Tiron ameliorates oxidative stress and inflammation in titanium dioxide nanoparticles induced nephrotoxicity of male rats

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A B S T R A C T

Although the widespread use of titanium dioxide nanoparticles (TiO$_2$ NPs), few studies were conducted on its hazard influence on human health. Tiron a synthetic vitamin E analog was proven to be a mitochondrial targeting antioxidant. The current investigation was performed to assess the efficacy of tiron against TiO$_2$ NPs induced nephrotoxicity. Eighty adult male rats divided into four different groups were used: group I was the control, group II received TiO2 NPs (100 mg\Kg BW), group III received TiO2 NPs plus tiron (470 mg\kg BW), and group IV received tiron alone. Urea, creatinine and total protein concentrations were measured in serum to assess the renal function. Antioxidant status was estimated by determining the activities of glutathione peroxidase, superoxide dismutase, malondialdehyde (MDA) level and glutathione concentration in renal tissue. As well as Renal fibrosis was evaluated though measuring of transforming growth factor-β1 (TGFβ1) and matrix metalloproteinase 9 (MMP9) expression levels and histopathological examination. TiO$_2$ NPs treated rats showed marked elevation of renal indices, depletion of renal antioxidant enzymes with marked increase in MDA concentration as well as significant up-regulation in fibrotic biomarkers TGFβ1 and MMP9. Oral administration of tiron to TiO$_2$ NPs treated rats significantly attenuate the renal dysfunction through decreasing of renal indices, increasing of antioxidant enzymes activities, down-regulate the expression of fibrotic genes and improving the histopathological picture for renal tissue. In conclusion, tiron was proved to attenuate the nephrotoxicity induced by TiO$_2$ NPs through its radical scavenging and metal chelating potency.

1. Introduction

As nanotechnology is developing rapidly, more concerns on the potential health hazards about exposure to nanoparticles (NPs) have been arising. NPs, according to the European Union, are natural or manufactured particles with one or more external dimensions in the size range 1–100 nm [1]. Owing to the extremely small sizes and large surface areas, NPs possess dramatic differences in the physicochemical properties compared to their fine size analogues [2]. Because of these peculiar features, NPs are able to penetrate cells and interfere with various subcellular mechanisms [3]. Some nanoparticles interact directly with cell macromolecules including proteins, lipids and DNA [4]. In the recent decades, nano-sized titanium dioxide (TiO2 NPs) have been mass-produced and used as a common additive in a wide range of applications including food-related industries, materials for air pollution control, pharmaceuticals, household products, cosmetics and other personal care products [2,5]. As a consequence, the level of human exposure to TiO2 NPs through multiple media and pathways has been increased. Inhalation and dermal exposure of industrially released TiO2 NPs are considered as the main routes of TiO2 NPs exposure, whereas oral exposure may occurs by direct consumption of products elaborated with high amounts of TiO2 NPs as nano-food or nanomedicine [5]. Administration of TiO$_2$ NPs through different routes results in their accumulation in the various tissues including liver, kidney, brain and spleen with potential toxicological impacts [6,7]. As kidney is a frequent target for toxic effects of xenobiotics, kidney is found to be one of the major targets of TiO2 NPs deposition, even in low level of TiO2 NPs exposure [7]. Subchronic TiO$_2$ NPs toxicity led to chronic nephritis

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with several pathological lesions such as proximal cell death [8], renal cell necrosis [9] and renal fibrosis [10]. Recently, the molecular mechanisms responsible for the renal toxicity of TiO2 NPs have been elucidated. Generation of intracellular reactive oxygen species (ROS) by TiO2 NPs and its subsequent oxidative damage play the key role in the mechanism underlying the TiO2 NPs-induced toxicity [12]. The implication of oxidative stress and inflammation in the etiology and progression of several clinical disorders has led to the suggestion that agents with antioxidant properties may have great health benefits. Some studies have disclosed that antioxidant pretreatment or co-treatment can reverse the toxicity of metallic NPs [4,13,14]. Tiron (4,5-dihydroxy-1,3-benzene disulfonic acid), a water soluble synthetic analog of vitamin E, has been known to be a potent antioxidant to rescue ROS-evoked oxidative damage [15,16]. Additionally, tiron is a non-toxic chelator that is effective in alleviation of various metal intoxications such as aluminum, lead and vanadium [17]. The renal protective influence of tiron against metal toxicity and diabetic nephropathy was reported previously [15–17]. Trion acts as a direct hydroxyl radical, superoxide scavenger as well as a metal chelator [18].

In this study, we present an overview on possible influences of tiron as antioxidants and metal chelator against nephrotoxicity and renal fibrosis in TiO2 NPs induced nephrotoxicity in rats.

2. Materials and methods

2.1. Chemicals

TiO$_2$ NPs: Anatase form, white in color, density 3.9 g\ml, particle size (10 nm), surface area ($>$150 m^2\g), purity 99.9 was purchased from Sigma Aldrich Chemical Co., Germany. The nanosized TiO$_2$ was suspended in phosphate buffer saline solution at a concentration of 1 mg\ml, shaken and dispersed via sonication for 10 min before use. Tiron: White odorless powder was purchased from Sigma Aldrich Chemical Co., Germany.

2.2. Animals

Eighty adult (average 55 days old) male albino rats weighting 180–200 g were obtained from department of Toxicology and Forensic Medicine's animal house, Faculty of Veterinary medicine Cairo University were used. All animals were subjected to two weeks of acclimatization. The animals were housed in separate well-ventilated cages, under standard conditions, with free access to standard diet and water. The local Committee approved the design of the experiments and the protocol conforms to the guidelines of the National Institutes of Health.

