Synopsis of the thesis entitled

Impact of nanosized particles on an Avian Embryo

Submitted to

The Maharaja Sayajirao University of Baroda,

Vadodara-390 002, Gujarat, INDIA

For the degree of Doctorate of Philosophy

In

Zoology

Ph.D student

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Synopsis of the PhD Thesis **August 2017**

INTRODUCTION:

In nature, Nanosized particles (>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 posses increased structural integrity as well as unique mechanical, optical, chemical, electrical and magnetic properties (Günter 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. Nanomaterials 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 is unique with respect to its properties and toxicity, it becomes imperative to investigate their toxicity in living systems.

Nano-sized metal oxides were more toxic than their bulk metal oxide counterparts. Hence, nanomaterial toxicity studies conducted so far have raised ample concern 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. The influence of the release of nanomaterials into the environment is relatively unknown and has recently become a focus in nanomaterials research

(Unesco, 2006). Lockman *et al.* (2003) and Günther Oberdörster *et al.* (2004) found that a decrease in particle size changed the structural and physicochemical properties of nanomaterials and increased its bioavailability and toxicity. They reported that nanoparticles can cross strong biological barriers such as the blood-brain barrier. Titanium dioxide nanoparticle (TiO_2 NP) is an important industrial material that is widely used as an additive in cosmetics, pharmaceuticals, and food colorants. Many consumer products such as sunscreens contain nanoparticles of metal oxides (TiO_2 and ZnO). Mice injected with TiO_2 NPs have shown signs of acute toxicity such as passive behaviour, loss of appetite, tremor and lethargy (Chen *et al.*, 2006). Sharma *et al.* (2012) showed cytotoxic effects and mitochondrial dysfunction after exposure to zinc oxide nanoparticles. Superparamagnetic iron oxide nanoparticles (SPION) are widely used in the biomedical field and have multiple mature and emerging applications such as magnetic resonance imaging (MRI) contrast agents, cell separation media, drug delivery carriers, and cancer hyperthermia (Krishnan, 2010; Pankhurst *et al.*, 2003). Apart from these, SPION are intensively explored in neuro-medicine because they can cross the blood−brain barrier (BBB)(Wang *et al.*, 2010). Zhang and Wang (2008) have reported subtle changes in cytotoxicity and genotoxicity of SPIONs with different sizes and functional groups.

There is limited understanding about the adverse effects of nanomaterials on nature and wildlife (Smith *et al.*, 2007). Though, there are a few studies that have investigated the impact of nanoparticles (NPs) on some species of invertebrates (Heinlaan *et al.*, 2008; Hu *et al.*, 2010; Wiench *et al.*, 2009) and vertebrates (Bai *et al.*, 2010; Kim *et al.*, 2005; Lei *et al.*, 2008), there are very few reports on their impact on an avian system. Ability of NPs to cross strong biological barriers such as the blood brain barrier renders the wild species of birds vulnerable to the NPs. The nanoparticles that make way into the natural environs and enter the pristine aquatic habitats render several species of birds especially the plankton feeders at a higher risk.

DEFINITION OF THE PROBLEM:

The proposed project aims at assessing the possible impact of popularly used industrial nanoparticles on an avian embryo. Fertilized eggs were subjected to

various doses of metal oxide NPs to decipher intricacies related to their entry into an avian embryo. Impact of nanoparticles on embryonic development was assessed by standardized protocols. This study includes assessment of nanoparticles induced tissue oxidative stress, DNA damage or teratogeny. In a nutshell, proposed study investigates the intricacies of avian- nanoparticle interaction at an embryonic level.

