

Toxicity, toxicokinetics and biodistribution of dextran stabilized Iron oxide Nanoparticles for biomedical applications



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ABSTRACT

Advancement in the field of nanoscience and technology has alarmingly raised the call for comprehending the potential health effects caused by deliberate or unintentional exposure to nanoparticles. Iron oxide magnetic nanoparticles have an increasing number of biomedical applications and hence a complete toxicological profile of the nanomaterial is therefore a mandatory requirement prior to its intended usage to ensure safety and to minimize potential health hazards upon its exposure. The present study elucidates the toxicity of in house synthesized Dextran stabilized iron oxide nanoparticles (DINP) in a regulatory perspective through various routes of exposure, its associated molecular, immune, genotoxic, carcinogenic effects and bio distribution profile. Synthesized ferrite nanomaterials were successfully coated with dextran (<25 nm) and were physicochemically characterized and subjected to *in vitro* and *in vivo* toxicity evaluations. The results suggest that surface coating of ferrite nanoparticles with dextran helps in improvising particle stability in biological environments. The nanoparticles do not seem to induce oxidative stress mediated toxicological effects, nor altered physiological process or behavior changes or visible pathological lesions. Furthermore no anticipated health hazards are likely to be associated with the use of DINP and could be concluded that the synthesized DINP is nontoxic/safe to be used for biomedical applications

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1. Introduction

The revolutionary bloom of nano scale technologies in the present era has set new horizons for the wide exploitation of this unique technology for an ample domain of biomedical applications. However the unique physico-chemical and dimensional characteristics of the nano-sized particles is of great concern as it can interact with the biological system and can scale up to unpredictable manifestations. This growing debate on the risk assessment of nanoparticles has to be addressed to unravel the safety issues to manpower associated with the research, production and end use of the nanoparticles. Nanotoxicology, a science that deals with toxicity studies of nanomaterials (Twie, 2012) is particularly appealing as well as demanding in the above context.

Super paramagnetic iron oxide nanoparticles, represents a promising nanoparticle system in nano medicine with numerous clinical as well as theranostic applications such as MRI contrast

enhancement, cell targeting and labeling, drug delivery and hyperthermia, cell separation, tissue repairing etc (Laurent and Mahmoudi, 2011). The potential adverse effects of metal and metal oxide nanoparticles are however mitigated/eliminated by appropriate biomimetic surface coatings (Yu et al., 2012). Dextran ($C_6H_{10}O_5$), a branched polysaccharide, coated Ferrite nanoparticles have been used extensively for health care prospects, including MRI contrast agents, cellular targeting probes, hyperthermia agents etc (Jha et al., 2014). Dextran being hydrophilic and biocompatible aids in the intra cellular uptake of ferrite nanoparticles (Uthaman et al., 2015). Despite the cautious engineering of DINP, their intended application can induce toxicity in biological system, due to the inherent nano scale properties. In the natural milieu, iron homeostasis is well maintained in mammalian cells and biological fluids (Cabantchik, 2014). In excess, the natural iron binding proteins may get saturated allowing free iron to circulate in systemic circulation. This when lodged into internal organs can exacerbate toxic effects due to the production of reactive oxygen species that increases lipid peroxidation with resulting damage to mitochondria and other cellular organelles (Adibhatla and Hatcher, 2010). Iron mediated enhanced oxidative damage to DNA is also reported (Chattopadhyaya and Goswami, 2012). Furthermore,

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absence of an excretory pathway for iron aggravates the toxic effects mediated by iron bioaccumulation (Peto, 2010).

Most intracellular and *in vivo* toxicity of nanoparticles are attributed to their ability of producing excess reactive oxygen species (ROS) (Manke et al., 2013). It has been reported that nanoparticle induced oxidative stress occurs due to the dissolution of iron from iron based nanoparticles which mediates generation of hydroxy and peroxy free radicals from H_2O_2 via the Fenton reaction (Thomas et al., 2009). Excess ROS are markers of oxidative stress and can damage the cells by altering proteins, modulating gene expressions, lipid peroxidation, DNA adduct formations etc finally culminating in pathological conditions (Dalle-Donne et al., 2006). Oxidative stress affecting the cell signaling cascades can either activate apoptotic pathways leading to apoptosis or necrosis (Fulda et al., 2010). It can also get associated with carcinogenic activity of cells. Hence a detrimental outcome is expected through iron nanoparticle interaction at the cellular level. However coating with biocompatible phases ameliorates these effects and hence toxicity evaluation of surface modified iron oxide nanoparticles requires attention.

Toxicity of any nanomaterial is also governed by the route and duration of the exposure. Inhalation, ingestion, injection and dermal contact form the basic exposure routes often encountered by researchers, manufacturing unit people and finally the end users (Ray et al., 2009). Hence a thorough put investigation on the significant biological responses at each exposure routes is needed. Interaction of nanoparticles with immune system is another potential area of research interest. Major consideration in the nanoparticle mediated immunotoxicity is that of inflammatory response and T cell response. Immuno response of DInP interaction needs to be comprehended further, as the primary cells that come in contact with nanoparticles would be the immune cells present in the blood upon systemic absorption. Furthermore, although there are reports that iron oxide particles does not truly cause any direct genotoxic effects (Singh et al., 2010), its consequences at the genetic level due to the indirect action of ROS needs to be elucidated.

Exclusive studies on toxicity of dextran stabilized ferrite nanoparticles are limited. It has been shown that dextran coating considerably decreased the iron nanoparticle mediated cytotoxicity in pulmonary artery endothelial cells (Mojica Pisciotti et al., 2014). A comparative study of neurotoxic potential of dextran coated iron-based magnetic nanoparticles reported that coated nanoparticles has no significant effect on synaptic vesicle acidification, glutamate levels as well as synaptic vesicular functions (Borysov et al., 2014). Acute toxicity and irritation studies following subcutaneous injection of a water-based dextran-coated magnetic fluid (dextran-magnetic fluid) in mice concludes that dextran-magnetic fluid is biocompatible and well tolerable (Yu et al., 2008). In a study on the effect of surface coatings on cell behavior and morphology of fibroblasts, it was shown that dextran-magnetite nanoparticles result in decreased proliferation rate and subsequently cell death analogous to that caused by uncoated iron oxide particles (Berry et al., 2003). The cytotoxicity was attributed to the breakdown of the dextran shell exposing bare iron oxide NPs to interact with the cellular systems. Our group has reported the acute dermal and acute oral toxicity studies of dextran coated ferrite nanomaterials (Syama et al., 2014; Mohanan et al., 2014). Furthermore, other than an *in vitro* investigation that demonstrated micronucleus formation in human MCL5 lymphoblastoid cells treated with dextran-coated $g\text{-Fe}_2\text{O}_3$ nanoparticles for 24 h (Valdiglesias et al., 2015), genotoxic evaluation of DInP is not been extensively studied. Hence the consequence of DInP interaction at the cellular and molecular level, genotoxic and immuno toxic effects needs to be elucidated in detail before rendering it safe for biomedical application. In the

present study, the toxicological profile including toxicokinetics and bio-distribution of an in house synthesized dextran stabilized iron oxide nanoparticles of less than 25 nm particle size is addressed in a regulatory perspective for projecting DInP as a prospective bio-nano material in nanomedicine.

2. Materials and methods

2.1. Synthesis and physicochemical characterization of Dextran stabilized Iron oxide nanoparticles

Iron oxide nanoparticles were synthesized by an alkaline co-precipitation method from heating an aqueous solution of $FeCl_3$ and $FeCl_2 \cdot 4H_2O$ (2:1) in a nitrogen atmosphere at 80 °C followed by precipitation with 3 M NaOH as described elsewhere (Pereira et al., 2012). Physical adsorption of a layer of dextran on to the surface of ferrite nanoparticle was done by stirring of ferrite nanomaterial in a solution of water: ethanol (7:3) containing 3% dextran. Black precipitate obtained is washed with deionized water and lyophilized to get dextran stabilized iron oxide nanoparticles. The synthesized nanoparticle is characterized for its hydrodynamic size by DLS (Malvern Zeta sizer, Nano ZS), particle size by TEM (Hitachi H-7650) component analysis by FTIR (Nicolet 5700 FTIR spectrometer), phase purity analysis by XRD (PAN analytical X'Pert Pro MRD) and TGA analysis (SDT-2960 TA).

2.2. Experimental animals

Albino rats (Wistar), Albino guinea pigs (Hartley) and Albino mice (Swiss) were procured from the Division of Laboratory Animal Sciences, BMT wing, SCTIMST. All the experiments were performed as per the CPSCEA guidelines and in accordance with OECD guidelines after obtaining approval from Institute animal ethics committee prior to the tests. The animals were handled humanely, without hurting or distressing them and with due care for their welfare. Individually ventilated cages were used for the housing of guinea pigs. Rats were housed in anodized cages and mice were grouped in 5 numbers and were housed in ventilated cages. A controlled environmental condition of temperature (22 ± 3 °C) and humidity (30–70%) and with a 12 h light and dark cycle was maintained for the animals. Commercially available food and filtered water were given *ad libitum* throughout the experimental period. Guinea pigs in the weight range 300–600 g, albino mice (19–23 g) and rats (200–220 g) were used for the *in vivo* experiments.