2.3. Experimental design

For two months the rats were divided into four equal groups. Group I (control group) injected IP with normal saline in last two weeks of the experimental period, Group II (TiO$_2$ NPs treated group) received 100 mg\Kg BW of TiO$_2$ via oral gavage once daily [19]. Group III (TiO$_2$ NPs + Tiron) this group received 100 mg\kg BW TiO$_2$ NPs once daily and the same rats injected IP with tiron 470 mg \kg BW [20] daily for the last two weeks of experimental period, group IV (tiron group) it was received tiron 470mg\kg BW only in the last two weeks. All symptoms and deaths were carefully recorded daily.

2.4. Sampling

At the end of the experiments, the rats were weighted, scarified and the blood and kidney tissues were collected. Blood samples were collected from the eye vein and the serum was collected by centrifuging blood at 3000 x g for 10 min for serum analysis. While kidney tissues were washed and stored for subsequent investigations.

2.5. Relative weight of kidney

After weighing the body and kidney of each animal, the relative weight of kidney was calculated as the ratio of kidney (wet weight, mg) to body weight (g).

2.6. Serum analysis

Kidney indices were evaluated by measuring of serum levels of blood urea according to Tietz [21], creatinine Tietz [22] and total protein concentration Tietz [23] using commercial Kits.

2.7. Titanium content analysis

The frozen kidneys tissues were thawed and $\sim$0.1 g samples were weighed, digested, and analyzed for titanium content according to method described by Gui et al. [24]. The detection limit of titanium was 0.074 ng/ml.

2.8. Malondialdehyde and antioxidants biomarkers determinations

Specimens from kidney tissue was weighted and homogenized in cold phosphate buffered saline (pH 7.4) using Teflon homogenizer. The homogenates were centrifuged at 14,000 $\times$ g for 15 min at 4 °C. The supernatant was used to measure the MDA level [25], superoxide dismutase (SOD) activity [26], glutathione peroxidase (GPX) activity [27], reduced glutathione (GSH) concentration [28] and protein content [29].

2.9. Quantitative real-time PCR for matrix metalloproteinase 9 (MMP9) and transforming growth factor β1 **(TGFβ1)**

Approximately 100 mg of kidney tissue was used for total RNA extraction using Qiagen Rneasy Mini Kit following the manufacturer's protocol. The RNA yields and purity were determined using Spectrophotometer (Thermo Scientific, USA). Ten μg of TRNA were treated with DNase I for 20 min at 37 °C. The cDNA synthesis was carried out using reverse transcriptase (Invitrogen) and oligo-dT following the manufacturer protocol. After initial heat denaturation of 1 μg of total RNA (65 °C for 5 min), the reactions (20 μl) were incubated for 60 min at 42 °C. cDNA was added to a SYBR Green qPCR Master Mix (Qiagen) containing 30 pg/ml of each primer with the following sequence MMP9 forward CACTGTAACTGGGGGCAACT, reverse CACTTCTTGTCAGCGTCGAA, TGFβ1 forward GGACTCTCCACCTGCAAGAC, reverse CTCTGCAGGCGCAGCTCTG. The cDNA was amplified by 40 cycles of denaturation at 95 °C for 45 s, annealing at 57 °C for 45 s for MMP9 and 60 °C for 45 s for TGFβ1 and extension at 72 °C for 45 s. The size of all amplicons was confirmed by 2% agarose gel electrophoresis stained with SYBR Safe DNA gel stain (Invitrogen). The GAPDH gene was amplified in the same reaction to serve as the reference gene. Each measurement was repeated 3 times, and the values were used to calculate the gene/GAPDH ratio, with a value of 1.0 used as the control (calibrator). The normalized expression ratio was calculated using the method described by Khalaf et al., [14].

2.10. Histopathological examination

The kidneys tissues from the different groups were fixed in 10% neutral buffer formalin then managed for obtain 4 μm paraffin embedding sections. The tissue sections were stained with

hematoxylin and eosin (H&E), periodic acid Schiff's (PAS) and Masson's trichrome (MT) stain for assessment of fibrosis [30]. The morphometric measurement of the glomerulosclerotic index (GSI) and the tubulointerstitial fibrosis index (TIFI) were performed following the method described by Saito et al. [31].

2.11. Immunohistochemical analysis for fibrotic proteins (MMP9 and TGFβ1)

The immunohistochemical analysis was done according to the methods described by Ogaly et al. [32]. The tissue sections were deparaffinized, rehydrated and pretreated with 10 mM citrate buffer for antigenic retrieval. Sections were incubated for two hours at 4 °C in a humidified chamber with one of the following primary antibodies: rabbit polyclonal anti-TGF beta 1 antibody with concentration of 20 μg/ml. (ab92486; Abcam, Cambridge, UK), and Rabbit polyclonal anti-MMP9 diluted 1:200 (ab38898; Abcam, Cambridge, UK). The tissue sections were incubated with a biotinylated goat anti rabbit antibody (Thermo scientific, USA), Streptavidin peroxidase (Thermo scientific, USA) and 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma). The slides were counterstained with Mayer's haematoxylin then dehydrated and mounted. Primary antibodies were replaced by PBS for negative controls. The stained sections were analyzed by Lieca Qwin 500 Image Analyzer (Leica, Cambridge, England). In each field, the immunopositive area (dark brown) was recorded. Percentage of positive stained area (%) was calculated as mean of 10 fields/slide.

2.12. Statistical analysis

The different analytical determinations in the biological samples were carried out in duplicate and results are expressed as the mean ± SE. Data for multiple variable comparisons were analyzed by one-way analysis of variance (ANOVA) test to analyze the significant differences ($P < 0.05$) between groups using SPSS version 16 package for Windows.

