# CHAPTER 2

# Biocompatibility test of metal oxide nanoparticles and their cytotoxicity against chicken embryonic fibroblasts

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## INTRODUCTION

In nature, nanoparticles (>100nm) can be found in volcanic dust, natural water and soil and hence, are called as **Natural nanoparticles** (Handy *et al.*, 2008). They also can be produced artificially by physical, chemical or microbial processes. Such man-made nanoparticles are called as **Engineered nanomaterials** that can be either carbon-based (carbon nanotubes) or metal based (metal oxides) (Adams *et al.*, 2006). Engineered nanoparticles are produced within the range of 1-100 nm ($10^{-9}$m) in length or diameter for commercial uses. Nanoparticles possess increased structural integrity as well as unique mechanical, optical, chemical, electrical and magnetic properties (Oberdörster *et al.*, 2005; Thomas & Sayre, 2005). Due to these reasons nanomaterials have gained enormous attention and are currently being widely used in modern technology. They have increasingly been applied in many products including cosmetics, electronics, food packaging, medical devices, catalysts, and water treatment technologies (Brody, 2006; Karnik *et al.*, 2005). It has been recognized that nanotechnologies have huge potential to affect drug development, water decontamination, and development of information and communication technologies (Adams *et al.*, 2006). Although the applications and benefits of these engineered nanomaterials are extensive (Hoet & Boczkowski, 2008; Thomas & Sayre, 2005), there is a severe lack of information concerning their implications on human health due to occupational exposure and on environment and wildlife. Since each engineered nanoparticle (ENP) is unique with respect to its

properties and toxicity, it becomes imperative to investigate their toxicity in living systems.

Nanomaterial toxicity studies conducted so far have raised ample concerns about their risks to the environment and humans (Roco, 2005). With increased use of nanomaterials in daily life, there is also an increased chance of their leaching into the environment during their production, application or disposal. Although several studies have been conducted on the toxicity of metal oxide nanoparticles, towards bacteria (Qu & Jiang, 2004; Rincón & Pulgarin, 2004; Wei *et al.*, 1994) and higher-order cells (Hussain *et al.*, 2005; Ivask *et al.*, 2015; Jeng & Swanson, 2006; Schneider *et al.*, 2017; R. K. Shukla *et al.*, 2013; S. Shukla *et al.*, 2015), there is limited understanding about the adverse effects of nanomaterials on chicken embryo. Hence, this study focuses on the impact of metal oxide nanoparticles ($TiO_2$, $Fe_2O_3$, ZnO, MgO or $Al_2O_3$) on chicken embryonic fibroblasts.

## MATERIALS AND METHODS

### Nanoparticles

Metal oxide nanoparticles Titanium dioxide ($TiO_2$ NPs), Iron oxide ($Fe_2O_3$ NPs), Zinc oxide (ZnO NPs), Magnesium oxide (MgO NPs) or Aluminium oxide ($Al_2O_3$ NPs) were procured from Aldrich (St. Louis, MO, USA). The characteristics of NPs are mentioned in Table 2.1.

**Table 2.1:** Standard specifications about metal oxide nanoparticles mentioned by Aldrich

| Chemical formula | Colour | APS [a] (nm) | TD [b] (g/cc) |
|---|---|---|---|
| $TiO_2$ | White | <100 | 4.26 |
| $Fe_2O_3$ | Dark Red-Brown | 35 | 5.15 |
| ZnO | Off-white | ≤40 | 1.7 |
| MgO | White | <50 | 3.58 |
| $Al_2O_3$ | White | <50 | 4.0 |

a Average particle size measured by Brunauer, Emmett, and Teler (BET) technique
b True density

**Preparation and Characterization of nanoparticles**

Nanoparticles tend to agglomerate upon addition of cell culture media if they are uncoated or functionalized (Ji *et al.*, 2010). If the agglomerates are used directly for nanotoxicity studies, it would result in inaccurate dose estimation and the interpretation of the results would be flawed (Ji *et al.*, 2010). Therefore, it is necessary to characterize the NP suspension prior to toxicity assessment. NPs (1 mg/ml) were suspended in water and probe sonicated (LMUC-4, Labman scientific instruments Pvt. Ltd., Kolkata, India) for 30 min. After sonication, the particle size distribution was measured using a 90 plus DLS (Dynamic light scattering) unit from Brookhaven (Holtsville, USA).

**Hemolysis Assay**

Chicken whole blood was procured from the slaughter house and 5 mL was added to 10 mL of calcium- and magnesium-free Dulbecco's phosphate buffered saline and centrifuged at 500 g for 10 min to isolate RBCs from serum. This purification step was repeated five times and then RBCs were diluted to 50 mL in PBS. To test the hemolytic activity of nanosized particles, 0.2 mL of diluted RBC suspension (around 4.5 x $10^8$ Cells/mL) was added to 0.8 mL of metal oxide nanoparticles suspended in PBS (1mg/ml). $dH_2O$ (+RBCs) and PBS (+RBCs) were used as positive and negative control, respectively. All the samples were placed on a rocking shaker in an incubator at 37$^o$C for 3 h. After incubation, the samples were centrifuged at 10,016 g for 3 min. The hemoglobin absorbance in the supernatant was measured at 540 nm, with 655 nm as a reference (Liao *et al.*, 2011). Percent hemolysis was calculated as:

$$\textbf{Percent hemolysis (\%)} = \frac{\textbf{sample abs}_{\textbf{540-655 nm}} - \textbf{negative control abs}_{\textbf{540-655 nm}}}{\textbf{positive control abs}_{\textbf{540-655 nm}} - \textbf{negative control abs}_{\textbf{540\_655 nm}}} * \textbf{100}$$

**Culturing of Chicken embryonic fibroblasts**

Fertilized eggs (55±2.1 g) of White leghorn (*Gallus gallus domesticus*) were obtained from Shakti hatcheries, Sarsa, Gujarat, stored for 2 days at 12°C and then incubated under standard conditions (37.5°C, humidity 60%) for 10 days and guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) were hereby followed. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC; MSU-Z/IAEC/03-2017) and the Committee for the Purpose of

