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Research Article

Standardization of Alternative Methods for Nanogenotoxicity Testing in *Drosophila melanogaster* Using Iron Nanoparticles: A Promising Link to Nanodosimetry

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The remarkable advancement of nanotechnology has triggered enormous production of metal nanoparticles and nanomaterials for diverse applications in clinical diagnostics and biomedical research. Nanotechnology has facilitated understanding and analysing nanotoxicology in a holistic approach. Iron nanoparticles have been of special interest in recent research owing to their dynamic, paramagnetic, and catalytic properties. Research studies (*in vitro* model) have demonstrated the lack of toxicity in nanoiron. The present study design involves *in vivo* toxicity assessment of nanoiron at specific concentrations of 0.1 mM, 1 mM, 5 mM, and 10 mM in *Drosophila*. DNA fragmentation assay in exposed and F1 population showed first-line toxicity to flies. Viability and reproductive ability were assessed at 24-hour and 48-hour intervals and thus indicated no statistical significance between the exposed and control groups. The wing spot assay has expressed transparent lack of toxicity in the studied concentrations of nanoiron. Protein profiling has demonstrated that the protein profiles have been intact in the larvae which confirm lack of toxicity of nanoiron. This leads to concluding that nanoiron at the defined concentrations is neither genotoxic nor mutagenic.

1. Background

Nanotechnology and Nanoparticles. Nanotechnology has grown tremendously at an unimaginable pace in less than a decade owing to its distinct advantages that it offers in terms of properties of its size, surface area, and quantum mechanics. This has triggered enormous production of nanomaterials for diverse applications in clinical diagnostics and biomedical research apart from engineering industries and information technology. This in turn has led the research in identifying newer methods to synthesize nanomaterials/nanoparticles with altered properties to meet the demands of the current market [1–3]. A major portion of nanoparticle research has been focused on delivering nanoparticles into the body for clinical diagnosis and cancer immunotherapy. The interaction, absorption, retention, and excretion properties of these nanoparticles in tissues and organs pose a health concern. It is therefore extremely important to address the toxicological issues that could arise from being exposed to nanoparticles in humans, animals including aquatic ecosystems, and environment [4–6]. Nanotechnology has necessitated analysing, understanding, and exploring nanotoxicology in a holistic approach. The need for standard operating procedures in evaluating the toxicity and genotoxicity of nanomaterials/nanoparticles is scientifically significant and indispensable to meet the health safety issues apart from risk assessments in the forthcoming years. This in turn shall build a stable, secure, and sustainable nanotechnology industry. Several research articles have highlighted the requirements of various tests and methods used to study nanotoxicity [7–9].

Iron Nanoparticles. Iron nanoparticles have been used with great interest in recent research owing to their dynamic, paramagnetic, and catalytic properties. Many research studies

conducted "hitherto" have used iron nanoparticles in cancer research predominantly apart from engineering industries (sensors, storage devices, etc.) and environmental remediation including effluents and waste water treatment. The magnetic property of nanoiron has enhanced its application in biomedical research towards drug delivery, tumour inhibition, and gene transfer methods into cells *in vitro* and *in vivo* apart from labelling of cells and molecules. Another promising area of nanoiron research includes its application in enhancement of contrast in MRI and hyperthermia based cancer therapy [10–12]. Use of nanoiron in hyperthermia has been proved advantageous for the fact that it is capable of localizing itself selectively to the desired tumour areas and these iron nanoparticles radiate heat to the cancer cells by the virtue of alternating magnetic field, eventually destroying the cancer cells without bystander effect to normal surrounding cells. The high magnetic saturation value of iron ensures that the patient is exposed to low doses with maximum desirable hyperthermia effect. The biocompatibility and dispersion properties of nanoiron have offered themselves favourable parameters for its application. This technology presents itself with the excessive use and exposure of nanoiron to human, animal, and environmental biota. It is therefore essential to evaluate the toxicity (*in vitro* and *in vivo*) with certain focus on genotoxicity [9, 13, 14].

