International Journal of Sciences

Research Article Volume 6 – March 2017 (03)

TiO_2 Nanoparticles Induce Lung Fibrosis and Proteinosis through Influence on Matrix Metalloproteinase Expression

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Abstract:

Background: nanotechnology applications, speared very quickly while very little has been done to measure and assess the hazard of nanoparticles (NPs) to an ecosystem and to the biological systems. Lung exposure to titanium dioxide nanoparticle (TiO_2 NP) may induce pulmonary alveolar proteinases and fibrosis through influence on matrix metalloproteinase expression (MMPs).

Methods: In order to study this, TiO_2 NP was instilled into the lung, then, histopathological alteration and MMPs (MMP-1, MMP-2, Collagen-I) expression using RT-qPCR were assessed at 4 days, a month and 3 months post-instillation. Data were analyzed using ANOVA test and gene expression was normalized to that of housekeeping gene, which was hypoxanthine phosphoribosyltransferase (HPRT).

Results: The results showed that TiO_2 NP induces acute inflammation in lung tissue after 4 days post-instillation with significantly decrease ($p<0.05$) in MMPs expression. While inducing fibrosis and proteinosis with significantly increase ($p<0.05$) in MMPs expression after a month post- instillation. Otherwise, after 3 months post-instillation the fibrosis and proteinosis were decreased and the expression significantly increased ($p<0.05$).

Conclusion: TiO2 NP induces many alterations in lung structure after 4 days and a month from intratracheal instillation this included metaplasia in bronchus epithelial and in alveolar epithelial, Fibrosis, angiogenesis and proteinaceous through affected on MMPs expression which decreased the expression of MMP-1, MMP-2, MMP-12 and Collagen-I in the lung.

Keywords: Lung fibrosis; Pulmonary alveolar proteinosis; TiO2 NPs; MMPs

Introduction

Nanoparticles are microscopic matters have one or more dimensions less than 100 nm and have attracted powerful scientific interest. Distinguishing volume-dependent properties of nanoparticles are mainly due to their relatively large surface area. They are utilized as a targeted transmission system for transport of tiny and major molecules by changing their pharmacokinetics and pharmacodynamics properties. Nanoparticles are old in the environment as their presence had been discovered a long time ago for e.g. air pollutants, but they had been reported and formulated for different beneficial purposes such as medicine delivery, tissue targeting, cancer therapy, diagnostic operator and for imaging purpose (1).

Titanium dioxide nanoparticles (TiO_2 NP), which generally utilized in prettifying, paints, sunscreens, and nutrition, induced emphysema and lung sore in mice (2). TiO_2 particles are not immunologically active and may be an essential supplement in beat normal gut cell hypo-responsiveness to endogenous luminal molecules. They assumed that the TiO_2 particles become immune prospect after adsorption of lipopolysaccharide (3). It is known that the extracellular matrix plays a key role in tissue architecture and function, also MMPs thought to be involved in the pathogenesis and improvement of many diseases including arthritis, lung injury, vascular disease, cancer and some neurodegenerative disorders. 20 MMPs are may be involved in key events relate to matrix degradation and/or inflammation. MMPs have activity in rheumatoid

Published at: http://www.ijsciences.com/pub/issue/2017-03/
DOI: 10.18483/ijSci.1197; Online ISSN: 2305-3925; Print ISSN: 2410-4477

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arthritis (RA) and damage the joints by the degeneracy of the cartilage and bone, as well as motivating angiogenesis and inflammation (4). The MMP prevent fibrosis or, for that issue, any illness or remedy process by controlled their catalysis. Contrasted with a normal remedy, the action of an effector MMP might be over/or under performed fibrosis, and conversion in overall activity can be created by two mechanisms: First, biosynthesis of the MMP might be varied. Expression of most MMPs managed at the level of transcription. Therefore, over/or downregulation of MMP would appear, the effects on regulated gene expression in a specific cell type are also modified. The second mechanism(s) that MMP activity form is different controls over enzyme efficiency, experience data demonstrating or submitting functional roles, both protective and injurious, for specific MMPs in lung, kidney and liver fibrosis. Although there are many mechanisms for obstetrics fibrosis diffuse among these organs, notably involving the constant activation of myofibroblasts from occupant interstitial cells and common immune characteristics, it is remarkable to note that the roles for specific MMPs are different among organ systems (5). Through the severe inflammatory stage of acute respiratory distress syndrome, irregular releasing of active cytotoxic moderators from penetrating leukocytes, including matrix metalloproteinases (MMPs), nitrogen species, proteolytic enzymes such as elastase and reactive oxygen, effects in injury to pulmonary epithelial and endothelial cells and, if acute, damages the lung scaffold (6).

Methods

Preparation of Nano-TiO2 Solution

Manufactured nano-TiO2 was purchased from Sigma, particles with a size 21 nm. Nano-TiO2 was sterilized in 121 °C for 20 min to decrease the danger of bacterial pollution, then powdered TiO2 was scattered into a suspension with 0.9% (w/w) sodium chloride (NaCl). For adequately scattered particles, solutions containing TiO2 particles were sonicated for 5 min by Ultrasonicator processor and vortexed before it's utilization in treatment (7).

Animals and Experiment Design

A total of 63 male rats (8weeks age) with average body weight of 123.87±22.47 g were used. Animals 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 *ad libituium*. Rats were divided into seven groups. The control group was treated with 0.9% *w/w* NaCl solution, and the treated groups with (0.5, 5, 50 mg/kg) (8), 1.5, 15, 150 mg/kg (B.W.) of nano-TiO_2 (9,10). All groups underwent repeat exposure (twice a week, for four consecutive weeks) by 0.1 ml/100 g (B.W) intratracheal instillation. Rats anesthetized with 16.5mg/kg xylazine and 112.5mg/kg ketamine until their breathing was slow, then placed in a supine position with the head elevated. A tracheal cannula was inserted through the vocal cord to reach the trachea bronchial and insert nano TiO_2 solution into the lungs (11). After 4 weeks of intra-tracheal instillation, three rats from each group were randomly sacrificed after being anesthetized with xylazine and ketamine in 4 days, 1 month and 3 months, animals and lung were weighted.

Histopathological Examinations

Histopathological examinations performed by using standard laboratory procedures. Tissue samples 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 as well as Van Geison stain, then examined under light microscope for histopathological alteration and collagen contents (12).