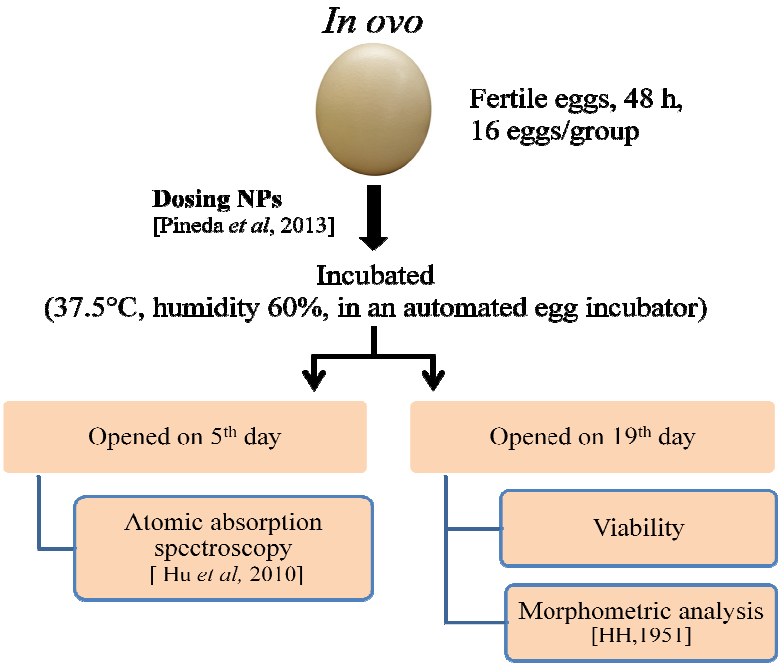
RELEVANCE OF THE WORK:

Development of an avian embryo and production of a progeny is the essence of survival of an avian species in the wild. After all, lack of production of a progeny by a particular bird species can be a crucial factor for its speedier extinction. NPs are known to cross strong biological barriers and hence it is assumed that it can possibly cross an avian eggshell also. Therefore, the ground and marsh nesting birds are a high risk group of species susceptible to the impact of NPs. Other studies have also reported impact of NPs on avian embryonic tissue such as the bursa of fabricius (Grodzik & Sawosz, 2006). These facts warranted a detailed study to decipher avian- nanoparticle interaction. Hence, the proposed study answers basic questions pertaining to avian- nanoparticle interaction in terms of its impact on avian embryonic development.

OBJECTIVES:

- Toxicological evaluation of NPs on chick embryo and chicken embryonic fibroblasts.
- Physicochemical and particokinetic evaluations of NPs
- To identify toxicity targets during NP-avian embryo interaction.
- To assess the ability of chosen NPs in crossing *in ovo* biological barriers.

1. Toxicological evaluation of NPs on chick embryo and chicken embryonic fibroblasts



The metal oxide nanoparticles viz. TiO_2, ZnO, Fe_2O_3, Al_2O_3 and MgO were screened for its impact on an avian egg (white leghorn, *Gallus gallus domesticus*). The eggs were procured from Shakti hatcheries, Sarsa, Gujarat, stored for 2 days at 12°C, and then incubated under standard conditions for 48 hours. The nanoparticles were suspended in normal saline (0.72% sodium chloride only) and injected (10-1000 µg/ml) in the air space of the egg and further incubated until day 19 of development. On 19th day, the experiment was terminated by opening the eggs and the viable embryos were weighed and decapitated. The morphology of the embryos was examined according to the Hamburger and Hamilton (1951) standards and ratios of live vs. dead/malformed were recorded. External malformations (of the head, limbs, body and tail) were observed under a dissecting microscope and photographed with a digital (Nikon coolpix p900) camera. The malformed embryos were processed for Alizarine-alcian blue staining (Lamb *et al.*, 2003).

The results showed that TiO_2 nanoparticles showed deformity in the form of omphalocele formation, haemorrhage and flexed limbs at the lower doses (10

and 25 µg/ml). Along with the deformity, there was overall reduction in the weight and the length of the embryos. Also, the numbers of mortal embryos were higher at the above mentioned doses. The other nanoparticles (ZnO, Al_2O_3 and MgO) did not cause any deformity but there was a reduction in the length and weight of the embryo and a greater percentage of mortality at all the doses as compared to the control. Fe_2O_3 nanoparticles did not account of deformities but at 200µg/ml dose there was 100% mortality.