Drosophila as a Model for Toxicity Assessment. Research studies conducted over the past decade have primarily focused on the toxicity assessment of nanoparticles on *in vitro* models (cell lines) extensively. However, few research studies have documented the toxicity assessment of nanoparticles especially nanoiron in mammals and zebra fish. *In vitro* endpoints include the assessment on cell lines (viability, hemolytic assay) apart from micronucleus assay, chromosomal aberration assay, and Ames test to evaluate genotoxicity and mutagenicity, respectively. Cell culture based research studies have been extensively used to replace the animal based experiments hugely owing to the multiple advantages that cell lines offer in terms of time and cost needed for maintenance. Cell culture systems are dynamic in their growth where the 2D and 3D systems help to study and understand cellular and molecular mechanisms with ease when compared to the complex animal models. These *in vitro* tools help researchers analyse and explore human cells to evaluate nanotoxicity in situations where an *in vivo* human model assessment is not possible [15, 16]. However, the results obtained in *in vitro* studies are incomplete and not holistic as the data obtained from cell culture studies depend on the various cell growth and differentiation parameters that vary significantly from "within the flask" as compared to "within the body." Also, the real time view and understanding of how a nanoparticle interacts with complex biological animal body cannot be evaluated apart from understanding tissue and organ toxicity. This warrants the role of entire living biological species research to help us understand the complex interactions between nanoparticle and animal model in terms of developmental changes, tissue-organ properties apart from behavioural changes [17–19].

Understanding the intricacy involved in multiple signalling systems which build a living organism is quite complex and the animal model research contributes significantly in this perspective. *Drosophila melanogaster* is one of the best known "tool box" used widely in evolutionary biology and developmental research apart from genetic studies. It is commonly referred to as the "Cinderella" or "work horse" in genetic research. The fruit fly is well characterized genetically, has a short life cycle, is inexpensive to handle in the laboratory, and requires simple equipment for breeding. An array of standard and specific molecular tools and techniques are available to explore the scientific supremacy it holds in its genes apart from possessing strong homologous conserved genetic sequences and pathways with humans. In spite of being an invertebrate model, it has contributed immensely to understanding the molecular genetics of Parkinson's disease, Alzheimer's disease, and cancer. However, its role is not limited to research in the human health and the fly has been used to understand evolutionary biology apart from animal growth, differentiation, development, and death. Most often, many biologists and toxicologists specifically prefer the use of rodent or mammalian model systems for toxicology studies. In recent years, the zebrafish research has gained significant momentum in toxicology. The fly however has not been used extensively to understand toxicology despite the several advantages it offers scientifically. The present study has focused on optimizing the protocols to develop the potential of *Drosophila* as an *in vivo* model system for nanotoxicity assessment. The low cost, ease of maintenance, and susceptibility to genetic manipulation apart from standard markers available for phenotypic analysis make it a high throughput animal model for higher order and statistically significant screening and analysis. On comparison, the vertebrate models involve ethical and technical issues for genetic manipulation studies and a good proportion of time and resources is required to be invested to address the scientific data the fly model can offer with the above-mentioned examples and advantages. Many research studies conducted in the past have demonstrated the similarity in response to nanoparticle exposure between the fly and the mammalian model systems. This has articulated the attraction to fly model research for nanotoxicity assessment [20–22].

In Vivo Toxicity Assessment. The present study evaluated the genotoxicity of the synthesized nanoparticle by *in vivo* studies using *Drosophila melanogaster* as the animal model. The end points used for the *in vivo* studies are as follows:

(1) Assessing the viability.

(2) Evaluation of phenotypic changes: exposed and F1 population.

(3) DNA fragmentation assay: exposed and F1 population.

(4) Protein profiling of exposed larva and adult flies.

(5) Assessing the reproductive ability.

(6) Wing spot assay.

(1) Assessment of Viability. The fruit fly has been a scientifically significant biological system in genetic research including classical and transmission genetics apart from its advantage in studying development and behaviour. The experimental advantages of the fly model have been discussed above. Longevity, viability, and aging have been investigated extensively on the fly model. The biology of aging has been characterized by description of genes involved in the process of aging apart from understanding of genetic portrait of the fly. Assessing the viability and longevity of the fly is quantitative and it is heavily influenced by the genetic and environmental machineries. The role of natural and artificial selection strategies cannot be ignored. Climatic conditions, reproductive success, and the diet influence the viability of flies to a great extent. The most common approach used to determine viability is by altering the diet through the introduction of the test compound into food under standard culture conditions. The ability of the fly to respond and tolerate the stress is reflected in its viability phenotype and is a direct measure of toxicity of the test chemical/nanoparticle. The data obtained are qualitative in the perspective that the genes that influence or direct loss of viability are not evaluated. Many molecular genetic studies in *Drosophila* have led to advances in investigating the lifecycle and development profile of the genes influencing them [15, 16].

