CHAPTER 6

Iron oxide nanoparticles induced neurotoxicity in chicken embryo

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INTRODUCTION

Engineered nanomaterials have witnessed an increase in their commercial, diagnostic, therapeutic and several other applications. The most important nanomaterials are simple metal oxides such as aluminium oxide (Al_2O_3), zinc oxide (ZnO), titanium oxide (TiO_2) manganese oxide (MnO_2) and iron oxide (Fe_3O_4, Fe_2O_3) (Balasubramanyam *et al.*, 2010; Oszlánczi *et al.*, 2010; Sárközi *et al.*, 2009). Owing to their very small size and resultant higher surface area than their bulk counterparts, nanometal oxides are extensively researched for their use in sunscreen, stain resistant clothing, paints, self-cleaning glass, sports equipment, drug delivery etc. (Card *et al.*, 2008; Iavicoli *et al.*, 2012; Rosi & Mirkin, 2005).

Maghemite nanoparticles (Fe_2O_3 NPs) are widely used in the biomedical field and have multiple applications such as magnetic resonance imaging (MRI) contrast agents, cell separation media, drug delivery carriers, and cancer hyperthermia (Krishnan, 2010; Pankhurst *et al.*, 2003). Recent developments of Fe_2O_3 NPs include the synthesis of enhanced contrast agents for early detection of brain tumors via MRI (Kumar *et al.*, 2010; Sun *et al.*, 2008; Yu *et al.*, 2010) as well as magnetic (Chertok *et al.*, 2010) and convection-enhanced (Perlstein *et al.*, 2008) drug-delivery systems targeting brain tumor or tissue. Magnetite nanoparticles (Fe_3O_4) are also intensively explored in neuro-medicine as they can cross the blood−brain barrier (BBB) (Wang *et al.*, 2010). But, due to their small size and novel characteristics they may be responsible for adverse biological effects. Hence, the health and environmental safety of nanomaterials have drawn increasing attention (Europea, 2004).

Superparamagnetic iron oxide nanoparticles have been reported to be metabolized easily and their resultant iron ions are known to generate reactive oxygen species (ROS) mediated toxicity and peroxidation of membrane lipids (Singh *et al.*, 2012; Stohs & Bagchi, 1995). A study has shown that carboxyl-coated iron oxide nanoparticles primarily distributed in the liver and spleen, smaller NPs (10 and 20 nm) effectively altered the expression level of oxidative stress, iron transport, metabolism, and apoptosis-related genes and the smallest size (10 nm) penetrated more readily into the brain and the uterus (Yang *et al.*, 2015). Also, iron oxide nanoparticles induced apoptosis by generating oxidative stress in immune cells (Couto *et al.*, 2014; E.-J. Park *et al.*, 2015) and altered immune homeostasis in body by remaining in the body for a long time (Ban *et al.*, 2013; E.-J. Park *et al.*, 2010). Reports on genotoxicity of Fe_2O_3 NPs describe their ability to induce DNA breaks and oxidative DNA damage (Singh *et al.*, 2009).

The number of studies assessing toxic effects of Fe_2O_3 NPs on embryos is limited. Fe_2O_3 NPs have been reported not to induce any mortality in *Xenopus laevis* embryos (Nations *et al.*, 2011) but significantly impact embryonic development in zebrafish in form of hatching delay, embryonic malformations and mortality (Zhu *et al.*, 2012). Studies on mammalian experimental models have reported contrasting effects of Fe_2O_3 NPs wherein, the duration of gestation and fetal growth were normal but, post-natal development, maturation and puberty were affected in experimental mice (Noori *et al.*, 2011) and rabbits (Bourrinet *et al.*, 2006). Additionally, health hazards in early-life following exposure to environmental risk factors has been considered as a major health problem worldwide (Caminati *et al.*, 2015; Huang *et al.*, 2015). There are no reports on Fe_2O_3 NPs toxicity in chick embryo and hence this study investigates the same with an objective of

understanding its interaction with egg albumen and its possible toxicity on embryonic development.

MATERIALS AND METHODS

Spectroscopic characterization

Egg albumen- iron oxide nanoparticles interaction was identified by Fourier transform infrared (FTIR) spectra (PerkinElmer spectrum 65 series, PerkinElmer, Inc., MA, USA). Sample was prepared by making pellet of 1.5 to 2 mg of sample (freshly collected albumen dispersed with Fe_2O_3 NPs) mixed with 200 mg KBr (AR, Sigma, USA) in the KBr press machine (model Mp-15) at 5 kg/cm^2 pressure for 2 min. After taking background scan, samples were analyzed at 400-4000 cm^{-1}. FTIR direct-transmittance spectroscopy (KBr) was used to indicate the degree to which oxygen groups were removed and the IR absorption of water from the air was mostly eliminated. Each measurement was repeated in triplicates to minimize the error.

