Iron oxide nanoparticles (Fe₂O₃-IONPs) have revolutionized the industry by significant economic and scientific impacts. Enormous increase in the usage of IONPs has raised concerns about their unseen adverse effects. In the current study, we investigated the effects of IONPs and its bulk on oxidative stress biomarkers, histopathology and biodistribution in rats after 28 days repeated oral treatment at 30, 300 and 1000 mg/kg body weight (b.w.). IONPs size in dry, wet forms and crystallinity was determined using TEM, DLS and XRD. The investigation of oxidative stress biomarkers demonstrated significant increase in lipid peroxidation and decrease in reduced glutathione content in the liver, kidney and the brain of the treated groups in a dose dependant manner. Further, antioxidant enzymes catalase, glutathione S transferase, glutathione peroxidase and glutathione reductase activities were significantly elevated along with significant decrease in superoxide dismutase activity in treated rat organs. ICP-OES analysis revealed dose and size dependant accumulation of IONPs in the liver followed by kidney and the brain than bulk. Moreover, accumulation of IONPs at high dose brought pathological changes only in liver. A large fraction of IONPs was eliminated in urine. Bulk material was substantially excreted in faeces than IONPs suggesting increased absorption of IONPs. In conclusion accumulated IONPs and bulk in organs trigger free radical generation, leading to the induction of oxidative stress condition in rats. The results obtained highlight the importance of toxicity assessments in evaluating the efficiency of IONPs for the safe implementation for diversified applications.

1. Introduction

Nanotechnology is the mere controlling and fabrication of materials at nano scale level, where characteristics vary with altered size, shape, density, aggregation and surface area. This technological leap leads to a revolution in the field of electronics and communications, optics, chemistry, energy and biology
Nanotechnology is a rapidly expanding field with an estimated market of $75.8 billion by 2020 (www.reportlinker.com/april2015). IONPs have promising characteristics like biocompatibility and magnetic behaviour that makes them good agents for magnetic resonance imaging, carriers for drug delivery, magnetic hyperthermia, tissue engineering, cell separation, enzyme immobilization, protein purification and biosensing (Mahmoudi et al., 2012; Rosen et al., 2012). IONPs are also used in many analytical techniques like PCR, immunoassay, HPLC and LC MS/MS (Cuo et al., 2012). Moreover, IONPs offer significant improvements in water purification and environmental remediation because of low cost technology (Baumann et al., 2014).

New approaches in nanotechnology and extensive production for numerous diversified applications of nanomaterials (NMs) might increase human exposure. A recent study on mice by Yang et al. (2015), reported size dependent bioaccumulation and transport of IONPs in the liver and spleen. Further, expression of genes associated with antioxidant defence system, transport and metabolism of iron were reported when treated with IONPs. Pregnant mice treated with multiple doses of IONPs reported biodistribution of iron in the foetal liver, placenta and increased foetal deaths (Di Bona et al., 2014). Intratracheal instillation studies of IONPs in mice reported increased cell disruption in the respiratory epithelium and affected haemostasis (Zhu et al., 2008). Initiation of microglial proliferation and pathological alterations in the brain was observed in mice treated with IONPs by intranasal route (Wang et al., 2013). IONPs induced higher levels of inflammation and immunosuppression in mice (Ban et al., 2015). This shows that IONPs can easily cross the intestinal linning and can enter into systemic circulation where it accumulates in different organs.

In vivo and in vitro studies of different NMs reported generation of free radicals and oxidative injury is the key mechanism to instigate adverse effects on cellular functions (Auffan et al., 2009). Overloading of cells with IONPs may trigger inimical cellular effects by initiating the fenton chemistry, which generates free radicals (Singh et al., 2009). Cells have antioxidant defence system to intercept the reactive oxygen species (ROS), however if the biological systems fail to nullify the overwhelming ROS, it will lead to oxidation of biomolecules like proteins, DNA and lipids (Prabhakar et al., 2012). Several studies demonstrated oxidative stress as an important biomarker in assessing NMs toxicity (Jain et al., 2008; Park and Park, 2009; Prabhakar et al., 2012; Negahdary et al., 2015; Arooj et al., 2015).

In the present investigation, the physicochemical properties of IONPs were determined using X-ray Diffraction (XRD), Transmission Electron Microscopy (TEM) and Dynamic Light Scattering (DLS). The purpose of the current study was to assess the differences in exerting toxic effects between IONPs and bulk after repeated oral treatment to Wistar rats. The liver, kidney and the brain were selected for our study, to investigate the oxidative stress induced by IONPs and its bulk using biomarkers like reduced glutathione (GSH), malondialdehyde (MDA), antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S transferase (GST). The histopathological analysis was done to detect tissue damage, if any. Biodistribution studies were undertaken to identify the potential target site of accumulation of IONPs and bulk.

