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RESEARCH ARTICLE

Alpha Lipoic Acid (ALA) Alleviates Hepatocytes Toxicity of Titanium Dioxide Nanoparticles in Rats

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Abstract

BACKGROUND: Titanium dioxide nanoparticles (TiO_2 NPs) uptake may primarily cause adverse effects by inducing oxidative stress, resulting in cell damage, genotoxicity, inflammation, and immune response. To date, there are limited studies investigating the adverse effect of TiO_2 NPs on liver health and no studies found a naturally occurring compound able to ameliorate such effect. Thus, this study investigated alpha lipoic acid (ALA) potential for reversing the biochemical and histopathological changes that TiO_2 NPs exposure causes in rat liver.

METHODS: Thirty adult male albino rats were divided into: control rats received distilled water, control rats treated with 50 mg/kg ALA, rats intoxicated with TiO_2 NPs, and TiO_2 NPs-intoxicated rats treated 50 mg/kg ALA. Rats were sacrificed before blood samples were collected to assess the liver function using parameters of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, albumin and total protein. Liver tissue homogenates were prepared to assess hepatic antioxidant and oxidative stress using parameters of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and malondialdehyde (MDA). Liver tissue sections were used for histopathological analysis and caspase-3 immunohistochemical analysis.

RESULTS: TiO_2 NPs produced deleterious effects on rat liver tissue, as confirmed through biochemical results, caspase-3 immunohistochemistry, and histological alterations. TiO_2 NPs intoxication induced hepatocyte vacuolation, blood vessels congestion, biliary proliferation, apoptosis, and fibrosis. However, ALA treatment of TiO_2 NPs-intoxicated rats significantly alleviated deleterious impact on the liver.

CONCLUSION: Administration of 600 mg/kg TiO_2 NPs to rats resulted in hepatic degenerative lesions, depletion of GSH, oxidative stress, and apoptosis. However, these changes were mitigated by ALA administration. Therefore, ALA offers protection against deleterious effects of TiO_2 NPs intoxication.

KEYWORDS: titanium dioxide nanoparticles, TiO_2 NPs, hepatotoxicity, alpha-lipoic acid (ALA), rat

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Introduction

Titanium dioxide nanoparticles (TiO_2 NPs) are utilized in the industrial production of many products used regularly by human, increasing the chance of human exposure to these nanoparticles. Thus, it is necessary to determine the possibility of its toxic impact on human. Several studies have demonstrated the deleterious effect of TiO_2 NPs. *In vivo* studies have shown the toxic impacts of TiO_2 NPs on the lungs, brain, liver, kidneys, embryo and neurons. Indeed, it is becoming discussions that daily oral intake of TiO_2 NPs might result in the enhanced likelihood of chronic neoplasm and inflamed intestines. Some toxic effects of TiO_2 NPs

on organs of animal models have also been extensively documented.(1,2)

The toxic effect of TiO_2 NPs may occur through production of reactive oxygen species (ROS) and oxidative insult, as well as genetic lesions via interaction at the genetic level. Nanoparticles are well-known for its ability to cause redox imbalance in cells, leading to oxidative stress. An antioxidant is a substance that helps to counteract the deleterious effect of free radical-induced oxidative stress. TiO_2 toxicity is associated with production of reactive oxygen species, thereby causing lipid peroxidation and alteration in the enzymatic antioxidant defense. Several herbs exert antioxidant and free radical-scavenging effects because of their flavonoid contents. Alpha lipoic acid (ALA) possesses similar properties (3), since it is a naturally co-enzyme occurring in multi-enzyme complexes and exists in mitochondria for essential oxidative metabolism. ALA enhances hepatoprotective effect by participation in the breaking down of free radicals, the regeneration of other antioxidants, the repair of oxidatively damaged proteins, and the prevention of lipid peroxidation.(4,5) ALA is mostly found in meat and liver, but only trace amounts may be present in plant-derived foods. Generally, the levels of ALA in animal and plant-derived foods are low. Thus, there is need to boost available ALA by supplementation.(6)

In the current study, a number of markers have been chosen to be examined in order to evaluate the activity of ALA as hepatoprotective agent. Albumin, an important protein produced by hepatocytes, and total bilirubin can provide insight about liver function. Several other markers that assess the oxidative stress and liver function were also examined, including aspartate transaminase (AST), alanine transaminase (ALT), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione (GSH).(7)

Limited number of studies have investigated TiO_2-induced toxicity following oral administration. However, no studies have dealt with TiO_2 NPs induced hepatotoxicity and effect of ALA in alleviating such toxicity. Therefore, the aim of this study was to evaluate the adverse impact of low-level TiO_2 NPs on rat's hepatocytes, and to assess the possible modulating effects of ALA on liver toxicity induced by TiO_2 NPs.

