Investigation of hematotoxic effect of nano ZnO, nano Fe$_3$O$_4$ and nano SiO$_2$ in vitro

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Objective(s): Evaluation of nanomaterials interaction with blood ingredients is a part of preclinical risk assessment of newly-synthesized materials, especially for nano-sized pharmaceuticals which are intravenously administrated. The red blood cells (RBCs) are susceptible to oxidative stress damage. This study was designed to evaluate induced oxidative hematotoxic effect of nano ZnO, Fe$_3$O$_4$, and nano SiO$_2$ on human red blood cells in vitro.

Methods: Blood samples were collected from healthy male volunteers. RBCs were exposed to different concentrations (50, 100, 250mg/ml) of nano ZnO, nano Fe$_3$O$_4$, and nano SiO$_2$ at 4°C for 24hours. Lipid peroxidation and intracellular Glutathione (GSH) level were studied as the biomarkers of oxidative stress.

Results: The results showed that the lipid peroxidation had significantly increased. However, after exposure to nanoparticles, the GSH level of RBCs considerably decreased compared to the controls (p<0.05).

Conclusions: This study suggests that nanoparticles could induce oxidative stress that eventually leads to cell toxicity and hemolysis of RBCs at certain doses. This study provides important experimental information for safety assessment and pharmacokinetics of high-doses intravenous administration of nanoparticles for design of clinical trials.

INTRODUCTION

Nanomaterials are defined as a new class of materials having unique physical and chemical properties. There is great interest in applying nanomaterials in different fields of medicine and industry. The potential benefit of nanomaterials in medicinal devices or drug delivery proposes new methods in diagnosis, medication, and treatment of complicated diseases such as cancers. Any nanomaterials applied as pharmaceuticals or medical devices must be assessed for potential risk for consumers before approval for prescription. Evaluation of nanomaterials interaction with blood ingredients is a part of preclinical risk assessment of newly-synthesized materials, especially for nano-sized pharmaceuticals which are intravenously administrated [1]. Several studies have shown that the unique structure and size of the nanoparticles could lead to oxidative injury and cell toxicity in different cell cultures and also in blood cells [2-3]. Formation of reactive oxygen and nitrogen species (RONS) at levels higher than the body’s antioxidant capacity has been known as the oxidative stress. Reactive oxygen and nitrogen species are generated in the body as the result of normal metabolism.
and either as the result of exposure to a variety of chemicals, drugs, and pollutants. Nucleic acids, proteins, lipids are cellular macromolecules that may be harmed by RONS. Oxidative stress also involves in pathogenesis of different diseases including cancer, neurodegenerative diseases, atherosclerosis, kidney and liver damage, rheumatoid arthritis, immunological disorder, and aging [4-5].

Lipid peroxidation of polyunsaturated fatty acids leads to production of malondialdehyde (MDA) that is a useful biomarker of oxidative stress. MDA is commonly measured by the thiobarbituric acid-reactive-substances (TBARS) assay. Small thiols including tripeptide glutathione (GSH) are considered as the protective sulphydryl antioxidants and radical scavengers. Glutathione protects sensitive thiol groups (-SH) of proteins against oxidative stress [6].

The RBCs are intrinsically susceptible to oxidative stress damage because of exposing to high oxygen pressure and the presence of polyunsaturated fatty acid in their cell membrane. Furthermore, RBCs do not have endoplasmic reticulum and nucleus to replace damaged proteins [7]. Some studies have investigated the hematotoxic effects of nanoparticles on the blood cells in vivo and in vitro [8-13]. Nano Fe₃O₄, nano ZnO, and nano SiO₂ are well-known nanoparticles that have promising biomedical applications and their oxidative stress effects have been reported in different studies [9, 14-15]. Zinc nanoparticles are used in cosmetics ointment, sunscreens, food additive, antimicrobial, fungicide, UV protection coatings, and as a catalyst [15]. SiO₂ nanoparticles have found extensive applications in chemical mechanical polishing and as the additives to drugs, cosmetics, printer toners, varnishes, and food. In recent years, the use of SiO₂ nanoparticles has been extended to biomedical and biotechnological fields [16]. Fe₃O₄ nanoparticles have been used as the contrast agents in Magnetic resonance imaging (MRI) angiography and perfusion imaging [17]. In this study, the induced oxidative hematotoxic effects of different doses of nano ZnO, nano Fe₃O₄, and nano SiO₂ have been studied on human RBC.

