Chemico-Biological Interactions 278 (2017) 54–64

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Recurrent exposure to ferric oxide nanoparticles alters myocardial oxidative stress, apoptosis and necrotic markers in male mice

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ARTICLE INFO

Keywords:
Apoptosis
Cardiotoxicity
Fe_2O_3-NPs
Necrosis
Oxidative stress

ABSTRACT

The cardiotoxicity of iron oxide nanoparticles (Fe_2O_3-NPs) in mice was investigated. The mice were intraperitoneally administered with Fe_2O_3-NPs at the dose of 25 and 50 mg/kg bw for 30 days at seven days interval. *In vivo* MRI analysis reveals the Fe_2O_3-NPs accumulation in the cardiac system. Also, serum iron estimation and Prussian blue staining confirms the iron deposition in circulatory system. Cardiac dysfunction was assessed by ECG analysis and further validated by evaluating the functional markers such as cardiac Troponin-1 (cTnI) expression, AChE activity and levels of LDH and CK-MB in cardiac tissue. Fe_2O_3-NPs exposure disturbs the balance between the oxidants and antioxidants resulting in oxidative myocardial damages. In consequence, damaged mitochondria, diminished ATP level and NOX4 over expression were observed in the intoxicated groups indicating the role of Fe_2O_3-NPs in oxidative stress. A dose dependant increase in oxidative stress mediates apoptosis through upregulation of Bax, cytochrome *c* and cleaved caspase 3 in the 25 mg/kg treated group. Sustained oxidative stress suggest the occurrence of necrosis in addition to apoptosis in 50 mg/kg treated group evidenced by altered expression pattern of cleaved PARP, cytochrome *c*, Bax and cleaved caspase 3. In addition, triphenyl tetrazolium chloride (TTC) staining confirms cardiac necrosis in 50 mg/kg Fe_2O_3-NPs treated group.

1. Introduction

Nanoparticles (NPs) have been widely used in various fields including nanomedicine. The uniqueness of the NPs arises specifically from higher surface-to-volume ratio. They can be engineered and such particles exhibit novel properties. These differences influence the physical and chemical behaviour of NPs in the biological system. NPs are present in tablets, sunscreen lotions, toothpastes and even in food products. As the use of NPs has been growing in recent years there is a major concern about the risks associated with its applications. More definitive research has to be carried out to frame suitable regulations to ensure safe usage of NPs.

NPs gain access into the human body through different routes such as inhalation, ingestion and injection, which is ultimately translocated to several systemic organs causing adverse biological effects [1,2]. Among the variety of NPs used in biomedical applications superparamagnetic iron oxide nanoparticles (SPIONs) have been used potentially as contrast agent in magnetic resonance imaging (MRI) in clinical diagnosis [3,4], hyperthermia in cancer therapy [5], magnetofection [6] and gene delivery [7]. Heart has lower levels of antioxidants and this makes it more vulnerable to oxidative stress related injuries by xenobiotics. NPs preferentially accumulate in the heart and produce cellular injuries [8]. Recent studies have shown that iron oxide nanoparticles (Fe_2O_3-NPs) damage the brain and kidney [9]. It has been demonstrated that Fe_2O_3-NPs injures endothelial cells and might play a key role in downstream cardiovascular diseases such as atherosclerosis, hypertension and myocardial infarction [10]. However, the impact of Fe_2O_3-NPs on cardiac function remains elusive. Thus, the aim of the present study was to determine the toxic effects of Fe_2O_3-NPs on cardiac system of mice model.

