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IN VIVO ASSESSMENT ON ACUTE TOXICITY OF IRON OXIDE NANOPARTICLES WITH DIFFERENT COATINGS

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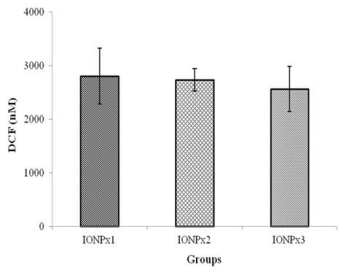
Graphical abstract



Abstract

Engineered nanoparticles have been extensively explored in various biomedical settings including nanoparticulate imaging agents due to its promising benefits to mankind. Iodine-intolerance patients have caused alarming concerns in searching new contrast media with lower toxicity effect. However, proper potential mechanism of nanoparticles has yet to be fully established despite its early acceptance and emerging usage. By using animal model system, our aim is to assess acute toxicity of 14 nm iron oxide nanoparticles (IONPs) coated with citric acid, nitric acid, perchloric acid and silane-polyethylene glycol (SiPEG). Eighteen male Wistar Rats were used in order to explore the underlying toxicity of IONPs in liver tissues after 24 hours. Hydroxyl radicals (˙OH) were elucidated by using reactive oxygen species (ROS) production assay and western blotting for the presence of p53 protein expression. The results revealed SiPEG coated IONPs have lower ROS production and lower expression of p53, however no statistical significant were observed. It can be hypothesized that SiPEG has blood-pooling contrast agent potential due to longer circulation period in blood. While, IONPs not coated with SiPEG tend to be phagocytosed by mononuclear phagocyte system and released Fe2+ ions initiative to acute cellular toxicity. The outcomes highlighted that administration of SiPEG coated IONPs believed to be a safer radiographic contrast media.

Keywords: Nanoparticulate, phagocytosed, SiPEG

1.0 INTRODUCTION

Nanotechnology has evolved since the last decades and a promising revolution in the 21st century to the extent that it is now possible to specially construct and characterize the functional properties of the nanoparticles to be incurred into plethora branches of science [1]. Recently, knowledge in this field aid in rapid development of nanoparticles as an alternative to iodinated conventional radiographic contrast enhancer [2]. National Nanotechnology Initiative (NNI) in the article by Wilczewska *et al*. [3] define nanoparticles as engineered particles with size of < 100 nm in one dimension. Despite its wide usage, many studies had been done to portray the toxicology of

nanoparticle. However, cells interactions with nanostructures remain as a knowledge gap.

Maghemite, γ-Fe2O3 or Magnetite, Fe3O4, a single dimension with 5-20 nm diameters, are among rising nanosized magnetic particles for mankind usage. Magnetite, Fe3O4 is the commonly used magnetic iron oxide [1]. Iron oxide nanoparticles (IONPs) consist of superparamagnetic particles can be used as contrast agents for Magnetic Resonance Imaging (MRI). Contrast agent made of metallic nanoparticles has lower toxicity level and with enhance imaging features [2]. Uncoated nanoparticles are not suitable to be used for various clinical applications due to its low solubility level and form free radical particles [4]. Bare IONPs can cause obstruction in blood vessel after administration due to high agglomeration rate under physiological circumstances and gravitation forces [5]. To avoid oxidation, causing agglomeration of IONPs, Fe3O4 nanoparticles must be coated with either organic or inorganic molecules while undergoing precipitation process besides to achieve ferrofluid biocompatibility and hydrophilicity [6]. However, to date, the toxicity of surface coating cannot be precisely weighted due to many conflicting outcomes.

Degradation rate of IONPs and release of iron ions are highly influenced by the surface coating physico-chemical properties and its chemical synthesis. Despite their biocompatibility contribution, little is known about their toxicity in vivo. Present study was conducted to investigate acute toxicity of 14 nm IONPs coated with citric acid, nitric acid, perchloric and silane polyethylene glycol (SiPEG) in rat's liver. Previous study by Li *et al*. proved that coating with material such as citric acid could stabilize IONPs by creating a hydrophilic surface on the nanoparticles, preventing agglomeration in target tissues and addition of functional groups aid further surface derivation [7]. The use of hydrophilic polymer such as polyethylene glycol (PEG) as a coating material for IONPs able to reduce opsonization process of colloidal particles thus potentially prolong the circulation time of IONPs in the body and provide effective accumulation in target tissues [8-10]. Toxicity mechanism was elucidated using cellular reactive oxygen species (ROS) and western blot. We used p53 as a marker in response to generic oxidative stress which activates cellular pathways such as cell cycle arrest, DNA damage repair, and apoptosis [11–13].

