International Journal of NanoScience and Nanotechnology.
ISSN 0974-3081 Volume 5, Number 1 (2014), pp. 25-35

http://www.irphouse.com

Nanotoxicology testing: Potential of *Drosophila* in toxicity assessment of nanomaterials

Deepa Parvathi V[1] and Rajagopal K[2]

1 Department of Human Genetics, Sri Ramachandra University, Chennai
2 Department of Biotechnology, Vels University, Chennai
Corresponding Author: Deepa Parvathi V
Lecturer, Department of Human Genetics, Sri Ramachandra University, Chennai 600116
deepakoushik305@gmail.com; +919884610225

Abstract

Nanomaterials have created a profound impact in terms of their application in the fields of biomedicine, commercial products and industrial practices. The technology in the synthesis, manipulation and application of materials/engineered particles in the nano scale has established itself as one of the key technologies in the new millennium. Many nano materials have gained demand in the market owing to their unique properties and this creates a necessity for the researchers to analyze and explore the toxicity perspectives along with establishing standard tools for toxicity assessment to avoid environmental hazards and health risks. The present paper summarizes on nano genotoxicity as a phenomenon and the various *in vivo* and *in vitro* methods currently available to test genotoxicity of NMs. The paper also highlights the importance of *Drosophila* as an effective *in vivo* model system and the advantages it offers over other animal models and *in vitro* systems.(1,2)

Key words: Nanotoxicology, Genotoxicity, *In vitro, In vivo*, Drosophila, SMART (Wing Spot Assay)

Introduction

Nanomaterials have created a profound impact in terms of their application in the fields of biomedicine, commercial products and industrial practices. The technology in the synthesis, manipulation and application of materials/engineered particles in the nano scale has established itself as one of the key technologies in the new millennium.

This technology referred to as nanotechnology promises to deliver a new revolution to the community. Their application into the field of biomedicine, healthcare and research is challenging, particularly in areas of medical imaging and diagnosis, pharmaceuticals, drug delivery, and therapy. This has lead to a phenomenal demand for nanomaterials(NMs) in the market worldwide. The technology is exponentially advancing with many thousand nano products available in the market. NMs demonstrate unique properties and functions that significantly differ from those observed or recorded in the corresponding bulk counterpart owing to their small size and large surface area. Many research studies have tried to establish that these novel properties of NMs which make them unique and dynamic could also be equally responsible for their potential toxicity. It is important for the scientific community to explore more than what is available about NMs in terms of environmental hazards and health risks. The increasing unintended exposure to NMs is of importance and the lack of standard regulatory guideline(s) on the testing/evaluation of nano materials makes it relevant and significant to employ *in vitro* and *in vivo* toxicological assessment tools. This has introduced to us the science of nanogenotoxicity which focuses on investigational studies in the toxicity of nano based materials, in particular, genotoxicity studies of NMs and nanoparticles (NP). It is important for researchers to explore and establish the varied factors that influence the toxicity of nanomaterials to avoid undesirable effects. This review aims at presenting the various *in vitro* and *in vivo* end points that are currently employed for nanotoxicology assessment and a brief extension to their advantages and limitations. The review importantly also focuses on the use of *Drosophila* in modern toxicology testing tools and its potential towards *in vivo* testing.(1,2,3)

Application of Nanotechnology

Nanomaterials have created a profound impact in terms of their application in the fields of biomedicine, commercial products and industrial practices. NMs are increasingly used in biomedicine as biosensors, imaging contrast molecules and drug delivery agents. NMs are used in combination with bio molecules like DNA, proteins or antibodies as “coated molecules” for drug delivery and imaging to avoid immunological reactions. Their application has extended in medicine involving ingestion or injection of NPs into the body. Commercially NMs are used as semiconductors, microelectronics, catalysts, water purification plants, textiles, sewage treatment etc.

