Toxicological evaluation of dextran stabilized iron oxide nanoparticles in human peripheral blood lymphocytes

Sheeja Liza Easo and Parayanthala Valappil Mohanan^{a)}

Division of Toxicology, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Poojapura, Thiruvananthapuram 695 012, Kerala, India

(Received 26 July 2016; accepted 24 August 2016; published 14 September 2016)

Iron oxide nanoparticles present an attractive choice for carcinogenic cell destruction via hyperthermia treatment due to its small size and magnetic susceptibility. Dextran stabilized iron oxide nanoparticles (DIONPs) synthesized and characterized for this purpose were used to evaluate its effect on cellular uptake, cytotoxicity, and oxidative stress response in human peripheral blood lymphocytes. In the absence of efficient internalization and perceptible apoptosis, DIONPs were still capable of inducing significant levels of reactive oxygen species formation shortly after exposure. Although these particles did not cause any genotoxic effect, they enhanced the expression of a few relevant oxidative stress and antioxidant defense related genes, accompanied by an increase in the glutathione peroxidase activity. These results indicate that under the tested conditions, DIONPs induced only minimal levels of oxidative stress in lymphocytes. Understanding the biological interaction of DIONPs, the consequences as well as the associated mechanisms *in vitro*, together with information obtained from systemic studies, could be expected to advance the use of these particles for further clinical trials. © 2016 American Vacuum Society. [<http://dx.doi.org/10.1116/1.4962268>]

I. INTRODUCTION

In recent years, there has been a drastic increase in research focusing on the development of nanoparticles (NPs) for application in drug delivery, cancer therapeutics, gene therapy, and molecular imaging. Specifically, iron oxide based NPs with superior magnetic properties and effective surface functionalization are being rigorously investigated to achieve highly efficient carcinogenic cell destruction through hyperthermia treatment.^{1–3} The precision of this technique is achieved by the higher sensitivity of tumor cells to temperature increases above 42 °C. At this temperature, the normal enzymatic processes that keep cells alive are destroyed, thus allowing their selective killing.^{4,5} Another advantage of using superparamagnetic iron oxide NPs for cancer therapy is that they may be specifically guided by a magnetic field toward a specific area of interest, thereby reducing the required dose and eliminating side effects.⁶ The successful translation of such an application to clinical use requires that these NPs possess several criteria such as small size and narrow size distribution, superparamagnetic behavior, and most importantly, biocompatibility and minimal toxicity.

Studies using various NPs have indicated that the most common mechanism for NP toxicity is via the formation of reactive oxygen species (ROS) and induction of oxidative stress.^{7–12} ROS such as the highly reactive hydroxyl radicals may arise at the surface of NPs itself. For example, iron oxide NPs have been shown to generate hydroxyl radicals at the NP surface by Fenton reaction; passivation of NP surface with oleate or Bovine serum albumin was rather ineffective in suppressing hydroxyl radical production.¹³ On the other hand, NPs could directly interact with nicotinamide adenine

dinucleotide phosphate (NADPH) oxidases from plasma membrane and/or mitochondria generating superoxide anion.⁹ The increase in pathological levels of intracellular ROS induces the activation of antioxidant enzymes such as heme oxygenase 1, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). At even higher levels of ROS, the activation of proinflammatory signaling pathways occur culminating in cytotoxicity.^{14,15}

The toxicity of different iron oxide NP preparations has been previously examined in various cells.^{12,16–18} It was shown that iron oxide nanoparticles have the potential to induce genotoxicity in human skin and lung epithelial cells, which appears to be mediated through ROS generation and oxidative stress.¹⁶ Murray *et al.* demonstrated that coexposure to UV radiation and superparamagnetic iron oxide NPs was associated with the induction of oxidative stress and release of inflammatory mediators in human epidermal keratinocytes and murine epidermal cells.¹⁸ Generally, cell lines utilized in culture are cancer or immortalized (virus transfected) cell lines that have altered properties with respect to cellular signaling, apoptosis induction, and deoxyribo nucleic acid (DNA) damage responses compared to primary cells. From a clinically relevant perspective, there remain concerns about the safety of iron oxide NPs with regard to primary human cells such as peripheral blood lymphocytes. Moreover, studies on the interaction of iron oxide NPs with primary lymphocytes are rather limited. For this reason, we have chosen human peripheral blood lymphocytes as an *in vitro* experimental model owing to its abundance in systemic circulation, lymph fluid, and tissue, and also because these cells are critical to immune surveillance and defense.

In the context of tumor hyperthermia application, we had previously synthesized and characterized dextran stabilized iron oxide nanoparticles (DIONPs).¹⁹ Hence, the aim of the present study was to examine the cellular uptake,

^{a)} Author to whom correspondence should be addressed; electronic mail: mohanpv10@gmail.com

cytotoxicity, and oxidative stress response induced by DIONPs exposure in human lymphocytes. Using *in vitro* techniques, we have tried to assess the extent of interaction of DIONPs with human peripheral blood lymphocytes.