2.0 MATERIALS AND METHODS

2.1 Chemicals

IONPs were obtained from NanoBiotechnology Research and Innovation, Institute for Research in Molecular Medicine (NanoBRI @ INFORMM), Universiti Sains Malaysia, Malaysia. Oxiselect In Vitro ROS/RNS Assay Kit (Green Fluorescence) were purchased from Cell Biolab, Inc. (San Diego, CA). Monoclonal anti-mouse p53 antibodies, monoclonal anti-mouse GAPDH loading control antibodies and ECL chemiluminescence detection kit were purchased from Thermo Scientific Pierce, USA; other chemicals were purchased from Merck Company unless otherwise specified.

2.2 Animals

Animal study was conducted in accordance with the ethical guidelines of Universiti Teknologi MARA Committee of Animal Research and Ethics (UiTM CARE) concerning the use of experimental animals (Ref:68/2015). Eighteen healthy four-week-old Wistar rats weighing about 200 grams were obtained from Laboratory Animal Facility and Management (LAFAM), UiTM Puncak Alam Campus. The animals were acclimatized for two weeks and maintained under 12-h light/dark scheduled at a temperature of 21 ± 2 °C. Normal pellet diet with filtered water was give ad libitum. Experiments were performed on healthy six-week-old Wistar rats weighing about 250 grams. The study consisted of three groups (n = 6) of six-week-old Wistar rats which are divided into iron oxide nanoparticles coated citric acid group (IONPx1), iron oxide nanoparticles coated nitric acid and SiPEG group (IONPx2) and iron oxide nanoparticles coated percloric acid and SiPEG group (IONPx3) group. IONPx1, IONPx2 and IONPx3 animals received 0.5 mL of 300μg/mL IONPs via intravenous administration. After 24 hours, all of the animals were euthanized by cervical dislocation. Liver tissues were excised immediately and stored at−80 OC prior to further analysis.

2.3 Measurements of Cellular Reactive Oxygen Species (ROS)

The liver cellular ROS generation level was assessed using the Oxiselect In Vitro ROS Assay Kit (Green Fluorescence) (Cell Biolabs, Inc, San Diego, CA, USA) according to the manufacturer's instructions. Tissue samples were resuspended at 20 mg/ml in PBS and homogenized on ice. Sample was spun at 10,000 g for five minutes. Supernatant was collected and assayed directly for ROS production determination. The presence of fluorescent dichlorodihydrofluorescein (DCF) from oxidation reaction of ROS samples with DCFH probe was measured fluorometrically at 480 nm excitation/530 nm emission using POLARstar Omega Plate Reader. Free radical content was determined by comparison with the predetermined DCF standard curve.

2.4 Western Blot Analysis of p53 Proteins

Tissues were homogenized in 20 mg/ml ice-cold RIPA lysis buffer containing 10 µl EDTA-free protease inhibitor cocktail and kept for 30 minutes. The EDTA-free protease inhibitor cocktail was mixed to homogeneity just before used by vortex a few seconds. Then, the lysates were centrifuged at 10 000 g for 20 minutes at 4oC to pellet cell debris and collected supernatants. Total protein concentration in the supernatants was

determined using the Modified Lowry Protein Assay Kit (Thermo Scientific). Aliquots from supernatant containing 30 μg of total protein concentration were mixed with 5X Laemmli at ratio 1:5(v/v). Sample were boiled for 5 minutes at 95°C followed by centrifugation at 11 000 g for 1 minutes. Samples were separated by denaturing SDS-PAGE and transferred to a PVDF membrane (0.45 μm, 26.5 cm x 3.75 cm, Thermo scientific) by electrophoretic transfer (Bio-Rad Laboratories, Inc., USA). The membrane was preblocked with 5% non-fat milk (Sunlack) for overnight at 4°C. Following this, washed with TBST (0.1% Tween-20 in Tris-buffered saline) for 3 times, 10 minutes for each and incubated the membrane with the primary antibody (anti p53, 1:500, Thermo Scientific) for 2 hours at room temperature. Then, membrane was washed 3 times for 10 minutes and incubated with the secondary horseradish peroxidase-linked antibodies (anti-GAPDH, 1:20000, Thermo Scientific) for 2 hours at room temperature. Membrane was exposed to enhanced chemiluminescence (ECL) for 5 minutes at room temperature. Protein band detection by using X-ray film in dark room and processed by automated film processor (Konica Minolta SRX-201). To verify equal protein loading and transfer, the blots was reprobed with GAPDH using anti-GAPDH antibody (1:5000 dilution) as a loading control.

3.0 RESULTS

3.1 ROS Production upon Coated IONPs Treatment

Reactive Oxygen Species (ROS) production assay was conducted to elucidate the oxidative stress induction in the liver tissues of experimental animals. Figure 1 depicts the level of ROS production in liver tissues 24 hours following administration different coated IONPs. ROS production in IONPx1 was observed to be highest among three groups. No significant differences noted in all three experimental groups.