(2) Assessment of Phenotypic Changes. The DNA at the molecular level is susceptible to attack by various physical, chemical, and biological agents. Thus, the assault to the DNA is defined as mutation. Mutation is caused due to a sudden heritable change in the genome of the organisms. Mutations are both beneficial and deleterious. They help generate variation and mediate evolution apart from causing terrible genetic diseases. These mutations are mostly deleterious and serve as an important cause to understand, study, and explore gene structure, location, and function. The central dogma of molecular biology drives the genotype and phenotype of an organism through transcription and translation, which mediates gene expression in an adult. The genotype is the total genetic content of the organism which controls and reflects its phenotype (collection of observable characters).

The girth and extent of genomic complexity, environmental influence, and phenotypic heterogeneity make genomic prediction difficult. Understanding the genotype-phenotype interaction and association is hard as it involves a constellation of molecular factors that controls and coordinates gene expression. The role of several genetic variants implicated in composite phenotypic expression cannot be ignored. Multiple genes and epitasis are the giants in any genetic laboratory. Though scientists recognize that their existence cannot be ignored, they include intrinsic difficulties in analysing and establishing balanced and equitable associations between multiple genes. The role of modifier genes still remains overlooked. A huge chunk of genome is transcribed; however, it is not translated. These genes may not code for specific products but have a critical role in gene regulation and expression. A mutation to this noncoding DNA may affect the structure and function of gene products that are translated. The genome of any organism is complex and dynamic and possesses inherent variability in gene expression. The mutagenic assault to the DNA triggers multiple molecular mechanisms including DNA repair at somatic and germ line levels before a phenotypic expression is generated. Analysis of phenotypic changes provides a qualitative data on the possible genotoxic and mutagenic effect caused by chemical/nanoparticle exposure [15–17].

(3) DNA Fragmentation Assay. It is one of the most regularly and extensively used molecular tools for qualitative assessment of DNA damage. Formation of DNA fragments (170–200 bp) is apparent of apoptosis in eukaryotic cells. The damage to nuclear morphology can be identified through post-DNA isolation and agarose gel electrophoresis which helps observe the ladder pattern due to fragmentation of DNA. However, the method is solely qualitative and can support the possible incidence of apoptosis. This however requires adjunct quantitative tools to understand the mechanism involved apart from evaluating the scale of damage. DNA fragmentation assay is widely used in *in vitro* cell culture based experiments [15, 18, 19].

(4) Protein Profiling. The central dogma of molecular biology involves transcription and translation which fundamentally includes protein expression to understand the gene structure and function. The phenotype of an organism is an outcome of protein expression. Proteomics is an upcoming field which studies the total protein content of an organism. Whole organisms *in vitro* cell lines exposed to chemicals/nanoparticles/biological stress exhibit structural and functional changes of cellular proteins. Cell metabolism is largely controlled by proteins as enzymes that regulate metabolic pathways are proteinaceous in nature. Proteomics also involves the understanding of structure and function of proteins from cellular levels; here posttranslational modifications are involved apart from gene expression studies. Molecular tools target DNA analysis to record mutagenic assaults. However, whole protein isolation from cells and their profiling by SDS PAGE provide useful information about loss or gain of specific proteins. Therefore, real time understanding of toxicity chiefly involves protein expression. SDS PAGE is a simple molecular tool used to separate proteins based on their molecular weight. Differential protein profiling is used to analyse protein levels in samples to understand the physiological and biochemical changes that have been induced or observed in an organism after being exposed to drug/chemical or nanoparticle. These expression patterns provide data to categorize and conclude on chemical challenges, phenotypic changes, or environmental responses [20, 21].