3. Results

3.1. Serum analysis

The biochemical indices monitoring are a useful markers for assessment of kidney tissue damage. Table 1 illustrates the changes in the serum kidney function tests in rat following the repeated administration of TiO$_2$ NPs for two months. Urea and creatinine concentrations were increased significantly in group II intoxicated

with TiO$_2$ NPs while total protein level was significantly reduced in group II compared to control. Oral administration of tiron for group III in the last two weeks of experimental period was significantly reduced the urea concentration by 59.87%, creatinine by 40.80% and significantly increased the total protein level by 55.6%.

3.2. Kidney coefficient and titanium accumulation

Results of table Table 2 indicated that administration of TiO$_2$ NPs and or tiron did not change the relative weights of kidney between different experimental groups. In the same sequence kidney of both group I and IV contained no titanium (Ti), which suggested that the rats were not exposed to metal from other sources. On the other hand significant elevation for Ti content in group II intoxicated with TiO$_2$ NPs compared to group I was detected. Administration of tiron in last two weeks of experimental period significantly reduced the Ti content in renal tissue by 58.08% Table 2.

3.3. Oxidative stress parameters

From the current results reported in Table 3, it was obvious that, MDA the main indicative marker for lipid peroxidation (LPO) showed a significant elevation in group II intoxicated with TiO$_2$ NPs in comparison to group I. Supplementation of tiron to group III caused significant reduction in the elevated MDA by 47.9%. In the current study, the administration of TiO$_2$ NPs to group II led to a significant reduction in GSH concentration, GPX, and SOD enzymes activities compared to the group I. Co-administration of tiron as antioxidant for group III caused a significant increase in GSH by 46.5%, GPX by 33.3% and SOD by 44.7% enzyme activities (Table 3).

3.4. TGFβ1 and MMP9 expression levels

Renal fibrosis is the common pathological foundation for several chronic kidney diseases. To investigate whether TiO$_2$ NPs induced renal fibrosis, the expression of TGFβ1 as an essential fibrogenic cytokine and MMP9 a novel fibrotic and inflammatory marker were evaluated in the present study. According to results detected in Table 4 and Fig. 1 TGFβ1 appeared to over expressed by more than 6 fold in group II treated by TiO$_2$ NPs compared to group I. Tiron treatment to group III induced significant down expression to TGFβ1 by 52.5% compared to group II. In the sequence, tissue MMP9 over-expressed significantly in group II compared with control and it decreased significantly with administration of tiron (group III) by 44.76% Table 4 and Fig. 1, but they did not return to the values of the control group.

3.5. Histopathological results

Group I and group IV revealed normal histological features of renal glomeruli, renal tubules and the interstitial tissue (Fig. 2A & D). Group II showed moderate to severe glomerular and tubulointerstitial changes in the form of segmental sclerosis, moderate mesangial expansion, moderate to marked thickening of the parietal layer of the Bowman's capsule, adhesion of glomerular

Table 1
Effect of tiron on serum kidney function tests of TiO2 NPs intoxicated rats.

	Group I	Group II	Group III	Group IV
Urea mg\dl	33.68 ± 0.82[a]	73.85 ± 0.936[b]	29.63 ± 3.4[ca]	28.2 ± 2.2[cda]
Creatinine mg\dl	0.49 ± 0.06[a]	0.963 ± 0.03[b]	0.57 ± 0.05[ca]	0.30 ± 0.035[d]
Protein g\dl	6.8 ± 0.045[a]	3.9 ± 0.089[b]	6.07 ± 0.022[c]	6.33 ± 0.09[d]

Table 2
Effect of tiron on relative weights and titanium content in kidney of TiO2 NPs intoxicated rats.

	Group I	Group II	Group III	Group IV
Relative kidney weight	1.263 ± 0.03[a]	1.32 ± 0.07[a]	1.40 ± 0.055[a]	1.36 ± 0.047[a]
Ti content (ng\ g tissue)	Not detected	298 ± 9.2[a]	125 ± 7.4[b]	Not detected

Data are presented as (Mean ± S.E). S.E = Standard error. Mean values with different superscript letters in the same row are significantly different at($p \leq 0.05$).

Table 3

Effect of tiron on oxidative stress parameters in kidney of TiO2 NPs intoxicated rats.

Kidney	Group I	Group II	Group III	Group IV
MDA μM\ mg	2.6 ± 0.05^a	4.8 ± 0.06^b	2.51 ± 0.061^{ca}	3.63 ± 0.14^{db}
GSH μg\ mg	11.92 ± 0.72^a	8.3 ± 0.35^b	12.16 ± 0.53^{ca}	8.6 ± 0.04^{db}
GPX U\mg	3.62 ± 0.17^a	2.11 ± 0.042^b	2.82 ± 0.069^{ca}	2.32 ± 0.069^{db}
SOD U\mg	5.6 ± 0.052^a	3.8 ± 0.06^b	5.51 ± 0.06^c	3.2 ± 0.14^d

Data are presented as (Mean ± S.E). S.E = Standard error. Mean values with different superscript letters in the same row are significantly different at($p \leq 0.05$).

Table 4

Effect of tiron on TGFβ1 and MMP9 expression levels in kidney of TiO2 NPs intoxicated rats.

Kidney	Group I	Group II	Group III	Group IV
TGFβ1 fold change (RQ)	1 ± 0.00^a	6.35 ± 0.39^b	3.37 ± 0.26^c	1.87 ± 0.09^{da}
MMP9 fold change (RQ)	1 ± 0.00^a	2.1 ± 0.09^b	1.19 ± 0.06^c	0.90 ± 0.04^{da}

Data are presented as (Mean ± S.E). S.E = Standard error. Mean values with different superscript letters in the same row are significantly different at($p \leq 0.05$).

tuft to parietal layer of the Bowman's capsule. The renal tubular epithelium showed moderate to marked necrobiotic changes with extensive protenious and cellular cast accumulation. (Fig. 2B). Excessive collagen depositions (Fig. 3B) with mononuclear inflammatory cell aggregation in the interstitial tissue were observed in most animals. These changes were markedly attenuated in group III (Fig. 2C & C).

