CHAPTER 3

In ovo toxicity assessment and HET-CAM analysis of metal oxide nanoparticles

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INTRODUCTION

Nanoparticles are emitted into the environment by primary sources such as natural phenomena, combustion processes or industrial activities (e.g. welding) or are released during generation and handling of engineered NPs. As NPs are transported through the environment they could be toxic to the animals. There are a few studies that have been performed which adequately address the toxicity of ENPs. The results of some of the studies that have been performed to test the toxicity of the ENPs are summarized below. The animal models mostly used are invertebrates, nematodes, earthworms, fish and mammals.

Crustacea: TiO_2 nanoparticles have been reported to cause acute toxicity in *Daphnia magna* and *Thamnocephalus platyurus*. Of the total *D. magna* cells exposed to a 20 mg/L concentration, 60% showed toxic effects, whereas marginal toxic effects were observed in *T. platyurus* after exposure to TiO_2 NPs (Baun *et al.*, 2008). *D. magna, T. platyurus* and *Tetrahymena thermophila* were also tested for toxic responses to metal oxide nanoparticles (ZnO and CuO) (Blinova *et al.*, 2010). In natural waters, LC50 values for ZnO NPs were lower than those of CuO nano- and bulk-particles. The acute toxicity of TiO_2 NPs (6 nm) and Al NPs (100 nm), was tested on *Daphnia magna* and *Vibrio fischeri* (Strigul *et al.*, 2009). TiO_2 displayed low toxicity to *D. magna*, the LD50 (48 h) value for TiO_2 in *D. magna* was 107.88 mg/L. This study also reported that nano-aluminium is more toxic than its bulk counterparts. An acute toxicity study was conducted with C_{60} and TiO_2 nanoparticles (48 h) on *D. magna* (Lovern & Klaper, 2006). Results indicated that

fullerene (C_{60}) was more toxic than TiO_2 NPs and a dose-dependent increase in mortality was observed.

Nematodes: *Caenorhabditis elegans* is the most commonly studied nematode for testing toxicity of nanoparticles. H. Wang *et al.* (2009) evaluated the toxicity of both nano and bulk forms of ZnO, Al_2O_3 and TiO_2 in *C. elegans*. Results indicated that LC50 values for ZnO nano and bulk forms were the same (2.3 mg/L). However, LC50 values for Al_2O_3 and TiO_2 bulk were almost double than that of their nano counterparts.

Earthworms: Heckmann *et al.* (2011) has assessed the toxicity of three pure metal-based nanoparticles (Ag, Cu and Ni), four metal oxide nanoparticles (Al_2O_3, SiO_2, TiO_2, and ZrO_2) and their bulk counterparts (i.e., metal and metal oxides) in earthworms (*Eisenia fetida*). The Ag NPs, Cu NPs, TiO_2 NPs and all their metal salts induced reproductive toxicity. Accumulation of ZnO NPs was reported in organelles and in cytosol of earthworms (*Eisenia fetida*). Also, increased SOD (Superoxide dismutase), CAT (catalase) and GSH-px (Glutathione peroxidase) activities occurred in worms treated with the NPs (Li *et al.*, 2011). Effects of different concentrations of TiO_2 and ZnO NPs on earthworms (*E. fetida*) were studied (C. Hu *et al.*, 2010). No significant change was observed in SOD activity, whereas CAT activity decreased with the increase in NP concentration. DNA damage was reported at the higher doses and ZnO NPs accumulated in the earthworms and caused cellular and organelle damage.

Fish: TiO_2 nanoparticles elicited a respiratory response in the fish and caused sublethal effects (Federici *et al.*, 2007).

Mammals: Handy and Shaw (2007) performed respiratory studies in rats with carbon nanotubes, ultrafine TiO_2 NPs, ultrafine cadmium particles (70 μg/L), and metal oxide

particles (1–5 mg). Results indicated significant lung damage, inflammation and fibrotic responses after intra-tracheal exposure to the said NPs. Intraperitoneal injection of polyalkyl sulphonated C_{60} to rats induced toxicity due to the accumulation of NPs in liver, spleen and kidney (Chen *et al.*, 1998). Gastrointestinal dosing in mice (B. Wang *et al.*, 2008), with either ZnO NPs or larger particles resulted in more severe symptoms (lethargy, vomiting, diarrhea and deaths) being produced by the nano-treated group. A significant decrease in lung function was observed by Conner *et al.* (1988) in guinea pigs that had inhaled zinc oxide nanoparticles (50 nm).

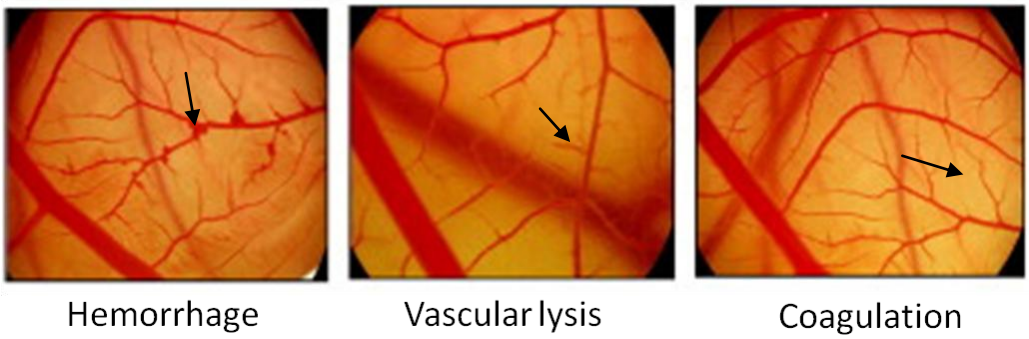
In this study, chicken embryo (*Gallus gallus domesticus*) has been used as an experimental model. Domestic chickens are important model organisms because they bridge the evolutionary gap between mammals and other vertebrates and their genome was the first to be sequenced among all agricultural animals (Hillier *et al.*, 2004). Due to the experimental advantages of *in ovo* embryogenesis, the embryo of the chicken is a useful vertebrate system for the developmental biologists and widely used experimental model for assessment of toxicity, developmental deformity, physiological alterations, drug delivery, etc. (Stern, 2004).

