PROTECTIVE EFFECT OF N-ACETYLCYSTEIN Against Titanium Dioxide Nanoparticles MODULATED IMMUNE RESPONSES IN MALE ALBINO RATS

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ABSTRACT

The protective effects of N-acetylcysteine (NAC) against orally administered titanium dioxide nanoparticles (TiO2) for 3 months on male albino rats were examined. Adult male albino rats were given saline as a control group, TiO2 (1200 mg kg⁻¹ BW), NAC (100 mg kg⁻¹ BW) and co-treatment of NAC and TiO2 as a protective group for 3 months. Blood was assayed for serum changes in GPT, GOT, lipid profiles, cytokines and immunoglobulins profiles. Moreover, spleen was examined for alterations in cytokines expression and histopathology. Administration of TiO2 significantly increased serum levels of GPT, GOT and increased lipid profiles. Administration of NAC to TiO2 rats improved significant changes induced by TiO2 alone. There were an increase in IL-1β and IL-6 secretion in TiO2 administered rats which is normalized by NAC administration. TiO2 administration down regulated IL-8 and IL-10 secretion, while co-administration of rats by NAC together with TiO2 normalized that down regulation. Moreover, TiO2 induced toxicity in spleen that accompanied by a decrease in IgA, IgG and IgM that are normalized by NAC administration. Finally, TiO2 up-regulated IL-1β, IL-6 and TNF-β expression in spleen and NAC administration together with TiO2 normalized cytokines expression. In conclusion, present findings confirmed the protective effect of NAC on TiO2 induced alteration in immune responses in male albino rats.

Keywords: Titanium Dioxide, N-acetylcysteine, Spleen, Immune Responses

1. INTRODUCTION

Titanium dioxide nanoparticles (TiO2 NPs) are widely used in a number of applications: as an additive, including as a white pigment in paint, as a food colorant, in sunscreens and in cosmetic creams as well as in the environmental decontamination of air, water and soil by the destruction of pesticides (Fisher and Egerton, 2001; Kaida et al., 2004; Choi et al., 2006; Medina et al., 2007). With the rapid development of nanotechnology, the potential health hazards and environmental impact of manufactured TiO2 NPs have gained increasing attention. The smaller the particles of TiO2, the more reactivity, effectivity and toxicity (Oberdorster, 2006). It has been shown that the degree of cellular damage and oxidative stress of nanoparticles is related to the particle size and its chemical composition (Hoet et al., 2004). It has been demonstrated that oxidative stress is one of the most important toxicity mechanisms in lung, kidney, brain and spleen (Li et al., 2010; Sang et al., 2012).
Intraperitoneal injection of 100-nm TiO2 NPs in high doses (324-2592 mg kg\(^{-1}\) bw) caused significant accumulation of particles, mainly in the spleen, but also in the liver, kidney and lung (Chen et al., 2009). Upon exposure to TiO2, TiO2 nanoparticles can induce pathological lesions in the liver, spleen, kidneys and brain (Shi et al., 2013).

The N-Acetylcysteine (NAC) as an antioxidant and free radical scavenger is used extensively in conditional nutrient (Moschou et al., 2008). NAC acts as a cysteine donor and maintains or even increases the intracellular levels of glutathione, a tripeptide which protects cells from toxins such as free radicals. Reports have shown the ability of antioxidants such as NAC to reduce cell damage induced by cadmium (Smith et al., 2009), or dental composite (Stanislawski et al., 2000; 2003). Zafarullah et al. (2003) reported that cell growth and survival rate increased in response to ROS-induced injuries that lead to growth arrest and apoptosis. As known, NAC is an antioxidant with free radical-scavenging properties, acts as the source of cysteine, the precursor of de novo GSH synthesis (Van de Poll et al., 2006; Sadowska et al., 2007; Atkuri et al., 2007).