Chicken embryo model, autopsy and morphometry

As described in chapter 2

Histopathology

Chicken brain from control and Fe_2O_3 NPs treated groups were fixed in 10% buffered formalin (pH 7.2) for 72 h. Fixation was followed by washing in running water for 6-8 h. Fixed tissues were dehydrated by a graded series of alcohol, cleared in xylene and embedded in paraffin wax. Paraffin blocks were sectioned in microtome to generate 5-6 μm sections and collected on slides. Sections were de-paraffinized in Xylene I, II and III

(5 min) and then dehydrated by serial gradation of ethyl alcohol (100%, 95%, and 70%) for 3min each. The slides were then rinsed in distilled water (5 min), and stained with hematoxylin (6 min). After staining with hematoxylin, slides were rinsed in running tap water (20 min). Thereafter, stained slides were decolorized in acid alcohol (1 sec) and rinsed again in tap water (5 min). Then slides were stained with Eosin (15 sec) and dehydrated with ethyl alcohol (95% and 100%) for 3 min each. Slides were cleared in Xylene I and II (5 min), finally mounted, observed and photographed (Leica DM 2000) at 100X and 400X. Each slide was scored for healthy and degenerating neurons. Multiple random areas in each tissue section was scored on a six-point scale: 0 = no dead neurons, 5 = >0-10 %, 30 = >10-50%, 70 = > 50-90%, 95 = 90≤ 100%, and 100 = 100% dead neurons and mean scores were calculated (George *et al.*, 2004). The stock and working solutions of Hematoxylin and Eosin are given below.

	Hematoxylin	**Eosin**
Stock Solution	HHS-32 (Hematoxylin)	1% aqueous Eosin-Y and 1% aqueous Phloxin B
Working Solution	95% Ethanol (2578 ml), dH_2O (950 ml) and HCL (9 ml)	Stock Eosin (100ml), stock Phloxin B (10 ml), 95% Ethanol (780 ml) and glacial Acetic Acid (4 ml)

Evaluation of Nanoparticles crossing biological barriers (Immersion studies)

The experimental protocol (MSU-Z/IAEC/03-2017) was approved by the Institutional Animal Ethical Committee (IAEC) and the Committee for the Purpose of Control and Supervision of Experiments on Animals (827/GO/Re/S/04/CPCSEA). Fertilized eggs of White leghorn (*Gallus gallus domesticus*) were obtained from Shakti hatcheries, Sarsa, Gujarat, India and incubated for 7 days. Fertilized eggs (7-day old) were weighed and randomly divided into groups, each with 16 eggs viz. Control, Placebo (normal saline) and Fe_2O_3 NPs treated (10-200 µg/ml). The eggs were immersed in the respective solutions for 30 minutes, air dried and placed back in the incubator (Varga *et al.*, 2002). 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. Brain sections (5–6-µm thick) were stained with hematoxylin–eosin (HXE), observed, photographed (Leica DM 2000) at 100X and 400X and scored as mentioned above.

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

Spectroscopic characterization

Secondary structural changes in native egg albumen were studied in absence or presence of Fe_2O_3 NPs using FTIR spectra (400-4000 cm^{-1}, Figure 6.1) at 100 µg/ml. Peaks at 3591 and 3534 cm^{-1} obtained in native albumen corresponded to H-O-H asymmetric and symmetric stretching whereas, a peak at 2083 cm^{-1} corresponded to linear C-O group present in amino acids. Peaks at 1643 and 1651 cm^{-1} corresponded to amide I region of albumen due to C=O stretching whereas, a peak at 1549 cm^{-1} implies at amide II region due to N-H bend in plane and C-N stretch. Peak at 1453 cm^{-1} is due to CH_2 scissoring vibration and around 1401 cm^{-1} corresponded to C=O stretch of COO^- group. Also, 674 cm^{-1} is due to mainly O=C-N deformation summing up the amide IV region in native albumen. In Fe_2O_3 NPs + albumen there was no distinguishable shift recorded in addition to the peak at 418 cm^{-1} which corresponds to the Fe-O metal oxygen vibrational mode of Fe_2O_3 NPs. An approximation based deconvolution method was used to investigate the secondary structural changes taking place in the amide I region from 1600-1700 cm^{-1}. Figures 6.2 (A, B) shows the secondary derivative Gaussian fitted spectra of the amide I region and the percentage of secondary structural component (Table 3).

Histopathology

Hematoxylin-Eosin stained sections of brain revealed neuronal loss (scattered dark, shrunken cell with picnotic or small, densely stained nuclei and eosinophilic cytoplasm) that was significantly higher in 10, 25, 50 and 100 µg/ml treated groups as compared to the control (Figure 6.3 A,B). The tissue sections were scored for degenerating neurons

(Figure 6.3 C) wherein, the results indicated higher percentage of neuronal loss at 10 (50%), 25 (60%), 50 (56.66%) and 100 µg/ml (60%) doses of Fe_2O_3 NPs.

Immersion Studies

Eggs immersed in Fe_2O_3 NPs solution accounted for ~20-40% mortality at 25-200 µg/ml doses (Figure 6.4A). Body weight and crown-rump length of viable embryos revealed significant decrement at 100 and 200 µg/ml doses (Figure 6.4B). Gravimetry of liver, brain, heart and spleen revealed non-significant decrements in their weights at all doses of Fe_2O_3 NPs (Table 6.2). Hematoxylin-Eosin stained sections of brain revealed neuronal loss that was significantly higher in 25, 50, 100 and 200 µg/ml treated groups as compared to the control (Figure 6.5A). The tissue sections were scored for degenerating neurons (Figure 6.5B) wherein, the results indicated higher percentage of neuronal loss at 10 (50%), 25 (60%), 50 (50%), 100 (60%) and 200 µg/ml (60%) doses of Fe_2O_3 NPs.