MATERIALS AND METHODS

HET-CAM Test (Hen's egg test-chorioallantoic membrane)

Fertile eggs (54-58g) of white leghorn were procured from nearby poultry farm and incubated at 37.7°C and 60% humidity. The eggs were incubated for 10 days and rotated manually every 3-4 hours to prevent the attachment of the embryo to one side of the egg and also checked for viability by candling the eggs, non-viable eggs were discarded. Eggs

were divided into 7 groups viz. control (0.9% NaCl solution), positive control (0.1 N sodium hydroxide) and metal oxide nanoparticles treated (1mg/ml). The egg shell was cut just above the air space and inner shell membrane was carefully moistened with 0.9% NaCl solution and carefully removed without injuring any underlying blood vessels using tapered forceps. The standards and test solution were freshly prepared before the assay at room temperature. 0.3 ml of the test chemical was added to the CAM and changes on the CAM were observed over a period of 5 minutes (Luepke & Kemper, 1986). The CAM was monitored for:



Chicken Embryo model and experimental groups

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 48 hours 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). Candling was done to confirm the fertility of eggs and unfertile eggs were discarded. Eggs were randomly divided into nine groups of 16 eggs/group viz. control (untreated), placebo (treated with normal saline) and NPs (TiO_2, Fe_2O_3, ZnO, MgO or Al_2O_3) treated (10-1000 µg/ml) groups. NPs powder was suspended in saline (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 and injected *in ovo* (0.3 ml/egg) in airspace using sterile 1 ml tuberculin syringe and incubated for 18 days.

Autopsy and Morphometry

On 19^{th} day, the experiment was terminated and shell of control and treated eggs were cut at the broad end by sterile scissors and inner shell membrane was removed to expose the embryo. Viable embryos were removed and made free of placental tissue and fluids prior to weighing (MS-600, American weigh scales Inc., GA, USA). Later, the embryos were examined according to the Hamburger and Hamilton (1951) standards and possible malformations (of the head, limbs, body and tail) were observed under a dissecting

microscope. Ratio of live vs. dead/malformed embryos was also recorded. Lengths for each embryo were recorded and all embryos were photographed with a digital (Nikon coolpix p900) camera. Later, embryos were decapitated and organs (brain, heart, liver and spleen) were dissected, weighed and processed for histopathology.

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

HET-CAM test

CAM of fertilized eggs were treated with metal oxide nanoparticles and observed for changes in the blood vasculature such as hemorrhage, lysis or coagulation. Results showed that Fe_2O_3 NPs caused hemorrhage to the blood vessels whereas; the other metal oxide nanoparticles (TiO_2, ZnO, Al_2O_3 and MgO) did not cause hemorrhage, lysis or coagulation. Qualitative data depicting the same is seen in Figure 3.1.

Natality and Morphometry of Chicken embryos

Lower doses (10 and 25 µg/ml) of TiO_2 NPs treatment accounted for 12.5% and 25% viable embryos respectively on 19^{th} day of incubation. Also, 56.25% and 43.75% embryos were found to be malformed at 10 and 25 µg/ml doses (Figure 3.2). However, higher doses (50 - 1000 µg/ml) recorded viable embryos ranging between 75-87.5% (Figure 3.3A). Morphometry of the embryos (whole weight and length) recorded significant decrement at all the doses (10-1000 µg/ml) (Figure 3.3B). Whole weights of liver, brain and heart showed non-significant decrement at all the said doses (Table 3.1). Fe_2O_3 NPs treated eggs recorded ~50% mortality at 10-1000 µg/ml doses but, 200 µg/ml accounted for 100% mortality (Figure 3.4A). Body weight and length of viable embryos revealed significant decrement at 50 and 100 µg/ml doses (Figure 3.4B). Gravimetry of liver, brain, heart and spleen revealed non-significant decrements in their weights at all doses of Fe_2O_3 NPs (Table 3.2). ZnO NPs treated eggs recorded 30-40 % mortality at 10-1000 µg/ml doses (Figure 3.5A). Body weight and length of viable embryos revealed significant decrement at 50- 200 µg/ml doses (Figure 3.5B). Gravimetry of liver, brain, heart and spleen revealed non-significant decrements in their weights at all doses of ZnO NPs

(Table 3.3). MgO NPs treated eggs recorded 50-60 % mortality at 10-1000 µg/ml doses (Figure 3.6A). Body weight and crown-rump length of viable embryos revealed significant decrement at 25-1000 µg/ml doses (Figure 3.6B). Gravimetry of liver, brain, heart and spleen revealed non-significant decrements in their weights at all doses of MgO NPs (Table 3.4). Al_2O_3 NPs treated eggs recorded 30-40 % mortality at 10-1000 µg/ml doses but, 200 µg/ml accounted for 100% mortality (Figure 3.7A). Body weight and crown-rump length of viable embryos revealed no decrement at 10-1000 µg/ml doses (Figure 3.7B). Gravimetry of liver, brain, heart and spleen revealed non-significant decrements in their weights at all doses of Al_2O_3 NPs (Table 3.5).