2.3. Cytotoxicity assay—*In vitro* studies using L929 fibroblasts

The cytocompatibility of synthesized DInP was assessed qualitatively by direct contact assay and quantitatively by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay based on established protocol (van Meerloo et al., 2011). Briefly, 96 well plates were seeded at a density of 1×10^4 cells per well with L929 cells and maintained in a 5% CO_2 incubator at 37 °C. After overnight incubation, DInP at a concentration range of 100–800 $\mu\text{g}/\text{mL}$ were kept in contact with cells for 24 h. After incubation, the cells were examined under microscope for altered morphology. The cellular response in comparison to negative and positive control was scored as non cytotoxic, mildly cytotoxic, moderate cytotoxic and severely toxic. For quantitative measurement, 25 μL of MTT dye (2 mg/mL) in PBS was added to each well and incubated for 4 h in dark at 37 °C. The soluble formazan crystal formed as a result of metabolic reduction of soluble MTT by mitochondrial dehydrogenase is solubilized with 200 μL of DMSO and measured at 540 nm in a micro plate reader.

2.4. Oral toxicity—In vivo studies in rats

2.4.1. Acute oral toxicity

A single oral dose of 300 mg/kg body wt and 2000 mg/kg body wt of DINP were administrated to experimental rats ($n=3$ each group) using gastric needle. Untreated rats were taken as control. The animals were observed for a period of 14 days for the evidence of adverse reactions. After observation period, the animals were sacrificed and various postmortem analyses were performed.

2.4.2. Chronic oral toxicity

Prolonged and repeated exposure of DINP was studied as per OECD 453 guidelines (OECD, 2016a). The animals were provided with commercially available feed mixed with DINP at a defined concentration (low dose 500 mg/kg bodyweight- Medium dose-1000 mg/kg bodyweight and High dose-2000 mg/kg bodyweight). Feed intake was monitored biweekly for a prolonged period of 12 months.

2.5. Dermal toxicity—In vivo studies in rats

Information on the possible health hazards likely to rise from the repeated exposure of nanomaterials through dermal route was evaluated by exposing different concentration of DINP (low-25 mg/kg, Medium-50 mg/kg and High-100 mg/kg) to the experimental rats ($n=3$ each group) 6 h daily for 28 days.

2.6. Immuno toxicity

2.6.1. Lymphocyte proliferation assay

100 mg/kg body wt of DINP were injected intraperitoneally in mice ($n=24$). The treated animals were sacrificed at 7th, 14th and 21 days of exposure. Untreated animals at day 0 were taken as control. Spleen was collected from the sacrificed animals. Splenocytes were isolated using histopaque gradient as per accepted protocol (Jungblut et al., 2008). T cells and B cells were separated with the aid of mouse T and B cell enrichment kit (Easy Sep, Stem cell technology) in an automated magnetic cell sorter (ROBOSEP, Stem cell Technologies) and lymphocyte proliferation assay were carried out using Tritiated Thymidine incorporation method (Bounous et al., 1992).

2.6.2. Delayed hyper sensitivity

Closed patch test (Beuhler test) was used to study the delayed hypersensitivity response of DINP as per ISO standards (10993-101, 2010). For induction and sensitization, a concentration of 80 mg/animal of DINP was applied topically on the clipped upper back region of guinea pigs ($n=10$). Similarly a patch of four ply gauze moistened in physiological saline alone was applied to the control animals ($n=5$). This procedure was repeated three times/week for three weeks. On the 14th day after the last application (sensitization period), all the experimental animals were challenged with the similar dose of DINP. The skin sensitization potential was evaluated at 24, 48 and 72 h and scored according to the Magnusson and Kligman scale (OECD, 2016b).

2.7. Genotoxicity

2.7.1. Detection of 8- hydroxyl-2-deoxyguanosine (8-OHdG) by ELISA

Mitochondrial as well as genomic DNA was isolated from 100 mg of liver collected from the rats used for acute oral toxicity studies using mitochondrial DNA extractor kit (mtDNA Extractor Kit, Wako, Japan) and Genelute mammalian genomic DNA mini prep kit (Sigma Aldrich, USA) as per kit protocol. Isolated DNA samples were confirmed by PCR analysis of mitochondrial DNA using specific primers for cytochrome c gene and genomic DNA

using specific primers for the gene β -actin. 8-OHdG was determined from the DNA samples by a competitive Immuno sorbent assay using new 8-OHdG check kit (Japan Institute of Control of Aging, Japan) as per the kit protocol.

2.7.2. Mammalian bone marrow chromosomal aberration study

The study was conducted as per OECD guidelines for the testing of chemicals (OECD, 2016c). Briefly 50 mg/kg, 100 mg/kg, 200 mg/kg body weight of DINP, and 50 mg/kg bodyweight cyclophosphamide (positive control) and 50 mL/kg body weight Physiological saline (negative control) was intraperitoneally injected into mice and after 24 h, the animals were sacrificed. All the animals were received colchicine 3 h before sacrifice. Bone marrow from the femur bones were collected into normal saline, centrifuged and treated with hypotonic solution. After fixing the cell button with methanol: glacial acetic acid solution smears were prepared and stained with Giemsa stain. The stained slides were subjected to microscopic evaluation for the evidence of chromosomal anomalies.

2.8. Carcinogenicity

The carcinogenic potential of DINP were also examined in experimental animals subjected to prolonged oral exposure of DINP as per OECD guidelines combining both chronic oral toxicity and carcinogenicity studies (OECD, 2016a).

2.9. Bio-distribution

Elemental analysis of the DINP exposed samples (liver, kidney and spleen) from chronic toxicity (rat) as well as immunotoxicity (mice) was determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS (Optima 5300 DV, Perkin Elmer). Briefly the samples were digested with concentrated nitric acid and perchloric acid. Clear solution obtained after digestion is analyzed using ICP-MS, results are recorded and processed using Win Lab 32 software.

2.10. Evaluations

2.10.1. Ante mortem evaluations: clinical observation

The result of the systemic toxicity was evaluated on the basis of toxic signs, symptoms, body weight reduction or death of animals. The animals were subjected to daily observation for overall health, behavior changes/patterns, mortality or morbidity signs. Individual body weights and feed intake were noted periodically. For long term animals, a throughput physical examination that included palpation for the presence of tissue masses was given biweekly.

2.10.2. Biochemical and hematological parameters

Experimental animal blood was collected in EDTA vials and analyzed for routine hematological parameters such as Hemoglobin (Hb, g/dL), total count ($WBC \times 10^3/\text{mm}^3$), red blood corpuscles count ($RBC \times 10^6/\text{mm}^3$), platelet count ($PLT \times 10^3/\text{mm}^3$), using automated Vet ABC Animal blood counter (ABX Diagnostics, France). Serum was analyzed for biochemical parameters such as urea, Serum Glutamic Oxaloacetic transaminase (SGOT), Serum Glutamic Pyruvate Transaminase (SGPT), Alkaline Phosphatase (ALP), Gamma-Glutamyl Transferase (GGT), glucose (GLU), cholesterol, triglycerides, total protein, albumin, calcium, phosphorus, chloride, total bilirubin and creatinine using automated biochemistry analyzer, ERBA Manheim XL 300 (ERBA, Mannheim, Germany).

2.11. Postmortem evaluation

2.11.1. Histopathological analysis

After the experimental period, all animals were euthanized and subjected to a postmortem examination. At necropsy, all organs and tissues were examined for grossly visible lesions, and the required tissues were fixed and preserved in 10% neutral buffered formalin for histopathological analysis. Tissues were trimmed, processed, embedded in paraffin, sectioned to a thickness of 4–6 μm , and stained with hematoxylin and eosin (H&E) and examined under light microscope.

2.11.2. Preparation of liver homogenate and anti oxidant assays

Liver of experimental animals after euthanasia was rapidly excised, washed in normal saline and were collected. 10% of tissue homogenate was prepared in phosphate buffer (0.1 M, pH 7.4) and was used for the estimation of total protein, LPO, GR, GSH, GPx, SOD as per established protocols (Geetha et al., 2013).

3. Results and discussion

The need for an exclusive investigation on the biological consequences of super paramagnetic iron oxide nanoparticles is imperative, particularly in the scenario of its significantly increased application in nano medicine. Detailed investigation of acute as well as potential long term effects of exposure to these nanoparticles is however needed, ensuring its safe and controlled usage to humans. The iron oxide nanoparticles were synthesized

by the classic alkaline co precipitation method which has an advantage of synthesizing large amount of nanoparticles. Iron oxides nanoparticles were prepared by heating a stoichiometric mixture of FeCl_3 and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (2:1) in aqueous solution. The co-precipitation process, involves 2 stages an initial burst of nucleation followed by the slow growth of the nuclei by diffusion of the solutes onto the crystal surface. The size of the particle should be controlled at the initial event of nucleation phase. The disadvantage of this method is that since there are only kinetic forces to govern the entire reaction, particle size distribution cannot be controlled. The method is detailed elsewhere (Peternele et al., 2014).