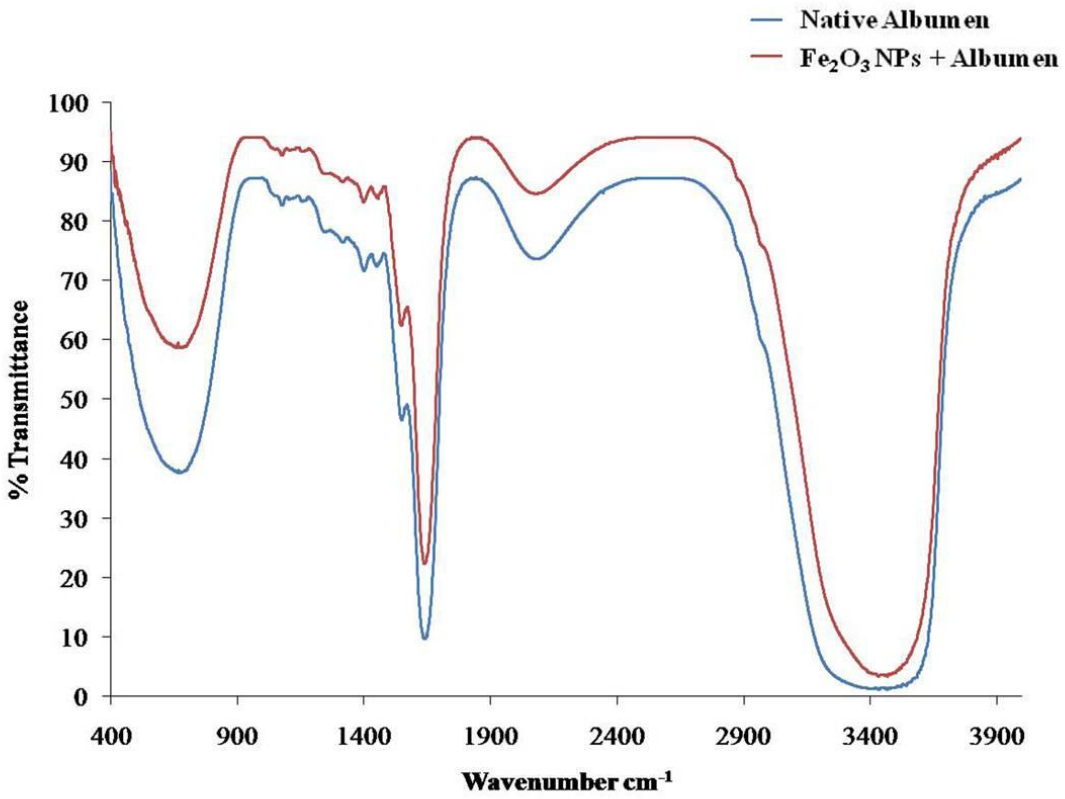

Figure 6.1: Comparative FTIR spectra of native egg albumen and Fe_2O_3 NPs + Albumen