Gene Expression of Extracellular Metalloproteinase

Total RNA was extracted from the lung of experimental groups according to manufacturer's instruction of RNeasy Mini Kit (QIAGEN Hilden, Germany). The template RNA was detected by electrophoresis on 0.8% agarose gel, then resolved and visualized by ethidium bromide staining (13). Complementary DNA (cDNA) was synthesized by Accupower Rocketscript RT PreMix (Bioneer, Korea). Real Time quantitative Polymerase Chain Reaction (RT-qPCR) was performed to examine the mRNA expression of extracellular metalloproteinase genes by using AccuPower^{R} 2X Greenstar^{TM} qPCR master mix (Bioneer, Korea) as described by the manufacturer. Genes analyzed were *MMP-1*, *MMP-12* (14), *MMP-2* and *Collagen -I* (15). The sequences of primers are given in table [1]. Gene expression was relative to that of hypoxanthine phosphoribosyltransferase (*HPRT*) as a housekeeping gene. The temperature profile was as 95˚C for 10min, 95˚C for 1min, 51.4˚C for 1min, 72˚C for 1min for 45 cycles, melting from 50˚C to 95˚C for 1sec each temp. for 1 cycle. The expression level was calculated from the qPCR cycle number (CT) where the increased fluorescence curve passes a threshold value. The relative expression of target genes was procured using reference gene (ΔCT method) as in

equation [1]. The ΔCT was calculated by subtracting target CT from that of *HPRT* genes while the NO. of copies was calculated according to the standard curve shown in appendix A (16). The RT-qPCR product was detected by melting curve.

$$\text{Ratio}\left(\frac{\text{reference}}{\text{target}}\right) = 2^{\text{Ct(reference)}-\text{Ct(target)}} \ldots.[1]$$

Table (1) Primers sequences used in RT-qPCR

Genes	Genebank accession number	Sequences (5´- 3´)	Size of products (bp)
MMP-1	NM_001134530.1	GCCAACAGGTGCAACAACAC GCATCAAGTTTACCTGGCAGATT	186
MMP-2	RNCOLL	ACAGTGACACCACGTGACAAGC CATTCCCTGCGAAGAACACAG	130
Collagen-I	NM_053356	TGCTGCTTGCAGTAACGTCG TCAACACCATCTCTGCCTCG	116
HPRT	NM_012583.2	TTGGAAAGGGTGTTTATTCCTCAT ATCCAGCAGGTCAGCAAAGAA	158

Statistical aAnalysis:

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

Coefficients of Tissue

The coefficients of tissue are shown in the table [2]. It was defined as grams (wet weight of tissue/body weight). In 4 days after intratracheal instillation, at low doses, there was significantly decreased in coefficients and increased coefficients at the high dose in lung tissue ($p<0.05$). After a month and 3 months of instillation, the coefficient was significantly decreased in lung tissues at all doses ($p<0.05$) compared with that of the control group which depended on the dose concentration.

Table (2) Ended weight and organs weight percentage of rats during the study.

		TiO_2NPs **(mg/kg) B.W.**						
Groups		**0**	**0.5**	**1.5**	**5**	**15**	**50**	**150**
4days	body	126.34±4.54	164.35±0.65	194.33±8.48	164.17±0.05	127.33±9.63	144.20±0.06	126.18±15.77
	lung	1.53±0.10	0.74±0.064*	0.85±0.112*	0.88±0.121*	1.12±0.297*	0.99±0.11*	1.99±0.34*
month	body	171.33± 1.3	324.56±1.99	176.13±7.37	318.63±11.06	184.33±0.25	317.36±11.49	199.33±0.58
	lung	1.404±0.07	1.058±0.13*	1.179±0.048*	0.895±0.03*	0.931±0.04*	0.814±0.01*	1.23±0.09*
3months	body	171.33±4.56	326.17±4.06	176.13±7.36	318.63±11.05	184.73±6.47	317.36±11.49	199.33±0.58
	lung	1.40±0.07	1.05±0.12*	1.18±0.48*	0.9±0.28*	0.93±0.43*	0.81±0.08*	1.26±0.049*

Histopathological Examinations:

The histological observation showed the normal structure of the lung in the control group. Histopathological observation after 4 days of intratracheal instillation, on the treated groups with (0.5, 1.5, 5, 15, 50, 150) mg/kg of TiO_2 included acute inflammatory cells infiltration (polymorph leukocyte and neutrophil) in alveolar septa with many lymphocytes and plasma cells, evidence which show that acute inflammatory reaction is superimposed on subacute or chronic lesion, thick alveolar septa with extravasation of red blood cells, with aggregation of lymphocytes and congested blood vessels, muscular layer becomes thicker than normal, deposition of titanium nanoparticles on interalveolar septa and inside macrophages due to phagocytosis, which was increased as the dose raised. Mature lymphocytes were aggregated in groups in the lymphoid follicle

with small round deeply basophil nuclei (fig.1), metaplasia occurred in some region of the bronchial epithelium which represented by the changes from pseudostratified ciliated epithelium to cuboidal or simple or stratified squamous epithelium, enlarge lymphatic nodule which extended into the bronchus epithelial Beginning of fibrosis featured by a heavy increase in density of collagen fiber surrounded the bronchus and the bronchioles also around the dilated veins with numerous fibrocytes (fig.2)

After a month from an intra-tracheal instillation, the treated groups with lower doses show some improvement in healing and this included the alveolar sacs return to the normal structure while there was still some alteration noticed such as enlarge lymphatic follicle around the bronchiole, aggregation of inflammatory cells around the dilated blood vessels. Lymphatic follicles frequently appeared in alveolar septa, An increase in density of collagen fiber as bundle and strands. TiO_2 NPs deposition was decreased in all groups compared with the preceding time, it was found in alveolar septa and inside macrophages. Metaplasia occurred in squamous epithelial of alveoli, which transferred to stratified cuboidal epithelial, there was an eosinophil material filled the alveoli and in interalveolar septa probably present a proteinaceous (fig.3). Big foamy macrophages fill the alveoli, TiO_2 induce a chronic granulomatous inflammation (fig.4).After 3 months of intratracheal instillation, the treated groups exhibited a healing improvement in their structure except the lower doses possess a developed of emphysema due to alveolar sacs extending, terminal bronchioles with normal collagen fiber detected, extending of tunica adventitia of pulmonary artery while the proteinaceous was decreased, but the immune response was still presented, including the forming of lymphoid follicles in the alveolar septa and around the blood vessels. The result showed an increase in the smooth muscle and tunica adventitia thickness due to increase collagen fiber(fig.5).