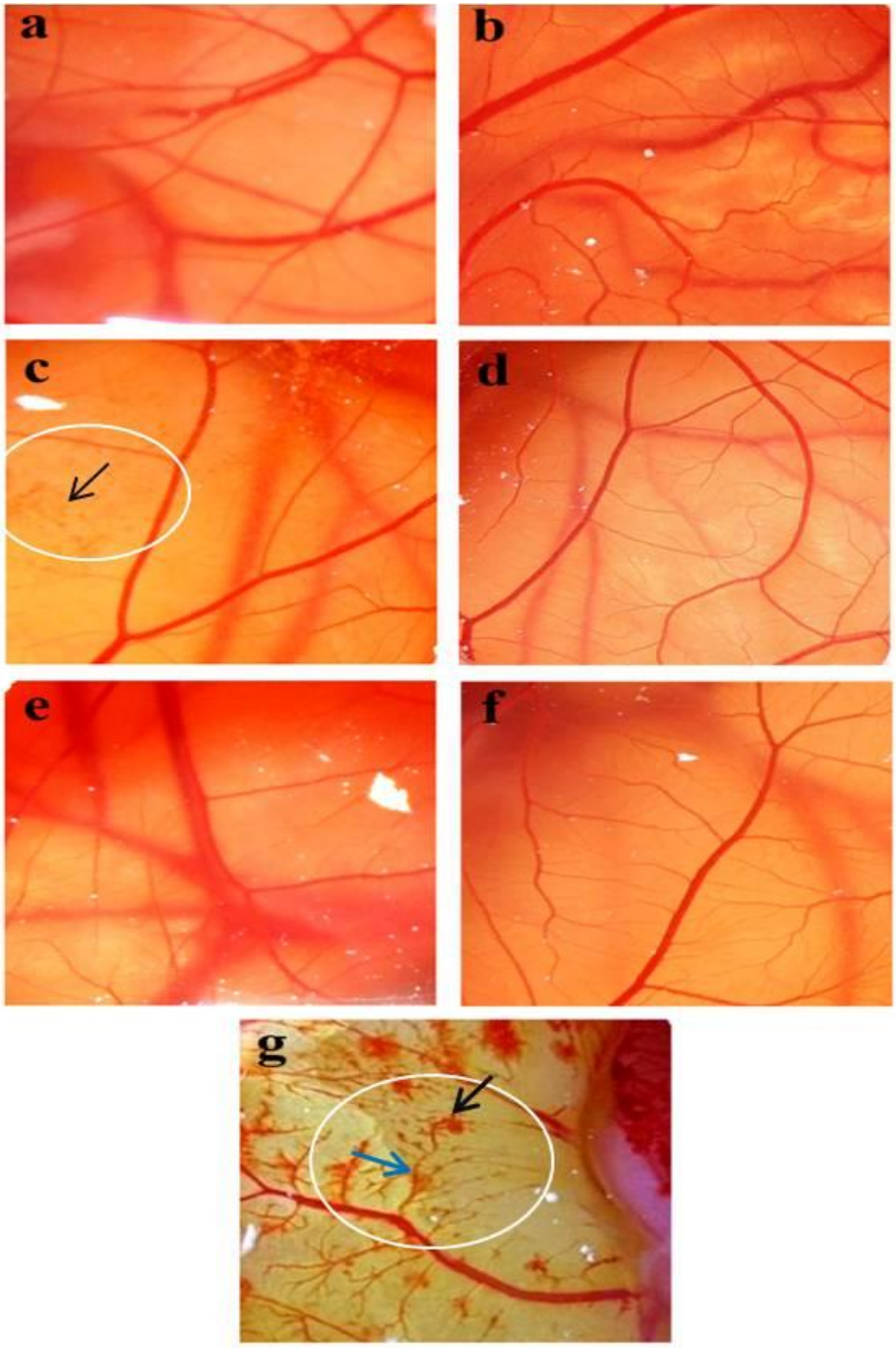
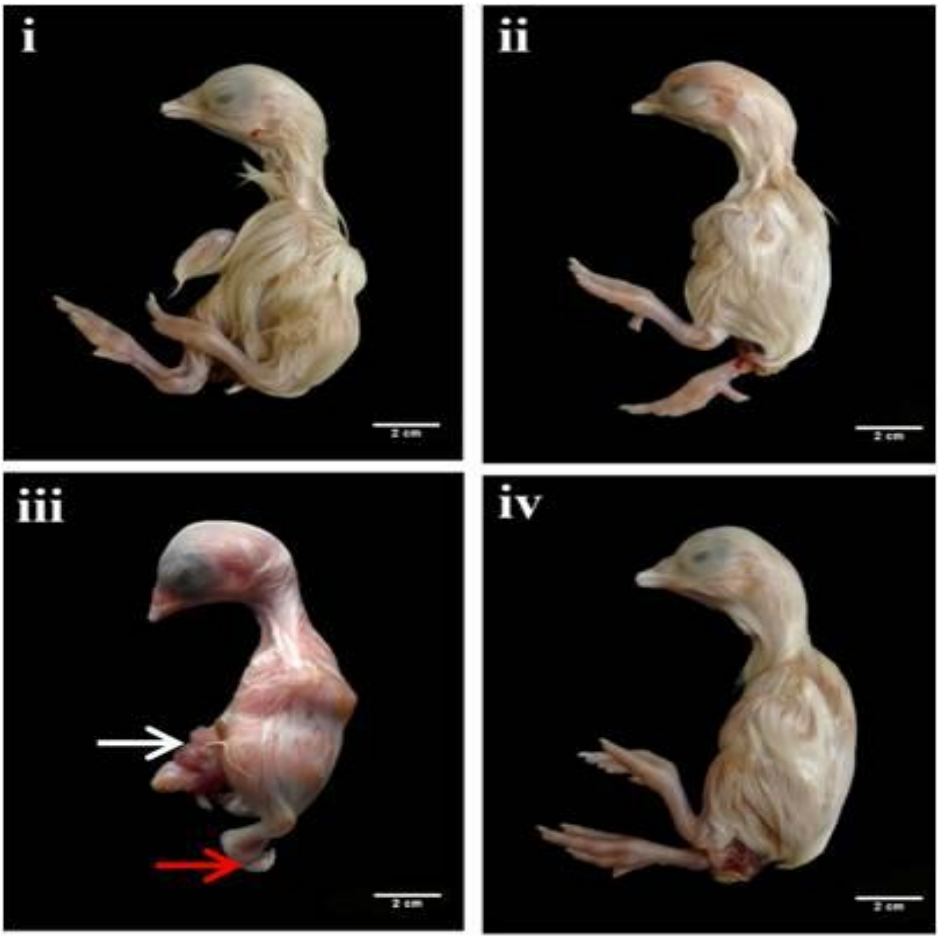


Figure 3.1: Reaction of CAM to metal oxide nanoparticles exposure. a- control, (b-f) TiO_2, Fe_2O_3, ZnO, MgO and Al_2O_3 NPs treated, g- positive control (0.1 N NaOH). Blue arrow indicates lysis of blood vessels and black arrow indicates hemorrhage.



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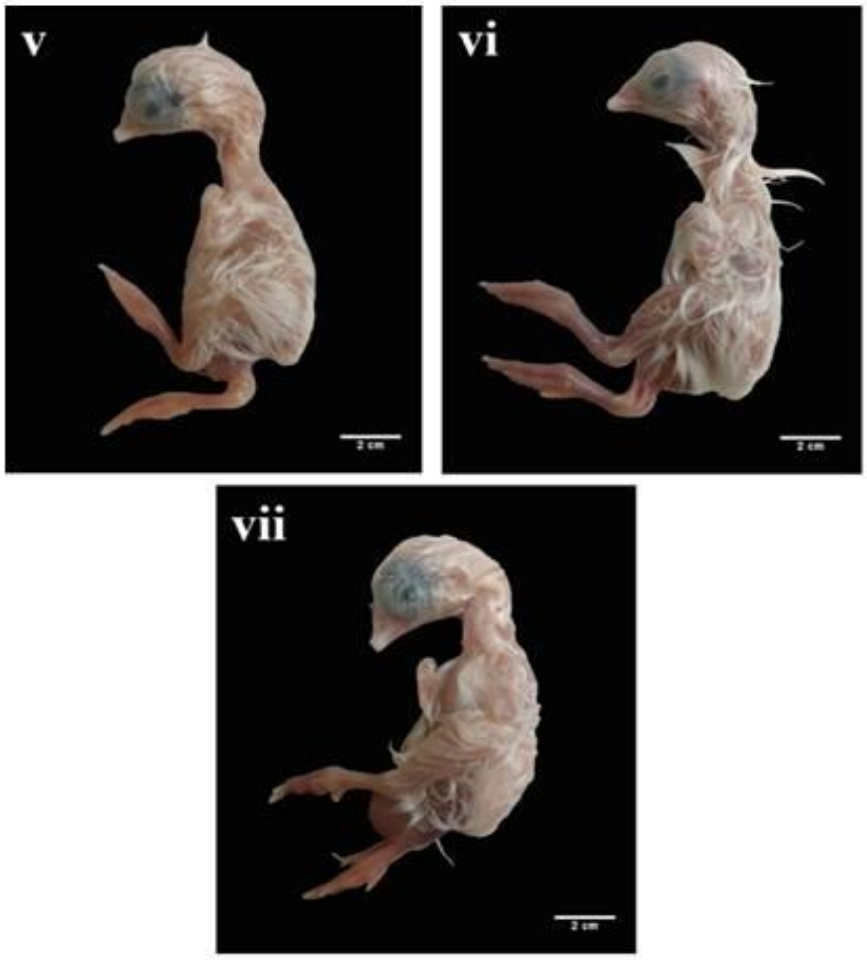

Figure 3.2: Chicken embryonic development after 19 days of incubation in metal oxide nanoparticles treated groups. i-control, ii-placebo, TiO_2, Fe_2O_3, ZnO, MgO and Al_2O_3 NPs (iii-vii). (Scale bar: 2 cm). Embryos treated with 10 µg/ml TiO_2 NPs (iii) exhibits omphalocele (white arrow) & flexed limbs (red arrow).

