ABSTRACT

Nanoparticles used for filling of teeth causes developmental and mechano-sensory defect in Drosophila melanogaster

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Nanotechnology is widely used in dental field as nano dentistry. While choosing the nanoparticle for the use in the field of nano dentistry its chemical, physical, along with the biological aspect of nanostructures are taken into account. Some of the nanoparticles widely used in dentistry includes, zirconia, hydroxyl apatite and titanium dioxide. These nanoparticle are used for filling of teeth, oral disease preventive drugs, prostheses and for teeth implantation. The wide use of nanoparticle enhances the chances of introducing the nanoparticle into the food chain. Thus it warrants checking the toxicity of nanoparticle using a model organism. The current study checks the toxicity of nanoparticle using *Drosophila* as a model organism.

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Key words: Hydroxyapatite nanoparticles; mechanosensory neurons; *Drosophila melanogaster*; Oxidative stress, Micronuclei, Phosphorous absorption

Introduction

Nanotechnology, from a decade or two, has become one of the most widely used technique in the pharmaceutical, biomedical and cosmetics as well as photothermal therapy(Reddy et al. 2012). Having tunable size, larger surface area, and label-free characterization methods, allow the wide use of nanoparticles in various fields(Albanese et al. 2012). Some of the nanoparticles (NPs) like silica nanoparticles and solid lipid nanoparticles (SLN) are used as drug carriers to target specific cells in cancer therapy and directed drug delivery(Cho et al. 2008b; Slowing et al. 2008; MuÈller et al. 2000; Cho et al. 2008a). Hydroxyapatite nanoparticles (HaPNPs) have extensive applications in the field of medicine by virtue of its good biocompatibility and bioactivity (Kantharia et al. 2014). It has a hexagonal structure with a Ca/P proportion 1.67, which is matching with bone apatite (Mostafa and Brown 2007; Kalita et al. 2007). Along with that, it is thermodynamically stable under various physiological conditions such as temperature and pH(Kalita et al. 2007). It's stability and biocompatibility allow it to be used in implanting, coating, bone filling, bone substitutes and oral treatment (Sun et al. 2001; LeGeros 1991). Despite having vast applications, the toxicity of hydroxyapatite nanoparticles has been reported in zebrafish (*Danio rerio*), where HApNPs cause axial deformation as well as developmental delay (Zhao et al. 2013). In osteoblasts-like cells HApNPs (a naturally occurring calcium apatite) can induce proliferation and apoptosis(Shi et al. 2009).The toxicity reported from HApNPs is alarming and warrants a thorough investigation using a model organism.

Besides nanoparticle toxicity study, *Drosophila* has long been used to study several neurodegenerative diseases like Alzheimer's disease and seizure disorders (Ong et al. 2015; Pandey and Nichols 2011). Low maintenance cost, short life cycle, all the well-studied developmental stages make it an ideal model to study the toxic effect of any chemical (Campos-Ortega and Hartenstein 2013). More importantly, it has only four chromosomes and 75% genes of the *Drosophila* genome share functional homology with the human disease-causing genes(Pandey and Nichols 2011).Compelling the extensive application of HApNP into contemplation, the aim of this work is to explore the toxic effect of HApNPs on development, behaviour and cellular physiology of *Drosophila* when exposed through oral route.

Material and Methods

Fly rearing: The fly media used for rearing flies consisted of sucrose, cornmeal, yeast powder and agar-agar (type-1). Propionic acid and Nipagin M were added to prevent mould and fungal contamination. The flies were incubated at 25°C temperature, 60% relative humidity (RH) and were exposed to a 12-hour dark-light cycle. The flies used were of Oregon-R type obtained from C-CAMP Bangalore.

Materials

Orthophosphoric acid, sodium hydroxide, hydrogen peroxide, ammonium hydroxide, calcium hydroxide, 2,7-Dichlorofluorescein, trypan blue, nitro blue tetrazolium and hydrochloric acid was

purchased from Himedia. All chemicals used in this study were of analytical grade. All the chemicals are used in the experiment as supplied by the company without any further purification.

Synthesis of hydroxyapatite (HaP)

For the production of unadulterated HaP, 37gm$Ca(OH)_2$ was liquefied in 100 mL of water. Next, H_3PO_4 was supplemented to get a proportion of Ca/P of1.67. The pH of the mixture was maintained 10 by the addition of NH_4OH. The resulting product was imperilled to microwave radiation with a machinating power of 800 W for 20 min. A precipitate was achieved as a product of the previous step. The precipitate was rinsed several times with distilled water to take away undesirable water-soluble ions. After washing the desired product is calcined at 500°C for 3hr. The anticipated product was grinded with the help of mortar and pestle to achieve pure hydroxyapatite.