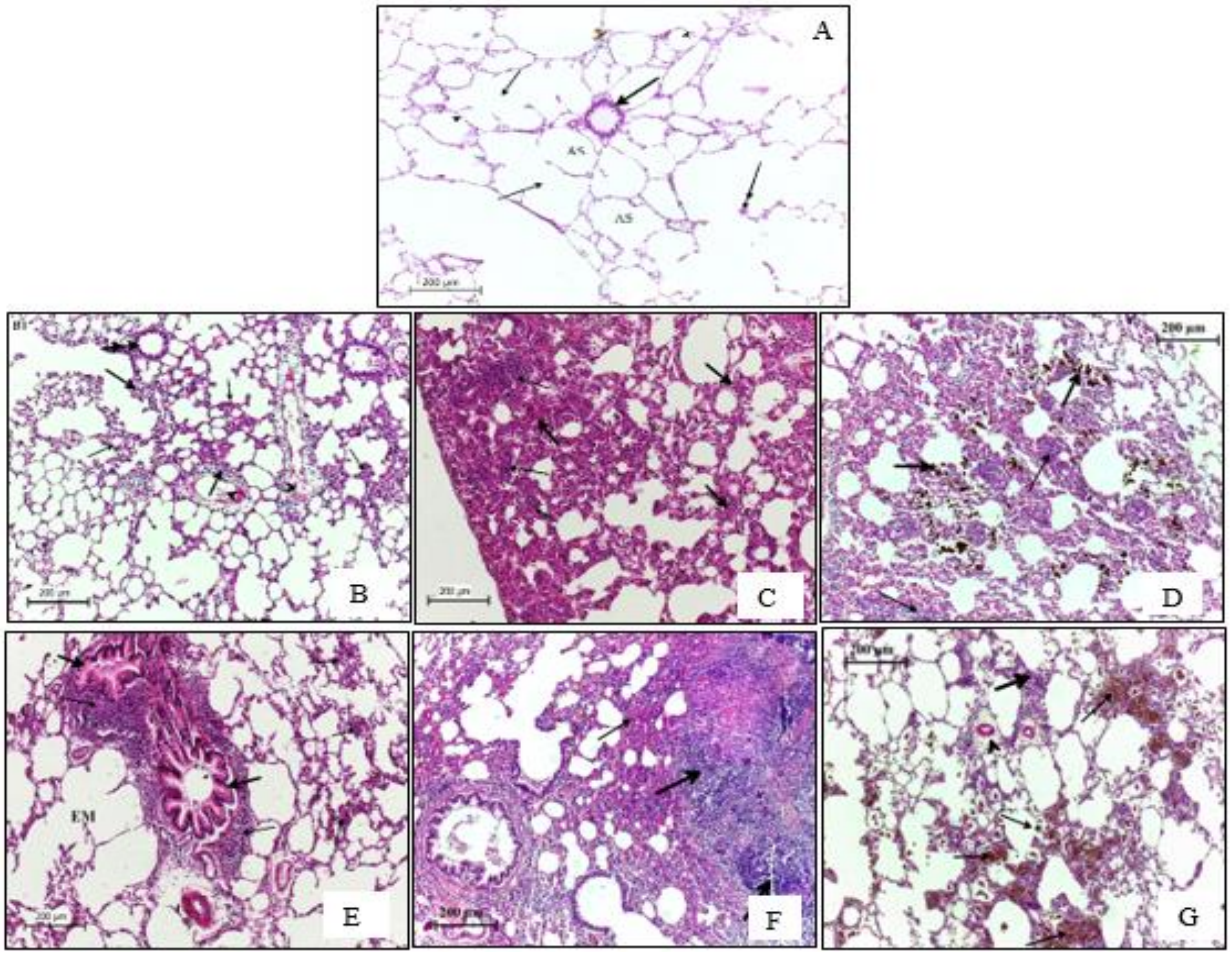

Fig. 1. Histological section of lung from (A) the control group and (B, C, D, F, G) treated group with (0.5, 1.5, 0.5, 5, 15, 50, 150) mg/kg of TiO_2 NP respectively, after 4 days from intratracheal instillation, (A) showing the normal structure of alveoli (thin arrow), terminal bronchiole (thick arrow) and normal alveolar lining epithelial (heads

arrows). (B-G) showing the alteration in lung structure, a heavy infiltration of lymphocyte in the alveolar space and septa (thick arrows), heavy bleeding in alveolar septa and deposition of TiO_2 NP in alveolar septa and macrophage). H&E stain.