Methods

Study Design and Animal Model

A total of 30 albino rats weighing between 120 and 150 g were included in this study. The rats were fed adaptively on pellet feed under standard laboratory conditions for 7 days, with unlimited access to water. Prior to commencement of the research, the rats were subjected to overnight fast. The rats were randomly divided into four groups: group I was the negative control group consisting of 5 untreated rats who received distilled water; group II was the positive control group consisting of 5 rats treated with 50 mg/kg ALA for 1 month (8); group III was the TiO_2 NPs-intoxicated group consisting of 10 rats that were given TiO_2 NPs at a daily dose of 600 mg/kg (Sigma-Aldrich Chemical Company, Amman, Jordan) (9); and group IV consisting of 10 TiO_2 NPs-intoxicated rats treated with 50 mg/kg ALA for 1 month. ALA was solubilized in distilled H_2O prior to oral administration at a level of 50 mg/kg.(10) Ethical approval for protocol of the study was obtained from The Institutional Ethics Committee, Faculty of Medicine, University of Mutah, Jordan (No. 1052023, dated April 13, 2022).

Assessment of Hepatotoxicity

Following the overnight fast, 2-3 mL of subjects' retro-orbital blood was obtained from each rat and centrifuged at 3000 g for 10 min at room temperature. After separation of the serum, the samples were used to estimate the liver markers and for biochemical tests. The biochemical analysis for ALT, AST, total bilirubin, albumin and total protein was performed by Roche Modular Chemistry Analyser (Roche, Rotkreuz, Switzerland) following the standard procedures.

Measurement of The Oxidative Stress Markers and Antioxidants

All rats were anesthetized with ether and sacrificed. The livers were then excised, rinsed in ice-cold saline, and subjected to gravimetry. Liver tissue specimens were washed in a solution of ice-cold 0.9 % NaCl and homogenized in 9% ice-cold phosphate-buffered saline of pH 7.5. The homogenate was centrifuged at 3000 rpm for about 15 minutes, and the supernatant was collected and kept at -80ºC prior to measurement of MDA, GSH, CAT and SOD. The measurement of MDA was based on the reaction of MDA with thiobarbituric and trichloroacetic acids, measurement of GSH was using reduction reaction of glutathione and 2-nitrobenzoic acid, measurement of CAT was using H_2O_2 reaction with 4-aminophenazone and 3,5-Dichloro-2-hydroxybenzene sulfonic acid, meanwhile the measurement of SOD was based on SOD inhibition of reduction reaction of nitro blue tetrazolium mediated by phenazinemethosulphate.(10)

Histological Analysis

Specimens of the liver tissues were fixed in 10% formalin. Liver tissues were removed from the fixing fluid and subjected to clearing and dehydration prior to paraffinization, sectioning, hematoxylin and eosin (H&E) staining, and light microscopy. Moreover, the paraffin-embedded blocks were immunohistochemically analyzed for the detection of caspase-3 cell markers in liver tissues. Another liver sample was stained in 3% glutaraldehyde and subjected to transmission electron microscopic analysis.(11)

Statistical Analysis

All statistical analyses were performed by SSPS 17 (IBM Corporation, Armonk, NY, USA). Data were expressed as mean±SD. The significant differences were analyzed using one-way analysis of variance (ANOVA), followed by LSD post-Hoc test. Values of $p<0.05$ indicated the statistical significance.

Results

Effect of ALA on TiO_2-induced Hepatotoxicity

The serum levels of ALT, AST, total bilirubin, albumin and total protein were used to assess liver function. As shown in Figure 1, the administration of TiO_2 NPs impaired (group III) liver function, as indicated by significant increase in serum levels of ALT, AST, and total bilirubin, but showed significantly lower in albumin and total protein levels, as compared to negative control (group I) ($p<0.05$). Interestingly, ALA treatment to TiO_2 NPs-intoxicated rats (group IV) significantly restored levels of these parameters to almost normal ranges (group I), as compared to TiO_2 NPs-intoxicated rats (group III) ($p<0.05$). It was also worth mentioning that ALA treatment for positive control rats (group II) did not significantly alter the levels of these parameters, as compared to negative control (group I).

