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Research Article

IRON OXIDE NANOPARTICLE (IONP): CHEMICAL SYNTHESIS AND NEUROTOXIC STUDIES IN WISTAR RAT

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ABSTRACT

In the present study, Iron oxide Nanoparticles (IONPs) were synthesized using a simple chemical co-precipitation method followed by systematic characterization and neurotoxicity assessment in Wistar rats. Transmission Electron Microscopy (TEM) analysis confirmed that the average particle diameter is 16±2 nm. X-ray diffraction (XRD) stick pattern confirms the crystalline cubic spinel structure of IONPs having average diameter of 15 nm. The UV–visible spectrum of IONPs showed absorption maxima at 307–345 nm. Rats were intraperitonealy administered with low dose, moderate dose and high dose of purified and functionalized IONPs dispersed in water. These rats were autopsied on 7, 14, and 28 days post exposure. For neurotoxicity assessment, activity of superoxide dismutase (SOD) and catalase (CAT) as well as levels of Thiobarbituric acid reactive substances (TBARS) were estimated in brain sub-regions namely, frontal cortex, hippocampus, corpus striatum and cerebellum. Activity of SOD and CAT increased in treated groups as compared to control group. Though activity of SOD was found to be significantly high in hippocampus and corpus striatum sub-regions on day 14th but there occurred no significant changes in CAT activity. TBARS level showed an elevation in all treatment groups at day 7th and day 14th with a significant increase in hippocampus and corpus striatum region of high dose group animals on 7th day, whereas there was a slight decline in its level post 28th day of IONP exposure. From this study, we conclude that more focused study is required to avoid adverse impacts of IONPs before it is used as a tool in biomedical applications.

Keywords: IONPs, SOD, CAT, TBARS, Wistar rat, Neurotoxicity.

INTRODUCTION

Nanotechnology is science, technology and Engineering conducted at the nanoscale which is about 1 nm to 100 nm. The unusual optical, electronic and magnetic properties of nanoscale science and engineering have attracted much attention (Tari *et al.,* 1979; Poizot *et al.,* 2000; Mahmoudi *et al.,* 2010). Nanomaterials can be applied in a wide range of fields from medical applications to environmental sciences due to their unique properties, altered dimensions and modification in their original state. Metal oxide nanoparticles (NPs) have great potential in numerous biomedical or diagnostics applications and *in vivo* clinical applications such as magnetic resonance imaging (MRI) contrast enhancement, tissue specific release of therapeutic agents, targeted destruction of tumor tissue through hyperthermia, magnetic field assisted radionuclide therapy, iron detection and tissue engineering (Liu *et al.,* 2013; Ito and Kamihira, 2011).

Along with unique nanoscale physico-chemical properties short blood half-life, non-specific targeting, higher relaxation values and small size which is enough to escape from the reticuloendothelial system (RES) and immune system make metal NPs more effective agents in biomedical applications (Talelli *et al.,* 2009; Landsiedel *et al.,* 2012). Interesting properties like super-paramagnetism and high field irreversibility truly makes Superparamagnetic Iron Oxide NPs (IONPs) theragnostic (Schleich *et al.,* 2015). IONPs are the only clinically approved magnetic nanoparticles which show stability towards oxidation and continuously show ferromagnetism

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as compared to other metal NPs which shows much sensitivity to oxidative environment. Thus, IONPs shows wide scope of biomedical applications.

The major concern about magnetic NPs is their biocompatibility and biosafety. Biocompatible IONPs with proper surface architecture, modified dimensions and altered original state facilitates their use in laboratory diagnosis and therapeutics (Gupta and Gupta, 2005; Shubayev *et al.,* 2009). IONPs application in biology, medical diagnosis and therapy requires that these NPs should be stable in water at neutral pH and physiological salinity. Thus, synthesis procedures of NPs needs more focused study to avoid adverse impacts due to agglomeration of particles in biological environment. To synthesize iron oxide nanoparticles, chemical co-precipitation method is the most preferred laboratory friendly method over other methods when considering the high yield, high magnetic property and biocompatibility. Biocompatibility and the hydrophilic nature of NPs make them more stable and these particles show greater potential for biomedical applications rather than particles in organic solvent.

Many studies have revealed that the same properties that make NPs so unique- that is primarily their small size, large surface area; geometry etc. could also be responsible for their potential hazard to human and animal health (Yang *et al.,* 2010; Sanvicens and Macro, 2008). Although IONPs are considered safe to use but there are several reports in the literature that have examined the cytotoxic potential of several different types of IONP with a range of sizes, shapes and with different surface coatings and have generally found low or dose dependent cytotoxicity associated with these NPs (Singh *et al.,* 2010; Mahmoudi *et al.,* 2011; Liu *et al.,* 2013; Li *et al.,* 2014; Valdiglesias *et al.,* 2015). Studies have proposed that accumulated iron NPs due to their unique nanoscale properties may also cross the blood brain barrier via olfactory nerve tract or systemic circulation and may translocate to brain (Wang *et al*., 2007; Kong *et al.,* 2012). Brain tissue is rich in polyunsaturated lipid and has high iron content. This increases vulnerability of the brain to abnormal iron regulation, which may trigger different neurodegenerative disorders (Wang *et al.,* 2009*;* Wu *et al.,* 2013; Borysov *et al.,* 2014).

