The Protective Effects of Antioxidant (Vitamin C) Against Hepatic Oxidative Damage Induced by Zinc Oxide Nanoparticles

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ABSTRACT: It has demonstrated that nanoparticles, may cause more inflammation than larger particles of the same materials at a same mass dose. The aim of this study was to investigate the role of vitamin C against the toxic effect of zinc oxide nano-particles (ZnO-NPs) induced oxidative stress in rat livers. ZnO-NPs were administered orally using two doses (300 mg and 1g/kg body weight/day for two weeks) in two test groups of rats, another two test groups were received the same doses in addition to vitamin C (30 mg/kg body weight) daily for four weeks. Some biomarkers of tissue damage were investigated. Indices of different antioxidant enzymes (namely, superoxide dismutase, catalase, glutathione-S-transferase) as well as lipid peroxidation and glutathione were determined in liver homogenates. Finally, histopathological studies were performed from liver sections of all rats of all groups. Administration of vitamin C significantly reversed these changes to almost normal. Apart from these, histopathological changes also revealed the protective nature of vitamin C against ZnO-NPs induced necrotic damage of the liver tissues. Vitamin C proved to be hepatoprotective agents against ZnO-NPs toxicity because they ameliorated the oxidative stress related to liver damage.

Keywords: ZnO-NPs, Vitamin C, Hepatic damages, Hepatic enzymes, Oxidative stress

INTRODUCTION

The fast growth of the nanotechnology industry has led to wide-range of production and application of engineered nanoparticles (NPs). NPs are not only used in industry and medicine but are also increasingly used in different consumer products such as cosmetics, sunscreens, and food products (1). However, the properties that make them useful are also the cause of concern (2-4). The ability of NPs to induce toxicity has been attributed to their increased surface reactivity (5,6). The small particle size of NPs creates a large surface area per unit mass and makes them more reactive in a cell. It has also been proposed that the increase in the surface area of NPs greatly increase their ability to produce reactive oxygen species (ROS) (7,8). NPs can enter the human body through different routes such as inhalation, ingestion and injection and dermal penetration. Among them, uptake of nanoparticles by the gastrointestinal tract is one of the most important routes (9,10). They may then translocate to blood causing different biological reactions in many organs, which are considered to be the secondary major sites of interaction (11,12).

Zinc oxide nanoparticles is one of the most utilized nanoparticles that have a wide range of applications due to their special characteristics, including semiconducting, optical, magnetic, catalytic, electrical, ultraviolet light absorption and antimicrobial properties (3,6).

With the wide range of application of ZnO nanoparticles in the industrial field, the human body may be intentionally or unintentionally exposed to nanoparticles through several routes, such as oral ingestion, inhalation and intravenous injection (11).

However, study of the toxicological effects of ZnO nanoparticles in biological systems has lagged behind the speed of their mass production and applications in different fields. Moreover, ZnO is generally considered as a material with low toxicity, because zinc is a principle trace element in the human body and is commonly present in foods or added as a nutritional supplement, so zinc has a little attention during assessment of toxicity of nanoparticles (9).
Therefore, the aim of our study is to investigate the toxicity effect of oral zinc oxide nano-particles in low (300 mg/kg) and high doses (1 g/kg body weight) on liver and the protective effects of antioxidant Vitamin C (Ascorbic acid) which acts as a potent antioxidant against oxidative stress that might be the mechanism of this toxicity.

MATERIALS And METHODS

Animals
90 Adult albino rats of either sex weighing 180 ±20 gm were used throughout the experiments. Animals were kept in raised mesh bottom cages to prevent coprophagy. The animals were maintained in colony cages at 25 ± 2°C, relative humidity 50-55%. The animals were fed with standard animal feed and water ad libitum. Animals divide into 5 groups 18 animals each.

Drugs and Chemicals
Zinc oxide nano-particles
Vitamin C
Fine chemicals
All chemicals were obtained from Sigma Chemical Co

Experimental Design

Animal Grouping
The study animals are divided into 5 groups (18) rats each:
Group I  control one and were receive vehicle orally
Group II were receive zinc oxide nano-particles orally in a low dose (300 mg/kg/day) Sharma et al. (13) for two weeks.
Group III were receive zinc oxide nano-particles orally in a high dose 1 g/kg body weight/day for two weeks.
Group IV were receive zinc oxide nano-particles orally in a low dose (300 mg/kg/day) for two weeks co-administered Vitamin C (30 mg/kg) daily orally, Rekha et al. (14) , to be followed by two weeks Vitamin C only (30 mg/kg) daily.
Group V were receive zinc oxide nano-particles orally in a high dose 1 g/kg body weight/day for 14 days co-administered Vitamin C (30 mg/kg) daily orally, to be followed by two weeks Vitamin C only (30 mg/kg) daily.