II. MATERIALS AND METHODS

A. DIONPs

DIONPs were synthesized as a stable aqueous ferrofluid, whose physicochemical characterization was performed previously.¹⁹ Briefly, ferrous and ferric chloride in a stoichiometric ratio of 1:2 was mixed with equal volume of urea and 2% dextran and the mixture was heated to 80–100 °C in order to completely decompose the urea. Subsequently, tetramethylammonium hydroxide [(Sigma), 25 wt. % in water] was added dropwise into the mixture at room temperature. The entire reaction was maintained in nitrogen atmosphere with vigorous stirring. Large aggregates were removed by centrifugation, and the remaining magnetic colloid was purified by dialysis against deionized water for 48 h, changing the water twice daily. A drop of the NP suspension was deposited on formvar coated grids, air-dried for several hours, and the core particle size and morphology was examined by transmission electron microscopy (TEM) (Hitachi H-7650) at an accelerating voltage of 80 kV. The hydrodynamic radius and polydispersity index (PdI) of NPs were determined by dynamic light scattering [DLS, Zetasizer Nano ZS (Malvern Instruments, Malvern, UK)] at 25 °C. Samples were taken from concentrated NP dispersion (35 mg/ml) and diluted in water as well as in complete culture medium [RPMI-medium (Sigma, USA) supplemented with 10% heat inactivated fetal bovine serum (Invitrogen), 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin] to obtain a homogeneous solution with a final concentration of 0.05 mg/ml. Similarly, the zeta potentials of freshly prepared 0.05 mg/ml nanoparticle dispersions in water and complete culture medium were also measured at 25 °C. All measurements were calculated as an average of three runs containing 12 measurements per run after 4 h incubation. Structural characterization of NPs was performed by powder x-ray diffractometry (XRD) (PANalytical X'Pert Pro MRD using Cu-K α radiation). Dextran coating on NP surface and the nature of its bonding was analyzed by FTIR analysis. Freeze-dried NP preparations were mixed with potassium bromide to form a fine powder, which was compressed into a thin pellet, and the FTIR spectrum was recorded between 4000 and 400 cm⁻¹ at a resolution of 4 cm⁻¹.

B. Lymphocyte culture

Human venous blood from three healthy volunteers was collected into heparinized tubes (10–20 U/ml). Written informed consent was obtained from all volunteers, and the study was approved by the Institutional Ethics Committee (ECR/189/Inst/KL/2013). Individual blood samples were pooled, and peripheral blood mononuclear cells were isolated based on density gradient using Histopaque-1077

(Sigma, USA). Lymphocytes at a density of 1×10^6 cells were resuspended in complete RPMI-medium. Cells were cultured at 37 °C, 5% CO₂ atmosphere.

C. Cellular uptake

Cellular uptake of DIONPs by peripheral blood lymphocytes was assessed by Prussian blue staining. 2×10^5 cells were incubated with different concentrations of NPs (8–1000 μ g/ml) at 37 °C for 24 h. Thereafter, cells were washed briefly with phosphate buffered saline (PBS), placed on coverslips coated with poly L-lysine solution in a six-well plate to allow cells to adhere onto coverslips. Cells were stained with 10% potassium ferrocyanide/10% HCl (ratio 1:1) for 30 min, washed with PBS, and counterstained with 0.5% neutral red solution. The coverslips with adhered cells were washed, allowed to dry, mounted with DPX (Distrene-80, plasticizer, xylene) mounting medium, and visualized under a light microscope (AxioStar plus, Carl Zeiss). The experiment was performed in triplicates.

D. Cytotoxicity

Lymphocytes were treated with varying concentrations of DIONPs for 24 h. The proportion of viable, apoptotic or necrotic cells was analyzed with the Alexa Fluor[®] 488 Annexin V/Dead Cell Apoptosis kit (Invitrogen) according to the manufacturer's instructions. Cell gating was performed to establish peripheral blood lymphocytes on the basis of forward scattered light and side scattered light. Four different population of cells was detected: viable cells that are annexin V⁻/PI⁻, early apoptotic cells that are annexin V⁺/PI⁻, late apoptotic/necrotic cells that are annexin V⁺/PI⁺, and necrotic cells that are annexin V⁻/PI⁺. Hydrogen peroxide (100 μ M H₂O₂) was included as positive control. The cytotoxicity analysis was performed thrice, with two replicates in each analysis.

E. Intracellular ROS generation

Intracellular ROS generation was measured using the hydrophobic dye 2,7-dichlorofluorescein diacetate (H₂DCFH-DA) (Invitrogen). Cells were exposed to increasing concentrations of NPs for 4 and 24 h in the presence and absence of 2 mM reduced glutathione (GSH) (Sigma, USA). H₂O₂ of 100 μ M was included as positive control. Following exposure, cells were washed twice with PBS and incubated with DCFH-DA dye (5 μ M in PBS) for 60 min. Further, the dye solution was replaced with 200 μ l of PBS and the fluorescence due to dichlorofluorescein (DCF) was read at 485 nm excitation and 530 nm emission wavelengths in a multiwell plate reader (Chameleon, Hidex, Turku, Finland). Results were expressed as % DCF fluorescence compared to control cells. The assay for ROS generation was performed on six different occasions with three replicates in each set.

F. Intracellular GSH levels

Lymphocytes were exposed to different concentrations of DIONPs for 24 h and were examined for changes in

FIG. 1. Characterization of DIONPs. (a) TEM image of DIONPs indicate particles to be spherical in shape with an average core diameter of ~ 9 nm. (b) Mean hydrodynamic diameter of DIONPs measured by DLS in water indicate a mean size of ~ 25 nm. (c) Mean hydrodynamic diameter of DIONPs in complete culture media measured by DLS show a mean size of ~ 123 nm. (d) XRD spectra of DIONPs show several distinct peaks characteristic of spinel ferrite configuration. (e) FTIR analysis demonstrates dextran coating on the surface of iron oxide nanoparticles.