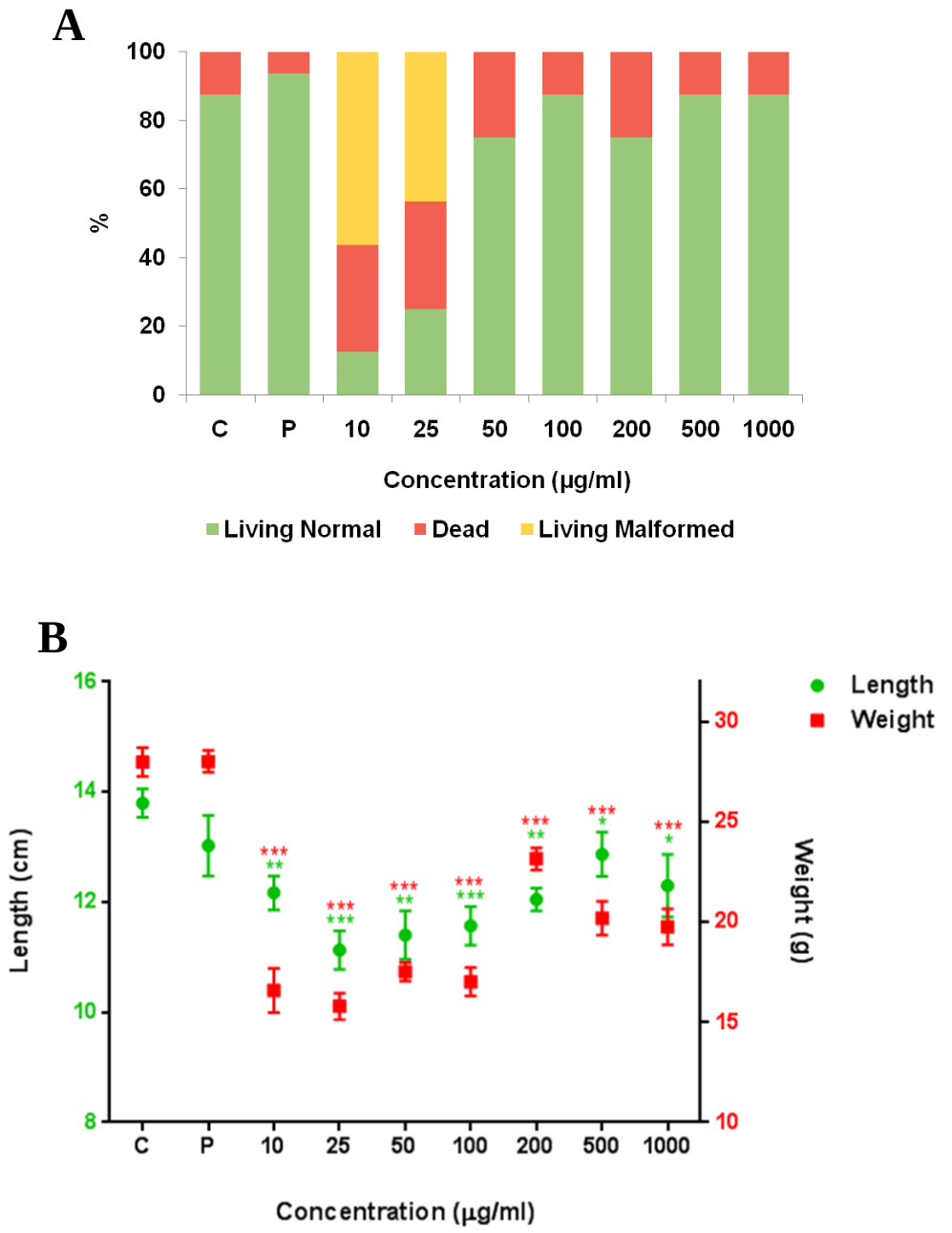

Figure 3.3: (A) Percentages of living normal, dead and malformed embryos, (B) Mean body weight (g) and lengths (cm) of living 19-day-old chicken embryos in control and treated groups. The data are expressed as Mean±SD. Statistical analysis was done by one way ANOVA, *=p ≤ 0.05, **p< 0.01, ***p< 0.001, C- control, P-placebo, TiO_2 NPs -treated groups (10-1000 µg/ml).

Table 3.1 : Organ weights of chicken embryos treated with Titanium oxide nanoparticles

	Control	Placebo	10 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml	500 µg/ml	1000 µg/ml	p value
No. of Viable embryos	12	13	2	3	10	12	10	12	11	-
Liver, g	0.48±0.05	0.49±0.04	0.35±0.08	0.43±0.04	0.44±0.10	0.42±0.04	0.50±0.04	0.43±0.04	0.44±0.04	ns
Brain, g	0.43±0.10	0.40±0.04	0.42±0.07	0.34±0.03	0.39±0.07	0.31±0.12	0.41±0.04	0.29±0.07	0.32±0.10	ns
Heart, g	0.17±0.03	0.15±0.03	0.15±0.03	0.16±0.02	0.16±0.02	0.16±0.01	0.16±0.02	0.17±0.01	0.18±0.01	ns
Spleen, g	0.02±0.01	0.01±0.00	0.01±0.00	0.02±0.01	0.01±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00	ns

Mean organ weights of embryos that were alive and without deformities in control and TiO_2 NPs -treated groups (10-1000 µg/ml). The data are expressed as Mean±SD. Statistical analysis was done by one way ANOVA. ns- non significant.