An approach to Nanotoxicology

The technology in the synthesis, characterization, manipulation and application of materials/engineered particles in the nano scale (i.e. materials that have at least one dimension less than 100nm in length) is referred to as nanotechnology. The name nanomaterials/nano particles refer only to engineered particles and does not apply to particles below 100nm that occur in nature or are by products of other processes. NMs exhibit exceptional physical properties (advanced magnetic, catalytic, electrical,

optical and mechanical) on comparison with their bulk counterparts. These enhanced properties may be attributed to their exceptionally small size and large surface area to volume ratio. This also confers on them the property of being highly reactive. This potential high reactivity could mediate harmful interactions of the nanomaterials with biological systems and environment.(2,3)

This warrants the scientific community to contribute adequately in exploring, understanding and addressing the toxicity issues that may be contributed by NMs. Apart from this, the mechanisms involved and appropriate testing tools at both *in vivo* and *in vitro* levels need to be established. The new branch of science in toxicology referred to as Nanotoxicology shoulders the responsibility to address the knowledge and devise research strategies to identify adverse environmental and health risks caused by NMs.

The discipline of nanotoxicology has its role in the advancement of sustainable and safe nanotechnology. Nanotoxicology involves characterization of physicochemical properties, assessing routes of exposure, bio distribution, genotoxicity assessment and regulatory perspectives. It also proposes a reliable, robust and data assured test protocols for health and environmental risk assessment of NMs.(3,4)

Nanogenotoxicology perspective

Many NMs and NPs in various research operations conducted “hitherto” using both *in vivo* and *in vitro* studies have demonstrated significant genotoxicity and adverse effects on biological systems. The mechanisms involved help researchers to categorize the effect as primary and secondary genotoxicity. Genotoxicity expressed due to direct exposure of the NM/NP is referred to as primary genotoxicity and an adverse effect caused to the genetic material due to the interaction of the NM/NP with biological cells or tissues and results in production of ROS (reactive oxygen species) which in turn reacts with nucleic acids, proteins, carbohydrates and lipids causing apoptosis or necrosis. This has evolved into a specialty referred to as nanogenotoxicology which involves study of toxic effects of NMs/NPs on the genetic material in terms of structure, stability and expression profiles of DNA, RNA and protein.(4,5)

***In vitro* approaches to assess nanogenotoxicology**

In vitro studies are performed using primary cells or established cell lines derived from target tissues to assess toxicity. The preliminary testing of any agent and its reactivity is tested using cell culture studies. *In vitro* studies are easy to control, compare, reproduce and most importantly ethically accepted compared to animal models. It is important to realize the absence of internationally accepted standard guidelines/protocols for toxicity testing of NMs/NPs. Currently researchers have extended experimental tools and techniques of cell biology and toxicology for nanotoxicological studies. The various techniques used are as under:

1. Microscopic observation/assessment of intracellular localization using SEM, TEM, AFM

2. Assays to determine cell viability/proliferation/ROS generation(Trypan blue assay, apoptosis)
3. Hemolytic assay to quantify the release of hemoglobin.
4. Ames test(Bacterial reversion mutation test)
5. Genotoxicity assays like Micronucleus assay/chromosomal aberration assay and Comet assay.
6. Gene expression analysis using sophisticated systems.

Cytotoxicity assays are susceptible to changes in culture conditions such as differences in temperature, pH and nutritional supplements. Hence, it is important to critically monitor the conditions and ensure that the measured cell death or toxicity directly corresponds to the exposure of the NM and not due to vulnerable culture conditions. It is equally important to choose an appropriate cytotoxicity assay and is important to be conducted in duplicates and multiple tests may be conducted to draw consensus and establish reliable and reproducible data.

Different NPs demonstrate varied biological responses and no single gold standard method can be advocated to provide all satisfactory data on toxicity. To study the mechanisms of toxicity a combination of multiple assays is required.

Hemolytic assay: The biocompatibility of nano particles has been widely tested using in vitro hemolysis where the physico chemical characteristics(size, surface action) of NM/NP on human RBCs is evaluated colorimetrically by quantifying the release of hemoglobin.

Ames Test (Bacterial Reversion Mutation Test): The mutagenicity of any chemical compound is assessed by this in vitro method. Histidine dependent auxotrophic mutant strains of *Salmonella typhimurium* is used in this test. These strains contain mutations in the genes that block synthesis of histidine which is essential for cell growth. The test NM/NP is added to different locations on the agar plate and the bacterium is inoculated onto minimal histidine media. The test NM/NP is declared to be mutagenic if it is capable of reverting the mutation and express the histidine synthesis ability. This test is often performed as an accessory tool because of the inability to extrapolate and translate the results obtained in a prokaryotic system for eukaryotic genotoxicity testing. It is also essential to note that the results could be ambiguous in cases where the NM/NP is unable to cross the cell wall or in express bactericidal activity by killing the test organism. This could deliver false positive results and the data obtained should be co related with other tests after initial screening.(5,6,7)

Genotoxicity assays.