The potential of the NPs to induce toxicity has been highly attributed to the increased surface area and reactivity. Large surface area of

Abbreviations: AChE, Acetyl choline esterase; Bax, Bcl-2, associated X protein, an apoptosis promoter; CAT, Catalase; CK-MB, Cartine kinase-MB; cTnI, Cardiac Troponin I; CuO, copper oxide; DAB3, 3′, Diaminobenzidine; ECG, Electrocardiogram; Fe_2O_3-NPs, Ferric oxide nanoparticles; GSH-Px, Glutathione peroxidase; LDH, Lactate dehydrogenase; MRI, Magnetic Resonance Imaging; NO, Nitric oxide; Nox4, Nicotinamide adenine dinucleotide phosphate oxidase 4; PC, Protein carbonyl; ROS, reactive oxygen species; SOD, Superoxidase dismutase; SPIONs, superparamagnetic iron oxide nanoparticles; TTC, 2,3,5-triphenyltetrazolium chloride

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http://dx.doi.org/10.1016/j.cbi.2017.10.003
Received 15 April 2017; Received in revised form 12 September 2017; Accepted 3 October 2017
Available online 06 October 2017
0009-2797/

NPs increases their ability to produce reactive oxygen species (ROS) [11]. Transition metals and transition metal oxide NPs such as Fe_2O_3, copper oxide (CuO) NPs generate ROS through Fenton's reaction [12]. ROS are highly active and when present in excess they damage biomolecules especially, lipids, proteins and DNA [13]. It is well established that Fe_2O_3-NPs cause oxidative stress both *in vitro* and *in vivo* [9]. Although oxidative stress induction by NPs was suspected, the consequent toxic impacts remain unclear. Hence this study was designed to explore the toxic effects of Fe_2O_3-NPs on cardiac function in albino male mice.

2. Materials and methods

2.1. Animals

Male albino mice (8 weeks old) in the body weight range of 25–30 g were procured from the Experimental Animal Centre, Kerala Agricultural University, Trissur, India. Animals were maintained with 12 h light/dark cycle and fed with commercially available pelleted mice chow (Sai Durga Feeds Pvt. Ltd., Bangalore, India) and tap water was supplied *ad libitum*. The experiments were conducted in accordance with ethical norms approved by The Ministry of Social Justice Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines (722/02/A/CPCSEA).

2.2. Nanoparticle characterization

Fe_2O_3-NPs (< 50 nm) purchased from Sigma-Aldrich (Cat#544884) were used in this study. The average size, crystal structure and energy dispersion of Fe_2O_3-NPs were analyzed.

2.3. Experimental design

NPs were dissolved in 0.9% saline and dispersed with sonication at 20 Hz for 5 min at 4 °C. The animals were randomly divided into three groups with six animals in each group. Control and experimental groups were intraperitoneally administered with 0.9% saline and Fe_2O_3-NPs with the dose of 25 mg/kg and 50 mg/kg respectively for 30 days (each dose at an interval of seven days). The doses of 25 and 50 mg/kg bw were chosen based on exposure of Fe_2O_3-NPs in biomedical applications [14,15] and our earlier studies [16,17]. Further, as contrast agent, a mass of more than 200 mg of Fe_2O_3-NPs was injected to human in magnetic resonance imaging [15]. But the doses used in our experiment were comparatively lesser than the dose administered in biomedical applications. Cerebral toxicity has been observed on the intraperitoneal administration of 2.5 mg L-Dopa coated Fe_2O_3-NPs per mouse [18] which is much higher than the doses chosen in the present study. Mice exposed with iron oxide NPs at the dose of 40 mg/kg for 2 weeks (i.p) has been shown to affect sperm count and leydig cells at molecular level [19]. In addition, apoptosis in liver and kidney were observed in rats upon exposure to 40 mg/kg of Fe_2O_3-NPs through i.p injection daily for one week [20]. In view of all these, we have selected 25 mg/kg bw as lower and 50 mg/kg bw as higher doses.

After 24 h of last treatment the animals were sacrificed by cervical decapitation. The blood was collected and serum was separated. Heart was excised out and washed with 0.9% saline. Cardiac tissue was homogenized (Polytron, PT 2500 E, Switzerland) with Tris-HCl buffer (pH 7.6) and was centrifuged at 10,000 rpm for 20 min at 4 °C. The tissue homogenate was recovered and the biochemical assays were carried out immediately.

2.3.1. Electrocardiogram analysis

Electrocardiogram (ECG) recordings were performed in anaesthetised mice using 3 lead-2 channel power labs (AD instruments, Australia). Alterations in the duration of RR Interval (s), Heart Rate (BPM), PR interval and QTc Interval (s) in the Fe_2O_3-NPs intoxicated mice were recorded [21].