FIG. 2. Cellular uptake of DIONPs in human peripheral blood lymphocytes. Internalized particles can be visualized as blue pigments by Prussian blue staining ($n = 3$). (a) control, (b) $8 \mu\text{g/ml}$, (c) $40 \mu\text{g/ml}$, (d) $200 \mu\text{g/ml}$ and (e) $1000 \mu\text{g/ml}$.

level of intracellular GSH according to a previous method.²⁰ The assay was carried out in triplicates, and the amount of GSH was expressed in terms of nM/mg protein.

G. Chromosome aberration

Heparinized venous blood of 0.5 ml was placed in PB-MAX™ karyotyping medium containing phytohemagglutinin

FIG. 3. Cytotoxicity of DIONPs in human peripheral blood lymphocytes. Comparative flow cytometry quadrant analysis of Annexin V/propidium iodide (PI) double stained lymphocytes exposed to 8, 40, 200, and 1000 µg/ml DIONPs for 24 h. The dot plots have been obtained from a single representative experiment of three individual experiments that gave very similar results. Low left quadrant: viable cells (annexin V⁻/PI⁻); low right quadrant: early apoptotic cells (annexin V⁺/PI⁻); upper left quadrant: late apoptotic/necrotic cells (annexin V⁺/PI⁺); and upper right quadrant: dead cells (annexin V⁺/PI⁺). Number of counted cells is 10 000.

(Invitrogen) and cultured in 5% CO₂ atmosphere, at 37 °C for 48 h. Cells were exposed to increasing concentrations of NPs for 24 h. Cells with media were included as negative control, whereas cells treated with 1 µg/ml mitomycin C (Biochem Pharmaceutical Industries, India) were considered as positive control. Two hours prior to the end of the incubation period, cultures were treated with spindle poison colchicine (Sigma, USA) to arrest cells in metaphase. Cells were harvested at the end of 72 h, treated with prewarmed hypotonic solution and fixed with 3:1 methanol-acetic acid solution. After fixation, the solution containing cells were dropped onto prechilled glass slides to facilitate the breaking of cells, with the release of metaphase chromosomes onto slides. Slides were heat fixed and allowed to air dry. Subsequently, the metaphase preparations were stained with Giemsa stain (Himedia Laboratories, India) and allowed to dry. Microscopic analysis of structural chromosome aberrations such as chromosome gap, chromosome break, chromatid gap, and chromatid break was performed on 50 cells per point using the fully automated metaphase finder (Carl Zeiss, Germany). The chromosome aberration frequency for each sample was calculated and presented as mean value ± standard deviation (SD) from two separate experiments. Mitotic indices were calculated by counting 1000 cells for each of the doses considered.

H. Oxidative stress and antioxidant defense gene expression

A pathway specific polymerase chain reaction (PCR) array comprising probes for 84 oxidative stress and antioxidant defense relevant genes (PAHS-065, SuperArray Bioscience, Frederick, MD, USA) was used to examine alterations in gene expression caused by exposure to DIONPs in lymphocytes. Cells were exposed to 1000 µg/ml DIONPs for 24 h. Ribonucleic acid (RNA) was extracted from cells using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Complementary DNA (cDNA) was reverse transcribed from RNA using RT² First Strand Kit (SuperArray). cDNAs were mixed with the RT² SYBR Green/Rox PCR master mix, following which quantitative real time PCR and data analysis were performed in triplicates according to the manufacturer's instruction. Data normalization was carried out using three reference genes: beta-2 microglobulin (*B2M*), ribosomal protein L13A (*RPL13A*), and beta-actin (*ACTB*). The relative gene expression changes were analyzed with the $\Delta\Delta C_t$ method and were determined as fold changes using the web-based analysis software available at <http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>. Differential expression values were identified using Student's *t*-test with a significant value of $p < 0.05$ and a fold-change cut-off of 1.5-fold.

I. Intracellular antioxidant enzyme activities

Peripheral blood lymphocytes exposed to 1000 $\mu\text{g/ml}$ DIONPs were washed three times in PBS and centrifuged at $1000 \times g$ for 5 min, 4°C to pellet down cells. The cell pellet was resuspended in three times the pellet volume in 50 mM phosphate buffer (pH 7.8) and sonicated on ice using a Vibra-CellTM sonicator (Sonics and Materials, Inc., USA) at 40% power. The resultant cell extract was used for the measurement of SOD, CAT, and GPx activities as described elsewhere.²¹ The experiment was carried out in triplicates for each of the antioxidant activities.

J. Statistical analysis

All data were expressed as mean $\pm$ SD. For statistical significance, values among groups were analyzed by one-way analysis of variance method. Comparisons between experimental group and control group were made by Student's *t*-test. The results were regarded as statistically significant at $p < 0.05$ unless mentioned otherwise.