METHODS

Rats of all groups were subjected to:
Blood samples were obtained from the retro-orbital vein plexus.
   Aspartate transaminase (AST) and Alanine transaminase (ALT) activities in serum were measured according to Reitman-Frankel colorimetric transaminase procedure (15), using standard diagnostic kits.
   Alkaline phosphatase (ALP) activity was done according to the method of Belfield and Goldberg (16), using standard diagnostic kits.
   Total bilirubin: Walter and Gerade (17), using standard diagnostic kits.
   Lipid peroxidation: Assay for malondialdehyde (MDA) was done through the method of Janeiro (18) by measuring thiobarbituric acid reactive substances produced during lipid peroxidation.
   Glutathione-S-transferase (GST): In all the experimental samples, the activity of the enzyme was measured according to the method of Habig and Jakoby (19). The reaction mixture contained suitable amount of enzyme (25 μg of protein in tissue homogenate), KH2PO4 buffer, EDTA, CDNB and GSH. The reaction was carried out at 37 C and monitored spectrophotometrically at 340 nm for 5 min. A blank was run in absence of the enzyme. One unit of GST activity is defined as 1 μmol product formation per minute.
   The catalase (CAT) activity was determined spectrophotometrically by the method of Pljesa-Ercegovac et al., (20) the activity was expressed as μmol/min/g tissue using the molar absorbance of 43.6 for hydrogen peroxide.
   The glutathione (GSH): Glutathione concentration was determined by the method of Tietze (21). Liver homogenates were deproteinated with trichloroacetic acid (TCA) by centrifugation and GSH released in the supernatant were derivatized with 5,5’ dithiobis-2-nitrobenzoic acid (DTNB). The development of color was measured at 412 nm. A standard curve was drawn using different known concentrations of GSH solution. With the help of this standard curve, GSH contents in all the experimental samples were calculated.
Tissue SOD activity: The assay of SOD is based using the nitroblue tetrazolium (NBT) method described by Sun et al. (22) and modified by Durak et al. (24) In this method, NBT is reduced to blue formazan by superoxide (O2−), which has a strong absorbance at 560 nm.

Histopathological studies by light microscope: After the end of the tested period, rats were sacrificed, livers will be excised and fixed in 10% formalin saline, Bouin’s and Carnoy’s fluids. Sections were prepared and stained with hematoxylin and eosin (H&E) for the histological investigations.

Histopathological studies by electron microscope (EM): EM was used to evaluate ultra-structural changes in the liver induced by ZnONP. Livers of control and ZnONP treated rats were fixed with 1.5% glutaraldehyde in 0.1 M cacodylate buffer, stained with uranyl acetate, and embedded in epoxy resin. Ultra-thin (60 nm) sections were cut, stained with uranyl acetate and lead citrate, and examined with an EM.

Statistical Analysis: Data were statistically analyzed by comparing values of different treatment groups with the values of individual controls. Results were expressed as mean ± SD. Students t-test was applied for detecting the significance of difference between groups. P values of 0.05 or less were considered significant.

RESULTS

Table 1 and 2 respectively showed that administration of ZnO-NPs in either in low and in high repeated doses significantly elevated liver function biomarkers compared with normal control value. The increase of these biomarkers was pronounced in high doses. Co-administration of vitamin C significantly attenuated ZnO-NPs induced elevation of serum liver function markers.

Effect of the ZnO-NPs on products of lipid peroxidation

The level of MDA in all groups from liver homogenates is shown in Table 3. The MDA levels in group II and III rats were found to be significantly elevated compared to control group. Co-administration of 30 mg/kg body wt of vitamin C in groups IV and V rats showed significant reduction in the MDA level compared to group II and III rats.

Effect on antioxidant enzymes

Effect of ZnO-NPs on CAT activity

The CAT activity levels in liver of all rat groups are shown in Table 3. The CAT activity in liver homogenates of group II and III was significantly lower than that of group I. CAT activity was found to be significantly increased when the animals treated with vitamin C compared to the only ZnO-NPs treated rats.

Effect of ZnO-NPs on SOD activity

The activity of SOD in the liver homogenates of all rats groups are shown in Table 3. The SOD activity in group II and III rats was significantly reduced compared to group I. In group IV and V rats there was an enhancement of SOD value corresponding to only ZnO-NPs treated groups.