MATERIALS AND METHODS

Nano particles characterization

Silicon Dioxide (SiO₂) Nanopowder (99+%, 20-30 nm, amorphous) was purchased from Nanosany Corporation, Iranian Nanomaterials Pioneers Company, Mashhad, Iran. Figure 1 and Figure 2 represent transmission electron microscope (TEM) and scanning electron microscope (SEM) images of nano SiO₂.

Iron Oxide (Fe₃O₄) Nanopowder (99+%, 20-40 nm, spherical) was purchased from Nanosany Corporation, Iranian Nanomaterials Pioneers Company, Mashhad, Iran. Figure 3 represent the TEM image of nano Fe₃O₄.

Nano-zinc metal powder (99.99+, 20-40 nm) was purchased from the Intelligent Materials Pvt. Ltd., Nanoshel LLC, Wilmington, DE, USA. Figure 4 represent transmission electron the TEM image of nano ZnO.

All the other chemicals used in this experiment were analytical grade with the highest purity.
Blood samples preparation
Blood samples were collected from healthy male volunteers in heparinized tubes and centrifuged at 3000 rpm for 25 min. Plasma and the buffy coats of blood were discarded. RBCs were washed three times with phosphate-buffered normal saline and then exposed to different concentrations (50, 100, 250 mg/ml) of nano ZnO, nano Fe$_3$O$_4$, and nano SiO$_2$ before incubation at 4°C for 24 h. Each experiment was performed in triplicate.

Lipid peroxidation assay
Lipid peroxidation in human erythrocytes was quantified by measuring the formation of TBARS. Erythrocytes were mixed with trichloroacetic acid 20% (1:1). Samples were centrifuged (600g×10 min) and then thiobarbituric acid 15% was added to the supernatants. Finally, the samples were heated at temperature 100°C for 15 min and the absorbance of the supernatant was measured at 532 nm. The quantities of TBARS were presented as the percentage of TBARS production over the control.

Quantification of intracellular GSH levels
Cellular level of reduced GSH was determined using the GSH colorimetric assay kit. This method is based on a chemical reaction between GSH and 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB) that results in generating glutathione disulfide (GSSG) and-nitro-5-thiobenzoic acid, a yellow colored product. Thus, GSH concentration in a sample solution can be determined by the absorbance measurement at 412 nm [18].

Statistical analysis
One-way analysis of variance (ANOVA) was used to compare the results. P value greater than 0.05 was considered insignificant.

RESULTS AND DISCUSSION
Effect of Zn, Fe$_3$O$_4$, and SiO$_2$ nanoparticles on lipid peroxidation
TBARS assay is a sensitive and simple method for detecting lipid peroxidation which is commonly used as an important biomarker of oxidative stress. As showed in table 1, the percentage of lipid peroxidation biomarker, malondialdehyde level, was significantly increased in treated red blood compared to the control groups after incubation with different concentrations of all nanoparticles.

Table 1. TBARS level of RBCs after 24 h exposure to ZnO, Fe$_3$O$_4$, and SiO$_2$ nanoparticles

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>TBARS (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nano ZnO</td>
</tr>
<tr>
<td>0 mg/ml (control)</td>
<td>100</td>
</tr>
<tr>
<td>50 mg/ml</td>
<td>151±9.3*</td>
</tr>
<tr>
<td>100 mg/ml</td>
<td>157±7.4*</td>
</tr>
<tr>
<td>250 mg/ml</td>
<td>183±11*</td>
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</tbody>
</table>
(p<0.05) and at higher doses of nano ZnO and Fe$_3$O$_4$ nanoparticles more significant effects were seen (p<0.005). Furthermore, exposure to the different doses of Fe$_3$O$_4$ nanoparticles has led to high lipid peroxidation level compared to controls. However, dose dependency of lipid peroxidation was not significant. In SiO$_2$-treated groups, significant increase in lipid peroxidation level was showed (p<0.05). Nanoparticle-free RBCs were used as the control groups. The significant data are indicated by* versus control p<0.05. ** shows significant data compared to other doses of that nanoparticle p<0.05.