III. RESULTS

A. DIONPs

TEM analysis indicated the synthesized NPs to be spherical in shape with an average core diameter of 9.08 ± 1.48 nm. The mean volume hydrodynamic size of particles in water as measured by DLS was 25.3 ± 0.97 nm. The Z-average intensity-weighted hydrodynamic radius of DIONPs was 38.68 ± 0.96 nm and the PdI was 0.2. DLS measurements of DIONPs in complete culture medium revealed the hydrodynamic size to be 123.1 ± 6.48 nm. The Z-average hydrodynamic radius was 119.1 ± 7.74 nm with a PdI value of 0.1. The zeta-potential of NPs measured in water (pH 7) and complete culture medium (pH 7.4) was -7.87 and -11.6 mV, respectively. The mean size of particles suspended in culture medium was higher than the mean size of particles in water, signifying that these NPs have a propensity to form larger complexes in culture medium, possibly due to the formation of a protein corona on the NP surface. FTIR analysis indicates that the NP surface has undergone modifications due to the dextran layer. The characteristic absorption bands of polysaccharide at 3319.9 cm^{-1} due to the O-H stretching and at 1643.3 cm^{-1} due to bending vibration that are present in the dextran spectrum are absent in DIONPs. This observation implies that the OH groups present in the glycosidic moieties play a vital role in the binding of dextran on the NP surface (Fig. 1).

B. Cellular uptake and cytotoxicity

NP internalization was analyzed using Prussian blue staining. Peripheral blood lymphocytes treated with lower concentrations of NPs showed very few iron positive stained cells, although at higher doses more particle internalization was observed. This indicates that lymphocytes internalize DIONPs only at high concentrations (Fig. 2). Utilizing flow cytometry quadrant analysis of Annexin V/propidium iodide (PI) double

FIG. 4. Intracellular ROS and GSH levels in human peripheral blood lymphocytes exposed to DIONPs. (a) ROS production in cells at 4 h (blue bars) and 24 h (pink bars). PC, positive control (H_2O_2). (b) ROS production in cells with and without GSH at 4 h. Data expressed as mean % DCF fluorescence $\pm$ SD ($n = 6$). (c) Intracellular GSH levels in cells at 24 h ($n = 3$). * $p < 0.01$ compared to control cells. # $p < 0.01$ compared to respective concentrations of NP treated cells in the absence of GSH.

stained lymphocytes, it was observed that the NP exposure did not cause significant levels of apoptosis in the NP treated lymphocytes compared to untreated lymphocytes even at doses that resulted in particle internalization. In contrast, the frequency of viable cells was significantly decreased in lymphocytes treated with positive control H_2O_2 ; the frequency of early apoptotic cells was markedly increased in these cells (Fig. 3).

C. Intracellular ROS and GSH

The ability of DIONPs to induce intracellular oxidant production was assessed using DCF fluorescence. ROS production increased in a dose-dependent manner upon NP exposure [Fig. 4(a)]. DCF fluorescence intensity increased to 105%, 112%,

FIG. 5. Metaphase spread of lymphocytes. (a) Control, (b) 8 $\mu\text{g/ml}$, (c) 40 $\mu\text{g/ml}$, (d) 200 $\mu\text{g/ml}$, (e) 1000 $\mu\text{g/ml}$, and (f) mitomycin C ($n = 2$). Arrowheads show the presence of chromosome aberrations in the metaphase preparations.

and 121% after 4 h exposure to 40, 200, and 1000 $\mu\text{g/ml}$ DIONPs, respectively, relative to control. At 24 h, the ROS levels for the same doses were significantly decreased (75%, 89%, and 37%, respectively). To further confirm that intracellular ROS generation was due to exposure to DIONPs, cells were cotreated with the antioxidant GSH. Cotreatment with GSH attenuated ROS levels at all concentrations [Fig. 4(b)].

Alteration in the total cellular GSH level in cells can be considered as an indication of adaptive response of the cell to oxidative damage. The levels of intracellular GSH increased with increasing particle concentration up to a dose of 200 $\mu\text{g/ml}$, indicating possible glutathione synthesis by cells. However, at the highest dose, there was a decrease in

cellular GSH levels, suggesting that cellular GSH was oxidized to GSSG (oxidized glutathione). However, the alterations in GSH levels were not statistically different from untreated cells [Fig. 4(c)].

D. Chromosome aberration analysis

To analyze whether DIONPs-induced intracellular ROS levels could cause genotoxic effects, chromosome aberration analysis was performed in human lymphocytes treated with DIONPs for 24 h. The metaphase spread of control cells, DIONPs exposed, and mitomycin C exposed cells are shown in Fig. 5. The chromosome aberration frequency calculated

TABLE I. Chromosome aberration frequencies and mitotic indices in human lymphocytes treated with DIONPs for 24 h. Collective data from two independent experiments expressed as mean $\pm$ SD. Mitotic indices obtained from 1000 cells.

Treatments	% Aberration frequency Mean $\pm$ SD	Mitotic index Mean $\pm$ SD
Negative control	0.022 $\pm$ 0.031	3.9 $\pm$ 0.49
8 μ g/ml	0.043 $\pm$ 0.01	4.0 $\pm$ 0.63
40 μ g/ml	0.108 $\pm$ 0.030	4.3 $\pm$ 0.28
200 μ g/ml	0.108 $\pm$ 0.09	4.3 $\pm$ 0.10
1000 μ g/ml	0.108 $\pm$ 0.030	4.2 $\pm$ 0.60
Positive control	2.021 $\pm$ 0.338 ^a	2.95 $\pm$ 0.06 ^a

^a $p < 0.05$ with respect to control cells ($n = 2$).

for each concentration is shown in Table I. Compared to untreated control cells, DIONPs had no significant effect on chromosome aberration frequencies in lymphocytes, nor affected the mitotic indices of cells. On the other hand, mitomycin treated cultures showed a significant increase in aberration frequency with respect to control; the mitotic index value was also substantially decreased.