As known, cytokines are low molecular weight proteins produced by various cell types (Feghali and Wright, 1997). They are pharmacologically active, exhibiting both beneficial and pathologic effects on the target cells. Imbalanced expression of cytokines has been implicated in the progression of many diseases (Arend and Gabay, 2004). Their expression reflect the immune and health state of the body. Therefore, the present study examined the effect of TiO2 administration for 3 months on liver and lipid profiles, serum changes of immunoglobulins and cytokines and cytokines expression in spleen and possible protection by N-acetylcysteine.

2. MATERIALS AND METHODS

2.1. Materials and Animals

2.1.1. Titanium Dioxide (TiO2)

Anatase form, particle size (25-70 nm) was purchased from Sigma Aldrich chemical Co., USA. Gum acacia and NAC were obtained from El-Nasr Co., Cairo, Egypt. Cytokines primers were from MACROGEN, Seoul, Korea. Forty adult male albino rats weighting 150-200 g were obtained from the Animal House in Zagazig University. Faculty of Veterinary Medicine. Rats were give free access to food and water with 12h/12h dark light cycle. All animals were left 2 weeks for adaptation. They were housed in separate well-ventilated cages, under standard conditions.

2.2. Experimental Design

Forty rats were divided into 4 groups (10 rats per group). Group I (control group), were kept under standard conditions, fed on balanced diet for 3 months. Group II (NAC treated group) received 100 mg kg\(^{-1}\) body weight NAC once daily by gastric gavage for 3 months. Group III (TiO2 treated group): received 1200 mg kg\(^{-1}\) body weight TiO2 by gavage (1/10 LD 50) in 1 mL of 5% gum acacia solution as a solvent once daily for 3 months. The dose of TiO2 was used based on studies of Wang et al. (2007). Group IV (NAC+TiO2) received 100 mg kg\(^{-1}\) body weight NAC one hour before TiO2 by gavage once daily for 3 months. At the end of the experiments, the rats were sciarified. Blood and spleen were taken for biochemical assays, histopathology and gene expression. Serum was extracted from blood and kept -20°C till assayed.

2.3. Serum Biochemical and Cytokines Assays

Commercial available kits for lipid profiles such as total cholesterol, Triglycerides (TG), Low Density Lipoprotein-c (LDL), High Density Lipoproteins-c (HDL), GPT and GOT were purchased from Clini Lab, El Manial, Cairo, Egypt. They were measured spectrophotometrically based on the instruction supported by kits. For serum IL-1, IL-6, IL-8 and IL-10 measurements, ELISA kits were from Mabaret Al-Asafra, Alexandria, Egypt, The kits were imported from Wako Pure chemicals, Osaka, Japan.

2.4. Serum Immunoglobulins Assay

Blood was collected by tail vein incision at the end of experiment. IgG, IgA and IgM levels were measured in serum using a sandwich Enzyme-Linked Immunosorbent Assay (ELISA) by kits imported from Clini lab, Douki, Giza, Egypt.

2.5. Histopathological Examination

The spleen of male albino rats was collected from the different groups after 3 months. The samples were fixed in Bouin’s solution, then dehydrated in ascending grades of alcohols, cleared in xylene and embedded in paraffin. The samples were casted, then sliced into 5 µm in thickness and placed onto glass slides. The slides were stained by general and specific stains (Wilson and Gamble, 2008).
Table 1. PCR conditions for cytokines expression