Recent studies support the concept that transitional metal NPs might play an important role in the neurodegenerative disorders. Experimental studies have demonstrated that nanopaticles with a small size, high surface area, high catalytic activities, have an ability to generate reactive oxygen species (ROS) (Li *et al.,* 2014). The iron accumulation in brain tissues is an initial event causing cellular disturbances which may lead to cell death because these free iron molecules may produce highly reactive hydroxyl radicals in the oxidative environment (Salvador *et al.,* 2011). These hydroxyl radicals could damage cellular components such as DNA, proteins polysaccherides and lipids etc. in the cells (Singh *et al.,* 2010; Soenen *et al.,* 2011; Szalay *et al.,* 2012; Wu *et al.,* 2013).

Therefore, there is need for broader understanding about the NPs and the associated risks. The choice of biocompatible and biodegradable coating used during the synthesis procedure is the most crucial step in NPs application (Shukla *et al.,* 2015). Here, we are highly concerned about the potential neurotoxicity of IONPs as well as their careful synthesis procedures.

In the present study, a chemical co-precipitation method is opted to synthesize ultrafine well-soluble hydrophilic IONPs. This method is the modification of "Large-Scale Fe_3O_4 Nanoparticles Soluble in Water Synthesized by a Facile Method" (Hui *et al.,* 2008). Synthesis was done to investigate the neurotoxic potential of the IONPs in rats after repeated exposure to IONPs. The particle induced oxidative stress in brain sub regions was evaluated by oxidative stress biomarkers estimation biochemically.

MATERIALS AND METHODS

Materials

Citric acid, trisodium salt dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) and sodium hydroxide (NaOH) were procured from HiMedia Laboratories (Mumbai, India), sodium nitrate ($NaNO_3$) and ferrous sulphate salt ($FeSO_4 \cdot 7H_2O$) were procured from Avantor (Thane, Maharashtra India) for the preparation of iron oxide nanoparticles. Ultra pure water prepared from Direct-Q® Water Purification System (Merck Millipore) was used throughout experiments to avoid metal contamination during synthesis and to prepare reagents and buffers for biochemical assays. All the chemicals and solvents were of analytical grade.

IONPs synthesis method

Iron Oxide nanoparticles (IONPs) were synthesized via chemical method (Hui *et al.,* 2008). For this purpose 1mmol of citric acid, trisodium salt dihydrate ($C_6H_5Na_3O_7 \cdot {}_2H_2O$), 3.99 mmolof sodium hydroxide (NaOH), 66.65 mmol of sodium nitrate ($NaNO_3$) was dissolved in 19 ml of deionized water. This solution was mixed using magnetic stirrer to obtain a pellucid solution for 20 minutes at 100°C. In the above solution freshly prepared 1.61 mmol of ferrous sulphate ($FeSO_4 \cdot 7H_2O$) was added and the solution was mixed on magnetic stirrer for 10-15 minutes and the temperature was maintained at 100°C as well. The final mixture was heated at 100°C on water bath for 1 hour to obtain a black precipitate. The solution was cooled to room temperature naturally. The precipitate mass was then separated and purified from solvent by a magnet. The final obtained precipitate was purified by water washing for 4-5 time followed by washing with 70% ethanol for 2-3 times. The alcohol dispersed black precipitate was collected on a petri plate and it was allowed to dry under a light lamp for overnight. The dried black powder was collected for further use.

