2014). Toxicity assessment of zinc oxide nanoparticles using sub-acute and sub-chronic murine inhalation models. *Particle and Fibre Toxicology*, 11, 15. https://doi.org/10.1186/1743-8977-11-15

Agarwal, H., Kumar, S. V., & Rajeshkumar, S. (2017). A review on green synthesis of zinc oxide nanoparticles—An eco-friendly approach. *Resource-Efficient Technologies*, 3, 406–413. https://doi.org/10.1016/j.reffit.2017.03.002

Alaraby, M., Annangi, B., Marcos, R., & Hernández, A. (2016). *Drosophila melanogaster* as a

suitable in vivo model to determine potential side effects of nanomaterials: A review. *Journal of Toxicology and Environmental Health, Part B*, 19, 65–104. https://doi.org/10.1080/10937404.2016.1166466

Anand, A. S., Prasad, D. N., Singh, S. B., & Kohli, E. (2017). Chronic exposure of zinc oxide nanoparticles causes deviant phenotype in *Drosophila melanogaster*. *Journal of Hazardous Materials*, 327, 180–186. https://doi.org/10.1016/j.jhazmat.2016.12.040

Anand, A. S., Verma, K., Amitabh, Prasad, D. N., & Kohli, E. (2023). The interplay of calponin, Wnt signaling, and cytoskeleton protein governs transgenerational phenotypic abnormalities in *Drosophila* exposed to zinc oxide nanoparticles. *Chemico-Biological Interactions*, 369, 110284. https://doi.org/10.1016/j.cbi.2022.110284

Cardozo, T. R., De Carli, R. F., Seeber, A., Flores, W. H., da Rosa, J. A. N., Kotzal, Q. S. G., Lehmann, M., da Silva, F. R., & Dihl, R. R. (2019). Genotoxicity of zinc oxide nanoparticles: An in vivo and in silico study. *Toxicology Research*, 8, 277–286. https://doi.org/10.1039/C8TX00255J

Demir, E., Akça, H., Kaya, B., et al. (2020). Nanoparticle toxicity in model organisms. *Chemosphere*, 244, 125496–125510 DOI 10.1016/j.chemosphere.2019.125496.

Fadeel, B., Farcal, L., Hardy, B., Vázquez-Campos, S., Hristozov, D., Marcomini, A., Lynch, I., Valsami-Jones, E., Alenius, H., & Savolainen, K. (2018). Advanced tools for the safety assessment of nanomaterials. *Nature Nanotechnology*, 13, 537–543. https://doi.org/10.1038/s41565-018-0185-0

Ji, L., Huang, Q., Qi, Y., Wang, Z., Kong, X., Zhu, X., Yang, B., Li, J., He, X., Deng, X., Cheng, X., Yu, H., Shi, Y., Lin, Z., Zhao, X., Wang, X., & Yu, J. (2024). Quercetin and Astragaloside IV mitigate the developmental abnormalities induced by gestational exposure to zinc oxide nanoparticles. *ACS Omega*, 9, 47802–47810. https://doi.org/10.1021/acsomega.4c08235

Khan, I., Saeed, K., & Khan, I. (2019). Nanoparticles: Properties, applications and toxicities. *Arabian Journal of Chemistry*, 12, 908–931. https://doi.org/10.1016/j.arabjc.2017.05.011

Kolesnikova, T. D., Klenov, M. S., Nokhova, A. R., Lavrov, S. A., Pokholkova, G. V., Schubert, V., Maltseva, S. V., Cook, K. R., Dixon, M. J., & Zhimulev, I. F. (2022). A spontaneous inversion of the X chromosome heterochromatin provides a tool for studying the structure and activity of the nucleolus in *Drosophila melanogaster*. *Cells*, 11, 3872. https://doi.org/10.3390/cells11233872

Kumari, M., Khan, S. S., Pakrashi, S., Mukherjee, A., & Chandrasekaran, N. (2011). Cytogenetic and genotoxic effects of zinc oxide nanoparticles on root cells of *Allium cepa*. *Journal of Hazardous Materials*, 190, 613–621. https://doi.org/10.1016/j.jhazmat.2011.03.095

Liao, K. H., Lin, Y. S., Macosko, C. W., & Haynes, C. L. (2017). Zinc oxide nanoparticles exhibit cytotoxicity and genotoxicity through oxidative stress responses in human lung fibroblasts and *Drosophila melanogaster*. *International Journal of Nanomedicine*, 12, 1621–1637. https://doi.org/10.2147/IJN.S124403

Liu, Y., Zhao, Y., Sun, B., & Chen, C. (2020). Nanoparticle toxicity mechanisms. *Environmental Science: Nano*, 7, 1452–1476.

Motulsky, H. (2014). *Intuitive Biostatistics: A Nonmathematical Guide to Statistical Thinking* (3rd ed.). Oxford University Press, New York.