Characterization of NPs:

FTIR spectroscopy (Perkin-Elmer) was used to define the functional groups present in the synthesised HAp. The crystallinity of the produced HAp was scrutinized by an X-Ray diffractometer (XRD-PHILIPS PW 1830) with CuKα source. The morphology of the Hap was explored by field emission scanning electron microscopy (FESEM), successively the chemical composition was restrained by EDX in Nova NANOSEM/FEI. Surface area of the Hap was deliberated by using the Brunauer–Emmet–Teller (BET) equation in QUANTACHROME AUTOSORB (IQ) model ASIQM0000-4. The distribution of pore size and volume were obtained by applying the Barrett–Joyner–Halenda (BJH) method. HApNPs were degassed in vacuum at 150 °C. Transmission electron micrographs (TEM) (PHILIPS CM 200) of HApNPs were taken on a carbon-coated copper grids. For the zeta potential, the hydroxyapatite nanoparticles were first dispersed in Milli-Q water and sonicated for 20 minutes. Then the zeta potential was measured by Malvern NANO-ZS-90.

Treatment: For the experiments, the setup consisted of one control having untreated flies and five setups of Hydroxyapatite treated flies of concentration 5mg.L^{-1}, 10mg.L^{-1}, 20mg.L^{-1}, 40mg.L^{-1} and 80mg.L^{-1}.Hydroxyapatite NPs were sonicated for half an hour in distilled water prior to the experiment to disperse properly. Afterward the desired concentration was added to the food. Later five females and three males were transferred to each vial.

Lifecycle: The flies, transferred to vials containing Hydroxyapatite in the food. The flies will feed and lay eggs on the food. The time taken to develop from egg to third instar larvae, from third instar larvae to pupae and to adult was noted. Also, the number of pupae formed and the fraction of pupae hatched into adult flies was noted every day (Panacek et al. 2011).

Larva crawling behavior: For larvae crawling behavior, six third instar larvae were taken from each concentration including the control. Larvae were allowed to crawl on a circular surface made from 2% agarose(Nichols et al. 2012). The background was kept black so that it would be easy to distinguish the white larvae. The crawling was recorded by a camera and kept for analysis. The

tracking was done by a software named 'Ctrax: The Caltech Multiple Walking Fly Tracker' and further analysis and plotting were done by MATLAB (R2015a). Finally, the speed of larvae was calculated in $mm.sec^{-1.}$

Nitro blue tetrazolium (NBT) assay: Larvae from different concentrations including control were subjected for nitro blue tetrazolium (NBT) test. Briefly, 15 third instar larvae were taken from each vial. Larvae were ruptured with a sterilized needle and the haemolymph was extracted. The extraction was done in cold chamber in order to prevent melanisation of the haemolymph. 5µL of haemolymph was taken and 10µL of 1X PBS was added to it. This step was followed by the addition of 15µL of 1.6 mM NBT solution. The sample was kept for 1-hour incubation in dark. With the onset of incubation, equal volume 100% glacial acetic acid was added to stop the NBT reaction, which was followed by centrifugation. Next 150 µL of 50% glacial acetic acid was added and the absorbance was taken at 595nm(Jambunathan 2010).

Trypan blue staining: Trypan blue staining was done following the given protocol (Krebs and Feder 1997; Carmona et al. 2015). Briefly, six third instar larvae from different vials were isolated and washed in 1X PBS solution in order to remove any traces of food from the surface of the larvae. The larvae were dipped in trypan blue solution(0.02%) and kept on a shaker for 30 minutes. The excess trypan blue was removed from the larvae surface by washing with 1X PBS solution. Now the larvae were observed under a stereomicroscope and imaged to check if there was any cell damage.

SEM and EDX: Six guts from each concentration were isolated in 4% PFA and kept at4°C. Guts were swept away with PBS to remove the excess of PFA. Guts were dehydrated by graded serial dehydration process by increasing the concentration of ethyl alcohol. Finally, the dehydrated guts were smeared with a layer of platinum and perceived under scanning electron microscope (SEM) (Nova NANOSEM). The amount of calcium and phosphate were checked from EDX analysis.