**Effect of ZnO, Fe$_3$O$_4$, and SiO$_2$ nanoparticles on intracellular GSH levels**

GSH is a special sulphydryl-contained molecule in cells that maintains homeostasis of cellular oxidation-reduction. Alterations in GSH homeostasis can be considered as the indication of functional damage to the cells. As can be seen in Table 2, compared to the controls, the level of GSH in RBCs significantly decreased after exposure to nanoparticles in all treated groups (p<0.05). Nanoparticle-free RBCs were used as the control groups. The significant data are indicated by * (p<0.05) versus control.

The data showed a significant decrease in GSH

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Table 2. GSH levels of RBCs after 24h exposure to ZnO, Fe$_3$O$_4$, SiO$_2$ nanoparticles

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>Nano zinc oxide</th>
<th>Nano Fe$_3$O$_4$</th>
<th>Nano SiO$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mg/ml (control)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>50 mg/ml</td>
<td>49±7.2*</td>
<td>32±4.4*</td>
<td>13±3.1*</td>
</tr>
<tr>
<td>100 mg/ml</td>
<td>43±8.8*</td>
<td>18±9.1*</td>
<td>48±15.7*</td>
</tr>
<tr>
<td>250 mg/ml</td>
<td>17±3.9*</td>
<td>10±2.6*</td>
<td>29±9.5*</td>
</tr>
</tbody>
</table>
level in nanoparticles-exposed RBCs. There was a reverse relation between MDA and GSH levels in our experiment.

The purpose of the present study was to investigate induced oxidative damage in RBCs and the possible mechanisms of hematotoxicity of nano ZnO, nano FeO₄, and nano SiO₂. Increased membrane lipid peroxidation (TBARS level) and decreased level of GSH are indicators of elevated oxidative stress. Many studies have shown that nanoparticles could induce oxidative stress and cause cell toxicity in cell cultures and animal models [19-24].

FeO₄ nanoparticles were used as contrast agents in MRI and imaging. Iron is known as the oxidative stress progenitor via the Fenton reaction and eventually causes cell toxicity at high doses [25]. Previous studies have indicated that the FeO₄ nanoparticles could induce oxidative stress, cell membrane damage, and cytotoxicity in a dose-dependent manner [10, 26-29]. Our study showed that in presence of iron oxide nanoparticles, the level of TBARS has increased while the level of GSH has decreased and the FeO₄. Nano SiO₂ have applied in food industry and also as drug additives and cosmetics. It was shown that cristalobalite nanoparticles induce oxidative stress and cell toxicity in different types of cell lines [30-31]. Our study revealed that SiO₂ could induce oxidative stress and cytotoxic effects in RBCs. However, the oxidative stress indicators, TBARS and GSH, did not show a dose dependent relationship. Nano ZnO particles were used in biomedical imaging, drug and gene delivery, and food/cosmetics industries. Some in vitro and in vivo studies revealed that oxidative stress has a principal role in nano ZnO cytotoxicity. Our study suggested that oxidative stress is a mechanism of RBCs cytotoxicity. Our experiment demonstrated significant increase in TBARS and reduced GSH levels, especially in higher doses [32-36]. Our results and previous studies have shown the pro-oxidant effects of certain doses of nanoparticles in RBCs which should be considered in people exposed to nanoparticles via pharmaceuticals especially through systemic administration routes. In population who are susceptible to oxidative stress, such as sickle cell anemic patients, anemic persons, and those who are suffering from chronic disease like diabetes, the nanoparticles should be prescribed carefully [37-38].

CONCLUSIONS

In conclusion, this study suggested that nanoparticles could induce oxidative stress, cell toxicity, and RBC hemolysis at certain doses. This study provides important experimental information for the safety assessment and pharmacokinetics of high doses intravenous administration of nanoparticles and for the design of clinical trials.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

REFERENCES

36. Kim, Y. H., Fazlollahi, F., Kennedy, I. M., Yacobi, N. R.,