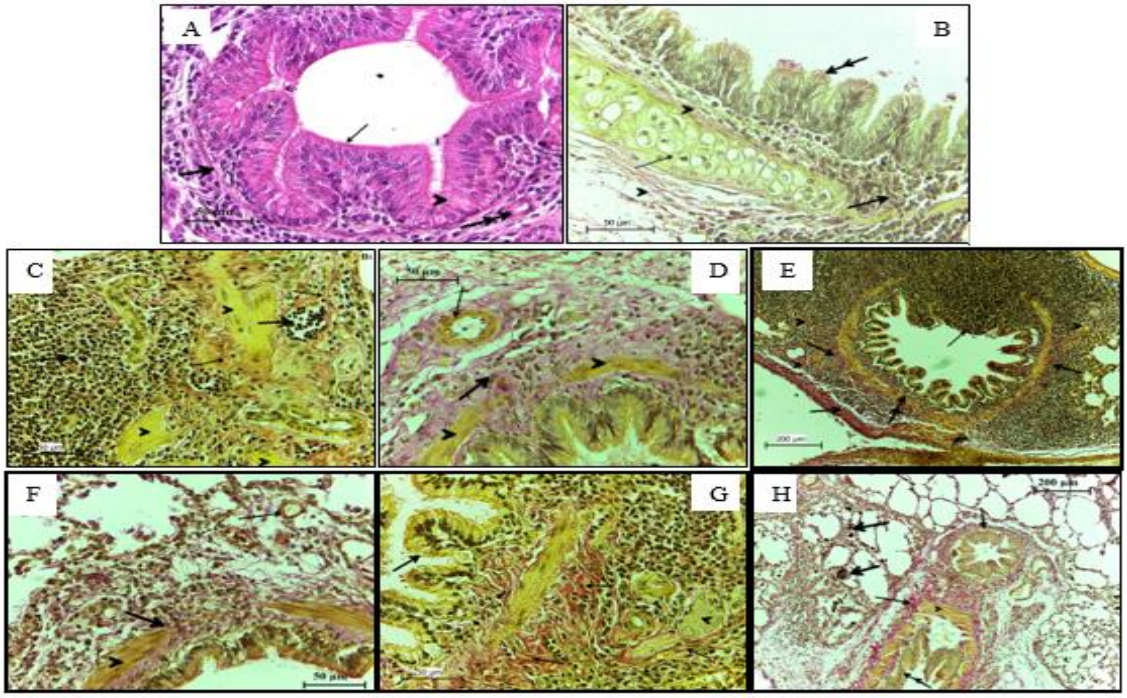

Fig. 2. Section of lung from the control group (A, B) and treated group (C, D, F, G, H) with (0.5, 1.5, 0.5, 5, 15, 50, 150) mg/kg of TiO_2 NP respectively, after 4 days post-instillation, (A, B) showing the normal bronchus lining epithelial (thin arrow), normal arrangement of collagen fiber (heads arrows). (C-H) showing greatly alteration of collagen bundles (thick arrows), metaplasia on lining epithelial (double arrows). A: H&E stain, C-H: Van Geison stain.

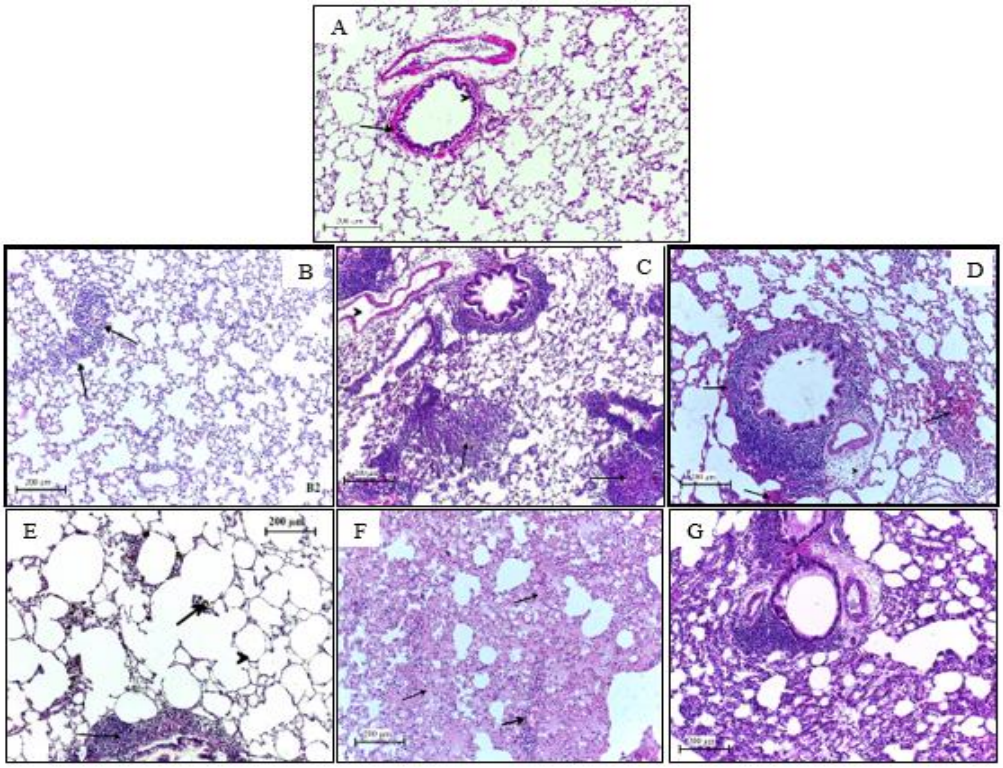

Fig. 3. Illustrated the alteration on lung of the treated groups (B, C, D, E, F, G) with (0.5, 1.5, 0.5, 5, 15, 50, 150) mg/kg of TiO_2 NP respectively comparing with control group (A) after a month post-instillation, showing lymphocyte cluster in alveolar (thin arrow), proteinaceous material present in alveoli (thick arrows). H&E stain

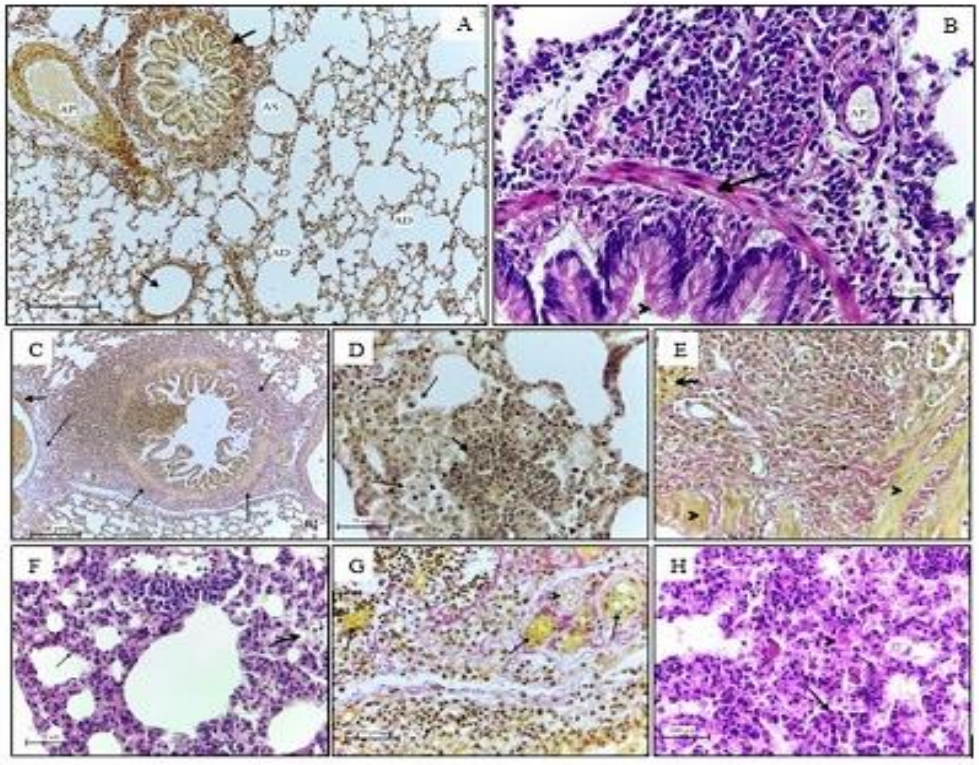

Fig. 4. A cross section o lung of the control group (A, B) and the treated group with (C, D, F, G, H) with (0.5, 1.5, 0.5, 5, 15, 50, 150) mg/kg of TiO_2 NP respectively, after a month post-instillation, showing metaplasia in alveolar epithelial which transferred from simple squamous epithelial to stratified cuboidal epithelial, a foamy macrophage

detected in alveoli, collagen bundle greatly increased and a chronic granulomatous inflammation also induced. A, C, D, E, G: Van Geison stain; B, F, H: H&E stain.