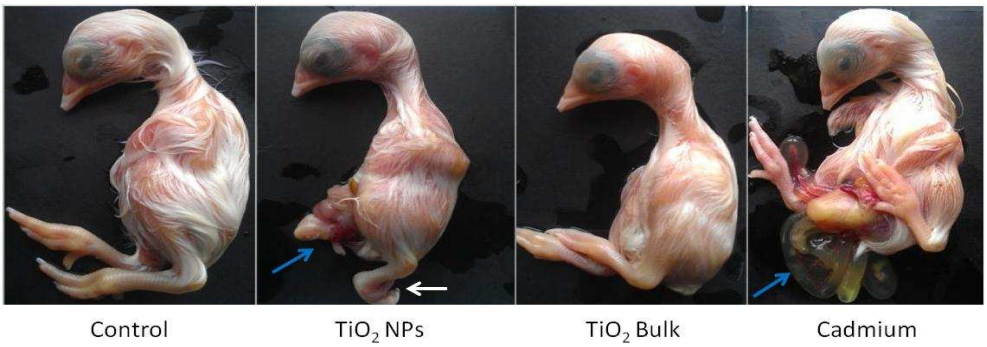


FIGURE 1: *In ovo* toxicity of TiO_2 nanoparticle. Embryos (**19 day old**) treated with 10 ppm TiO_2 NPs exhibits omphalocele with some viscera externally located (blue arrow) & flexed limbs (white arrow).

Chicken embryonic fibroblasts were cultured according to Hernandez and Brown (2010). MTT assay was performed to assess the viability of the cells. ZnO nanoparticles showed maximum cell death, inhibitory concentration being at 10µg/ml. The other nanoparticles did not show much of toxicity as they agglomerated and could not enter the cells. Also, the nanoparticles reacted with the dye and showed false positive results.

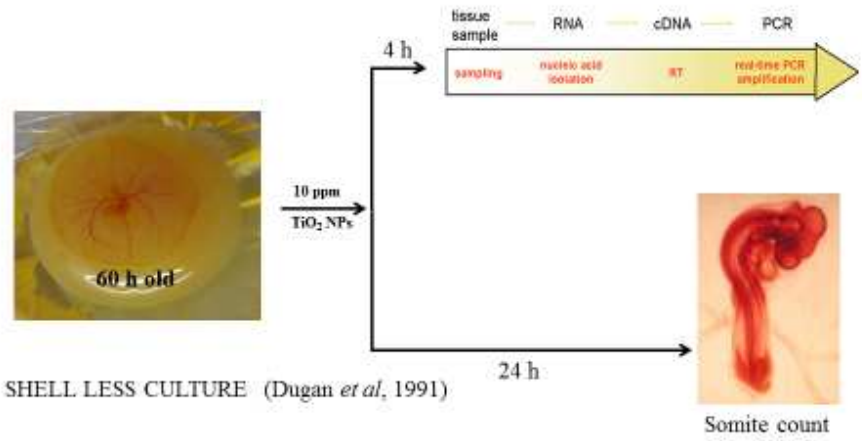
2. Physicochemical and particokinetic evaluations of NPs

The physicochemical properties such as density, viscosity, surface tension, activation energy, and friccohesity elucidate dispersion activities of nanoparticles in water and saline. Densities were determined with Anton Paar Density and Sound velocity Meter (DSA 5000 M). Three mL suspension was filled in DSA Quartz U tube. Pendent drop numbers (PDN) and viscous flow times (VFT) were measured with Borosil Mansingh Survismeter (BMS) for assessment of surface tension and viscosity measurements respectively. To maintain physiological

temperature of 37 °C, an auto temperature control LAUDA ALPHA RA 8 thermostat was used. In TiO_2 nanoparticles, the hydrodynamic radii increased with the concentrations indicating that the particles were no longer in the nano form and hence resulting into toxicity at the lower doses rather than at the higher ones. Also, the nanoparticles agglomerated at higher doses and did not interact with saline, which also could be a reason for toxicity at the lower doses.

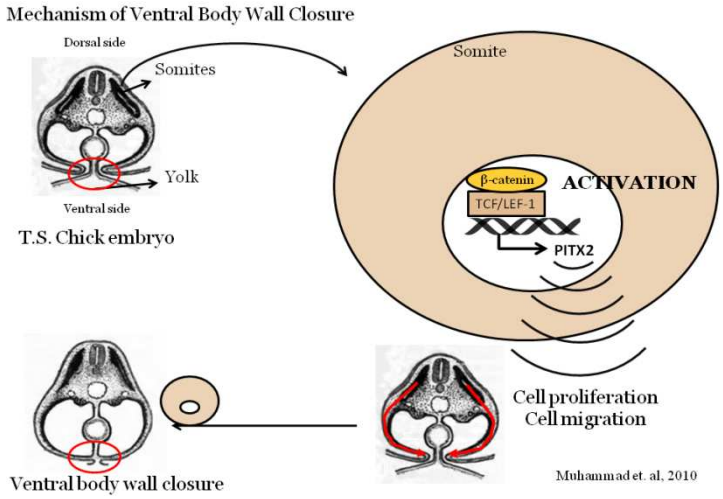
3. To identify toxicity targets during nanoparticle- avian embryo interaction