The intrinsic instability of nanoparticles with a tendency of agglomeration over long periods of time, a common problem associated with any nanoparticle, holds good for iron oxide nanoparticles as well. The hydrophobic interactions between Iron oxide nano particles inherently possessing large surface area- to volume ratio will cause them to aggregate forming larger clusters. Bare iron oxide nanoparticles are easily trapped by immune system preventing them reaching the target. Moreover they are easily oxidized in air resulting in the loss of dispensability and magnetic properties rendering them inactive for their intended application (Lu et al., 2007). To minimize these undesired effects, polymeric surface coatings are given to stabilize the nanoparticles as well as to provide biomimicity. The polymers coated on iron oxide nanoparticles will augment the repulsive forces to counteract with the vanderwaals attractive forces acting on the nanoparticles and hence polymer functionalized iron oxide nanoparticles have

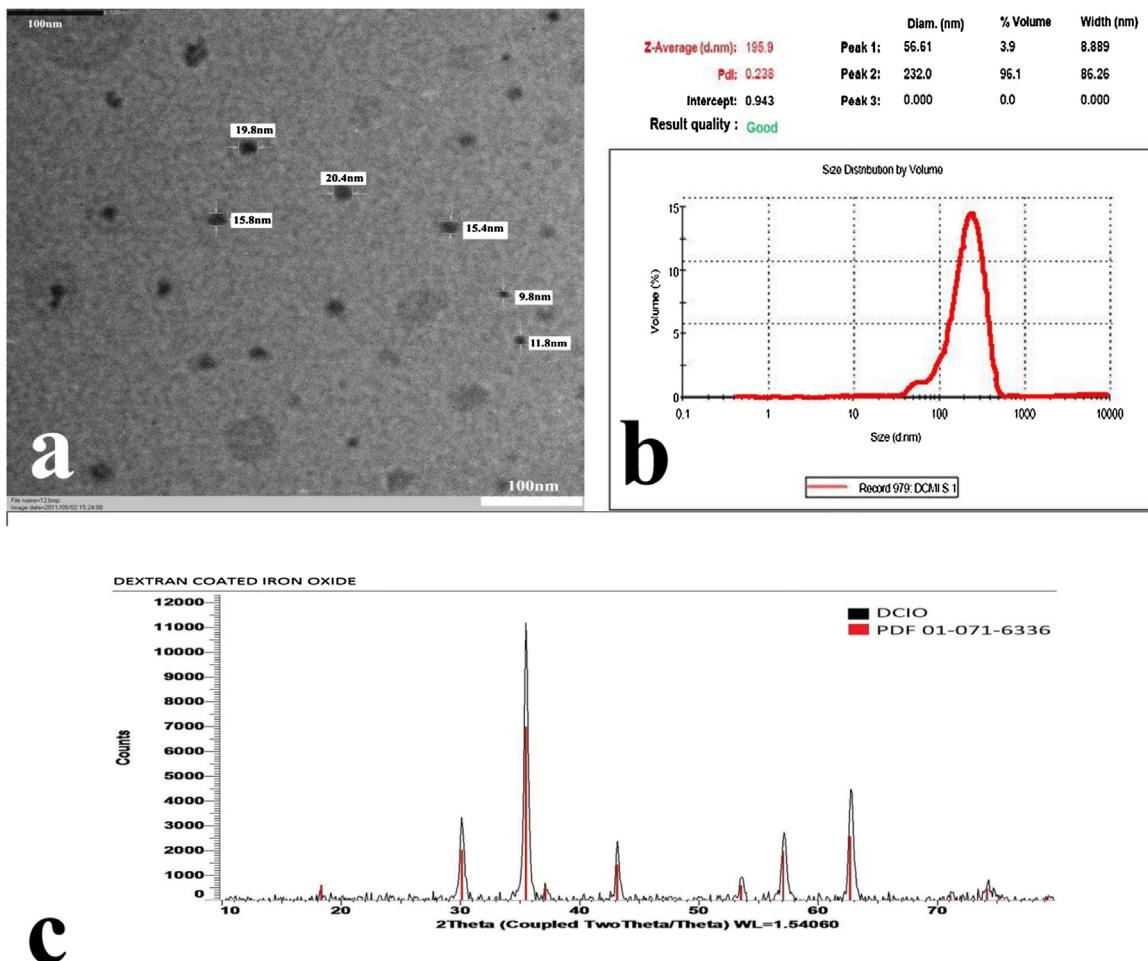


Fig. 1. Physicochemical characterization of DInP (a) TEM analysis (scale bar corresponds to 100 nm) (b) hydrodynamic size measurement using DLS (c) XRD analysis.

gained much attention in applied research. However the coating will increase the particle size and decrease the saturation magnetization value of iron oxide nano particles. Even though the bio-distribution profile of the nanoparticle is improved, limited clearance as well tissue penetration is seen associated with it. Commonly used polymers for functionalization are dextran and its derivatives, starch, arabinogalactan, glycosaminoglycan, sulfonated styrene-divinylbenzene, polyethylene glycol (PEG) and polyvinyl alcohol (PVA) (Boyer et al., 2010). Dextran, a polysaccharide has been widely used for functionalization of iron nanoparticles because of its biocompatibility. Ferumoxtran-10 and ferumoxides are modified dextran functionalized iron oxide nanoparticles (Di Marco et al., 2007). It has been reported that the physical adsorption of dextran moiety on to the surface of iron oxide core is primarily attributed by the polar interactions, especially the hydrogen bonding between the hydroxyl group present in dextran chains and the iron oxide particle surface (Laurent et al., 2008). The large number of hydroxyl groups in the dextran chains significantly increases the total bonding energy of hydrogen bonds which upsurge the adsorption. It is demonstrated that, the dextran polymer coating is desorbed from the iron oxide surface on dilution or heating at 121 °C. In this context, sterilization methods

have to be modified taking this matter into consideration. Surface bound dextran is biomimetic as it mimics the glycocalyx of the cell. Furthermore, dextranase an enzyme that degrades dextran is present in bacteria and not in mammalian tissue and hence is resistant to *in vivo* enzymatic degradation making it more biocompatible.

The size of the particle core as determined by TEM showed spherical nanoparticles within the range of <25 nm with an average core diameter of 15.4 ± 4.5 nm (Fig. 1a). However the dynamic light scattering method that measures the hydrodynamic size of the particles in dispersion medium showed slightly larger sized particles between the ranges of 40–160 nm with an average hydrodynamic size of 86 nm in diameter. (Fig. 1b). X ray diffraction (XRD) pattern matches with the standard pattern of Fe_3O_4 with definite peaks at 220, 311, 400, 511, 440 respectively. (Fig. 1c). The average crystalline size corresponding to the highest peak observed is 21.8 nm as calculated from Scherrer equation which almost matches with the average core diameter obtained by TEM imaging. The phase purity was comparable to that of standard magnetite. FTIR spectrum showed the characteristic absorption peak due to the stretching vibration of Fe-O bond in iron oxide particles and DINP at 580 cm^{-1} . The peculiar absorption bands of