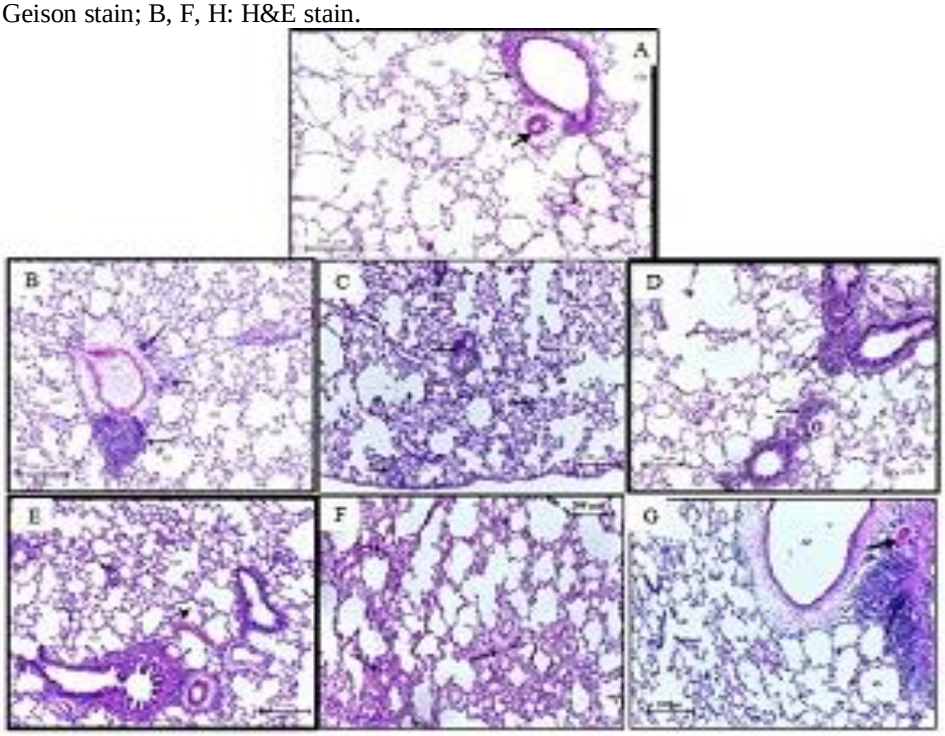

Fig. 5. Histopathological alteration on lung section of the treated groups (B, C, D, E, F, G) with (0.5, 1.5, 0.5, 5, 15, 50, 150) mg/kg of TiO_2 NP respectively comparing with control group (A) after 3 months post-instillation, the section revealed cluster of mature lymphocytes, smooth muscle and tunica adventitia of pulmonary artery increased in thickness, decreased in proteinaceous in the alveoli. H&E stain.

Gene expression of Extracellular Metalloproteinase:

Purification of Total RNA:

Agarose gel electrophoresis was performed to identify the cleansed all out cell's RNA from rat lung tissue (Fig. 6). The concentration of RNA was 202.43 ng/µl measured on A 260˚ nm by NanoDrop spectrophotometer.

Amplification of Extracellular Metalloproteinase Gene:

RT-qPCR was used to evaluate the effect of TiO2 NP on the metalloproteinase and *collagen I* mRNA expression. Specificity of RT-qPCR product was archived a single band, which detected by a lightcycler melting curve was performed to detect the melting temperature for each gene (fig.7).

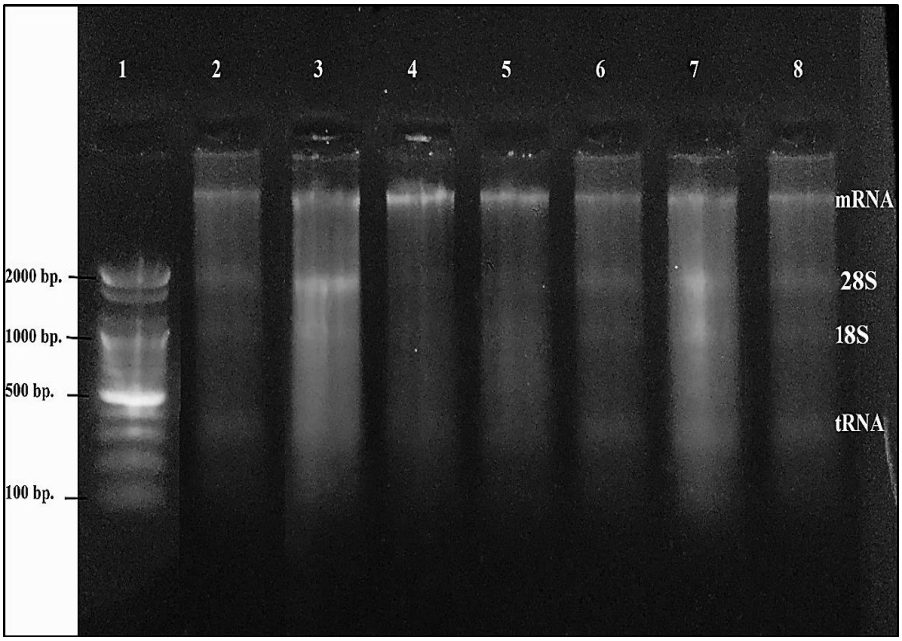

Fig. 6. 0.8% Gel electrophoresis (at 60 volts for 30 min) analysis of total RNA extract from all lung's groups; lane 1: ladder (100 bp DNA marker), lane 2 to 8: total RNA extracted from the experiment groups (control and treated groups).