Larva gut staining: Third instar larvae gut were dissected and kept in 4% PFA at4°C.For staining, guts were rinsed with PBS for 10 minutes to remove excess PFA. Afterward, PBS was removed and the same amount of PBST was added and kept for 10 minutes. The addition of PBST was repeated thrice.Subsequently2,7-Dichlorofluorescein (a marker for ROS) was added and guts were incubated in dark for 30 minutes as the dye is light-sensitive. Lastly, the guts were eroded with PBS and mounted with mounting media containing DAPI.

Adult climbing assay: For the climbing assay, 30 adult flies were isolated from each vial and transferred to a 100 ml measuring cylinder having a mark up to 10 cm. The mouth of the cylinder was closed by a cotton plug. Then the cylinder was tapped two to three times so that all the flies would come down to the bottommost of the cylinder. Now the flies were permitted to ascend for 10 seconds. The number of flies that were able to climb up to the 10 cm mark and which stayed at the bottom was noted(Nichols et al. 2012). This experiment was repeated for six times and the mean value was calculated.

Adult phenotype: For phenotypic analysis, 50 flies from each vial were observed under a stereomicroscope. Flies were checked for abnormality in eyes, bristles, and wings. Amount of abnormalities were represented statistically.

Adult weight: For weight measurement, 50 adult flies containing 25 males and 25 females were collected from each experimental vial. The weight of 50 flies from each concentration was measured by weighing balance. The average weight for each vial was calculated and plotted in a graph.

Adult H_2O_2 assay: For H_2O_2 assay, the adult flies were fed with a paper soaked in a mixture of 6% sucrose and 9% Hydrogen peroxide. Then they were observed in an interval of two hours till flies were left alive.

Statistical analysis: All experiments were reiterated three times and the statistical values (unpaired p-test) were analysed using Graph pad prism 6.0 software. All the values were compared statistically against the control.

Results:

Material characterization

To check the functional groups in the synthesized material, the samples were evaluated by FTIR spectrum (Fig. 1A). The spectra retain sharp and strong band at 3451cm^{-1}, conforming to stretching mode of OH group, while the peak at 1653 cm^{-1} corresponds to bending vibration of OH group. Strong bands at 1051 cm^{-1}, 880 cm^{-1}, and 579 cm^{-1} were due to PO_4^{3-} group. The bands obtained for PO_4^{3-} and OH groups in HAp, were in good agreement with other published data(Chaudhry et al. 2006; Yao et al. 2003; Puvvada et al. 2010). A sharp band at 1412 cm^{-1} was due to CO_3^{2-}, which might be incorporated to HAp from the atmosphere during the sample preparation. Since carbonates are the constituents of bone tissues, the presence of CO_3^{2-} may contribute to a vibrant role towards the bioactivity of the synthesized NP. The synthesized NPs were further analyzed with X-ray diffraction (XRD) patterns as shown in Fig. 1B. All the diffraction peaks of the HApNPs can easily be cataloged to the pure hexagonal phase of HAp, which counterparts with the JCPDS card no. 09-0432. XRD pattern reveals the crystalline nature of the material.

To learn about the surface charge and steadiness of the nanoparticles, zeta potential was measured. The dispersant used was Milli-Q water and the temperature was 25°C. The Zeta potential value was found to be -21.6 mV (Fig. 1G). This value signifies that; the nanoparticles are moderately stable in the dispersant medium. So agglomeration might only happen when a higher concentration of the nanoparticles is present in the dispersion medium.

Larva crawling behavior: The larvae crawling assay was accomplished to observe the amount of confusion in the crawling behavior of the third instar larvae. This corroborates the extent of neuronal damage occur in an early developmental stage(Dominick and Truman 1986). The

crawling speed of the control larvae was 1.342 ± 0.03 mm.sec^{-1} (=8.052 cm.min^{-1}). With the increase of hydroxyapatite concentration, the average crawling speed decreased. In 5 mg.L^{-1}, the speed was 1.123 ± 0.05mm.sec^{-1}(=6.738 cm.min^{-1}), which ultimately decreased to 0.9450 ± 0.03mm.sec^{-1}(=5.67 cm.min^{-1}) in the case of 80 mg.L^{-1}(Fig. 2A). The larvae tracking paths are demonstrated in Fig.2B.

