RENOPROTECTIVE EFFECT OF BAICALIN AGAINST TITANIUM DIOXIDE NANOPARTICLES INDUCED NEPHROTOXICITY IN RATS

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Abstract. The safety of titanium dioxide nano-particles (TiO2NPs) is currently being questioned. TiO2NPs have multiple uses in disinfectants, plastics, cosmetics, and food coloring. Our goal was to determine if administering Baicalin (50 mg/kg body weight) could help lessen the harmful effects caused by TiO2NPs (100 mg/kg body weight) in rats. By reducing kidney damage from TiO2NPs, treatment with Bai led to lower levels of creatinine (Cr), urea (Ur), and uric acid (UA). The harmful effects of TiO2NPs can be counteracted by Baicalin, which has demonstrated its ability to protect the kidneys. Additionally, it can restore balance between oxidation and antioxidants by increasing CAT, SOD, GSH, and reducing malondialdehyde levels. Not only does it exhibit anti-inflammatory effects by downregulating IL-6 and TNF- and increasing IL-10, but it also contributes to maintaining equilibrium in the body.

Keywords: renoprotactive, baicalin, TiO2NPs, nephrotoxicity.

List of Abbreviations

TiO2NPs – Titanium dioxide nanoparticles
ROS – Reactive oxygen species
MDA – Malondialdehyde
SOD – Superoxide dismutase
CAT – Catalase
GSH – Glutathione
TNF-α – Tumor Necrosis factor –alpha
LPO – Lipid peroxidation
NO – Nitric oxide

Introduction

The growing use of Titanium dioxide nanoparticles (TiO2NPs) has raised safety concerns. TiO2NPs is utilized as a food dye, cosmetic ingredient, plastic additive, and antibacterial agent (Cao *et al.*, 2018). Titanium dioxide (TiO2NPs) with a nanometer-scale size distribution has become commercially available and widely used as an additive in numerous sectors over the past few decades (Center, 2007). These sectors include the food industry, pollution control materials, the pharmaceutical industry, the personal care industry, and the cosmetics industry. As a result, more people are being exposed to TiO2NPs via a variety of channels. Oral exposure may occur by direct eating of goods developed with large quantities of TiO2NPs as nano-food or nano-medicine (Cao *et al.*, 2018). Inhalation and dermal exposure of industrially produced TiO2NPs are regarded the major pathways of TiO2NPs exposure. The buildup of TiO2NPs in diverse tissues after their administration through different routes has been linked to possible toxicological consequences (Li *et al.*, 2016; Lockwood *et al.*, 2010). By altering molecular complex structure and cellular membrane permeability (Diego *et al.*, 2013; Gao *et al.*, 2008), Under oxidative stress, TiO2NPs have the potential to induce genetic toxicity and cause oxidative damage. This is due to the production of reactive oxygen species (ROS), specifically hydroxyl radicals, which can result in DNA oxidation. This leads to the formation of 8-OHG and subsequently replication errors and mutations (Li *et al.*, 2016; Rajab & Ali, 2015). Genetic instability and cytotoxicity occur due to TiO2NPs releasing oxygen-free radicals, which in turn, cause DNA damage (Lockwood *et al.*, 2010). Due to the role that oxidative stress and inflammation play in various clinical diseases; some studies have suggested that agents with antioxidant properties could be beneficial. As a result, TiO2NPs have been found to trigger inflammatory processes and apoptosis, potentially causing kidney damage. It has been demonstrated in multiple studies that pretreatment or co-treatment with antioxidants can be effective (Gao *et al.*, 2008; Popovic *et al.*, 2008) to mitigate the harmful effects of metallic NPs.

After being initially isolated, baicalin has found extensive use in several countries, such as China and Southeast Asian nations. It has been discovered through research that baicalin possesses various biological effects including anti-apoptotic, antioxidant, anti-inflammatory, and immunological modulatory properties. Our team's previous studies have successfully shown baicalin's effectiveness in managing inflammation and protecting against oxidative damage (Liang *et al.*, 2023a). Baicalin has been shown to reduce Pb-induced oxidative damage, although its exact effects and mechanisms have not been reported. Therefore, we looked at how baicalin affected oxidative damage caused by Pb in living organisms. This paper provides a review of Baicalin's potential effects as an antioxidant and metal chelator in protecting against nephrotoxicity and renal fibrosis in rats exposed to TiO2NPs.

Materials and Methods

Chemicals

We ordered two special things from Sigma Aldrich in St. Louis, MO, USA- Baicalin (Bai) and Titanium dioxide nanoparticles (TiO2NPs). All the materials and solutions we used were up to par with lab-grade standards or better.

Animals

This inquiry, a-okayed by the ethical board, tracked world-renowned principles and regulations for the care and use of research critters. After two weeks to get used to their lab abode, adult male Wistar albino rats – weighing from 180 to 200 grams – were borrowed from the Laboratory Animal Center at Veterinary Medicine school in Baghdad University. They were placed in plastic cages with new wood shavings every day, as well as provided pellet food and a water basin that was always full.