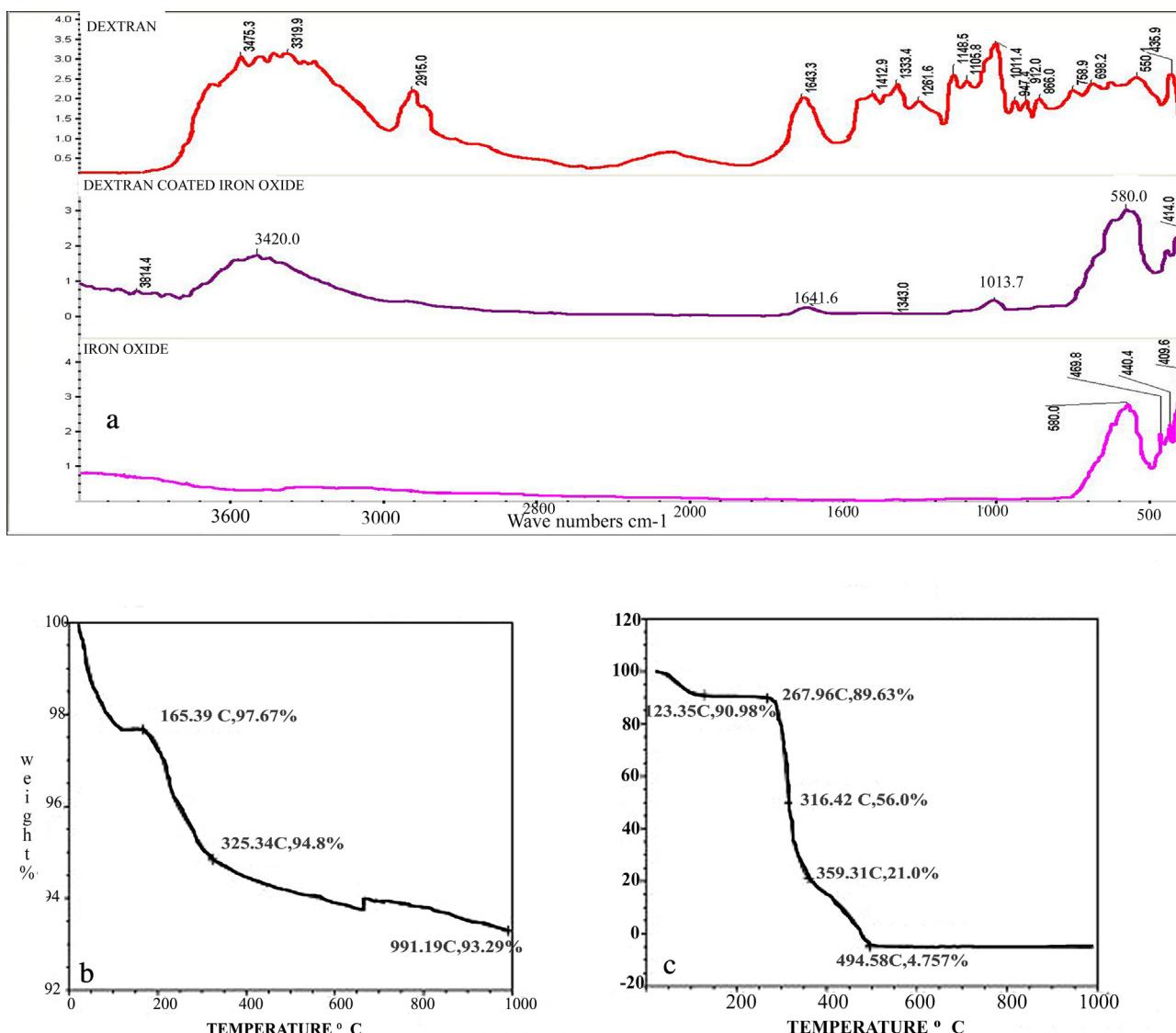


Fig. 2. Physicochemical characterization of DINP (a) FTIR analysis (b) and (c) TGA analysis.

polysaccharide at 3420 cm^{-1} is due to the hydroxyl stretching vibration of the polysaccharide (O—H). The band in the region of 1631 cm^{-1} is due to carboxyl group and peak at 1013 cm^{-1} corresponds to the great chain flexibility present in dextran around the glycosidic bonds. The spectrum exhibited bands characteristics of dextran as well as iron oxide confirming the adsorption of dextran on iron oxide nanoparticle. (Fig. 2 a). Initial weight loss of 2% at $\sim 200^\circ\text{C}$ in thermo gravimetric analysis is due to the decomposition of physically adsorbed water. The rest 5% weight loss at a temperature range between 200°C and 400°C may accounts for the breakdown of polymer while bare iron oxide nanoparticle persists throughout the temperature range. The data was confirmed by TGA analysis of pure dextran which shows degradation between the 200°C and 400°C (Fig. 2b and c).

The in-house synthesized dextran stabilized iron oxide nanoparticles were evaluated for their cytocompatibility. The direct contact assay with L929 fibroblast showed that, cells tolerated DINP even up to a concentration of $800\text{ }\mu\text{g/mL}$. No phenotypic changes were observed in the cells exposed to different concentrations of DINP. The cells retained their spindle shaped morphology with cell membrane integrity. This is attributed by the biocompatibility of dextran (Fig. 3a). Furthermore MTT assay which evaluates the mitochondrial activity of viable cells also substantiated the cytocompatibility of DINP as more than 80% of the cells were metabolically active when spectrophotometrically examined (Fig. 3b). This also gives the evidence that, the coating of polymer is not degraded exposing the bare iron oxide nanoparticle which would have been otherwise detrimental to the cells.

3.1. Acute oral toxicity

The safety issues of iron oxide based nanoparticles especially when using as ingestible therapeutic medication should be

addressed before clinical translation. Acute oral toxicity studies are therefore conducted for DINP that might provide information on the possible health imperilment likely to arise from single exposure to a material within a period of 24 h up to 14 days. Clinical signs and symptoms were monitored carefully throughout the experimental period. Peculiar animal behavior like tremors, excessive salivation, piloerection, convulsions, lethargy, coma, morbidity and mortality were not evident in untreated as well as treated groups. Blood parameters were comparable to that of untreated control sample. Some of the biochemical parameters analyzed showed alteration from the untreated samples. The slight elevation in the levels of total protein observed in DINP treated animals at a conc of 300 mg/kg body weight ($7.7 \pm 0.2\text{ g/dL}$) and at high concentration of 2000 mg/dL (8.23 ± 0.87) when compared to the untreated control animals ($7.23 \pm 0.21\text{ g/dL}$) may be due to the increased levels of iron binding proteins in blood as the amount of albumin was not elevated. Total blood glucose were also slightly elevated in high dose administered animals ($117.75 \pm 2.05\text{ mg/dL}$) compared to that of control ($87.1 \pm 2.82\text{ mg/dL}$) that accounts for the enzymatic degradation of dextran to the final product glucose. However not much noticeable changes occurred in the biochemical and hematological assessment following oral administration of DINP. The data has already been reported (Syama et al., 2014). The major paradigm for nanoparticle toxicity is related to oxidative stress due to the increase in reactive oxygen species and its deleterious consequences. The partially reduced oxygen metabolites like superoxide anions, hydrogen peroxides and hydroxyl radicals formed as a result of oxidative stress can interact with components of cell resulting in their structural modifications. Lipids are the major site of modification by ROS causing its peroxidation. Measurement of MDA (malondialdehyde), the byproduct of LPO provides an index of such oxidative damage. A slight increase in malondialdehyde was observed in both

a. Direct contact assay

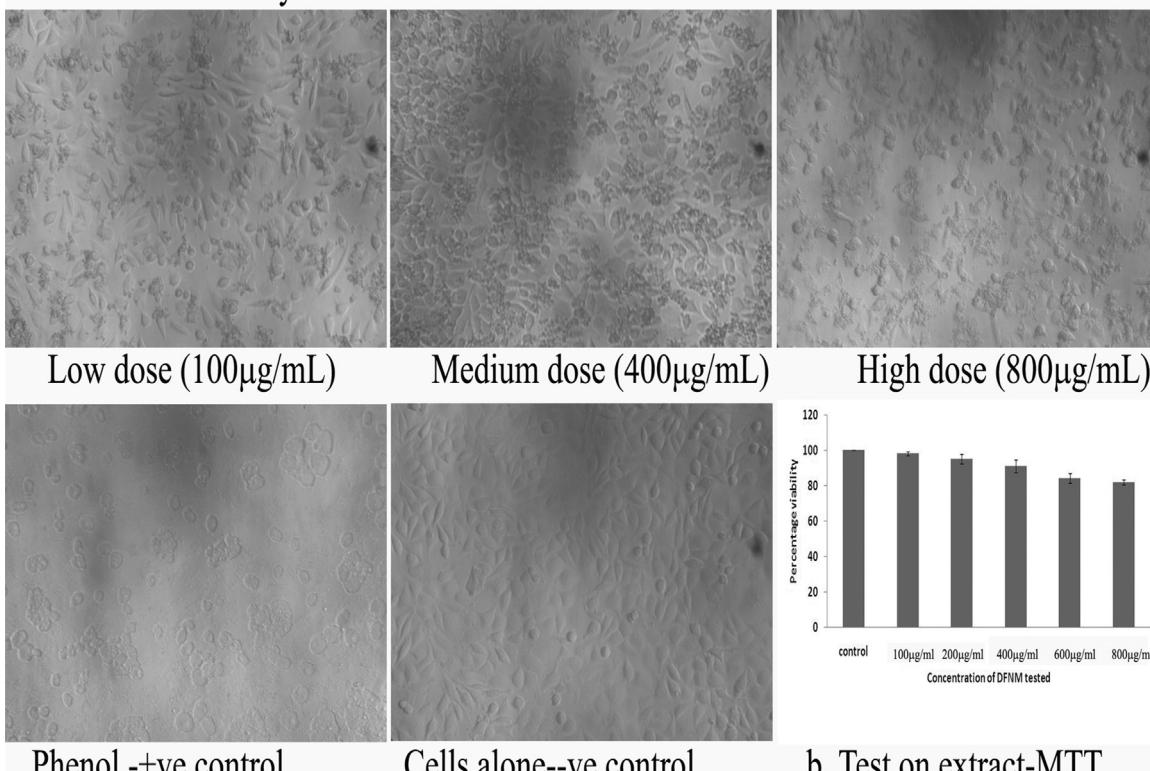


Fig. 3. (a) Morphological analysis of cells exposed to different concentration of DINP. (b) Assessment of metabolic activity by MTT assay.

concentrations of treated animals compared to that of control. The values were 3.42 ± 0.17 , 4.61 ± 0.71 and 4.82 ± 0.07 nmol/mg protein for control, low dose and high dose respectively. Nevertheless no pathologies were seen associated with lipid peroxidation. Similarly it was observed that the tested concentrations of Dinp are not impairing the normal anti oxidant defense mechanism of the cells as understood from the antioxidant status of liver.

3.2. Chronic oral toxicity

In Chronic toxicity studies the compound under study is administered over more than 90 days and the cumulative/long term effect of that material is evaluated. This is particularly important for nanoparticles as their toxic effects are often attributed to the prolonged persistence inside the body. The possible health consequences likely to emerge following repeated oral exposure of Dinp up to a prolonged period of 12 months is evaluated in rodents. The animals were given feed mixed with dextran stabilized iron oxide nanoparticles daily. During the experimental period, feed intake was normal. The animals did not show any abnormality or behavioral changes. There was a regular increase in body weight. The hematological and biochemical values showed no variation among the groups. All parameters were normal when compared with the control group. (Tables 1A and 1B). Histopathological examination also does not reveal any abnormalities in the organs examined (Fig. 4). Oxidative stress parameters evaluated are presented in Table 2.

