

Hepatoprotective Effect of N-Acetylcystiene on the Toxic Hazards of titanium Dioxide Nanoparticles

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ABSTRACT

The present study was directed to evaluate the toxic effects of orally administered titanium dioxide nanoparticles (TiO₂) on liver of male albino rats and to evaluate the ameliorative effects of N-acetylcysteine (NAC). Forty adult male albino rats were divided into 4 groups; control group, NAC group, TiO₂ group and TiO₂/ NAC group. Rats were administered either TiO₂ (1200 mg kg⁻¹ BW) or NAC (100 mg kg⁻¹ BW) alone or together for 9 months. Blood was taken to evaluate serum changes in GPT, GOT and MDA levels. Liver tissues were examined for changes in MDA, GSH and changes in liver histopathology. Administration of TiO₂ increased serum GPT, GOT and decreased MDA levels. Co-treatment of rats with NAC and TiO₂ improved such significant changes induced by TiO₂ alone. Moreover, significant time dependent increase in MDA and decrease in GSH levels in liver tissues were recorded. Liver histopathology showed vacuolar, hydropic degeneration and cell death of some hepatic cells. In conclusion, results confirmed the protective effect of NAC in amelioration of the biohazard effects induced by TiO₂ in rats.

Keywords: Titanium Dioxide, N-Acetylcysteine, Liver, Rat, Hepatoprotective

1. INTRODUCTION

Nanosized-TiO₂ is used in widespread applications such as cosmetics, food colorant and white pigment as well as in the environmental decontamination of air, soil and water. Every person has been exposed to nano size TiO₂ as we inhale them with every breath and consume them with every drink (Medina *et al.*, 2007). While the small size of particles is what makes nanotechnology so

useful in medicine and industry, it is also one of the main factors that might make them potentially dangerous to human health. The smaller the particles, the more reactive and toxic are their effects (Nighswonger, 1999; Oberdrster, 2006). The cellular damage and oxidative stress of nanoparticles in the liver cells were related to the particle size and chemical compositions of nanoparticles. Nanoparticles that enter the rat liver induce oxidative stress locally (Hoet *et al.*, 2004). The

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toxicity of nanosized-TiO₂ in mouse liver was demonstrated by the enhanced activities of liver damage-related enzymes, alterations of defense-related enzymes, accumulation of nanoparticles as well as histopathological changes. Recent evidence, however, suggest that nanosized-TiO₂ can cause inflammatory response in airways of mouse and mice. The hepatocyte apoptosis and DNA cleavage of the mouse liver occurred by nanosized-TiO₂ (Jeon *et al.*, 2013). Aggregated TiO₂ particles were clearly seen in the sinusoids of liver and were phagocytized by Kupffer cells that inhabit liver sinusoids (Okaya *et al.*, 2012). Administration of nanoparticles resulted in depletion of reduced glutathione and oxidized glutathione, as well as these nanoparticles were distributed in the hepatocytes. The literature on rodent models *in vivo* strongly indicates that most nanoparticles tend to accumulate in the liver (Zhou *et al.*, 2006; Kamruzzaman *et al.*, 2007; Sadauskas *et al.*, 2007). They have been shown to be retained by the liver leading to tissue injury in mice (Wang *et al.*, 2007). In acute and sub acute toxicity study, Wang *et al.* (2007) reported that mice treated with TiO₂NP showed pathological changes in the liver in the form of the large-area of hydropic degeneration with fatty change, hepatocellular necrosis and apoptosis, with hepatic fibrosis around the central vein. This study evaluated the role of NAC in TiO₂ induced oxidative stress and liver damage in male albino rats. N-Acetylcysteine (NAC) is a thiol-containing amino acid with free radical-scavenging properties, powerful neuro-protective and anti-oxidant actions (Atkuri *et al.*, 2007).

2. MATERIALS AND METHODS

2.1. Materials

Titanium Dioxide (TiO₂): Anatase form, particle size (25-70 nm), surface area (20-25) m/g, purity 99.9 was purchased from Sigma Aldrich chemical Co., Germany. Gum acacia and N-acetylcysteine were obtained from El-Nasr Co., Egypt.