Experimental design

Rats were randomly separated into four groups of ten animals each after acclimatization, as follows:

1. Control (Cont) group: simply a regular diet and tap water were given to the rats.

2. Baicalin (Bai) group: Bai (50 mg/kg.bw) was administered orally to rats using a gastric tube for four weeks.

3. Titanium dioxide nanoparticles (TiO2NPs) group: rats were orally given TiO2NPs with gastric tube (100 mg/kg.bw) for 4 weeks.

4. Titanium dioxide nanoparticles (TiO2NPs) + Baicalin (Bai) group: rats were orally given TiO2NPs (100 mg/kg.bw) and Bai (50 mg/kg.bw) for 4 weeks.

Sample collection

At the end of our experiment, we hypnotized the rats with ketamine/xylazine through their veins (0.1 ml/100g b.w). Then we bled them, putting the serum in a freezer at -20 °C for further research. The kidneys were taken too and cleansed. We also chopped off a bit of liver and whizzed it in a blender. Lastly, the remaining pieces were preserved in formalin to do more studies.

Biochemical analysis

1. Determination of kidney function:

Using kits purchased from Spinreact (Girona, Spain), the serum's uric acid, creatinine (Cr), and urea (Ur) levels were determined to monitor kidney function.

2. Determination of oxidative stress markers and antioxidants:

The Kidney homogenate contained levels of malondialdehyde (MDA) that were measured using the BioDiagnostic Kit. This kit was also utilized to estimate activities of superoxide dismutase (SOD) and catalase (CAT) in addition to the levels of glutathione (GSH) in liver tissue. Dokki, Giza, Egypt provided the aforementioned Bio Diagnostic Kit.

3. Determination of pro-inflammatory cytokines:

Serum levels were assessed for TNF-α, IL-6, and IL-10 using BosterBio kits from California, USA.

Histological examination of Kidney sections

Samples of rats kidneys were preserved in a buffered formalin solution for 24 hours. Following this, the kidneys underwent dehydration

and were subsequently coated with paraffin. This unique procedure facilitated the examination of histological changes through the application of Hematoxylin and eosin (H&E) staining on 5µ-section samples. The slides were carefully inspected using a light microscope, and photographs were taken to document the observed alterations.

Immunohistochemical examination of Kidney sections

The kidney sections of rats were first processed by removing paraffin and adding water, then put in xylene and ethanol to be hydrated. To retrieve the antigens, a microwave was used. To prevent the enzyme peroxidase from being active, a solution of hydrogen peroxide and methanol was applied at a temperature of the room for 20 minutes. To stop any non-specific binding, serum was used. The primary antibodies, Caspase-3 from Invitrogen, and Bcl-2 from Santa Cruz Biotechnology, were then left to incubate with the sections overnight at a temperature of 4 °C. Following incubation with a 3,3′-diaminobenzidine-tetrahydrochloride-hydrogen peroxide solution to induce color development, the sections were observed under a light microscope. Secondary antibodies were then introduced after a wash with PBS. Mayer's hematoxylin was used as a counterstain.

Statistical analysis

Statistical analysis, including one-way analysis of variance and Duncan's multiple range tests, was performed using GraphPad Prism 7.02. The mean along with the standard error of n = 5 was reported to assess the significance of differences among groups.

Results

Kidney function tests

In comparison with control group, the oral administration of TiO2NPs for 4 weeks has shown significant increases in the serum levels of Kidney function such as Cr, Urea, and UA. However, rats treated with Bai exhibited significant decreases in the serum levels of the mentioned diagnostic markers of kidney injury, as compared to those treated with TiO2NPs.

Oxidative stress and antioxidants markers in kidney

Analysis of kidney MDA levels aimed to evaluate the effects of Bai treatment on lipid peroxidation in different groups. Findings indicated a significant rise in MDA levels in TiO2NPs-treated rats' kidneys compared to control rats. However, administration of Bai successfully alleviated this increase in MDA levels in TiO2NPs-treated rats. Interestingly, rats exclusively receiving Bai showed no alteration in MDA levels when compared to the normal control group. The rats that were given TiO2NPs experienced a significant drop in GSH levels and the functioning of SOD, CAT in their kidneys, which was not seen in the control rats. However, when the TiO2NPs treated rats were given Bai supplementation, their GSH levels and the functioning of SOD, CAT in their kidneys were protected. This effect was not seen in the untreated TiO2NPs treated rats.

Pro- and anti-inflammatory cytokines

Rats, after a period of 4 weeks, demonstrated a remarkable decrease in the presence of the pro-inflammatory elements TNF- and IL-6, a stark contrast to the control group. Conversely, the mice administered TiO2NPs exhibited noticeably elevated levels of TNF- and IL-6, while concurrently experiencing diminished levels of IL-10 when juxtaposed with the control group. Furthermore, the rats treated with Bai encountered a surge in the occurrence of anti-inflammatory cytokines (IL-10).