Control and Supervision of Experiments on Animals (827/GO/Re/S/04/CPCSEA). 10-day-old eggs were candled and eggs without embryos or with dead embryos were discarded. Eggs were sterilized by swabbing with 70% ethanol, placed in hood and a large circular hole was made at the blunt end of the egg. The shell above the air sac and the membrane were removed with sterile scissors or forceps, exposing the embryo. The embryo was removed with blunt ended curved forceps, placed into a sterile petridish containing 10 ml of 1X PBS with calcium and magnesium, the head, wings, feet and body viscera were dissected and discarded. The body was taken into a 50-ml conical tube, fragmented with dissecting scissors and washed with 1X PBS to remove red blood cells. 25 ml of thawed 0.25% trypsin was added to the embryo and incubated at 37◦C for 20-25 min. Larger pieces were allowed to settle and the supernatant containing the cell suspension was transferred to a tube containing 25 ml of complete DMEM-F-12 (1:1, 5% FBS and 1% antibiotic antimycotic solution) media. Centrifuged for 10 min at 1000 rpm at room temperature, supernatant was discarded and the pellet was resuspended in complete DMEM-F-12 media (Hernandez & Brown, 2010) and maintained at 37°C with 5% $CO_2$. Cells were trypsinized using 1X TPVG every third day.

**Viability Asssay**

Cell viability of NPs in Chicken embryonic fibroblasts (CEFs) was measured using MTT to detect mitochondrial activity. In the presence of succinate dehydrogenase enzyme, yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced to a water insoluble purple formazan crystal in metabolically active cells bearing intact mitochondria. The resulting intracellular purple formazan can be solubilised

in an organic solvent and quantified by spectrophotometer. Chicken embryonic fibroblasts (CEFs) were seeded in 96 well plate ($10^4$ cells/well) and allowed to grow overnight. NPs were suspended in PBS (1 mg/ml) and sonicated (LMUC-4, Labman scientific instruments Pvt. Ltd. Kolkata, India) for 30 min. Further, NPs were diluted to 10-1000 µg/ml doses with complete media and dosed to the cells. After 24 h of incubation, MTT (5 mg/ml) was added in each well and incubated in dark for 4 h. The resultant purple formazan crystals were dissolved in DMSO (150 µl/well) and absorbance was measured at 540 nm using ELX800 universal Microplate Readers (Bio-Tek instruments, Inc., Winooski, VT) and % viability was calculated with respect to control using the following formula:

$$\textbf{\% Viability} = \frac{\textbf{Average OD of Treated Cells}}{\textbf{Average of Untreated Cells (control)}} \textbf{ X 100}$$

**LDH assay**

Lactate dehydrogenase is expressed extensively in body tissue and is released during tissue damage. LDH catalyzes the oxidation of lactate to pyruvate accompanied by the simultaneous reduction of NAD to NADH. The level of lactate dehydrogenase (LDH) released from CEFs was measured to evaluate the cytotoxicity of ZnO NPs. CEFs were treated with 10 µg/ml of ZnO NPs for 12 h. The cell-free supernatant was separated by centrifuge (2,000 rpm, 5 min). The activity of LDH in the supernatants was determined using LDH detection kit (Reckon diagnostic Pvt. Ltd.) according to the manufacturer's instructions. All samples were assayed in duplicates for LDH content by clinical chemistry analyser (Micro lab 300, Merck, Germany).

**Assessment of Nuclear morphology (DAPI)**

Nuclear condensation in cells was studied by DAPI staining. 4'-6-Diamidino-2-phenylindole (DAPI) is a popular nuclear counterstain which stains nuclei specifically, with little or no cytoplasmic labeling. The blue-fluorescent DAPI stain preferentially stains dsDNA; it appears to associate with AT clusters in the minor groove. Binding of DAPI to dsDNA produces a ~20-fold fluorescence enhancement, apparently due to the displacement of water molecules from both DAPI and the minor groove. CEFs were seeded in 6 well plate and after 24 h, the cells were exposed to ZnO NPs (10 μg/ml) for 1 h. The ZnO NPs containing media was aspirated and the cells were washed twice with PBS. After washing, DAPI (1 μg /ml) was added and incubated for 15 min. Excess stain was removed by washing with PBS. 200 μl of PBS was then added to each well and cells were observed in Floid cell imaging station (Invitrogen, USA).

**Assessment of intracellular oxidative stress**

The level of Intracellular ROS generation was estimated by staining with 2', 7'-dichlorfluorescein-diacetate (DCF-DA; Sigma Aldrich, USA). Dichlorofluorescein diacetate (DCF-DA) is an intracellular ROS detecting probe which is non-fluorescent but, when it enters into the cells undergoes deacetylation in the presence of esterase enzyme. Subsequently, the non fluorescent DCFH is oxidized by free radicals present in the cells to form a fluorescent species DCF, which can be estimated by micro-plate reader, flow cytometry or confocal microscopy. CEFs were seeded in a 6 well plate and after 24 h, the cells were exposed to ZnO NPs (10 μg/ml) for 1 h. The ZnO NPs containing media was aspirated and the cells were washed twice with PBS. Thereafter, culture medium containing DCFDA dye (10 μM) was added to each well. The plate was incubated for 30

min at 37ºC and the medium containing DCFDA was discarded. Excess stain was removed by washing with PBS. 200 µl of PBS was then added to each well and cells were observed in Floid cell imaging station (Invitrogen, USA).

**Assessement of mitochondrial membrane potential**

Intracellular mitochondrial membrane potential ($\Delta\psi_m$) was assessed by Rhodamine 123 (RHO 123; Sigma Aldrich, USA) dye. RHO 123 is lipophilic cationic dye that equilibrate across membranes in a Nernstian fashion and accumulate into the negative (i.e., more polarized) mitochondrial membrane matrix space. A more negative $\Delta\psi_m$ will accumulate more dye in case of healthy cells, while in apoptotic cells depolarization leads to loss of dye. CEFs were seeded in 6 well plate and after 24 h, the cells were exposed to ZnO NPs (10 µg/ml) for 1 h. The ZnO NPs containing media was aspirated and the cells were washed twice with PBS. After washing RHO 123 stain (10 µg /ml) was added and incubated for 15 min. Excess stain was removed by washing with PBS. 200 µl of PBS was then added to each well and observed in Floid cell imaging station (Invitrogen, USA).