Table 1. The Protective effect of vitamin C on serum liver function markers of intoxicated rats with low doses of ZnO-NPs.

<table>
<thead>
<tr>
<th></th>
<th>ALT</th>
<th>AST</th>
<th>ALP</th>
<th>Total bilirubin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.2±1</td>
<td>10.3±1</td>
<td>70.0±6.5</td>
<td>3.75 ± 0.96</td>
</tr>
<tr>
<td>ZnONPs</td>
<td>47.8±3.7</td>
<td>21.2±0.8</td>
<td>165.6±16.1</td>
<td>7.21 ± 1.01</td>
</tr>
<tr>
<td>Vitamin C +ZnONPs</td>
<td>28.3±3.6</td>
<td>11.1±0.4</td>
<td>93.2±7.3</td>
<td>4.64 ± 0.96</td>
</tr>
</tbody>
</table>

Data represents mean ± SD, (Pa and Pb< 0.05). Pa indicates statistically significant difference of the values of serum liver markers of ZnO-NPs treated rats with respect to control; Pb indicate the same with respect to the values of serum liver markers of vitamin C treated rats respectively with respect to ZnO-NPs treated rats.

Table 2. The protective effect of vitamin C on serum liver function markers of intoxicated rats with high doses of ZnO-NPs.

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<td>ZnONPs</td>
<td>95.9±2.2</td>
<td>77.8±2.1</td>
<td>447.0±11.5</td>
<td>7.74 ± 1.72</td>
</tr>
<tr>
<td>Vitamin C +ZnONPs</td>
<td>37.6±3.8</td>
<td>16.5±1.67</td>
<td>110.1±5.3</td>
<td>5.63±1.21</td>
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Data represents mean ± SD, (Pa and Pb< 0.05). Pa indicates statistically significant difference of the values of serum liver markers of ZnO-NPs treated rats with respect to normal control; Pb indicate the same with respect to the values of serum liver markers of vitamin C treated rats respectively with respect to ZnO-NPs treated rats.

Effect of the ZnO-NPs on products of lipid peroxidation

The level of MDA in all groups from liver homogenates is shown in Table 3. The MDA levels in group II and III rats were found to be significantly elevated compared to control group. Co-administration of 30 mg/kg body wt of vitamin C in groups IV and V rats showed significant reduction in the MDA level compared to group II and III rats.

Effect on antioxidant enzymes

Effect of ZnO-NPs on CAT activity

The CAT activity levels in liver of all rat groups are shown in Table 3. The CAT activity in liver homogenates of group II and III was significantly lower than that of group I. CAT activity was found to be significantly increased when the animals treated with vitamin C compared to the only ZnO-NPs treated rats.

Effect of ZnO-NPs on SOD activity

The activity of SOD in the liver homogenates of all rats groups are shown in Table 3. The SOD activity in group II and III rats was significantly reduced compared to group I. In group IV and V rats there was an enhancement of SOD value corresponding to only ZnO-NPs treated groups.

Table 3. The Protective effect of vitamin C on products of lipid peroxidation.

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Effect of ZnO-NPs on GST activity

GST activity as measured from liver tissue homogenates of all experimental rats is shown in Table 3. As seen from the table there is a reduction in GST activity of groups II and III rats in liver homogenates. In co-administration of vitamin C treated group (IV and V rats), GST activity was higher compared to groups II and III treated rats.

Effect of ZnO-NPs on GSH level

As seen from Table 3 there was a reduction of GSH in ZnO-NPs treated liver homogenates compared to normal controls. However, co-administration of vitamin C restored the GSH levels both in group IV and V rats.