In the morphometric analysis, the GSI and TIFI were much higher in group II than the group I and group IV. Group III showed significant reduction in GSI and TIFI comparing to the TiO2 NPs treated group (Fig. 4)

3.6. Immunohistochemical analysis of TGF beta 1and MMP9

TGFβ1 immunostaining was observed in the interstitial tissue, proximal convoluted tubules and glomerular mesangial area (Fig. 5). Group II showed significant increase in the TGFβ1 immunostaining (Fig. 5B) comparing to group I (Fig. 5A) and group IV. Group III (Fig. 5C) showed significant reduction in the TGFβ1 immunostaining than group II (Fig. 5B). MMP9 immunoreactivity was seen in mesangial glomerular area, proximal and distal convoluted tubules with little extent to the interstitial tissue (Fig. 6). Group II showed significant increase in the MMP9 immunostatining than group I (Fig. 6A), group IV and group III (Fig. 6C). Administration of tiron to group III showed significant reduction in the TGFβ1 immunostaining than group II. Non-significant differences in TGFβ1 and MMP9 immunostatining were detected between group I and group IV.

4. Discussion

After any route of exposure nanoparticles (NPs) attain to the systemic circulation. They can distribute to several organs like kidney, liver and spleen [33]. As kidneys are particularly susceptible to xenobiotics and renal excretion, it considered as an expected and possible elimination route for NPs in living organisms [34]. The present investigation was conducted to evaluate the possible therapeutic role of tiron (potent antioxidant and metal chaleter) against the nephrotoxicity induced by TiO2 NPs. According to the obtained results we found that oral administration of 100 mg/kg BW of TiO2 NPs(10 nm) for 60 consecutive days induced nephrotoxicity marked by significant increases in kidney indices (urea, creatinine and total protein) (Table 1), significant elevation of Ti content in kidney tissue (Table 2), severe oxidative stress (Table 2), indicated by significant elevation of MDA the indicative for LPO and significant reduction for GSH concentration as well as antioxidant enzymes activity (SOD and GPX), severe renal pathological changes, particularly renal inflammation, necrosis and or renal apoptosis and renal fibrosis () coupled with significant elevation in tissue fibrosis related genes (TGFβ1 and MMP9) (Table 4, Figs. 1, 5, 6). As the measurement of serum concentrations of kidney indices such as creatinine, urea is usually a marker of kidney function and its other pathological condition s [35]. The elevated renal indices detected in our study indicated the renal injury induced with TiO2 NPs. The
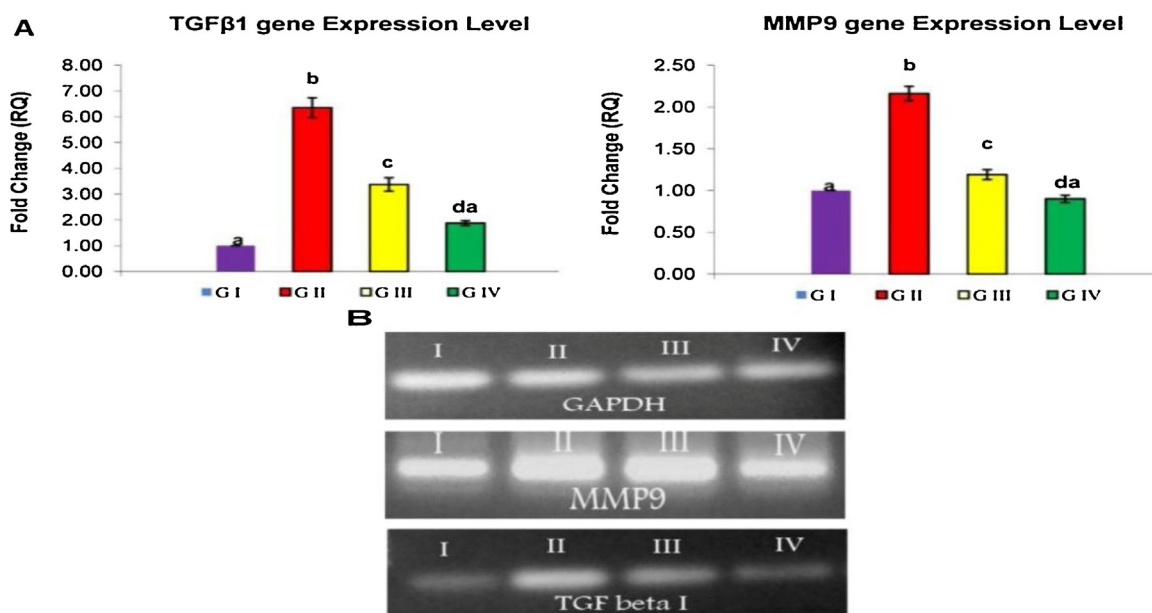
Fig. 1. Effect of tiron on TGFβ1 and MMP9 expression level in kidney intoxicated with TiO2 NPs. (**A**) Fold change of mRNA expression of TGFβ1 and MMP9 in rat kidney of different experimental groups using qPCR. *Data are presented as (Mean ± S.E). S.E = Standard error. Mean values with different superscript letters in the same row are significantly different at (p ≤ 0.05).* (**B**) The results obtained by qPCR analysis were confirmed by agarose gel electrophoresis analysis of the PCR product compared to GAPDH.
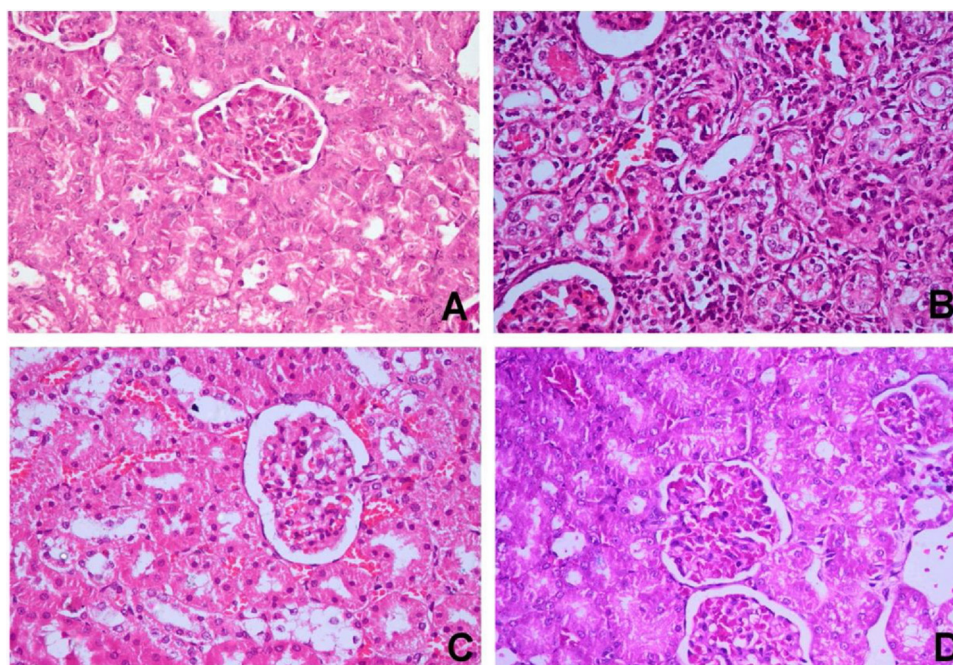
Fig. 2. Histopathological changes in the kidneys of the different groups (H&E X400). **A**. Group I showing normal histological picture; **B**. Group II showing marked interstitial fibrosis with mononuclear inflammatory cell aggregation, necrobiotic changes of renal tubular epithelium, thickening of the parietal layer of the Bowman's capsule and mesangial expansion (arrow); **C**. Group III showing partial adhesion of glomerular tuft to parietal layer of the Bowman's capsule, mild thickening of the parietal layer of the Bowman's capsule and mild necrobiotic changes of renal tubular epithelium; **D**. Group IV showing mild vacuoloation of renal tubular epithelium.