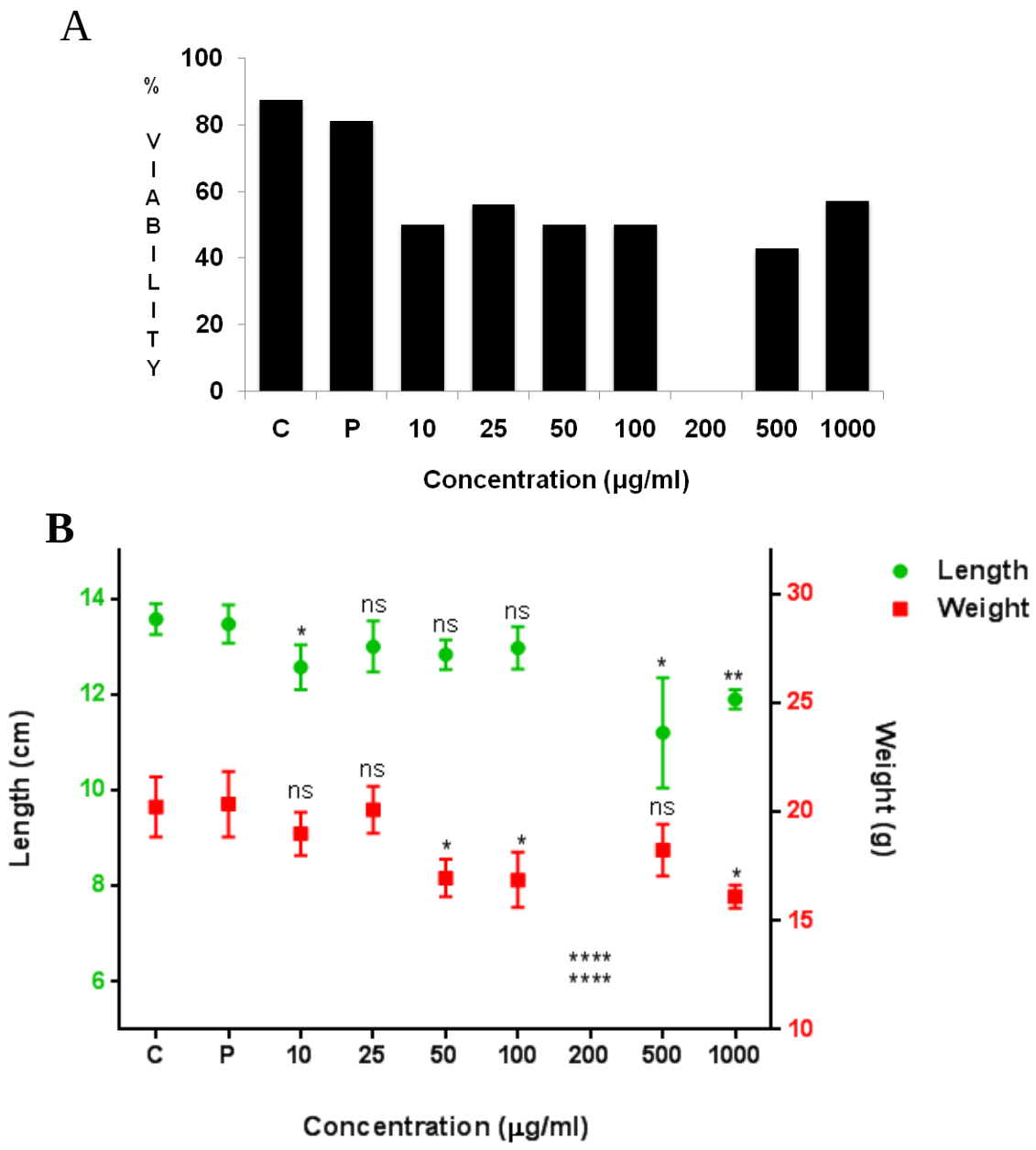

Figure 3.4: (A) Percentage viability, (B) Mean body weight (g) and lengths (cm) of living 19-day-old chicken embryos in control and treated groups. The data are expressed as Mean±SD. Statistical analysis was done by one way ANOVA, *=$p \leq 0.05$, ****=$p \leq 0.0001$, ns- non significant, C-control, P-placebo, Fe_2O_3 NPs-treated groups (10-1000 µg/ml).

Table 3.2 : Organ weights of chicken embryos treated with Iron oxide nanoparticles

Organs	Control	Placebo	10 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	500 µg/ml	1000 µg/ml	p value
No. of Viable embryos	14	13	8	9	8	8	6	8	-
Liver, g	0.50±0.04	0.49±0.07	0.34±0.09	0.42±0.03	0.43±0.09	0.42±0.07	0.43±0.08	0.43±0.07	ns
Brain, g	0.45±0.10	0.41±0.04	0.39±0.10	0.35±0.03	0.38±0.08	0.30±0.08	0.40±0.08	0.31±0.07	ns
Heart, g	0.17±0.03	0.16±0.01	0.14±0.02	0.16±0.02	0.16±0.02	0.14±0.01	0.17±0.01	0.16±0.01	ns
Spleen, g	0.02±0.01	0.02±0.00	0.01±0.00	0.02±0.01	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00	ns

Mean organ weights of embryos that were alive and without deformities in control and Fe_2O_3 NPs-treated groups (10-1000 µg/ml). The data are expressed as Mean±SD. Statistical analysis was done by one way ANOVA. ns- non significant.