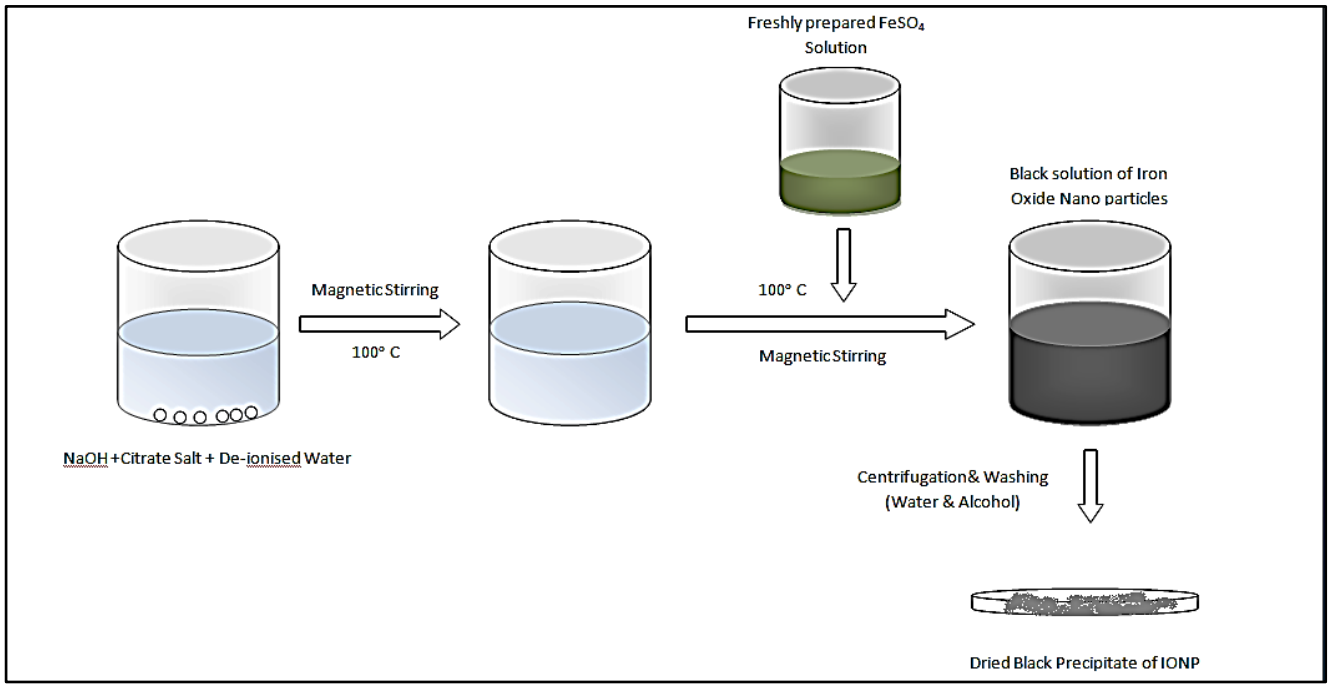


Figure 1. Schematic block diagram of Iron Oxide nanoparticles synthesis.

Particle characterization

The structure of hydrophilic NPs was characterized by using PANalytical X-ray Diffractometer having Cu Kα as a radiation source of wavelength 1.54606 Å within 2θ = 10–70˙ at the scan speed 0.4˚ per min for structural confirmation of IONPs /Diffraction with Cu Kα radiation (1.5418Å) generated at 40 kV and 30 mA. UV-Vis absorption spectra were acquired in the 250-700 nm wavelength range using an UV-Vis spectrophotometer (Varian) operating at a resolution of 2 nm for the analysis of optical properties of colloidal solution. Transmission Electron Microscopy (TEM) images of the NPs were obtained by a HRTEM FEI Technai 20 U Twin Transmission Electron Microscope (TEM) microscope operated at an acceleration voltage of 120 kV. The nanoparticle powder samples were dispersed in ultra-pure water by sonication and then dropped onto a copper grid for TEM observation. The size distribution and morphology of NPs were determined by measuring diameters of one hundred NPs randomly selected on the TEM images.

Animal handling

Healthy Wistar rats of proven fertility, 6-8 weeks-old and 90–120 grams body weight were used in this study. The rats were fed sterilized standard rodent pellet diet (Aashirwaad Industries, Chandigarh, India) and water *ad libitum* throughout the experiment. They were kept in an air cooled room under standard hygienic laboratory conditions of temperature (25 ± 2˚ C) and 50–70% relative humidity with a 12h light-12h dark photoperiods in polypropylene cages with clean dust free wooden shavings (procured locally) as bedding throughout the experiment. The animal care and handling was done according to the guidelines set by the Committee for the Purpose of Control and Supervision on Experiments on Animal, New Delhi, India (1678/GO/a/12 CPCSEA). The departmental animal ethical committee (DAEC) approved the experimental protocols for this study.

Experimental schedule

Animals were randomly divided into four groups (18 animals per group) including control group (0.9 % saline water), low dose group (L.D., 20.322 mg/kg body weight), moderate dose group (M.D., 40.644 mg/kg body weight) and high dose group (H.D., 81.288 mg/kg bodyweight). Adult male rats were given dose of IONPs intraperitonealy once daily for 28 days. All the experimental groups were monitored daily for general activities including appetite and body weight. Six rats per group were sacrificed by cervical dislocation, on day 7th, 14th and 28th post exposure for studying biochemical variables superoxide dismutase (SOD), catalase CAT and Thiobarbituric acid reactive substances (TBARS).

In vivo studies: SOD, CAT activity analysis and TBARS level estimation

Rats were sacrificed by cervical dislocation and their brain were taken out carefully, immersed in cold saline, blotted with filter paper and weighed quickly. After which they were quickly placed on a chilled glass plate resting over ice for the separation of four brain sub-regions viz., frontal cortex, hippocampus, corpus striatum and cerebellum for biochemical estimation (Scheuhammer and Cherian, 1982). All the four separated brain sub regions were homogenized in ice cold buffer (1mM Tris–HCl+0.1mM EDTA–2Na+0.8% NaCl, pH=7.4) to yield 10% (w/v) homogenate. This homogenate was then centrifuged at 2000rpm and 4°C for 10min for removal of cellular debris. Supernatant was immediately used for estimation of SOD, CAT and TBARS level. Activity of SOD and CAT were estimated by the methods suggested by Marklund and Marklund (1974) and Aebi (1974) respectively whereas TBARS level estimation was done to assess levels of lipid peroxides in the brain tissue (Ohkhawa *et. al.,* 1979).