Trypan blue staining: Trypan blue stains only the dead cells. So, it is possible to distinguish between dead and alive cells. Positive trypan blue staining was seen in 5mg.L^{-1}, 10 mg.L^{-1}, 20mg.L^{-1} and 40mg.L^{-1}. In 5mg.L^{-1} and 20mg.L^{-1}prominent staining was seen whereas mild stain was observed in 10mg.L^{-1} and 40mg.L^{-1}. The damaged cells which took up the blue stain are marked by the dashed circle (Fig.2C).

NBT assay: NBT assay was done to govern the extent of ROS produced in the hemolymph of 3rd instar larvae. In control, the absorbance value at 595 nm was found to be 0.21. In 5mg.L^{-1} the value increased to 0.286 ± 0.0037. In 10mg.L^{-1} the absorbance was 0.249 ± 0.019 and for 20mg.L^{-1} the absorbance was 0.265 ± 0.018. In 40mg.L^{-1} concentration the absorbance was 0.291 ± 0.014 and for 80mg.L^{-1} the absorbance was 0.224 ± 0.0023. For NBT assay the absorbance is directly proportionate to the amount of ROS produced and ultimately corresponds to the extent oxidative damage to the cells. The amount of increase occur with respect to control are plotted in graphs (Fig.2D).

SEM and EDX: Scanning electron microscopy was done in order to determine the amount of calcium and phosphorus within the gut of 3rd instar larvae. In control, the calcium percentage in the gut was 2% and that of phosphorus was 35%. In 5mg.L^{-1}treated vial the calcium amount was negligible and phosphorus was 7%. In case of 10 mg.L^{-1} and 20 mg.L^{-1} also, there was a negligible amount of calcium whereas phosphorous amount was 14.2% and 5.82% respectively. In 40 mg.L^{-1}treated vial 1.29% calcium and 7.7% phosphorus was found and in 80 mg.L^{-1} treated vial the calcium and phosphorus level found to be 6.89% and 8.0% respectively (Fig.3).

Larva gut staining: The gut of 3rd instar larvae was stained with DAPI to check the nuclei of the gut. Except for control, the nuclear damage was seen in all the treated vials. There were instances of clustered micronuclei dispersed all over the gut. Most amounts of these broken nuclei were seen in case of 20mg.L^{-1}, 40mg.L^{-1}, 80mg.L^{-1}. The nuclei of different concentrations including control are represented in Fig.4. (Fig.4A-F).The amount of ROS was further measured from gut using 2,7-Dichlorofluorescein dye. The intensity of the dye indicates the extent of stress level (Fig.4 G-L).

Lifecycle: The lifecycle of eggs hatched in the experimental vials was monitored to analyse if there was any developmental defect. For the same, the time taken from egg to 3rd instar larval stage was recorded. In control the time is taken to form 3rd instar larvae were about 72hours, whereas in 5mg.L^{-1}, 10mg.L^{-1} and 20mg.L^{-1}, the time taken was around 84hours. In 40mg.L^{-1} the time is taken for the 3rd instar larvae was around 90hours and for 80mg.L^{-1} the duration was about 96hours (Fig.5A).

Percentage of pupae formed: A number of pupae was counted to check for any developmental defect due to which fewer pupae could form. The obtained data was normalized so that the total number of pupae in control would be 100 and all the other data are obtained with respect to the control. In 5mg.L^{-1} the number of pupae hatched was 86.40 ± 1.84 and in 10mg.L^{-1}, it was 95.01 ± 0.8926. For 20mg.L^{-1}, the obtained number was 83.76 ± 2.48. For 40mg.L^{-1} and 80mg.L^{-1} concentration, the pupae count was 82.50 ± 2.01 and 73.75 ± 3.33 respectively. The numbers of pupae are plotted as graph (Fig.5B)

Percentage of adult flies: The number of adult flies hatched from pupae was counted form all the vials. All flies hatched from the control was normalized to 100. Number of flies hatched from other vials was compared with respect to the control. In 5mg.L^{-1} treated vial, the number of adult flies was 87.35 ± 4.11. In 10mg.L^{-1} the number was 93.15 ± 0.8072, also for 20 mg.L^{-1}, the number of adult flies was 86.42 ± 2.647. In higher concentration like 40mg.L^{-1} and 80mg.L^{-1} the value was 85.85 ± 1.625 and 66.48 ± 4.652 respectively (Fig. 5C).