3.2 p53 Protein Expression

Higher expression of p53 protein seen in IONPx1 compared to IONPx2 and IONPx3. The levels of expressions are indicative to DNA damage, growth arrest or apoptosis after 24 hours administration of IONPs with 3 different coating. Figure 2 shows in comparison to GAPDH control expression, p53 expression in all groups was considered mild.

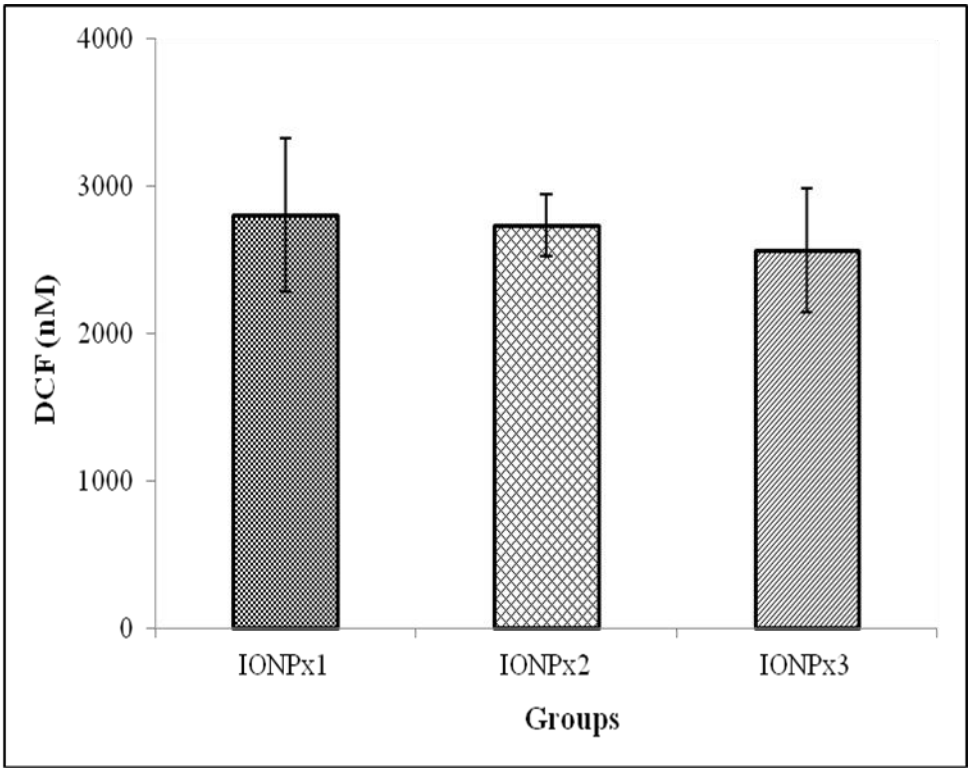


Figure 1 ROS production values. The line chart shows ROS production value of IONPx1, IONPx2 and IONPx3 groups in liver tissues. Values were expressed as mean ± S.E.M (n=6) (p = 0.05). No significant differences seen among group (p < 0.05)

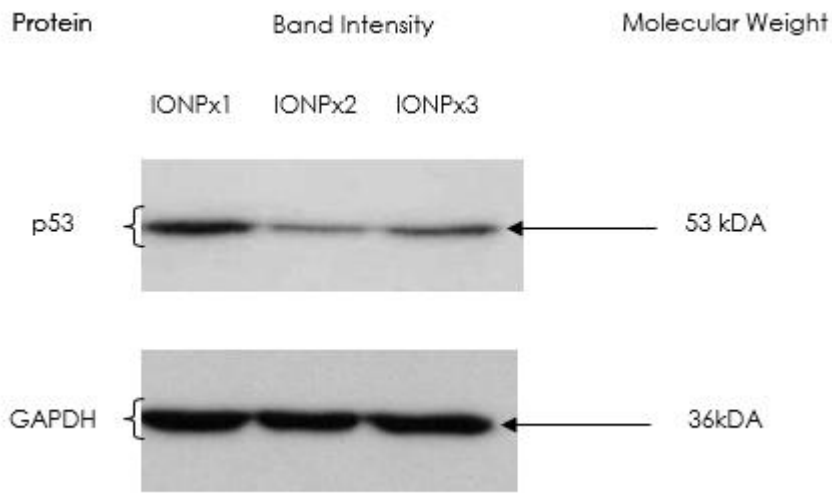


Figure 2 Autoradiographs representing expression of p53 and GAPDH proteins in all IONPx group, i) Expression of p53 proteins observed with molecular weight of 53 kDa in all groups., ii) Expression of quality control GAPDH proteins observed with molecular weight of 36 kDa in all groups