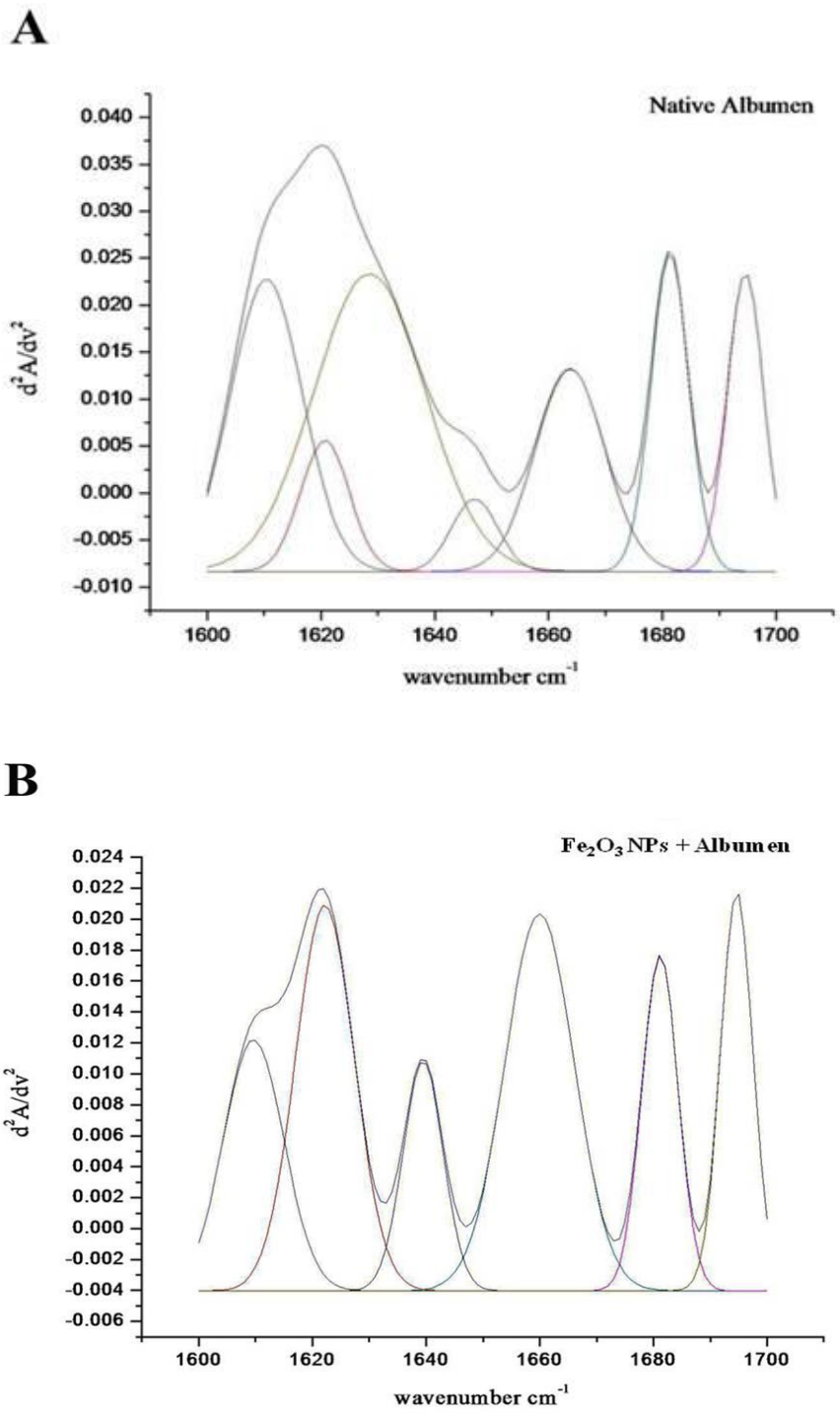

Figure 6.2: Gaussian curve fitting of secondary derivative of Native egg Albumen (A) and Fe_2O_3 NPs + egg Albumen (B)

Table 6.1: Percentage of secondary structure components in Native egg albumen and Fe_2O_3 NPs + egg albumen at 100 µg/ml

	% Secondary Structure					
	Side chain	β sheet	Random coil	α helix	β turn	β sheet
Native Albumen	20.93	39.11	3.30	13.94	11.95	10.76
Fe_2O_3 NPs + Albumen	15.41	23.06	9.47	26.56	12.26	13.23

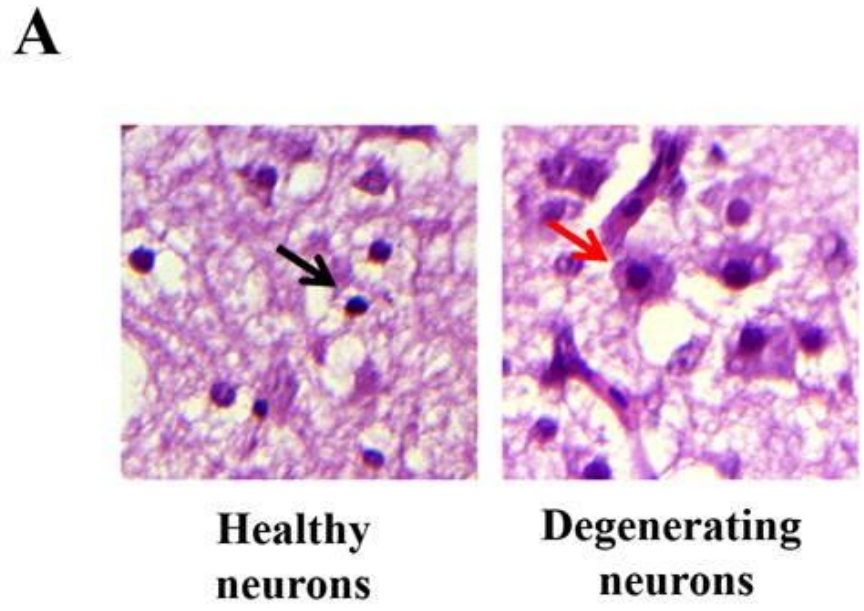

Figure 6.3 A: Digitally enlarged images of healthy neuron (black arrow) and degenerating neuron (red arrow) (600X).