Kidney histopathology

In the control group, the histological results revealed the kidney's normal structure. Bai's group showed nearly normal histological features, with slight glomerular hypertrophy. The TiO2NPs group displayed numerous histological impairments, such as glomerular atrophy, content fragmentation, and a widened Bowman's capsule. Some glomeruli experienced ruptures in their Bowman's capsule walls. Additionally, there was desquamation in certain urinary tubule linings and necrosis in some tubule cells, along with degeneration in others. The fourth group exhibited milder tissue lesions

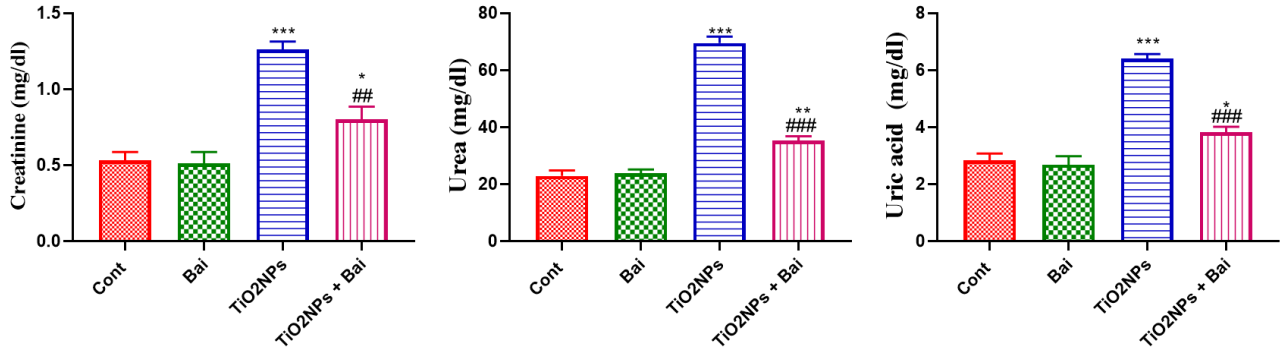

Fig. 1. The effect of (TiO2NPs) and Bai on the average concentration of Cr, Urea and UA (mg/DL) in the Rat's serum in different classes:
✓ Values are expressed as "mean ± SD" n = 7
✪ Relative to the Bai group
* versus those in the TiO2NPs group
\# comparison of TiO2NPs + Bai group

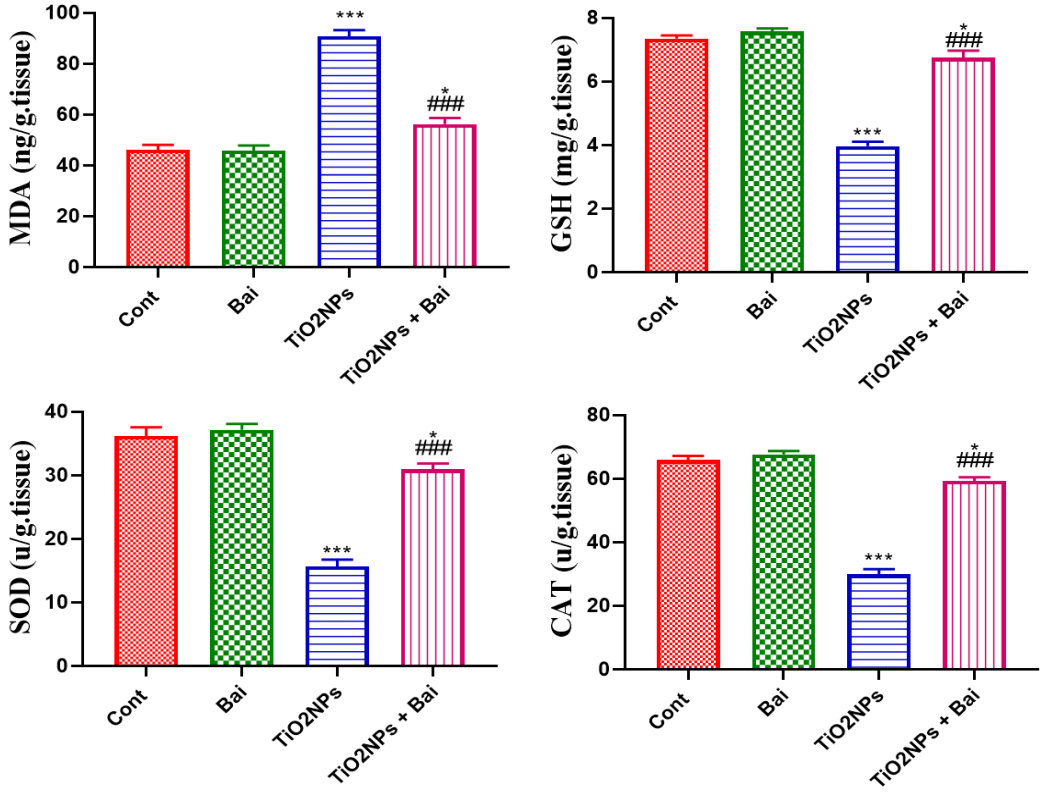

Fig. 2. Various parameters including lipid peroxidation products MDA, superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) concentration were assessed in different groups of rat serum to evaluate the impact of TiO2NPs and Bai:
✓ Values expressed as "mean ± SD" n = 7
✪ Relative to the Bai group.
* versus those in the TiO_2NPs group.
\# comparison of TiO2NPs + Bai group