2.2. Animals

In this experiment, 40 adult male albino rats weighting 150-200 gm were obtained from the Animal House in Taif Univ. Faculty of Science. All animals were subjected to 14 days of passive preliminaries in order to adapt themselves to their new environment

and to ascertain their physical wellbeing. They were housed in separate well-ventilated cages, under standard conditions, with free access to standard diet and water. The experiment was performed in accordance with the guidance of ethical committee for research on laboratory animals of (ILAR, 1996).

2.3. Experimental Design

Forty rats were divided into 4 groups (10 rats per group). Group I (control group), the rat were kept under standard condition, fed on balanced diet for 9 months. Group II (N-Acetylcystiene treated group) the rat received 100 mg kg⁻¹ body weight N-Acetylcystiene once daily by gavage for 9 months. Group III (titanium dioxide 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 9 months. The LD50 of TiO₂ for mouse is larger than 12,000 mg kg⁻¹ body weight after oral administration (Wang *et al.*, 2007). Group IV (titanium dioxide and N-Acetylcystiene treated group) received (100 mg kg⁻¹ body weight N-acetylcysteine1h before 1200 mg kg⁻¹ body weight TiO₂) by gavage once daily for 9 months. At the end of the experiments, the rats were scarified. Blood and liver were taken for biochemical assays and histopathology.

2.4. Serum and Tissue Biochemical Analysis

Commercial available kits for GPT, GOT and MDA were purchased from Clini Lab, El Manial, Cairo. They were measured spectrophotometrically based on the instruction supported by kits. For tissue measurements of MDA and GSH, liver tissues was homogenized with 10 times (w/v) ice-cold 0.1 M phosphate buffer pH (7.4). MDA estimation was based on the method of Ohkawa *et al.* (1979). In brief, one milliliter of homogenate was incubated at 37°C for 10 min. One milliliter of 10% (w/v) chilled Trichloroacetic Acid (TCA) was added to it and centrifuged at 2500 rpm for 15 minutes at room temperature. One milliliter of 0.67% Thiobarbituric Acid (TBA) was added to 1 mL of supernatant and kept in a boiling water bath for 10-15 min. The tubes were cooled under tap water and followed by an addition of 1 mL of distilled water. Absorbance was recorded at 530 nm and the results were expressed as nmol MDA/g tissue. For reduced glutathione (GSH), it is estimated by the method described by (Ellman, 1959) One milliliter of 10% crude homogenate was mixed with 1 mL of 5% TCA (w/v). The mixture was

allowed to stand for 30 min and centrifuged at 2500 rpm for 15 min; 0.5 mL of the supernatant was taken and 2.5 mL of 5'5'-Dithionitrobenzoic acid (DTNB) was added, mixed thoroughly and the absorbance was recorded at 412 nm. The results were expressed as nmol/g tissue.

2.5. Histopathological Examination

The liver of male albino rats was collected from the different groups after 9 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, 2002).

2.6. Immunohistochemical Study

Immunohistochemical reaction was carried using avidin biotin peroxidase system. The primary antibody used was a rabbit polyclonal antibody (Sigma Laboratories). Universal kit used avidin biotin peroxidase system produced by Nova Castra Laboratories Ltd, UK. The same method was applied to prepare negative control sections but the primary antibody was not added. Mayer's hematoxilin was added as counter stain. Tonsil was used as positive control tissue (Kiernan, 2008).

2.7. Statistical Analysis

Data were represented as means ± SE. The differences were compared for statistical significance by ANOVA and post hoc Turkey's tests. Difference was considered significant at $p<0.05$. The statistical analysis was performed using Epi-Info version 6.1 (Dean *et al.*, 2000).