**Gene expression studies**

Fibroblast cells were cultured in 6 well plate and exposed to 10 µg/ml ZnO NPs for 1 h. At the end of exposure total RNA was isolated using TRIzol reagent (Invitrogen, CA, USA) wherein; RNA isolation involves four steps viz. Homogenization, Phase separation, RNA precipitation and RNA wash. RNA pellet was dissolved in DEPC water and the purity of RNA was measured by analyzing $A_{260}/A_{280}$ ratio in UV-Vis spectrometer (Bio-Tek instruments, Inc., Winooski, VT). RNA samples with $A_{260}/A_{280}$

ratio (an indicator of protein contamination) between 1.9 and 2.1 were used for the analysis. The integrity of RNA samples was also assessed by agarose gel electrophoresis. OD at 260 was further used to calculate concentration of RNA (µg/µl) in sample using the formula given below:

$$\frac{\boldsymbol{OD\ at\ 260\ nm \times 40 \times 200}}{\boldsymbol{1000}}$$

Where, 40 = extinction coefficient, representing 1 = 40 µg/ml of RNA

200 = dilution factor for 5 µl of RNA in 995 µl of RNase free water

1000 = factor for conversion from µg/ml to µg/µl

cDNA was synthesized by reverse transcription of 1 µg of total RNA using iScript cDNA Synthesis kit (BIORAD, California, USA). The reaction protocol was as follows:

| | |
|---|---|
| Priming | 5 min at 25ºC |
| Reverse transcription | 20 min at 46 ºC |
| RT inactivation | 1 min at 95 ºC |
| Hold at 4 ºC | |

PCR was carried out using Dream Taq Green PCR Master Mix. Total volume was 12.5 µl: 1 µl c-DNA, 6.25 µl Dream Taq™ (Invitrogen, CA, USA), 0.5 µl of each primer and 4.25µl ultrapure water. Cycler conditions were as follows: Initial denaturation at 95°C for 3 min further it was followed by PCR cycle of Denaturation at 95°C for 30 sec, annealing at 60°C (GAPDH), 57.8°C (p53), 57.8°C (BAX), 56.5°C (CASP 3), 58.9°C (BCL2) for 30 sec and strand extension at 72°C for 30 sec and ends with final extension at 72 °C for 8-10 mins. The number of PCR cycles was 35. The PCR products were separated on 2%

agarose gel which was stained with ethidium bromide and quantified by ImageJ. Primers used for this study are listed below:

| Gene | Gene Name | Primer sequence (5'-3') |
|---|---|---|
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase | F:-ACTGTCAAGGCTGAGAACGG<br>R:-ACCTGCATCTGCCCATTTGA |
| CASP3 | Caspase 3 | F:-TGGCCCTCTTGAACTGAAAG<br>R:-TCCACTGTCTGGTTCAATACC |
| p53 | Tumor protein 53 | F:-GCCGTCGCCGTCTATAAGAA<br>R:-GGTCTCGTCGTCGTGGTAAC |
| BAX | Bcl-2-associated X | F:-TCCTCATCGCCATGCTCAT<br>R:-CCTTGGTCTGGAAGCAGAAGA |
| BCL2 | B-cell lymphoma 2 | F:-CTTCCGTGATGGGGTCAACT<br>R:-GGTACTCGGTCATCCGAGGTG |

**Statistical analysis**

Data analysis was carried out by unpaired Student's t-test or one way analysis of variance (ANOVA) using Graph Pad Prism 6.0 (CA, USA). Differences between control and treatment groups were deemed to be significant when $P \leq 0.05$.

## RESULTS

### Characterization of nanoparticles by DLS

The mean hydrodynamic diameter of $TiO_2$ NPs, $Fe_2O_3$ NPs, ZnO NPs, MgO NPs and $Al_2O_3$ NPs dispersed in Milli Q water as measured by DLS was 88.6, 71.2, 85, 65.2 and 73.6 nm respectively (Figure 2.1)

### Hemolysis assay

The hemolysis assay of chicken whole blood was employed to evaluate the *in vitro* blood compatibility of NPs. The results showed that MgO or ZnO NPs were able to manifest very little (10-20%) hemolysis. Whereas, $Al_2O_3$, $TiO_2$ or $Fe_2O_3$ NPs did not induce hemolysis (Figure 2.2)

### Cell Viability Assay

Chicken embryonic fibroblasts were dosed with metal oxide nanoparticles (10-1000 µg/ml) viz. $TiO_2$ NPs, $Fe_2O_3$ NPs, ZnO NPs, MgO NPs or $Al_2O_3$ NPs and cytotoxicity was studied by MTT assay. Cytotoxicity studies with chicken embryonic fibroblasts revealed that $TiO_2$, MgO and $Fe_2O_3$ nanoparticles showed 50% toxicity up to the highest dose (1000 µg/ml). However, $Al_2O_3$ was relatively more toxic and accounted for 50% cell death at 500 µg/ml. ZnO nanoparticles were the most toxic and accounted for 50% cell death at 10 µg/ml (Figure 2.3). Hence, all the further parameters were assessed with only ZnO NPs (10 µg/ml).

**LDH assay**

The level of LDH released from the cells was significantly increased after 12 of exposure to ZnO NPs (10 μg/ml) (Figure 2.4) as compared to the control cells.

**Nuclear staining with DAPI**

To detect nuclear condensation, CEFs were stained with DAPI. As shown in Figure 2.5, a typical image of untreated cells featuring round intact nuclei was observed. In contrast, the chromatin of nuclei condensed and nuclear apoptotic bodies were formed when the cells were treated with 10 μg/ml ZnO NPs.

**Intracellular reactive oxygen species (ROS) measurement**

The level of ZnO NPs induced intracellular oxidative stress was measured by DCF fluorescence as a marker of ROS generation in fibroblast cells (Figure 2.6A). As shown in Figure 2.6B, an increase in intracellular ROS generation (green fluorescence) was observed after 1h of exposure to ZnO NPs as compared to control.

**Assessement of mitochondrial membrane potential**

The $\Delta\psi_m$ of cells was assessed by monitoring the intensity of fluorescence staining of rhodamine 123 (Figure 2.6A). A significant decrease in mean fluorescence intensity was observed following treatment of cells with ZnO NPs (Figure 2.6C). The data showed that apoptosis induced by ZnO NPs was accompanied by alterations in the mitochondrial membrane potential.