(5) Assessment of Reproductive Ability. Fly research has identified the homology in genes governing development between flies and higher animal species including humans. The conservation of these genes across such species in evolution has been proved advantageous in helping researchers understand, analyse, and explore the possible mechanisms involved in the developmental stages. Adult flies have high fecundity rates and a single female is capable of producing more than

3500 progeny in her short life span. In a genetic research laboratory, males and females are mated in the ratio of 1 : 3, as a single male is capable of generating over 10,000 progeny by mating with many females. Fertilization occurs during egg laying and the embryonic development gets completed in less than 24 hours. Embryogenesis is followed by larval emergence and moulting to pupal stages is some evidence of metamorphosis. The final phase of metamorphosis is marked by the emergence of imago or an adult. The developmental stages in the lifecycle of a fly largely depend on the diet and temperature under which they are maintained. The ideal temperature is 22–24°C. Prolonged or continuous exposure to higher or lower temperature leads to altered fertility rates, impairment of viability, and distorted lifecycle durations. Fatality is recorded in unfavourable conditions [22–24].

(6) The Wing Spot Assay. It is also referred to as SMART (somatic mutation and recombination test) serves as a standard to quantify assault of DNA (due to exposure to chemical/physical agent) caused due to any of the following molecular interactions like chromosome rearrangement, chromosome breakage, gene mutations, or chromosome loss. LOH (loss of heterozygosity) is a measure of genotoxicity and mutagenicity which is analysed in wings of *Drosophila* (transheterozygous larvae of *mwh/flr3* crossover). SMART uses *mwh* and *flr3* (recessive markers) to detect mutations, recombination (somatic), and deletions. This test is sensitive and quantitative. The imaginal cell clusters of *D. melanogaster* are divided by mitosis during the larval phase of metamorphosis till the differentiation of adult body structure is complete. Any genetic assault or alteration to one of the imaginal disc cells will spread consecutively to all the remaining cells that follow the mutated cell by mitosis. This results in the clone formation of mutant cell. These induced mutations express themselves as spots on the wings of the adults that survive from the transheterozygous larvae. These spots are observed as multiple wing hair or flare phenotype. *mwh* and *flr3* twin spots and *mwh* single spots are expressed as a consequence of induced recombination. SMART involves preparation of adult wing blade pairs on slides and systematic and extensive documentation of size, pattern, and frequency of the different spots to quantify mutagenic and recombinogenic effects. The popularity of SMART is attributed to its inexpensive nature involved regardless of the extensive statistical data and relevance it offers for quantitative analysis. Observing the spots under the microscope is tedious; however, specific software for data analysis is also available [25].

2. Materials and Methods

2.1. Culture and Breeding of Drosophila. Canton S (wild type strain) was procured from *Drosophila* Stock Center, University of Mysore. The flies were bred (in the ratio of 3 females : 1 male) in corn meal agar medium and maintained at 22°C in an incubator. The composition of corn meal agar includes corn flour, sugar, dextrose, agar agar, and yeast extract as some of the key ingredients. Propionic acid, orthophosphoric acid, and benzoic acid combinations were also used as antifungal agents.

2.2. Viability Assay (Exposed and F1). Two-day-old adult flies were exposed (24 hours and 48 hours) to different concentrations (0.1 mM, 1 mM, 5 mM, and 10 mM) of iron NPs along with a positive (25 μL ethyl methane sulphonate) and a negative control (sterile water). Instant food (Carolina Biologicals, USA) in sterile water was used for exposure where the defined concentrations of NPs were mixed and the flies were allowed to feed on them. 100 flies were added in each vial. After 24 hours and 48 hours of exposure, the number of viable and nonviable flies was observed and the data was tabulated. The recorded data was then expressed in graphs for viability.

The experimental set-up was run in duplicate. One set of experiment was used for evaluating viability in the exposed population while the other reaction allowed the flies to breed in the food with the defined concentrations of nanoparticles and the flies obtained from the first filial generation were evaluated in number (on comparison with the controls) for viability similar to that of the exposed flies.

2.3. Phenotypic Changes (Exposed and F1). Two-day-old adult flies were exposed (24 hours and 48 hours) in different concentrations (0.1 mM, 1 mM, 5 mM, and 10 mM) of iron NPs along with a positive (25 μL ethyl methane sulphonate) and a negative control (sterile water). Instant food (Carolina Biologicals, USA) in sterile water was used for exposure where the defined concentrations of NPs were mixed and the flies were allowed to feed on them. After 24 hours and 48 hours of exposure, the exposed and F1 population flies were evaluated for phenotypic changes under the stereo zoom microscope. The analysis revealed varied phenotypic changes like orange discoloration of thorax, shrunken wings, bigger and pale abdomen of female, curling of abdomen in males, and so forth. The percentage of the flies exhibiting each of the morphology has been represented in table for the exposed and F1 analysis at both 24 and 48 hours, respectively.