3. RESULTS

3.1. Serum Changes in GPT, GOT, MDA in Blood

The protective effect of NAC on TiO₂ induced changes GPT, GOT and MDA levels, serum was assayed for changes in GPT, GOT and MDA. Serum GOT, GPT were increased in TiO₂ administered rats. While MDA levels were decreased in TiO₂ administered group relative to control. Overall results as in **Table 1**, show that TiO₂ increased GPT, GOT and decreased MDA levels. Moreover, co-treatment with NAC together with TiO₂ for 9 months normalized the increase in GPT, GOT

and the decrease in MDA levels confirming the protective effect of NAC (**Table 1**).

3.2. Liver Changes in MDA and GSH Contents after Exposure to TiO₂ in Male Rats

Next, we tested the tissue changes in MDA and GSH after 9 months of TiO₂ exposure. As seen in **Table 2**, no significant difference between control and NAC administered rats. Administration of rats by TiO₂ induced alteration in both oxidative stress and antioxidant activities. TiO₂ induced significant increase in MDA and decrease in GSH activity in liver. Co-administration of NAC with TiO₂ administered rats normalized the alteration shown in TiO₂ administered group.

3.3. Histopathological Findings

The histological structure of control and NAC groups revealed the normal structure of the liver. The liver consisted of central vein surrounded by hepatic cords. Each hepatic cords consists of hepatocytes with centrally basophilic nuclei and eosinophilic cytoplasm (**Fig. 1**). The liver cells showed positive immunostaining with BCL-2 (**Fig. 2**).

The histopathological examination of the liver of titanium dioxide group revealed, vacuolar and hydropic degeneration in the liver cells (**Fig. 3**). The portal areas showed fibrosis and congestion of the blood vessels (**Fig. 4**). Some cells of the hepatocytes showed necrosis (**Fig. 5**). The hepatocytes showed faint positive immunostaining with BCL-2 stains (**Fig. 6**).

The histological structure of treated group (Tio-2+NAC). The liver showed disappearance most of the degenerative changes induced by Tio-2, while some dilated blood vessels still persist (**Fig. 7**). Some hepatocytes take positive immunostaining for BCL-2 (**Fig. 8**).

Table 1. Serum changes in GPT, GOT and MDA in rats

	GPT	GOT	MDA
Control	38.3±2.1	21.±0.95	85.5±4.5
N-Acetylcysteine	44.5±2.1	25.45±2.6	96.2±10.1
TiO ₂	73.5±1.16*	78.03±2.08*	60.8±10.9*
N-Acetylcysteine+ TiO ₂	48.6±1.6#	47.1±2.5#	86.2±8.15#

Male rats were exposed to TiO₂ for 9 months and protected by N-acetylcysteine together with TiO₂ for 9 months. Blood was assayed for changes in GPT, GOT and MDA. Data were represented as means ± SE for 10 different rats per group. * $p<0.05$ Vs control and # $p<0.05$ vs. TiO₂

Table 2. Hepatic changes in MDA and GSH contents (nmol/g tissue) after TiO₂ exposure in male rats

N/ groups Parameters	GroupI Negative control	GroupII NAC treated	Group III (TiO ₂ treated)	Group IV (TiO ₂ /NAC)	F	P
Duration	9 months	9 months	9 months	9 Months		
MDA nmol/gm tissue	76.7±3.47	72.3±3.25	160±13.9 ^{ac}	88.6±2.91 ^b	145.8	<0.001
GSH NmoL/gm tissue	105±3.93	109±2.72	29.6±3.92 ^{ac}	95.7±6.5 ^{ab}	317.6	<0.001

Male rats were exposed to TiO₂ for 9 months and protected by N-acetylcysteine together with TiO₂ for 9 months. Liver were assayed for changes in MDA and GSH as in materials and methods. Data were represented as means ± SE for 10 different rats per group. Letter a is significant at p<0.05 Vs. control; b is significant at p<0.05 Vs. TiO₂ and c is significant at p<0.05 vs. TiO₂ and NAC

