Histological Effects of Titanium Dioxide Nanoparticles in Adult Male Albino Rat Liver and Possible Prophylactic Effects of Milk Thistle Seeds

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Abstract: Titanium dioxide nanoparticles (TiO_2 NPs) are manufactured worldwide in large quantities for use in a wide range of applications including pigment, plastics, papers, inks, food colorants, toothpastes and cosmetic manufacturing. Milk thistle is a herbal supplement used to treat liver and biliary disorders. Silymarin, an active ingredient of Milk thistle, is a strong antioxidant that promotes liver cell regeneration and stabilizes cell membranes. Our aims were to investigate the biochemical and histological changes in the liver after administration of different doses of TiO_2 NPs and if there is a protective role of Milk thistle against these changes. The present study was carried out on fifty adult male rats divided into five groups: control group**,** group IIa injected by 100mg/kg TiO_2, group IIb treated by oral Milk thistle during injection by 100mg/kg TiO_2, group IIIa injection by 150mg/kg TiO_2 and group IIIb treated by oral Milk thistle during injection by 150mg/kg TiO_2. There were a decrease in the body weight and an increase in the coefficients of the liver in all treated groups. Changes in hepatocytes include; ballooned vacuolated cytoplasm and congested dilated portal vessels with inflammatory cellular infiltrations. Hepatocytes showed early signs of apoptosis and degeneration with nuclear changes. There were excessive amount of collagen fibers and marked depletion in the amount of glycogen. The histologic alterations observed might be an indication of hepatocyte injury due to TiO_2 NPs toxicity that interacts with proteins and enzymes in hepatic tissue. This interferes with antioxidant defense mechanisms and generation of reactive oxygen species induce hepatocytes apoptosis and generates inflammatory process resulting in cellular degeneration. These changes were improved by Milk thistle supplement as increase body weight, normal coefficient and normal morphology of the liver.
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Key Words: TiO2 nanoparticles, rats, liver, histology, Milk thistle

1. Introduction

Nanotechnology has been used in the areas of health care, consumer products, clothes, electronics and sporting goods. The number of nanotechnology-based consumer products available on the world market exceeds 1000. Nanomaterials have chemical, mechanical, optical, magnetic and biological properties that make them desirable for commercial and medical applications. Their toxicological impact is still under investigation and their effects on biological systems remain incomplete **(Peralta, 2011).** Nanoparticles (NPs) are characterized by their small size and large surface area with an active group. These characters increase their chemical reactivity to enable them to penetrate into living cells. The impacts of NPs on human and the environment have been put forward by some scientists and organizations **(Warheit *et al.*, 2007).** The toxic effects of NPs distributed mainly in important organ systems as lymph nodes, brain, lung, liver and kidney **(Li and Chen, 2011).** Titanium dioxide (TiO_2) is among the manufactured NPs and the earliest industrial product of nanomaterials in the world. TiO_2 is a natural insoluble non silicate mineral oxide occurs in different forms and is widely used in the cosmetics, pharmaceutical and paint industries. TiO_2 NPs can be absorbed into the body by inhalation, ingestion and dermal penetration due to their small size **(Wang *et al.*, 2007).** TiO_2 NPs occur in different sizes, shapes, chemical compositions and four crystalline polymorphic forms. Rutile and anatase are the most common forms; some studies indicate that anatase TiO_2 NPs is more cytotoxic than rutile TiO_2 NPs **(Li *et al.*, 2012).**

The liver is an active organ for detoxification and TiO_2 NPs can penetrate liver cell. **Liu and his colleagues (2009)** showed that the body weight of the rat treated with nano-anatase TiO_2 was significantly decreased while the liver weight was increased. The toxic effect of TiO_2 on the liver has been studied previously biochemically, physiologically, however the effect of TiO_2 NPs on the morphology of the liver was found to be few in the literatures. **M*a et al.* (2009)** proved that TiO_2 NPs damage liver function and induce an oxidative stress attack leading to liver toxicity. **Li *et al.* (2012)** stated that TiO_2 NPs increase gene expression levels of reactive oxygen species (ROS) and cytochrome p450 (CYP1A)**.** Bioactivation of CYP1A results in the formation of free radicals and ROS, which initiate lipid peroxidation and protein oxidation lead to damage of hepatocellular membranes. This

process is followed by the release of inflammatory mediators from activated hepatic macrophages lead to hepatic necrosis.