Statistical Analysis

All the results were statistically analyzed using Graphpad Prism 7 (Version 7.00) for Windows 7. Each group contained 6 animals and values represented as mean ± SEM. Comparisons of mean were analyzed by one-way ANNOVA followed by Dunnet's multiple comparison test. P values ≤0.05 were considered as statistically significant.

RESULTS

Particle characterization

Ultrafine well-dispersed hydrophilic Fe_3O_4NPs were synthesized by using chemical co-precipitation method. The synthesized particles can be re-dispersed in water and have high stability in water. Particle's magnetic nature is shown in figure 2, 3 and 4. XRD stick pattern of IONP, as shown in figure 5, confirms the crystalline cubic spinel structure of IONPs. The XRD pattern indicates that the product synthesized is iron oxide nanoparticles having average diameter 15nm. The diffraction peaks at 30.1, 35.6, 42.6, 53.1, 57.0, 62.8 responded to (220) (311) (400) (422) (220) (440) planes of Fe_3O_4 lattice respectively as shown in the figure. This pattern shows similarity with the XRD pattern of Fe_3O_4 NPs reported in literature.

Figure 6 shows the TEM images of varied sized NPs present in the solution. The particle size of IONP was recorded in between 15nm- 30nm range (16±2). The images obtained in TEM, supports the fact that the nanoparticles are stable in dispersed form and hence do not show agglomeration. The size distribution was analyzed by measuring the diameter of more than 50 NPs randomly selected in TEM image using computer program. The UV–visible spectrum of Fe_3O_4 nanoparticles shows an absorption band in the region of 307–345 nm as shown in figure 6, which originates primarily from the absorption and scattering of UV radiation by magnetic nanoparticles in accordance with the previously reported literature.

Figure 2. Gram-scale well-soluble hydrophilic Fe3O4 NPs were prepared by chemical coprecipitationmethod. (a) Samples of solid state hydrophilic magnetic nanoparticles powder on a Petri plate. (b) Dispersion in water, which can be moved by a magnet.

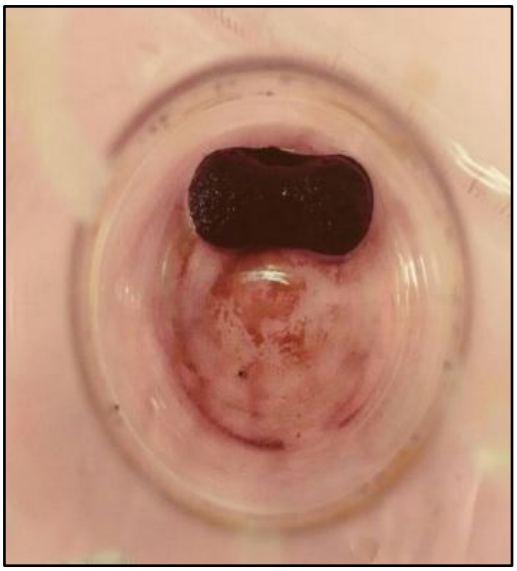


Figure 3. shows Flask showing IONP during chemical synthesis and magnetic bead showing IONP sticking on it showing particle's magnetic nature.

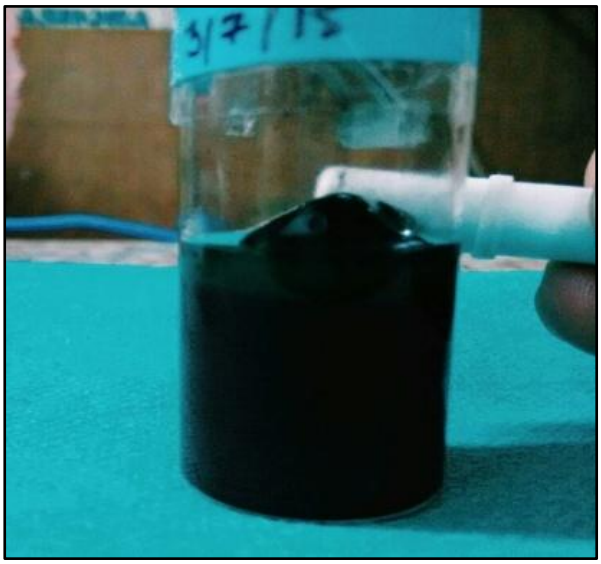


Figure 4 shows IONP in dispersed form showing magnetic attraction to magnetic bead. This confirms that the fluid is magnetic fluid due to presence of magnetic NPs.