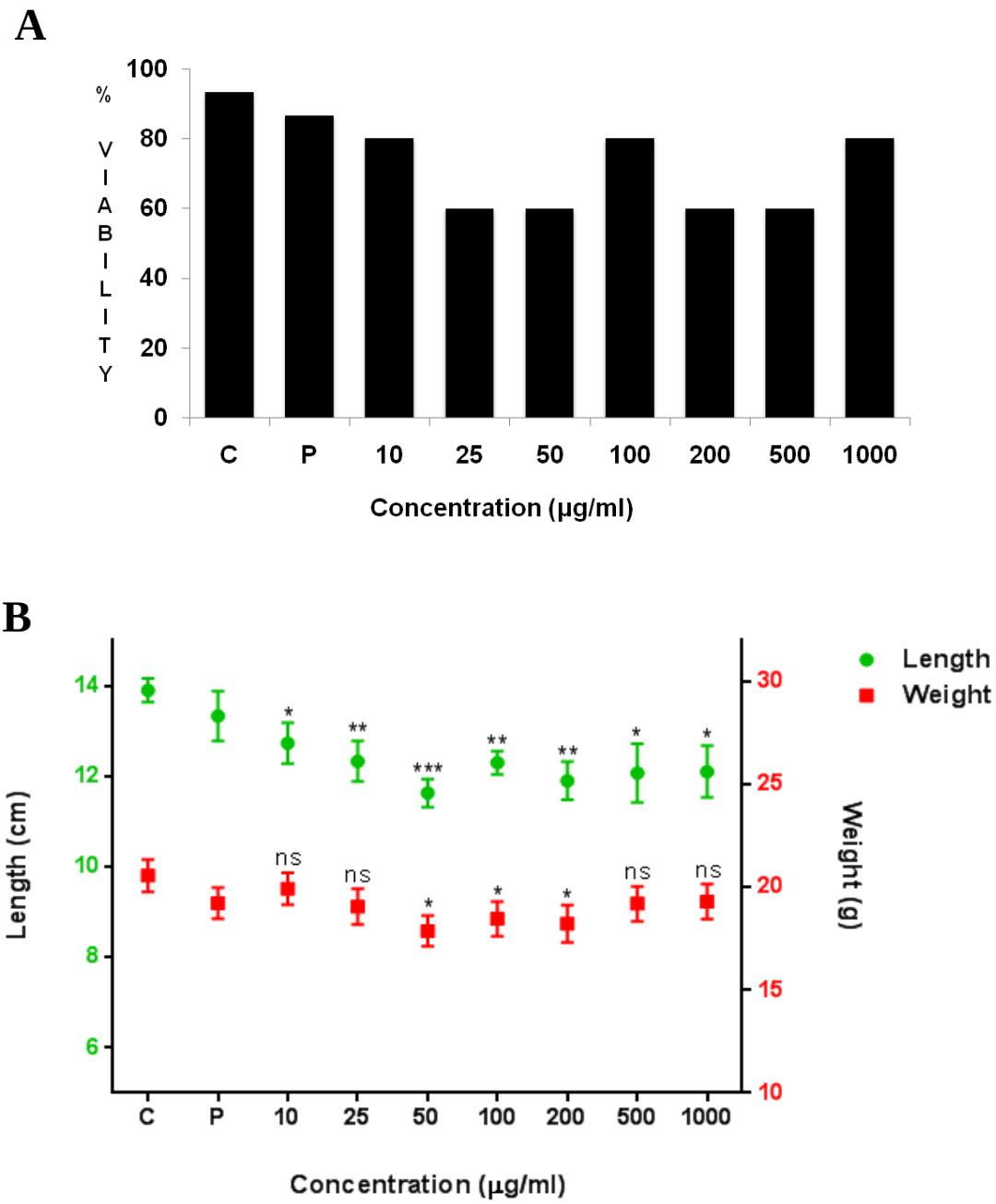

Figure 3.5: (A) Percentage viability, (B) Mean body weight (g) and lengths (cm) of living 19-day-old chicken embryos in control and treated groups. The data are expressed as Mean±SD. Statistical analysis was done by one way ANOVA, *=p ≤ 0.05, **=p ≤ 0.01, ***=p ≤ 0.001, ns-non significant, C- control, P-placebo, ZnO NPs-treated groups (10-1000 µg/ml).

Table 3.3: Organ weights of chicken embryos treated with Zinc oxide nanoparticles

Organs	Control	Placebo	10 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml	500 µg/ml	1000 µg/ml	p value
No. of Viable embryos	14	13	11	8	8	11	8	8	11	-
Liver, g	0.49±0.03	0.48±0.05	0.44±0.03	0.44±0.05	0.46±0.05	0.43±0.05	0.45±0.05	0.43±0.05	0.46±0.04	ns
Brain, g	0.43±0.07	0.40±0.02	0.40±0.08	0.35±0.02	0.40±0.07	0.36±0.04	0.40±0.08	0.35±0.05	0.36±0.03	ns
Heart, g	0.16±0.03	0.16±0.01	0.14±0.01	0.15±0.02	0.15±0.01	0.15±0.01	0.17±0.01	0.16±0.01	0.15±0.01	ns
Spleen, g	0.02±0.00	0.01±0.00	0.01±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00	ns

Mean organ weights of embryos that were alive and without deformities in control and ZnO NPs -treated groups (10-1000 µg/ml). The data are expressed as Mean±SD. Statistical analysis was done by one way ANOVA. ns- non significant.

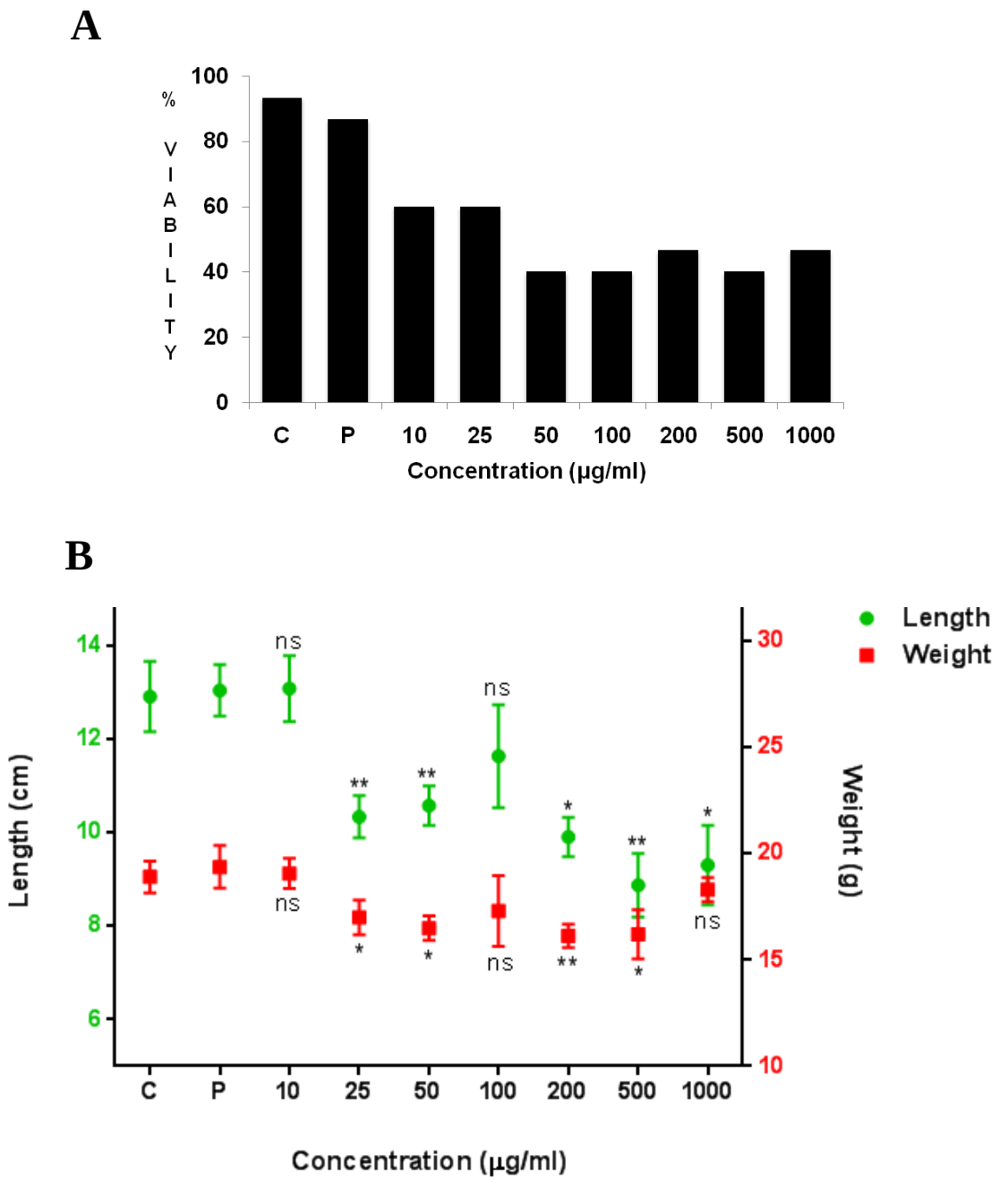

Figure 3.6: (A) Percentage viability, (B) Mean body weight (g) and lengths (cm) of living 19-day-old chicken embryos in control and treated groups. The data are expressed as Mean±SD. Statistical analysis was done by one way ANOVA, *=$p \leq 0.05$, **=$p \leq 0.01$, ns- non significant, C- control, P-placebo, MgO NPs-treated groups (10-1000 µg/ml).