4.0 DISCUSSION

Engineered nanoparticles have been extensively explored especially in improving current biomedical settings due to its promising functions and benefits. Mankind stands to get huge benefit from nanotechnology; however, the physicochemical properties of these particles have caused new concerns and debates about the potential risk on human health. Proper potential toxicity mechanism of nanoparticles has not yet been established despite early acceptance of nanotechnology and emerging usage [14]. Paradoxically, risks cannot be weighted accurately because different employment of experimental model systems in which comparison of results are difficult to be made [15]. These considerations have led to this study, which is to assess the nanotoxicological properties of iron oxide nanoparticles (IONPs) coated with citric acid, nitric acid perchloric acid and SiPEG. The toxicity of IONPs was interpreted by using ROS production assay and p53 expression.

All IONP used in this study have a diameter of 14 nm. Based on the diameter, it is non-toxic when internalized in vivo. A research conducted by Pan and coworkers affirmed that nanoparticles greater than or equal to 14 nm were proven to be non-toxic regardless the cell type examined, whereas nanoparticles with diameter 1 to 2 nm were normally able to induce toxicity to the cells [16]. IONPs in this experiment were coated with three different materials, which are citric acid, nitric acid and SiPEG and perchloric acid and SiPEG. It is well known that surface modification of nanoparticles is essential in improving its biocompatibility, hydrophilicity and stability in biological environment [10]. However, the potential risks of coated IONPs with vital organ such as liver need to be elucidated.

Liver has been known as a major organ of detoxification process. Nanoparticles are mainly deposited in the liver due to Kupffer cell intakes which act as a host defense and its toxicological effects can be measured [2]. Rats were euthanized 24 hours after nanoparticles administration. ROS production following nanoparticles administration may suggest early cellular response leading to cytotoxicity [17]. 24 hours after administration of different IONPs, IONPs coated with citric acid (IONPx1) had the highest ROS production compared to SiPEG coated IONPs (IONPx2 and IONPx3). Internalization of IONPs may lead to enzymatic degradation in lysosome due to high acidic environment and release iron ion in ferrous form. In mitochondria, ferrous ion react with hydrogen peroxide induces generation of reactive oxygen species (ROS) as hydroxyl particles through mechanism called Fenton reaction [18, 19]. Induction of intracellular ROS by IONPs has been ascribed as the main cause of oxidative stress. Study result showed that SiPEG-coated IONPs have lowest ROS production and the outcome parallel with studies indicating that SiPEG have steric repulsion effect that able to reduce intracellular interactions including decrease opsonins adsorption and other serum proteins [20, 21].

Western blot analysis was further used to decipher molecular mechanism of cell after 24 hours administration of coated IONPs with p53 as a marker. p53 has a major function to inhibit proliferation of abnormal cells [12]. In this study, western blot data showed expression of p53 protein in all sample, with stronger band signal was observed in IONPx1 compared to IONPx2 and IONPx3. SiPEG-coated IONPs showed lighter expression of p53 band. Mild expressions were seen in all IONPs groups compared to GAPDH control expressions. Higher expression of p53 is an indicator to cell growth arrest or apoptosis

triggered by cells in response to stress. Therefore, high p53 accumulation in IONPx1can be considered as toxicity [22].

Prior to administration of foreign materials such as nanoparticles into the body, serum proteins such as immunoglobulins, lipoproteins, complement and coagulation factors will absorb these foreign materials to be removed by mononuclear phagocyte system (MPS) which take place in liver and spleen [23]. In order to be active targeting contrast media in vivo, nanoparticles must have long circulating period. PEGlyted nanoparticles were known to have sustained half-life in blood. In correlation to our results, IONPs coated with SiPEG showed lower ROS productions and lower expression of p53. PEG able in producing opposing effects in which PEG coated nanoparticles become invisible to MPS and tend to circulate longer in the bloodstream [24]. Study conducted by Cho *et al*. [22] stated PEG coated nanoparticles can be considered as blood-pool contrast agent due to its long blood half-life. Indeed, study by Yu *et al*. proved that PEG coated IONPs able to reduce its cytotoxicity [25].

5.0 CONCLUSION

Present study highlighted acute toxicity assessment of IONPs coated with citric acid, nitric acid, perchloric acid and SiPEG. The outcomes proposed SiPEG coated IONPs have lower intracellular toxicity from ROS production and expression of p53. SiPEG coated IONPs may be a safer choice for alternative contrast media. These assay have given limited information and not sufficient to build predictions on toxicity of similar nanoparticles. Instead, we urge further research to prove the circulation period of SiPEG coated nanoparticles in blood stream, histopathological changes in vivo, and acute inflammatory responses to affirm present study.

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