E. Oxidative stress and antioxidant defense gene expression

Further, the potential of DIONPs to modulate the expression of oxidative stress dependent signaling and metabolism genes was analyzed using a human oxidative stress and antioxidant defense PCR array that included 84 oxidative stress responsive genes. The genes investigated were categorized as superoxide release and metabolism genes, peroxide metabolism genes, oxidoreductase genes, inflammation related genes, apoptosis inducer genes, and cell cycle related genes. Changes in gene expression relative to control cells in a 24 h exposure paradigm are presented in Table II. The expression of four genes: dual oxidase 2 (*DUOX2*), myeloperoxidase (*MPO*), glutathione peroxidase 1 (*GPX1*), and glutathione transferase zeta 1 (*GSTZ1*) was found to be significantly upregulated by at least 1.5-fold compared to untreated cells. The genes that were upregulated mainly belong to classes of genes that are involved in oxidation-reduction, peroxidation, and superoxide metabolism, whereas genes relevant to inflammation or associated with cell division and apoptosis were not affected. Gene expression alterations determined by fold changes are presented in the form of a scatter plot in Fig. 6.

F. Intracellular antioxidant enzyme activities

Further measurement of antioxidant enzyme activities in cells exposed to 1000 μ g/ml DIONPs for 24 h showed that there was no significant variation in cellular SOD levels between treated and control cells [Fig. 7(a)]. CAT activity was slightly yet significantly decreased [Fig. 7(b)], whereas cellular GPx activity showed a significant increase [Fig. 7(c)] in cells exposed to DIONPs. The enhanced GPx

antioxidant activity level is consistent with the increased expression of *GPX1* gene.

IV. DISCUSSION

The toxicity of NPs is highly dependent on the cells that represent the exposure route as well as the target organs. Intravenous administration of DIONPs would invariably allow their interaction with blood lymphocytes, an important component in the regulation of the immune system. NP uptake by leukocytes such as neutrophils and monocytes has been reported to occur mainly through the mechanism of phagocytosis, and to a small measure by pinocytosis.^{22,23} On the contrary, lymphocytes do not possess the intrinsic ability of phagocytosis.²⁴ Lymphocytes internalize NPs by the mechanism of endocytosis. The ability of human lymphocytes to internalize NPs by receptor-mediated endocytosis was first demonstrated by Bulte *et al.* using antilymphocyte directed monoclonal antibody-linked to dextran coated magnetic iron oxide NPs.²⁵ Later, labeling of lymphocytes by iron oxide NPs was also carried out by modifying the NP surface with Tat peptide.²⁶ A recent study demonstrated that amino-functionalized NPs were efficiently ingested by T-cells, while NPs with surface carboxyl groups or protein conjugates such as transferrin showed lesser uptake in T-cells.²⁷ Such an observation implies that surface functionalization is a critical factor for entry of NPs into cells.^{28–30} In addition to surface functionalization, other factors such as particle size, shape, surface charge, and surface functionality also affect the interactions between particles and cells.^{31,32} In our study, iron oxide NP surface was modified with dextran as confirmed by FTIR analysis. Utilizing DIONPs, we observed that the size of NPs in culture media was greater than its primary size, which is most likely due to the formation of the protein corona on the NPs in the buffer medium. This protein corona would in turn modify the surface charge and chemical nature of DIONPs. However, we observed that internalization of DIONPs occurred only at higher concentrations. This may be indicative of the involvement of a nonspecific endocytic process that does not use membrane receptors, since receptor-mediated endocytosis is more efficient than nonspecific endocytosis.³³ It would appear that resting lymphocytes are reluctant to internalize particles unless stimulated or exposed to NPs conjugated to antibodies or ligands that recognize specific receptors on the cell surface. For instance, it has been demonstrated that after 30 min of incubation, only 8% of endocytic vesicles in resting lymphocytes internalize cationized ferritin. T-cells internalize the equivalent of their entire surface area in approximately 54 h; this is a longer time than is required by phytohemagglutinin-stimulated human lymphocytes.³⁴ Thus, higher concentration of NPs may be required to allow nanoparticle internalization in resting peripheral blood lymphocytes.

The biocompatibility of DIONPs toward lymphocytes was evaluated using flow cytometry. Cytotoxicity analysis showed that the frequencies of viable, early apoptotic and late apoptotic/necrotic cells were not affected by DIONPs at any of the

TABLE II. DIONPs-mediated changes in cellular pathway-specific gene expression associated with oxidative stress and antioxidant defense in human peripheral blood lymphocytes.