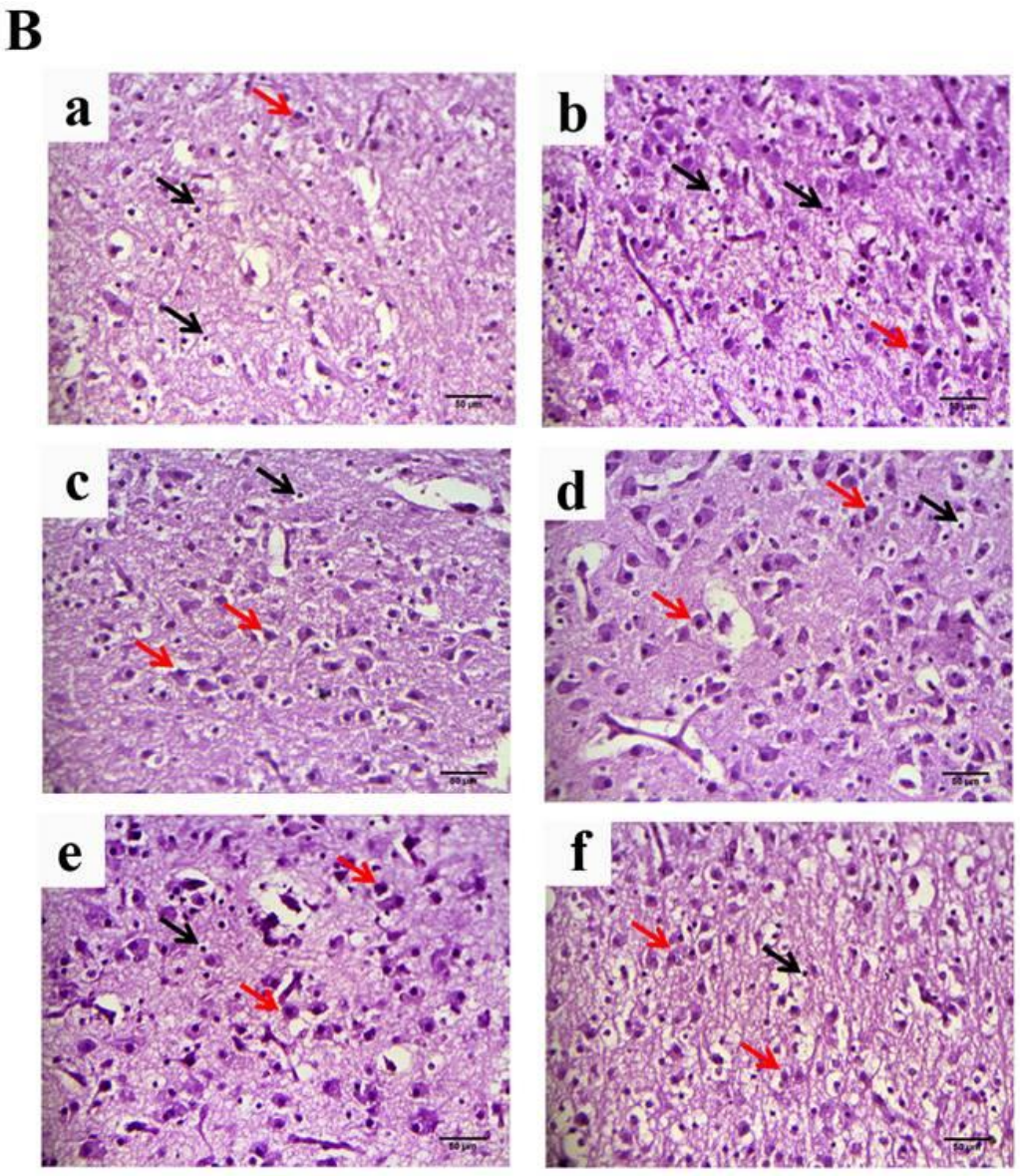

Figure 6.3 B: Histopathological evaluation of brain sections (5 µm) stained with HXE (400X). Degenerating neurons are scattered dark and shrunken cells with picnotic nuclei and eosinophilic cytoplasm. a-control, b-placebo and 10-100 µg/ml iron oxide nanoparticles treated groups (c-f) (Scale bar: 50µm).

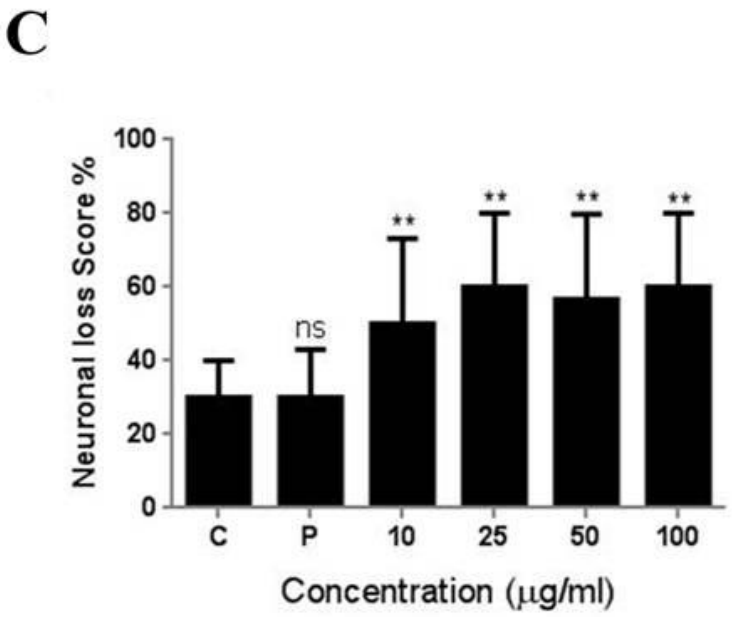

Figure 6.3 C: Percentage neuronal loss in the brain. The data are expressed as Mean±SD. Statistical analysis was done by one way ANOVA, **=$p \leq 0.01$, ns- non significant, C- control, P-placebo.