<table>
<thead>
<tr>
<th>mRNA expression</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>PCR cycles and Annealing Temp.</th>
</tr>
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<tbody>
<tr>
<td>IL-1β (218 bp)</td>
<td>5'-ATGGCAACCGTACCTGAAACCA-3'</td>
<td>5'-GCTCGAAAAATGCCAAGGAAA-3'</td>
<td>30 cycles, 60°C 1 min</td>
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<tr>
<td>IL-6 (450bp)</td>
<td>5'-AGTTGCTCTTCTTGGGACTGATGT-3'</td>
<td>5'-ACCACATTTGTTGCTTCT-3'</td>
<td>30 cycles, 57°C 1 min</td>
</tr>
<tr>
<td>TNF-α (256 bp)</td>
<td>5'-CCACCACGGTCCTCCTGAC-3'</td>
<td>5'-GACCACATTTGTTGCTTCT-3'</td>
<td>30 cycles, 58°C 1 min</td>
</tr>
<tr>
<td>IL-10 (320 bp)</td>
<td>5'-GGACTGAAGACCAAAGG-3</td>
<td>5'-TCTCCAGGAATTCAAAATG-3'</td>
<td>30 cycles, 57°C 1 min</td>
</tr>
<tr>
<td>GAPDH (309 bp)</td>
<td>5'-AGATCCACAACGGATACATT-3</td>
<td>5'-TCCCTCAAGATTGTCAGCAA-3'</td>
<td>25 cycles, 52°C 1 min</td>
</tr>
</tbody>
</table>

2.6. RNA Extraction

For preparation of total RNA, spleen tissue (approximately 100 mg per sample) were collected from rats, flash frozen in liquid nitrogen and subsequently stored at -70°C in 1 mL Qiagen (QIAGEN Inc., Valencia, CA). Frozen samples were homogenized using a Polytron 300 D homogenizer (Brinkman Instruments, Westbury, NY). Then 0.3 mL chloroform were added to the homogenate. The mixtures were shaken for 30 sec followed by centrifugation at 4°C and 12,500 rpm for 20 min. The supernatant layer was transferred to a new set of tubes and an equal volume of isopropanol was added to the samples, shaken for 15 sec and centrifuged at 4°C and 12,500 rpm for 15 min. The RNA pellets were washed with 70% ethanol, briefly dried up then, dissolved in Diethylpyrocarbonate (DEPC) water. The prepared RNA integrity was checked by electrophoresis. RNA concentration and purity were determined spectrophotometrically at 260 nm. The ratio of the 260/280 optical density of all RNA samples was 1.7-1.9.

2.7. cDNA Synthesis

For synthesis of cDNA, mixture of 2 µg total RNA and 0.5 ng oligo dT primer in a total volume of 11 µL sterilized DEPC water was incubated in the PeX 0.5 thermal Cycler (Thermo Electronic Corporation, Milford, MA) at 65°C for 10 min for denaturation. Then, 4 µL of 5X RT-buffer, 2 µL of 10 mM dNTPs and 100 U Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase (SibEnzyme Ltd. Ak, Novosibirsk, Russia) were added and the total volume was completed up to 20 µL by DEPC water. The mixture was then re-incubated in the thermal Cycler at 37°C for 1 h, then at 90°C for 10 min to inactivate the enzyme.

2.8. Semi-Quantitative PCR Analysis

Specific primers for tested genes (Table 1) were designed using Oligo 4 computer program and synthesized by Macrogen (Macrogen Company, Gasa-dong, Geumcheon-gu, Korea). PCR was conducted in a final volume of 25 µL consisting of 1 µL cDNA, 1 µL of 10 picomolar (pM) of each primer (forward and reverse) and 12.5 µL PCR master mix (Promega Corporation, Madison, WI) the volume was brought up to 25 using sterilized, deionized water. PCR was carried out using a PeX 0.5 thermal Cycler with the cycle sequence at 94°C for 5 min one cycle, followed by 25 cycles each of which consisted of denaturation at 94°C for one minute, annealing at the specific temperature corresponding to each primer (Table 1) and extension at 72°C for one minute with additional final extension at 72°C for 5 min. As a reference, expression of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA was detected by using specific primers (Table 1). PCR products were electrophorized on 1% agarose gel (Bio Basic INC. Konrad Cres, Markham Ontario), stained with ethidium bromide in TBE (Tris-Borate-EDTA) buffer. PCR products were visualized under UV light and photographed using gel documentation system. The intensities of the bands were quantified densitometrically using NIH image program (http://rsb.info.nih.gov/nih-image/).

