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RENAL HISTOPATHOLOGICAL AND MCP-1 MODULATION IN MALE RAT (*Rattus norvigicaus*) FOLLOWING REPEATED TiO_2 NPs INTRATRACHEAL INSTILLATION

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ABSTRACT

The rapidly expanding field of nanotechnology is becoming a possible source for human exposure to nanoparticles. Titanium dioxide nanoparticles (TiO_2-NPs) is one of an important nanoparticle which has been widely manufactured in developed processes for several years. The aim of this study was to investigate the effects of TiO_2-NPs on some biochemical parameters and the renal histopathology alterations in adult Wistar rats. 63 male rats were used and treated with different doses (0.5, 5, 50, 1.5, 15, 150 mg/kg) of TiO_2-NPs (21 nm) twice a week for 4 weeks. Each group separated into three subgroups then sacrificed at 4 days, month and 3 months post intratracheal instillation. The IL-10 and MCP-1 estimation in BALF and histopathological examination of kidney were done. The results showed serve histological alteration in renal elements post 4 days post-instillation, which got an increase in a month post instillation as the concentration of MCP-1 and IL-10 increased in BAlF and in lung tissues homogenate. The histopathological examination revealed decreasing in glomeruli number in cortex of the kidneys with hemorrhage and inflammatory cells infiltration at 4 days post-instillation while after a month of instillation, the changes like swelling, dilatation of Bowman's capsule and degeneration changes in renal tubules were observed. At 3 months post-instillation, some regeneration in renal elements were observed.

INTRODUCTION

Titanium dioxide (TiO_2), a natural non-silicate mineral oxide, which found in different forms, is widely used in the pharmaceutical, cosmetic, and paint industries as a coloring material, However, its special characteristics such as small size, large surface per mass and high reactivity make it easy to enter into the human body, therefore, poses potential risk to human health and the environment [1]. TiO_2 NPs have two major arrangements of crystal structures, named rutile and anatase. Both are toxic but the rutile NPs may produce fewer toxic than anatase NPs and these particles mutually linked with oxidizing mechanisms of an organism, which eligible to produce reactive oxygen species (ROS) [2]. Fine TiO_2-NPs have been counted safe and affectation little hazard to humans, suggesting that exposed to this material was reasonably harmless. However, presented data revealed that TiO_2-NPs could cause numerous adverse influences on mammalian cells such as an increase of ROS production and cytokines levels, decrease of cell viability and proliferation, stimulation apoptosis and genotoxicity [3].

TiO_2 NPs formulated products are facilely getting into the human body via diverse paths with different formats and may object the body metabolism; most of the toxicological studies of TiO_2 NPs in mammalian models have concentrated on the hepatotoxicity through dermal or inhalation exposure [4]. In 2008, Fabian *et al* investigated the deposition and removal cycles of nano-TiO_2 in various organs and found that the liver cleaning cycle is the longest (28 days). The influences of TiO_2 in the spleen, lungs, and kidneys are far smaller than in the liver [5]. For instance, Geyu *et al*. showed that transbronchial exposure to TiO_2 nanoparticles (with a size of 50 nm, and 0.5, 5.0, and 50.0 mg/kg nano-TiO_2) can stimulate oxidative stress in the liver and kidney but has no effect on the liver and kidney function that causes pathological changes [6].

The toxic effects of nano-TiO_2 in adult mice have been expert, proposing that higher dose nano-TiO_2 (25 and 80 nm) raising the ratio of alanine aminotransferase to aspartate aminotransferase, the activity of lactate dehydrogenase and the liver weight, and caused the hepatocyte necrosis [7]. Recent studies have indicated that TiO_2-NPs are toxic on lung, liver, spleen, kidney, and a gill of animals [8-12].

Moreover, a pathological examination by Chen *et al* revealed that nano-TiO_2 is deposited in the liver, where it caused liver cell apoptosis, necrosis, and liver fibrosis, and in the kidney, where it

causes glomerular swelling so nano-TiO_2 has been shown to produce toxicity in the liver and kidneys[13].

Broncho-Alveolar Lavage Fluid (BALF) is usually sampled during bronchoscopy in individuals with suspected lung cancer, it contains a varied diversity of cellular material such as macrophages and neutrophils, a large number of proteins produced by epithelial and inflammatory cells, and tumor cells if present [14]. Proximal biofluids have much reduced the active range of protein abundances and in some cases are in direct interaction with the site of the disease [15].

In the present study, our aim was to identify altered levels of protein biomarkers in BALF samples and as the functional integrity of the mammalian kidney is vital to the total body homeostasis, because the kidney plays a principal role in the excretion of metabolic wastes and in the regulation of extracellular fluid volume, electrolyte composition, and acid–base balance, so we investigated the renal histopathological caused by repeated TiO_2 NP intake.