Table 3.4 : Organ weights of chicken embryos treated with Magnesium oxide nanoparticles

Organs	Control	Placebo	10 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml	500 µg/ml	1000 µg/ml	p value
No. of Viable embryos	14	13	8	9	5	6	7	6	7	-
Liver, g	0.50±0.04	0.48±0.06	0.48±0.04	0.45±0.04	0.45±0.04	0.45±0.03	0.46±0.03	0.45±0.03	0.45±0.02	ns
Brain, g	0.44±0.08	0.44±0.03	0.43±0.05	0.41±0.04	0.41±0.06	0.41±0.05	0.42±0.03	0.41±0.06	0.41±0.02	ns
Heart, g	0.17±0.03	0.16±0.00	0.16±0.01	0.15±0.02	0.15±0.01	0.15±0.01	0.15±0.00	0.15±0.01	0.15±0.01	ns
Spleen, g	0.02±0.00	0.02±0.00	0.01±0.01	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	ns

Mean organ weights of embryos that were alive and without deformities in control and MgO NPs -treated groups (10-1000 µg/ml). The data are expressed as Mean±SD. Statistical analysis was done by one way ANOVA. ns- non significant.

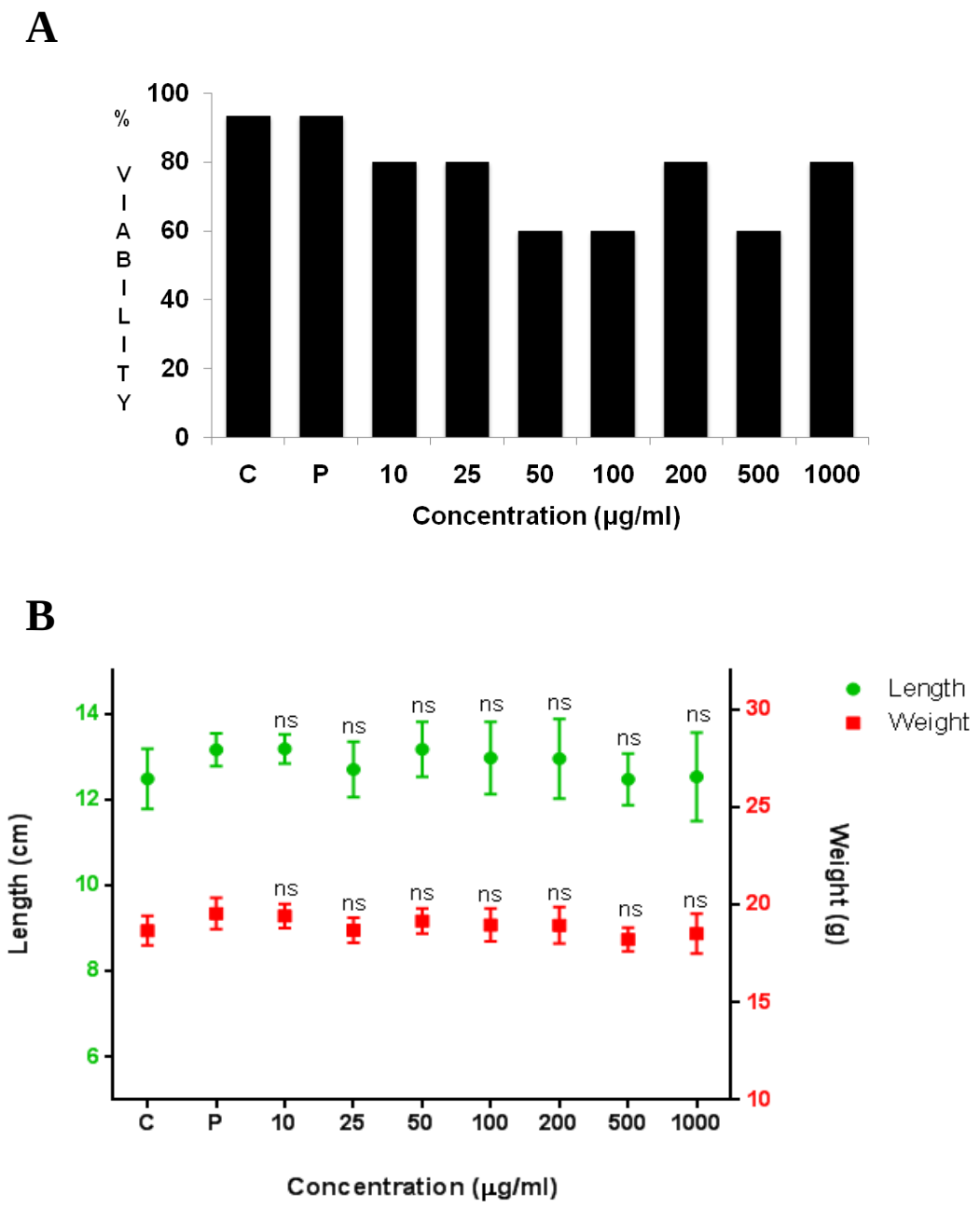

Figure 3.7: (A) Percentage viability, (B) Mean body weight (g) and lengths (cm) of living 19-day-old chicken embryos in control and treated groups. The data are expressed as Mean±SD. Statistical analysis was done by one way ANOVA, ns- non significant, C- control,P-placebo, Al_2O_3 NPs-treated groups (10-1000 µg/ml).

Table 3.5: Organ weights of chicken embryos treated with Aluminium oxide nanoparticles

Organs	Control	Placebo	10 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml	500 µg/ml	1000 µg/ml	p value
No. of Viable embryos	14	14	11	12	7	8	12	7	8	-
Liver, g	0.48±0.04	0.48±0.04	0.47±0.01	0.48±0.05	0.48±0.05	0.47±0.04	0.48±0.03	0.48±0.05	0.49±0.06	ns
Brain, g	0.47±0.10	0.47±0.08	0.47±0.06	0.43±0.05	0.43±0.09	0.44±0.06	0.45±0.09	0.44±0.05	0.45±0.06	ns
Heart, g	0.16±0.01	0.16±0.01	0.15±0.01	0.16±0.02	0.16±0.00	0.15±0.01	0.16±0.00	0.15±0.00	0.15±0.00	ns
Spleen, g	0.02±0.00	0.01±0.00	0.01±0.01	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	ns

Mean organ weights of embryos that were alive and without deformities in control and Al_2O_3 NPs -treated groups (10-1000 µg/ml). The data are expressed as Mean±SD. Statistical analysis was done by one way ANOVA. ns- non significant.