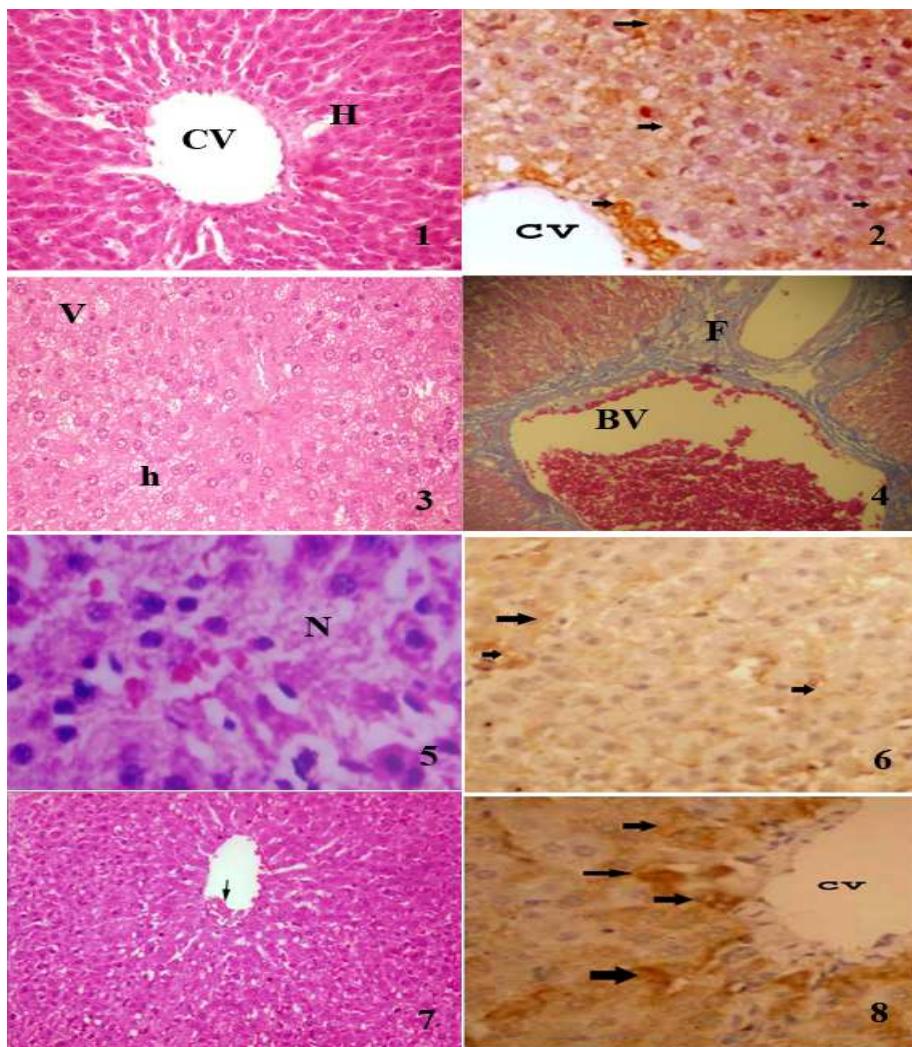


Fig. 1. Photomicrograph of the liver of the control and (NAC) group showing, Central Vein (CV) and Hepatic cords (H) (1) H&E X10. Liver cells stained positive by Immunostains (Arrows) (2). BCL-2 stains X20. Liver of the TiO₂ group showing vacular (V) and hydroptic degeneration (h) (3). H&E X10. Fibrosis in the triad area (F) and congestion in the Blood Vessels (BV). (4). Masson trichrome X40. The hepatic cells showed necrosis (arrow) (5) H&E X40. Negative immunostains with BCL-2 of the liver tissue while few cells take faint positive reaction (Arrows) (6) BCL-2 X10. Liver of the treated group (TiO₂+NAC) showing regeneration of the hepatic tissues with some dilatation in the central vein (arrow) (7) H&E X10. Numerous hepatic cells take positive immunostains (Arrow) (8) BCL-2 X10

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 therefore be taken in the handling of higher dose nano-TiO₂. Many *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, nano particles can promote enzymatic activities and the mRNA expression of cytokines during pro inflammatory responses in mice (Muller *et al.*, 2005) and in human dermal fibroblasts and human lung epithelial cells (Sayes *et al.*, 2006).