**Gene expression studies**

Semi-quantitative RT-PCR analysis indicated the involvement of various apoptotic signaling genes in ZnO NP mediated cell death. Results showed that ZnO NPs significantly altered the mRNA expression levels of p53, BAX, BCL2 and CASP3. We observed that the expression of tumor suppressor gene p53 and proapoptotic genes (BAX and CASP3) were upregulated while the expression of antiapoptotic gene BCL2 was downregulated in ZnO NPs (Figure 2.7).

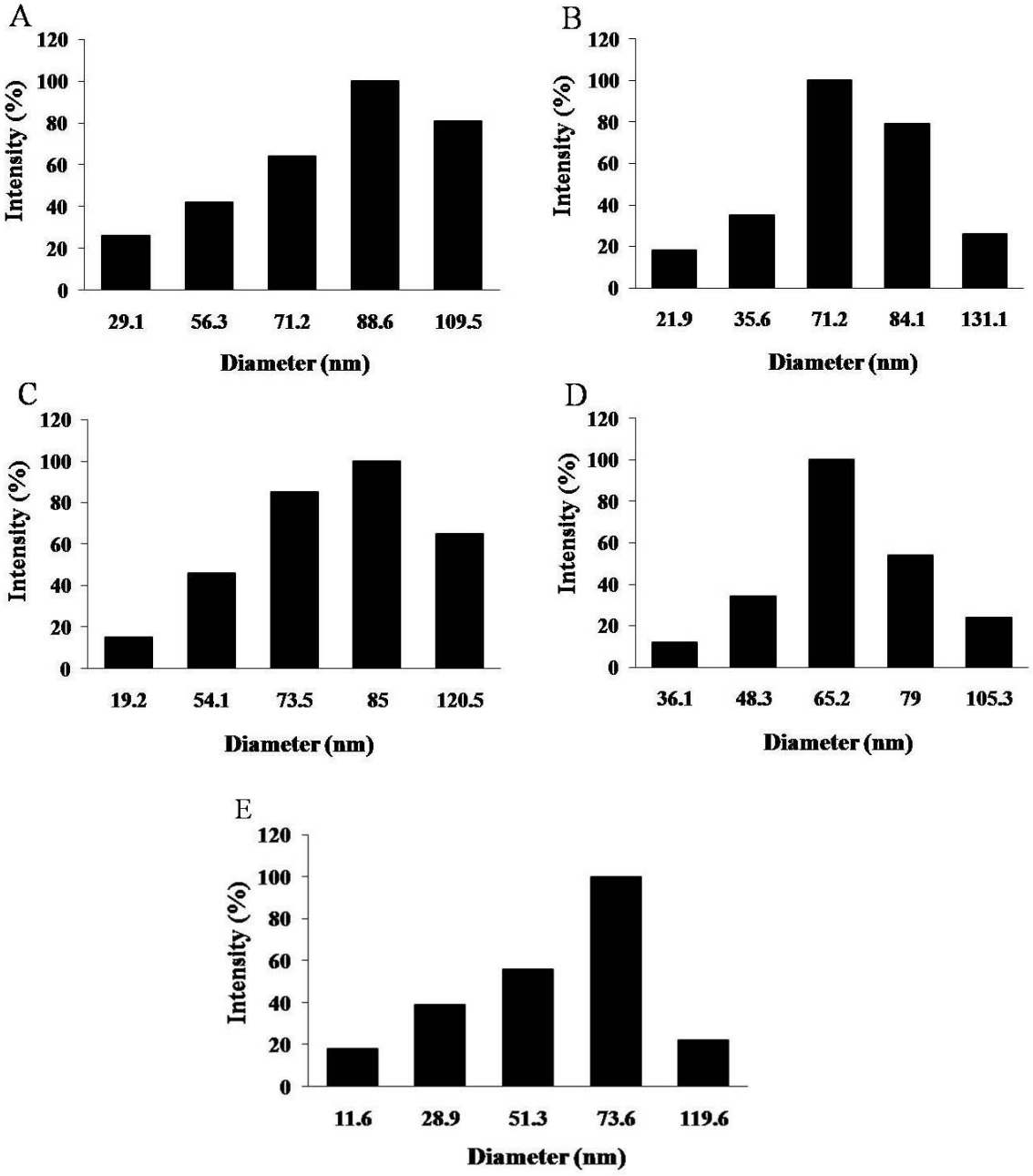


**Figure 2.1:** The mean hydrodynamic diameter of metal oxide nanoparticles, A ($TiO_2$), B ($Fe_2O_3$), C (ZnO), D (MgO) and E ($Al_2O_3$) measured by DLS.

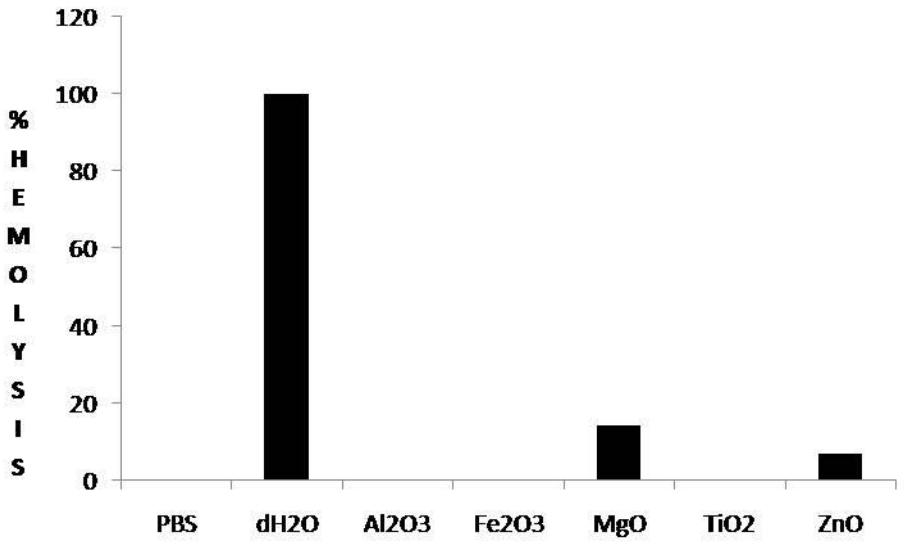


**Figure 2.2:** Percent hemolysis of chicken RBCs incubated with different metal oxide nanoparticles ($Al_2O_3$, $Fe_2O_3$, MgO, $TiO_2$ and ZnO).