The use of herbal medicines from documented medicinal plants had a wide variety of clinical conditions including acute and chronic liver diseases. *Silybum marianum* (Milk thistle) is an effective developing therapeutic agent from natural products which may reduce the risk of hepatotoxins and also has a hepatoprotective effect **(Post-White *et al.*, 2007).** It is used as an herbal supplement treating liver and biliary disorders. It has been investigated for use as a cytoprotectant, an anti-carcinogen and a supportive treatment for liver damage from toxins. Silymarin is an active ingredient of *Silybum marianum* which has a strong antioxidant and anti-inflammatory properties that promotes liver cell regeneration and reduces blood cholesterol **(Vaknin *et al.*, 2007).**The protective antioxidant effects of *Silybum marianum* may be through impairing TiO_2 NPs mediated oxidative stress. It decreases production of free radical derivatives, decreases CYP1A activity and increases in the hepatic glutathione level **(Moon *et al.*, 2008).**

This research was done to address two main points:

1- Clarify the morphological changes in the liver caused by TiO_2 NPs.

2- Whether the use of Milk thistle with TiO_2 NPs ameliorates these changes or not.

2. Materials and methods

I-Preparation of TiO_2 nanoparticles

In this method, the titanium (IV) tetraisopropoxide, Ti $(OC_3H_7)_4$, (TTIP) used was 98% pure liquid Aldrich (USA)). Isopropanol (C_3H_7OH) was 99.9% pure liquid product of Aldrich. Preparation of nano titania particles, $TiO_2(s)$, was carried out following the procedure described earlier by **(Khalil *et al.*, 1998)**. X-ray diffraction (XRD) nitrogen adsorption/desorption isotherms and electron microscopy of TiO_2 NPs were performed.

II-Preparation of TiO_2 solution

Hydroxypropylmethyl cellulose K4M 0.5% (HPMC, K4M) was used as a suspending agent. TiO_2 NPs powder was dispersed onto the surface of 0.5% w/v HPMC and then the suspending solutions were treated by ultrasonic for 30 min and mechanically vibrated for 5 min **(Choi *et al.*, 2006).**

III-Preparation of Milk thistle suspension

Milk thistle seeds were purchased from El Masria for herbal medicine in Sohag. They were grinded into powder 15g suspended in 100cm boiled water for 15 minutes followed by filtration.

IV-Animals and treatment:

A total number of 50 adult male Albino rats were used in the present study with average weight 150-200g and purchased from Assuit's Experimental Animal Facility, Assuit University. Animals were housed in stainless steel cages in a ventilated animal room. All animals were given *ad libitum* access to tackled rodent chow diet and water from sanitized bottle fitted with stropper and sipper tubes. They were acclimated to this environment for 5 days prior to the experiment. All procedures used in this experiment were approved with the local Ethics Committee. Animals were randomly divided into five groups, 10 animals each as the following:

Group I: control group subdivided into three subgroups (3 rats each). Subgroup (i) kept without treatment, subgroup (ii) injected only by Hydroxypropylmethyl cellulose and subgroup (iii) treated only by oral Milk thistle.

Group IIa: treated by daily intraperitonial injection of 100mg/kg TiO_2 for two weeks.

Group IIb: treated by 15% oral Milk thistle suspension twice daily for four weeks, one week prior and two weeks during 100mg/kg TiO_2 treatment then one week after.

Group IIIa: treated by daily intraperitonial injection of 150mg/kg TiO_2 for two weeks.

Group IIIb: treated by 15% oral Milk thistle suspension twice daily for four weeks, one week prior and two weeks during 150mg/kg TiO_2 treatment then one week after.

V-Coefficients of liver

After weighing the body and liver, the coefficients of liver to body weight were calculated as the ratio of liver (wet weight, mg) to body weight (g) **(Liu *et al.*, 2009)**.

VI- Liver samples

The animals were sacrificed after being anaesthetized by ether. The liver were excised and weighed accurately. Liver samples of 0.5 cm thick were taken from all groups and immediately fixed in a 10% formalin solution and embedded in paraffin blocks, then sliced into 5µm in thickness mounted onto glass slides and stained with the following stains.

1- Heamatoxyline and Eosin for general histological studies

2- Masson trichrome for demonstration of collagen fibers.

3- PAS for demonstration of glycogen.

VII- Statistical analysis

Analysis of Variance (ANOVA) with a statistical significance of $P<0.05$ was used randomized designed according to other groups. Computations were performed with STATA version 9.2 software. All the analyses were performed in a blinded fashion.

3. Results

I- TiO_2 NPs Characterization results

1- XRD Diffraction patterns: The hydrolysis products and its dry products at 120C, as shown in **(Figure 1)**, were non crystalline to XRD **(JCPDS, 1995).** .

2- Nitrogen adsorption isotherm: Nitrogen adsorption isotherm determined on the NanoT400 material and The BET plot is shown in **(Figures 2,3)**. The isotherm is of type IV of isotherms and exhibits a hysteresis loop of type H2, thus, indicating mesoporous materials.