Nath, B. B., Khanna, P. K., Phatak, K. A., et al. (2024). ZnO nanoparticles: A key ingredient of sunscreen shows absence of adverse effects on *Drosophila* melanization pathway. *Nano-Structures & Nano-Objects*, 38, 101145. https://doi.org/10.1016/j.nanoso.2024.101145

Nel, A., Xia, T., Mädler, L., & Li, N. (2006). Toxic potential of materials at the nanolevel. *Science*, 311, 622–627. https://doi.org/10.1126/science.1114397

Pandey, U. B., & Nichols, C. D. (2011). Human disease models in *Drosophila melanogaster* and the role of the fly in therapeutic drug discovery. *Pharmacological Reviews*, 63, 411–436. https://doi.org/10.1124/pr.110.003293

Roco, M. C. (2019). Nanotechnology future. *Journal of Nanoparticle Research*, 21, 1–14.

Sharma, V., Singh, P., Pandey, A. K., & Dhawan, A. (2012). Induction of oxidative stress, DNA damage and apoptosis in mouse liver after sub-acute oral exposure to zinc oxide nanoparticles. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 745, 84–91. https://doi.org/10.1016/j.mrgentox.2011.12.009

Sirelkhatim, A., Mahmud, S., Seeni, A., Kaus, N. H. M., Ann, L. C., Bakhori, S. K. M., Hasan, H., & Mohamad, D. (2015). Review on zinc oxide nanoparticles: Antibacterial activity and toxicity mechanism. *Nano-Micro Letters*, 7, 219–242. https://doi.org/10.1007/s40820-015-0040-x

Sood, K., Kaur, J., Singh, H., Kumar Arya, S., & Khatri, M. (2019). Comparative toxicity evaluation of graphene oxide (GO) and zinc oxide (ZnO) nanoparticles on *Drosophila melanogaster*. *Toxicology Reports*, 6, 768–781. https://doi.org/10.1016/j.toxrep.2019.07.009

Srivastava, A., Kumar, A., Sharma, B., et al. (2022). ROS-mediated toxicity. *Environmental Research*, 204, 112001–112015.

Valdiglesias, V., Costa, C., Kiliç, G., Costa, S., Pásaro, E., Laffon, B., & Teixeira, J. P. (2013). Neuronal cytotoxicity and genotoxicity induced by zinc oxide nanoparticles. *Environment International*, 55, 92–100. https://doi.org/10.1016/j.envint.2013.03.005

Wang, Z., Li, X., Chen, J., et al. (2023). Transgenerational nanotoxicity. *Journal of Hazardous Materials*, 452, 131245–131260.

Xia, T., Kovochich, M., Liong, M., Mädler, L., Gilbert, B., Shi, H., Yeh, J. I., Zink, J. I., & Nel, A. E. (2008). Comparison of the mechanism of toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress properties. *ACS Nano*, 2, 2121–2134. https://doi.org/10.1021/nn800511k

Zhao, Y., Liu, J., & Chen, C. (2024). Mechanisms of metal oxide nanoparticle-induced oxidative stress and toxicity: Recent advances. *Journal of Hazardous Materials*, 468, 133744.

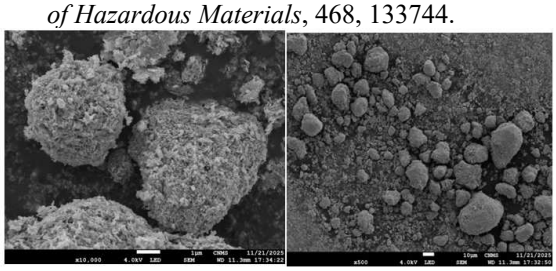
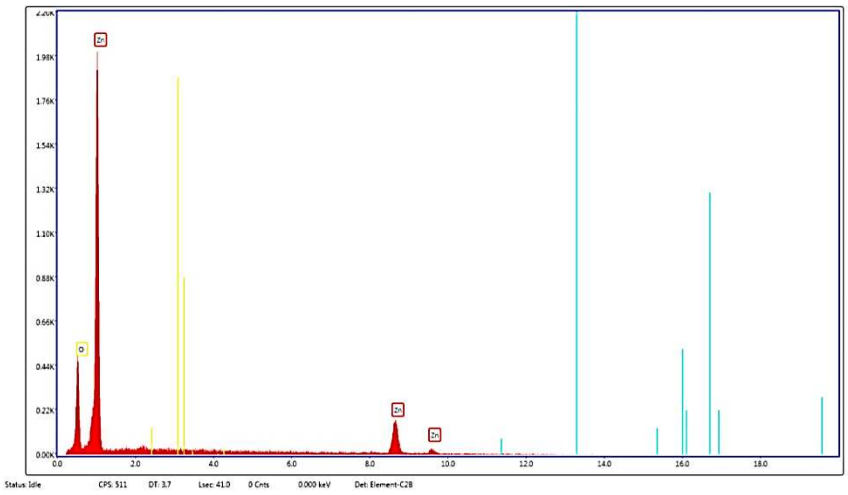


Fig 1: Different-scale SEM images of the ZnO nanoparticles

a)

b)



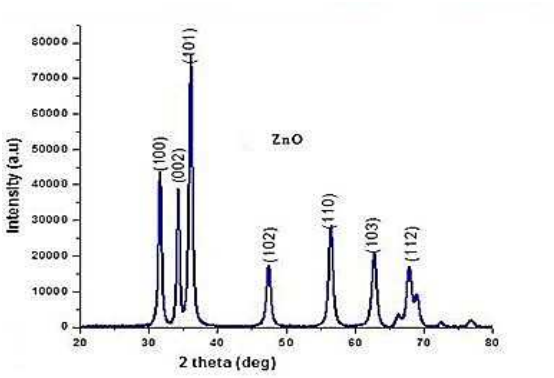


Fig 2: XRD spectrum of the ZnO nanoparticles (a), pattern of the ZnO nanoparticles (b).