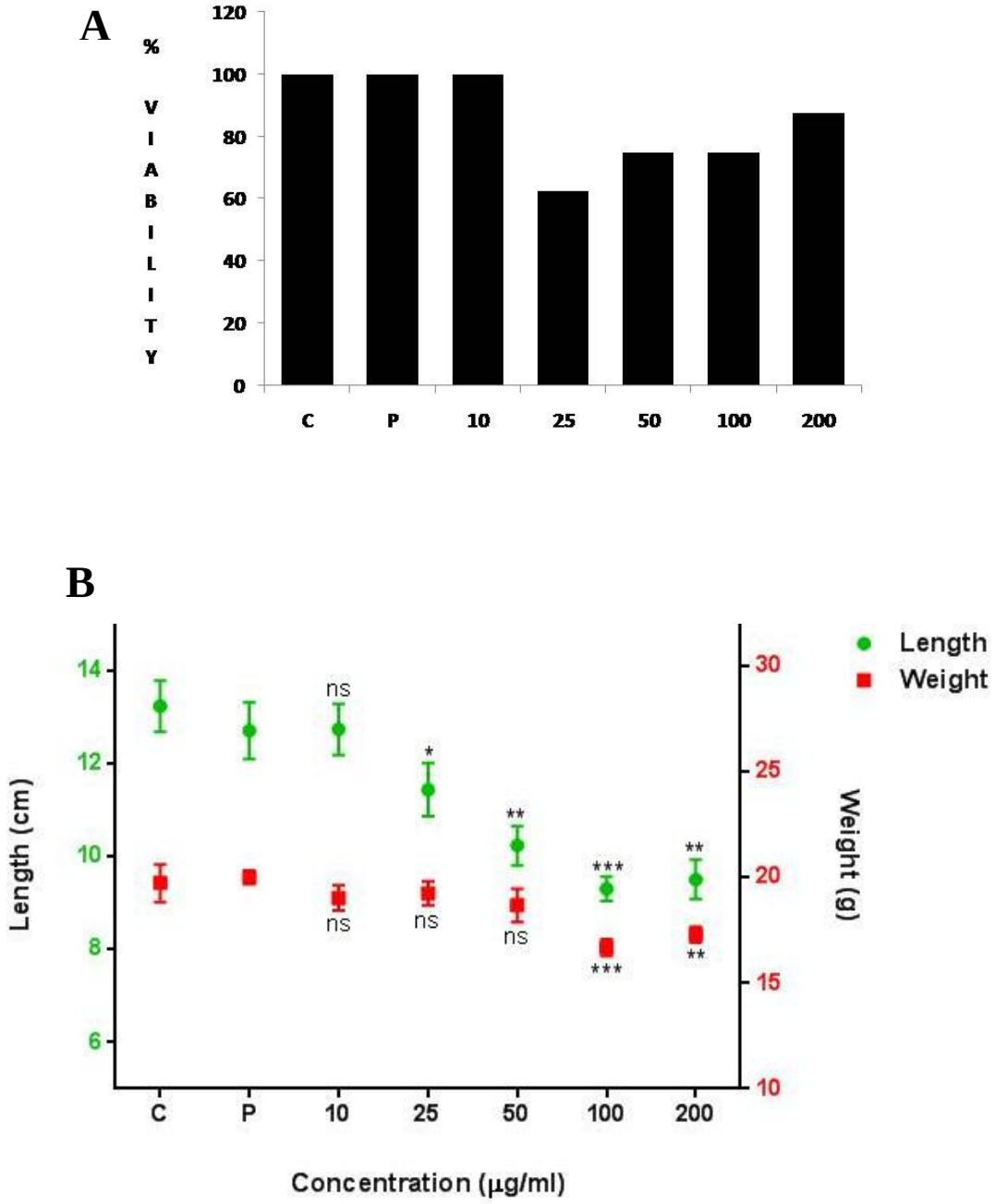

Figure 6.4: (A) Percentage viability, (B) Mean body weight (g) and lengths (cm) of living 19-day-old chicken embryos in control and Fe_2O_3 NPs -immersed groups. The data are expressed as Mean±SD. Statistical analysis was done by one way ANOVA, *=$p \leq 0.05$, **=$p \leq 0.01$, ***=$p \leq 0.001$, ns- non significant,C-control,P-placebo, Fe_2O_3 NPs -immersed groups (10-200 µg/ml).

Table 6.2 : Organ weights of chicken embryos treated with Iron oxide nanoparticles (immersion)

	C	P	10	25	50	100	200	p value
No. of Viable embryos	14	13	8	9	8	8	6	-
Liver, g	0.49 ±0.04	0.48 ±0.07	0.33 ±0.09	0.41 ±0.03	0.42 ±0.09	0.41 ±0.07	0.42 ±0.08	ns
Brain, g	0.42 ±0.10	0.42 ±0.04	0.40 ±0.10	0.36 ±0.03	0.38 ±0.08	0.38 ±0.08	0.40 ±0.08	ns
Heart, g	0.18 ±0.03	0.17 ±0.01	0.15 ±0.02	0.16 ±0.02	0.14 ±0.02	0.15 ±0.01	0.17 ±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	ns

Mean organ weights of embryos that were alive and without deformities in control and Fe_2O_3 NPs -immersed groups (10-200 µg/ml). The data are expressed as Mean±SD. Statistical analysis was done by one way ANOVA. ns- not significant, C-control, P-Placebo.