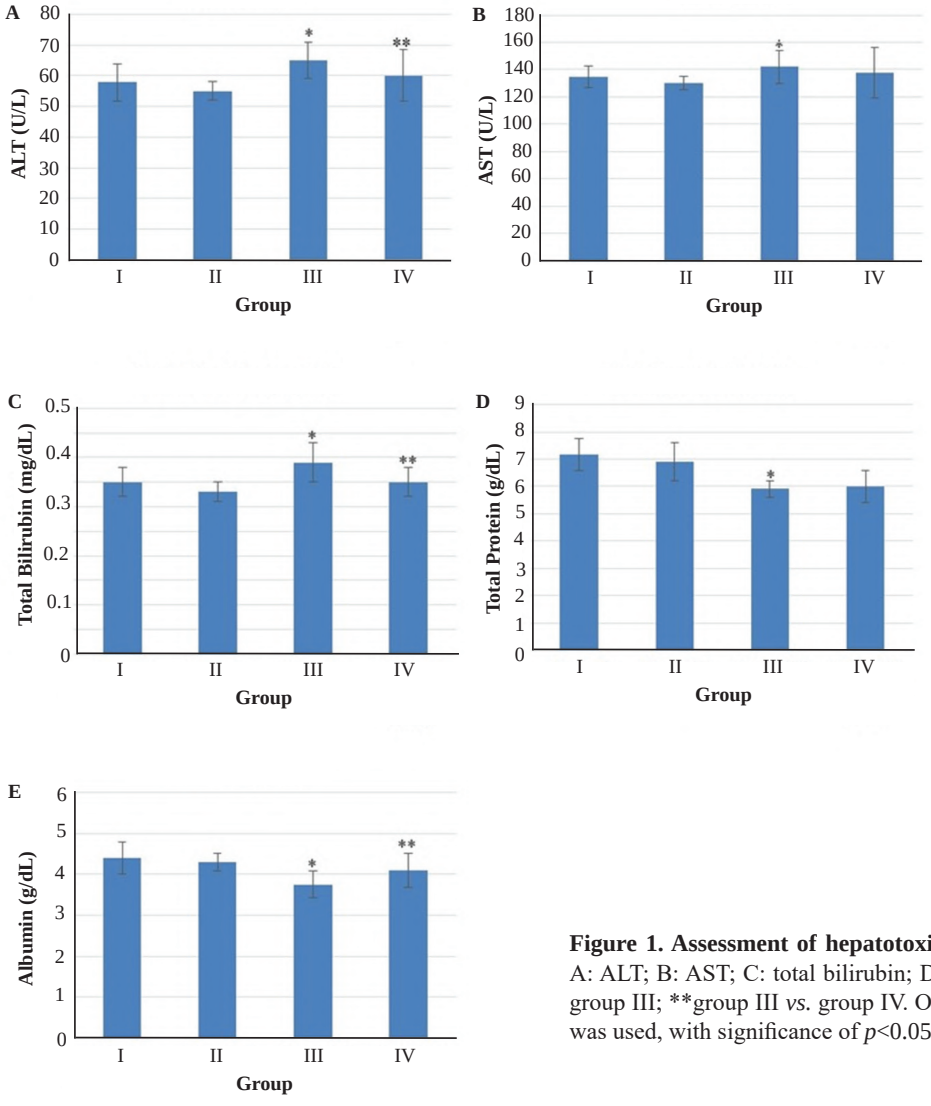

Figure 1. Assessment of hepatotoxicity parameters in treatment groups. A: ALT; B: AST; C: total bilirubin; D: total protein; E: albumin. *group I *vs.* group III; **group III *vs.* group IV. One-way ANOVA with LSD post-hoc test was used, with significance of $p<0.05$.

Effect of ALA Treatment on Hepatic Antioxidant and Oxidative Stress Markers

As shown in Figure 2, ALA treatment to positive control rats (group II) did not induce any significant change to the levels of SOD, CAT, GSH, and MDA. However, TiO_2 NPs-intoxication (group III) resulted in significant reduction in levels of SOD, CAT and GSH, as well as high levels of MDA in rats, as compared to the negative control (group I) ($p<0.05$). Surprisingly, ALA treatment to TiO_2 NPs-intoxicated rats demonstrated a significant effect on restoring the levels of these parameters to normal level, as compared to TiO_2 NPs-intoxicated rats (group III).