MATERIALS AND METHODS

Animals and Treatments

Sixty-three mats rats (*Rattus norvigicus* at age 8 weeks) were housed in cages kept in standard conditions in animals' room, 25˚C temperature with relative humidity at 60% and a 12 hour light/dark cycle, distilled water and sterilized food for rats were available. Rats were divided into seven groups (9 rats each). The control group was treated with 0.9% w/w NaCl solution. The experimental groups treat with 0.5, 5, 50, 1.5, 15, 150 mg/kg of nano-TiO_2 (size 21 nm) [11]. All groups perform to repeated exposure (twice a week, for four consecutive weeks) by 0.1 ml/100 g (B.W) intratracheal instillation. Animals were sacrificed at 4 days, a month and three months post-instillation; animals weighted then lungs lavage to collect bronchoalveolar lavage fluid (BALF). The kidneys were removed, weighted and processed for histopathological study. The coefficients of tissue to body weight were calculated and it was defined as grams (wet weight of tissue/body weight).

Collection of Broncho-Alveolar Lavage Fluid (BALF)

The left lung clamped, and the right lung lavage 3 times with a 3 ml of 0.9% sterile buffer saline (physiological buffer solution PBS) using a single 3mL Monoject syringe. Lavage fluid collected in EDTA tubes. Tubes centrifuged at 4°C and 1500 rpm for 3 minutes to isolate alveolar macrophages from the BALF [16]. BALF supernatant collected for immunological assay.

Preparation of lung homogenates

Lungs sample incised and weighted, immediately freeze in -80˚C for later used. The samples were hold at 2-8□C. then PBS was added, the samples were homogenized thoroughly by Homogenizer, and centrifuged at 2000-3000 rpm for 20 minutes, the supernatants collected carefully, kept and frozen for later used in an immune assay.

Measurements of inflammatory mediators

The levels of interleukin 10 **(**IL-10), monocyte chemoattract protein -1 (MCP-1), were measured in the lung homogenate and the BAL fluid supernatants enzyme-linked immunosorbent assay (ELISA) according to the manufacturer of ELISA Kits (Asiagene, Chania).

Histopathological Examination

Histopathological examinations performed by using standard laboratory procedures. Kidneys were removed from experimental groups and rinsed thoroughly for 1 mint in normal saline. Then, the tissue was fixed in a Carnoy's fluid for 60 minutes and transferred to 95% percent or absolute alcohol for 1 hours. Thereafter, processed to paraffin embedding routine. Sections of 5-7 µm were stained with hematoxylin and eosin stain, collagen stain (Van Geison) and Periodic Acid Schiffs PAS then examined under light microscope to determine the histopathological changes and collagen contents [17].

Statistical Analysis:

Statistical analysis of all data was carried out using the ANOVA test with differences less than 0.5 ($p<0.05$) considered to be statistically significant. This calculation was carried out according to the Statistical Package for Social Science (SPSS version 20) and the least significant difference (L.S.D) at a level less than (0.05) was also used.

RESULTS

Kidneys coefficients

The results revealed there was a significant decreasing ($p<0.05$) in kidneys coefficients at 4 days post-instillation in all groups comparing with the control, with no significant differences between groups. After a month of instillation, the coefficients were increased significantly ($p<0.05$) in low doses while the moderate and high doses increased with no significant ($p<0.05$). After 3 months of instillation, there was decreasing in the coefficients of some groups (0.5, 5, 15&50 mg/kg) and increased significantly ($p<0.05$) in both doses (1.5& 150 mg/kg) (table: 1)

Table (1): the alteration in kidneys coefficients in treated groups through the experiment.

	TiO_2 (mg/kg) BW						
	0	**0.5**	**1.5**	**5**	**15**	**50**	**150**
4 days	.555±.012	.3498±.021*a	.3615±.037*a	.3638±.045*a	.4221±.073*a	.3676±.09*a	.3819±.018*a
Month	.5847±0.011	.3957±.026*b	.4600±.05*b	.3723±.015*a	.4526±.032*a	.3757±.03*a	.4233±.025*a
3 months	.6043±.032	.3723±.012*b	.5333±.025*c	.3606±.029*a	.4325±.055*a	.3146±.03*b	.5000±.05*b

Values represent mean ± SD, (n=3)

* The mean difference is significant ($p < 0.05$) compared with control

Different letters mean is significant ($p<0.05$) between periods

Measurements of inflammatory mediators

After 4 days of TiO_2 intra-tracheal instillation, the results showed that IL-10 concentration in tissue homogenate was significantly increased ($p<0.05$) in lowest doses (0.5, 1.5 mg/kg) while significantly decreased in moderate and high doses (15, 50, 150 mg/kg), and it was significantly increased ($p<0.05$) in BALF in all groups compared to the control group, the highest levels of IL-10 were in high doses (50, 150 mg/kg) (Table: 2). After a month and 3 months post-intratracheal instillation, there was a significant increased ($p<0.05$) of IL-10 concentration in both homogenate tissue and BALF of all groups compared with the control group. (Table:2).

