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Iron oxide nanoparticles modulate heat shock proteins and organ specific markers expression in mice male accessory organs

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ABSTRACT

With increased industrial utilization of iron oxide nanoparticles (Fe_2O_3-NPs), concerns on adverse reproductive health effects following exposure have been immensely raised. In the present study, the effects of Fe_2O_3-NPs exposure in the seminal vesicle and prostate gland were studied in mice. Mice were exposed to two different doses (25 and 50 mg/kg) of Fe_2O_3-NPs along with the control and analyzed the expressions of heat shock proteins (HSP60, HSP70 and HSP90) and organ specific markers (*Caltrin*, *PSP94*, and *SSLP1*). Fe_2O_3-NPs decreased food consumption, water intake, and organo-somatic index in mice with elevated iron levels in serum, urine, fecal matter, seminal vesicle and prostate gland. FTIR spectra revealed alterations in the functional groups of biomolecules on Fe_2O_3-NPs treatment. These changes are accompanied by increased lactate dehydrogenase levels with decreased total protein and fructose levels. The investigation of oxidative stress biomarkers demonstrated a significant increase in reactive oxygen species, nitric oxide, lipid peroxidation, protein carbonyl content and glutathione peroxidase with a concomitant decrement in the glutathione and ascorbic acid in the male accessory organs which confirmed the induction of oxidative stress. An increase in NADPH-oxidase-4 with a decrease in glutathione-S-transferase was observed in the seminal vesicle and prostate gland of the treated groups. An alteration in HSP60, HSP70, HSP90, *Caltrin*, *PSP94*, and *SSLP1* expression was also observed. Moreover, accumulation of Fe_2O_3-NPs brought pathological changes in the seminal vesicle and prostate gland of treated mice. These findings provide evidence that Fe_2O_3-NPs could be an environmental risk factor for reproductive disease.

1. Introduction

Human exposure to nanomaterials is inexorable as the advancement and introduction of nanoproducts for a wide variety of applications are entering a broad array of markets worldwide. This is due to physicochemical properties and novel characteristics that researchers are applying in many areas including biomedical applications (Gupta et al., 2007). Fe_2O_3-NPs are being increasingly utilized in molecular labeling, pigments, storage devices, sensors, and water treatment (Perez, 2007; Gonzalez et al., 2011; Ge et al., 2012). With the expansion of Fe_2O_3-NPs applications, the sustainable production and utilization of Fe_2O_3-NPs with a likelihood of human exposure are on the ascent which brings an increased concern on reprotoxicity. Though there exists speculation that Fe_2O_3-NPs might cause reprotoxicity but the meager evidence available to affirm or rebut these concerns, which necessitate addressing and precisely evaluating the reprotoxicity of Fe_2O_3-NPs.

Nickel nanoparticles (NPs) were shown to cause reprotoxicity in both the male and female rats with reduced motility of the sperm and alterations in seminiferous tubule histology of the former and increased apoptosis in ovarian tissues of the latter (Kong et al., 2014). Reproductive function of the male offspring was affected with vacuolation in the seminiferous tubules and gradual diminution in the daily sperm production with age when carbon black NPs are intratracheally administered to the gestational mother mice (Yoshida et al., 2010). Similarly, reduced daily sperm production in the offspring was observed with subcutaneous administration of nano-sized titanium dioxide to pregnant mice (Takeda et al., 2009). Though there are scanty scientific reports that reveal the reprotoxicity of NPs, none looked into the male accessory organs - seminal vesicle and prostate gland. In addition to testis, these two accessory organs are essential for the survival and fertilizing potential of the spermatozoa and once intoxicated they affect the fertility of the organism (Mann and Lutwak-Mann, 1981).

Co-administration of sodium fluoride and aluminium chloride decreased fructose levels in the seminal vesicle and acid phosphatase in the prostate gland of mice (Chinoy et al., 2005). A significant decrease in absolute and organ-to-body weight ratio of the seminal vesicle and prostate gland with altered histology of the seminal vesicles was reported in mice on exposure to nickel (Pandey et al., 1999). Yet another study revealed the pathological alterations in the seminal vesicle and prostate gland on the administration of cadmium to adult male rats (Kamel et al., 2011). So far, the nanotoxic effects on the seminal vesicle and prostate gland in mice have not been adequately investigated. Considering the

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significance of the male accessory organs in fertility of mammals, we studied the Fe_2O_3-NPs effects of the seminal vesicle and prostate gland. This is the first work revealing the *in vivo* toxicity of Fe_2O_3-NPs to the seminal vesicle and prostate gland by assessing the heat shock proteins and organ specific markers.

2. Materials and Methods

2.1. Animals

Adult male Albino mice (*Mus musculus*), body weight 25-30 g and age 8 weeks old were procured from the Experimental Animal Centre, Kerala Agricultural University, Trissur, India. The animals were fed with balanced, sterilized commercially available pelleted chow (Sai Durga Feeds Pvt., Bangalore, India), water *ad libitum*, and maintained under standard laboratory conditions. The animal experimental protocols were approved by the Institutional Animal Ethical Committee. All animal experiments were carried out in accordance with the approved protocols.