Position	Gene bank	Gene symbol	Description	Fold change	p value	Class ^a
A01	NM_000477	<i>ALB</i>	Albumin	1.28	0.66	4
A02	NM_000697	<i>ALOX12</i>	Arachidonate 12-lipoxygenase	1.09	0.46	1, 3
A03	NM_021146	<i>ANGPTL7</i>	Angiopoietin-like 7	0.83	0.08	4
A04	NM_001159	<i>AOX1</i>	Aldehyde oxidase 1	1.06	0.98	1
A05	NM_000041	<i>APOE</i>	Apolipoprotein E	0.98	0.86	4
A06	NM_004045	<i>ATOX1</i>	ATX1 antioxidant protein 1 homolog (yeast)	1.25	0.02 ^b	4
A07	NM_004052	<i>BNIP3</i>	BCL2/adenovirus E1B interacting protein 3	1.05	0.49	6
A08	NM_001752	<i>CAT</i>	Catalase	1.36	0.02 ^b	3
A09	NM_002985	<i>CCL5</i>	Chemokine (C-C motif) ligand 5	1.06	0.63	5
A10	NM_005125	<i>CCS</i>	Copper chaperone for superoxide dismutase	1.32	0.08	1
A11	NM_007158	<i>CSDE1</i>	Cold shock domain-containing E1, RNA-binding	1.20	0.14	4
A12	NM_000101	<i>CYBA</i>	Cytochrome b-245, alpha polypeptide	1.10	0.52	1
B01	NM_134268	<i>CYGB</i>	Cytoglobin	1.39	0.11	1
B02	NM_001013742	<i>DGKK</i>	Diacylglycerol kinase, kappa	0.99	0.84	4
B03	NM_014762	<i>DHCR24</i>	24-dehydrocholesterol reductase	1.49	0.01 ^b	3, 6
B04	NM_175940	<i>DUOX1</i>	Dual oxidase 1	0.88	0.52	1, 2, 3
B05	NM_014080	<i>DUOX2</i>	Dual oxidase 2	2.12	0.01 ^b	1, 2, 3
B06	NM_004417	<i>DUSP1</i>	Dual specificity phosphatase 1	1.43	0.01 ^b	4, 7
B07	NM_001979	<i>EPHX2</i>	Epoxide hydrolase 2, cytoplasmic	1.13	0.02 ^b	3
B08	NM_000502	<i>EPX</i>	Eosinophil peroxidase	1.01	0.97	2
B09	NM_021953	<i>FOXM1</i>	Forkhead box M1	1.14	0.36	7
B10	NM_197962	<i>GLRX2</i>	Glutaredoxin 2	1.14	0.43	1, 6
B11	NM_153002	<i>GPR156</i>	G protein-coupled receptor 156	0.93	0.76	2, 3
B12	NM_000581	<i>GPX1</i>	Glutathione peroxidase 1	1.75	0.02 ^b	2, 3
C01	NM_002083	<i>GPX2</i>	Glutathione peroxidase 2	0.98	0.83	2, 3
C02	NM_002084	<i>GPX3</i>	Glutathione peroxidase 3	1.02	0.79	2, 3
C03	NM_002085	<i>GPX4</i>	Glutathione peroxidase 4	1.21	0.18	2, 3
C04	NM_001509	<i>GPX5</i>	Glutathione peroxidase 5	1.06	0.78	2, 3
C05	NM_182701	<i>GPX6</i>	Glutathione peroxidase 6	1.12	0.45	2, 3
C06	NM_015696	<i>GPX7</i>	Glutathione peroxidase 7	0.87	0.37	2, 3
C07	NM_000637	<i>GSR</i>	Glutathione reductase	0.93	0.49	2, 3
C08	NM_000178	<i>GSS</i>	Glutathione synthetase	0.93	0.70	4
C09	NM_001513	<i>GSTZ1</i>	Glutathione transferase zeta 1	1.65	0.002 ^b	2, 3
C10	NM_001518	<i>GTF2I</i>	General transcription factor II, i	1.12	0.20	1
C11	NM_006121	<i>KRT1</i>	Keratin 1	0.63	0.03 ^b	4
C12	NM_006151	<i>LPO</i>	Lactoperoxidase	0.89	0.441	2, 3
D01	NM_000242	<i>MBL2</i>	Mannose-binding lectin (protein C) 2	1.48	0.30	5
D02	NM_004528	<i>MGST3</i>	Microsomal glutathione S-transferase 3	1.32	0.15	2
D03	NM_000250	<i>MPO</i>	Myeloperoxidase	2.32	0.008 ^b	2, 3
D04	NM_002437	<i>MPV17</i>	MpV17 mitochondrial inner membrane protein	1.01	0.94	1
D05	NM_012331	<i>MSRA</i>	Methionine sulfoxide reductase A	1.51	0.002 ^b	3
D06	NM_005954	<i>MT3</i>	Metallothionein 3	1.08	0.53	4
D07	NM_004923	<i>MTL5</i>	Metallothionein-like 5	1.04	0.79	4
D08	NM_000265	<i>NCF1</i>	Neutrophil cytosolic factor 1	1.00	0.997	1
D09	NM_000433	<i>NCF2</i>	Neutrophil cytosolic factor 2	1.20	0.13	1
D10	NM_003551	<i>NME5</i>	Nonmetastatic cells 5	1.15	0.10	7
D11	NM_000625	<i>NOS2A</i>	Nitric oxide synthase 2A	1.22	0.20	3
D12	NM_024505	<i>NOX5</i>	NADPH oxidase	0.75	0.06	1
E01	NM_002452	<i>NUDT1</i>	Nudix-type motif 1	0.87	0.54	4
E02	NM_181354	<i>OXR1</i>	Oxidation resistance 1	1.16	0.03 ^b	4
E03	NM_005109	<i>OXSRI</i>	Oxidative-stress responsive 1	0.90	0.41	4
E04	NM_020992	<i>PDLIM1</i>	PDZ and LIM domain 1 (elfin)	1.00	0.92	4
E05	NM_015553	<i>IPCEF1</i>	Interaction protein cytohesin exchange factors 1	1.08	0.38	2
E06	NM_007254	<i>PNKP</i>	Polynucleotide kinase 3'-phosphatase	1.02	0.88	4
E07	NM_002574	<i>PRDX1</i>	Peroxiredoxin 1	0.92	0.37	2, 3
E08	NM_005809	<i>PRDX2</i>	Peroxiredoxin 2	0.93	0.45	2, 3
E09	NM_006793	<i>PRDX3</i>	Peroxiredoxin 3	1.18	0.04 ^b	2, 3