2.4. DNA Isolation (Exposed and F1). Two-day-old adult flies were exposed (24 hours and 48 hours) to different concentrations (0.1 mM, 1 mM, 5 mM, and 10 mM) of iron NPs along with a positive (25 μL ethyl methane sulphonate) and a negative control (sterile water). Instant food (Carolina Biologicals, USA) in sterile water was used for exposure where the defined concentrations of NPs were mixed and flies were allowed to feed on them. After 24 hours and 48 hours of exposure, the DNA was isolated using the phenol : chloroform : isoamyl alcohol (25 : 24 : 1), PCI method.

The experimental set-up was run in duplicate. One set of experiment was used for DNA isolation while the other reaction allowed flies to breed in the food with the defined concentrations of nanoparticles and the flies obtained from the first filial generation were subjected to DNA isolation and DNA fragmentation assay as performed for the exposed flies.

2.5. Quantification of DNA Using Nanodrop and Fragmentation Assay (Exposed and F1). The DNA thus obtained from the exposed and F1 along with their respective positive and negative controls was quantified using nanodrop using

Table 1: Percentage of viability (exposed and F1), 24 and 48 hours.

	Number of flies exposed	Exposed		F1	
		24 Hrs	48 Hrs	24 Hrs	48 Hrs
0.1 mM	100	98	95	97	96
1 mM	100	98	95	97	95
5 mM	100	97	94	96	95
10 mM	100	96	94	96	94
Negative control	100	98	96	97	96
Positive control (EMS)	100	67	32	60	30

nucleic acid measurement. The isolated DNA was run on a 2% agarose gel to check for any damage caused to the DNA, using DNA fragmentation assay.

2.6. Protein Profiling Using SDS-PAGE (Exposed and F1). Second instar larvae were isolated from culture in sterile phosphate buffer saline. Instant food with defined concentrations of iron NPs (0.1 mM, 1 mM, 5 mM, and 10 mM) along with a positive (25 μL ethyl methane sulphonate) and a negative control (sterile water) was prepared and 50 larvae were added in each vial. Protein was extracted from the treated and controls using RIPA buffer after exposure. The extracted protein was estimated by Bradford method at 595 nm. Protein profiles were studied using the Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) method. 8% : 5% (separating : stacking gel) was used for profiling.

The experimental set-up was run in duplicate. One set of experiment was used for protein isolation while the other reaction allowed flies to breed in the food with the defined concentrations of nanoparticles and the larvae obtained from the first filial generation were subjected to protein estimation and protein profiling as performed for the exposed flies.

2.7. Reproductive Ability Assessment. Two-day-old adult flies were exposed (24 hours and 48 hours) to different concentrations (0.1 mM, 1 mM, 5 mM, and 10 mM) of iron NPs along with a positive (25 μL ethyl methane sulphonate) and a negative control (sterile water). Instant food (Carolina Biologicals, USA) in sterile water was used for exposure where the defined concentrations of NPs were mixed and the flies were allowed to feed on them. The exposed flies were split into two and were transferred on days 3, 5, and 7 to food with NP and food without NP. For analysis of reproductive ability, all the life cycle stages of *Drosophila* were examined daily and the total number of flies emerged in each transfer (counted and tabulated with and without nanoparticle).

2.8. Wing Spot Assay. Second instar transheterozygous larvae (*mwh* + *flr3*) were isolated from culture in sterile phosphate buffer saline. Instant food with defined concentrations of iron NPs (0.1 mM, 1 mM, 5 mM, and 10 mM) along with a positive (25 μL ethyl methane sulphonate) and a negative control (sterile water) was prepared and 100 larvae were added in each vial. The larvae were allowed to feed on the NPs and moult into adults. Wings were dissected from the adults and fixed on glass slides using Faure's solution. 100 wing pairs per each concentration were scored for the presence of spots as per the scoring criteria and the results were tabulated for statistical analysis.