Histopathological Assessment of Hepatotoxicity Induction and Recovery by ALA Treatment

H&E results showed that liver specimen from negative control (group I) revealed a normal appearance characterized by radially arranged anastomotic hepatocyte cords radiating from the central veins, with blood sinusoids that were lined by Kupffer cells present in-between, as well as portal tract containing branches of portal vein, hepatic artery, and bile duct (Figure 3A). Similarly, liver specimens from positive control (group II) showed normal hepatic architecture (Figure 3B), as seen in liver specimen from negative control (group I). The TiO_2 NPs-intoxication (group III) resulted in deteriorations of hepatic architecture, as evidenced by vacuolation of hepatocytes, prominent Kupffer cells, congestion of central vein and blood vessels in portal tracts associated with biliary proliferation, as well as portal infiltration of inflammatory cells (Figure 3C, 3D and 3E). Importantly, ALA treatment to TiO_2 NPs-intoxicated rats (group IV) showed marked recovery and restoration of almost normal hepatic appearance and architecture, with mild congestion and mild biliary proliferation (Figure 3F, 3G and 3H).

The ultrastructural results showed that liver cells from negative control (group I) revealed healthy and normal ultrastructure seen as normal euchromatic nuclei of hepatocytes, numerous rough endoplasmic reticulum, and many mitochondria (Figure 4A). Moreover, normal healthy liver cells were also seen in liver section treated with ALA alone (group II) (Figure 4B). The TiO_2 NPs-intoxication (group III) resulted in an evident damage and injury of the liver cells, destruction of mitochondria and rough endoplasmic reticulum, with raised cytoplasmic fat droplets, cytoplasmic vacuoles, biliary proliferation, congestion, apoptotic cells, and fibrosis (Figure 4C, 4D, 4E and 4F). Interestingly, ALA treatment to TiO_2 NPs-intoxicated rats (group IV) revealed marked recovery and restoration of healthy liver cell ultrastructure, with the mitochondria appearing similar to those of negative control (group I) (Figure 4G, GH and 4I).

Effect of ALA on TiO_2 NPs-induced Apoptosis in Liver

Immunohistochemical study showed normal healthy liver from negative control (group I) demonstrated negative

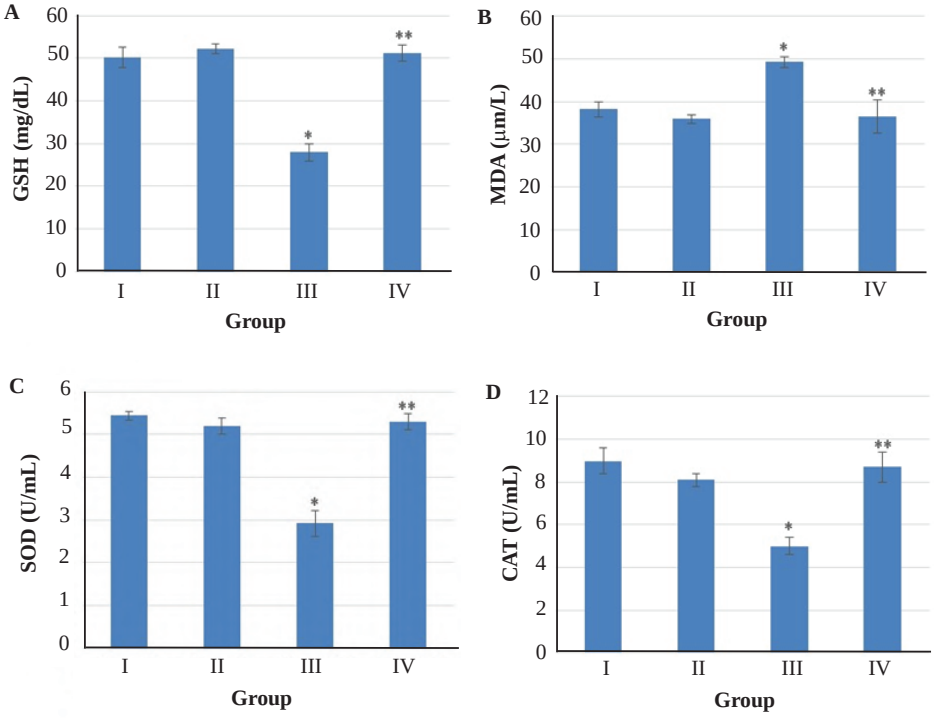

Figure 2. Measurement of hepatic antioxidant and oxidative stress markers in treatment groups. A: GSH; B: MDA; C: SOD; D: CAT. *group I *vs.* group III; **group III *vs.* group IV. One-way ANOVA with LSD post-hoc test was used, with significance of $p<0.05$.