current findings come in accordance with results reported by Escárcega-González et al. [13] and Fartkhooni et al. [36]. Those elevations might by due to over deposition of TiO_2 NPs in kidney tissue (Table 2). Several evidences suggested the main role of oxidative stress in TiO_2 NPs induced toxicity [24,37]. Generation of ROS by metallic NPs such as TiO_2 NPs and subsequent depletion of antioxidant cell defenses can result in disruption of prooxident \antioxidant balance which increased the permeability of mito-chondrial membrane and induction of mitochondrial membrane depolarization [11].Under normal condition, the over production of ROS were neutralized by the antioxidant defense mechanisms which included both enzymatic and non enzymatic antioxidants.
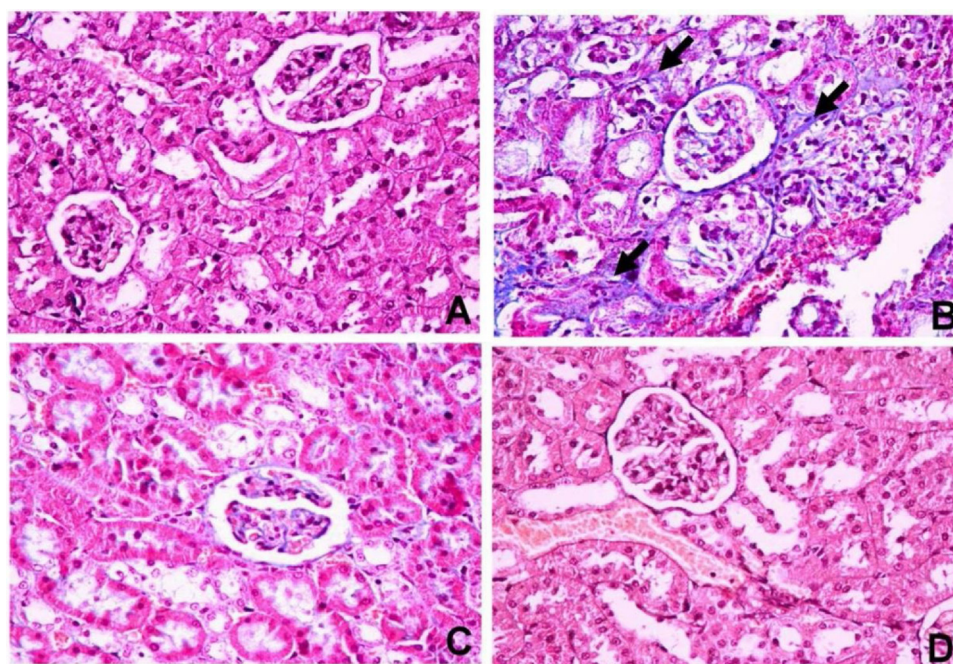