Table 3. The Effect of vitamin C on oxidative stress indices such as MDA content, CAT activity, SOD activity, GST activity and GSH level in liver homogenates, of ZnO-NPs treated rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CAT (Units/mg protein)</th>
<th>SOD (Units/mg protein)</th>
<th>GST (Units/mg protein)</th>
<th>GSH (mg/total protein)</th>
<th>TBARS (nmoles/gm tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>110 ± 5.2</td>
<td>295 ± 12</td>
<td>6.2 ± 0.27</td>
<td>46 ± 2.1</td>
<td>41 ± 1.1</td>
</tr>
<tr>
<td>300 mg/kg ZnO-NPs</td>
<td>66 ± 3.2(^a)</td>
<td>220 ± 9.2(^a)</td>
<td>3.4 ± 0.13(^a)</td>
<td>19 ± 0.80(^a)</td>
<td>72 ± 3.2(^a)</td>
</tr>
<tr>
<td>1g/ kg ZnO-NPs</td>
<td>41 ± 1.7(^a)</td>
<td>211 ± 7.1(^a)</td>
<td>2.9 ± 0.12(^a)</td>
<td>12.9 ± 0.66(^a)</td>
<td>91 ± 3.5(^a)</td>
</tr>
<tr>
<td>Vit. C +300 mg/kg ZnO-NPs</td>
<td>101 ± 4.3(^b)</td>
<td>278 ± 12.5 (^b)</td>
<td>6 ± 0.20(^b)</td>
<td>35 ± 1.5(^b)</td>
<td>50 ± 2.0(^b)</td>
</tr>
<tr>
<td>Vitamin C +1g/ kg ZnO-NPs</td>
<td>83 ± 3.8(^b)</td>
<td>267 ± 11.3 (^b)</td>
<td>5.6 ± 0.21(^b)</td>
<td>30 ± 1.2(^b)</td>
<td>55 ± 2.1(^b)</td>
</tr>
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</table>

Data represents mean ± SD, (Pa and Pb< 0.05). Pa indicates statistically significant difference of the indices of oxidative stress of ZnO-NPs-treated rats with respect to normal control and Pb indicates the same for the indices of oxidative stress of vitamin C treated rats with respect to ZnO-NPs-treated rats.

Histopathological results

Light Microscope

In control group, the livers have no histopathological alteration with normal histological structure of the central vein and surrounding hepatocytes were recorded (Fig. 1 A).

In group II dilatation and congestion were detected in the portal vein associated with diffuse kupffer cells proliferation in between the hepatocytes (Fig. 1 B).

While in group III sever dilatation and congestion were detected in the central (Fig. 1 C) and portal veins as well as hepatic sinusoids associated with oedema and inflammatory cells infiltration in the portal area (Fig. 1 D). Fatty change was observed in few individual hepatocytes (Fig. 1 E), associated with diffuse kupffer cells proliferation in between the hepatocytes (Fig. 1 F).

In groups IV that received low doses of ZnO with vitamin C showed normal histological appearance. While rats administrated high doses of ZnO-NPS with vitamin C (group V), there was mild congestion of central vein (Fig. 1 G).
Figure 1. Photomicrographs of liver sections, stained with H&E from rats. (A) Liver section from control rat showing normal architecture of hepatocytes. (B) Liver section from rat received low doses of ZnONPs showing dilatation and congestion in the portal vein associated with diffuse kupffer cells proliferation in between the hepatocytes. (C, D, E & F) Liver section from rat received high doses of ZnO showing dilatation and congestion were detected in the central (C) and portal veins with oedema and inflammatory cells infiltration in the portal area (D) with fatty change in individual hepatocytes (E) and diffuse kupffer cells proliferation in between the hepatocytes (F). (G) Liver section from rat received high doses of ZnO with vitamin C showing mild dilatation of central vein.
**Electron Microscope**

The liver belonging to control group showing the hepatic cell (H) of normal morphological appearance having large vesicular nucleus (N), mitochondria (m), rough endoplasmic reticulum (RER), glycogen granules (G), and bile canaliculi (Bc) (Fig. 2 A). While in group II, the hepatic cells (H) contained fat globule (f) and the mitochondria (m) swollen and lost its crests. The kupffer cell (kc) hypertrophied and contained large vacuoles (v) that contained light electron dense granular material, nucleus (N) and the other cell organelles (Fig. 2 B). In the meantime liver belonging to group three showing the hepatic cell (H) contained swollen mitochondria (M) with disintegration of its crests and infiltration of moderate amount of glycogen granules (arrow). The wall of the sinusoid contained fat storing cell containing numerous fat globule (F) and the sinusoidal lumen contained RBCs (Fig. 2 C). In group IV the liver showing the hepatic cell (H) with large vesicular nucleus (N) well distinct mitochondria (m) with its normal morphological appearance, RER and moderate amount of glycogen granules (arrows) (Fig. 2 D). Liver belonging to group V showing slight dilatation of the bile canaliculi (Bc) and contained moderate electron dense material. The hepatic cell (H) contained large vesicular nucleus (N), mitochondria (m) and moderate amount of glycogen (arrow) (Fig. 2 E).