### 2. Materials and methods

#### 2.1. Materials

IONPs were produced by chemical coprecipitation method following the method of Lee et al. (2004). The purity of IONPs was performed according to the method given by Yokel et al. (2009). The spectra analysis of IONPs was obtained using Siemens/D-5000 X-ray diffractometer. The size and morphology of IONPs were characterized using TEM (Hitachi H-7600). Hydrodynamic size distribution and zeta potential of IONPs were measured using Malvern Zetasizer. Probe sonicator (UP100H Heilscher, Germany) was used to disperse the NMs. Dispersion of NMs in the solution is measured by the polydispersity index (PDI).

#### 2.2. Synthesis and characterization of IONPs

IONPs were synthesized by adding 1.2 M iron (II) chloride hexahydrate and 0.9 M sodium hydroxide in 0.9% sodium chloride solution with continuous stirring for 1 h. The resultant solution was then washed several times with distilled water and dried at 80°C. The produced IONPs were characterized using X-ray diffraction, Transmission Electron Microscopy and Dynamic Light Scattering.

#### 2.3. Animals and treatment

According to the OECD (Organization for Economic Cooperation and Development) guidelines, the preferred rodent species is the rat, preferably females because of their sensitivity in comparison to males. Hence, female rats were used in our study. Institutional Animal Ethics Committee (IICT, Hyderabad) approved the present investigation. Female albino wistar rats of 8–10 weeks were procured from the National Institute of Nutrition, Hyderabad, India. Animals were acclimatized under standard laboratory conditions for 7 days prior to the start of experiments. The animals were fed on commercial pellet diet and water *ad libitum*.

The animals were randomly selected, marked and divided into following groups:

| Group | Compound | Dosage          |
|-------|----------|-----------------|
| Control | Water    | –               |
| Low    | Nano     | 30 mg/kg b.w.   |
| Medium | Nano     | 300 mg/kg b.w.  |
| High   | Nano     | 1000 mg/kg b.w. |
| Low    | Bulk     | 30 mg/kg b.w.   |
| Medium | Bulk     | 300 mg/kg b.w.  |
| High   | Bulk     | 1000 mg/kg b.w. |

The rats were treated daily with IONPs and bulk for 28 days and sacrificed on the 29th day after being anesthetized. The study and dose design were according to OECD guideline 407 (2008). High dose was chosen to illustrate the lethal effects and symptoms without mortality, whereas the low dose was selected to demonstrate no observed adverse effect levels (NOAEL). The animals were checked for toxic symptoms and mortality (if any) every day, whereas, weight gain and feed intake were recorded once in a week.
2.4. Oxidative stress biomarkers

Lipid peroxidation (LPO) in rat organs was estimated according to the method described by Wills (1969); reduced glutathione (Jollow et al., 1974); superoxide dismutase (Marklund and Marklund, 1974); catalase (Aebi, 1984); glutathione peroxidase (Paglia and Valentine, 1967); glutathione reductase (Carlberg and Mannervik, 1985); glutathione S transferase (Habig et al., 1974) and detailed procedures were described in Reddy et al. (2015). The protein estimation was done using Lowry et al. (1951) method.

2.5. Analysis of iron content in tissues

A part of the liver, kidney, and the brain from each group was collected at the end of the study. Individual urine and faecal samples were collected daily and pooled, analysed for the metal content following standard procedures as described in our earlier publication Reddy et al. (2015). ICP-OES (IRIS Intrepid II XDL, Thermo Jarrel Ash) was used to quantify Fe concentration at alternate wavelengths (232 nm and 238 nm). Quantification of Fe was done using a calibration curve.

2.6. Histopathological study

The liver, kidney and the brain collected from each rat were fixed in 10% formalin and processed following standard procedures. Detailed protocol was described in Reddy et al. (2015).

2.7. Data analysis

All the data were expressed as mean ± standard deviation (SD). Graphpad prism 5, a statistical software system was used to perform a post hoc multiple comparison test (Dunnet’s test) after ANOVA. \( P < 0.05 \) was considered to be significant.