The increasing of IL-10 concentration in the homogenate tissue of all groups was significant during the interval experiment ($p<0.05$). While IL-10 concentration in BALF of all treated groups was significantly increased ($p<0.05$) at 4 days and month post-instillation then it was decreased with no significantly ($p<0.05$) at 3 months post-instillation (Fig.1).

The results showing that the MCP-1 concentration in tissue homogenate after 4 days post-intratracheal instillation was significant ($p<0.05$) decreased, while the MCP-1 concentration in BALF was increasing significant ($p<0.05$) in all groups compared with the control group (Table:1). After a month and 3 months of TiO_2 intra-tracheal instillation, there was a significant ($p<0.05$) difference in the mean concentration of MCP-1 in both homogenate tissue and BALF of all groups compared with the control group as an adjective in the table (2).

There were noticing the concentration of MCP-1 was increased significantly ($p<0.05$) during the experiment interval reached the highest after 3 months of instillation in the homogenate tissue of treated groups compared with the values in 4 days after installation. There was no significant ($p<0.05$) increase in the MCP-1 concentration in BALF of treated groups after a month of instillation compared with their concentration at 4 days of installation, while there was a significant ($p<0.05$) difference after 3 months of instillation compared with the concentration at 4 days of installation. (Fig. 2)

Table (2) IL-10 and MCP-1 concentration in all group after 4 days, 1 month and 3 months of intratracheal installation TiO_2 in BALF and tissue homogenate.

	TiO_2 NP (mg/kg) body weight						
Periods	0	0.5	1.5	5	15	50	150
4days							
BALF IL-10 (pg/ml)	87.62±9.32	137.31±11.26[a]	165.63±27.53[a]	194.32±10.07[a]	202.34.±49.56[a]	255.48±20.81[a]	339.26±12.51[a]
***BALF MCP-1 (pg/ml)**	109.67±31.82	229.33±32.71[a]	248.67±25.11[a]	237.67±53.03[a]	280.33±28.28[a]	241.67±28.86[a]	276.33±20.78[a]
Homog IL-10 (pg/ml)	136.±33.94	163.24±9.26[a]	228.61±20.15[a]	175.45±12.27[a]	74.05±8.12[a]	57.09±14.93[a]	50.08±11.91[a]
***Homog .MCP-1 (pg/ml)**	142.67±35.35	84.67±8.32[a]	112.67±17.67[a]	112.67±26.16[a]	125.33±20.52[a]	95.33±21.57[a]	124.33±8.38[a]
1month							
BALF IL-10 (pg/ml)	89.00±5.29	181.98±15.98[a]	225.38±68.81[a]	274.88±27.24[a]	266.49±10.74[a]	323.89±24.95[a]	360.89±31.01[a]
***BALF MCP-1 (pg/ml)**	102.33±2.83	233.33±38.19[a]	216.67±14.43[a]	284.67±74.19[a]	237.33±75.43[a]	242.33±38.30[a]	235.33±60.01[a]
Homog IL-10 (pg/ml)	147.18±21.04	250.18±20.66[a]	257.32±37.14[a]	271.56±29.77[a]	246.187±10.67[a]	264.24±16.19	242.74±20.44[a]
***Homog .MCP-1 (pg/ml)**	157.67±37.1	282.67±61.85[a]	386.0±85.71[a]	283.67±83.33[a]	286.33±86.97[a]	402.67±25.11[a]	171.33±15.55[a]
3months							
BALF IL-10 (pg/ml)	87.75±8.07	181.28±32.09[a]	175.90±33.43[a]	143.39±12.82[a]	304±10.11[a]	265.31±29.73[a]	308.62±21.85[a]
***BALF MCP-1 (pg/ml)**	107.67±51.08	364.67±50.2[a]	436.33±27.78[a]	374.67±9.23[a]	446.0±14.42[a]	326.33±1.52[a]	324.67±67.88[a]
Homog IL-10 (pg/ml)	153.87±11.84	235.98±11.81[a]	258.6±35.52[a]	296.77±34.53[a]	370.78±22.69[a]	177.31±40.01[a]	254.36.±34.3[a]