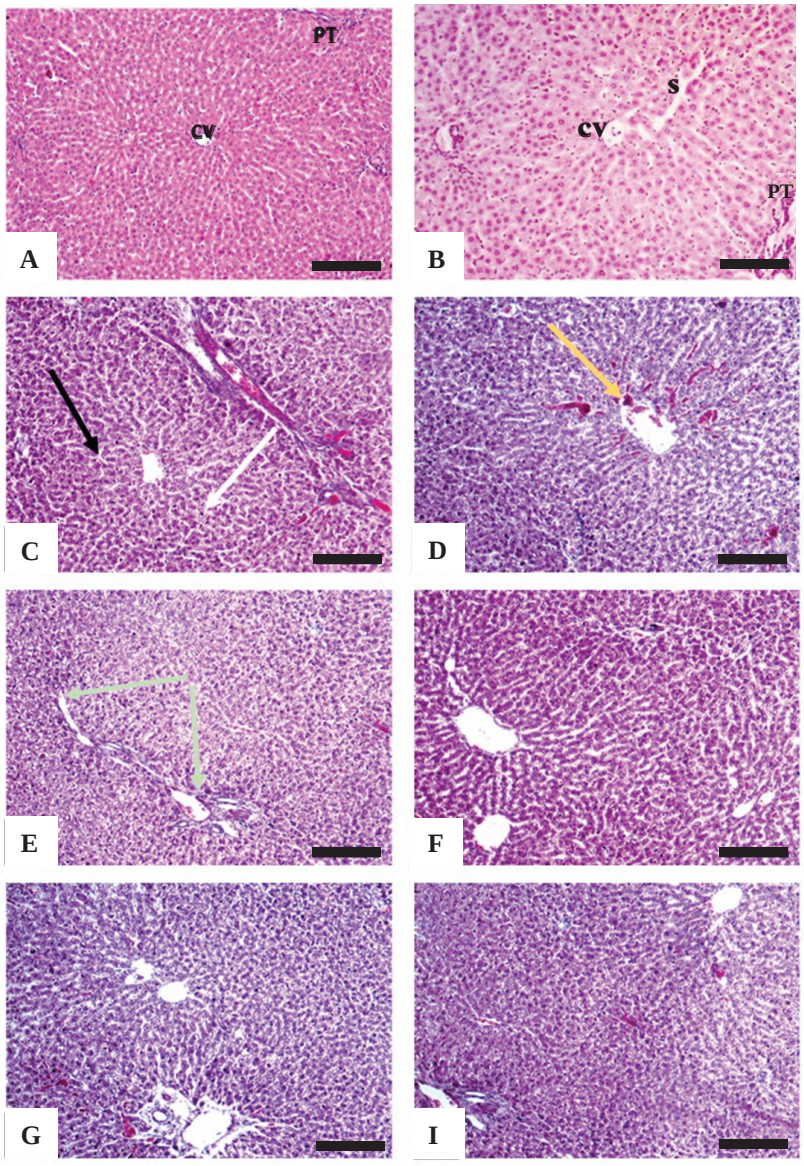

Figure 3. Photomicrograph of the rat liver in treatment groups. A: Group I, showing normal hepatic architecture with hepatocytes radiating from central vein (CV) and portal tract (PT); B: Group II, showing normal hepatic architecture with hepatocytes radiating from central vein (CV), sinusoid (S) and portal tract (PT); C: Group III, showing changes of hepatic architecture as evident by vacuolation of hepatocytes (black arrow) and prominent Kupffer cells (white arrow), D: Group III, showing changes of hepatic architecture as evident by portal infiltration of inflammatory cells (yellow arrow); E: Group III, showing changes of hepatic architecture as evident by congestion of central vein and blood vessels (grey arrow); F: Group IV, showing preserved hepatic architecture with mild vacuolation, G: Group IV, showing preserved hepatic architecture with mild congestion; H: Group IV, showing preserved hepatic architecture with mild biliary proliferation. Observed after H&E staining. Black bar: 50µM.

expression for caspase-3 (Figure 5). Similar results were also seen for liver section from rats treated with ALA alone (group II). Importantly, a strong positive cytoplasmic caspase-3 immuno-expression was displayed in liver sections taken from TiO_2 NPs-intoxicated rats (group III). However, minimal caspase-3 expression was exhibited in liver section taken from ALA treated TiO_2 NPs-intoxicated rats (group IV).