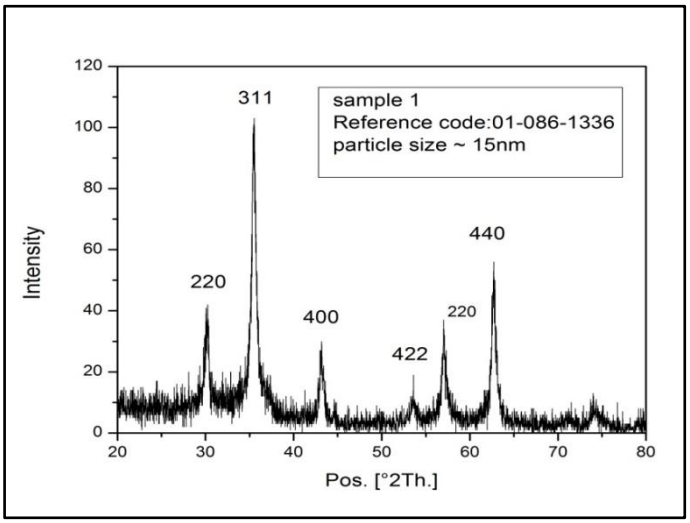
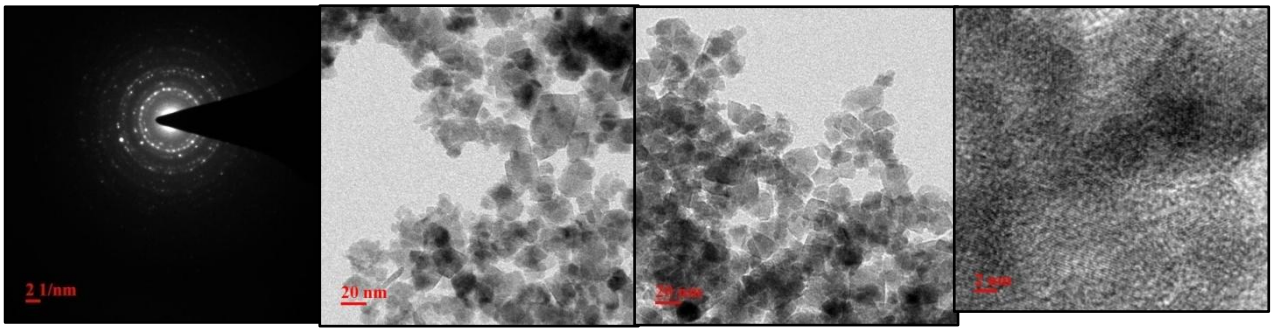
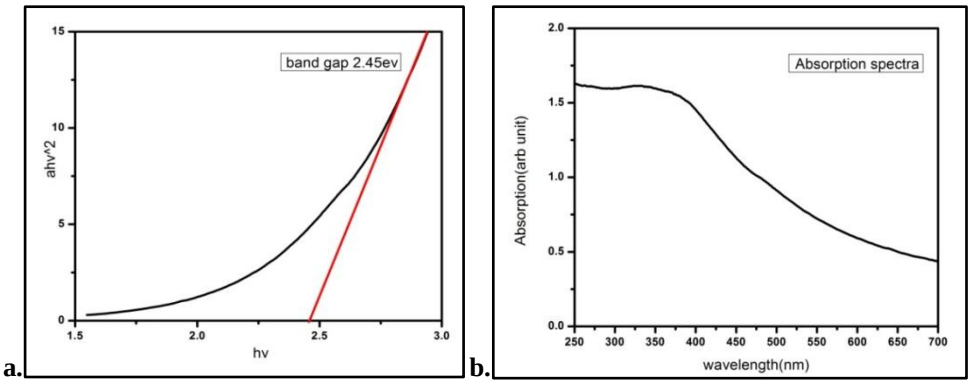


Figure 5. Shows XRD stick pattern of IONP (analyzed by PANalytical X-ray Diffractometer) that confirms the crystalline cubic spinel structure of IONPs. The XRD pattern indicates that the product is iron oxide.



a. **b.** **c.** **d.**

Figure 6 (a,b,c,d). TEM images of different sized NPs present in the solution. The particle size of IONP was recorded in between 15nm- 30nm range. The size distributions show that the synthesized Fe_3O_4 NPs had a narrow size distribution.



a. **b.**

Figure 7 (a, b). Shows the UV–visible spectrum of Fe_3O_4 nanoparticles showing an absorption band in the region of 307-345 nm.

General toxicity of IONPs

The exposure of animals to IONPs did not elicit any change in vocalization, breathing, moving and inter-relations among the cage mates. Many animals showed itching generally at the injection site especially in initial 1 hour of injection. Some of the treated animals however became lethargic and congregated at the corner of the cage immediately following dose administration. No significant difference was observed in the body weight gain of animals as shown in the table 1 given below.

Table 1: Effect on body weight gain in Wistar rats post IONP exposure. Each group contained 6 animals and values represented as mean ± SEM**.**

Group	Body weight gain (g)
Control	24.4 ± 2.619
Low Dose	19.4 ± 1.691
Moderate Dose	18 ± 3.302
High Dose	16.2 ± 3.734

Effects on Tissue Superoxide dismutase (SOD), Catalase (CAT) activities and TBARS level in brain sub-regions

The results obtained shown an increase in SOD activity in all brain sub regions of treated group animals as compared to control group post 7th, 14th and 28th day of IONP exposure (Figure 8). However, there occurred a significant elevation in SOD activity in hippocampus region in M.D. and H.D. group animals and corpus striatum region in H.D. group animals, post 14th of IONP exposure. The test compound increased catalase activity in all treated groups as compared to control group but no significant change was observed, as shown in figure 9. TBARS levels, which is the indicative of extent of lipid peroxidation, increased in all treatment groups post 7th and 14th day of IONP exposure. It was found to be significantly high in hippocampus and corpus striatum sub regions of only H.D. group animal post day 7th. On the other hand, TBARS levels showed a slight decline post 28th day of exposure in all treated groups as compared to control group (Figure 10).