3- Transmission Electron Microscopy: Typical electron micrographs represent NanoT400 material is shown in **(Figure 4)**. The micrographs exhibit titania aggregates composed of very small poly-angular elementary particles of about 10 nm in diameter.

II- Growth and the coefficients of liver results:

There was very highly significant difference $P<0.0001$ between group IIIa versus the control and highly significant differences ($P<0.001$) between group IIa and group IIIb versus the control but significant difference ($P<0.05$) between group IIb versus the control as regard to body weight before and after treatment **(Table 1)**. There were very highly significant differences ($P<0.0001$) between group IIIa and group IIIb versus the control and moderately significant difference ($P<0.05$) between group IIa versus the control but no significant difference **(NS)** between group IIb versus the control as regard to liver coefficients (weight liver mg / body weight g) **(Table 2).**

III- Histological results by light microscope:

1- Hematoxylin& Eosin:

In comparison with the control group, histological changes especially in higher dose in the form of distortion of the liver cell plate, congestion, prominent vasodilatation with destruction of endothelial membrane and inflammatory cellular infiltrations. Some hepatocytes were ballooned and vacuolated cytoplasm with nuclear changes, others appeared with early signs of apoptosis as hazy vacuolated cytoplasm and indistinct cell boundaries and small condensed nucleus. Histological observations were improved in group IIb in the form of normal architecture with acidophilic hepatocytes and few vacuolated cytoplasms. It showed normal vesicular nucleus and mild congestion in central vein and blood sinusoids. In group IIIb, minimal improvement has been observed in histological changes and adverse side effects was still reported **(Figure 5;A-E)**.

2- Masson's Trichrome stain:

TiO_2 NPs caused deposition of excessive amount of collagen fibers and thickening in basement membrane of portal vasculature and blood sinusoid in all treated groups compared to the control one. There was a marked decrease in the amount of collagen fibers in group IIb compared to those demonstrated in group 11a **(Figure 6;A-E)**.

3- Periodic acid Schiff's (PAS) stain:

Control group showed strong positive reaction for PAS in the cytoplasm of the hepatocytes in the form of dark purple granules specially in the cells around the central vein.There was a mild decrease in the intensity of the positive reaction for PAS in the cytoplasm of the hepatocytes in group IIa while group IIIa showed negative reaction in peripheral zone 1. In group IIb there was a strong positive reaction for PAS compared to control group however group IIIb showed increase in the intensity of the positive reaction for PAS compared to group IIIa **(Figure 7; A-E).**

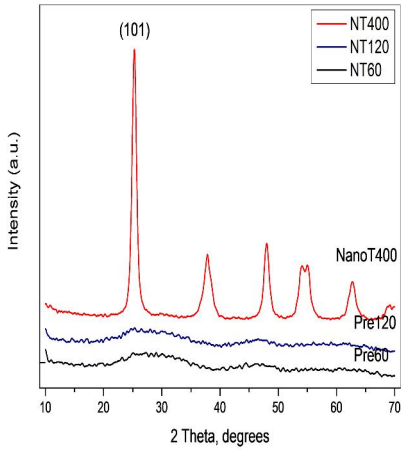


Figure (1): XRD diffraction pattern.

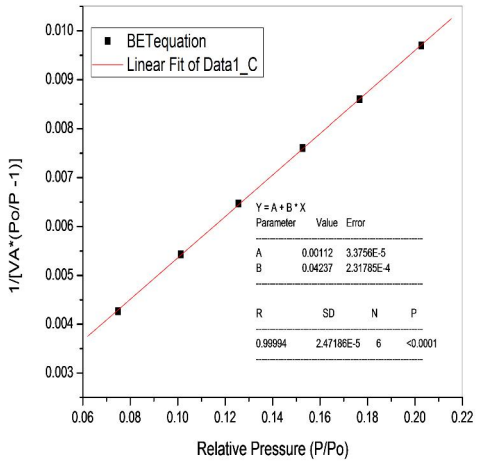


Figure (2): N2 adsorption isotherm of NanoT400

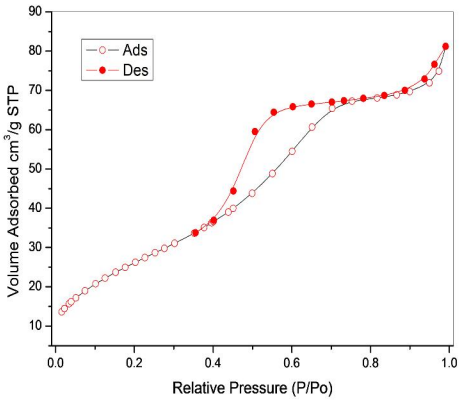


Figure (3): BET plot for NanoT400.