***Homog. MCP-1 (pg/ml)**	149.08±17.67	337.33±17.67[a]	366.33±56.3[a]	393.67±64.5[a]	323.33±47.52[a]	480.33±25.42[a]	558.33±18.01[a]

Values represent mean ± SD, (n=3)
[a] The mean difference is significant ($p < 0.05$)

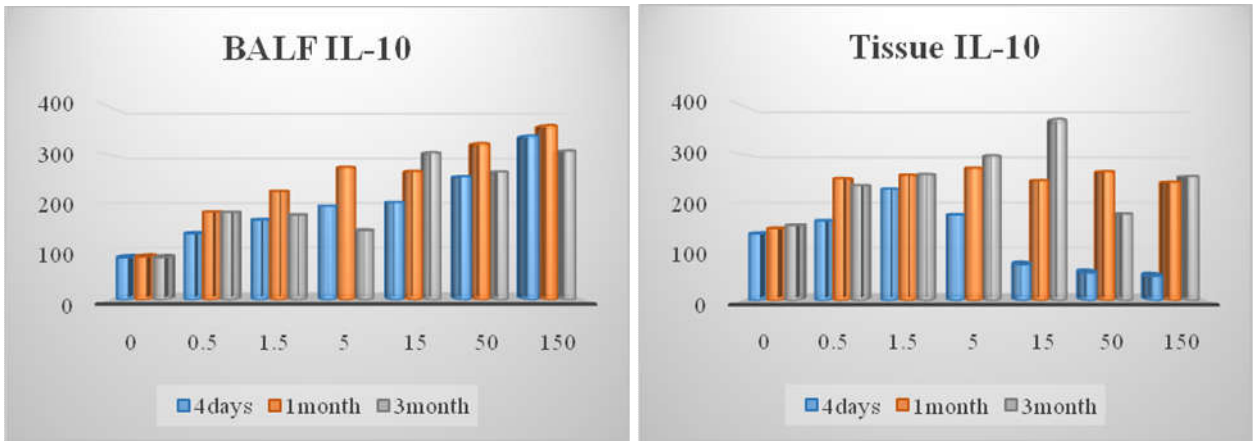

Fig. (1) IL-10 concentration in BALF and homogenate lung of all group after 4 days, 1 month and 3 months post-intratracheal installation.

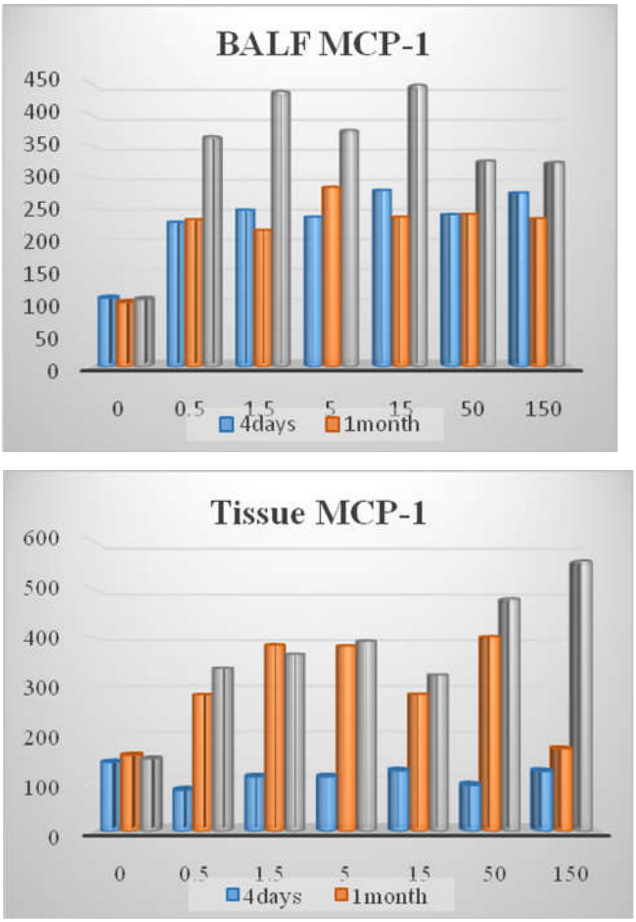

Fig. (2) MCP-1 concentration in BALF and homogenate lung of all group after 4 days, 1 month and 3 months post-intratracheal installation.