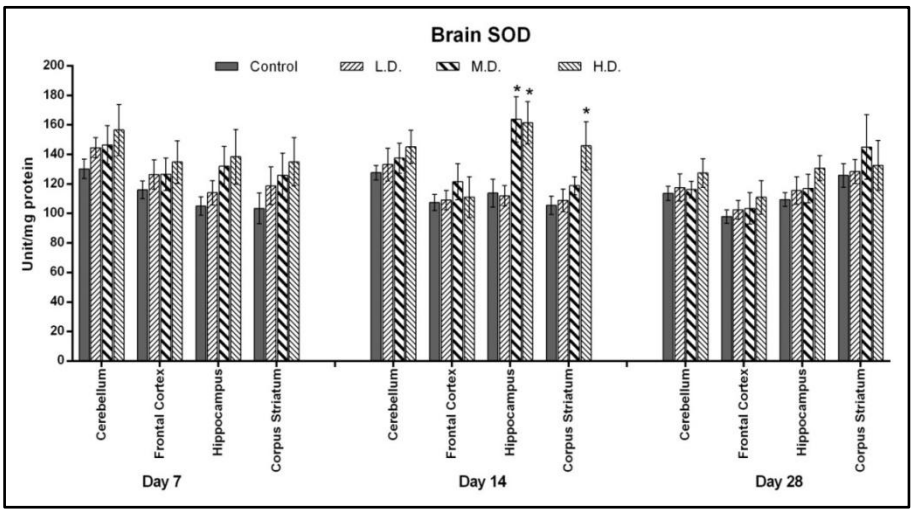


Figure 8: SOD activity in Cerebellum, Frontal Cortex, Hippocampus and Corpus Striatum of rats following administration of IONP. Each group contained 6 animals and values represented as mean ± SEM**.** Data analyzed by one way ANNOVA followed by dunnet's multiple comparison test. Abbreviations and unit used: SOD, Superoxide Dismutase; 1 Unit is equals to the amount of enzyme inhibiting the rate of autoxidation of pyrogallol by 50%. *P≤0.05 indicates statistical difference from Control group.

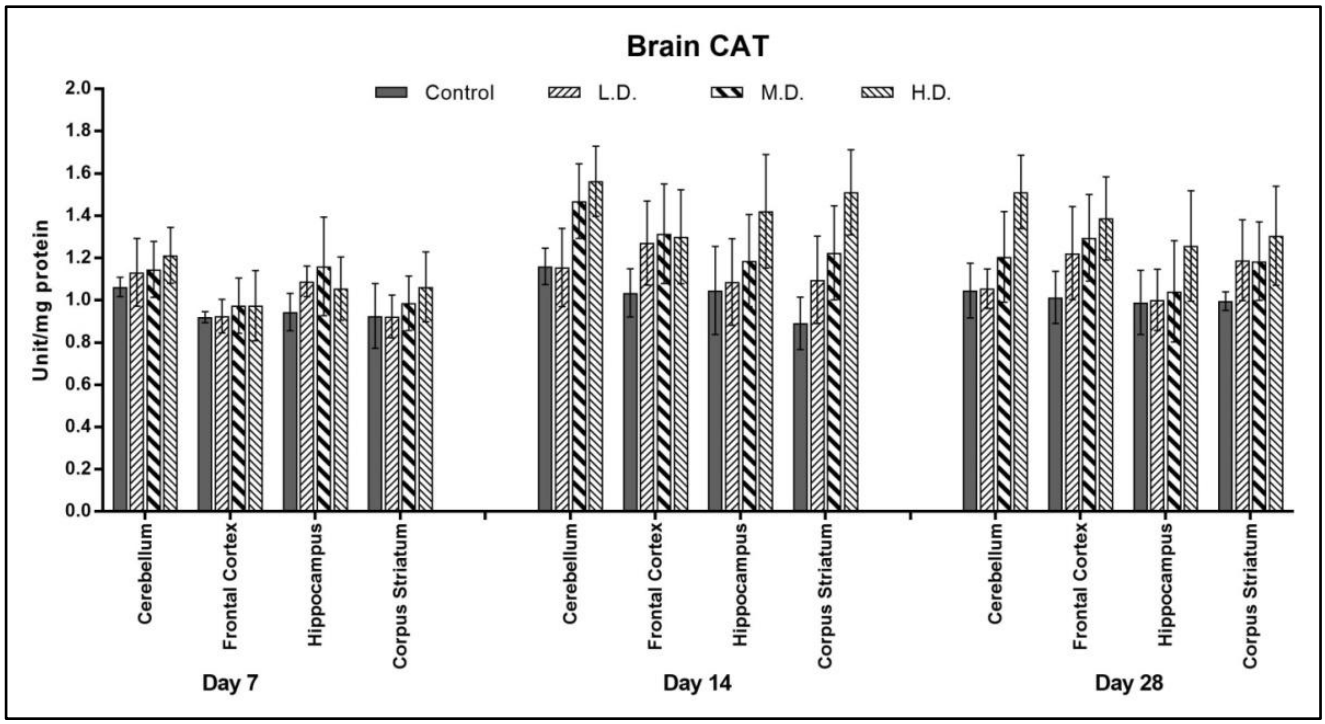


Figure 9: CAT activity in Cerebellum, Frontal Cortex, Hippocampus and Corpus Striatum of rats following administration of IONP**.** Each group contained 6 animals and values represented as mean ± SEM. Data analyzed by one-way ANNOVA followed by Dunnet's multiple comparison test. Abbreviations and Unit used: CAT: Catalase; 1 Unit is equals to 1 millimol H_2O_2 decomposed per minute per milligram protein.