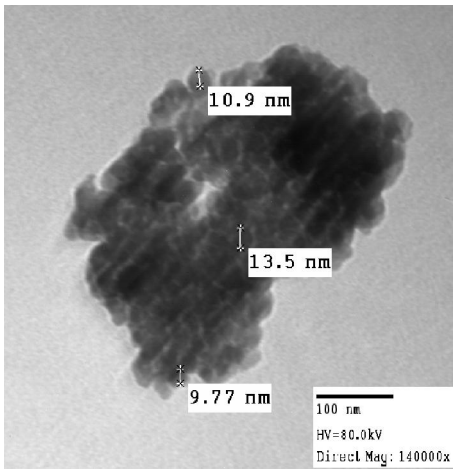


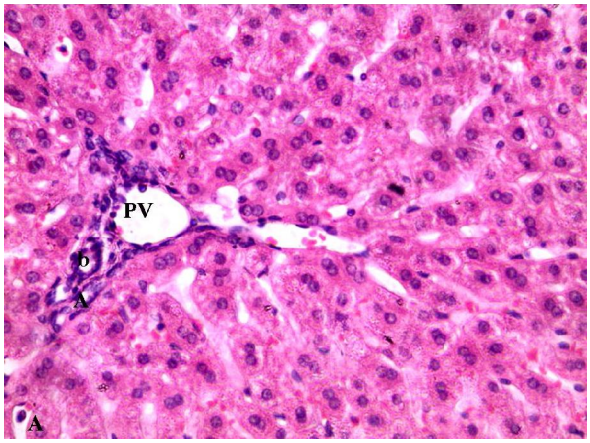
Figure (4): TEM NanoT400 10 nm. (X140000)

Table (1): Body weight before and after treatment in different groups.

Groups	Weight before	Weight after	Difference	*P* value
Controls	157.22 (12.78)	184.44 (13.09)	27.22	<0.0001(****)
Group IIa	100.56 (10.74)	90.22 (10.9)	-10.34	<0.001(***)
Group IIb	126.11 (25.95)	134.11 (20.44)	8	0.05(*)
Group IIIa	133.33 (25.74)	100.67 (20.42)	32.66-	<0.0001(****)
Group IIIb	153.89 (23.95)	140.22 (21.05)	13.67-	<0. 001(***)

Table (2): Liver weight (mg) / body weight (g) ratio.

Groups	Liver weight(mg) /body weight(g)	*P* value
Controls	2.97 (0.53)	<0.0001
Group IIa	3.11 (0.40)	0.003(**)
Group IIb	2.96 (0.11)	0.29(NS)
Group IIIa	4.42 (0.74)	<0.0001(****)
Group IIIb	4.13 (0.51)	<0.0001(****)