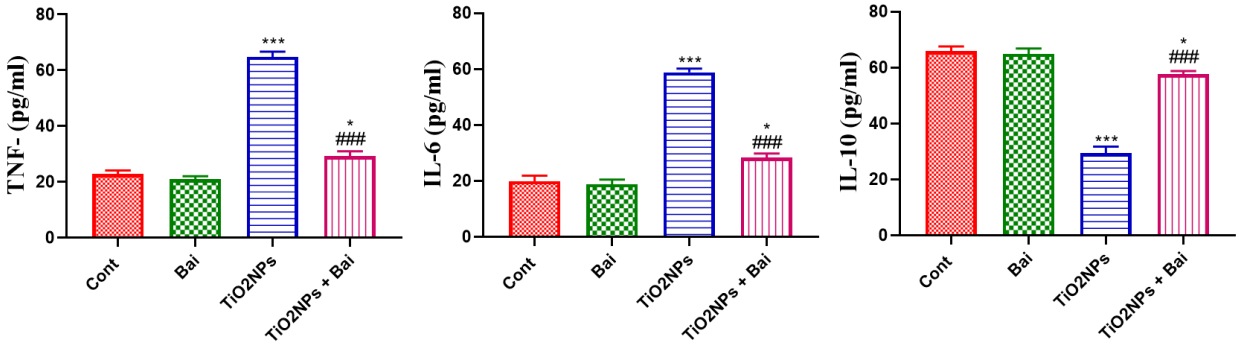

Fig. 3. Serum levels of IL-6, IL-1b, and TNF-α were examined to determine the impact of Bai and (TiO2NPs). Varying among the groups of rats, the expression levels were observed in pg/ml:
✓ Values expressed as the "mean ± standard deviation" of n = 7.
✪ Relative to the Bai group
* versus those in the TiO_2NPs group
\# comparison of TiO2NPs + Bai group