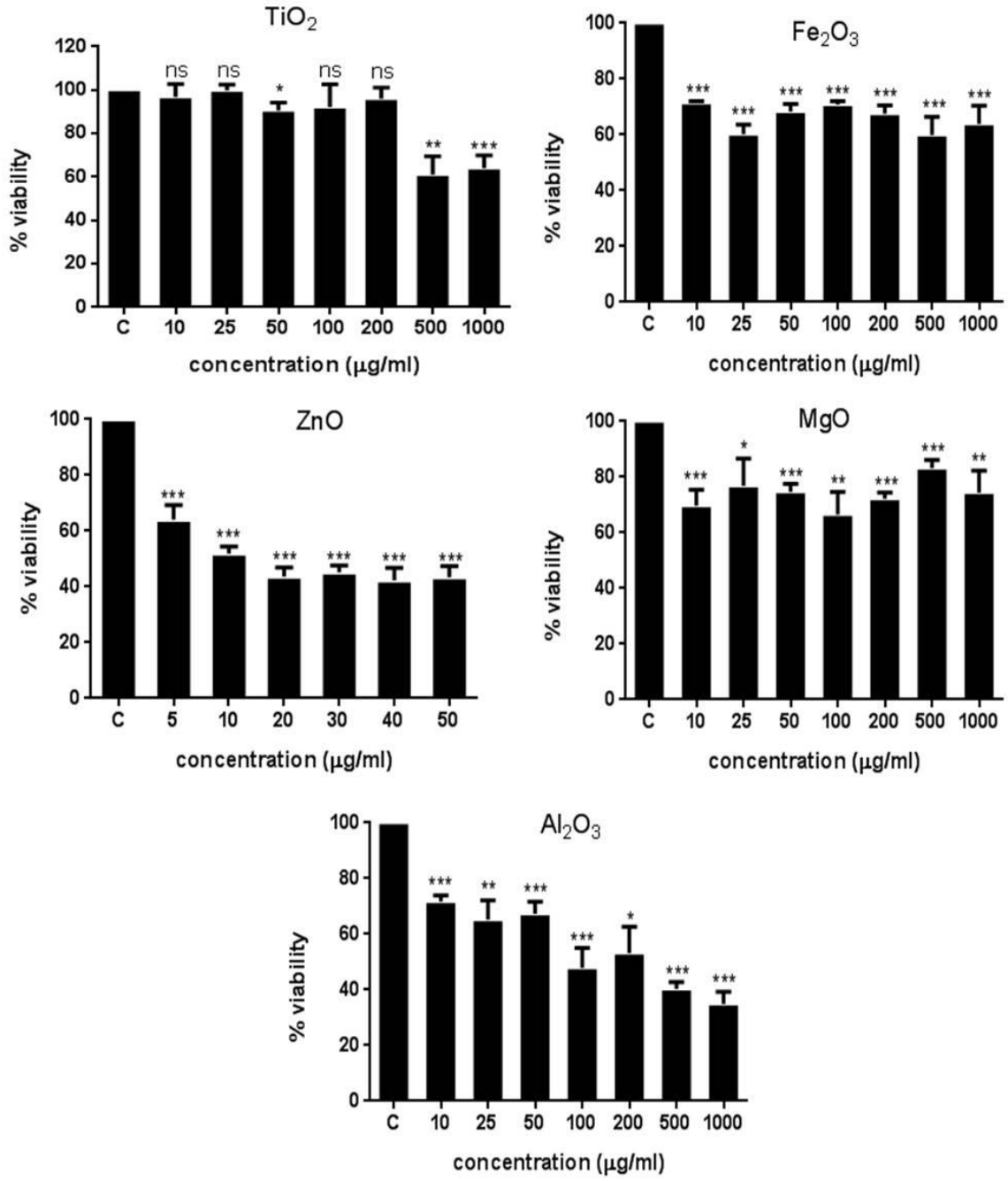


**Figure 2.3:** The cytotoxic potential of metal oxide nanoparticles on chicken embryonic fibroblasts. Cells were incubated with different concentrations of the nanoparticles (10-1000 µg/ml) for 24 h. Cell viability was determined by MTT Assay. Viability curves plotted against the concentration of nanoparticles. The data are Mean±SD.*=p≤0.05,**=p≤0.01,***=p≤0.001, ns-non significant.

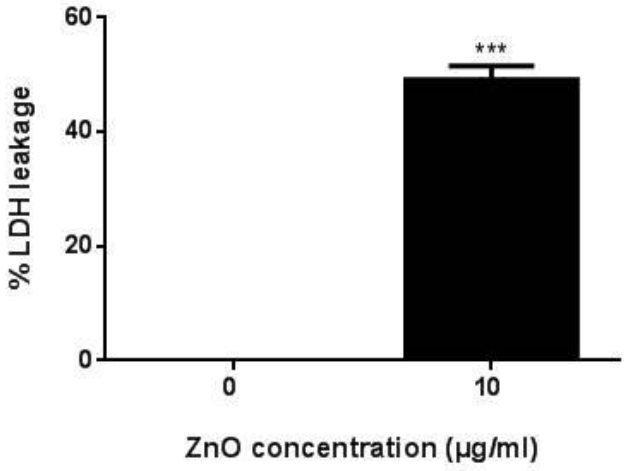


**Figure 2.4:** LDH release in chicken embryonic fibroblasts treated with ZnO NPs (10 µg/ml) for 12 h. The data are Mean±SD.***=p≤0.001.