Compared to acute oral exposure, the amount of malondialdehyde, a marker for lipid peroxidation in Dinp treated animals was lower relative to control. Glutathione is the major endogenous antioxidant molecule of the cell which resists oxidative stress by neutralizing reactive oxygen species and other free radicals. In healthy cells, more than 90% of total glutathione pool is in the sulfhydryl form (GSH) and disulfide form (GSSG) contributes to the remaining 10%. An increase in GSSG to GSH ratio is considered as an indicator of oxidative stress related toxicity. Normal cellular glutathione level was maintained in the Dinp treated group. Glutathione reductase enzyme, an anti oxidant enzyme, catalyses the reduction of glutathione disulfide (GSSG) to glutathione (GSH), which often compensates the loss of GSH during free radicals/ROS scavenging. Glutathione reductase activity was decreased in low dose group animals but increased in high dose treated animals when compared to control. The increase in glutathione reductase activity in high dose treated animals suggests that the system is combating some oxidative stress but in a controlled manner. Glutathione peroxidase (GPx) is a special class of antioxidant enzyme family with peroxidase activity that reduces generated hydrogen peroxides to water and lipid hydro peroxidases to their corresponding alcohols thereby limiting the oxidative stress related toxicity of the cell. Decrease in GPx activity was observed in male rats exposed to Dinp when compared to control. Superoxide dismutases (SOD) are enzymes that catalyses dismutation of superoxide radical into molecular oxygen or hydrogen

peroxide. When coming to SOD, increased activity was seen in treated animal groups compared to the control. This shows the active scavenging of superoxide radicals. However the differences in the above antioxidant parameters are insignificant and do not imply any serious oxidative stress related toxicity pertaining to dextran stabilized iron oxide nanoparticles.

3.3. Dermal toxicity

With the considerable increase in the production and use of nanomaterials for various industrial as well as biomedical applications, there exists the potential for increased exposure of workers to these nanomaterials, the risk associated with its exposure in terms of health and safety is still not fully known. Human skin with its large surface area is more prone to unintended exposure to these nanomaterials particularly during its research, engineering or production phase. Owing to the nanoscale size of Dinp, it is possible for the nanomaterial to evade the barrier raised by the stratum corneum and underlying layers, making its way to reach the systemic circulation or lymphatic circulation. The majority of dermal absorption is via passive diffusion. A study by [Berry et al. \(2004\)](#) found that non functionalized iron oxide nanoparticles (8–15 nm) were seen endocytosed by dermal skin fibroblasts ([Berry et al., 2004](#)). Another *in vitro* study showed that mechanical stretching of human skin caused penetration of fluorescent dextran particles of micro size through the stratum corneum ([Schneider et al., 2009](#)). Hence any adverse systemic effects occurring within a short time of dermal application is evaluated by dermal toxicity studies. The cage side observations of experimental animals on the details such as changes in fur, eyes and mucous membranes did not show any abnormality. Similarly respiratory, somato motor activity and behavior, circulatory system, central and autonomic nervous system were normal in all the animals treated. There were no possible signs of convulsions, salivation, diarrhea, lethargy and coma. There was no noticeable change in the hematological and biochemical parameters of treated animals. Gross and histopathological examinations do not reveal any abnormalities in the sections examined and are represented in Fig. 5. There was no pathologic evidence of iron overload in any of the organs examined. However fine brown particles were evident in the keratohyalin layer of the skin which may be the due to consequence of cutaneous iron deposition, which enhances melanin production by melanocytes. Concentration of malondialdehyde, a marker for oxidative stress related lipid peroxidation was high (39.41 ± 9.18 nmoles/mg protein) in liver homogenates of rats exposed to high dose Dinp through dermal route when compared to the control animals. The values of low dose and medium dose treated animals were comparable with that of control (34.49 ± 9.86 , 35.94 ± 5.56 and 34.81 ± 9.18 nmoles/mg protein for low, medium and control respectively) Glutathione reductase enzyme activity of treated animals were 0.307 ± 0.04 , 0.366 ± 0.01 and 0.307 ± 0.06 units/mg protein for low, medium and high dose group and 0.336 ± 0.02 units/mg protein for the control group. GSH values were unaltered

Table 1A
Hematological parameters of experimental animals exposed orally with Dinp.

Parameters	Control	Low	Medium	High	High Recovery
RBC ($10^6/\text{mm}^3$)	8.38 ± 0.32	$7.84 \pm 0.41^*$	8.61 ± 0.36	8.36 ± 0.17	$6.59 \pm 0.60^*$
HB (g%)	14.60 ± 0.54	14.03 ± 0.57	15.32 ± 0.84	14.55 ± 0.31	$15.67 \pm 0.95^*$
PLT ($10^6/\text{mm}^3$)	6.49 ± 0.68	7.18 ± 2.13	$7.87 \pm 0.77^*$	6.43 ± 0.27	6.44 ± 0.66
MCV (μm^3)	56.57 ± 1.48	57.13 ± 4.29	56.07 ± 3.31	56.22 ± 1.87	$54.33 \pm 1.75^*$
MCH (pg)	17.35 ± 0.31	17.95 ± 1.20	17.80 ± 0.56	17.42 ± 0.57	$23.92 \pm 2.03^*$
MCHC (g/dl)	30.73 ± 0.48	31.42 ± 0.68	31.23 ± 0.33	31.02 ± 0.08	$43.98 \pm 2.46^*$

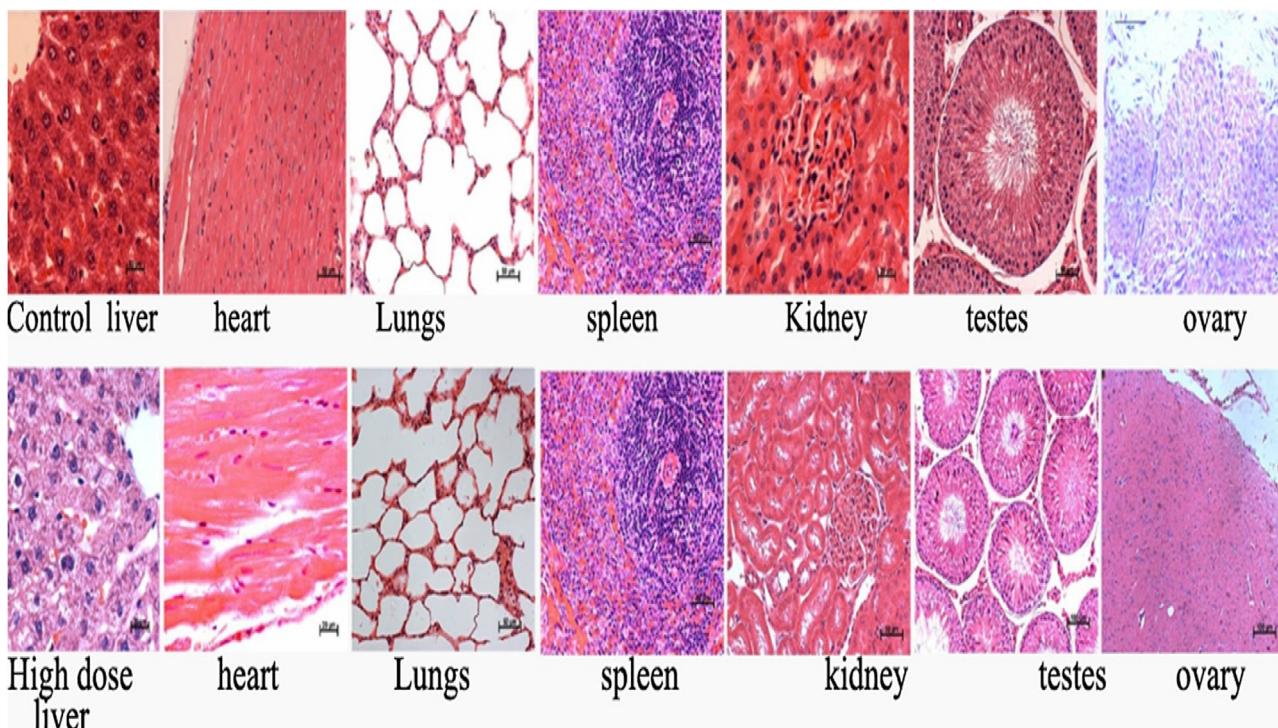
* Statistically significant corresponding to a p value of <0.05 .

Table 1B

Biochemical Parameters of experimental animals orally exposed to DINP- chronic exposure.