2.3.2. In-vivo Magnetic Resonance Imaging (MRI)

MRI was performed using Bruker BioSpec 7.0 T (70/30 USR). The MRI was done for the control and Fe_2O_3-NPs treated groups. All mice were anaesthetised during MRI, using 2–4% of isoflurane/oxygen mixture. Isoflurane concentration was adjusted to maintain the animal at the breath rate of 60 ± 10 breaths/min throughout the MRI sessions. T_2 Weighted MRI with repetition time [TR] = 3000 ms, echo time [TE] = 33 ms, flip angel [FA] = 180°, field of view [FOV] = 10 mm × 5 mm was done for whole body mice to assess the presence of Fe_2O_3-NPs.

2.3.3. Iron estimation and staining

In an individual experimental set up, 0.1 ml of serum and tissue homogenate of liver and heart was added to 1 ml of 0.1 M acetate buffer and 1 ml of 0.1% of phenanthroline in isopropanol [22]. After 5 min of incubation at room temperature the absorbance was read at 510 nm. For Prussian blue iron staining, the paraffin embedded myocardial tissue sections were deparaffinized, hydrated and placed in 10% potassium ferrocyanide solution with 20% HCl solution for 30 min. They were then dehydrated through series of decreasing concentration of alcohol, cleared in xylene and sealed with a cover slip [23].

2.3.4. Assessment of cardiac functional markers

Cardiac functional markers in serum such as lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB) were measured using commercially available kits (Agappe diagnostics Ltd, Agappe hills, Kerala, India).

2.3.5. Estimation of total protein and AChE activity in cardiac tissue

The total protein concentration was measured [24] using bovine serum albumin standards and expressed in mg/g tissue. The activity of AChE was determined spectrophotometrically [25] and expressed as μM/min/mg protein. The method involves the use of 5,5-dithiobis nitrobenzoic acid (DTNB) to quantify the amount of thiocholine produced from the hydrolysis of acetylthiocholine iodide by AChE. The absorbance at 412 nm was measured and the activity was calculated based on the change in absorbance during the reaction.

2.3.6. Biochemical analysis of oxidants in cardiac tissue

Nitroblue tetrazolium (NBT) assay was performed for the estimation of ROS [26]. ROS generation was calculated by the comparing the OD values with NBT standard curve and expressed as μM NBT reduction/10 mg tissue. Lipid peroxidation (LPO) was analyzed by measuring malondialdehyde (MDA) [27]. Thiobarbituric acid (TBA) reacts with the final product of LPO and forms MDA to produce a pink adduct. This was measured at 532 nm and expressed as nM of MDA formed/mg protein. Protein carbonyl (PC) content was estimated using 2, 4-dinitrophenylhrdrazine (DNPH) [28]. Carbonyl groups of proteins react with DNPH to form 2,4 dinitrophenylhydrazones and this can be quantified spectrophotometrically by absorbance at 360 nm and expressed as nM/mg protein. Nitric oxide (NO) level was estimated based on the formation of azo dye [29]. Griess reagent contains sulfanilic acid and N-(1-naphthyl) ethylenediamine dihydrochloride is employed to estimate the nitrite produced from the degradation of NO. Sulfanilic acid reacts with nitrite to form diazonium salt which is then coupled to N-(1-naphthyl) ethylenediamine forming azo dye. This can be quantified spectrophotometrically at 546 nm and expressed as μM/g tissue.

2.3.7. Biochemical analysis of antioxidants in cardiac tissue

Total superoxide dismutase (SOD) activity was measured by the rate of auto-oxidation of pyrogallol in the presence of SOD which was measured at 470 nm for 3 min at one min interval and expressed in U/mg protein [30]. Catalase (CAT) activity was measured based on the rate of conversion of hydrogen peroxide (H_2O_2) into water and oxygen [31]. Potassium dichromate reacts with CAT and destroys its activity. H_2O_2 that has not been split by the CAT when reacts with dichromate to