3. Results

3.1. Characterization

Fig. 1 summarizes the characterization of IONPs by XRD, TEM and DLS. The purity analysis by ICP-OES revealed that the purity of IONPs was >96%. This analysis was in support of XRD data obtained. Peaks obtained signify size, purity and crystallinity of IONPs and revealed similar characteristics with \( \gamma \)-Fe\(_2\)O\(_3\). TEM characterization of IONPs showed spherical morphology with mean size distribution of 7.02 ± 1.58 nm. The hydrodynamic sizes of IONPs were measured using DLS in MilliQ water showed an average diameter of 155 nm with a PDI measurement of 0.526. These hydrodynamic sizes in dispersion vehicle were larger than their original size in powder form signifying nanoparticles agglomeration. Zeta potential measurements for IONPs were -29.9 mV at pH 7. For bulk material size distribution intensity was found to be beyond detection limits.
3.2. Morphology, feed intake and body weight of animals

Fig. 2 represents body weight (A) and feed intake (B) of animals treated with IONPs and bulk. Signs of toxicity and mortality were not seen in the treated rats. However, rats treated with a high dose of bulk and IONPs showed significant loss in body weight in weeks 3 and 4. Average feed intake per animal was significantly decreased in high dose IONPs treated rats in comparison to other treatment groups and control.

3.3. Oxidative stress biomarkers

The effect of IONPs and bulk on LPO in the liver, kidney and the brain is presented in Fig. 3A. IONPs and bulk treatment caused significant and a dose dependent increase in MDA levels in the liver at high and medium doses. Only high dose of IONPs and bulk increased LPO level in kidney and the brain. IONPs treated rats showed increased MDA levels than the bulk. The changes in GSH content in different tissues of treated and control rats are summarized in Fig. 3B. IONPs and bulk brought dose dependent depletion in GSH content of the liver, except low dose group of bulk material. However, IONPs at 1000 mg/kg b.w. and 300 mg/kg b.w. and bulk at 1000 mg/kg b.w. significantly decreased GSH content in kidney and the brain. Furthermore, depletion in GSH content was found high in IONPs treated rats than its bulk. High and medium dose of IONPs and bulk treatment caused significant and dose dependent inhibition of SOD in liver. However, 1000 mg/kg b.w. treatment of IONPs and bulk caused depletion in SOD activity in kidney and the brain (Fig. 4A). A significant and dose dependant elevation in activity of CAT was observed in the liver, when rats were treated with IONPs and bulk at 1000 mg/kg b.w. and 300 mg/kg b.w. Similarly statistically significant increase in CAT activity was observed in kidney and the brain after the treatment of IONPs and bulk.
Figure 4  Effect of 28 day oral treatment of IONPs and Bulk on antioxidant enzyme activities in rat organs. Each value represents the mean ± SD; n = 5 rats. *P < 0.05.
at 1000 mg/kg b.w. (Fig. 4B). IONPs and bulk treatment at 1000 mg/kg b.w. elevated GPx activity significantly only in the liver and kidney. The activity of GPx was not altered in the brain at all the doses of IONPs and bulk treatment. The activity of GPx was not altered in the brain at all the doses of IONPs and bulk treatment (Fig. 4C). GR activity was significantly elevated in the liver in a dose dependent manner after IONPs treatment at 300 mg/kg b.w. and 1000 mg/kg b.w. However, bulk treatment at 1000 mg/kg b.w. increased GR activity only in the liver. Further, significant change in activity of GR was observed in kidney and the brain at mg/kg b.w. of IONPs and bulk treatment (Fig. 4D). Treatment of IONPs and bulk at 1000 mg/kg b.w. caused a substantial increase in GST activity in the liver, kidney and the brain (Fig. 4E). Antioxidant enzymes SOD, CAT, GPx, GR and GST were modulated significantly in IONPs treated rats than its bulk.

3.4. Analysis of iron content in tissues

A dose dependent increase in accumulation of Fe content was seen in the liver (188 µg/g, 224 µg/g, 303.9 µg/g), followed by kidney (58.8 µg/g, 67.4 µg/g, 88 µg/g) and the brain (28.6 µg/g, 38.3 µg/g, 45.8 µg/g) in IONPs treated rats. Similarly uptake of bulk was high in the liver (151.5 µg/g, 178.5 µg/g, 191.2 µg/g) followed by kidney (49.2 µg/g, 57.3 µg/g, 65.2 µg/g) and the brain (28 µg/g, 30.8 µg/g, 37.5 µg/g). However, accumulation of IONPs in organs was higher than bulk. Elimination of Fe through urine was found to be more significant in IONPs (65.3 µg/ml, 74.6 µg/ml, 83.6 µg/ml) treated rats than its bulk. In contrary, in faeces bulk (2390 µg/g, 8849.7 µg/g, 11072.4 µg/g) was eliminated more in comparison to IONPs (1116.7 µg/g, 3233.5 µg/g, 5366.9 µg/g) as shown in Fig. 5A.

3.5. Histopathological study

The histopathological changes in IOPNs and bulk treated the liver, kidney and the brain are presented in Fig. 6. Only high dose treatment of IONPs caused congested portal tract in liver whereas, normal histology was observed in the remaining groups of IONPs and bulk.