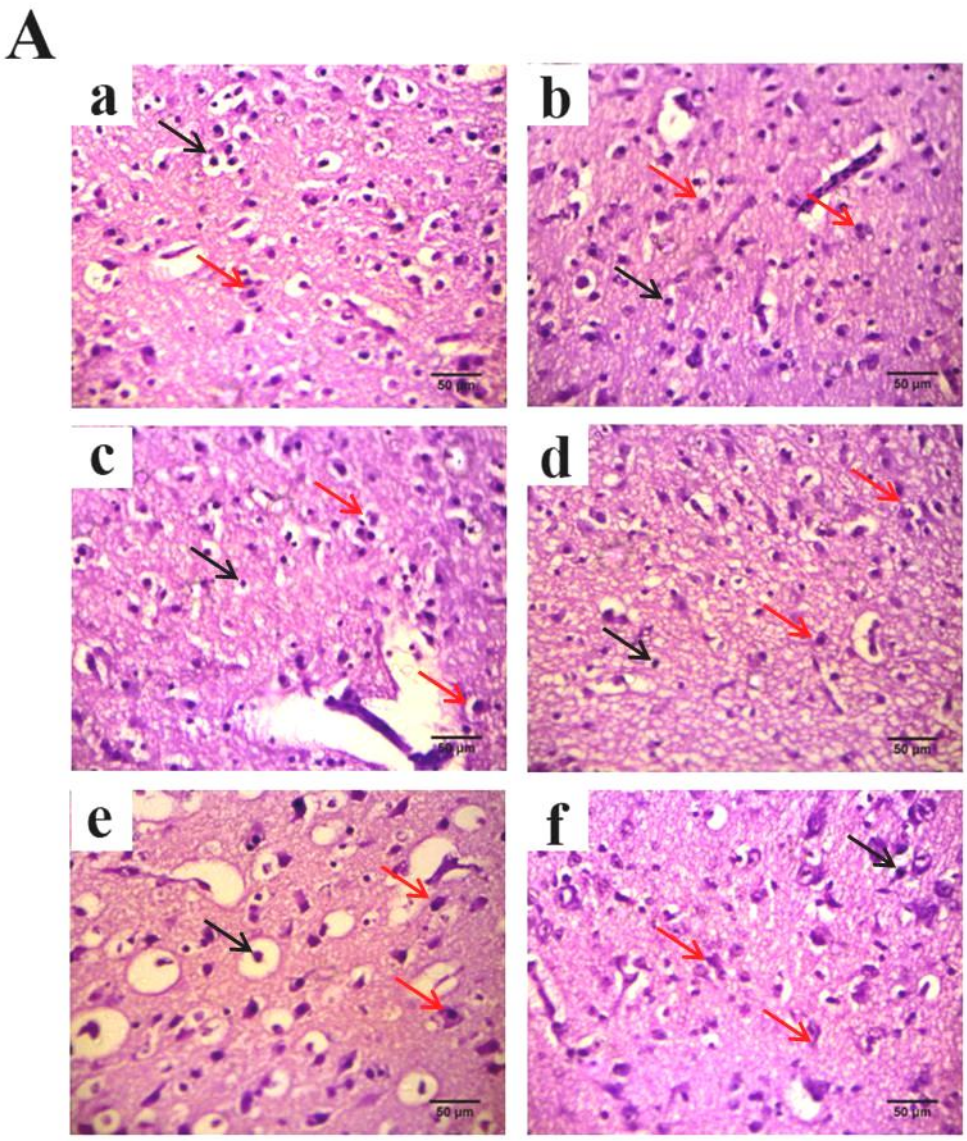

Figure 6.5 A: Histopathological evaluation of brain sections (5 µm) stained with HXE (400X) depicting healthy neurons (black arrow) and degenerating neurons (red arrow) control (a), and 10-200 µg/ml iron oxide nanoparticles immersed groups (b-f) (Scale bar: 50µm).

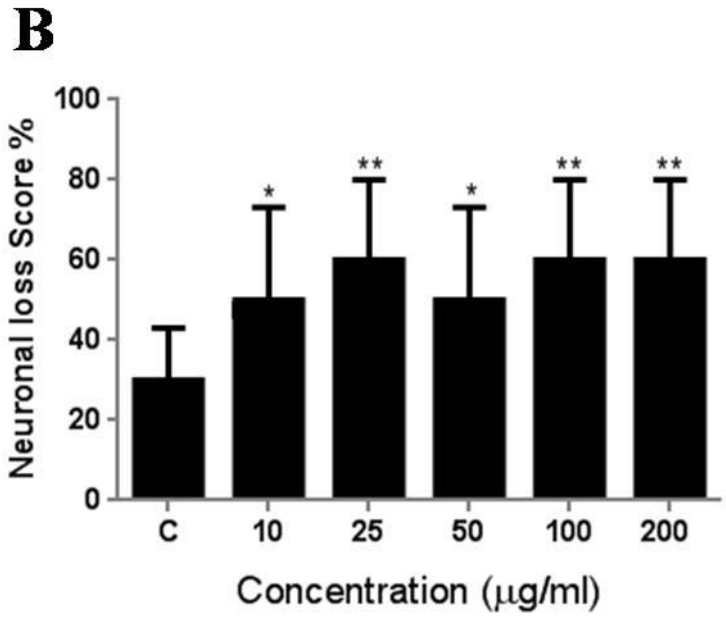

Figure 6.5 B: Percentage neuronal loss in brain. The data are expressed as Mean±SD. Statistical analysis was done by one way ANOVA, *=$p \leq 0.1$,**=$p \leq 0.01$, ns- non significant, C- control, P-placebo.