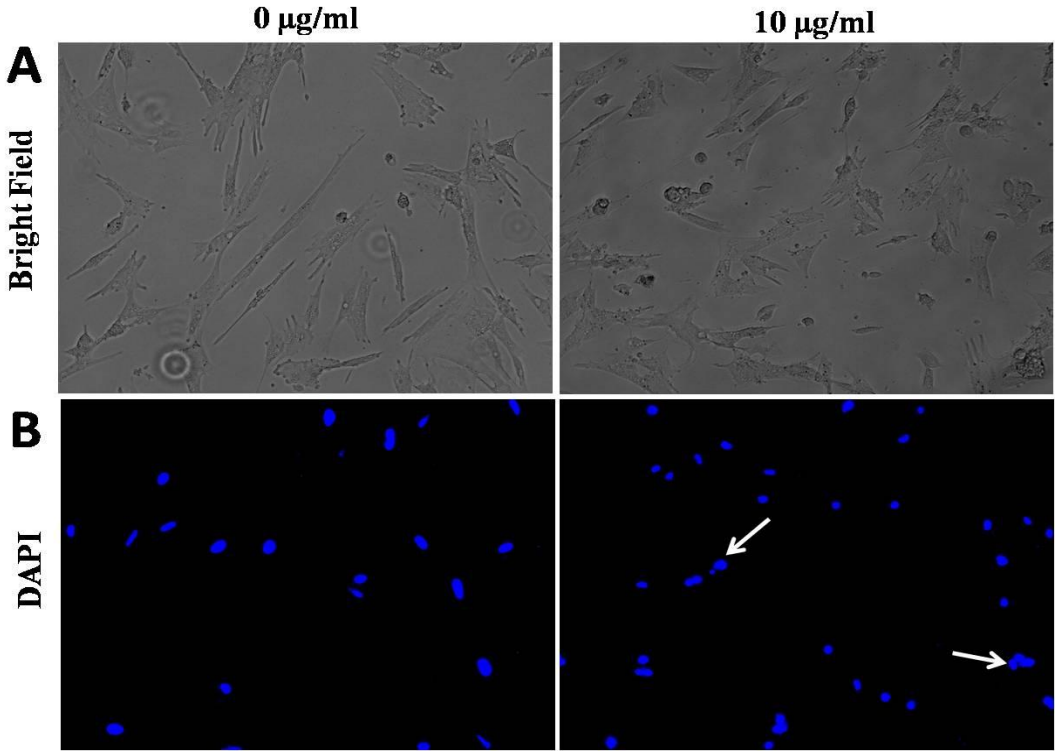


**Figure 2.5:** (A) Phase contrast images depicting the changes in the morphology of the cells treated with ZnO NPs (10 µg/ml). (B) Fluorescent images of chromosome condensation by DAPI staining in fibroblast cells exposed to ZnO NPs (10 µg/ml). White arrows indicate nuclear fragmentation. (Magnification, ×200).

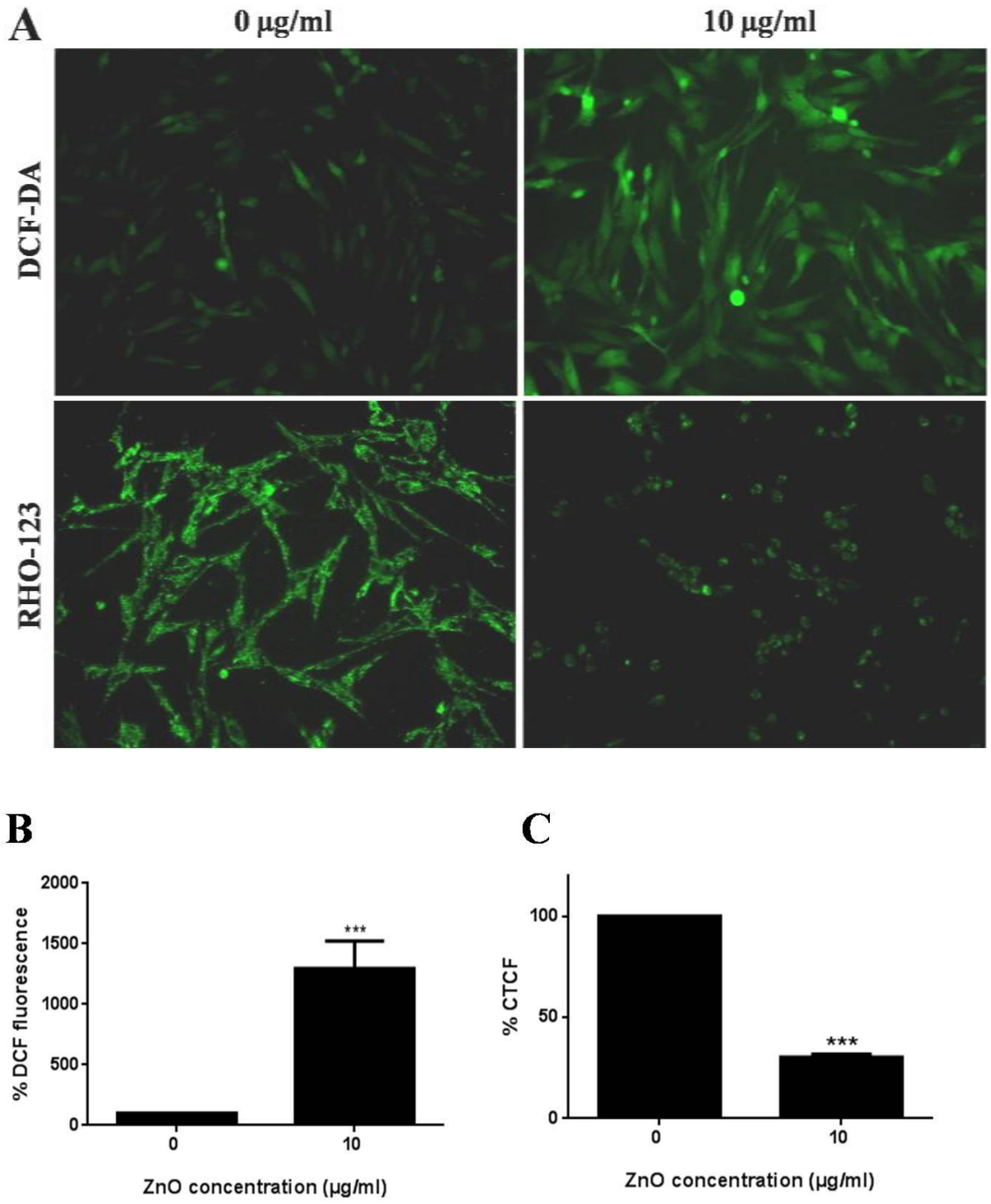


**Figure 2.6:** (A) Florescence photomicrographs of DCF-DA and RHO-123 stained fibroblast cells exposed to ZnO NPs. (B) ROS generation and (C) mitochondrial membrane potential in chicken embryonic fibroblasts treated with ZnO NPs (10 µg/ml) for 1 h. The data are Mean±SD.***=p≤0.001 (Magnification, ×200).