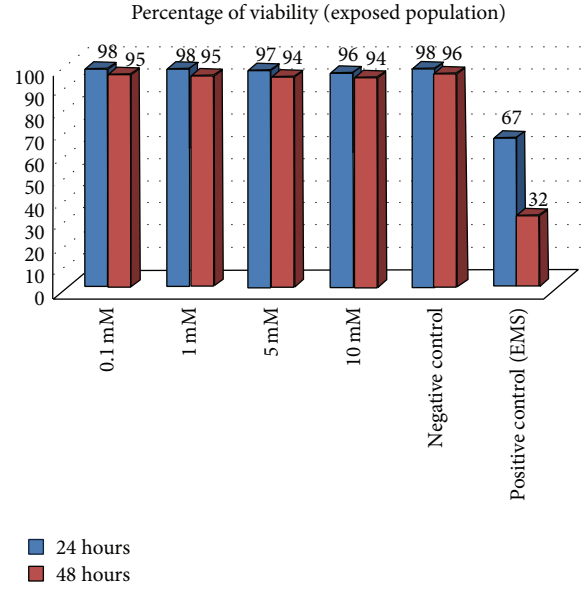

Figure 1: Percentage of viability of exposed population postexposure to nanoiron.

3. Results

3.1. Viability Assay (Exposed and F1). The percent viability of exposed population was calculated for the exposed population and F1 population after 24 hours and 48 hours of exposure (Table 1 and Figures 1 and 2).

3.2. Phenotypic Changes (Exposed and F1). The phenotypic changes of exposed population after 48 hours of exposure were observed and documented under a stereo zoom microscope. The details of the phenotypic changes are tabulated in Table 2.

100 flies in the F1 population in each of the exposed concentrations of the iron NPs along with the controls were evaluated under stereo zoom microscope. No significant phenotypic changes were observed. All the flies (both male and female) showed normal morphology.

Table 2: Phenotypic changes observed in exposed and F1 population.

Concentration of nanoiron	Number of flies exposed	Exposed		F1	
Time of exposure		24 Hrs	48 Hrs	24 Hrs	48 Hrs
0.1 mM	100	Normal morphology in males and females, 100%	Normal morphology in males and females, 100%	Normal morphology in males and females, 100%	Normal morphology in males and females, 100%
1 mM	100	Orange discolouration of thorax in males and females, 50%	Orange discolouration of thorax in males and females curling of abdomen in males, 40%	Normal morphology in males and females, 100%	Normal morphology in males and females, 100%
5 mM	100	Orange discolouration in males and females of thorax, shrunken wings, bigger and pale abdomen of female, 55%	Orange discolouration of thorax in males and females curling of abdomen in males, 40%	Normal morphology in males and females, 100%	Normal morphology in males and females, 100%
10 mM	100	Orange discolouration of thorax in males and females curling of abdomen in males, 62%	Orange discolouration of thorax in males and females curling of abdomen in males, 40%	Normal morphology in males and females, 100%	Normal morphology in males and females, 100%
Negative control	100	Normal morphology in males and females, 100%	Normal morphology in males and females, 100%	Normal morphology in males and females, 100%	Normal morphology in males and females, 100%
Positive control (EMS)	100	Orange discolouration of thorax, shrunken wings, bigger and pale abdomen of female, curling of abdomen in males, 97%	Orange discolouration of thorax, shrunken wings, bigger and pale abdomen of female, curling of abdomen in males, 95%	Orange discolouration of thorax, shrunken wings, bigger and pale abdomen of female, curling of abdomen in males, 95%	Orange discolouration of thorax, shrunken wings, bigger and pale abdomen of female, curling of abdomen in males, 93%

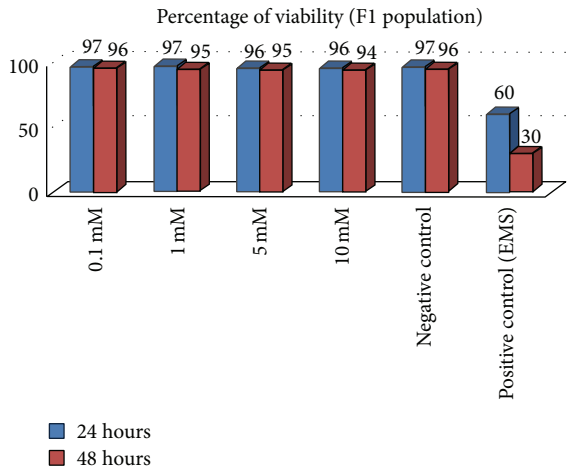

Figure 2: Percentage of viability of F1 population postexposure to nanoiron.