Adult weight: The weight of the adult flies was calculated from different vials to check the body growth and size. The weight of 50 (1:1 ratio male and female) control flies was found to be 19.5 mg whereas in 5mg.L^{-1} it was 16.85 ± 0.492 mg. Likewise, the weight of 50 flies was 17.89 ± 0.020 mg. For 10mg.L^{-1}, it was 17.47 ± 0.1745 mg and for 20mg.L^{-1}, the value was 16.85 ± 0.043 mg. In 40mg.$L^{-1}$the value was18.49 ± 0.236 mg for 80 mg.L^{-1} where compared to control, the 80 mg.L^{-1} data was non-significant. The body weight is plotted in the form of a graph (Fig.5D)

Adult climbing assay: Climbing assay denominates the behavioral changes that occur in the flies with respect to gravity. This assay is analysed by the number of flies that were able to climb up to the 10 cm mark in the time interval of 10 seconds. The number of flies that could climb up to 10cm in control vial was normalized to 100% in the due course of time. In 5 mg.L^{-1}, 84.31 ± 3.15 % of flies were able to climb whereas 90.06 ± 0.46 % in 10 mg.L^{-1}, 89.23 ± 0.62 % in 20 mg.L^{-1},86.33 ± 2.46 % in 40mg.L^{-1} and 80.74 ± 0.61 % in 80mg.L^{-1} were able to climb up to the 10cm mark. The result of the climbing assay is plotted in the form of a graph (Fig.5E).

Adult Phenotype:

In the HApNP treated flies, abnormalities were found in bristles, eyes and wings.

Wings: The wing characteristics like venation and size of the wings were observed in the adult files. In 5mg.L^{-1} treated vial, the posterior cross vain (PCV) was not continuous thus indicating incomplete venation. In 10mg.L^{-1}, 40 mg.L^{-1} and 80 mg.L^{-1} dark patches were observed comprising of comparatively thick hairs on the wing surface. The size and number of the patches increase with increase in treated concentration of HAp. All the phenotypic variations are represented in figures (Fig. 6A). Statical analysis of the abnormal wings were represented in S1.

Bristles: The bristles of all the adult flies were observed to find any abnormality which could affect their sensation. In 5 mg.L^{-1} there were a loss of left aSC, pSC and left aDC bristles. 10 mg.L^{-1} showed loss of right aDC, pDC and both the left- right pSC bristles. In 20 mg.L^{-1} there was

no right pSC bristle.40 mg.L^{-1}showed loss of left aDC bristle. In 80 mg.L^{-1} there was broken pSC bristle. All the bristle phenotypes from different concentrations are depicted in figure (Fig. 6B). Statical analysis of the abnormal bristles were represented in S1.

Eyes: The external structure of the eye of adult flies was observed to check for any abnormality. In 5ppm dark patches were found and small eye phenotype was seen in all the treated flies. In 10 mg.L^{-1} rough-eye phenotype along with blisters was observed. In 20 mg.L^{-1}, 40 mg.L^{-1} and 80mg.L^{-1}irregular margin in the eye was found indicating missing ommatidia from the boundary. The eye phenotypes are illustrated in Fig. 7. Statical analysis of the abnormal eyes were represented in S1.

Adult H_2O_2 assay: The H_2O_2 assay was done to determine the ability of the adult flies to endure stress. Ten adult flies from each treated vials were given H_2O_2 as a food and a number of survivors were noted down till 14 hours after the experiment started. Various treated flies were compared with control to measure their stress resistant property. The survivability was plotted in the form of graphs (Fig.8).

Discussion:

The increased use of HaPNPs in biomedical applications has raised great concern about its potential risk to human health. To investigate the toxic effect of nanoparticle, *Drosophila* serves to be an excellent model. The current study investigated several behavioral, developmental and biochemical assays to prove the toxic effect of HApNPs.

The current study reveals delay in development at higher concentration of HApNP treatment. Developmental delay is associated with a disturbance in the oogenesis period, ovarian defect and delayed egg chamber development. Nanoparticles like Fe, Ca, Cu and Ag are previously reported to cause such developmental delay in *Drosophila*(Chen et al. 2015; Gorth et al. 2011). NPs due to its small size can easily pass through the placental barrier(Chu et al. 2010) and tend to cause cellular and oxidative damage to DNA(Bhabra et al. 2009). In the current study, developmental delay is observed from larvae to the adult stage.