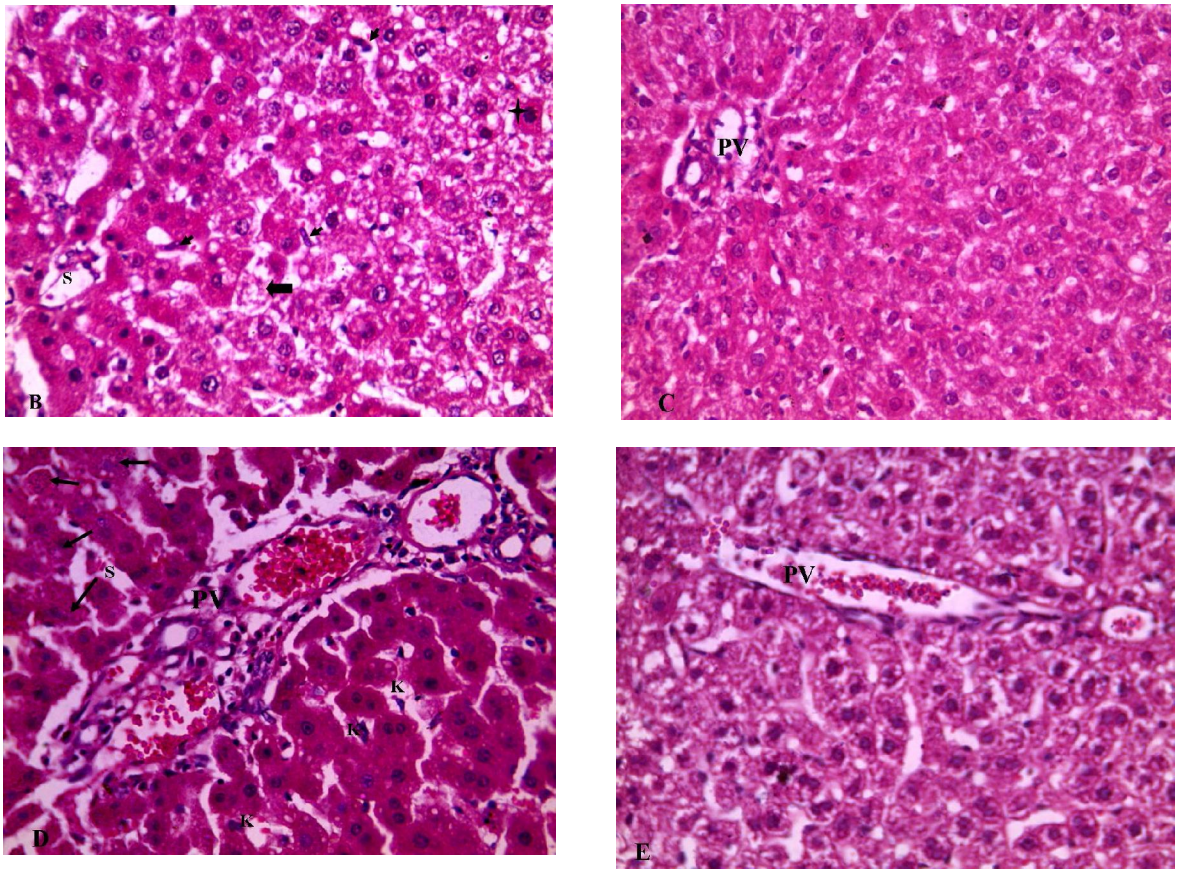
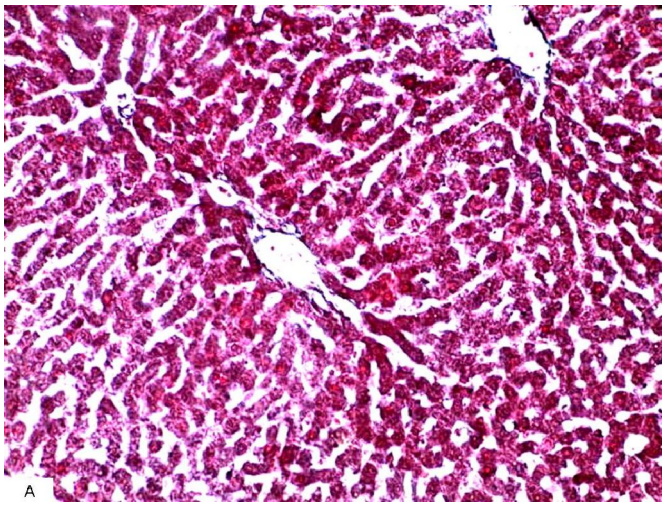


Figure (5): A- group 1,control, a photomicrograph of a liver section showing portal triad, **B-** (group IIa), showing congestion and dilatation in portal vessels **(PV)** and cell infiltration of lymphocytes, macrophages and fibroblasts **(arrows), C-** (group IIb), showing normal portal vessels **(PV)** and normal acidophilic hepatocytes with` vesicular nuclei, **D-** (group IIIa), showing congestion, dilatation and cellular infiltration around the portal vessels **(PV),** dilation in blood sinusoids **(S)** and **E-** (group IIIb), showing portal vessels congestion and cellular infiltration **(arrows). (H&E stain X400)**