TABLE II. (Continued)

Position	Gene bank	Gene symbol	Description	Fold change	p value	Class ^a
E10	NM_006406	<i>PRDX4</i>	Peroxiredoxin 4	1.09	0.18	2, 3
E11	NM_181652	<i>PRDX5</i>	Peroxiredoxin 5	0.95	0.72	2, 3
E12	NM_004905	<i>PRDX6</i>	Peroxiredoxin	0.99	0.93	2, 3
F01	NM_020820	<i>PREX1</i>	PIP3-dependent RAC exchanger 1	0.98	0.93	1
F02	NM_006093	<i>PRG3</i>	Proteoglycan 3	0.78	0.10	4
F03	NM_183079	<i>PRNP</i>	Prion protein (p27-30)	0.90	0.58	4
F04	NM_000962	<i>PTGS1</i>	Prostaglandin-endoperoxide synthase 1	1.24	0.24	2
F05	NM_000963	<i>PTGS2</i>	Prostaglandin-endoperoxide synthase 2	1.22	0.24	2
F06	NM_012293	<i>PXDN</i>	Peroxidasin homolog (Drosophila)	0.91	0.38	2
F07	NM_144651	<i>PXDNL</i>	Peroxidasin homolog (Drosophila)-like	0.68	0.04 ^b	2
F08	NM_014245	<i>RNF7</i>	Ring finger protein 7	1.23	0.008 ^b	4
F09	NM_182826	<i>SCARA3</i>	Scavenger receptor class A, member 3	0.85	0.41	4
F10	NM_203472	<i>SELS</i>	Selenoprotein S	0.97	0.75	4
F11	NM_005410	<i>SEPP1</i>	Selenoprotein P, plasma, 1	0.97	0.78	4
F12	NM_003019	<i>SFTPD</i>	Pulmonary-associated protein D	2.91	0.95	1, 5
G01	NM_016276	<i>SGK2</i>	Serum/glucocorticoid regulated kinase 2	1.08	0.92	3, 7
G02	NM_012237	<i>SIRT2</i>	Sirtuin 2	1.01	0.86	4, 6
G03	NM_000454	<i>SOD1</i>	Superoxide dismutase 1	1.25	0.002 ^b	1, 3
G04	NM_000636	<i>SOD2</i>	Superoxide dismutase 2	0.92	0.60	1, 3
G05	NM_003102	<i>SOD3</i>	Superoxide dismutase 3	1.67	0.43	1, 3
G06	NM_080725	<i>SRXN1</i>	Sulfiredoxin 1 homolog (<i>S. cerevisiae</i>)	0.69	0.04 ^b	3, 4
G07	NM_006374	<i>STK25</i>	Serine/threonine kinase 25	1.10	0.35	4
G08	NM_000547	<i>TPO</i>	Thyroid peroxidase	1.02	0.97	2, 3
G09	NM_003319	<i>TTN</i>	Titin	0.88	0.13	2
G10	NM_032243	<i>TXNDC2</i>	Thioredoxin domain-containing 2	1.30	0.24	3
G11	NM_003330	<i>TXNRD1</i>	Thioredoxin reductase 1	1.28	0.08	3
G12	NM_006440	<i>TXNRD2</i>	Thioredoxin reductase 2	1.13	0.48	3

^a1—genes involved in superoxide metabolism; 2—genes with peroxidase activity; 3—genes with oxidoreductase activity; 4—additional genes involved in oxidative stress; 5—inflammation relevant genes; 6—genes involved in apoptosis; and 7—cell division relevant genes.

^b $p < 0.05$ with respect to untreated cells ($n = 3$).