Biochemical Parameters	Control	Low	Medium	High	High Recovery
Total Protein (g/dL)	8.30 ± 0.85	8.05 ± 0.22	8.18 ± 0.49	8.24 ± 0.39	8.50 ± 0.37
Creatinine (mg/dL)	0.99 ± 0.06	0.91 ± 0.07*	0.99 ± 0.07	0.95 ± 0.05	0.87 ± 0.06*
Urea (mg/dL)	41.23 ± 4.73	43.15 ± 3.46	42.12 ± 3.15	40.69 ± 1.92	37.15 ± 1.67*
SGPT (U/L)	90.09 ± 16.53	83.55 ± 12.83	104.03 ± 11.82	80.39 ± 10.45*	99.55 ± 9.49
SGOT (U/L)	109.55 ± 31.77	97.30 ± 17.68	181.48 ± 114.55	95.15 ± 33.06	128.72 ± 18.62
Glucose (mg/dL)	98.04 ± 12.93	89.07 ± 11.25	94.82 ± 31.97	96.67 ± 15.00	114.67 ± 7.27*
Cholesterol (mg/dL)	55.21 ± 8.40	66.17 ± 7.78*	76.00 ± 7.59*	67.60 ± 11.83*	80.67 ± 8.43*
Bilirubin Total (mg/dL)	0.13 ± 0.03	0.15 ± 0.04	0.15 ± 0.02	0.17 ± 0.04*	0.14 ± 0.03
Albumin (BCG) (g/dL)	4.42 ± 0.83	4.45 ± 0.29	4.55 ± 0.42	4.55 ± 0.24	4.66 ± 0.21
Phosphorus (mg/dL)	7.80 ± 1.48	6.37 ± 1.07*	7.60 ± 0.57	6.64 ± 1.27*	4.99 ± 2.26*
Chlorides (mEq/L)	110.99 ± 1.54	112.03 ± 2.74	112.38 ± 1.72	113.45 ± 2.20*	110.07 ± 2.36
GGT (U/L)	4.05 ± 3.28	1.80 ± 1.42*	3.87 ± 1.65	2.64 ± 1.40	2.13 ± 1.55

* Statistically significant corresponding to a p value of <0.05

**Fig. 4.** Microphotographs of histopathological analysis of organs chronically exposed to DINP.**Table 2**

Antioxidant parameters evaluated for chronic oral toxicity studies.

Parameters	Control	Low	Medium	High	High recovery
Malondialdehyde (nmol/mg protein)	23.86 ± 13.82	9.10 ± 2.86	15.71 ± 5.00	18.06 ± 7.26	25.75 ± 13.79
Total Glutathione (nmol/mg protein)	0.893 ± 0.19	0.94 ± 0.21	0.92 ± 0.16	1.16 ± 0.31	1.05 ± 0.11
Glutathione reductase (units/mg protein)	0.42 ± 0.15	0.39 ± 0.006	0.48 ± 0.09	0.47 ± 0.16	0.36 ± 0.06
Glutathione peroxidase (units/mg protein)	0.31 ± 0.19	0.28 ± 0.07	0.25 ± 0.02	0.36 ± 0.21	0.36 ± 0.34
Super oxide dismutase (units/mg protein)	0.14 ± 0.002	0.15 ± 0.008	0.18 ± 0.005	0.15 ± 0.001	0.17 ± 0.007

in control and treated animals groups (0.283 ± 0.02 , 0.268 ± 0.02 , 0.232 ± 0.04 units/mg protein for low, medium and high and 0.301 ± 0.01 units/mg protein for control animals respectively). GPx activity followed a dose dependent increase in treated animals. The values were 0.100 ± 0.015 , 0.129 ± 0.012 and 0.131 ± 0.016 and 0.104 ± 0.01 units/mg protein for 25 mg/kg (low), 50 mg/Kg(medium) and 100 mg/Kg(high) of DINP treated animals and control respectively. Super oxide dismutase activity

was insignificantly decreased in treated animals compared to control. In summary the liver antioxidant parameters were not significantly altered by DINP exposure through dermal route.

3.4. Immunotoxicity

The normal Immune surveillance of the body is critical in maintaining healthy homeostasis (D' Alessandri and Strid, 2014).

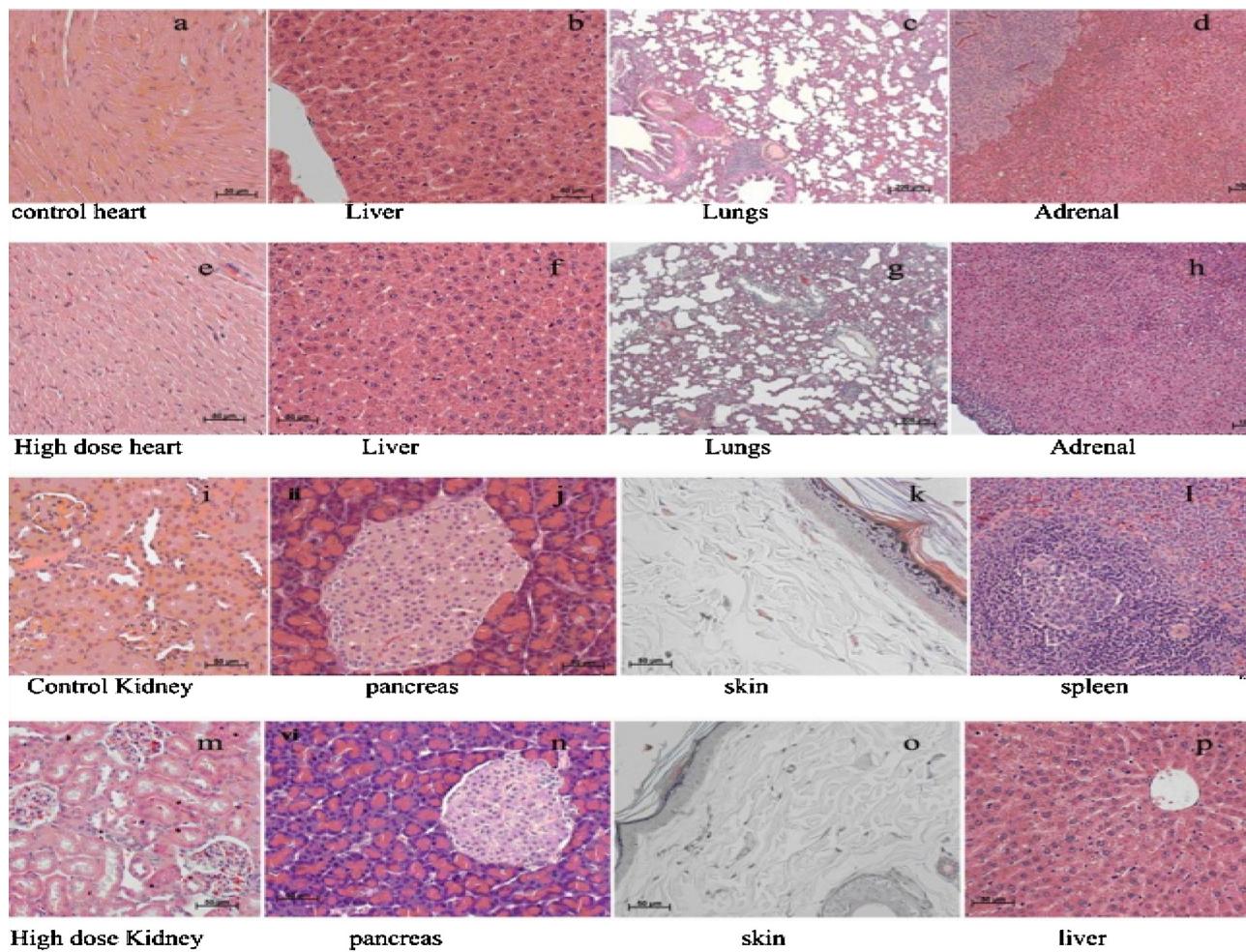


Fig. 5. Micro photographs of histopathological analysis of organs following dermal exposure of DINP.

Any change in this homeostasis therefore may progress to various pathological conditions. Nanomaterial interaction with the

immune system can lead to undesirable causes such immuno suppression or immuno stimulation, hypersensitivity reactions

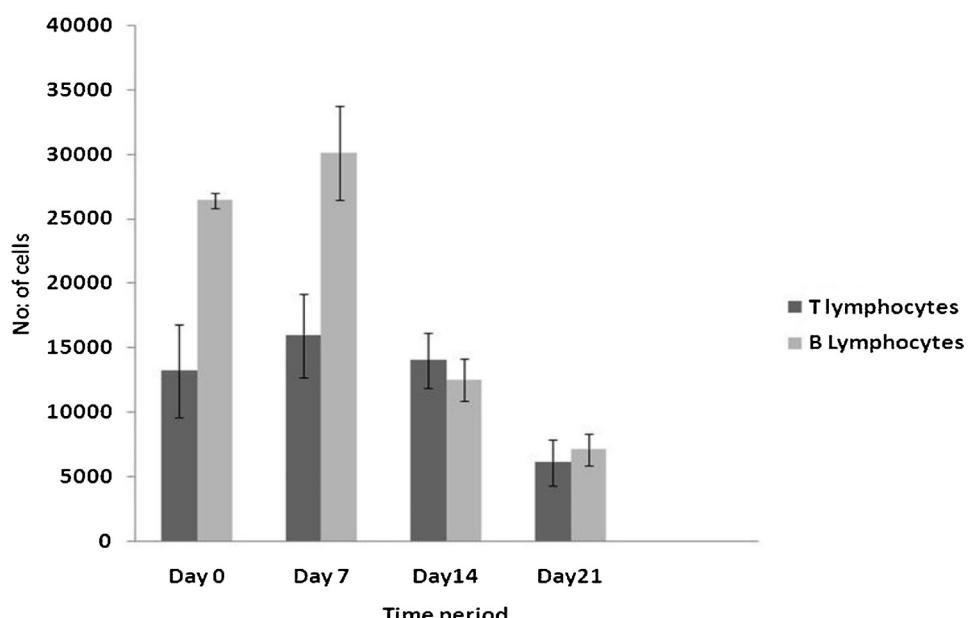


Fig. 6. Proliferative activity in spleen cells exposed to DINPs (values are mean \pm SD).