2.9. Statistical Analysis

Results are expressed as means ± S.E of 5 different rats per each group. Statistical analysis was done using ANOVA and Fischer’s post hoc test, with p<0.05 being considered as statistically significant.

3. RESULTS

3.1. Serum Changes in GPT, GOT and Lipid Profiles after TiO2 and NAC Administration in Male Albino Rats

The protective effect of NAC on TiO2 induced changes GPT and GOT levels was seen in Fig. 1. TiO2 administration increased GPT, GOT and all lipid profiles. Such changes was ameliorated compared to control and NAC groups when NAC was co-administered together with TiO2. Administration of NAC together with TiO2 prevented the changes in liver and lipid profiles confirming the NAC protective effect (Fig. 1). TiO2 administration decreased HDL levels and NAC normalized the decrease in HDL induced by TiO2 administration (Fig. 1).
Fig. 1. Serum changes in liver and lipid profiles after administration of either NAC or TiO2 alone or together in rats. Rats were administered TiO2 or NAC for 3 months and blood was assayed for changes in GPT, GOT, TG, cholesterol, LDL and HDL using commercial kits. Values are expressed as means ± SE for 5 different rats per group. *p<0.05 Vs control; #p<0.05 Vs TiO2 group.
Fig. 2. Serum changes in cytokines levels after administration of either NAC or TiO2 alone or together in rats. Rats were administered TiO2 or NAC for 3 months and blood was assayed for changes in IL-1β, IL-6, IL-8 and IL-10 levels using commercial ELISA kits. Values are expressed as means ± SE for 5 different rats per group. *p<0.05 Vs control; #p<0.05 Vs TiO2 group.
**Fig. 3.** Serum changes in immunoglobulins levels after administration of either NAC or TiO2 alone or together in rats. Rats were administered TiO2 or NAC for 3 months and blood was assayed for changes in IgA, IgG and IgM levels using commercial ELISA kits. Values are expressed as means ± SE for 5 different rats per group. *p<0.05 Vs control; #p<0.05 Vs TiO2 group.
**Fig. 4.** RT-PCR analysis of IL-β, IL-6, TNF-α and IL-10 expression administration of either NAC or TiO2 alone or together in rats. NAC and TiO2 were administered for 3 months as described in materials and methods. RNA was extracted and reverse transcribed (1 µg) and RT-PCR analysis was carried out for IL-β, IL-6, TNF-α and IL-10 genes. Densitometric analysis was carried for 3 different rats. *p<0.05 Vs control while # p<0.05 Vs TiO2 group.

**Fig. 5.** Photomicrograph of the male albino rat spleen of the control (a) and NAC (b) groups show; CT capsule (C) from which short trabecula (t) extend to the archtexture of the spleen. White pulp (W) consisted of aggregated lymphocytes around central vein (arrow C). Red pulp consisted of lymphocytes, blood cells and macrophages (* R). X100 H&E. The spleen of Tio-2 group (c) shows; lymphocytic proliferation around central vein (arrow), congestion in the blood vessels (*). The spleen of the treated group (d) showing; regression of the lymphocytic proliferation around central vein (c arrow) while the congestion still persist (star). X100 masson’s trichrome.
3.2. Serum Cytokines and Immunoglobulins Changes after TiO2 and NAC Administration in Male Albino Rats

Next, we examined the changes in IL-1β, IL-6, IL-8 and IL-10 after TiO2 administration for 3 months. As seen in Fig. 2, TiO2 administration stimulated inflammatory cytokines secretion (IL-1 and IL-6) compared to control and NAC administered rats. Co-administration of NAC with TiO2 in protective group normalized the changes in IL-1 and IL-6 secretion. Regarding the effect of TiO2 on chemo-attractant cytokine (IL-8) and regenerative cytokine (IL-10), Fig. 2 shows that TiO2 increased IL-8 and IL-10 secretion, administration of NAC with TiO2 normalized and stimulated their secretion confirming the immune-stimulatory effect of NAC during TiO2 toxicity. TiO2 administration decreased IgA, IgG and IgM secretion and NAC normalized their secretion when co-administered with TiO2 (Fig. 3).

