In Vitro and In Vivo Toxicity Studies of Engineered Nanoparticles

Synopsis
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**In Vitro and In Vivo Toxicity Studies of Engineered Nanoparticles**

**INTRODUCTION:**

Manufacture of nanomaterials of various shapes and compositions has increased in the last few years, with a vast potential of use ranging from diagnostic imaging to molecular construction. Its rapid proliferation promises to revolutionize not only the key areas of science and technology but also the fields of industries and biomedical applications (1).

Engineered nanomaterials are commonly defined as materials which have at least one dimension of 100 nm or less (1). Such materials possess unique properties (e.g., chemical, mechanical, optical, magnetic, biological) which make them desirable for commercial and medical applications (2). Fullerene, zinc oxide, titanium dioxide, chromium oxide, iron, aluminum, and silicon dioxide are the most common and frequently used nanoparticles. Fullerenes are used as imaging probe, antioxidant, and antibacterial agents, as a filler or pigment in cosmetics and also for drug delivery (3). ZnO and TiO₂ are commonly used in sunscreen and cosmetics as an ingredient, due to their antimicrobial and antifungal properties (4).

Due to vast production, increasing exposure to nanomaterials is a threat to the environment as well as humans. However, there are limited toxicity studies on manufactured nanomaterials, which include nanoparticles, nanotube, nanowire, fullerene derivatives, and other nanoscale materials (5). Nanomaterials are in the transitional zone between individual atoms or molecule and their counterpart bulk material. This can modify the properties (size, surface area, solubility, and shape) of the nanomaterial, which create the
opportunity for increased uptake and their interaction with the biological system (6).

Limited studies have been conducted earlier on toxicity of nanomaterials (7-10). Current knowledge of the potential toxicity of these engineered nanoparticles on human health and environment remains unclear. It is of utmost importance to evaluate their fate in the cell as well as to understand their mechanism of toxicity. Therefore the aim of this study is to elucidate the toxicity of engineered nanoparticles (eg. metal oxides) in biological systems.

**Objectives:**

1. Characterization of nanomaterials
2. *In vitro* and *in vivo* cytotoxicity and genotoxicity studies of metal oxide nanoparticles.
3. Molecular mechanism of Toxicity of metal oxide nanoparticles

**Methodology:**

**Models:**

*In vitro*: Cell lines of animal / human origin

*In vivo*: Mouse/Rat

To achieve the above objectives, standard methodology will be used.

**Particle Characterization:**

The toxicity of nanoparticles has been strongly correlated with several physical properties, such as specific surface area, the zeta (ζ) potential and particle size.

TEM, Dynamic light scattering and Phase analysis light scattering (Zetasizer Nano-ZS), will be used to determine size, size distribution and zeta potential respectively.
1. Cytotoxicity assay:
MTT assay will be carried out according to the method of Mosmann et al (12), with minor modifications to assess cytotoxicity under in vitro conditions. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of viable cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable cells.
Neutral red uptake assay is another reliable cytotoxicity assay. This assay will be done according to the method of Borenfreund et al (13). In this assay neutral red dye, a weak cationic supra vital dye is accumulated in the lysosomes of viable, undamaged cells and gives red color precipitate which is an indicator of viability of cells.

2. Cell Viability Assay:
To assess the cell viability Trypan blue dye exclusion assay will be performed according to the method of Philips et al (14). Viability assays measure the percentage of cells that are viable. The principal of this assay is that a vital dye, trypan blue, when put in the cell suspension gets excluded from the live cells while the cells whose membrane has been disrupted take up the colouring agent. The dye used for exclusion stain is usually trypan blue. The trypan blue dye exclusion is commonly used and a protocol for this procedure is included here:
A cell suspension of the cells to be assayed will be prepared (about \(10^6\)cells/ml) and a 1:1 dilution of the suspension with 0.4% trypan blue solution made. 20 µl of the above cell suspension will be loaded on the counting chambers of a haemocytometer and allowed to sit for 1-2 minutes
after which the number of stained cells and total number of cells will be counted using standard counting procedure. The unstained cells will represent the percentage of viable cells.

3. Genotoxicity Studies:
Genotoxicity studies will be conducted using standard techniques which are described below:

a. Single cell gel electrophoresis or Comet assay:
The single cell gel electrophoresis (SCGE or Comet assay) will be conducted according to the method of Singh et al. (15). Briefly, a suspension of single cells of interest will be embedded in an agarose layer on a microscopic slide and then lysed using high salt conditions and detergent, hence leaving only the nuclei in the agarose. The nuclei will then be subjected to unwinding (for relaxing the DNA super coils) and electrophoresis under highly alkaline condition, in order to express the single strand breaks and alkali labile sites. After neutralizing, these slides will be stained with ethidium bromide and visualized under fluorescent microscope using an image analysis system. The extent of the DNA damage in the cells will be ascertained by the Comet parameters viz. Olive tail moment, tail length and % tail DNA. This method provides qualitative and quantitative estimation of DNA damage.

b. Micronucleus Assay:
Micronucleus assay will be carried out according to the method of Fenech (18) and OECD guideline No.487 (22).
For in vitro micronucleus assay, cytochalasin B will be added to cells to get binucleated cells. After harvesting of the cells, slide will be prepared as per standard protocol (19). Binucleated cells will be evaluated for the
determination of micronucleus frequency.

*In vivo* micronucleus assay will be carried out according to Schmid (20) and OECD guideline No.474 (23). In this test animal will be treated with the nanomaterials and bone marrow cells will be isolated in fetal bovine serum. Slides will be prepared and stained with May-Grunwald and Giemsa. Slides will be scored for 1000 polychromatic erythrocytes (PCEs) per animal. The ratio of PCE/(PCE+NCE) will be determined for each dose to assess the effect of treatment on bone marrow proliferation.

4. Mechanistic Toxicity Studies:

**Reactive oxygen species (ROS) Assay:**

Reactive oxygen species such as hydrogen peroxide (H$_2$O$_2$), hydroxyl radicals (OH), superoxide (O$_2^-$) and singlet oxygen ($^1$O$_2$). This assay will be carried out according to the method of Wang *et al* (21), using 2, 7'-dichlorofluorescein diacetate (H$_2$ DCFDA), is a fluorescent dye with minor modifications.

In the presence of reactive oxygen species (ROS), DCFH is oxidized to highly fluorescent dichlorofluorescein (DCF). Therefore, the intracellular DCF fluorescence can be used as an index to quantify the overall Oxidative stress in cells. In this study, we used a fluorescent microplate reader to evaluate Oxidative stress in cells, induced by applying various free radical generators extracellularly, using DCFH as the probe.

Molecular mechanism of studies will be done by pathway focused western blotting.

**Statistics:**

Statistical significance of the results will be assessed by appropriate statistical methods.
References:


influenced by the menstrual cycle: a study in healthy Indian females. *Mutation Research* 565, 163-172.


23) OECD guideline for the testing of chemicals Mammalian erythrocyte micronucleus test 474: 1997.

Signature of Student

Signature of Supervisor

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