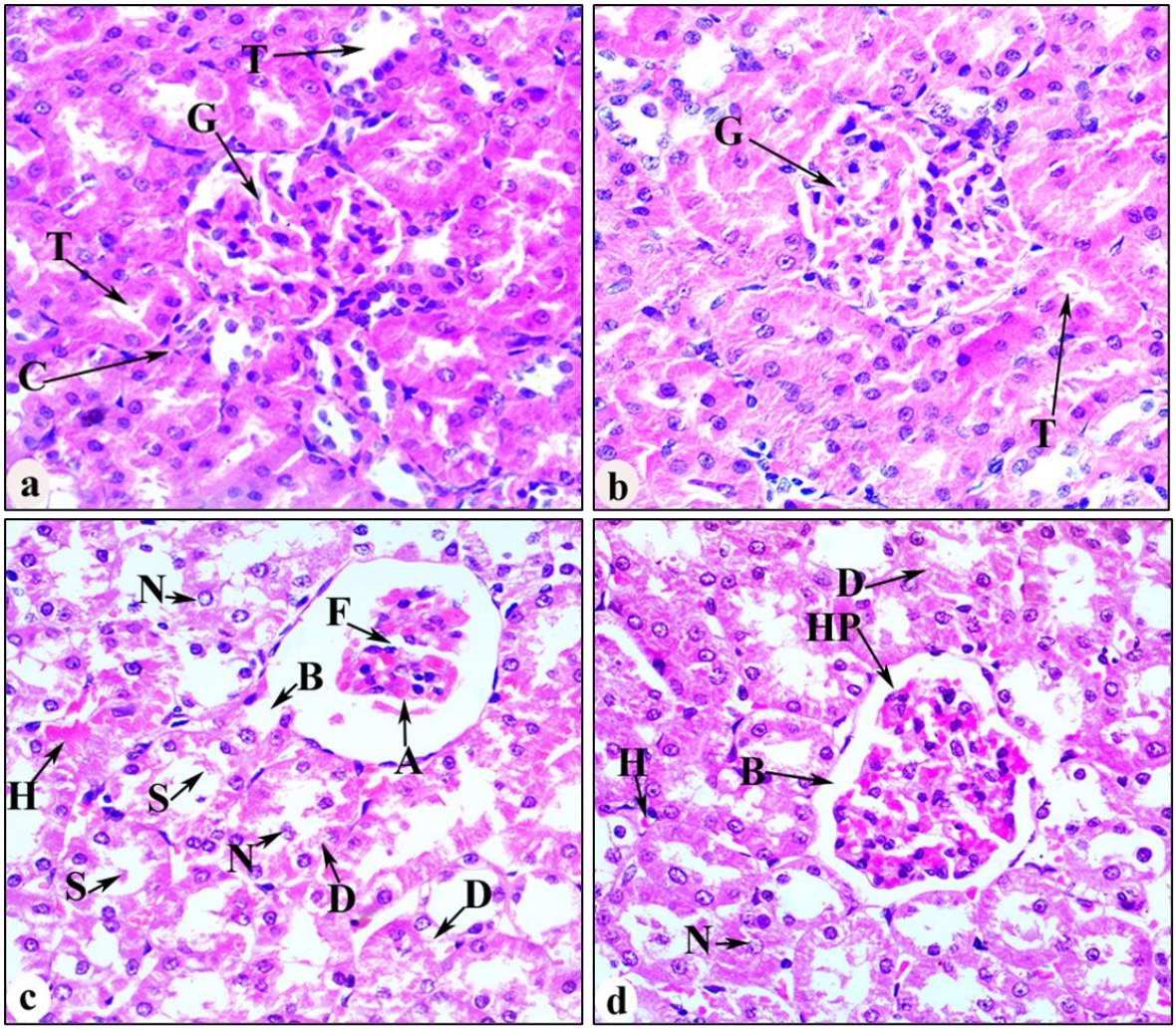

Fig. 4. Histological structure of the rat kidney. (a) Control group. (b) The Bai Group. (c) Group of TiO2NPs. (d) TiO2NPs + Bai groups. Abbreviations: G (glomerulus); T (tubule); C (interstitial connective tissue); N (necrosis); H (hemorrhage); D (degeneration); S (desquamation); B (Bowman's capsule); A (atrophy); F (fragmentation); HP (hypertrophy). Hematoxylin and eosin staining (400X); (scale bar is 25 µm)

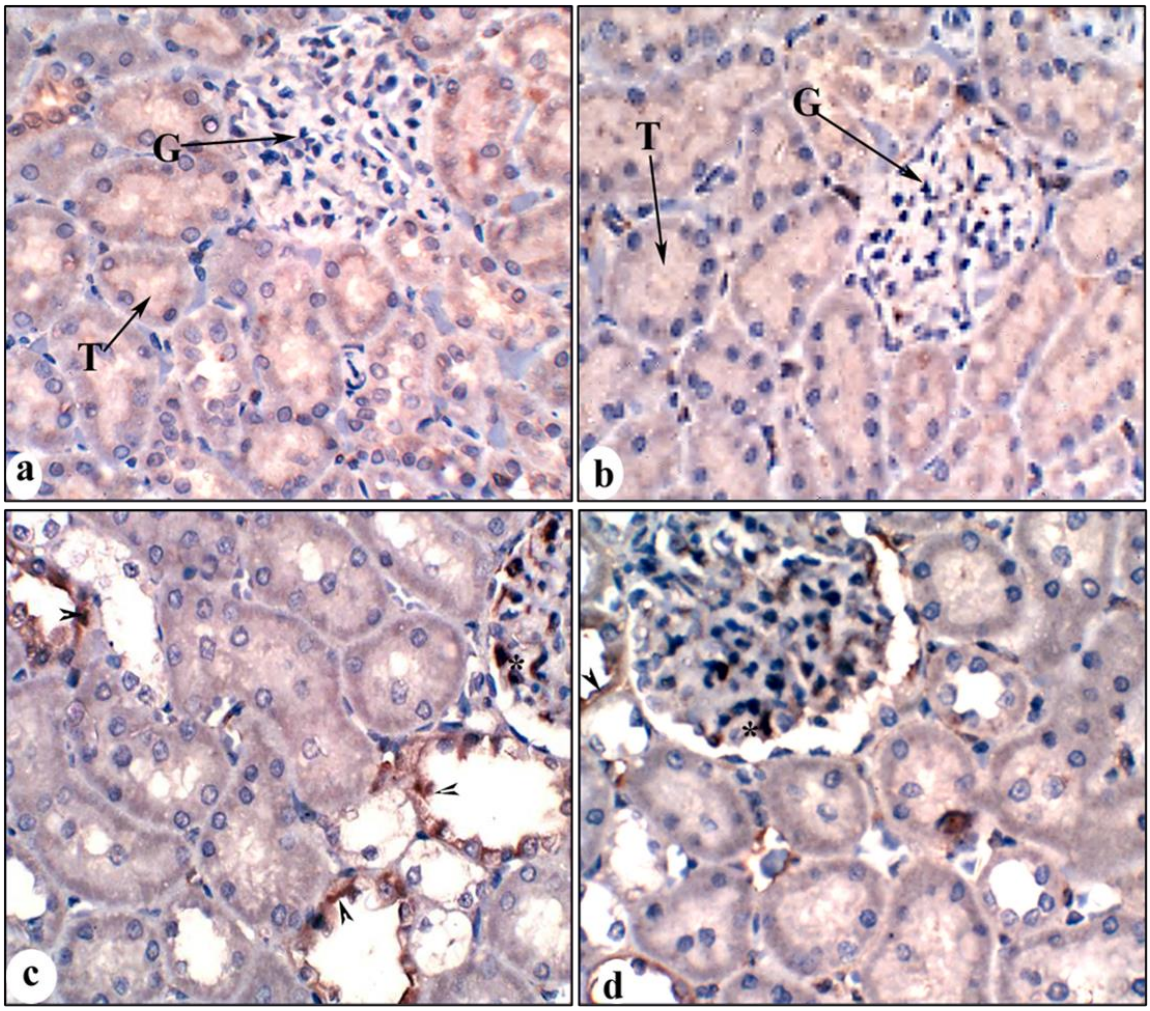

Fig. 5. Immunohistochemical Localization of Casp-3 in Rat Kidney Tissue. (a) Control kidney section. (b) Kidney sections treated with Bai. (c) Kidney slices treated with TiO2NPs. (d) Kidney slices treated with TiO2NPs and Bai. Abbreviations: G (glomerulus); T (tubule); arrow (positive response to T); one star (positive response to G) (400X); (scale bar 25 μm)

compared to the previous group, including an enlarged kidney and expansion of Bowman's space. Additionally, there was evidence of necrosis, degeneration, and hemorrhage within the kidney tissue (Fig. 4), albeit in a lesser extent compared to the third group. Interestingly, hemorrhage was observed between the contents of the kidney tissue, further contributing to its appearance.