4. Discussion

Nanotoxicological studies performed in vivo using different routes of exposure hold greater impact in evaluating the adverse biological effects. In the present investigation, we hypothesized oxidative stress as a crucial parameter in IONPs induced toxicity in treated rats. The current study illustrates that the MDA levels were increased in tissues in a dose dependent manner in both IONPs and bulk treated rats. Elevated levels of MDA indicate the formation of hydroxyl radicals which in turn is associated with LPO of tissues. Our results illustrated a dose dependent depletion of reduced glutathione (GSH) after IONPs and bulk treatment, possibly due to enhanced utilization of GSH in both enzymatic and non-enzymatic reactions. Srinivas et al. (2012) reported elevated LPO and depleted GSH in rat lung tissue after inhalation exposure of IONPs. Similarly, oral toxicity study of aluminium oxide NMs and bulk demonstrated elevated levels of MDA and decreased GSH levels in a dose dependant manner in the liver, kidney and the brain of treated rats (Prabhakar et al., 2012). Hence, it is suggested that MDA and GSH contents are good biomarkers in assessing toxicity, which may be possibly due to free radical generation.

![Figure 5](image_url)
Individual antioxidant enzymes play different strategies to counteract oxidant insult. In this study, a significant increase in CAT, GR, GPx, GST and decrease in SOD activity in treated rat tissues were observed. Superoxide radicals in association with adventitious metal like Fe, triggers the oxidative damage of proteins, lipids and nucleic acids (Chevion, 1988). Current study revealed a dose dependent decrease in SOD activity in rat organs. This might probably be due to the heavy influx of superoxide radicals that in turn resulted in high levels of H$_2$O$_2$ generation, which subsequently inhibited SOD activity (Li et al., 2010b). IONPs and bulk treatment caused elevation in enzyme kinetics of CAT as a defensive mechanism against excess H$_2$O$_2$ levels. GPx initiates H$_2$O$_2$ breakdown only at low concentrations, whereas CAT needs high levels of H$_2$O$_2$ to commence its activity. An increased GR activity was found in our study, this result reflects the recycling of oxidized GSH to reduced GSH by the pro-oxidant system which involves a coupled reaction engaged by GR (Nordberg and Arnér, 2001). Elevated GST activity and depleted GSH levels suggest increased utilization of GSH in conjugation reactions as part of detoxification mechanism. Altered antioxidant status as reported in our study is supported by findings from other investigations. Prabhakar et al. (2012) reported increased enzyme activities of CAT and GST, with depleted SOD activity in rat tissues exposed to aluminium oxide NMs. Administration of TiO$_2$ NMs to mice for 14 days induced significant oxidative stress than its bulk (Ma et al., 2010). An altered antioxidant status revealed imbalance in oxidant and antioxidant levels in the tissues.

In the present study ICP-OES analysis revealed that IONPs and bulk material were absorbed and extensively deposited in the liver, kidney and the brain, and excreted through urine and faeces. Wang et al. (2010) reported accumulation of IONPs in major systemic organs of mice at an oral dose of 600 mg/kg b. w., which is in support of our results. In the current investigation, IONPs with a hydrodynamic size of 155 nm and with a charge of $-29.9$ mV were readily taken by liver for phagocytosis, which clearly indicates size and charge also influence the uptake and clearance mechanisms. Our results demonstrated that uptake of IONPs was more in the liver, kidney and the brain in a dose dependent manner. The IONPs have differential organ distribution pattern which seems to be influenced by Fe binding proteins like transferrin and ferritin, which serve as carrier of Fe to different tissues. Increased Fe levels in brain in the present investigation might be due to binding of Fe to transferrin, that triggers up regulation of Fe receptors in the brain, thus transporting Fe across the blood brain barrier (Descamps et al., 1996). Inhalation exposure study in mice demonstrated that IONPs <50 nm reached the brain without provoking any adverse effects (Kwon et al., 2008). Higher localization of IONPs in liver might have caused histopathological changes, causing congestion in portal tract. However, histological changes were not observed in kidney and the brain of both the treated groups. A dose dependant and significant amount of IONPs was eliminated in urine. Trace amount of bulk passed through the intestinal epithelium and remaining being excreted in faeces. However, IONPs excreted in faeces were less than the bulk.

Taken together, the findings from the current investigation, confirm that IONPs accumulation in tissues triggered ROS generation, which significantly altered the antioxidant enzyme levels. The altered antioxidant status and bioaccumulation of
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IONPs caused histomorphological changes, which demonstrate that increase in oxidative stress affects the cellular structure. Moreover, the results of this study can be utilized by the regulatory bodies in defining safety limits for the usage of IONPs in biomedical applications.

Conflict of interest
The authors declare that there are no conflicts of interest.

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