Histopathological Examination

Histopathological examination of kidney at 4 days post intratracheal instillation revealed that the first three doses (0.5, 1.5 & 5 mg/kg) show more signs of alteration included inflammatory cells infiltration, hemorrhage in renal tissue, decreased in glomeruli number in cortex. Bleeding in the glomerulus and epithelial of proximal tubular were necrotic. Dilated and congested blood vessels and edema appeared in all doses but it was more in high doses (fig.3). When doses (15, 50 & 150 mg/kg) were increased, capsule thickness and giant cells with deposition of collagen fiber between renal tubules in the cortex (fig. 4). The wall of capillaries in glomeruli showed no thickening with division into two or three lobules (fig. 5).

After a month of instillation, swelling and dilatation of Bowman's capsule and degeneration changes in the epithelium of the proximal tubules were observed, which increased as the doses raised. In addition, bleeding and infiltration of the inflammatory cell were detected in all doses. The edema sacs became bigger comparing with those in 4 days (fig.3). The collagen fibers were increased

between renal tubules and around the congested vessels (fig.4). The thickening of the wall of capillaries in glomeruli increased with eosin materials deposition (fig. 5).

Some regeneration in renal tissue were detected at 3-month post instillation but still infiltrated with inflammatory cells especially in high doses. The lower doses revealed more progress in renal tissue remodeling (fig. 3). The collagen fibers were detected in moderate and high doses (fig.4). The wall of capillaries in glomeruli showed more thickening with division into two or three lobules in high doses (fig.5)

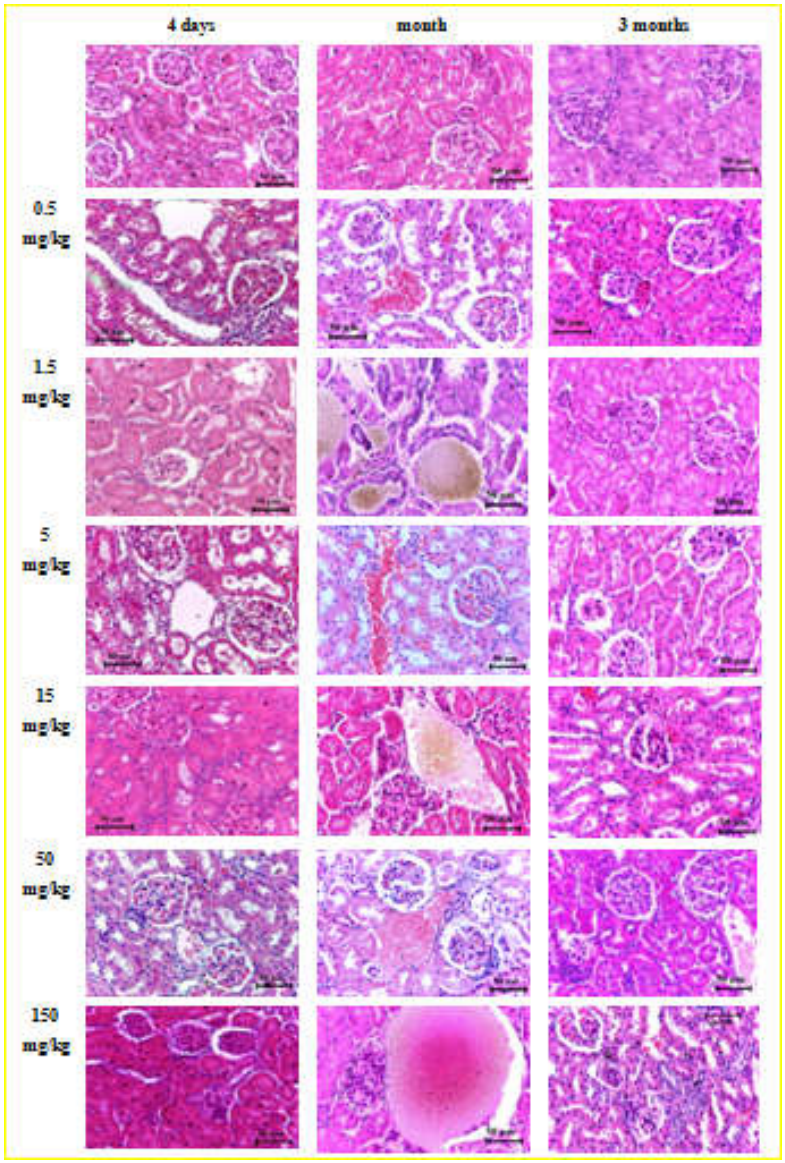

Fig. (3) Sections in the kidney from the control and the group treated with (0.5, 1.5, 5, 1
150) mg/kg of TiO_2 NP post-instillation, showing decreased in glomeruli number in cor
medulla. Bleeding and epithelial of proximal tubular were necrotic post 4 days while sw
dilatation of Bowman's capsule and degeneration changes in the epithelium of the proxi
tubules at a month and Some regeneration in renal tissue were detected at 3 months post
installation. H & E stain, 40X