FIG. 6. Gene expression pattern in lymphocytes treated with DIONPs. Red spots indicate genes that were significantly upregulated ($p < 0.05$) by at least by 1.5-fold ($n = 3$).

concentrations tested, in contrast to lymphocytes treated with H_2O_2 . Contrary to our data, there have been reports on the cytotoxicity of iron oxide NPs with different surface coatings in human lymphocytes. Magdolenova *et al.* demonstrated dose-dependent cytotoxicity and genotoxicity for oleate-coated iron oxide NPs.³⁵ Similarly, NP size-dependent and surface coating-dependent toxicity effects were reported in human T-lymphocyte.³⁶ In a comparative study involving different NPs, iron oxide NPs were shown to decrease cell viability and induce oxidative stress and DNA damage.³⁷ Therefore, we examined the ability of DIONPs to potentiate oxidative stress response in lymphocytes. A dose-dependent increase in ROS production was observed at an earlier time point, with subsequent reduction of the response at 24 h. Coincubation with the antioxidant GSH could prevent DIONPs-induced ROS generation. Moreover, intracellular ROS production was accompanied by alteration in intracellular GSH levels, with reduction in GSH levels observed at the highest dose. GSH represents one of the major nonenzymatic cellular defense mechanisms against oxidative stress,³⁸ which cycles between an oxidized (GSSG) and reduced (GSH) state to detoxify hydroperoxides in concert with glutathione peroxidase using the reducing equivalents in NADPH and GSH reductase. GSH directly participates in the scavenging of free

FIG. 7. Antioxidant activities in lymphocytes treated with DIONPs. Enzyme activities of (a) SOD, (b) CAT, and (c) GPx. Values are calculated as mean $\pm$ SD (n = 3). * p < 0.05 compared to control cells.

radicals, including superoxide anion and hydroxyl radical. Genotoxicity evaluation using the chromosome aberration assay showed that DIONPs did not induce oxidative DNA damage in lymphocytes.

Since DIONPs was observed to increase ROS production and alter GSH levels, we assessed the ability of DIONPs to modulate the expression of oxidative stress responsive genes. The expression of four genes was altered in DIONPs treated cells. *DUOX2* belong to the NADPH oxidase (NOX) gene family, which encode transmembrane proteins that transfer electrons across biological membranes. NOX enzymes reduce molecular oxygen to superoxide anion that can be further converted to various secondary ROS.³⁹ Although the NADPH oxidase system was initially identified only in phagocytic leukocytes such as neutrophils and macrophages,⁴⁰ recently NOX activity was also demonstrated in nonphagocytic cells. Different from other NOX enzymes (NOX 1–5) which produce superoxide anion, the two DUOX

enzymes (DUOX1, 2) produce H_2O_2 as the primary product.⁴¹ Unexpectedly, the expression of NOX5 gene was not detected in our analysis. A search of literature revealed that although NOX5 mRNA is abundant in tissue lymphocytes, it is almost absent in circulating blood lymphocytes.⁴² *MPO* codes for the heme enzyme myeloperoxidase, which is released by leukocytes and plays a crucial role in inflammation and oxidative stress at the cellular level. MPO is involved in ROS generation by catalyzing the conversion of H_2O_2 to secondary species such as hypochlorous acid.⁴³ The differential expression of *MPO* in T-cells has been suggested in a recent study.⁴⁴ *GPX1* is the most abundant isoform of the glutathione peroxidase family expressed in most cell types, with the preference for H_2O_2 as its substrate.⁴⁵ *GSTZ1* encodes a major phase II detoxification enzyme found mainly in the cytoplasm; it belongs to the glutathione S-transferase family which functions to catalyze the conjugation of electrophilic substrates to glutathione.⁴⁶

Further analysis of antioxidant enzyme activities in lymphocytes exposed to DIONPs revealed a significant increase in GPx activity with no marked alteration in SOD or CAT activities. SOD functions to inactivate the highly reactive superoxide anion by converting it to H_2O_2 and water in the presence of a metal ion acting as the redox agent;⁴⁷ it is the most important of the enzymatic antioxidant pathways in cellular homeostasis. GPx enzyme catalyzes the reduction of H_2O_2 by oxidizing glutathione to its reduced form.⁴⁸ CAT catalyzes the same hydrogen peroxidation reaction; this enzyme works alongside GPx and is generally responsible for removing H_2O_2 generated within cells. It has been proposed that GPx-CAT cooperativity is important in handling the wide range of H_2O_2 concentrations encountered by cells, in which GPx with much higher affinity for H_2O_2 operates at low concentrations, whereas CAT with low affinity is recruited at high concentrations.⁴⁹ Thus, it appears that GPx is the predominant enzymatic antioxidant defense involved in response to DIONPs exposure, and the role of CAT enzyme may be limited due to lack of GPx saturation by H_2O_2 .

V. CONCLUSION

In summary, DIONPs synthesized and characterized previously for hyperthermia application were used to investigate the extent of nano-bio-interactions between these particles and human peripheral blood lymphocytes *in vitro*. The data obtained indicate that under the conditions tested, DIONPs were not cytotoxic toward human peripheral blood lymphocytes; cellular toxicity response was limited to inducing minor levels of oxidative stress which was unaccompanied by genotoxic effects. The results of the study are undoubtedly significant, considering that lymphocytes would be among the first cell type exposed upon intravenous administration of DIONPs in a therapeutic setting. Since DIONPs have been fabricated for the application of hyperthermia, a further line of investigation would involve analyzing the possibility of accelerated generation of free radicals in lymphocytes in the presence of an alternating magnetic field.

ACKNOWLEDGMENTS

The authors are grateful to the Director of Sree Chitra Tirunal Institute for Medical Sciences and Technology and Head of Biomedical Technology Wing for their support. The authors acknowledge the financial support provided by Department of Science and Technology, India (Grant No. SR/NM/NS-90/2008). Technical assistance from Legi B, Anju Mohan, and Renjith Kartha are kindly acknowledged. Sheeja Liza Easo acknowledges the Council of Scientific and Industrial Research (CSIR), New Delhi, India, for financial support.

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