Larva crawling experiment is considered as a gauge to monitor the functionality of neurons (Kitamoto 2001; Pulver et al. 2009). Previously silica nanoparticles have been reported to interfere with the neuronal cells, cause toxicity and affect the calcium homeostasis (Ariano et al. 2011). The larvae crawling experiment points that with an increase of HApNP treatment, the crawling speed decreases. The track made by the HApNP treated larvae while crawling, evident that HApNP induces confusing behavior, leading to more sharp turns, non-linear crawling and lesser crawling speed. The crawling speed of wild-type third instar larvae is about 6-8 cm.min^{-1}(Chyb and Gompel 2013). The slower speed and more turns could be due to damage in the sub-oesophageal ganglion and poor coordination between the brain and the mechanosensory neurons(Riedl and Louis 2012). A similar larva crawling defect is reported with the treatment of

titania, silica, gold and carbon nanoparticles (Han et al. 2014; Liu et al. 2009; Raj et al. 2016; Sabat et al. 2016).

Nanoparticles such as silver, gold, and titanium cause oxidative stress, by the generation of more amount of ROS (Ong et al. 2016). NBT assay is a known method to detect the amount of ROS (Sabat et al. 2016; Ahamed et al. 2010). In the current study after HApNP treatment, oxidative damage occurred, which is detected by NBT assay. The amount of oxidative stress generated at lower concentration i.e. 5 $mg.L^{-1}$ of HApNP is comparatively more than that of highest one i.e. 80 $mg.L^{-1}$. The lower amount of ROS at higher concentration could be due to agglomeration of the nanoparticles (Yang et al. 2012). Due to agglomeration, NP loses its small size and larger surface area (Bose and Saha 2003) resulting in less production of ROS. A similar mechanism is reported in the case of Cerium Oxide NP treated to *Drosophila*(Alaraby et al. 2015). Nanoparticles produce oxidative stress in a cell by several means(Xia et al. 2008; Kim et al. 2009; Li et al. 2008). One of the possible mechanisms is the endocytosis mediated mechanism in the acidic pH of the lysosome, which triggers a Lysosome Enhanced Trojan Horse effect result release of toxic ions. These ions cause toxicity to several cell organelles and result in oxidative stress (Sabella et al. 2014; Manke et al. 2013).Since the foregut of *Drosophila* has acidic pH a similar mechanism is anticipated with exposure after HApNPs.

To check whether oxidative damage leads to cellular damage or not, trypan blue staining was performed. Trypan blue staining depicts highest dead cells in the gut treated with the lowest concentration of HApNPs. To check the effect of HApNPs in cellular pathways, larval gut was checked under scanning electron microscope. Surprisingly the amount of phosphorous decreased from the gut after the treatment of low concentration of HApNPs indicating that it affects the phosphorous absorption (Heaney and Nordin 2002). Similarly, the amount of calcium found to be decreased in case of lower concentration treated samples whereas it steadily increases from 40 $mg.L^{-1}$ concentration onwards. Now the question that arises is why low supplementation of HApNPs decreases the level of calcium and phosphate within the gut? The possible explanation could be when the concentration of NP is low, probably the NPs can easily penetrate through the gut epithelium and enter into the hemolymph, resulting in no calcium inside the gut. However, at high concentration, due to agglomeration, the particle size increases, and the HApNP stays inside the gut resulting increase in calcium and phosphorus within the gut which occurs after 40$mg.L^{-1}$ onwards. Does the change in the percentage of calcium and phosphate interfere with the normal activity of the cell? To check this structure of the gut cell was overviewed. The *Drosophila* gut is lined with the single layered epithelial cell. To check if there is any nuclear damage in the gut cells, the gut was stained with DAPI. In treated samples, clustered small nucleus and micronuclei like structure were observed in the gut epithelium. This might be due to apoptosis of the gut cells which is pretty obvious in the late third instar larvae as during pupae formation the whole larval gut will disintegrate and will ultimately be replaced by the adult gut. Since such structures are not observed in the case of the control, we anticipate that HApNP induces apoptosis in larval gut earlier than its actual time resulting developmental delay and abnormal phenotypes. Although no

such report available in the literature from *in vivo* study, but the *in vitro* study reports HApNP as a known inducer of apoptosis in osteoblasts and human hepatoma cells (Shi et al. 2009; Yuan et al. 2010).