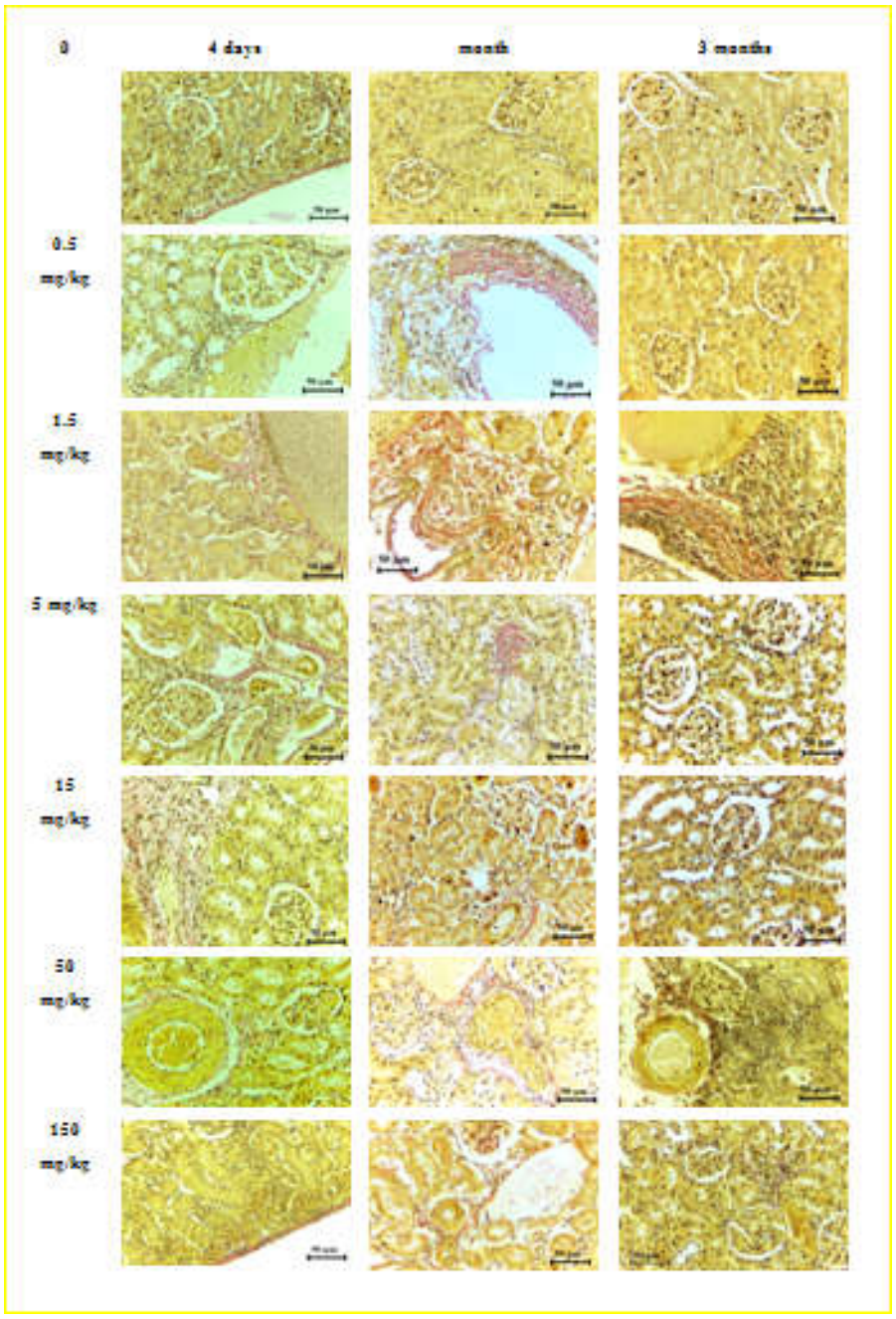

Fig.(4) Sections in the kidney from control group and the group treated with (0.5, 1.5, ˙TiO_2 NP post-instillation, thicken in capsule and giant cells with deposition of collagen f ın renal tubules in the medulla post 4 days while The collagen fibers were increased betwe ınd the congested vessels at a month. The collagen fibers were detected in moderate and l