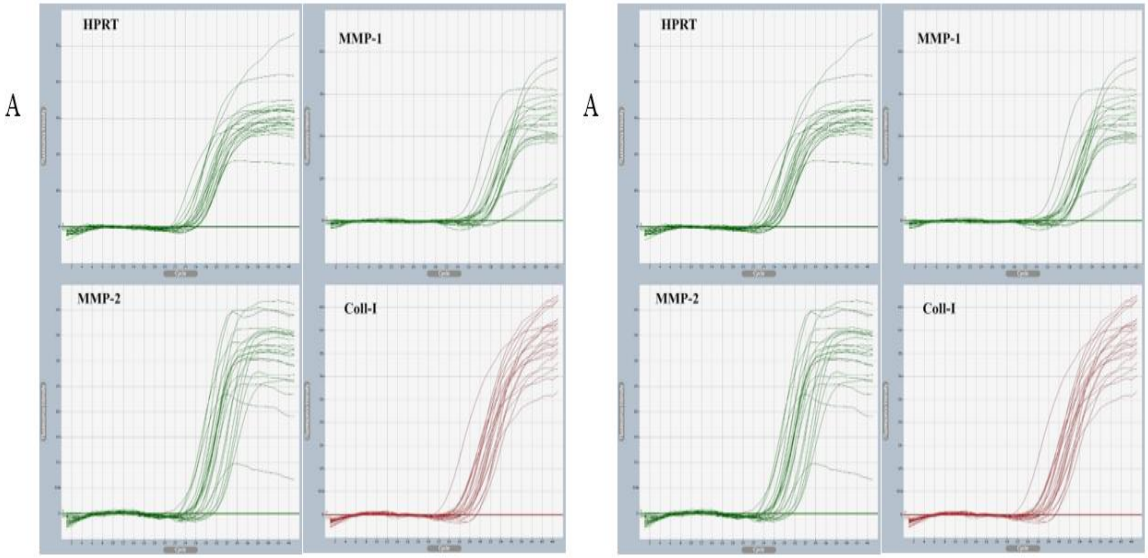

Fig. 7. A: Quantification of mRNA level by real-time quantitative PCR (RT-qPCR) for genes, B: Melting point for all genes.

Real Time efficiency was calculated from the given slope in lightcycler software (appendixA). The quantity of amplification was figured by equation $=10^{(CTgene\text{-}b)/m}$. While the ratio genes to the reference gene were calculated from threshold cycle (CT) that determined in lightcycler. Table (3-5)

The metalloproteinase and collagen I mRNA expression was determined according to the ΔCt method using a reference gene. Real-time analysis showed that *MMP-1*, *MMP-2* and *collagen I* expression were significantly down-regulated in treated groups after 4 days from intratracheal instillation ($p<0.05$) as shown in the table (3-5). After a month of intra-tracheal instillation, *MMP-1*, *MMP-2*, and *Collagen-I* expression were up-regulated with no significant difference ($p>0.05$) in all treated groups, while MMP-2 expression had a significant increase ($p<0.05$).

The increase in expression of *MMP-1*, *MMP-2*, and *Collagen-I* was continued after 3 months of intra-tracheal instillation in all experiment groups with no significant different ($p>0.05$). (fig.8)

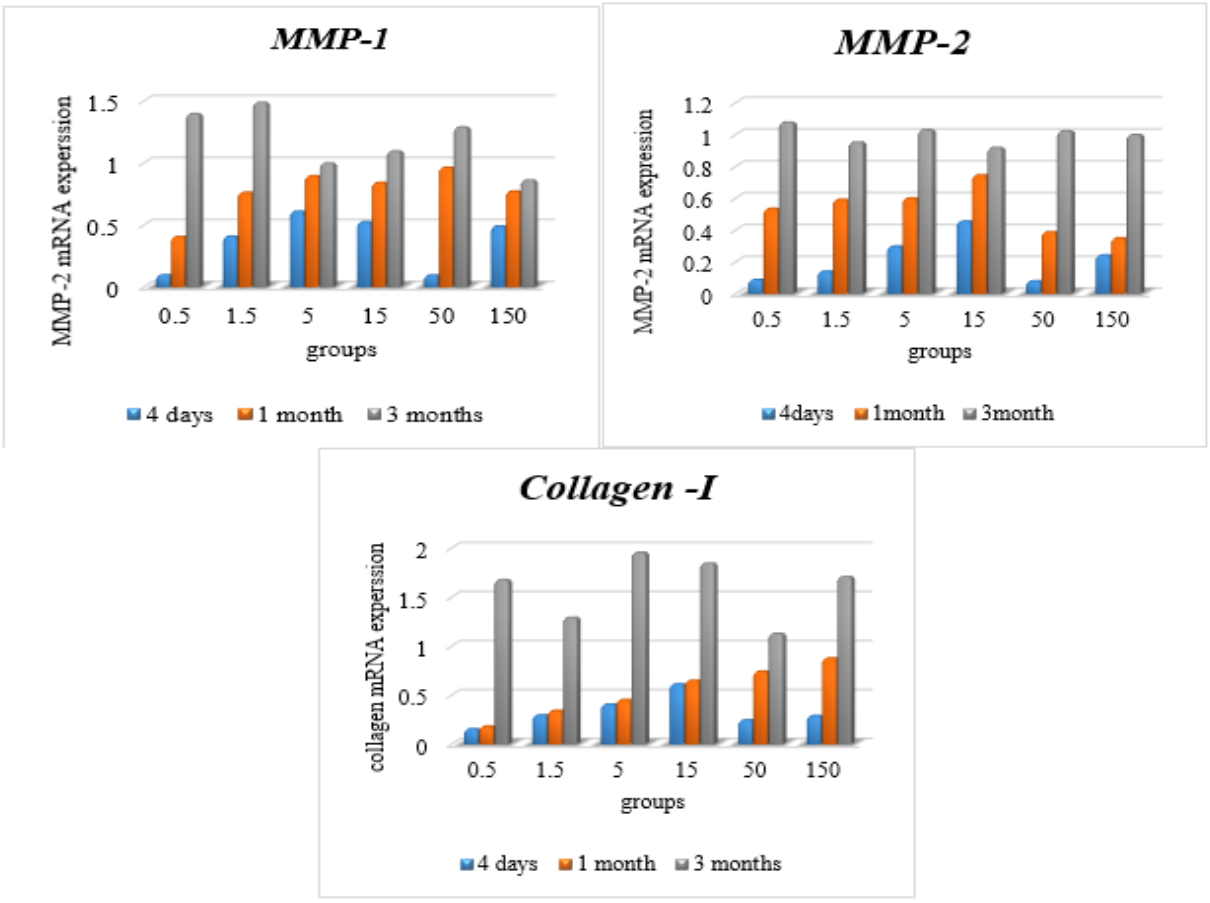

Fig. 8. Effect of TiO_2 NP on the mRNA expression of *MMP-1*, *MMP-2*, and *Collagen-I* in the lung after 4 days, a month and 3 months of intra-tracheal instillation.