Fig. 3. Histopathological evaluation of kidneys stained with Masson's trichrome (MT) stain in different experimental groups (X 400). **A**. Group I showing normal collagen deposition; **B**. Group II showing marked interstitial fibrosis (arrow); **C**. Group III showing mild interstitial fibrosis; **D**. Group IV.
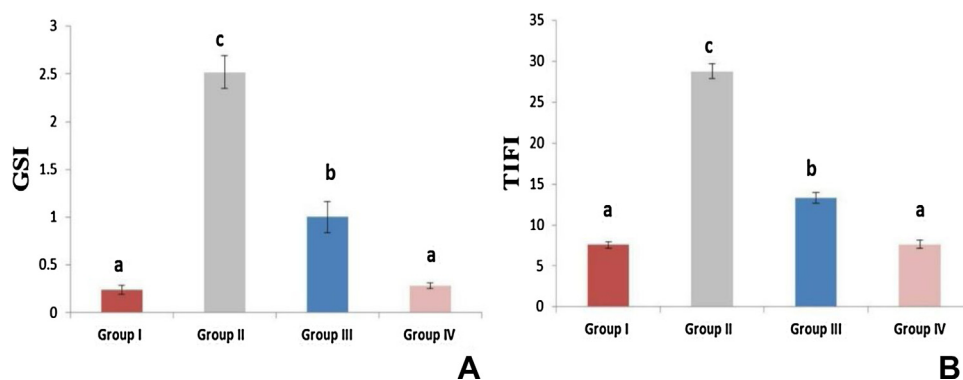
Fig. 4. The glomerulosclerotic index (A) and the tubulointerstitial fibrosis index (B) in the kidneys of different treated groups. Values with different superscripts are significantly different ($p < 0.05$).
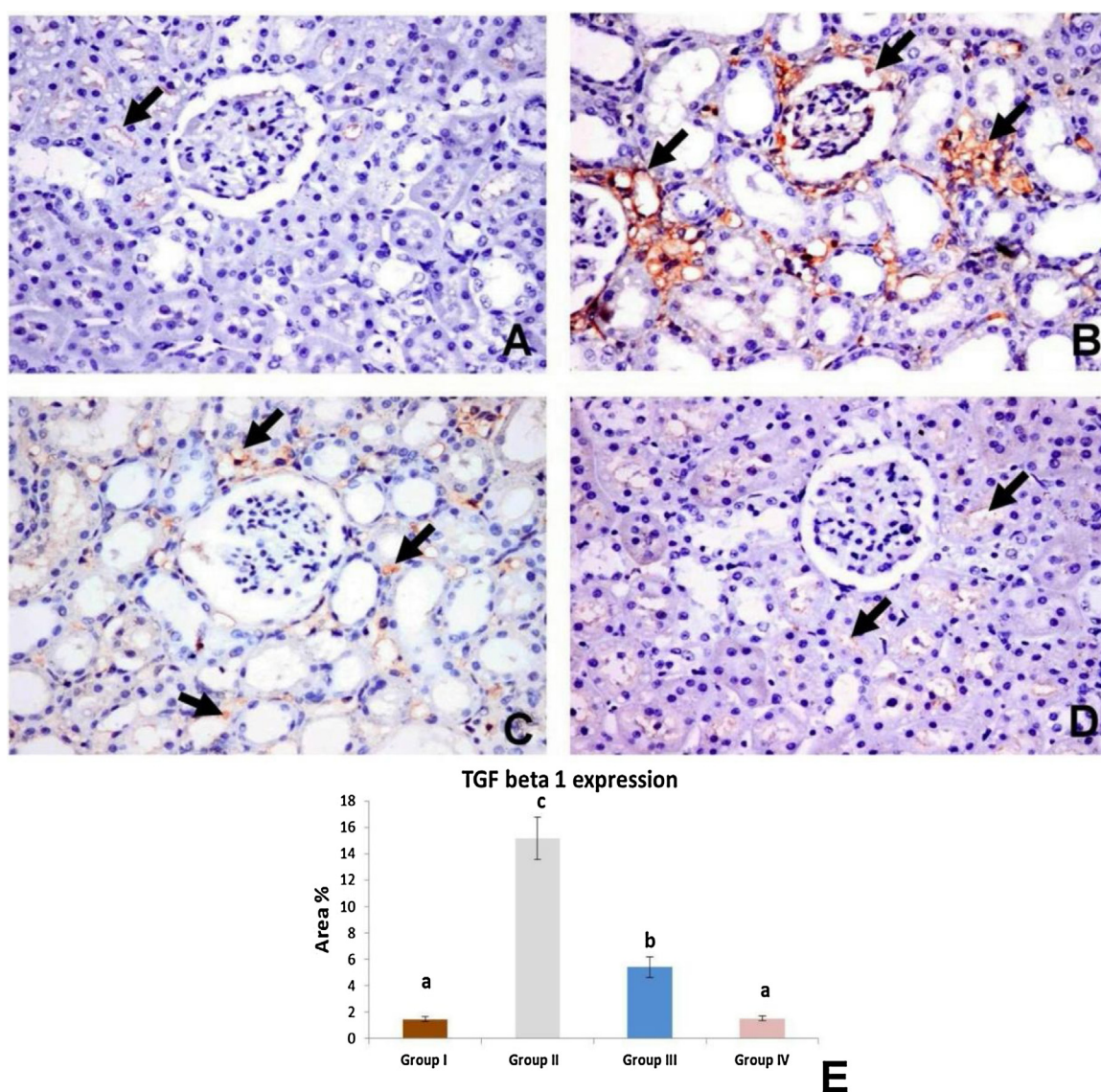
Fig. 5. Representative TGFβ1 immunohistochemistry in the kidney tissues of different experimental groups (X400). **A**. Group I showing little immunoreactivity (arrow); **B**. Group II showing intense immunopositive reaction (arrow) in the interstitial tissue and glomerular mesangial area; **C**. Group III showing reduced immunostaining reaction (arrow) in interstitial tissue; **D**. Group IV showing very weak immunostaining reaction (arrow); **E**. The bar chart represents TGFβ1 immunostaining expressed as area%. Values with different superscripts are significantly different ($p < 0.05$).