Many NMs/NPs have been assessed using common cytotoxic assays. It is critical to establish the toxicity of nano materials in terms of its genotoxic potential. Many in vitro genotoxic tools are performed.

Micronucleus Test (MN)/Cytokinesis Block Micronucleus Test (CBMN). MN assay is one of the standard cytogenetic tools used widely in dosimetry screening to evaluate and establish radioactive doses that cause DNA damages. Human peripheral

blood lymphocytes are vulnerable to clastogenic agents and are the sample of choice for MN assay. A mutagenic agent which causes damage to the genetic material and during anaphase of cell division, chromosomal fragments or whole chromosomes which are left behind form structures called Micronuclei(MN). The MN can be evaluated by blocking the cell cycle using a cytokinesis block referred as cytochalasin B that produces binucleated cells. This allows scoring the MN accurately and excluding the dividing cells from non dividing cells to enhance the reliability by reducing the incidence of false positive data. The MN assay has been routinely practiced to measure chromosome loss, breakage, apoptosis and non disjunction. This assay is based on scoring the number of micronuclei (MN) in treated cells which in turn gives a measure of genotoxicity. *In vitro* MN assay has been widely accepted to screen NPs/NMs for genotoxicity.(6,7,8,9)

Chromosomal Aberration Assay: Nanoparticles using chromosomal aberration with an in vitro cytogenetic assay may be identified. Cell culture is treated with test NPs and using colchicines as inhibitor the mitosis is arrested in metaphase. Using light microscope the metaphase was observed to detect the chromosome, chromatid aberration, sister chromatid exchange or polyploidy cells. Using control group the increase in frequency of structural and numerical aberration can be identified which indicates that NPs is toxic.(10)

Comet Assay (Single-Cell Gel Electrophoresis Assay): The comet assay is a simple, inexpensive widely used sensitive in vitro assay to assess DNA damage and repair. It has found application in genotoxicity testing of novel chemicals and pharmaceutical products apart from environmental bio monitoring. Its application has been employed for toxicity assessment of nanoparticles. The technique involves suspending cell samples in low melting agarose and casting them onto microscope slides. The cells are subjected to lysis and electrophoresed to separate the DNA strands based on their molecular weight. The DNA is stained with an appropriate fluorochrome and observed under fluorescence microscope. Damage cause to the DNA is expressed as fragments and the length of the tail of the comet is proportional to the extent of genetic damage. Studies conducted ‘hitherto’ have proved that most NMs/NPs exhibit high reactivity and cause DNA strand breaks. The assay is extremely sensitive and required careful handling to ensure reproducibility of results. It is also important to employ additional methods to investigate mutagenicity to yield valuable results.(10,11)

In vivo approaches to assess nanogenotoxicology

Drosophila as an animal model

Our understanding in terms of measurement of single strand and double strand breaks, mutations, deletions, chromosomal aberrations, DNA repair, tumorigenesis and carcinogenicity is highly advanced owing to research in genetic and molecular levels in various animal models and in vitro approaches in last few decades. The roundworm (*Caenorhabditis elegans*), the fruit fly (*Drosophila melanogaster*) and the zebrafish (*Danio rerio*) are the predominant alternative models that have been proposed through the genomic and post-genomic revolution of the last decade. The distinct advantages

of each of these models are well understood and appreciated with respect to their short generation time, cost involved in laboratory maintenance, their susceptibility to genetic manipulation, efficiency of screening methods and most importantly their homology and conservation of genome with higher organisms.(1,11,12)

In this paper we propose the use of *Drosophila* as a model organism in the current regime of toxicology testing of NMs/NPs emphasizing on its distinctive attributes. The ease of screening phenotypes and their susceptibility for genetic manipulation identifies *Drosophila* as an ideal model for mutagenicity and toxicological screening. *Drosophila* is a dynamic animal system which has carved a niche for itself in the field of biomedical research. *Drosophila* has proved effective to model many human neurodegenerative diseases and in drug discovery as well. (12,1,3,14)

The national research council committee on toxicology had put forward its recommendations to declare *Drosophila*, *C. elegans* and Zebra Fish as animal models to toxicological risk assessment and testing. The unique developmental biology with respect to life cycle, genome and experimental manipulation of the fly when compared with the worm or fish in the area of toxicology testing has evolved to suggest the name "Drosophotoxicology".