Table (3) Effect of TiO_2 NP on the amplification of extracellular metalloproteinase mRNA by RT-qPCR analysis after 4days, a month and 3 months of intra-tracheal instillation for consecutive 4 weeks.

Genes		**TiO_2 NP (mg/kg BW)**						
4days		**0**	**0.5**	**1.5**	**5**	**15**	**50**	**150**
refer-HPRT	Ct	23.106	22.701	22.774	23.105	22.997	23.275	23.067
	Copies	7.36E+3	3.28E+3	6.0E+3	14.63E+3	10.87E+3	11.23E+3	13.91E+3

MMP-1	Ct	23.515	26.514	24.227	24.235	24.877	27.123	24.517
	Relative Copies	32.705	3.669E+3	4.00E+2	1.738E+2	3.854E+3	1.36E+5	7.189E+2
	Ratio of MMP-1/HPRT	0.755±0.049	0.072±0.014[a]	0.306±0.112[a]	0.508±0.282	0.393±0.36[a]	0.07±0.012[a]	0.367±0.02[a]
MMP-2	Ct	22.040	25.538	25.144	24.278	23.625	26.254[a]	25.292[a]
	Relative Copies	28.1838	4.68E+4	1.395E+4	4.94E+3	5.457E+2	2.519E+5	4.13E+7
	Ratio of MMP-2/HPRT	1.661±0.432	0.145±0.0432[a]	0.209±0.089[a]	0.482±0.210[a]	0.721±0.374[a]	0.128±0.018[a]	0.408±0.33[a]
Coll-I	Ct	28.721	31.049	30.17	30.045	29.103	31.0519	30.511
	Relative Copies	7.27E+2	1.179E+5	2.76E+4	1.856E+4	4.872E+3	1.364E+6	7.647E+4
	Ratio of Coll-I/HPRT	0.021±0.007	0.003±0.0006[a]	0.006±0.0008[a]	0.008±0.001[a]	0.011±.002[a]	0.005±0.0035[a]	0.006±0.0001[a]
1month								
refer-HPRT	Ct	22.773	22.701	23.441	22.772	22.997	23.734	22.734
	Copies	2.85E+7	2.43E+8	4.17E+6	2.43E+8	2.61E+6	5.79E+3	2.62E+6
MMP-1	Ct	21.995	23.333	22.423	22.257	22.106	22.694	22.993
	Relative Copies	1.01E+5	2.696E+5	3.28E+4	1.19E+4	1.49E+4	7.08E+4	1.61E+5
	Ratio of MMP-1/HPRT	1.83±0.75	0.656±0.15[a]	1.419±0.68	1.461±0.39	1.943±0.75	1.537±0.39	0.822±0.95
MMP-2	Ct	20.005	20.897	20.846	20.764	20.795	22.020	21.587
	Relative Copies	1.56E+3	9.75E+3	1.91E+4	6.28E+3	9.31E+3	1.46E+5	6.74E+4
	Ratio of MMP-2/HPRT	6.844±0.807	3.602±1.045[a]	3.874±0.92[a]	4.065±0.74[a]	4.954±2.3	2.564±1.1[a]	2.341±1.00[a]
Coll-I	Ct	24.214	26.412	26.355	25.808	26.804	24.858	24.5166
	Relative Copies	3.84E+3	2.24E+5	2.01E+5	1.98E+5	1.35E+7	1.34E+4	1.72E+4
	Ratio of Coll-I/HPRT	0.386±0.386	0.077±0.016[a]	0.134±0.02	0.157±0.12	0.168±0.24	0.347±0.11	0.347±0.171
3months								
refer-HPRT	Ct	22.739	23.275	24.065	23.526	22.976	22.752	23.186
	Copies	4.35E+3	2.82E+6	1.39E+5	4.19E+4	1.12E+5	2.19E+6	2.14E+5
MMP-1	Ct	23.303	24.109	24.302	24.129	24.562	24.718	24.224
	Relative Copies	22.005	1.38E+3	5.53E+2	1.36E+2	1.18E+3	5.53E+2	1.23E+3
	Ratio of MMP1/HPRT	0.679±0.062	0.944±0.359	0.969±0.625	0.668±0.133	0.749±0.415	0.927±0.183	0.56±0.319
MMP-2	Ct	21.084	23.453	21.004	22.268	20.214	22.211	21.232
	Relative Copies	3.22E+6	5.59E+6	2.56E+4	5.46E+5	1.39E+4	2.41E+5	2.03E+4
	Ratio of MMP2/HPRT	0.926±0.103	0.984±0.087	0.873±0.036	0.946±0.022	0.843±0.044	0.938±0.051	0.919±0.065
Coll-I	Ct	26.464	26.373	27.553	26.301	25.829	26.600	26.494
	Relative Copies	6.51E+4	3.24E+4	4.98E+5	9.01E+4	1.15E+4	5.52E+4	2.18E+5
	Ratio of Coll-I/HPRT	0.078±0.022	0.124±0.048	0.092±0.026	0.156±0.065	0.139±0.019	0.089±0.042	0.151±0.138

Values present mean ± Sd, (n=3)

[a] The mean difference is significant ($p < 0.05$)

Discussion

Dependent on the variant endpoints, we detected the concerted toxic influence of exposure to TiO_2 NP. Probably TiO2 NPs causes cell damage related to the time and dose. Toxins exposure can happen through skin touch, inhalation, injection, and ingestion. The clusters of TiO2 NPs that deposited in lungs observed in high dose groups and some of them were not phagocytosed by alveolar dust cell that resembles with Li *et al*.(17) study. TiO2 -NPs treatment decreased the body weight significantly but no significant differences appeared in the coefficients of liver, kidney, and testis but, the coefficient of the brain was significantly higher in TiO2-NPs- exposed rats than the control group (18). In Abu-Dief *et al*. (11) study, intraperitoneal injection of different doses of TiO2 NPs can significantly decrease body weight and increase coefficients of the liver. Bermudez *et al*. who explained decrease body weight and shorter lifetime due to retention and overload of TiO_2 NPs in vivo (19). Duan and his associates stated that TiO_2 NPs were difficult to clearance *in vivo* result in its deposition in the liver and hepatic lesion (20).