MMP9 expression
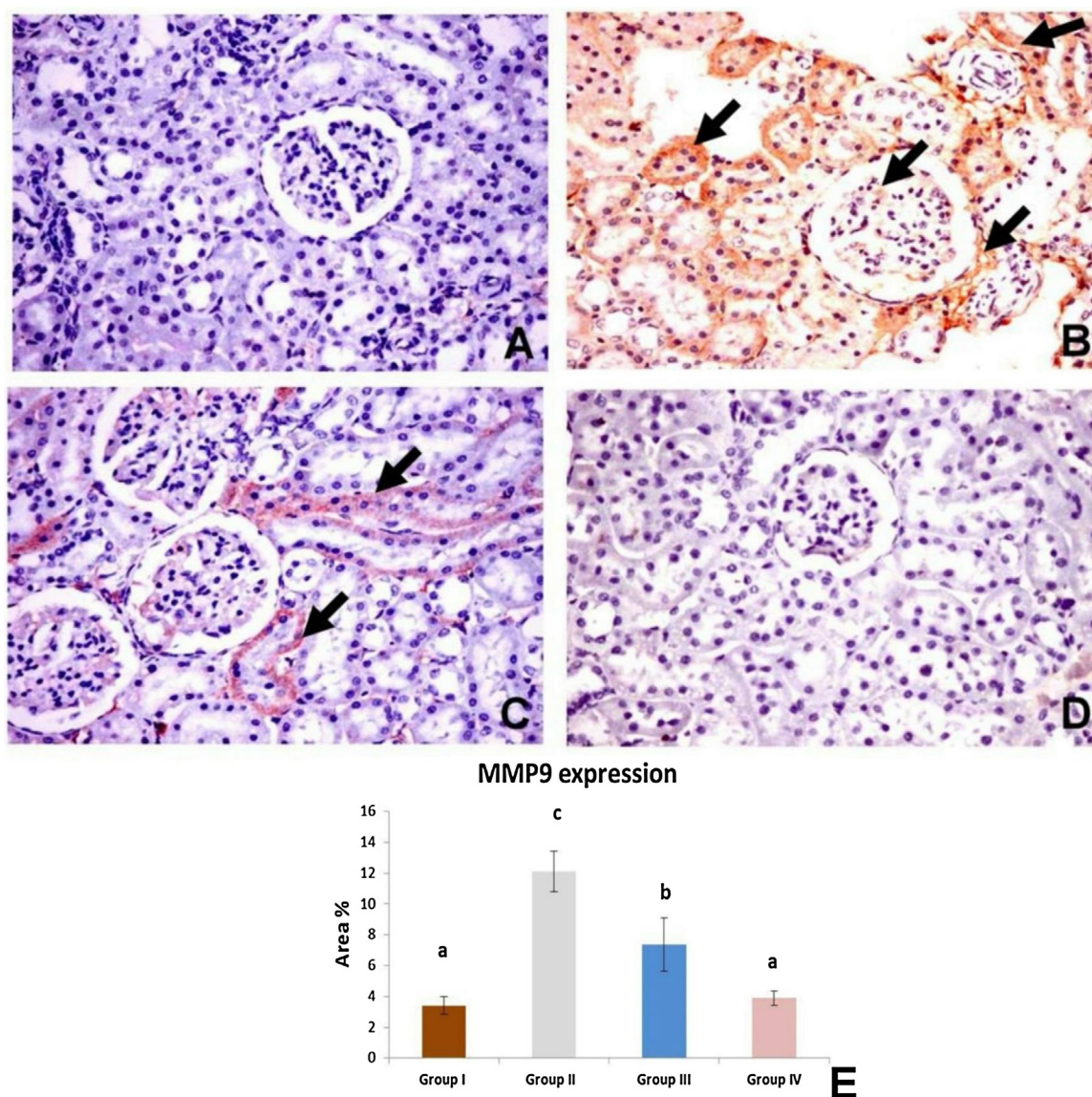
Fig. 6. Representative MMP9 immunohistochemistry in the kidney tissues of different experimental groups (X400). **A**. Group I showing slight immunostaining reaction; **B**. Group II showing strong tissue interstitial tissue; **C**. Group III showing mild immunopostive reaction in the renal tubules (arrow) and in the interstitial tissue; **D**. Group IV showing very weak immunostaining reaction(arrow); **E**. The bar chart represents MMP9 immunostaining expressed as area%. Values with different superscripts are significantly different (p < 0.05).

GSH is an important non enzymatic antioxidant that plays a crucial role in the detoxification of ROS. SOD and GPX are potent enzymatic antioxidants that scavenge harmful ROS, bringing the first line of defense against free radicals formed by metallic NPs. In the current study, the decreased GSH concentration, SOD and GPX enzyme activities and elevated MDA levels in kidney indicated that the generation of oxidative stress upon TiO$_2$ NPs intoxication. Natarajan et al. [38] reported that, TiO$_2$ NPs could induce oxidative stress and decreased the total antioxidant capacity and thus mediate LPO. Furthermore TiO$_2$ NPs stimulates cells to produce ROS and interferes with mitochondrial energy production [37].The mechanism of the generation of the oxidative stress after NPs treatment is not clear, but may related to its large particle surface area [39]. Jeon et al. [40] speculated that part of the ROS generation might be due to the catalytic properties of TiO$_2$ NPs. At the molecular level TiO$_2$ NPs significantly activated p38, c-Jun N-terminal kinase, nuclear factor kappa B, Nuclear factor-like 2 (Nrf-2) and heme oxygenase-1 expression, which in turn, led to