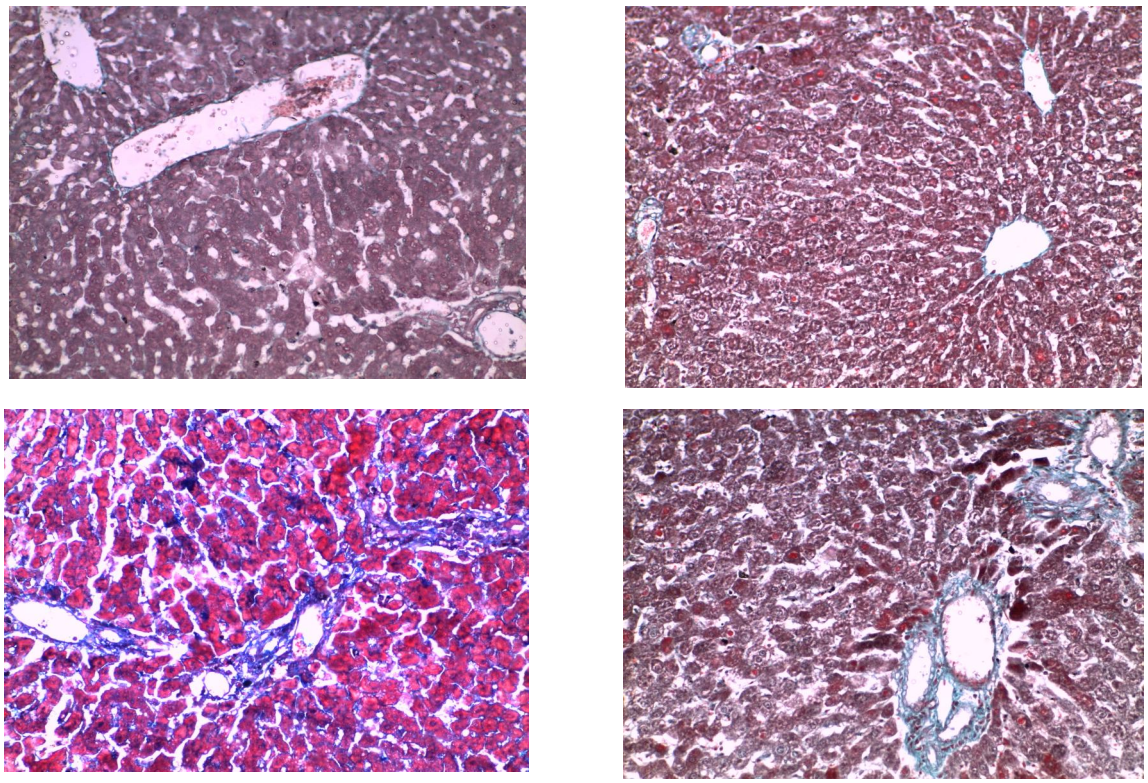
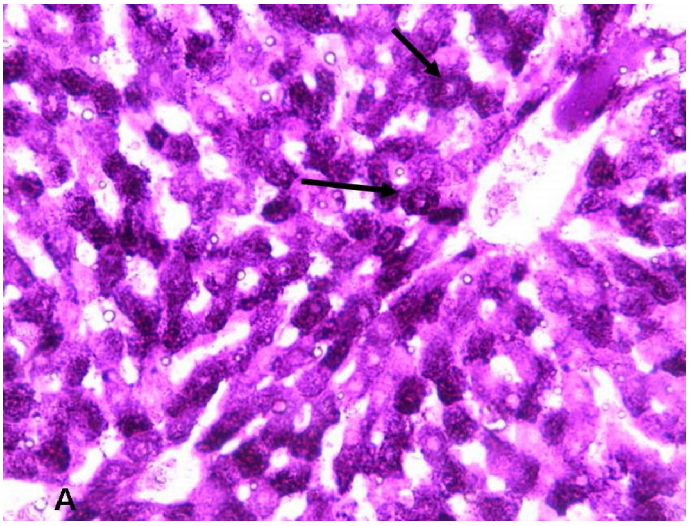


Figure (6): A- group 1,control, a photomicrograph of a liver section showing normal distribution of fine collagen fibers around the central vein **(CV)** and around the portal vessels **(PV), B-** (group IIa), showing normal amount of collagen fibers around the central vein **(CV)** and relatively thicker collagen fibers around the portal vessels **(PV), C-** (group IIb), showing fine collagen fibers around central vein **(CV)** and around portal vessels **(PV**), **D-** (group IIIa), showing numerous collagen fibers around the portal vessels **(PV)** and the blood sinusoids **(S)**, **E-** (group IIIb), showing apparent increase amount of collagen fibers within the portal vessels **(PV)** and the blood sinusoid **(S). (Masson's trichrome stain X200)**