Immunohistochemical results

Casp-3 data for the control group and the second group treated with Bai did not reveal any interaction. However, Bowman's capsule and a few glomeruli cells responded strongly and favorably to the TiO2NPs group. Notably, the walls of particular urinary tubules showed a significant reactivity. Regarding the fourth group, some glomerulus cells and urinary tubules showed a moderate contact.

When using Bal-2, no interaction was seen in the control group and the Bai group as well (Fig. 6a, b). While a strong interaction appeared in the cells of the wall of Bowman's capsule and some cells of the glomerulus, as well as the cells of the walls of some urinary tubules in the TiO2NPs group (Fig. 6c). While in the group treated with TiO2NPs with Bai, the reaction was weak in the above components (Fig. 6d).

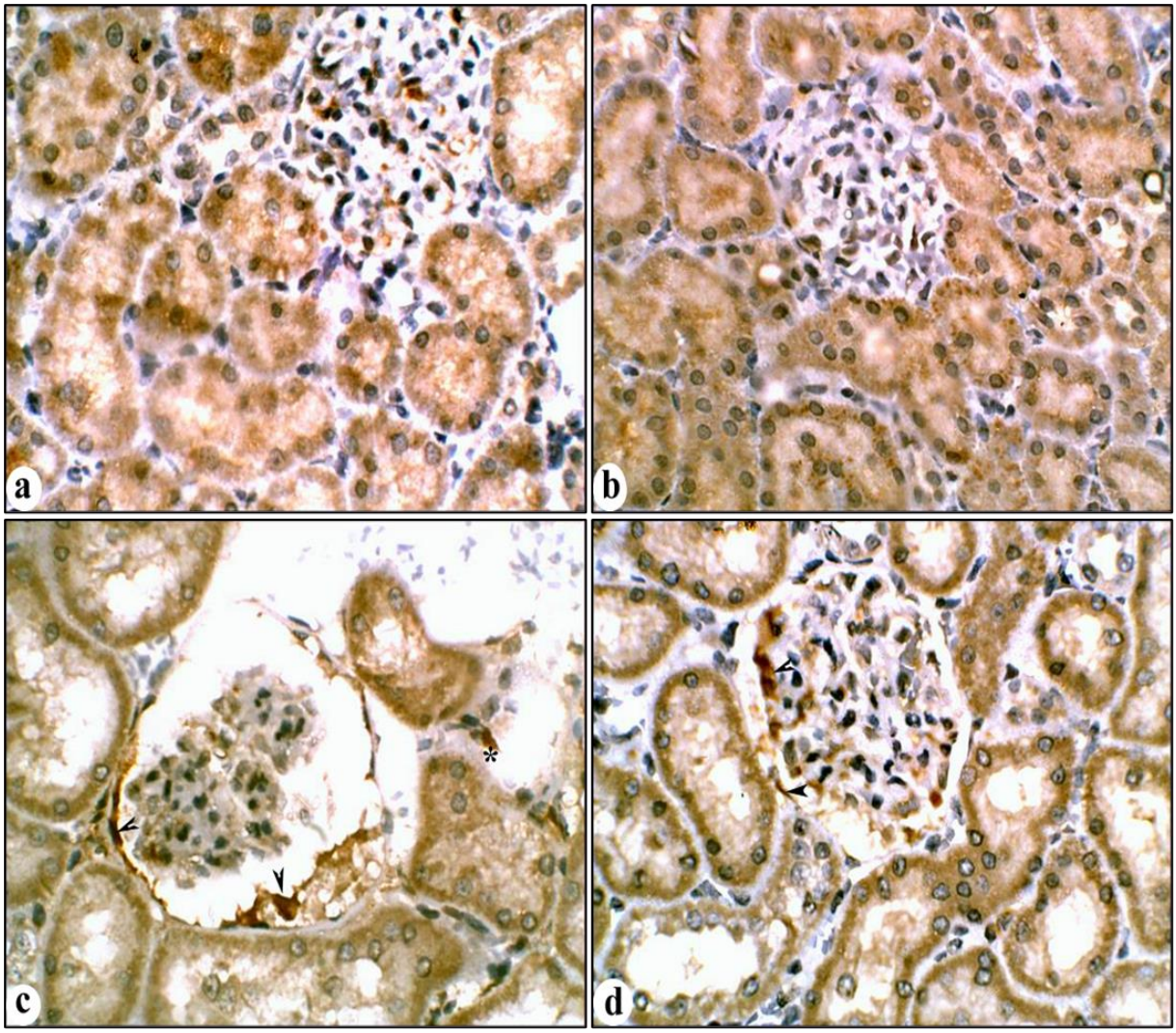

Fig. 6. Immunohistochemical localization of Bcl-2 in rat kidney tissue. (a) Control kidney section. (b) Kidney sections treated with Bai. (c) Kidney slices treated with TiO2NPs. (d) Kidney slices treated with TiO2NPs and Bai. Abbreviations: Arrow (positive reaction in glomeruli); one asterisk (positive reaction in tubules) (400X); (scale bar is 25 μm)

Discussion

TiO2NPs is widely used as a second generation of macrolide antibiotic. However, accumulating evidence indicates that prolonged use of TiO2NPs increase risk of Nephrotoxicity as elevation in activities of kidney function enzymes and proteins (Martinez *et al.*, 2015). The current study describes the natural flavonoid Baicalin's nephroprotective properties against TiO2NPs-induced nephrotoxicity through reducing oxidative stress and inflammation.