Figure 2. Photomicrographs of liver sections from rats. (A) Liver section from control rat showing normal architecture of hepatocytes. (B) Liver section from rat received low doses of ZnONPs showing fat globule (f) and the mitochondria (m) swollen. The kupffer cell (kc) hypertrophied and contained large vacuoles (v). (C) Liver section from rat received high doses of ZnO showing hepatic cell (H) contained swollen mitochondria (M) with disintegration of its crests and infiltration of moderate amount of glycogen granules (arrow). The wall of the sinusoid contained fat storing cell containing numerous fat globule (F) and the sinusoidal lumen contained RBCs. (D) Liver section from rat received low doses of ZnO with vitamin C showing normal morphological appearance. (E) Liver section from rat received high doses of ZnO with vitamin C showing slight dilatation of the bile canaliculi (Bc).
DIscussion

Number of studies proved that NPs, including ZnO-NPs, may cause more inflammatory tissue damage than larger particles of the same materials at a same mass dose administration (23). The present study revealed that both two doses of ZnO-NPs (300 mg/kg/day and 1 g/kg/day for 2 weeks) induced liver damage demonstrated by the elevation of all serum liver function markers compared to control group, proving cellular leakage and loss of the functional integrity of cell membranes in liver (24). This finding reflects that liver is one of the target organs of ZnO-NPs toxicity. It has been reported that liver damage could be induced by excess oral zinc salt and zinc powder administration (24). Another report demonstrated that high dietary zinc caused liver toxicity of rats and resulted in inhibiting the activity of GOT in liver homogenate of rats (25). This study provided evidence that co-administration of vitamin C to ZnO-NPs intoxicated rats with either low or high doses, significantly reduced serum liver enzymes level compared with intoxicated rats. This may indicate that the used agents act as effective hepatoprotective against liver dysfunction caused by nanomaterials toxicity.

Our experimental data also demonstrated that treatment of rats with ZnO-NPs increased the level of hepatic lipid peroxidation. Moreover, SOD, CAT and GST activities were decreased in livers of ZnO-NPs treated rats. The reduced activity of SOD, CAT, GST could be due to enhanced lipid peroxidation or inactivation of the antioxidative enzymes. A number of evidence pointed to the potential involvement of oxidative stress via production of ROS in ZnO-NPs-induced organ toxicity (26, 27).

In other researches, it was demonstrated that oxidative stress induction is the major toxicological mechanism of ambient NPs. Large amounts of ROS could be generated even when only small amounts of ZnO-NPs are incorporated into cells (28). Upon entering the cell, particles may induce intracellular oxidative stress by disturbing the balance between oxidant and anti-oxidant processes. Excessive oxidative stress may also modify proteins, lipids, and nucleic acids, which further stimulates the anti-oxidant defense system or even leads to cell death. Meanwhile, with increased ROS production, NPs can cause DNA damage and increase gene expression of the death receptor (29). In addition, increased ROS induced by NPs in lysosomes can cause DNA point mutations or induce single- or double-strand breaks (30).

The intake of vitamin C immediately with ZnO-NPs ingestion presented in this study was beneficial in the prevention of this NPs induced liver peroxidation as well as increases the total antioxidant power of hepatocytes. These results are accordant with other investigations which reported that vitamin C supplementation protects against oxidative damage caused by different pathological conditions through inhibition of ROS production (31, 32). Hepatic GSH levels were significantly reduced after ZnO-NPs treatment, while treatment with vitamin C increased GSH content in liver cells when compared with ZnO-NPs intoxicated rats. This was attributed to the antioxidant properties of vitamin C.

Combining these results it can be said that vitamin C significantly prevented the alterations of the liver damages caused by ZnO-NPs probably by enhancing the activities of endogenous antioxidants.

Our results were supported by the histopathological examination of liver tissues, which clarified that animals received either low or high doses of ZnO-NPs showed severe congestion and dilation along the central vein. ZnO-NPs treated liver sections had massive inflammation and out flow of polymorphonuclear infiltrates with fatty degeneration. On the other hand, the inflammations in the livers of co-administration of vitamin C treated rats were less. These results also confirm the protective response of the vitamin C against the hepatic damage caused by ZnO-NPs.

Vitamin C acts and combats the toxicity of ZnO-NPs as it could manage the acute oxidative stress imposed by ZnO-NPs by decreasing ROS generation, lipid peroxidation and increasing the total antioxidant enzymes of hepatocytes.

conclusions

These findings proved that vitamin C supplementation as prophylactic agents may be beneficial against inflammatory responses induced liver injury and oxidative stress caused by toxic effect of zinc oxide nano-particles.

reFerences