Discussion

ALA, also known as a co-factor thioactic acid, exists in many multi-enzyme complexes. ALA supplementation has been utilized for managing several types of hepatic disorders. (12) ALA was found to activate the nuclear factor erythroid 2-related factor (Nrf2), subsequent signaling of additional anti-oxidant factors, as well as a number of essential anti-oxidant enzymes to alleviate oxidative stress.(13) During chronic injury of liver, harmful reactive oxygen species are generated by hepatocytes and Kupffer cells, which causes these cells to die and activate stellate cells. Activation of these cells stimulates the formation of extracellular matrix, resulting in fibrosis.(14) ALA, through an unidentified mechanism, can cause hepatic stellate cells to undergo apoptosis, which protects against liver fibrosis. ALA has been shown to not only protect but also promote both the proliferation and survival ability of stem cells in an oxidative stress microenvironment for regeneration and repair of damaged liver tissues.(15)

Higher AST and ALT levels could indicate cellular leakage and damage to the hepatocyte cell membrane functional integrity. Due to this hepatocellular leakage, these enzymes leaked from the liver into the bloodstream.

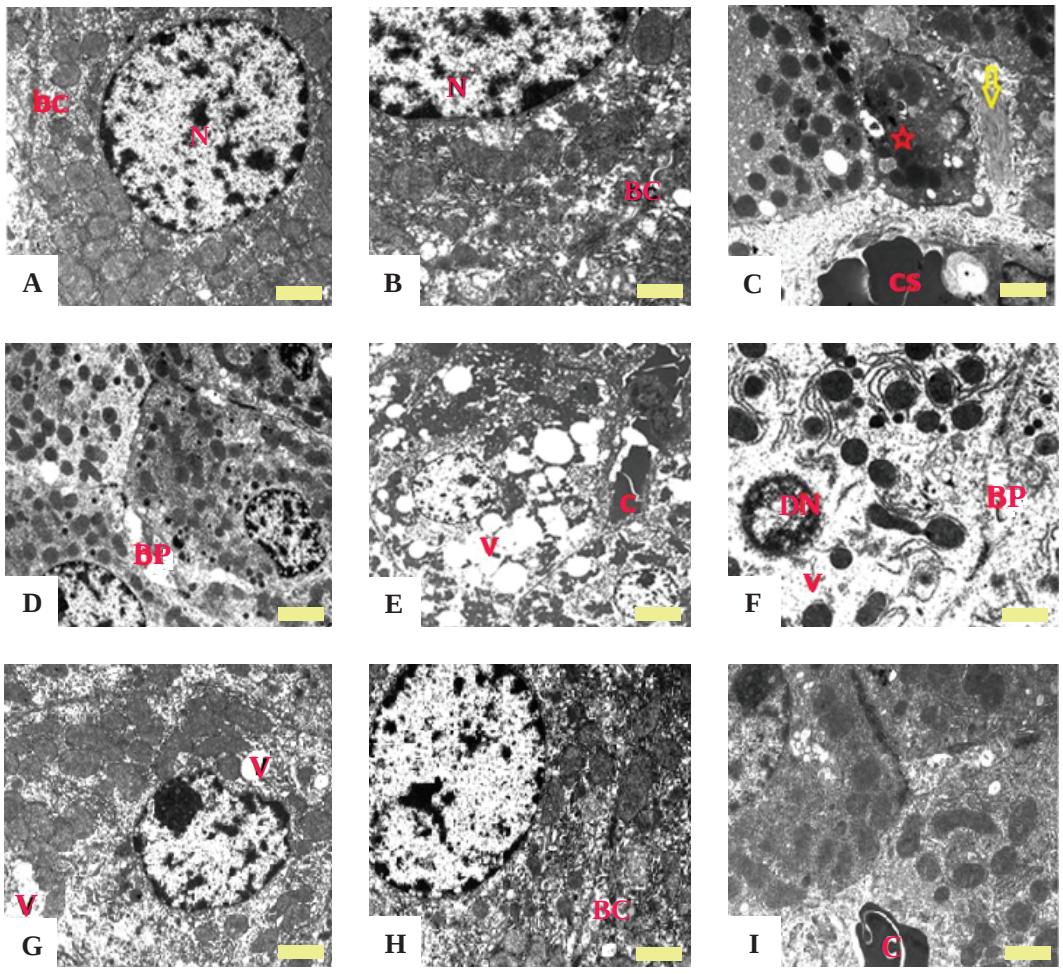

Figure 4. Electron micrograph picture of rat liver sections in treatment groups. A: Group I, showing classical hepatic ultrastructure with hepatocytes nuclei (N) had regular smooth contour and cytoplasm consisting of abundant organelles, the bile canaliculi (BC) were seen as narrow space firmly bounded by desmosomes; B: Group II: showing classical hepatic ultrastructure with hepatocytes nuclei (N) had regular smooth contour and cytoplasm consisting of abundant organelles; the bile canaliculi (BC) were seen as narrow space firmly bounded by desmosomes; C: Group III, showing evident ultrastructural changes with congested sinusoids (CS), fibrosis (yellow arrow) and apoptotic cell (red star); D: Group III, showing evident ultrastructural changes with biliary proliferation (BP); E: Group