at 3 months post-installation. Van Geison stain, 40X

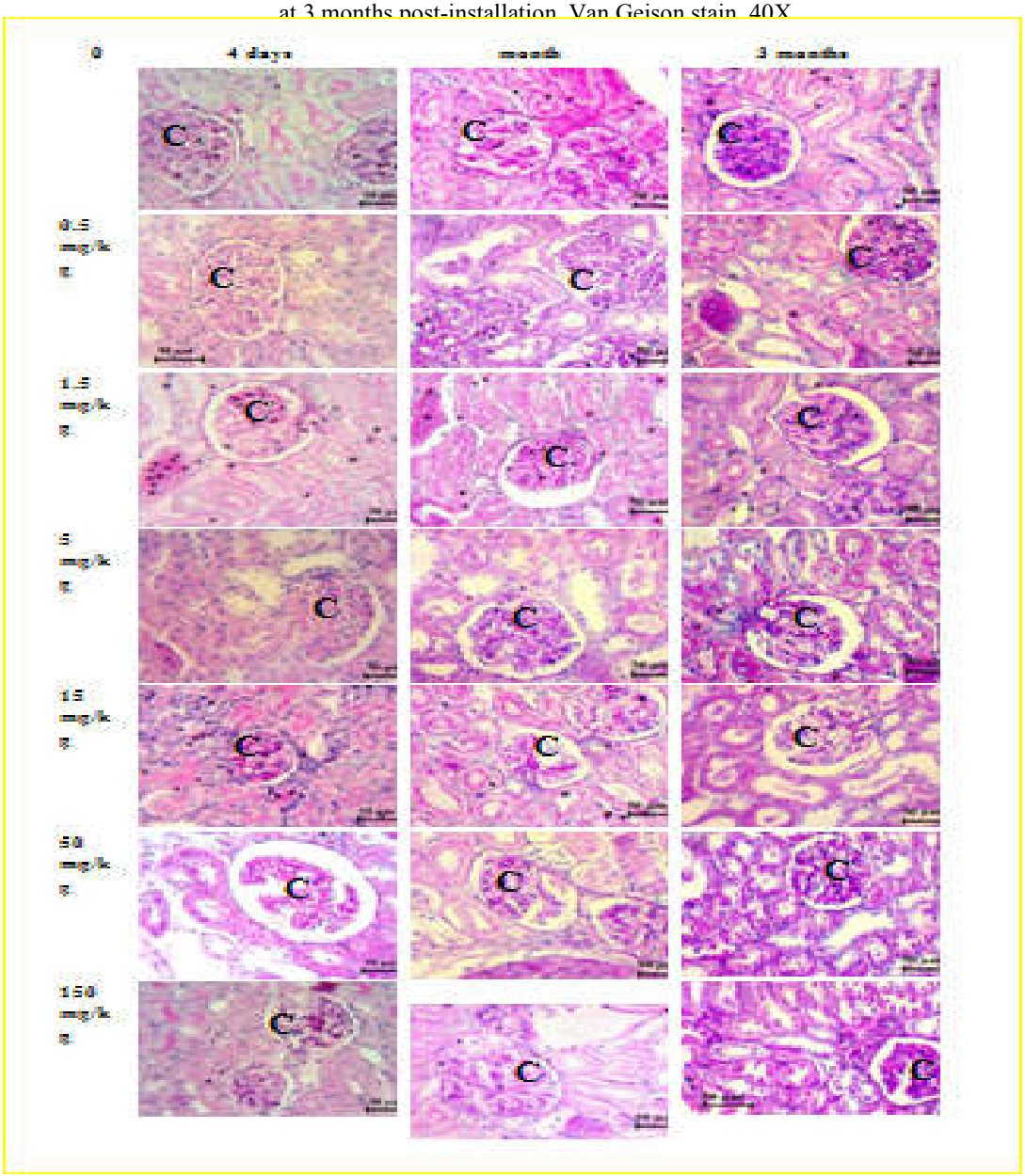

Fig.(5) Sections in the kidney from control group and the group treated with (0.5, 1.5, 5, 15, 50, 150) mg/kg of TiO_2 NP post-instillation, showing no thicken in the walls of capillary in glomeruli (C) post 4 days while the thicken increased at a month and 3 months post-instillation which was deeply pink . PAS stain, 40X.

DISCUSSION

TiO_2 NPs were used in a variety of applications including dyeing, plastics, papers, inks, food colorants, toothpaste and cosmetic manufacturing. Our aims were to investigate the biochemical and histological alterations in the kidney after administration of different doses of TiO_2 NPs. The results at 4 days and a month post instillation revealed that kidneys coefficient was decreased in all doses due to glomeruli degeneration, which detected through histopathological examination as well as to the decreasing in glomeruli numbers in both cortex. While after 3 months there was different in the coefficients due to the increase in edema sacs in high doses. Meena and Paulraj reported that lower and medium dose nano-TiO_2 did not show any significant differences in the organ to body weight ratios of the liver, kidney, spleen, and brain of treated animals from control group ,whereas 50mg/kg nano-TiO_2, caused a significant increase in ratio of the liver, kidney, and spleen to bodyweight [18]. There is agreement in suggesting that nano-TiO_2 might damage the organs.