At higher concentration of HApNP treatment, developmental delay is observed along with less number of pupae count. The decreased pupae count is a direct consequence of NP egg-pupae developmental delay (Panacek et al. 2011; Ong et al. 2016). Highest decrease in pupae count (66%) is observed in 80 $mg.L^{-1}$concentration vial. At 80 $mg.L^{-1}$ agglomeration of NP is occurring resulting a delay in the pupae-adult development. A similar abnormal delay in pupae-adult transition is reported from nanoparticles like Ca, Cu and Fe (Chen et al. 2015; Gorth et al. 2011). Besides developmental delay, climbing behavior was also found to be affected in the adult flies which are associated with the functionality of antenna. Behavioral assay specifies that with an increase of HApNP treatment, flies become more positive geotropic. This further suggests that HApNP exposure induces positive geotaxis suggesting the possibility of a defective antenna. This result is in agreement with the earlier report where oral ingestion of carbon, silver and gold nanoparticles resulted in anomaly in developmental and behavioral defect (Key et al. 2011; Liu et al. 2009; Raj et al. 2016).

An antenna is the largest mechanosensory organ of the fly. Besides antennae, bristles also contribute for the mechanosensation. In HApNPs treated adults, abnormal bristles were observed in the thorax region(Mackay 1995). In different concentration aSC, pSC, aDC, pDC bristles were missing. The arrangement of bristles on the thorax is regulated by several genes and signaling pathways(Peña-Rangel et al. 2002; Culí et al. 2001). One of the major genes which is involved in the development of bristles is *achaete-scute* complex (Georgiev and Gerasimova 1992). Thus any mutation in this family of genes during the pupae-adult development will result in bristle abnormality. Furthermore, EGFR and Notch signaling pathway also play a role in bristle development(Furman and Bukharina 2007). Missing bristle in HApNPs treated flies indicates an anomaly in the regulation of above two pathways.

Besides bristle, HApNP also induces wing phenotype (incomplete venations) at a lower concentration. An incomplete venation is the result of idiosyncrasy in the wing imaginal disk during the metamorphosis (de Celis 2003).A mutation in the posterior cross vein(PCV) gene induces similar abnormal wing pattern. Besides PCV, signaling pathways like Bone morphogenic protein (BMP) and Notch also affect the spacing and marginal fate of the wing pattern (de Celis 2003).Thus any interference with either of these pathways will result in an abnormal wing phenotype. Besides venation defect, brown patches were found on the wing surface due to the formation of comparatively thicker wing hairs. The wing hairs of *Drosophila* are composed of actin(Ren et al. 2006; Fristrom et al. 1993). During wing development, the thickness, and organization of wing hairs depends on the planar polarization of cells and the amount of actin present in the hair (Eaton et al. 1996). The formation of brown patches in the wing indicates the HApNPs induces defective planar cell polarity in the wing. Besides wings, the eye size also seems to be affected and irregular margins are found in the eye along with damaged ommatidia. Finally,

we checked the survivability of the flies in order to check the response of the adults towards stress(Grover et al. 2009). HApNP treated flies were less resistance to stress and thus died earlier than the control flies when treated with hydrogen peroxide. All the phenotypic defects handicap the treated flies to perform better during stress condition.

Conclusion: These findings collectively point to the fact that HApNPs can distress the neuronal development and behavior of *Drosophila* in a concentration-dependent manner. HApNPs bring these variations dramatically by altering the amount of calcium and phosphorous within the gut, increasing the ROS, and by inducing the apoptosis within the gut. Although HApNP is widely used to deliver various drugs and in dental purposes, only very few study focuses on the toxic property of these NPs. Till now only very few research works discuss the developmental defect caused by HApNPs. Although many NPs are known to affect the developmental cycle of Drosophila, no such report was available in the literature regarding the effect of HApNPs. The developmental and behavioral defects observed is a clear consequence of alteration of signaling pathways during development as well as the alteration of the amount of calcium and phosphorus during the developmental process. The toxic effect caused by HApNPs described herein needs further investigation. How HApNPs interfere with the imaginal disc, a polar organization of cells and bring structural and functional variation, will be the focus of our further investigations.

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Figure legends

Figure 1.Characterisation of nanoparticles (A) FTIR spectra, (B) XRD pattern, (C) FESEM image, (D) TEM image (inset size distribution curve), (E) HRTEM image, (F) SAED pattern of hydroxyapatite nanoparticles, (G) Zeta potential distribution curve of hydroxyapatite nanoparticles.