DISCUSSION

The present study demonstrates the impact of metal oxide nanoparticles (TiO_2, Fe_2O_3, ZnO, MgO and Al_2O_3 NPs) on chicken embryo. HET-CAM analysis is performed to obtain a quick qualitative assessment of the test compound to a biological membrane and hence this test is widely used for various test compounds (Luepke & Kemper, 1986). In our study, HET-CAM analysis was employed to quantitatively assess the biological response of nanometals to chorioallantoic membrane. Since it was a qualitative assessment the same preceeded the *in ovo* toxicity analysis. In our study, TiO_2, ZnO, MgO and Al_2O_3 NPs failed to elicit a hemorrhagic response as the visual evidences were comparable to that of control. However, Fe_2O_3 NPs inflicted moderate hemorrhage on CAM. Though the results obtained with iron oxide nanoparticles was a much weaker impact as compared to the positive control (0.1 N NaOH), these observations are the first to report hemorrhagic potential of iron oxide nanoparticles. Use of Fe_2O_3 NPs for diagnostic purpose is common as magnetic resonance imaging (MRI) contrast agents, cell separation media, drug delivery carriers, and cancer hyperthermia (Krishnan, 2010; Pankhurst *et al.*, 2003). Fe_2O_3 NPs in prescribed doses is approved by the medical fraternity, but results obtained herein calls for profound studies with diverse animal models to set a benchmark for their toxicity and avoid side effects.

Chicken embryo is a sensitive and fast responding model (Abdolmaleki & Zahri, 2016; Coleman, 2008; Rashidi & Sottile, 2009) and hence it is widely used for developmental studies. TiO_2 NPs caused higher percentage mortality in developing chicken embryos at lower doses of TiO_2 NPs (10 and 25 µg/ml). But, the higher doses of TiO_2 NPs (50- 1000

µg/ml) failed to elicit a dose-dependent toxicological response possibly because TiO_2 NPs underwent physicochemical alterations. These results are in agreement with studies of graphene oxide and pristine graphene (pG) on chicken embryo wherein; results showed that pG administrated to the chicken embryos at levels from 50-10,000 µg/ml reduced chicken embryo survival but not in a dose dependent manner. The same can be attributable to the natural tendency of agglomeration of nanoparticles, compounded by proteins present in the embryo surroundings (Sawosz *et al.*, 2014). TiO_2 NPs caused significant reduction in morphometric indices (body weight and length) but the weight of the examined organs (liver, heart, spleen, and brain) was not significantly affected. It is hypothesized that the lower concentrations (10 and 25 µg/ml) of TiO_2 NPs penetrated the embryo more efficiently, compared to the higher concentrations (50-1000 µg/ml) due to different dispersion level of nanoparticles, hence causing deformities in the embryo like omphalocele and flexed limbs. In this study, 10-1000 µg/ml dose range of Fe_2O_3 NPs induced ~50% mortality of chicken embryos. Morphometry of viable embryos revealed significant decrement in body weight and crown rump length at 50 and 100 µg/ml doses. These results are in agreement with the observed decrement in snout vent length in *X. laevis* embryos following Fe_2O_3 NPs treatment (Nations *et al.*, 2011). Silver, copper and palladium nanoparticles have been reported to increase metabolic rates of embryos without inducing deformity (Sawosz *et al.*, 2009; Studnicka *et al.*, 2009). Since, no skeletal deformity was observed in 10-1000 µg/ml dose range of Fe_2O_3 NPs the same is in partial agreement with reports on other nanometals. But, 100 % mortality recorded at the highest dose of Fe_2O_3 NPs (200 µg/ml) could be attributable to Fe^{2+} ions overload

coupled with complex interactions of Fe_2O_3 NPs with albumen and other biomolecules which is discussed in detail in chapter 5.

Zinc oxide nanoparticles induced ~40% mortality of chicken embryos between doses 50-500 µg/ml. These results are in agreement with the observed dose dependent mortality of zebra fish embryos following ZnO NPs treatment (Y.-L. Hu *et al.*, 2011). Morphometry of viable embryos revealed significant decrement in body weight and crown rump length at 50, 100 and 200 µg/ml doses. Similar body weight loss was reported in mice after intratracheal instillation of zinc oxide nanoparticles (D. Wang *et al.*, 2017) and shortened body length of the zebra fish larvae. Zinc ions released from the nanoparticles probably contributed to their toxicity (Y.-L. Hu *et al.*, 2011). Other reported deformities caused by ZnO NPs include hatching retardation and tail malformations in zebra fish embryo and inflammatory and hyperplastic changes in the lungs of mice, which are in contrast to the results obtained in this study.

Treatment with magnesium oxide nanoparticles resulted in ~50-60% mortality of chicken embryos at the higher doses (100-1000 µg/ml). Also, there was a significant decrement in the weight and lengths of the embryo at the said doses but the weight of the examined organs (liver, heart, spleen, and brain) was not significantly affected. A study on zebra fish embryo showed toxic effects of magnesium oxide nanoparticles resulting in cellular apoptosis and intracellular reactive oxygen species along with a delay in hatching, increased mortality and developmental deformities (Ghobadian *et al.*, 2015). It is already known that MgO NPs release Mg ions but these ions did not result in any significant toxicity as their behavior is different from that of Zn^{2+} ions released from ZnO. Also, our

results are in agreement with the studies conducted in mice wherein; acute exposure of MgO did not show change in behavior and organ index after deposition of NPs but the sub-chronic exposure appears to influence the behavior and affect the organs (Shaikh *et al.*, 2015).

Aluminum oxide nanoparticles induced ~20-40% mortality of chicken embryos between doses 50-500 µg/ml. Morphometry of viable embryos revealed no change in body weight and crown rump length at all the doses of treated groups. These results are in agreement with the non toxic effect of Al_2O_3 NPs on zebra fish embryos (Y.-L. Hu *et al.*, 2011)

SUMMARY

Metal oxide nanoparticles witness a wide range of applications which can have an impact on human health and wildlife. The aim of the present study was to evaluate the impact of chosen metal oxide nanoparticles (TiO_2, Fe_2O_3, ZnO, MgO and Al_2O_3) on an avian embryo (*Gallus gallus domesticus*). HET-CAM analysis revealed Fe_2O_3 NPs induced moderate hemorrhage to the vasculature of CAM. The nanoparticles were dosed in the airspace of fertilized eggs and incubated until 19^{th} day of development. Nanoparticles caused an increase in the mortality of embryos and caused a decrement in their body lengths and weights without having an impact on the organ weights. The results showed that metal oxide nanoparticles could cross the inner shell membrane and the chorioallantoic membrane and affect the development of the embryo. Malformations were visible in TiO_2 nanoparticles treated embryos in the form of omphalocele and flexed limbs whereas; Fe_2O_3, ZnO, MgO and Al_2O_3 nanoparticles did not affect the embryonic development.