To understand better how TiO_2 nanoparticles interacted with the egg and caused deformities a separate experiment was performed in which procured eggs were incubated for 60 h in standard conditions and later were explanted into shell-less culture as per Dugan *et al.* (1991).

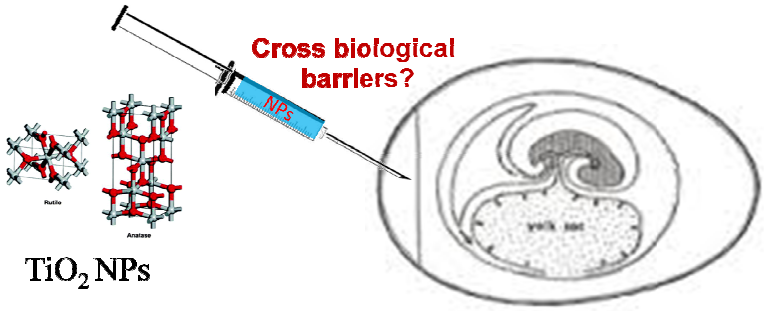


The deformities observed after the treatment of TiO_2 nanoparticles were omphalocele formation, flexed limbs and hemorrhage. Omphaloceleis a midline defect with herniation of abdominal contents. It is therefore a primary failure of fusion of the ventral body wall. It has been postulated that one of the potential contributors in movement of the lateral body folds are derived from somites. Somite derived cells migrate into the parietal layer of LPM to assist in forming the lateral body folds. PITX2, a bicoid-related homeobox gene, is expressed in the somite and lateral plate mesoderm during embryogenesis. It has been known to be regulated by ß-catenin dependent Wnt pathway. In the Wnt/ß-catenin pathway, the accumulation of ß-catenin in the nucleus converts DNA-binding factor, lymphoid enhancing factor-1 (LEF1), to a transcriptional activator. Downregulation of PITX2 may induce abnormal apoptosis in the somite and

LPM, interfering with the movement of the lateral body wall folds ventrally. Downregulation of LEF1 genes may interfere with ventral body wall formation in chick embryo causing omphalocele by disrupting somite myogenesis. ß-catenin gene is known to be a key component of adherens junctions and its downregulation causes omphalocele by distrupting the calcium signaling and Adherens junctions. Rho-associated coiled-coil containing protein kinase I (ROCK-I) and ROCK-II mediate actin-cytoskeleton assembly, cellular adhesion, and cell death in the Wnt/PCP pathway. In this study, downregulation of ROCK-I and ROCK-II gene expressions may cause omphalocele by disrupting Wnt non-canonical pathway. Recently, it has been demonstrated that Wnt11, a member of the noncanonical Wnts, is an important epithelialization factor acting on the dermomyotome interacting with Wnt6 and the noncanonical Wnt pathway activated by Wnt11 may be associated with ventral body wall formation. Calcium activates CaMKII that phosphorylates the light chain, initiating actin-myosin interaction. Protein kinase C and CaMKII control actin-cytoskeleton organization and cell contractility, and thereby contribute to several physiologic processes, including tissue closure. Downregulation of Wnt11, PKCα and CaMKII gene expression during the narrow window of early embryogenesis may cause omphalocele, interfering with cell movement and adhesion, disrupting Wnt/Ca2+ pathway. Also, the somite numbers were counted after 24 h of treatment with nanoparticles and there was a significant reduction in the number of somites in the lumbosacral region in the treated embryos as compared to control.