As a result of the kidneys' diminished capacity to filter creatinine and non-protein waste products, we discovered that rats treated with TiO2NPs had impaired renal function, with noticeable proximal tubule damage, elevated serum creatinine and BUN levels, and an increase in total protein excretion in urine.

The crucial markers for kidney damage, elevation levels of creatinine and BUN, were prevented and ameliorated by treatment with Baicalin, thus preserving histological integrity. The activities of creatinine and BUN enzymes, which indicate injury to renal tubules and are associated with cell death (Liu *et al.*, 2023; Wu *et al.*, 2023), saw an increase. In agreement with

previous studies, it was found that rats treated with TiO2NPs had higher levels of creatinine and BUN in their serum. These elevated levels are indicative of kidney dysfunction, which is believed to be caused by the disruption of nephron membrane architecture and cellular leakage caused by TiO2NPs administration (Wang *et al.*, 2023).

Nephrotoxicity caused by TiO2NPs has been documented in numerous investigations. Smaller TiO2NPs have been found to be widely distributed throughout the body, especially in the kidneys and plasma. According to our research, male rats exposed to TiO2NPs for 7 days develop kidney damage, which is shown by elevated plasma levels of creatinine and urea nitrogen (BUN), as well as abnormal changes in the proximal and distal convoluted tubules (PCTs) like dilatation, vacuolization, and necrosis.

Bai treatment in rats treated with TiO2NPs resulted in a noticeable reduction in creatinine and urea activities, while albumin levels increased in comparison to TiO2NPs-treated rats. These findings suggest a positive effect on the overall health of the kidneys. Previous research has also shown the strong ability of Bai to protect against kidney damage caused by various toxins and medications (Louis *et al.*, 2023; Qin *et al.*, 2023; Wang *et al.*, 2023).

The cause of TiO2NPs-induced nephrotoxicity is not completely known, but it is thought to be caused by oxidative stress and inflammation. Zhu *et al.* (2023) discovered that TiO2NPs can lead to kidney injury by producing an excess of ROS, which triggers lipid peroxidation (LPO). This damages the Nephron membrane and causes kidney enzymes to be released into the bloodstream.

The liver relies on Cytochrome P450, an enzyme type, to control the breakdown of drugs and other foreign substances (Jastrzębska & Daniel, 2023; Stanley, 2024). One interesting finding is that while cytochrome P450 metabolizes TiO2NPs, it produces reactive oxygen species (ROS) and unstable molecules. These reactive compounds create oxidative stress, which damages key membrane elements like lipids and proteins, resulting in the creation of end products such as malondialdehyde (MDA) and protein carbonyl (Pc) (Mhadhbi *et al.*, 2020).

TiO2NPs has been shown in rat experiments to cause lipid peroxidation in renal tissue. Recently, it was discovered that rats given TiO2NPs had significantly higher levels of hepatic MDA as a result of lipid peroxidation. In addition, compared to the control group, the levels of antioxidant enzymes like SOD and CAT were much lower, and there was less GSH present in the kidneys (Du *et al.*, 2023; Ibrahim *et al.*, 2023).

The harmfulness of TiO2NPs has been associated with an increase in the generation of reactive oxygen species (ROS) and free radicals (Ghorbani *et al.*, 2023; Khalid *et al.*, 2023). This leads to damage in the kidney and other organs (Du *et al.*, 2023), as the proteins, lipids, and nucleic acids are directly impacted by ROS. Our study reinforces earlier discoveries, indicating that exposure to TiO2NPs results in a noticeable rise in LPO, as evidenced by elevated levels of MDA (a byproduct of LPO), and a decrease in GSH and total protein thiols (-SH). TiO2NPs caused a decrease in LPO and GSH and total SH were also affected by NSO. After TiO2NPs treatment, a decrease in antioxidant enzyme activity was observed in the renal cortex and medulla, indicating a compromised renal antioxidant defense system and reduced effectiveness against ROS.

An increase in SOD and CAT activities and an elevation in GSH levels were observed after administering Bai to animals, leading to a decrease in MDA levels. These results strongly indicate that Bai has the ability to boost the liver's antioxidant defense system, resulting in a decline in lipid peroxidation. Furthermore, the enhancement of kidney structure and the reduction in kidney function enzymes may be attributed to the improved liver health. Extensive research has consistently demonstrated that oral consumption of Bai can effectively alleviate oxidative stress and bolster the antioxidant defense system, thereby potentially safeguarding the kidney (Majeed *et al.*, 2023; Park and Song, 2019; Shanmugam *et al.*, 2016).