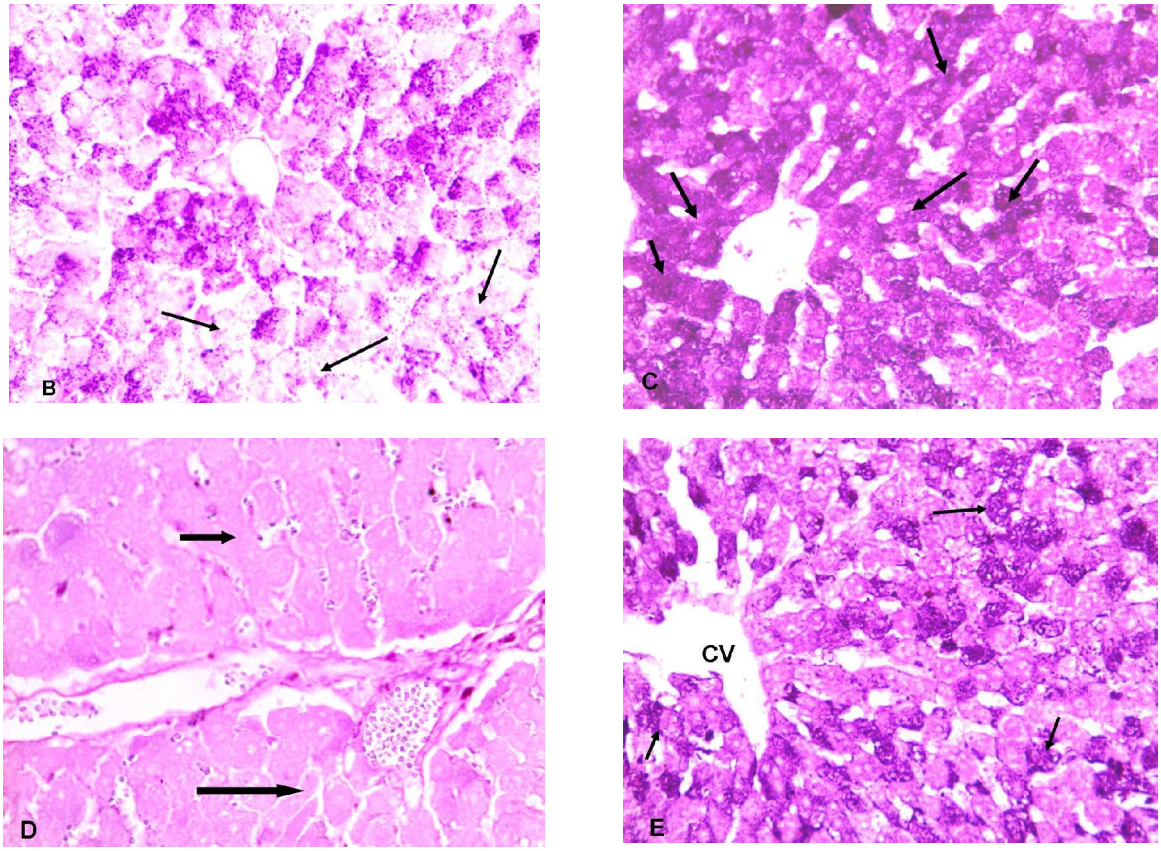


Figure (7): A- Group 1, control showing strong positive reaction for PAS in the cytoplasm of the hepatocytes in the form of dark purple granules especially in the cells around the central vein **(arrows). B-** (Group IIa) showing mild decrease in the intensity of the positive reaction for PAS in the cytoplasm of the hepatocytes **(arrows). C-** (Group IIb) showing strong positive reaction for PAS in the cytoplasm of the hepatocytes around the central vein and moderate positive reaction in those peripheral within the hepatic lobules **(arrows). D-** (Group IIIa) showing show negative reaction for PAS in the cytoplasm of the hepatocytes in peripheral zone 1 **(arrows). E-** (Group IIIb) showing increase in the intensity of the positive reaction for PAS within some hepatocytes **(arrows).** (PAS stain X400)

4. Discussion

TiO_2 NPs are used in a wide range of applications including pigment, plastics, papers, inks, food colorants, toothpastes and cosmetic manufacturing. Our aims were to investigate the biochemical and histological changes in the liver after administration of different doses of TiO_2 NPs and if there is a protective role of Milk thistle , is a herbal supplement used to treat liver and biliary disorders, against these changes. In our study, intraperitoneal injection of different doses of TiO_2 NPs can significantly decrease body weight and increase coefficients of the liver. These results are in line with **Bermudez *et al.* (2002)** who explained decrease body weight and shorten life-time due to retention and overload of TiO2 NPs in vivo. **Wang *et al.* (2007)** reported that increase liver weight due to accumulation and retention of TiO_2 NPs mainly in liver leading to inflammation and congestion especially in groups treated by high doses. **Oberdorster *et al.* (2005)** reported that the retention halftime of TiO_2 particles *in vivo* was long because of its difficult excretion. TiO_2 halftimes in rat lung were 117 days for fine particles (250 nm) and 541 days for ultrafine particles (20 nm). **Duan and his associated (2010)** stated that TiO2 NPs were difficult clearance in vivo result in its deposition in the liver and hepatic lesion. Moreover **Liu *et al.* (2009)** observed higher coefficients not only of the liver but also kidney and spleen of mice.

In this study histological alterations in the morphology of the liver were demonstrated in treated group. There were vasodilatation and congestion of both portal area and blood sinusoids.