3.3. Cytokines Expression in Spleen after TiO2 and NAC Administration in Male Albino Rats

Expression of IL-1β, IL-6 and TNF-α in spleen was increased after administration of TiO2 and their expression was normalized in rats administered NAC together with TiO2 (Fig. 4a-c). IL-10 expression was not altered in NAC and TiO2 administered rats, while co-administration of NAC with TiO2 stimulated IL-10 expression (Fig. 4d).

3.4. Histopathological Findings

The spleen of control and NAC treated groups was consisted of capsule of CT from which short trabecula extended into the architecture of the spleen. The spleen parenchyma consisted of white pulp and red pulp. The white pulp consisted of numerous lymphocytes aggregated around central vein. The red pulp consisted of numerous lymphocytes, blood cells and macrophages (Fig. 5a and b). The white pulp in the TiO2 treated group showed lymphocytic proliferation around the central vein, peri arterial lymphocytic sheath (PALS) with congestion in the blood vessels (Fig. 5c). The spleen of the NAC and TiO2 co-administered rats (protective group) showed decrease in the lymphocytic proliferation especially around the PALS, while the congestion still persist (Fig. 5d).

4. DISCUSSION

The increased biological activity of nanoparticles could be useful to penetrate cells for drug delivery. However, undesirable effects of nanoparticles could include generation of oxidative stress and/or impairment of antioxidant defense responses. Extra caution should be taken in the handling of higher dose TiO2 nanoparticles. In vivo studies showed that nanoparticles can be accumulated in the liver, kidney, spleen, lung, heart and brain, whereby generating various inflammatory responses (Brown et al., 2002). For instance, nanoparticles can promote enzymatic activities and the mRNA expression of cytokines during pro-inflammatory responses in mice (Muller et al., 2005) and that explain the increase in IL-1β, IL-6 and TNF-α secretion and expression.

Acute toxicity induced by various doses of TiO2 in mice (Chen et al., 2009) showed that accumulation of TiO2 NPs (80 nm, 100 nm, anatase) was high in spleen, liver, kidneys and lung in a decreasing manner. Some of the particles were excreted from the kidney (Chen et al., 2009). These results indicated that TiO2 NPs could be transported to and deposited in other tissues or organs (Shi et al., 2013). The inflammatory cytokines cascade may cause inflammatory cell chemotaxis and apoptosis, resulting in serious spleen injury (Linglan et al., 2009). The cellular damage and oxidative stress of nanoparticles in the spleenocytes were related to the particle size and chemical compositions of nanoparticles (Shi et al., 2013; Sycheva et al., 2011).

The increased level of hepatic enzymes (GPT and GOT) indicated liver damage or injury as reported by Wang et al. (2007); Chen et al. (2009) and Attia et al. (2013). Most of nanoparticles tend to accumulate in the liver (Zhou et al., 2006; Kamruzzaman et al., 2007; Sadauskas et al., 2007) as well as spleen and kidney (Xue et al., 2011). It has been shown that inhalation of toxic substances modulated the secretion and/or peripheral sensitivity of cytokines and considered to be a controller of various peripheral metabolic functions including the control of lipid profiles (Xue et al., 2011). Moreover, It has been confirmed that TiO2 toxicity increased liver profiles, total cholesterol and triglycerides (Duan et al., 2010) in accordance with our findings.