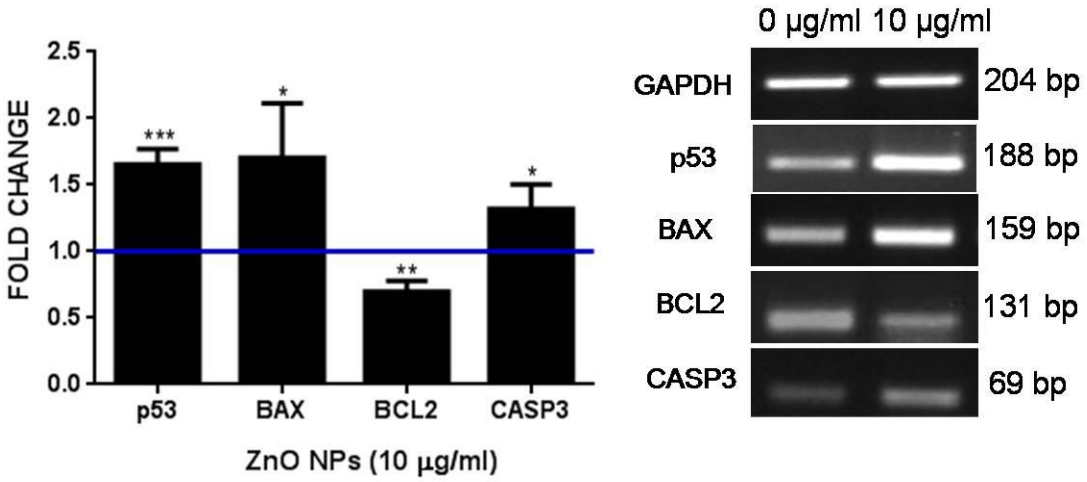


**Figure 2.7:** Semi-quantitative RT-PCR analysis of apoptotic genes (p53, BAX, BCL2, CASP3) in chicken embryonic fibroblast cells treated with ZnO NPs at 10 µg/ml for 1h. Data is normalized with GAPDH. The data are Mean±SD. .*=p≤0.05,**=p≤0.01,***=p≤0.001

## DISCUSSION

The use of NPs has been increasing in various fields and it is important to investigate the toxic effects of NPs on human health and environment. A large number of studies have reported that nanomaterials induce cytotoxicity (Murray *et al.*, 2013; Rajiv *et al.*, 2016; Sharma *et al.*, 2012). The toxicity of NPs was mainly due to its structure, solubility, shape and surface/mass ratio (Greish *et al.*, 2012). Hence, particle characterization is a crucial step because the shape, size, surface area, surface charge, monodispersity affects the physicochemical properties and biological functions of ENPs (Dhawan *et al.*, 2009). DLS was used to determine the size of the particles in suspension (Milli Q water), the results demonstrated that the particles were stable and monodispersed.

Hemolysis testing is a type of acute toxicity screening assay mainly used to evaluate the haemocompatibility of the materials when in contact with blood (Alves *et al.*, 2014). This method rapidly provides a direct quantitative estimate of the sensitivity of nanoparticles on the erythrocyte membrane. During hemolysis, rupture of erythrocyte membrane causes release of cellular contents including haemoglobin. Free haemoglobin released by materials on hemolysis in *vitro*, is read by spectrophotometer and extent of hemolysis can be calculated (Fischer *et al.*, 2003). In this study, red blood cells treated with MgO and ZnO NPs caused hemolysis whereas, $Al_2O_3$, $Fe_2O_3$, $TiO_2$ NPs did not induce hemolysis.

*In vitro* toxicity testing is an alternative method used instead of animal experiments for studying toxicity of nanoparticles (Holder *et al.*, 2012). The NPs damage the cells and stimulate the release of cytoplasmic LDH, which further catalyzes the conversion of the tetrazolium salt to formazan (Vijayakumar & Ganesan, 2012). Similarly in our study,

MTT assay showed that exposure of fibroblast cells to ZnO NPs significantly reduced cell viability in a concentration-dependent manner with 50% cell viability at 10 µg/ml. However, no reduction in cell viability was observed after treating with the other metal oxide nanoparticles ($Al_2O_3$, $Fe_2O_3$, MgO or $TiO_2$). This could be due to the nanoparticles not being internalized possibly due to agglomeration of nanoparticles or nanoparticles interfering with the assay probe molecules causing inaccurate results (Fisichella *et al.*, 2009; Monteiro-Riviere & Inman, 2006). The morphology of CEFs changed from spindle shaped to round after treatment with nanoparticles indicating nanoparticles induced damage to the cells. LDH is an enzyme which is released from the cell due to disruption of its membrane. Leakage of LDH due to cell membrane damage is also a prominent marker of detecting cytotoxicity. LDH leakage was found to increase in astrocytes (Wang *et al.*, 2014) treated with ZnO NPs, similar results were obtained in this study wherein significant increase in LDH was recorded in ZnO treated group.

The level of ZnO NPs induced intracellular oxidative stress was measured by DCF fluorescence as a marker of ROS generation in fibroblast cells. An increase in intracellular ROS generation (green fluorescence) was observed after 1 h of exposure to ZnO NPs which was due to the elevated production of hydrogen peroxide and peroxyl radicals that bring about intracellular oxidation of DCF-DA dye. Oxidative stress as a common mechanism for cell damage induced by nanoparticles is well documented (Heng *et al.*, 2010; R. K. Shukla *et al.*, 2013; R. K. Shukla *et al.*, 2011; Wang *et al.*, 2014).