These results are in accordance with **Donaldson *et al.* (2001), Duan and his associated (2010) and Alarifi *et al.* (2013)** who explained that nanoparticles

may affect permeability of the hepatocytes cell membranes and the endothelial lining of blood vessels leading to sequestration of red cells and platelets that could impair circulation and promote thrombosis. **Gerlofs *et al.* (2005)** reported that nanoparticles may affect the pulmonary and vascular system in spontaneously hypertensive rats.

Swelling of some hepatocytes may be a sign of hydropic degeneration of these cells. **Donaldson and his colleagues (2001) and Wang *et al.* (2007)** explained that swelling by occurrence of disturbed membrane function that lead to massive influx of water and Na+ that lead to an increase of intracellular water due to TiO_2 NPS effects. **Alarifi *et al.* (2013)** also reported that the swelling of hepatocytes on exposure to nanoparticles was due to adaptation of cell transporters.

Cellular degeneration may be due to leakage of lysosomal hydrolytic enzymes that lead to cytoplasm degeneration **(Wang *et al.*, 2007).** This is in accordance with the highly cytoplasmic acidophilia of these cells demonstrated in this study as rupture of lysosomes lead to amorphous eosinophilic cytoplasm as it is an initial sign of hepatocytes necrosis before shrinking and dissolution of nuclei **(Giray *et al.*, 2011).**

In the present study, inflammatory mononuclear cellular infiltrations between the hepatic tissue and around the portal area were demonstrated. These results are in accordance with the results of **Johar *et al.* (2004) and Giray *et al.* (2011)** who suggest that TiO2 NPs could interact with proteins and enzymes of the hepatic interstitial tissue interfering with the antioxidant defense mechanism and leading to reactive oxygen species (ROS) generation which in turn may imitate an inflammatory response. **Warheit *et al.* (2007)** observed that in vivo TiO2 NPs increased the inflammatory indicators and cell proliferation in bronchoalveolar fluid. In addition a study of **Wang *et al.* (2007)** on rat reported that TiO2 of smaller size (5-10 nm) would allow easier entry to liver cells and cause inflammatory cascade by releasing some cytokines. Furthermore; **Li *et al.* (2012)** proved that TiO2 NPs impair the function of macrophages and cause persistent inflammatory reactions in lungs. TiO2 NPs was proved to induce inflammatory process causing an increase in different inflammatory cytokine leading to inflammation of many organs as lung, liver and brain **(Win *et al.*, 2008).** The present work revealed that TiO_2 NPs caused deposition of excessive amount of collagen fibers and thickening in basement membrane of the portal vasculature and the blood sinusoid. There was also accumulation of extracellular matrix in interstitial regions of the liver. Hepatic fibrosis is a consequence of various chronic liver diseases caused by hepatitis viruses, drugs, alcohol, parasites, and autoimmune mechanisms. **Poli (2000)** explained that activated hepatic stellate cells are primarily responsible for the excess production of extracellular matrix and differentiate into myofibroblast to synthesis collagen I and III. **Yoshiji *et al.* (2002)** found that TGF-β1 increases the production of collagen and other extracellular matrix proteins during liver damage.

Treatment with TiO_2 NPs caused marked depletion in the amount of glycogen firstly from the peripheral zone 1. These results previously described by **Zhonghua *et al.* (2000)** who proved that toxins may cause anorexia lead to depletion of glycogen stores in the liver and this explained the loss of weight with the increase in liver coefficients.. However, **Tasci *et al.* (2006)** explained that these results were due to lipid peroxidative damage in mitochondria present in large amount in zone1 leading to compensatory enhancement of glycogenolysis.

Hepatoprotective effects of Milk thistle supported by **Post-White et al. (2007)** who reported that it impaired oxidative stress through decreased production of free radical derivatives, by the decreased CYP enzyme activity, MDA level and glutathione level which has antioxidant properties that protects against liver damage. **Jia et al. (2001)** explained our results that silymarin suppresses expression of profibrogenic procollagen alpha1 (I) and tissue inhibitor of metalloproteinases-1 (TIMP-1) most likely via down-regulation of transforming growth factor-beta (TGF-β1) messenger RNA (mRNA) in rats with biliary fibrosis and can also suppress lipid peroxidation. **Sung et al. (2009)** suggested that Milk thistle not only provides conjugation with injurious free radicals and diminishes their toxic properties, but also suppresses the proinflammatory response of a carbon tetrachloride (CCl4) induced liver injury by attenuate TGF-β1 levels and modulate expressions of TNF-α, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) mRNA.

In conclusion

The results of this study add our understanding of TiO_2 NPs induced histologic changes resulting in serious liver injury which is dose dependent. Milk thistle is a safe hepatoprotective herbal dietary supplement could be used to prevent hepatocellular damage and liver fibrosis by TiO_2 NPs.

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