Several cytokines are produced by various cells and tissues in response to infection and/or toxicity as TiO2 such as IL-6, IL-8 and IL-10. IL-6 is an interleukin that acts as pro-inflammatory and anti-inflammatory cytokine. It is secreted by T cells and macrophages to stimulate immune response to trauma, especially burns or other tissue damage leading to inflammation. It increased during various diseases and metabolic disorders (Smolen and Maini, 2006). IL-8 is a chemokine produced by macrophages and other cell types such as epithelial cells. It is also synthesized by endothelial cells,
which store IL-8 in their storage vesicles. This chemokine is secreted by several cell types. It acts as a chemoattractant and is also a potent angiogenic factor (Baggiolini and Clark-Lewis, 1992). In our finding we reported that TiO2 decreased IL-8 secretion and NAC administration normalized it in a way to initiate chemoattractant mechanism. On the same line IL-10 a cytokine produced primarily by monocytes and to a lesser extent by lymphocytes, has pleiotropic effects in immunoregulation and inflammation. It down-regulates the expression of Th1 cytokines and acts as anti-inflammatory cytokine. Knockout studies in mice suggested that IL-10 acts as an essential immunoregulator (Pestka et al., 2001). IL-10 inhibits IL-1 and IL-6 production from macrophages (Fiorentino et al., 1991). IL-10 plays a critical role in shaping the development of the immune response by blocking class II major histocompatibility complex expression and decreasing pro-inflammatory cytokine expression (Donnelly et al., 1999; Moore et al., 2001). So, the increase in IL-10 secretion and expression is to control degree of toxicity induced by TiO2 and to counteract the increase in expression and secretion of IL-1 and 6. It has been shown that long term exposure to low dose of TiO2 NPs may cause spleen injury, resulting from alteration of inflammatory and apoptotic cytokines expression and reduction of immune capacity (Shi et al., 2013).

Regarding immunoglobulins, IgG and IgA constitute 75% of serum immunoglobulins in humans. IgG molecules are synthesized and secreted by plasma B cells. IgG can bind to many kinds of pathogens, for example viruses, bacteria and fungi and protects the body against them by agglutination and immobilization, complement activation (Mallery et al., 2010), opsonization for phagocytosis and neutralization of their toxins. Here, TiO2 decreased IgM, IgG and IgA secretion and the exact mechanism is not clear and further studies are needed to confirm such effect probably due to general toxicity induced by TiO2. One possible explanation is the involvement of cytokines. The decrease in antibody secretion is coincided with the decrease in IL-8 and IL-10, because it has been reported that T cells and B cells besides antibody production they can secrete various interleukins as IL-8 and IL-10 (Heinrich et al., 2003; Smolen and Maini, 2006). IL-10 has pleiotropic effects in immunoregulation and inflammation. It enhances B cell survival, proliferation and antibody production (Pestka et al., 2004). So the increase in IL-10 expression is counteracting mechanism to overcome the decrease IgG and IgA production and inflammation related immune responses and that support our findings. In this study, all cytokines were ameliorated by TiO2 administration and normalized by NAC co-administration with TiO2 confirming the protective effect of NAC on spleen and body immune response. In spleen, TiO2 nanoparticles administration caused an increase in proliferation of local macrophages (Xu et al., 2013). Moreover, The damages of hepatic enzymes occurred by nanosized-TiO2, is evidenced by the increased activities of GOT and GPT. Hepatic enzymes increased during liver dysfunction indicate severe inflammation or liver injury (Wang et al., 2007; Chen et al., 2009). As known, the catalytic properties of nanosized-TiO2 lead to generation of reactive oxygen species (ROS). The over production of ROS would break down the balance of the oxidative/antioxidative system in the liver, resulting in lipid peroxidation via ROS production and hepatocytes apoptosis, which may be closely related to the reduction of anti-oxidative enzymes (Jeon et al., 2013).

5. CONCLUSION

N-acetylcysteine has a protective effect on biohazards induced by TiO2 through modulation of cytokines expression and secretion. N-acetylcysteine normalized the increase in liver and lipid profiles induced by TiO2 biohazards.

6. AKNOWLEDGMENT

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