4. To assess the ability of NPs in crossing *in ovo* biological barriers



There are three barriers in the avian egg namely the egg shell, shell membrane and the chorioallantoic membrane and the main aim of the study was to check whether the nanoparticles could cross these biological barriers. In this study, the eggs were immersed in nanoparticle solution (TiO_2), injected in the airspace or albumen, incubated for 4 days and then the embryos were processed for inductively coupled plasma atomic emission spectroscopy (ICP-OES) (Hu *et al.*, 2010). The amount of NPs crossing biological barriers and reaching the developing embryo was quantified by ICP-OES. A dose dependent increment was recorded with the highest dose of 500 µg/ml accounting for 0.06 µg/ml reaching chick embryo and the lowest dose 100 µg/ml accounting for 0.04 µg/ml following injections of NPs in albumen. Whereas, dosing of nanoparticles in the air space resulted in 0.04 µg/ml and 0.29 µg/ml accumulation at 500 and 100µg/ml doses respectively. Eggs when immersed in NPs solution resulted in reduction in size of embryos and deformed embryos indicating at their ability to cross the shell and the shell membranes.

CONCLUSION:

Our study concludes that all the metal oxide nanoparticles used for the study caused an impact on the overall morphometry of the developing embryo. TiO_2 NPs are the most toxic and cause deformities like omphalocele, flexed limbs and brain haemorrage. Omphalocele is caused by disrupting somite myogenesis. In our study, IONs were found to interact with egg albumen as evidenced by the observed changes in the secondary structure of IONs + egg albumen. These interactions could possibly allow IONs to traverse the biological barriers within

chicken egg and reach the brain. *In vitro* studies with the chicken embryonic fibroblasts reveal the effect of nanoparticles on cell viability, wherein, Zinc oxide nanoparticles were the most toxic. The results of density, viscosity and friccohesity measurement show that intermolecular interactions of TiO_2 NPs with albumen and saline are higher as compared to water, indicating greater dispersion and molecular interaction. Also, immersion studies showed the presence of nanoparticles in the embryo indicating that the nanoparticles can cross the biological barriers. Hence, metal oxide nanoparticles should be discarded properly to avoid harming the ground nesting birds.

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CONFERENCE PRESENTATIONS:

- Annual meeting of the Society for Reproductive Biology and Comparative Endocrinology; Holy cross College, Trichy, 7-9th January, 2014.
- International Conference On Bioactive Chemicals For Reproduction And Human Health & 33rd Annual Meeting Of The Society For Reproductive Biology And Comparative Endocrinology; Davangere University, Davangere, 26-28th February 2015.

- Abstract accepted for poster presentation at "11th International conference on the Environmental Effects of Nanosized particles and Nanomaterials",august 14-18, 2016, U.S.A.
- Received the Prof. N. J. Chinoy (third prize) award for Oral presentation on paper entitled "**TiO_2 nanoparticles induces Omphalocele formation in chick embryo: A focus on ß-Catenin, PITX2 and LEF1"** at the "International Conference on Reproductive Biology and Comparative Endocrinology and 35th Annual Meeting of the Society for Reproductive Biology and Comparative Endocrinology" held during 9-11th February, 2017 organized by the dept. of Animal biology, University of Hyderabad, Hyderabad, India.
- Oral presentation on "Collaborative research in biological sciences: Our journey so far" at the science conclave-2017 on science day (28th February) organized by the faculty of science, The Maharaja Sayajirao University of Baroda, Vadodara.

PAPERS PUBLISHED:

- Jana, Sarmita, Dipak Patel, **Shweta Patel**, Kapil Upadhyay, Jaymesh Thadani, Rahul Mandal, Santasabuj Das, and Ranjitsinh Devkar. "Anthocyanin rich extract of Brassica oleracea L. alleviates experimentally induced myocardial infarction." *PloS one* 12, no. 8 (2017): e0182137.
- Solanki, Ankita, Mehul H. Sadhu, **Shweta Patel**, Ranjitsinh Devkar, and Sujit Baran Kumar. "Ternary complexes of copper (II) and cobalt (II) carboxylate with pyrazole based ligand: Syntheses, characterization, structures and bioactivities." *Polyhedron* 102 (2015): 267-275.
- Sharma, Radhika, Shubhangi S. Soman, **Shweta V. Patel**, and Ranjitsinh V. Devkar. "Studies in the synthesis and applications of 4-(aminomethyl) quinolin-2 (1H)-one derivatives as anti-cancer agents.

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