and auto immunity (Zolnik et al., 2010). Hence it is very much essential to understand the immune compatibility of dextran stabilized iron oxide nanoparticles in the (pre)clinical development. Lymphocyte proliferation occurs in response to mitogens or sensitized antigens. Hence Lymphocyte proliferation assay is commonly used test for evaluating nanomaterial immuno stimulatory properties (Dobrovolskaia and McNeil, 2007). The proliferation of immune cells is directly related to the state of immune function. In the present study, the primary splenocytes proliferation assay was evaluated in both T and B lymphocytes isolated from spleen of animals exposed to DINPs at various time periods. The results are summarized in Fig. 6. At 7 days after exposure, a slight increase in cell number when compared to control in both T and B lymphocytes were observed. However, after 14 days and 21 days, proliferation of T cells and B cells were reduced when comparable to that of day 0 suggesting that DINP is not interfering with the immune system.

Delayed hypersensitivity or skin sensitization is the secondary cellular response mediated by T cells and monocytes/macrophages (Tsuji et al., 2002). When any material comes in contact with the cutaneous layer; they can induce proliferation, clonal expansion of allergen responsive T lymphocytes and provide immunological memory. On subsequent exposure, the memory immune cells elicit an increased immune response within 24–48 h, known as elicitation phase, which manifests as erythema to edema to necrosis. The present study evaluated the delayed immunological response of DINP and the data recorded in this assay is purely based on visual perception. The appearance of the challenge skin sites of both the test and control guinea pigs were observed at 24 h, 48 h and 72 h after removal of dressings and patches after challenge dose. The specific clinical manifestations/skin reactions were scored and the numerical grading was recorded as per ISO 10993-10: 2010 (E). The topically exposed DINP does not induce any skin irritation after a challenge dose. The skin irritations score of DINP treated and control areas were designated as '0'. (The skin irritation grades of 1 or greater in the test group generally indicates sensitization, provided grades of less than 1 are seen on control animals. Also the reactions of test animals that exceed the most severe control reaction are presumed to be due to sensitization).

3.5. Genotoxicity

Owing to the relatively small size and high surface area, nano materials may have unpredictable genotoxic properties. Nano-materials can damage genetic material either directly or indirectly. Directly, they could accumulate within the cell so that they gain access to interact directly with DNA whereas indirectly, they can damage DNA by increasing oxidative stress and inflammatory responses. Indirect genotoxic effects exerted by nanomaterials because of oxidative stress is represented by, base modifications (e.g. formation of 8-hydroxydeoxyguanosine adducts), DNA breaks and DNA cross-links, all of which if gone un-repaired have the potential to initiate carcinogenesis (Singh et al., 2009). Formation of 8-hydroxydeoxyguanosine (8-OHdG) adducts on the

mitochondria as well as genomic DNA of DINP treated rat liver homogenates were assayed by competitive ELISA. The levels of 8OHdG in mitochondrial DNA of DINP exposed groups were comparable with that of control values (1.756 ± 0.01 ng/mL for test and 1.726 ± 0.01 ng/mL for control respectively). Similarly no significant increase in 8OHdG levels were observed in genomic DNA of $100 \mu\text{g}/\text{mL}$ treated animals compared to un treated ones (8.76 ± 1.23 ng/mL for test and 9.00 ± 1.16 ng/mL for control)

Chromosomal aberration study was performed to analyze the potential of DINP to induce any structural chromosome aberrations because of single or double stranded DNA breaks. The induced DNA breaks are often repaired by the intrinsic repair mechanism. However when chromosomes are not reverted back to the original state, resulting structural defects could be demonstrated in the metaphase preparations. Induced chromosomal aberrations could be either chromatid type (asymmetric aberrations) like chromatid gaps/breaks produced during on/after replication or chromosome type(symmetric aberrations) like inversions, deletions, dicentrics, ring chromosomes that usually occur before replication (Anderson et al., 2009). Karyotyping of mouse bone marrow cells of DINP treated animals revealed that the total chromosome number remains unaltered (19 pair of autosomes and X and Y sex chromosomes). The chromosome aberration frequency calculated for DINP treated animals at various concentrations (50 mg/Kg, 100 mg/kg and 200 mg/kg body weight) is shown in Table 3 and representative figure of aberrations scored is given in Fig. 7. It was found that dextran stabilized iron oxide nanoparticles had no significant effect on the chromosome aberration frequencies in bone marrow cells and it has not affected the mitotic indices of cells.

There are many literature reports on the genotoxic potential of bare iron oxide nanoparticles. It has been reported that Iron oxide nanoparticles functionalized with polyethylene glycol (PEG) coating exhibit mutagenic activity without chromosomal and clastogenic abnormalities (Mahmoudi et al., 2011). Ma et al. in 2012 has demonstrated the presence of 8-hydroxy-deoxyguanosine in hepatic and renal tissues following intraperitoneal injection of Fe_3O_4 nanoparticles (Ma et al., 2012) and reported that a single dose of iron oxide nanoparticles is not causing any genotoxic effects in leucocytes (evaluated by the comet assay), chromosomal aberrations in bone marrow cells, or micronucleus in either of these cell types. However contradictory results were also reported in evaluating frequency of micronucleus in mouse bone marrow cells exposed to iron oxide nanoparticles (Song et al., 2012).

3.6. Carcinogenicity study

Bare iron metal nanoparticles have long been associated with cancer as iron-induced carcinogenesis is primarily due to the indirect DNA damage as a result of oxidative stress. It was reported that intra-muscular injections of iron–dextran complex in rats induced spindle cell sarcoma and pleomorphic sarcoma due to iron overload (Richmond, 1959). This neoplasm could be the result of solid-state carcinogenesis wherein the implanted foreign body

Table 3

Percentage frequency of chromatic aberration calculated for DINP treated animal groups.

	Chromatid gap	Chromatid break	Chromosome gap	Chromosome break
Low dose (50 mg/kg body weight)	3.5 ± 2.1	3.5 ± 2.1	1.2 ± 1.7	1.0 ± 1.4
Medium dose (100 mg/kg body weight)	1.3 ± 2.3	0.6 ± 1.1	0.6 ± 1.1	0.6 ± 1.1
High dose (200 mg/kg body weight)	4.0 ± 2.8	3.0 ± 1.4	4.0 ± 2.8	4.0 ± 5.6
Cyclophosphamide (50 mg/kg body weight)	20.0 ± 5.6	54.5 ± 26.16	9.5 ± 2.12	13.5 ± 4.9
Negative control (Physiological saline)	5.0 ± 1.4	1.0 ± 1.4	3.0 ± 4.2	2.0 ± 0.0

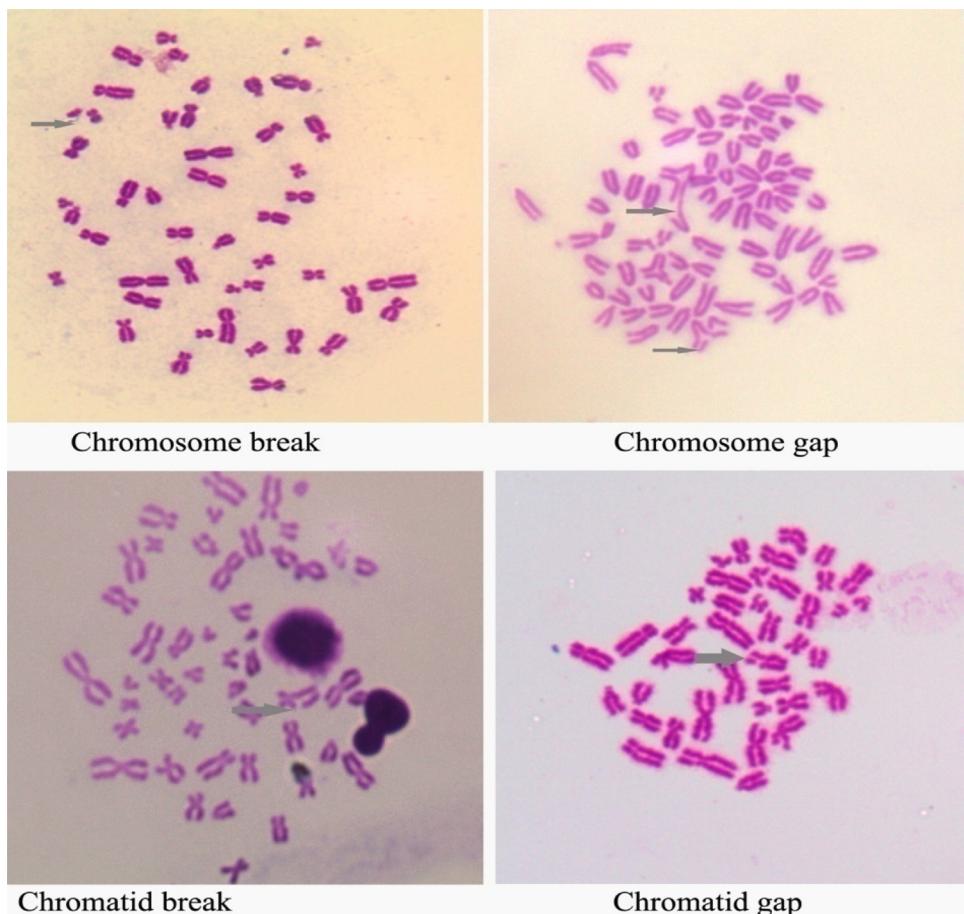


Fig. 7. Aberrations scored for evaluating chromosomal abnormalities.