The TiO_2 NPs caused increase in IL-10 concentration in the homogenate tissue of all groups during the interval experiment, while in BALF increased at 4 days and month post-instillation then it was decreased at 3 months post-instillation, thus indecated the lung inflammation resulted from TiO_2 treatments miaght there were increasing in the experssion levels of inflammatory factors.

The concentration of MCP-1 was increased during the experiment interval reached the highest after 3 months of instillation in the homogenate tissue of treated groups compared with the values in 4 days after installation. However, no significant increase in the MCP-1 concentration in BALF of treated groups after a month of instillation compared with their concentration at 4 days of installation, while there was a significant difference after 3 months post instillation. These alteration in MCP-1 secreation due to TiO_2 NPs treatment.

It had been reported that nanoparticles (diameter<100 nm) maight cause inflammation easier than the same mass of fine particles in spite of their chemical properties [19, 20]. Also it has been demonstrated that nano-TiO_2 could promote the expression of several cytokines and chemokines in the lung of rat and mice, including placenta growth factor (PlGF), MCP-1, IL-1b, and TNF-a [21,22].

The real-time quantitative PCR (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) analyses showed that TiO_2 NPs can significantly alter the mRNA and protein expression of several inflammatory pathways, including nuclear factor kappa,light-chain, enhancer of activated B cells (NF-κB), macrophage migration inhibitory factor (MMIF), TNF-α, interleukin (IL)-6 (IL-6), IL-1β, cross-reaction protein, IL-4, and IL-10 [23]. Intraperitoneal injection for 14 days indicated that the titanium content increased in mouse livers, resulting in liver cell damage, mitochondrial swelling, and other pathological changes, while the expression levels of inflammatory factors (NF-κB, MIF, IL-6, IL-1β, CRP, TNF-α, etc) were altered, indicating that nano-TiO_2 causes liver inflammation that results in liver injury [10]. The expression of the inflammation-associated molecule, monocyte chemoattractant protein-1 (MCP-1) and macrophage marker-CD11b was decreased in glomeruli in mice [24].

The results of histopathological showed decreasing in glomeruli number in cortex. Bleeding, epithelial of proximal tubular were necrotic post 4 days while swelling, dilatation of Bowman's capsule and degeneration changes in the epithelium of the proximal tubules at a month while some regeneration in renal tissue were detected at 3 months post-installation, thus because of the TiO_2 NPs accumulate in such organs which practically eliminate the toxic compounds. Accumulation of these substances caused abnormal pathological changes in the tissues of the lung and liver [11, 12]. Wang *et al*. consider that nanoparticles of TiO_2 have been deposited in the cells of kidney and caused the pathological alterations and nephron-like toxicity the kidney. Moreover, particles of 25 nm TiO_2 can significantly increase the urea level of serum compared with the control group [7]. Vasantharaja *et al*. suggested that oral administration of TiO_2 NPs aggregated in the liver and kidney [4]. After inhalation of nano-TiO_2, this nanoparticle accumulates in the kidneys and causes renal fibrosis via oxidative stress [25]. The effects of TiO_2 on Kidney included infiltration of inflammatory cells, fatty degeneration, and apoptosis; degeneration of superficial adipocytes; apoptosis of renal tubules [25]. The lymph nodes was described as the first target of NPs after lung translocation [27,28]. However, Pujalté, *et al*. study, like other published studies, failed to distinguish between direct transmit of NPs into the interstitium and then the blood, and transmit through lymphatic circulation to blood, nevertheless, with the exposure conditions used, the TiO_2 NPs translocation average to the lymphatic system and the blood circulation was found to be low compared to total amounts of NPs hooked up in the lungs through time [29].

CONCLUSION

The high doses (15, 50, 150) mg/kg of TiO_2 NPs had more effects on immune response which remain for 3 months post-instillation through its stimulating to secretion of IL-10, and MCP-1 where it raised in BALF and decrease in tissue homogenate. After post instillation, the low doses caused more alteration in the renal histological structures (included decreasing in glomeruli number, necrotic proximal convoluted tubules, division the glomeruli into more than one lobules and

collagen fibers deposition) which raised after month post-instillation in all doses. There were some regeneration in renal tissue after 3 months post-instillation.

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