III, showing evident ultrastructural changes with congested sinusoids (CS), as well as vacuoles and rarefaction (V); F: Group III, showing evident ultrastructural changes with biliary proliferation (BP) and vacuoles and rarefaction (V) associated with diminished size of the nucleus (DN); G: Group IV, showing preserved hepatic architecture with more or less normal nucleus and organelles associated with mild vacuolation (V); H: Group IV, showing preserved hepatic architecture with more or less normal nucleus and organelles associated with mild biliary proliferation (BC); I: Group IV, showing preserved hepatic architecture with more or less normal nucleus and organelles associated with mild congestion (C). Yellow bar: 500 nm.

Serum ALT levels are thought to be more specific and a better parameter for determining the extent of liver damage. (7,16,17) Moreover, albumin is a protein synthesized by hepatocytes and its low levels are indicative of hepatotoxicity. Furthermore, bilirubin is a marker indicative of liver diseases. High levels of total bilirubin may mean that liver suffers from decreased hepatic bilirubin clearance and consequently liver dysfunction.(18) Here, intoxication with TiO_2 resulted in significant elevated levels of AST, ALT and total bilirubin as well as a decrease in albumin levels indicative of TiO_2-induced hepatotoxicity. Such effects on these parameters have been reported before, where the primary mechanism of TiO_2-induced hepatotoxicity is the generation of cellular oxidative stress, which oxidizes lipids in the hepatocyte cell membrane, resulting in cellular leakage and loss of hepatocyte functional integrity.(19,20) Interestingly, treatment with ALA has significantly restored the levels of AST, ALT, albumin and total bilirubin to nearly normal levels. This is in line with other studies reporting the restoration of these liver function biomarkers following ALA treatment.(4,21,22)

Lipid peroxidation is believed to have associated directly with TiO_2 intoxication.(23) In this study, TiO_2 intoxication markedly elevated serum levels of MDA and