In a prior work, the addition of TiO2NPs affected the cytokine IL-6's expression levels (Kamal *et al.*, 2023). Overproduction of ROS results in oxidative stress, which is mediated by inflammation (Yao *et al.*, 2023). Our findings are consistent with this earlier investigation, as rats given TiO2NPs showed a significant rise in serum levels of the pro-inflammatory cytokine IL-6 and a decline in the anti-inflammatory cytokine IL-10. Free radical-induced inflammation is commonly acknowledged to be a key factor in the nephrotoxicity of TiO2NPs (Yang *et al.*, 2023). According to a study, TiO2NPs caused kidney injury by overproducing nitric oxide (NO) in hepatic tissue, which then triggered the release of inflammatory cytokines and ultimately led to cell death (Li & Tang, 2023). TiO2NPs-treated Bai rats that received supplements displayed a substantial rise in IL-10 levels. In the meantime, IL-6 levels dropped.

Baicalin supplementation reduces levels of proinflammatory cytokines IL-1β, IL-6, and TNF-α, as shown in a study by Lv *et al.* (2023).

According to Wang *et al.* (2023), there is evidence that treatment with Bai can reduce damage in the renal tubules and repair the structure of kidney cells. This finding supports our examination of kidney tissue and biochemical data. In particular, we observed a significant improvement in the structure of the kidneys in rats treated with Bai following exposure to TiO2NPs, compared to rats treated only with TiO2NPs.

In this study, it was observed that the group treated with TiO2NPs showed histological lesions, and Bai was found to be effective in reducing these lesions. Abdulkareem and Rabee (2020) conducted an experiment where mice were treated with her TiO2NPs substance for 30 days, at a concentration of 600 mg/kg body weight. This treatment resulted in glomerular congestion, tubular congestion, atrophy, and chronic inflammatory cell infiltration. Additionally, late squamous cell atrophy was observed in the tubules. Salama *et al.* (2023) attributed these complications and pathological changes in kidney structure to the excessive accumulation of these nanoparticles within the kidney tissue, leading to the development of oxidative stress, which he stated to be a common pathological mechanism responsible in its formation and development for cell damage. While Hussain *et al.* (2023) consider that ROS are produced in excess or antioxidant defenses necessary for metabolism are reduced when a cell is under oxidative stress. Damage from ROS includes changes in cellular macromolecules such as membrane lipids, nucleic acid, and protein. Cell function may be altered by changes in intracellular calcium, sodium, or pH as a result of damage, which may ultimately result in cell death (Park *et al.*, 2023). Therefore, it is believed that the histopathological changes that appeared in this study are due to the ability of TiO2NPs to trigger oxidative stress within the cells of the glomeruli and urinary tubules, which led to the occurrence of these histological damages. Bai has an important role in reducing it by reducing oxidative stress and inhibiting free radicals formed from TiO2NPs.

Immunohistochemical results

The TiO2NPs group had considerably greater levels of caspase-3 expression, which diminished when Bai was added. This suggests that the apoptotic effect of TiO2NPs on the renal glomerulus and urinary tubules may be lessened by Bai. The activation of caspase-3, also known as cleaved caspase-3, is crucial for the process of apoptosis, which is mediated by apoptosis genes and caspases. Its significance in starting and maintaining apoptosis was highlighted by Unnisa *et al.* (2023). According to a study carried out by Bi *et al.* (2023), TiO2NPs were frequently observed to negatively affect renal tubular cells by caspase-based or cytotoxic action.

The Bcl-2 immunohistochemistry examination revealed that there was no positive in the renal tissue control group. This implies that the control group's cells were going through a typical life cycle. The immunohistochemistry response to Bcl-2 in the kidneys of the Bai group, on the other hand, was mild, suggesting that Bai may help to reduce apoptosis in these kidneys. This data implies that pairing Bai with Bai has a protective effect. Additionally, in the TiO2NPs group, the immunohistochemistry re-

action for Bcl-2 became more intense, showing that TiO2NPs had an apoptotic effect.

In order to regulate apoptosis in healthy cells and prevent cell death caused by oxidative stress, it is crucial to control the expression of the Bcl-2 protein, as stated by Liang *et al.* (2023b). The research conducted observed that the TiO2NPs groups had lower levels of Bcl-2 protein expression compared to the control groups, which could have led to cellular harm. However, after undergoing treatment with Bai, Bcl-2 expression levels increased, ultimately reducing oxidative stress. Bai's potential role in safeguarding the kidneys from TiO2NPs damage is suggested.

The kidney's defense system can be protected by Baicalin against TiO2NPs-induced oxidative stress, inflammation, and nephrotoxicity, as suggested by this study. Baicalin's mechanism for nephroprotection involves suppressing inflammation and restoring the antioxidant defense system in renal cells. It also prevents nephrocellular leakage and the increase in kidney function enzymes. Additionally, histological investigation confirms the beneficial effects of Baicalin on the kidney of rats. In summary, Baicalin is a potent antioxidant, pharmacological, and nutritional agent that can alleviate renal damage and injury.

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