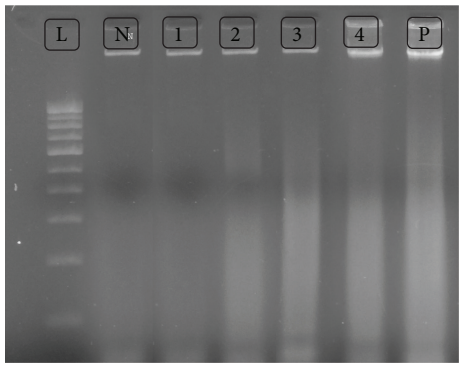

Figure 3: DNA fragmentation assay (exposed population). L: Molecular Ladder; N: Negative Control, (1) 0.1 mM, (2) 1 mM, (3) 5 mM, and (4) 10 mM; P: Positive Control.

3.3. DNA Isolation (Exposed and F1). DNA was successfully isolated from exposed and F1 population along with their respective controls after 24 hours and 48 hours of exposure using PCI method.

3.4. Quantification of DNA Using Nanodrop and Fragmentation Assay (Exposed and F1). Thus, the isolated DNA from exposed and F1 population along with their respective controls after 24 hours and 48 hours exposure was quantified using nanodrop. The DNA thus isolated and quantified (from exposed population) was analysed on 2% agarose which revealed DNA damage in higher concentrations. The intensity of shearing (DNA damage) was mild and dose dependent indicating minimum genotoxicity. Also, the DNA isolated from F1 population was quantified and analysed on 2% agarose. In the F1 population, only the higher concentrations of 5 mM and 10 mM showed mild shearing (Figures 3 and 4).

3.5. Protein Profiling Using SDS-PAGE (Exposed). Protein was successfully isolated from exposed larval population along with their respective controls after 48 hours of exposure using RIPA buffer and was estimated by Bradford method.

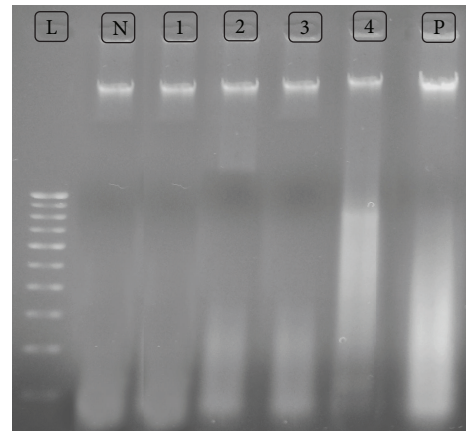

Figure 4: DNA fragmentation assay (F1 population). L: Molecular Ladder; N: Negative Control, (1) 0.1 mM, (2) 1 mM, (3) 5 mM, and (4) 10 mM; P: Positive Control.

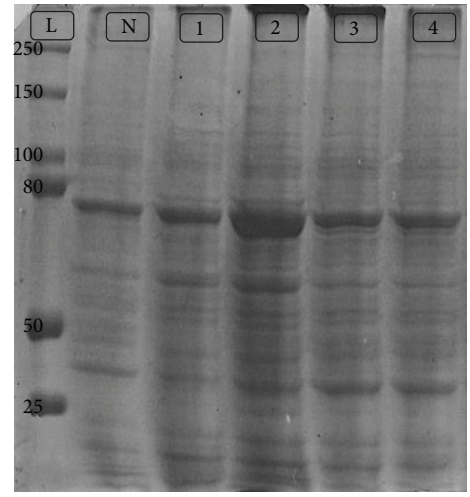

Figure 5: Protein profile (exposed larva). L: Molecular Ladder; N: Negative Control, (1) 0.1 mM, (2) 1 mM, (3) 5 mM, and (4) 10 mM.

Protein profiles of the exposed test and control samples of the whole tissue obtained from the larvae performed using SDS-PAGE revealed an expression of an array of proteins between 22 kDa and 250 kDa. The profile pattern revealed the significant expression of a protein at 70–80 kDa in all concentrations of the treated and control. The protein profile remained the same between the varying concentrations employed and the controls as well. Also, the consistent protein profile is indicative of the lacking toxicity towards protein expression (Figure 5).

3.6. Reproductive Ability Assessment. The emergence time taken by each population (control and exposed) was taken as a measurement to assess the reproductive ability. The total time taken for emergence with details of the time interval for each developmental stage (larval stages, prepupa, pupa, and adult) was documented. Also the total number of flies that emerged in each group is documented in Table 3. The

Table 3: Number of flies emerged postexposure and three line transfer.