DISCUSSION

Nanomaterials interact with protein in unique ways and undergo changes in their "biological identity" (size, shape and surface chemistry (Walkey & Chan, 2012) and the same was observed herein, with albumen and Fe_2O_3 NPs. The percentage relative areas of α helices for native egg albumen and Fe_2O_3 NPs + albumen were recorded at 1664 and 1660 cm^{-1}, respectively and were found to be 13.94% and 26.56%. Also, the percentage for β sheet of egg albumen and Fe_2O_3 NPs + albumen were recorded at 1621-1622cm^{-1} and 1695cm^{-1}, respectively and were found to be 49.87% and 36.29%. These results show an increment in α helices and a decrement in β sheet after interaction of albumen with Fe_2O_3 NPs as revealed by their percentage relative areas. The loops or β turn calculated for relative area at 1681cm^{-1} increased after interaction of albumen (12.26%) as compared to native Albumen (11.95%).The bands at 1647 and 1639 cm^{-1} were assigned for random coil and was found to be 3.30% and 9.47% respectively before and after interaction. The changes in above mentioned physicochemical parameters of albumen imply towards loss of its molecular activity following their interaction with Fe_2O_3 NPs culminating in imperative changes in their functional moieties. Hence, it is hypothesized that Fe_2O_3 NPs reach the developing embryo because of its interactions with egg albumen. Biological applications of Fe_2O_3 NPs are on the rise and hence, it was imperative to assess its interaction with biomolecules. This study is the first to investigate physicochemical changes in egg albumen following its interaction with Fe_2O_3 NPs.

In the next phase of the study, brain sections revealed neuronal loss (scattered dark, shrunken cell with picnotic or small, densely stained nuclei and eosinophilic cytoplasm).

Bare and surface modified Fe_2O_3 NPs have been reported to cross blood brain barrier (Wang *et al.,* 2010) and induce toxicity by production of excess ROS. ROS can damage cells by producing lipid peroxidation, mitochondrial damage, DNA disruption and gene transcription modulation, which can then trigger a cascade of Ca^{+2}-dependent signaling mechanisms resulting in cell apoptosis/death (Stroh *et al.,* 2004). High content of easily peroxidable unsaturated fatty acids, high oxygen consumption rate and a primordial antioxidant system in embryonic brain makes it highly vulnerable to ROS induced damage (Skaper *et al.,* 1999). Fe_2O_3 NPs have been reported to produce Fe^{2+} ions in biological systems (Yarjanli *et al.,* 2017). Another study had reported loss of neurons in hippocampal CA1 region due to Fe^{2+} ions overload (U. J. Park *et al.,* 2011; Salvador, 2010). Hence, the mortality and neuronal loss observed at the lower doses (10-100 µg/ml) and 100% mortality at a higher dose (200µg/ml) are attributable to complex interactions of Fe_2O_3 NPs with biomolecules *in ovo* coupled with Fe^{2+} ions overload.

The penetration of pesticides through the eggshell after application by immersion or by spraying has been reported for DDT, organophosphates and 2,4-D in different species (David, 1979; Somers *et al.,* 1974; Varga *et al.,* 2002). Since, Fe_2O_3 nanoparticles are known to cross biological barriers this study assessed the ability of Fe_2O_3 NPs to cross barriers (egg shell, shell membrane and chorioallantoic membrane) in an avian egg. Results obtained in the immersion study suggest that Fe_2O_3 NPs can penetrate the eggshell and impact the developing embryo. It is well known that chorioallantoic membrane helps in the uptake of particles in contact with the eggshell after immersion and transfers it to the embryo (Hoffman, 1990). Similarly in this study, the CAM and the egg albumen are responsible for the uptake of nanoparticles into the embryo.

Morphometry of viable embryos revealed significant decrement in body weight and crown rump length at 100 and 200 µg/ml doses. These results are similar to the studies wherein Fe_2O_3 NPs were injected in the air space of the chicken egg. Significant decrement in length and body weight of the embryos and an increase in mortality at the higher doses corroborated with the *in ovo* injection results, but no 100 % mortality recorded at the highest dose of Fe_2O_3 NPs (200 µg/ml) could be attributable to less Fe^{2+} ions interacting with the egg albumen and reaching the chicken embryo. Brain sections revealed dose dependent neuronal loss as observed in the *in ovo* studies.

Both methods (injection and immersion) are used in toxicology for various reasons. Immersion tends to mimic the exposure in nature and allows us to assess the threat of chemicals to avian embryos whereas; injection method is widely used for evaluating the teratogenic potency of drugs. The results obtained in this study indicate the toxic effect of Fe_2O_3 NPs irrespective of the method of dosing. Changes in physicochemical properties following interactions of Fe_2O_3 NPs with other biomolecules *in ovo* might have had subtle impact that needs further scrutiny.

SUMMARY

Nanomaterials based on their size, shape and surface chemistry, interact with protein in unique ways and undergo changes in their "biological identity". Hence, the aim of the present study was to evaluate the interaction of egg albumen with Fe_2O_3 NPs and its subsequent impact on an avian embryo. Fe_2O_3 NPs were dosed in the airspace of 48 h old eggs or immersed in Fe_2O_3 nanosuspension on 7^{th} day of incubation of fertilized eggs of *Gallus gallus domesticus* and incubated until 19 days. Fe_2O_3 NPs were found to interact with egg albumen as evidenced by the observed changes in the secondary structure of Fe_2O_3 NPs + egg albumen. These interactions possibly allowed Fe_2O_3 NPs to traverse the biological barriers within chicken egg and reach the brain. The observed neuronal loss is attributable to production of Fe^{2+} ions by Fe_2O_3 NPs. Egg immersion studies showed similar results indicating Fe_2O_3 NPs could cross *in ovo* biological barriers viz. egg shell, shell membrane and chorioallantoic membrane.