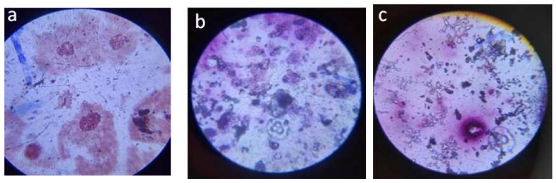


Fig 3: Polytene chromosomes of *Drosophila melanogaster* a) Normal, b) Low and c) Medium concentration of ZnO nanoparticles

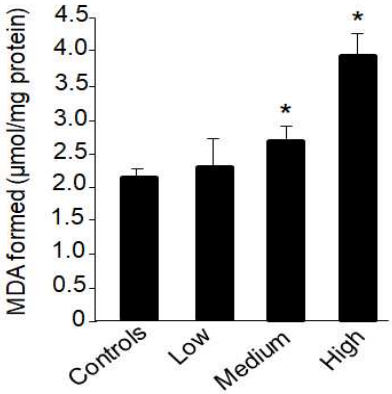


Figure 4. Effect of ZnO nanoparticles on larva lipid peroxidation. Values represent mean ± SD of three independent experiments. 3 to 5 larva were used from each group. *Indicates significant difference compared to controls,

Table 1: Overall developmental trends of *Drosophila melanogaster* under ZnO exposure.

Table 1: Overall developmental trends of *Drosophila melanogaster* under ZnO exposure.

Generation	Treatment	Peak Larvae (Day 10)	Peak Pupae (Day 10)	Key observation
G1	Control	24	93	Normal gradual development
	High-0.1mgml^{-1}	73	91	Reduced stability, irregular growth
	Medium-0.01mgml^{-1}	43	130	Highest pupation observed
	Low -0.001mgml-1	26	70	Moderate development
G2	Control	16	12	Reduced but stable growth
	High-0.1mgml-1	-	-	Complete mortality by day 2
	Medium - 0.01mgml^{-1}	12	160	Maximum pupal output
	Low -0.001mgml^{-1}	49	52	Balanced development
G3	Control	25	29	Normal development
	Medium-0.01mgm^{-1}	55	140	High pupation maintained
	Low -0.001mgml^{-1}	60	51	Increased larval count
G4	Control	38	58	Strong recovery and growth
	Medium- 0.01mgml^{-1}	-	-	Development suppressed
	Low - 0.001mgml^{-1}	-	-	Development suppressed

.

Values are mean of three independent experiments.

Table 2: Statistical analysis of larval counts under ZnO nanoparticle treatment

ZnO Concentration	Larva count	Coefficient of Variation (CV%)
Control	25.75 ± 7.89	30.6
High	36.50 ± 0.5*	100.0
Medium	27.50 ± 2.34	81.2
Low	33.75 ± 3.02*	68.2

Values are mean ± SD of three independent experiments. *indicates statistically significant from controls. A $P<0.05$ was statistically significant.

Table 3. Effect of ZnO nanoparticles concentration on the larval counts using one way ANOVA analysis.

Source of Variation	SS	df	MS	F	*p-value*
Between Groups	2156.75	3	718.92	4.87	0.018
Within Groups	2360.50	16	147.53		
Total	4517.25	19			

Table 4: Statistical analysis of pupa counts under ZnO nanoparticle treatment

ZnO Concentration	Pupa count	Coefficient of Variation CV%)
Control	48.00 ± 3.75	64.1
High	45.50 ± 5.50	100.0
Medium	107.50 ± 3.02	58.6
Low	43.25 ± 2.09	60.3

Values are mean ± SD of three independent experiments. *indicates statistically significant from controls. One way ANOVA was performed and A $P<0.05$ was statistically significant.

Table 5: Effect of ZnO nanoparticles concentration on pupal counts statistical analysis using one-way ANOVA.

Source of Variation	SS	df	MS	F	p-value
Between Groups	8925.60	3	2975.20	6.42	0.006
Within Groups	7410.30	16	463.14		
TOTAL	16335.90	19			

Table 6: Two-Way ANOVA Effect of Concentration and generation

Source of Variation	SS	df	MS	F	p-value
Concentration	10450.20	3	3483.40	7.15	0.002
Generation	8750.10	3	2916.70	5.98	0.004
Interaction	6520.75	9	724.53	3.21	0.012
Error	13240.60	24	551.69		
Total	38961.65	39			