Figure 2: **Assessment of toxicity in late 3rd instar larvae** (A) Graphical illustration of the distances traveled by the larvae in different concentrations of HaPNPs (Mean ± S.D) with the significance values against control are represented as asterisk (n=30;** P Value = 0.0042, *** P Value = 0.001, **** P value = < 0.0001). (B) Tracking path of larva crawling (C) Trypan blue staining to mark any damage in the gut. Red circle indicates the damaged part of the gut. D) NBT assay from hemolymph to measure the amount of free radicals in the third instar larvae. The graphs are plotted against the respective concentration ofHaP nanoparticles present in the food. The level of significance are marked in comparison to control with asterisks(* P Value=0.0142, ** P Value=0.0056,**** P Value < 0.0001, NS P Value= 0.1409).

Figure 3: **SEM analysis of gut of 3rd instar larvae** EDX analysis of gut of control as well as various concentrations of HaPNPs treated 3rd instar larvae depicting the amount of calcium and phosphorus within the gut.

Figure 4: Fluorescence imaging of gut of 3rd instar larvae (**A-F**) Guts were stained with DAPI and observed under fluorescent microscope. Nucleus damage was seen in all the cases except the control. In figure C, D and E more number of damaged nuclei were observed in comparison with control. The magnified image of the damaged nuclei are shown in the inset **(D'-F')** Guts were **s**tained with 2,7-Dichlorofluorescein dye to detect the amount of ROS generated within the gut.

Figure 5: Assessment of lifecycle, adults and behavior (A) The lifecycle of various concentration of HaPNP treated flies. From 5, 10, 20 mg.L^{-1} vial a delay of 12hrs in third instar larvae formation was found in comparison to control. At higher concentration of 40, 80 mg.L^{-1} a delay of 18 and 24hrs observed respectively **(B)** Percentage of pupae formed from third instar larvae in different concentration of HaPNP treated vial. The level of significance was marked in comparison with the control. (** P Value = 0.0014, *** P Value < 0.0007) (**C)** Percentage of adult flies hatched from pupae in each concentration of HaPNP treated vials are plotted with respected to the control flies. The level of significance was marked in comparison with the control (* P value = 0.0372, *** P value= 0.0010, ** P value <0.007) (**D**) Weight of adult flies:The total weight of 25 male and 25 female is plotted with respect to its corresponding concentration. The levels of significance were marked in comparison with the control (* P value = 0.0328, ** P value= 0.0073, *** P value< 0.0005, ns = 0.05) **(E)** Climbing behavior: The percentage of flies that climb in 5 and 80 mg.L-1 vial was significantly lower in comparison to control. The climbing of flies in 10, 40, 80 mg.L^{-1} treatment was found to be decreasing but were less affected in comparison to control (* P Value< 0.04, ** P value < 0.004).

Figure.6Bristle and wing phenotype (A) Control shows the normal wing venation in *Drosophila.* In 5 mg.$L^{-1}$the posterior crossvein is discontinuous. The abnormality is indicated by a black square. In 10 mg.L^{-1}, 40mg.L^{-1}, and 80 mg.L^{-1} there is a formation of brown patches due to thick wing hairs. The magnified patches are shown in the inset below the actual image. **(B)** Control shows the regular distribution of bristles in the thorax. In 5 mg.L^{-1} there is loss of left aSC, left pSC and left aDC bristles.10 mg.L^{-1} shows loss of right aDC, right pDC and both the left and right pSC bristles. In 20 mg.L^{-1} there is no right pSC bristle.40 mg.L^{-1} shows loss of left aDC bristle. In 80 mg.L^{-1} there is broken pSC bristle. The black arrow points to the missing or affected bristle. **aDC**- anterior dorso-central; **pDC**-posterior dorso-central; **aSC**- anterior scutellar; **pSC**-posterior scutellar; **pPA**- posterior post-alar; **aPA-** anterior post-alar; **pSA**-posterior supra-alar.

Fig.7 Eye Phenotype: The eyes of adult flies of different treated concentration are imaged and the damaged areas are shown by an arrow. The big circle shows the damaged ommatidia and the arrows show the irregular margin of the eye.

Figure.8 Survivorship assay: The control and HaPNP treated flies were exposed to 5% sucrose and 10% H_2O_2. In 5mg.L^{-1} and 10 mg.L^{-1} the HaP treated flies died within 12 hours, whereas in 20 mg.L^{-1}, 40 mg.L^{-1}, and 80 mg.L^{-1} treated vials flies died gradually within 14 and 16 hrs. The control flies could endure the stress up to 20hrs and in negative control, all the flies were alive for more than 24 hrs.

This manuscript is compliance with Ethical Standards:
Conflict of Interest: No conflict exists: The authors declare that they have no conflict of interest.

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