The Fly Biology

Flies are easy to maintain and propagate in laboratory on simple food medium containing corn meal, yeast and antifungal agents. They grow and breed between 22-25°C and their generation time is 12-14 days. The life cycle consists of four developmental stages: embryo, larva, pupa and adult. Each of these stages is exploited for various research studies. The imago(newly hatched fly) acquires characteristic behaviors of flight, chemo and photo taxis, foraging and mating which are very commonly used as phenotypic end points in screening and assessment. The fruit fly serves as a useful and adaptable model to explore toxicological tools.(14,15,16)

Features of *Drosophila*

Drosophila has been widely and primarily employed as a model organism for the study of genetics and has contributed to the fundamental principles of genetics such as chromosome theory of heredity and mutagenicity. Few significant features of the fly model are highlighted below:

1. Physical mapping of genes can be performed on large polytene chromosomes isolated from their salivary glands.
2. The simple genome content of the fly consisting of 4 chromosomes encoding 13,600 genes approximately with more than 95% of it genetic content on I(sex),II and III(autosome) chromosome has made genetic analysis easy and consistent.
3. Ease of mutagenesis screening in terms of distinguishing phenotypes in wing, eye, body color, shape etc was and is still conventionally practiced. This has rapidly advanced the knowledge of gene function. However, current advancements allow us to observe and analyze finest cellular details within developing tissues of embryo, larva and pupal stages using immunohistochemistry, use of fluorescent

biomarkers(GFP) and improved microscopy techniques(confocal microscopy).

4. The advent of molecular cloning and recombinant DNA technology has led to DNA sequencing and identification of genes and permitted *in vivo* analysis of transgenic systems.
5. The bioinformatics tools available for *Drosophila* research are comprehensive for genomic, proteomic and functional molecular studies. The virtual library and fly base contains details on all known genes, mutant phenotypes, published/unpublished references and links to stock centers and reagent sources.
6. The advances in creating transgenic flies through P element transformation have revolutionized molecular studies in *Drosophila.* This technique established stable integration of foreign DNA into the chromosome and permitted manipulation and expression changes of the introduced genes in the course of developmental cycle. Modifications in this technique have been used to create reporter gene constructs which are important in establishing the function of a given gene in response to a xenobiotic. This technique has evolved in creating 'humanized' versions of flies that express disease genes and mimic human disease in the fly.(15,16,17,18)

Quantifiable end points in *Drosophila* toxicology

Lethality

Drosophila has been classically used for genotoxicity assessments. The most common genotoxicity is referred to as Sex linked recessive lethal (SLRL) test where the mutagenicity of the test compound towards the X chromosome of the sex cells of the males is tested. The parental males are fed with food containing the test compound and the lethality of males is assessed in second generation (F2). This test has been performed for many compounds but though it demonstrated high specificity it was less sensitive in identifying carcinogenic compounds. This test has been replaced largely with *In vitro* Ames test which is more efficient and less time consuming.

Determination of larval and adult lethality post chemical exposure has been widely employed in many research studies. The larval to adult transition in flies is complex and is least homologous development and perhaps the least homologous developmental stage with respect to mammals. Adult lethality remains a potent screening tool for tolerance or susceptibility testing for a given chemical and thus presents many applications to toxicological testing.

Reproductive Ability

The fertility of the flies on exposure to NM/NP is assesses using the reproductive ability assay where the virgin females are isolated from unexposed and treated vials(with the defined concentration of NM/NP) and are pair mated in vials containing normal food. Pair mating is done using two different strategies for each treatment group. 50 pairs of flies are taken in each treatment group.(1). NM/NP exposed males are paired with normal females and (2) Normal males are treated with NM/NP exposed females. All the flies in each treatment group are transferred into fresh vials with normal food for next 10 days and the number of eggs laid during the ten days in

each vial is scored. The total fecundity rate (in terms of number of eggs) and mean value for egg production is calculated and the total number of flies emerged from the eggs laid in the 10 days of pair mating is counted. The mean number of flies emerged /pair(10 days of exposure) gives a measure of the reproductive ability.(19)