Table 4

Relative organ weights of Dinp treated animals (% body weight).

	Acute exposure (mice)		Chronic exposure (rats)	
	Control	Dinp Treated	Control	Dinp Treated
Body weight (g)	22.75 ± 0.31	23.75 ± 0.12	367.66 ± 6.30	349.12 ± 8.37
Liver	1.2 ± 0.2	1.3 ± 0.4	3.81 ± 0.09	3.54 ± 0.61
Spleen	0.07 ± 0.003	0.09 ± 0.001	0.23 ± 0.01	0.21 ± 0.01
Kidney	0.05 ± 0.004	0.07 ± 0.001	0.27 ± 0.01	0.22 ± 0.13

initiates tumor formation. Under circumstances where bare iron oxide particles are exposed due to damage to the outer dextran shell, Fe ions can lead to an imbalance in iron homeostasis and cause aberrant cellular responses that could further lead to cancer pathologies. For this reason, long term exposure of dextran stabilized iron oxide nanoparticles should be evaluated for their potential to induce carcinogenicity. Gross examinations of the carcasses of the dextran stabilized iron oxide nanoparticle treated groups (low, medium, high and high recovery groups) and control rats did not reveal any gross abnormality in the organs examined (data not shown) pertaining to the nanomaterial studied.

3.7. Bio-distribution

Bio-distribution of nanoparticles is of particular concern as metal-containing particles are quickly cleared from the blood by the reticulo endothelial system but remain in organs, such as the liver and spleen, for prolonged duration. Furthermore bio-distribution of nanoparticles depends on their physicochemical

characteristics such as size, shape and surface coating agents particularly the coating chemistry that influence opsonization which in turn directs uptake by reticuloendothelial system. Dinp treated animal organ weights were determined and are depicted in Table 4. No significant body weight changes or organ weight changes were observed in both of Dinp exposed (acute & chronic) animals. Fe content by ICP analysis is depicted in Table 5. The deposition of Iron was higher in liver and spleen when compared to kidney. Increased amount of iron content was observed in liver samples of chronically exposed animals when compared to the control. Also high dose treated animals showed an increased Iron content in kidney compared to control. However there were no observable signs of tissue damage in kidney, liver or spleen (Fig. 8). Generally, the toxicity of *in vivo* exposed magnetic ferrite nanoparticles is primarily associated with the degradation of these particles into free iron and subsequent sequestration of excess irons in the tissues, as mammals lack a defined pathway for iron excretion. From the study, it is clear that the accumulation of

Table 5
ICP analysis of Iron content.

	Acute Exposure			Chronic Exposure	
	Liver ($\mu\text{g/g}$)	Kidney ($\mu\text{g/g}$)	Spleen ($\mu\text{g/g}$)	Liver ($\mu\text{g/g}$)	Kidney ($\mu\text{g/g}$)
Control	33.19	119.73	14.48	108.9	55.17
Dinp treated group	57.19	164.34	27.08	195.22	104.31

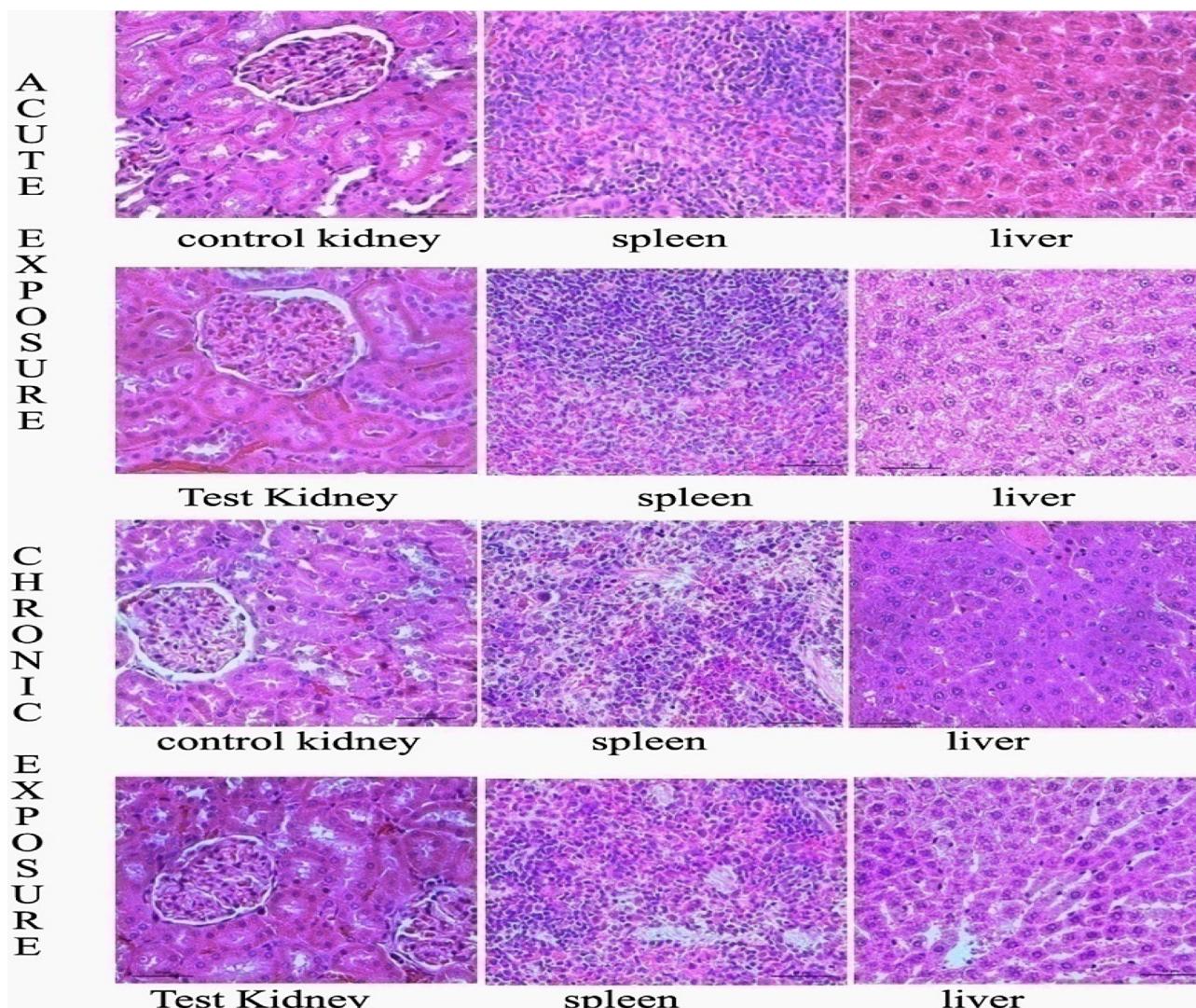


Fig. 8. Microphotographs of histopathological analysis of organs following acute and chronic exposure of DINP.

DINP is not causing any tissue damage or prolonged effects as evidenced from the acute as well as chronic exposure studies.

4. Conclusion

Dextran stabilized magnetic iron oxide nanoparticles of size <25 nm were successfully synthesized and physico chemically characterized. The synthesized nanoparticles were nontoxic when exposed to laboratory experimental animals under various exposure conditions. The nanoparticles do not seem to induce oxidative stress mediated toxicological effects, nor altered physiological process or behavior changes or visible pathological lesions. Allergic or delayed hypersensitivity reactions were not observed on exposure to dextran stabilized iron oxide nanoparticles. Thus it can be concluded that Dextran stabilized iron oxide nanoparticle is non irritant, non toxic and biocompatible under laboratory conditions. These nanoparticles therefore can be considered as unclassified compounds as per globally harmonized system for classification (category 5) for chemical substances and mixtures. Absence of any molecular toxicity authenticates the development of safe nanomaterials to be intended for biomedical applications. Furthermore, use of safe and biocompatible

nanomaterials is highly beneficial to mankind as it greatly reduces the toxicity/adverse side effects associated risks.

Conflict of interest

The authors declare that they have no conflict of interests.

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