TiO₂-induced liver toxicity and inflammatory responses in liver of mice. Both are complicated multifactorial disease processes. The inflammatory cytokines cascade may cause inflammatory cell chemotaxis and apoptosis, resulting in serious liver injury (Linglan *et al.*, 2009). The cellular damage and oxidative stress of nanoparticles in the liver cells were related to the particle size and chemical compositions of nanoparticles. Nanoparticles that enter the rat liver induced oxidative stress locally (Hoet *et al.*, 2004).

The increased level of hepatic enzymes (GPT and GOT) indicate liver damage or injury as supported by the work of (Wang *et al.*, 2007; Chen *et al.*, 2009). Most nanoparticles tend to accumulate in the liver (Zhou *et al.*, 2006; Kamruzzaman *et al.*, 2007; Sadauskas *et al.*, 2007). They have been shown to be retained by the liver leading to tissue injury in mice (Wang *et al.*, 2007).

The damages of liver function occurred by nanosized-TiO₂, as evidenced by the increased activities of GOT, GPT and ALP. Hepatic enzymes increase during liver dysfunction indicating severe inflammation or liver injury (Wang *et al.*, 2007; Chen *et al.*, 2009). It has been speculated that part of the Reactive Oxygen Species (ROS) generation might be due to the catalytic properties of nanosized-TiO₂. The overproduction of ROS would break down the balance of the oxidative/antioxidative system in the liver, resulting in the lipid peroxidation via ROS and MDA production and the hepatocyte apoptosis, which may be closely related to the reduction of antioxidative enzymes (Jeon *et al.*, 2013).

The results showed time dependent significant generation of oxidative stress in the liver. This was evident by an increased Malondialdehyde (MDA), the

end product of lipid peroxidation and decreased GSH level in the liver of rat treated with TiO₂ NP when compared to the liver of control rat. These findings were coincided with (Long *et al.*, 2007; Wang *et al.*, 2008; Ma *et al.*, 2010) who stated that TiO₂NP have more biological activities to produce ROS.

The results of the present study revealed time dependent depletion in GSH level of the liver of TiO₂NP rat. Pompella *et al.* (2003) stated that GSH is an endogenous, peptidal, antioxidant, which prevents damage to the cellular components by ROS and peroxides. In addition to working as a direct free-radical scavenger, GSH also functions as a substrate for GPx and GST.

TiO₂ can increase coefficients of the liver and its significant accumulation in the mouse liver can induce histopathological changes of liver, including congestion of vasculum, prominent vasodilatation, wide-bound basophilia and focal ischemia, hepatocyte tumescent mitochondria, vacuolization and apoptosis, thus leading to the damage of liver function. Wang *et al.* (2007) observed that the hydropic degeneration around the central vein was prominent and the spotty necrosis of hepatocytes in the liver tissue hepatocyte injury and apoptosis. These findings augmented our results in liver of male albino rats.

These findings were in accordance with (Zafarullah *et al.*, 2003). NAC had promoted the cell growth and survival in response to ROS-induced injuries which normally lead to growth arrest and apoptosis. Van De Poll *et al.* (2006); Sadowska *et al.* (2007) and Atkuri *et al.* (2007) stated that NAC is an antioxidant with free radical-scavenging properties. Additionally, NAC is a source of cysteine, the precursor of de novo GSH synthesis. So, administration of NAC replenishes intracellular GSH levels. On the same context, (Xue *et al.*, 2011) stated that that NAC strongly inhibited ROS production in TiO₂ treated cells and suppressed TiO₂ induced apoptosis.

Concomitant use of NAC along with TiO₂ significantly restored the values of MDA& GSH. Light microscopic examination of liver sections revealed regression of the degenerative changes of the hepatic cells. Immunostaining by BCL-2 showed positive reaction of hepatic cells.

5. CONCLUSION

At the end, it can be concluded that the TiO₂ has health hazard on the liver as, it affected the architecture of the hepatic cords. The addition of NAC has

hepatoprotective and ameliorative effect on the biohazard caused by TiO₂.

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