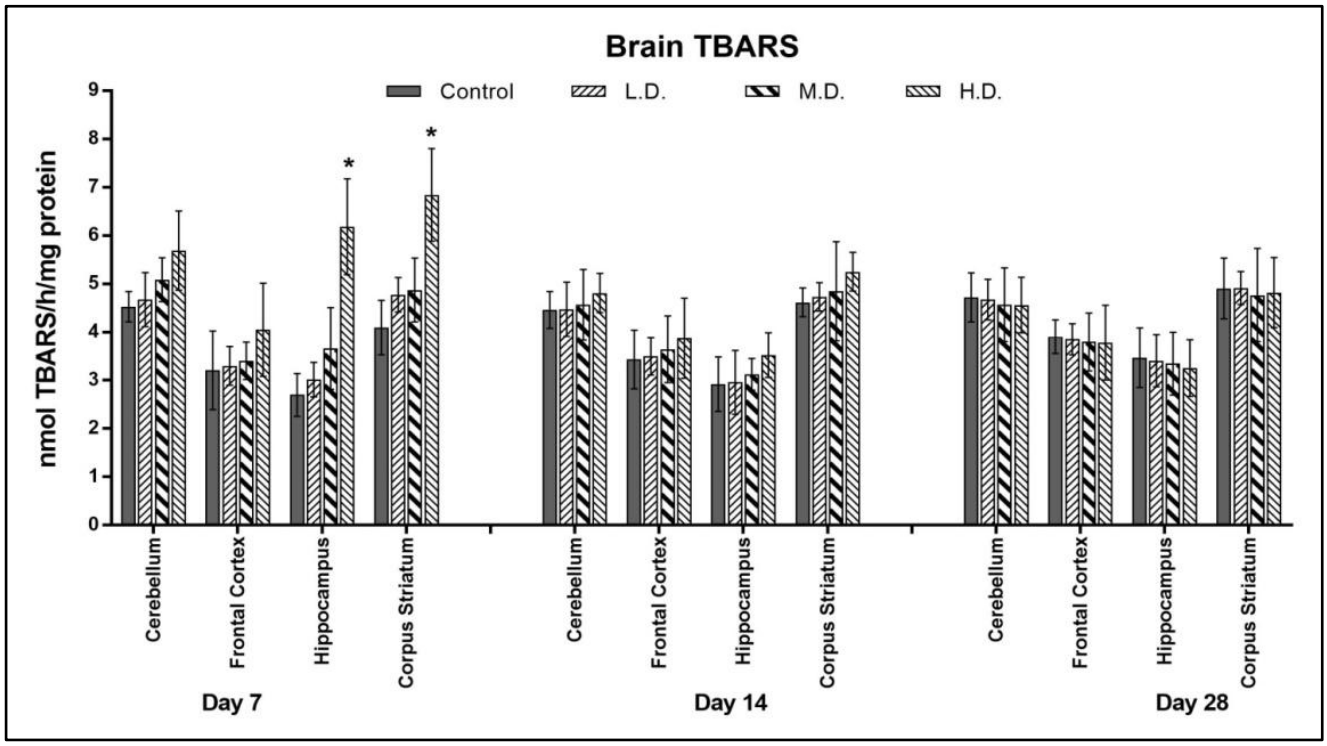


Figure 10: TBARS levels in Cerebellum, Frontal Cortex, Hippocampus and Corpus Striatum of rats following administration of IONP**.** Each group contained 6 animals and values represented as mean ± SEM. Data analyzed by one way ANNOVA followed by dunnet's multiple comparison test. Abbreviations and concentration used TBARS, Thiobarbituric Acid Reactive Substances as nmol/h/mg protein. *P≤0.05 indicates statistical difference from Control group.

DISCUSSION

In the present study, IONPs were synthesized by chemical co-precipitation method. Their solubility and handling was enhanced using citric acid salt ($C_6H_5Na_3O_7{\cdot}2H_2O$). These functionalized IONPs were dispersed uniformly in water using sonication for intraperitoneal exposure to rats. No behavioral changes were observed in IONPs treated rats. No significant change was observed in body weight gain in treated animals.

The intent of the current study was to investigate the hypothesis that IONPs exposure induces oxidative stress in brain and to know a possible mechanism of neurotoxicity. The front liners against toxic reactive oxygen species are anti-oxidative enzymes. During normal cellular function, reactive oxygen species (ROS) are produced which include hydroxyl radicals, superoxide anion, hydrogen peroxide and nitric oxide. They are highly unstable species due to their high chemical reactivity which leads to lipid peroxidation and oxidation of DNA and proteins. Under normal conditions, antioxidant systems of the cell minimize the perturbations caused by ROS. When ROS generation is increased to an extent that overcomes the cellular antioxidants, the result is oxidative stress (Mates, 2000). Even a small change in the activity of anti-oxidant enzymes would imply a disturbance in normal oxidative state of the cell and hence can lead to accumulation of ROS.