	Fundamental exposure		First transfer			Second transfer			Third transfer	
	Day of emergence	Number of flies emerged	Day of emergence	Number of flies emerged (food with NP)	Number of flies emerged (food without NP)	Day of emergence	Number of flies emerged (food with NP)	Number of flies emerged (food without NP)	Day of emergence	Number of flies emerged (food with NP)	Number of flies emerged (food without NP)
0.1 mM	20	205	20	201	199	21	190	190	21	191	193
1 mM	20	201	20	199	196	21	190	190	21	190	190
5 mM	21	199	21	198	195	21	187	188	21	188	189
10 mM	21	195	21	192	192	21	186	186	21	185	185
Negative control	20	230	—	—	—	—	—	—	—	—	—

Table 4: Results of wing spot analysis of nanoiron.

Concentration of nanoiron	*flr3*	*mwh*	*mwh*/*flr3*	Observations	Pictorial representation
Negative control	—	—	—	Absence of spots	
0.1 mM 1.0 mM 5 mM 10 mM	—	—	—	Absence of spots	
Positive control (EMS)				Presence of *mwh* and *flr3* spots	

table reveals that the time taken by the control population to emerge is 20 days (~3 weeks) which is in line with the normal developmental cycle of *Drosophila* on comparison with the control, 0.1 mM and 1 mM concentration emerged in 20 days (which is on par with normal life cycle) and 5 mM and 10 mM concentration emerged in 21 days.

3.7. Wing Spot Assay. *In vivo* genotoxicity of nanoiron was determined by SMART in *Drosophila melanogaster*. In this trial, 3rd instar transheterozygous larvae (*mwh*/*flr3*) were used for the recessive genetic markers of *mwh* and *flr3*. The scoring was done in 100 wing pairs. Statistical analysis using Dunnett's *t*-test (2-sided) showed that there were no statistical significance between the untreated and nanoiron treated groups ($p > 0.765$) as compared to the positive control EMS ($p < 0.023$). This shows that the nanoiron compound did not induce any genotoxic effect at the defined concentrations (Table 4).

4. Discussion

There has been an extensive use and relevance of nanoparticles and nanomaterials in the recent past showing an exponential growth in nanotechnology. Engineered nanoparticles tend to exhibit a wide range of applications due to their novel properties, as compared to their bulk counterparts. These nanoparticles are known to interact with several biological systems leading to possible toxicity to the system.

In vivo toxicity assessment methods used in the present assay have demonstrated dose dependent toxicity in the qualitative assay like DNA fragmentation assay in exposed population. However, the F1 population shows reduced toxicity in comparison with the exposed population. Distinct phenotypic changes in the flies were also observed in the exposed population; however, the F1 generation showed normal phenotype. This indicates that nanoiron has induced a possible change in gene expression but the same is not heritable and the role of DNA repair mechanisms cannot be ruled out. Viability and reproductive ability were assessed at 24-hour and 48-hour intervals for both exposed and F1 populations and have indicated no statistical significance between the exposed and control groups indicating lack of influence of nanoiron on the viability or longevity of flies. Also, the reproductive ability analysed using multiple transfer technique revealed no change in the treated and control groups with regard to the day of emergence and the number of flies

emerged indicates that the reproductive ability of the flies was not hindered due to nanoiron exposure. The quantitative method of evaluation wing spot assay has expressed transparent lack of toxicity in the studied concentrations of nanoiron. Shearing of DNA observed in DNA fragmentation assay is such evidences to show the first line of toxicity expression of the compound. However, the damage thus caused can be managed by repair mechanisms and the toxicity to the structure of chromosomes or expressions of spots as in wing spot assay was not significant. Also, protein profiling has clearly demonstrated that the protein profiles have been intact in the larvae which confirm the lacking toxicity of nanoiron. This leads to concluding that nanoiron at the defined concentrations is not genotoxic or mutagenic and it has a good potential for application in clinical diagnosis and biomedical research.

Disclosure

The corresponding author Venkatachalam Deepa Parvathi is a part time research scholar at Vels University, Chennai 600117, India.

Competing Interests

The authors would like to declare that there is no conflict of interests with regard to funding of the present research work.

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