It has been demonstrated that TiO_2 excite special apoptotic pathways (21), Roulet *et al*. (14) recognized inflammatory histological changes included vascular and bronchial infiltration of inflammatory cells. Angiogenesis occurring in tissues beyond the area of existing blood vessels as the results of reaction between fibroblasts with endothelial cells (22). TiO_2 NPs trends to conglomerate when administered by aerosol, especially at high doses, the size of nanoparticle conglomerates in the physiological environment may have a crucial influence on macrophage phagocytosis, as it represents the real size that could elucidate a pulmonary immune cell response, besides that, the inflammation produced stimulated by an elevate positive zeta potential which may induce NPs to injury the safety of the phagolysosomal membrane inside the phagolysosome under acid conditions (23). Porter *et al*. (24) reported histopathology scores for alveolitis, phagocytosed NPs, alveolar histiocytosis, and interstitial fibrosis, phagocytosed TiO_2 NPs in alveolar macrophages were significantly higher for mice exposed to nanospheres (7.5 and 30 µg) and short nanobelt (30 µg) at 28 and 112 days post-exposure compared with vehicle exposed controls, there was also a tendency for the long nanobelt to cluster or aggregate within cell cytoplasm of alveolar macrophages compared with the nanospheres, phagocytosis of nanospheres (30 µg dose) was also more evident in tracheobronchial lymph nodes at 112 days post-exposure compared with 28 days post-exposure. Research with high-aspect ratio nanoparticles shows blocked rescue from the lungs after inhalation exposure leading to the pathogenesis of diseases such as pulmonary fibrosis and mesothelioma (24). At present, there is evidence that high-aspect ratio TiO_2 NPs are relatively more pathogenic, and may elicit "frustrated phagocytosis" by macrophages, lysosome, and impaired lung clearance. Indeed, long-term studies show a link between particle retention and pulmonary pathology (25). Scarino *et al*. showed that lung tissue of chicken egg ovalbumin exposure to TiO_2 NPs inducing pulmonary inflammation, as shown by the leukocytes surrounding bronchioles and extent into alveoli (26).

Rats also were unique in the development of gradual fibroproliferative lesions and alveolar epithelial metaplasia in response to exposure to a high concentration of p-TiO_2 particles for 90 days (27). Rats and mice, but not hamsters, experienced overload at 10 mg/m2 nano-TiO2. Moreover, only rats had fibroproliferative lesions and alveolar epithelial bronchiolization (28). An inflammatory response is then observed and develops into active chronic inflammation, an increase in collagen deposition (coming from fibroblast proliferation) and epithelial cell proliferation, as well as metaplasia, were observed in rats subjected to a high dose of carbon black (29). The physical contact between the epithelium surface and the NPs may have been a source of injuries and restrain the enough gas exchange, leading to the high mortality rates after TiO_2 NP instillation.

Matrix metalloproteinase expression was affected by TiO_2 NP which demonstrated in this study. There was decreased in the expression of (*MMP-1, MMP-2, MMP12* and *Collagen I)* genes in 4 days post-instillation then gradually increased in a month and 3 months post-instillation comparing with the control group. It has long been consent that MMPs have a remarkable role in the pathogenesis of pulmonary fibrosis, but the accurate mechanisms are not well characterized. There are different interconnected processes such as basement membrane disruption, extracellular matrix remodeling, epithelial cell apoptosis, cell migration, and angiogenesis in which MMPs may have a central role, either by extracellular matrix direct cleavage or by producing bioactive mediators. TIMPs can modify cellular processes such as cell growth, migration, and apoptosis, and can be both anti- and pro-tumorigenic (30). MMPs have emerged as critical roles in the pathogenesis of lung fibrosis. However, their participation is not only limited to their role as extracellular matrix

modulators but also the determination of cell behavior. Crucially, some MMPs seem to encourage a fibrotic response while others appear to play a protection role (31).

In consequence of the degradation of the extracellular matrix by metalloproteinases, healthy cells can also be influenced by proliferation, apoptosis, or pathological morphogenesis. MMPs can also change the activity, they can even modify other proteins expressions (32). Armand *et al*. (33) demonstrated that five TiO_2 NPs stimulated a significant dose-dependent increase in MMP-1 mRNA expression 48 hours after the initial exposure but not at earlier time points, micrometric TiO_2 also stimulated MMP-1 mRNA-protein expression., so MMP-1 expression modulation by TiO_2 particles was mirrored by a significant dose-dependent decrease. Furthermore, Armand and coworkers reported that a dissociation between MMP-1 expression (mRNA and protein) and its activity.

In normal tissues, MMPs are expressed at low levels, and their participation manifests to play an important role in the development of a several of pathological processes including fibrosis. the involvement of MMPs demonstrated in the pathogenesis of pulmonary and hepatic fibrosis. For instance, broncho-alveolar lavage fluids from individuals with sarcoidosis or pulmonary fibrosis contain high levels of collagenase, concept to be neutrophil-derived, a fact that has been proposition to be related to the development of fibrosis in these subjects, the involvement of MMP-2 in extracellular matrix precipitation was also suggested in a model of bleomycin stimulated pulmonary fibrosis in rabbits (34). MMP-2 is a protease have gelatinolytic activity (hence its alternate name, gelatinase A), which is demonstrated to be expressed constitutively in various lungs cell types. This enzyme has a broad spectrum of substrates and is involved in modulating different cellular functions, including angiogenesis, tissue remodeling, and potentiation of the inflammatory response, MMP-2 is thought to contribute to the pathogenesis of a variety of pulmonary disorders, including chronic obstructive pulmonary disease, asthma, lung cancer and pulmonary fibrosis (35). Geraghty *et al*. (36) recently demonstrated that neutrophil elastase may raise MMP-2 expression from epithelial cells, potentially leading to increased remodeling and inflammatory response in cystic fibrosis. For example, erasure of MMP2 is preventive in allotransplant models because it significantly decreases cellular infiltration and fibrosis (37).

Conclusion

TiO2 NPs are widely used, many applications as the additive, including drugs deliver, a white pigment in paint, a food colorant, in cosmetic creams and sunscreens as well as in the environmental purification of air, water and soil as pesticide destruction products. So the potential health of TiO2 NPs had gained increasing attention and because of the smaller particles of TiO2 has more reactivity, effectively and toxicity; in this study we demonstrated that TiO_2 NP affected on MMPs expression and histopathological alteration on lung including fibrosis, acute inflammation, and proteinaceous.

Acknowledgments

This article was part of a Biostatistics PhD thesis entitled "Histopathological and immunohistochemistry effects of TiO2 NP after repeated intra-tracheal instillation and evaluation of metalloproteinase expression in animal model" in University of Basrah, College of science.

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