2.2. Characterization of Fe_2O_3-NPs

Fe_2O_3-NPs XRD patterns were recorded on a PANalytical X'PERT PRO powder X-ray diffractometer by using monochromatic Cu Kα1 (k = 1.54056 Å) radiation. Joint Committee on Powder Diffraction Standards - International Centre for Diffraction Data (JCPDS-ICDD) database was used to identify and compare the crystallographic planes of the Fe_2O_3-NPs (Scardi, 1999). Fe_2O_3-NPs (<50 nm) in powder form were obtained from Sigma-Aldrich (Cat #544884). The morphology and sizes of Fe_2O_3-NPs were examined under field emission scanning electron microscopy (FESEM) with Quanta 250 FEG, Czech Republic. An acceleration voltage of 10 kV was used to capture the digital images. The chemical bonding and surface chemistry of the Fe_2O_3-NPs were determined by Fourier transform infrared (FTIR) spectra obtained from with a bench-top spectrometer (Tensor, Bruker Optics GmBH, Ettlingen, Germany). The spectra obtained in a range between 400 and 4000 cm^{-1}. Data storage and quantitative analysis of the recorded spectra were done with the OPUS 7.2 (Bruker Optics, GmbH, Ettlingen, Germany). Aggregation of NPs is usually monitored by dynamic light scattering (DLS). The Fe_2O_3-NPs concentrated stock suspension was prepared at a concentration of 50 mg/L in saline. 10 mL of the aqueous suspension was sonicated at 20 Hz for 20 min at 4°C. Hydrodynamic size and zeta potential (ζ) of this sonicated Fe_2O_3-NPs suspension was determined with Zeta Sizer-Nano (Version 2.3; Malvern Instruments Ltd., Malvern, UK).

2.3. Experimental design

Animals were randomly divided into a control group and experimental groups (25 and 50 mg/kg BW of Fe_2O_3-NPs) with 6 each. The dose setting was based on exposure of Fe_2O_3-NPs in biomedical applications (Winer et al., 2012). Fe_2O_3-NPs suspension was prepared by dissolving 25 mg in 0.9% of 1 mL saline, sonicated at 20 Hz for 20 min at 4°C. This suspension was orally administered to mice once a day for 30 consecutive days at a dose of 25 and 50 mg/kg body weight to experimental groups, whereas the control mice received saline alone. After 24 h on last treatment, the mice were then euthanized by cervical dislocation, blood was collected, and serum was obtained by centrifugation at 3500 rpm for 10 min and stored at -80°C until use. The male accessory organs - seminal vesicle and prostate gland were excised, weighed accurately and stored at -80°C for further analyses until mentioned otherwise. Tissues were dissected, washed with 0.9% saline, fixed in a 10% formalin solution and processed with graded ethanol series and used for histological and immunohistochemical analysis. The organs were homogenized with Tris-HCl buffer (pH 7.4) and the homogenate was then centrifuged at 10,000 rpm for 20 min at 4°C, and the clear supernatant was recovered, and biochemical assays – iron content, total protein, LDH, fructose estimation were performed immediately and other parameters were studied later from the stored tissue samples.

2.4. Food consumption, water intake, body weight, and organo-somatic index (OSI)

Food consumption, water intake, body weights of mice were monitored during the experiments. The seminal vesicle and prostate gland were dissected out and weighed for the determination of OSI.

2.5. Iron content and iron accumulation

The level of iron content was estimated (Peters et al., 1956) in serum and urine and expressed as μg/mL, whereas in the feces, seminal vesicle and prostate gland were expressed as μg/g. The seminal vesicle and prostate gland tissue sections were subjected to Prussian blue reaction for histochemical demonstration of the ferric iron accumulation (Bancroft and Stevens, 1982).

2.6. FTIR

The seminal vesicle and prostate gland tissues were lyophilized and pelleted using potassium bromide. Under similar beam conditions, the pellets were analyzed using FTIR. The spectra were between 500 and 4000 cm^{-1}. The recorded absorption intensities were analyzed.

2.7. Total protein, lactate dehydrogenase (LDH) and fructose levels

Total protein content in the seminal vesicle and prostate gland was measured following the method of Lowry et al. (1951) and expressed as mg/g tissue. LDH was assayed in the serum, seminal vesicle and prostate gland adopting Megraw (1971) method and expressed as % leakage. Fructose was estimated in the seminal vesicle, according to Foreman et al. (1973) method and expressed as μg/mL.

2.8. Oxidants and antioxidants

Reactive oxygen species (ROS) generation was measured in the serum, seminal vesicle and prostate gland, according to Beauchamp and Fridovich (1971) and expressed as %. The nitric oxide (NO) level in the serum, seminal vesicle and prostate gland was assayed as described by Green et al. (1982) and expressed as μM nitrite released for serum, whereas μM nitrite/g tissue for the seminal vesicle and prostate gland. Lipid peroxidation (LPO) was estimated by measuring the generation of malondialdehyde (MDA) following the method of Devasagayam and Tarachand (1987) and expressed as nM MDA released/mg protein. Protein carbonyl content (PCC) was determined following Levine et al. (1990) and expressed as nM carbonyl/mg protein. The glutathione peroxidase (GPx) was assayed following Rotruck et al. (1973) and expressed as U/mg protein. Glutathione (GSH) levels were determined spectrophotometrically by following Ellman (1959) and expressed as μM/g tissue. Ascorbic acid was estimated colorimetrically according to the method of Roe and Kuther (1943) and expressed as mg/g tissue.

2.9. Western blotting

For protein analysis, the seminal vesicle, and prostate gland tissues were homogenized with ice cold radioimmunoprecipitation assay lysis buffer. Proteins were extracted from the tissue homogenates, and total protein content was estimated following Bradford method using bovine serum albumin (BSA) as a standard. 40 μg of protein samples was electrophoresised in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels with 5% stacking gels using the discontinuous buffer system and transferred onto a polyvinylidene fluoride membrane. The blots were washed in tris-buffered saline and tween