Behavioral Traits

Flies demonstrate a range of behaviors that may be applied to understand human responses to environmental changes. Behaviors that are commonly observed and documented include feeding, flight, locomotion, circadian rhythm, sleep cycles, courtship etc. Toxicity of NM may be tested using each of these behaviors as an end point. However it is extremely difficult to quantify the changes observed.(20)

Wing spo Assay

Several number of mutagenicity testing have been extensively performed on *Drosophila melanogaster*. These tests have been conducted in all the stages of the fly's life cycle and several changes have markedly observed in the region of its head, body, abdomen and wings. Among these tests, one of the most common and promising mutagenicity testing is SMART or otherwise referred to as, "Wing Somatic Mutation and Recombination Test". It is also called as the 'Wing Spot Assay', and is one of the gold standard assays for mutagenicity in *Drosophila*. The ability to assess loss of heterozygosity (LOH) as a consequence of gene mutation, chromosome breaks/re arrangement or chromosome loss can be detected by SMART.

This bio-tool employs wing cell recessive markers; namely multiple wing hairs (mwh,3-0.3) and flare(flr3, 3-38.8). Transheterozygous larvae (*mwh* +/+ *flr*3 animals) are used. The principle behind the bioassay involves expression of clone(s) of mwh and/or flr3 cells ["spot(s")] due to a mutation(upon chemical exposure) induced in a mitotic cell of a developing wing disc. These spot(s) are visible on the wing surface of the emerged adult fly under the microscope. The types of clone(s) expressed helps us identify the mechanism(s) involved in production of the clone; whereas the quantitative data obtained from the total number of clones induced(due to chemical exposure) reveals the genotoxic activity of the compound/chemical under test. (21,22,23,24,25)

The SMART system has been dynamically modified to help measure LOH frequencies in mutants as a consequence of defective meiotic recombination, disjunction or DNA repair. The pattern of spots reveals specific information(Table 1):

Type of Mutation	**Details**	**Pattern of spot(s) expressed**
Point Mutation Chromosome alteration Mitotic recombination	In flr+ or mwh+ Deletion of flr+ or mwh+	Single *flr3* or *mwh* spots(small and large clones)

Exclusive mitotic recombination	Twin spots indicate recombinagenic action of compound.	Twin spots (i.e., patches of adjacent *flr*3 and *mwh* cells)
Small Spots: are expressed due to chromosomal aberrations regardless of the time of initiation as the affected cells don't proliferate else proliferate slowly.	Small spots are produced during the last one to two rounds of cell division in the pupa	Small single spots (one to two mutant cells)
Large Spots	Large spots are produced earlier, during larval feeding	large single spots (more than three mutant cells)

Many hundreds of chemicals have been tested using SMART including various alklylating agents, antimetabolites, anticancer drugs etc.

DNA fragmentation Assay

DNA fragmentation assay is employed for qualitative assessment of DNA degradation upon exposure of DNA (*in vitro/in vivo*) to genotoxic agents. Similar to the principle applied in blood and cell lines, the flies are exposed for a stipulated time to various concentrations of any genotoxic agent followed by isolation of DNA by phenol-chloroform method and finally subjecting them to electrophoresis. This assay allows for comparison of damages observed in *in vitro* test in a dose dependent manner.

Summary and Conclusion

The science of Nanotoxicology predominantly investigates the toxic effects of NMs/NPs. However it also shoulders the responsibility to continuously monitor and assess the risks involved with NM/NP exposure in biomedicine and commercially. The increasing unintended exposure to NMs is of importance and the lack of standard regulatory guideline(s) on the testing/evaluation of nano materials makes it relevant and significant to employ *in vitro* and *in vivo* toxicological assessment tools. This has introduced to us the science of nanogenotoxicity which focuses on investigational studies in the toxicity of nano based materials, in particular, genotoxicity studies of NMs and nanoparticles (NP). Though many nanotoxicological research studies have been conducted there exists a prominent lacunae in interpreting data obtained from variable parameters and end points. The lack of studies towards understanding real time NM exposure and long term NM exposure in perspectives of understanding mutagenesis or tumorigenesis is a dangerous gap. Appropriate studies must be initiated to devise standard strategies which are consistent and reproducible for toxicity assessment in industries and environment. The safety and certainty of using NM/NP in biomedicine in spite of its overwhelming advantages remains cynical due to lack of appropriate toxicity assessment for health risk perspective. A definite

guideline on NM/NP synthesis and application is vital to ensure safety standards for individuals and environment.