The produced highly reactive hydroxyl radicals ($^{\bullet}OH$) could damage cellular components such as DNA, proteins polysaccharides and lipids etc. and may leads to oxidative damage. Therefore it becomes crucial to draw attention towards the aspect regarding the safety of these IONP with regards to the oxidation state and compositional changes that might occur over time and affect its shelf-life and toxicity.

Based on the previous studies about the ability of NPs to cross the blood brain barrier via olfactory route or via systemic distribution, we are highly concerned about the potential neurotoxicity of iron oxide nanoparticles (Kim *et al.*, 2006; Wang *et al*., 2007; Hu and Gao, 2010). For this, we evaluated the level of the most sensitive and specific oxidative stress biomarkers superoxide dismutase (SOD) and catalase (CAT**).** We observed an increase in SOD activity post 7^{th}, 14^{th} and 28^{th} day of IONP exposure in all brain sub regions as compared to control group but this enhanced activity was found to be significant only in hippocampus of M.D. and H.D. group animals and in corpus striatum region of H.D. group animals post 14^{th} day of IONP exposure. The significantly increased SOD activity in hippocampus and corpus striatum post 14^{th} day of IONP exposure may indicates that the increased production of superoxide anions post treatment leads to activation of SOD which in turn detoxifies these free radicals into hydrogen peroxide (H_2O_2) and hydroxyl radical. Moreover, the activity of CAT enzyme was also found to be increased in all brain sub-regions in treatment group animals as compared to control group animals which may suggest the fact that functioning of CAT enzyme is synergistic to SOD activity for detoxifying free radicals in a cascade fashion.

The results show that corpus striatum and hippocampus brain sub regions are more prone to iron led cellular distress in a delayed and concentration dependent manner (Wu *et al.,* 2013). These findings supports the fact that antioxidant enzymes shows differential regional distribution **(**Brannan *et al*., 1980; Goss-Sampson *et al.,* 1988; Ansari *et al.,* 1989; Verma *et al.,* 1992**)** and hence the dissimilar vulnerability to oxidative damage **(**Baek *et al.,* 1999**).**

Brain tissue is rich in polyunsaturated lipid and has high iron content, thus, highly vulnerable to ROS mediated oxidative damage (Siegel *et al.,* 1985). It is well known that transition metal provokes oxidative stress by generating reactive oxygen species through Fenton reaction, thus causing brain lipid peroxidation (Sayre *et al.,* 1997). This increases vulnerability of the brain to abnormal iron regulation. We instigated lipid peroxidation post exposure of IONPs and found non-significant increase in TBARS levels except in hippocampus and corpus striatum sub-regions post 7^{th} day of IONP exposure in H.D. group animals. Thiobarbituric acid reactive substances are formed as a result of lipid peroxidation which can be detected by the TBARS assay by means of thiobarbituric acid as a reagent which measures malondialdehyde (MDA), the end product of lipid membrane peroxidation (Patton and Kurtz, 1951). The increase in the level of TBARS was more pronounced post 7^{th} day of exposure in the moderate and high dose groups showing dose dependent response. These findings were concomitant with the previous studies conducted on liver cells on exposure to IONPs (Priprem *et al.* 2010). Since, there was no significant change in TBARS levels post 14^{th} and 28^{th} day of IONP exposure, it might indicate other antioxidants such as glutathione and glutathione related enzymes started working to effectively combat lipid peroxidation in the cells and thus providing a protection against oxidative stress (Wang *et al.,* 2009).

Therefore wide use of NPs in present biomedical applications warrants a cautious assessment of the likely negative effects associated with these particles. The use of NPs should be dealt with great concern and time and efforts should be spent on the biocompatible and rigid suitable coatings as well as on the optimized cellular NP toxicity studies in order to permit them to be used in a secure and well controlled manner for the advantage of mankind.

CONCLUSIONS

Ultrafine well-soluble hydrophilic Fe_3O_4 nanoparticles functionalized by citrate-group were prepared using a facile one-step method for evaluation of their *in vivo* toxicity. Particles were found to be well dispersed and non-agglomerative**.** Highly crystalline cubic spinel structure and magnetic properties induced by surface and finite-size effects suggest a promising future of the NPs in practical

applications. The altered levels of SOD, CAT and TBARS suggest that these NPs induces oxidative stress initially, which on subsequent functioning of antioxidant system, subsides post 14th and 28th day. The results obtained from oxidative stress related biomarker estimation indicate that NPs should be coated so as to avoid cytotoxicity. Therefore, **s**tandardization of incubation conditions, careful characterization of NPs in their biologically relevant environment and large scale comparative studies should be the first step in nanotechnology. Especially in case of *in vivo* applications, a lot of research still needs to be done to generate sufficient data to increase our understanding in the field of nanotoxicology.

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