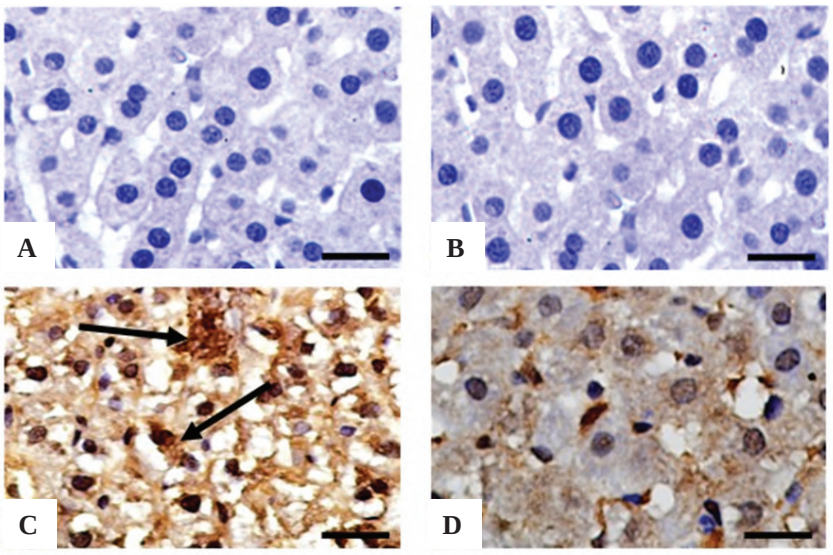

Figure 5. The immunohistochemical localization of caspase 3 expression of rat livers in treatment groups. A: Group I, showing negative expression of caspase-3; B: Group II, showing negative expression of caspase-3; C: Group III, showing a strong positive cytoplasmic caspase-3 immuno-expression (black arrow) in liver section; D: Group IV, showing weak positive cytoplasmic caspase-3 immuno-expression in liver section. Black bar: 5 µm.

significantly reduced levels of SOD, CAT and GSH. This indicates the generation of free radicals in the liver tissues resulting in lipid peroxidation and induction of oxidative stress. This is consistent with other studies reported detection of high significant level of MDA and decreased levels of SOD, CAT and GSH in TiO_2-intoxicated rats.(19,20) Importantly, ALA treatment demonstrated a significant effect on restoring SOD, CAT, GSH and MDA levels to almost normal ranges in TiO_2 NPs-intoxicated rats. Such results indicate the ability of ALA treatment to mitigate lipid peroxidation and oxidative stress induced by TiO_2 intoxication.

The microscopic findings in liver sections of the TiO_2 NPs-intoxicated rats in the present work showed various degrees of structural changes in the liver as evident in hydropic and fatty infiltration and patchy necrotic changes. These changes were in the region of the central vein, which has the least degree of oxygenation in the liver lobule. This might explain the high degree of susceptibility of liver cells near the centrilobular vein to TiO_2 NPs-induced pro-oxidative lesions.(24,25) Oxidative insult primarily affects Kupffer cells due to their high capacity to take up TiO_2 NPs close to sinusoidal region. In the same manner, cellular puffiness and vacuoles have been reported in experimental animals given harmful materials. The swelling results from the failure of membrane-located ATP-driven ion pumps, leading retention of Na^+ and water.(25,26) Furthermore, the periportal localization of NPs-induced hepatic injury appears due to rapid internalization of NPs from the portal blood and local oxidation in this liver zone.(27,28) Importantly, the present study also revealed evident vasodilatation and biliary proliferation, hepatic fibrosis, cell apoptosis, and infiltration of inflammatory cells. Moreover, evidence of TiO_2 NPs-induced cell apoptosis came from our immunohistochemical study showing high caspase-3 expression in TiO_2 NPs-intoxicated rats compared to no expression seen in negative control rats. This induced cell apoptosis might be attributed to ROS, which is a potential inducer of apoptosis. Such explanation was supported by many recent studies on some NPs reporting induced ROS-mediated cell apoptosis.(29,30) Overall, all of these findings are indicative of TiO_2 NPs induced hepatotoxicity. Such findings are similar to those observed in liver tissues examined by other studies demonstrating TiO_2 NPs induced hepatotoxicity.(19,20,31)

Surprisingly, these TiO_2 NPs-induced histopathological alterations of the liver were significantly alleviated by ALA treatment. This was evident by appearance of healthy liver cell ultrastructure and significant decrease in rate of caspase-3-mediated apoptosis of hepatocytes. Such ALA effect was explained by its ability to scavenge ROS and inhibition of inflammatory mediators as well as its anti-apoptotic activity due to inhibition of the transient receptor potential ankyrin 1 (TPRA1).(21,32,33)

Conclusion

Administration of 600 mg/kg TiO_2 NPs to rats resulted in hepatic degenerative lesions, depletion of GSH, oxidative stress, and apoptosis. However, these changes were mitigated by co-administration of 50 mg/kg ALA. Therefore, the toxic effects of chronic TiO_2 NPs intake may be neutralized with ALA.

Authors Contribution

AQAM was involved in the conceptualization of the study. SAD and AA were involved in the data curation. AA performed the formal analysis. FK and AS conducted the study investigation and were involved in the creation of figures/visualization. ALD provided study material and resources. AQAM drafted, edited, and revised the manuscript.

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