Drosophila has been a wonder animal model and has stamped its signature in many fundamental research discoveries in classical genetics, developmental biology, drug discovery and disease modeling. The attributes which make this organism so appropriate are well understood and appreciated and it expresses itself as a suitable animal model system to expose the toxicity and biological activity of chemicals which has been extended towards NMs/NPs. The conservation of fundamental genome and cellular and developmental sequences between humans and flies is elaborate and warrants its application in assessing nanogenotoxicology today.

References:

[1] T.N. Cheng, J.Jasmine, L.B.H. Bay, and L.Y.L.Yung, J Nucleic Acids. 2010(2010).
[2] M.D. Rand, Neurotoxicol Teratol.32,74 (2010).
[3] K.E.Drexler, Proc. Natl. Acad. Sci. 78,5275(1981).
[4] S. Arora, J.M. Rajwade and K.M. Paknikar, Toxicol Appl Pharmacol.258(2012).
[5] A. Poma and M.L.D. Giorgio, Curr Genomics. 9,571 (2008).
[6] V.D. Parvathi, A.S. Amritha,S.F.D.Paul, Sri Ram J of Med.2,33(2009)
[7] X.Z. Mail, S.Tian and Z.C.Mail, PloS ONE 7,9(2012)
[8] C.Hoskins, A.Cuschieri and L.Wang, J Nanobiotechnology. 3155(2012).
[9] D.N.Williams, S.H. Ehrman and T.R.P. Holoman, J Nanobiotechnology.4,3 (2006).
[10] K.Klien and J.godnić-cvar, Arh Hig Rada Toksikol.63,133(2012).
[11] P.P.Pompa, G.Vecchio, A.Galeone, V.Brunetti, S.Sabella, G.Maiorano, A.Falqui, G.Bertoni, and R.Cingolani, Nano Res. 4,405(2011)
[12] E.S.Demir, G.Vales, B.Kaya, A. Creus and R.Marcos, Nanotoxicology.5,417(2011).
[13] G.Vales, E.S.Demir, B.Kaya, A.Creus and R.Marcos, Nanotoxicology.7,462(2012).
[14] J.G.Affleck and K.V.Walker, Cytotech.57,1(2008).
[15] M.Ahamed, R. Posgai, T.J.Gorey, M.Nielsen, S.M.Hussain, J.J.Rowe, Toxic Applied Pharma.242,263(2010).
[16] N.G. Cheng-Teng, J.Li.Jasmine, B.H.Bay and L.Y. Lanry, J Nucleic Acids.2010,12(2010).
[17] J.H.Mohammad, M.F.Katharina, A.A. Ashkarran, J.A. Dorleta, R.Z. Ruiz, T.Rojo, V.Serpooshan,W.J.Parak and M.Mahmoudi, Trend Biotechi. 30,10(2012)
[18] E.R.Carmona, E.Kossatz, A.Creus and R. Marcos, Chemosphere.70,1910(2008).
[19] K.P.Anita, B.Ashley, L.B.May and P.B.Tchounwou, Int J Environ Res Public Health.*9,1649(2012)*.

[20] N.Tran, A.Mir, D.Mallik, A.Sinha, S.Nayar and J.W.Thomas, Int J Nanomedicine.5,277(2010).
[21] K.Wu. Y.Tan, H.Mao and M.Zhang, International J Nanomedicine.5,385(2010).
[22] P.V.Deepa,V.Priyanka, R Swarna A.S. Akshaya, Int J Med Clin Res 3,195(2012).
[23] X.J.Liang, C Chen, Y Zhao, L Jia and PC Wang, Curr Drug Metab.9,697(2008)
[24] D.J.Gorth, D.M.Rand and T.J.Webster, Int J Nanomedicine.6,343(2011)
[25] L.Xinyuanliu, D.Vinson, D.Abt, R.H.Hurt and D.M.Rand, Environ Sci Technol.43,6357(2009).