Healthy cells with functional mitochondria stain with green fluorescent lipophilic Rhodamine 123 dye. ZnO NPs exposed cells exhibited marked impairment of

mitochondria which is observed as decrease in green fluorescence and also change in morphology of the cells. However, the MMP level was maintained in control group. Previous studies have identified ROS as the main cause of mitochondrial damage and initiated mitochondrial mediated apoptosis (Ahmed *et al.*, 2017). Mitochondria are well known organelles responsible for ROS generation in cell. Therefore, it is envisaged that interactions of ZnO NPs with electron transport system of mitochondria may lead to the alterations in MMP, which ultimately cause a consequent increase in the ROS level of cell. Chromatin condensation in fibroblast cells suggest that the ZnO NPs cause cell death via apoptosis and the same has been observed in A375 cells (Alarifi *et al.*, 2013).

It is well-known that oxidative stress leads to cell death, either by apoptosis or necrosis depending on its extent of severity. Accumulating evidences have indicated that nanoparticles can induce apoptosis by targeting the mitochondrial apoptosis pathway, which includes activation loop phosphorylation, cytochrome c release from the mitochondria, decrease in Bcl-2 protein expression, activation of PARP and caspase cascades and DNA fragmentation (Lai *et al.*, 2008; Liu *et al.*, 2010; Sharma *et al.*, 2012). In the presence of DNA damage or cellular stress, p53 triggers cell cycle arrest to provide the time for damage to be repaired or for self-mediated apoptosis (Farnebo *et al.*, 2010). Our data exhibited an increase in the expression of BAX (pro-apoptotic) and decrease in levels of BCL2 (anti-apoptotic). This could be due to increased p53 levels which results in the modulation in the BAX/BCL2 ratio. Also, higher activity of caspase3 was recorded which is capable of autocatalysis as well as cleaving and activating other members of caspase family, leading to rapid and irreversible apoptosis (Jänicke *et al.*, 1998).

The present study systematically demonstrates the role of mitochondrial intrinsic pathway for ZnO NPs induced apoptosis in chicken embryonic fibroblasts which could be attributed to ROS mediated damage. The mechanism for the same is demonstrated in Figure 2.8.

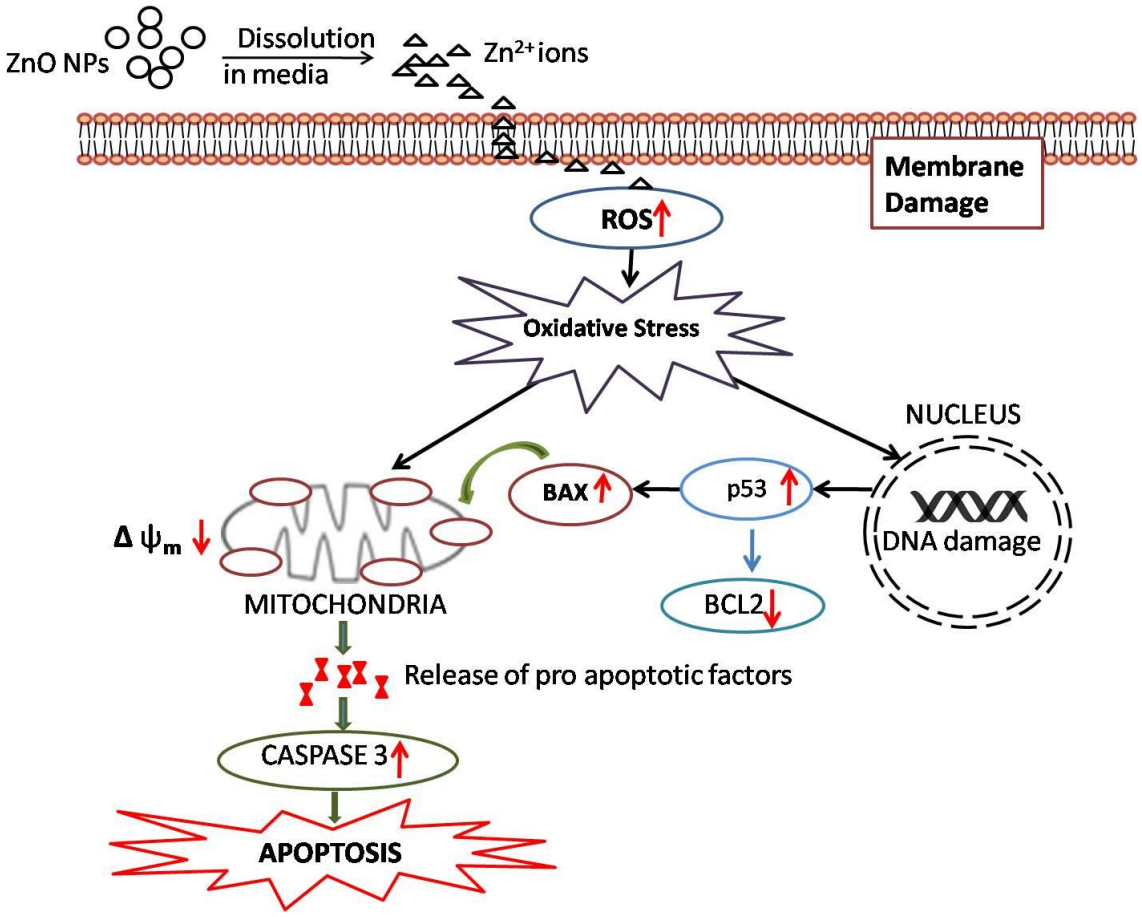


**Figure 2.8:** Possible mechanism of ZnO NPs induced cellular toxicity

## SUMMARY

Increasing exposure of ENPs due to their overuse is a threat to the environment as well as humans. Hence, this study is mainly focused on the adverse effects of metal oxide ENPs to understand the possible mechanism of toxicity in chicken embryonic fibroblasts. Out of five metal oxide nanoparticles ($Al_2O_3$, $Fe_2O_3$, MgO, $TiO_2$ and ZnO), ZnO resulted to be the most toxic with 50% cell death at 10 µg/ml. $Al_2O_3$ NPs accounted for 50% cell death at 500 µg/ml and the others ($Fe_2O_3$, MgO, $TiO_2$) resulted in 50% cell death upto the highest dose (1000 µg/ml). Hence, further studies were conducted on ZnO NPs, which induced toxicity in chicken embryonic fibroblast by distortion of cellular morphology, LDH leakage, mitochondrial dysfunction, increased intracellular oxidative stress and nuclear condensation. ZnO NPs also triggered apoptosis as evidenced by changes in caspase3, BAX, p53 and BCL2 genes.