increased production of ROS, as well as lipid, protein and DNA peroxidation, these suggest that, TiO$_2$ NPs induced oxidative damage may occur via the p38-Nrf-2 signaling pathway [41]. The induction of oxidative stress in renal tissue upon TiO$_2$ NPs intoxication had been reported by several studies [9,11]. Accumulating studies showed that the exposure to TiO$_2$ NPs able to cause several renal pathological changes in the form of inflammation of the glomeruli, cell necrosis, degenerative changes and fibrosis [36,42] which come in accordance with the current data ( ). Renal fibrosis is a common pathological feature of all kinds of chronic kidney diseases. It is characterized by interstitial leukocyte infiltration, fibroblast accumulation and increased interstitial matrix deposition. To identify the ability of TiO$_2$ NPs to induce renal fibrosis, Masson trichrome staining, periodic acid Schiff's (PAS), glomerulosclerotic index (GSI),tubulointerstitial fibrosis index and the expression of main fibrotic genes (TGFβ1 and MMP9) were performed. The present data showed widespread renal fibrotic injury in histological examination of rat kidney tissue

exposed to TiO$_2$ NPs. To explore the potential renal fibrosis mechanism, TGFβ1 and MMP9 content were measured by immunohistochemistry and real time PCR. The TGFβ1 and MMP9 mRNA expression level were significantly elevated in TiO$_2$ NPs intoxicated group. Also marked increased for TGFβ1 and MMP9 protein expression indicated by increased number of immune-stained nephritic cells was observed.TGFβ1 a known profibrotic cytokine, it has been suggested to be a vital mediator in the pathogenesis of renal fibrosis [43]. It regulates progressive renal fibrosis by stimulating extracellular matrix production while suppressing its degradation [43]. Previous studies proved the over expression of TGFβ1 in renal fibrosis [10,42]. Huang et al. [42] and Gui et al. [9] demonstrated that, TiO$_2$ NPs exposure induced over expression of TGFβ1 in renal tissue. Renal inflammation and fibrosis following exposure to TiO$_2$ NPs may be triggered by the TGFβ1/Smads/p38 mitogen-activated protein kinase (MAPK) pathway [10]. TiO$_2$ NPs appeared to significantly inhibited Smad7 expression, resulting in up-regulation of TGFβ1, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-jB) and enhancing renal inflammation and fibrosis and this implies the potential role of Smad7-NF-jB crosstalk pathway in the renal inflammation and fibrosis following exposure to TiO$_2$ NPs [10]. Huang et al. [42] concluded that TiO$_2$ NPs might induce renal fibrosis through a ROS/reactive nitrogen species-related hypoxia inducible factor 1α (HIF-1α) up-regulated TGFβ1 signaling pathway. In the same sequence a pro-fibrotic role of MMP9 well established in renal fibrosis [44]. It promotes epithelial-mesenchymal transition of tubular epithelial cells leading to kidney fibrosis in both tubule-interstial and glomeruli compartments [45,46]. Wan et al. [47] reported that some transition metal NPs had the ability to stimulate an imbalance of matrix metalloproteinase (MMP)\tissue inhibitor matrix metalloproteinase that cause MMP over expression. The over expression of MMP9 upon TiO$_2$ NPs intoxication was reported by Ambalavanan et al. [48]. Oxidative stress, apoptosis, and the inflammatory response are believed to be the main mechanisms underlying the toxicity of metallic NPs [49]. Several papers have disclosed that antioxidant co-treatment can attenuate the toxicity induced by metallic NPs [4,14,49]. The efficiency of tiron the mitochondrial targeting antioxidant in the acceleration of TiO$_2$ NPs elimination and in the reversal of renal fibrosis has been discussed in the present study. Tiron at the investigated dose (470 mg/kg) significantly improved the renal indices, antioxidant content and histopathological picture of renal tissue as well as decrease renal Ti content and the expression level for the main renal fibrotic markers (TGFβ1 and MMP9). Tiron significantly reduce the serum level of urea and creatinine the markers for renal dysfunction inducing improvement of kidney function and this may be due to the metal chelating properties of tiron which reduce the deposition of TiO2 NPs in kidney tissue. Tiron treatment could markedly increase the renal antioxidant capacity by restraining the content of MDA and simultaneously enhancing the activities of GPx and SOD (Table 3). Tiron is considered as a beneficial protective agent against the inducer of oxidative stress [50]. The small size of tiron allows it to easily enter inside the cells and therefore modifies intracellular electron transfer reactions by antioxidant mechanism via scavenging free radicals [51]. It is an effective antioxidant that can also prevent the inactivation of antioxidant enzymes [17]. The diphenolic nature of tiron allows it to forms water soluble complexes with a large number of metals leading to its elimination [17].Tiron appeared to improve the expression of Klotho gene which increases the resistance of kidney against oxidative stress [15]. In the same line such enhanced expression and activation of fibrotic markers (TGFβ1 and MMP9) due to TiO$_2$ NPs were significantly reversed upon co-treatment with tiron antioxidant (Figs. 1, 5, 6). Tiron blocked the over expression of TGFβ1 in renal

proximal tubular cell and this may be postulated to its antioxidant action [52]. In the same sequence Lu et al. [53] reported that tiron is a potent inhibitor of the expression of MMPs via the inhibition of the MAPK/Activator protein 1 (AP-1) signaling pathway.

5. Conclusion

Our findings revealed the tiron ability to abrogate renal pathological changes coupled with severe oxidative stress and fibrosis induced by long period of exposure to TiO$_2$ NPs. Tiron potency may be mediated through its radical scavenging and metal chelating antioxidant properties. Extra caution should be taken in the handling of higher dose TiO$_2$ NPs.

Competing interests

"The authors declare that they have no competing interests to disclose."

Authors' contributions

Prof Dr. Ashraf Morgan and Dr Peter Noshy participated in the study design, Dr. Marwa Ibrahim performed the molecular genetic studies, and Dr. Hanan Abdelsalam evaluated the biochemical parameters and helped to draft the manuscript. Dr Mona Khames evaluated the biochemical parameters and drafted the manuscript. Dr Reham Mohamad performed the pathological and immunohistochemical analysis. All authors read and approved the final manuscript.

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