

Original Research Article

# Repeated intratracheal instillation of TiO<sub>2</sub> nanoparticles effect on *MMP-12* expression in rat's lung

Dhamia K. Suker<sup>1</sup>, Fatimah A. Jasim<sup>2\*</sup> and Saad Abdullah<sup>3</sup>

Abstract

<sup>1</sup>University of Basra, College of Science, cell and biotechnology research unit

<sup>2</sup>Ministry of Education

<sup>3</sup>University of Basra, College of medicine

\*Corresponding Author's E-mail: [alifaith28@yahoo.com](mailto:alifaith28@yahoo.com)

Titanium dioxide (TiO<sub>2</sub>) nanoparticles have biological effects that may be caused by fibrosis or emphysema through effects on matrix metalloproteinase expression. The aim of this study investigates the effect of TiO<sub>2</sub> nanoparticles on MMP-12 expression. Sixty-three rats were intratracheally instilled with (0.5, 1.5, 5, 15, 50, 150) mg/kg body weight of TiO<sub>2</sub> nanoparticles for 4 weeks. Animals were sacrificed at 4 days, a month and 3 months post-instillation, immunohistochemistry of MMP-12 antibody and lung mRNA of *MMP-12* gene expression. At 4 days post-instillation, TiO<sub>2</sub> nanoparticles decreased macrophage MMP-12 expression with emphysema at high doses and fibrosis at low doses caused fibrosis while after a month and 3 months post-instillation macrophage MMP-12 increased and induce pulmonary response with emphysema at low doses. TiO<sub>2</sub> nanoparticles aggravated elastase-induced emphysema and caused histological inflammation and downregulate MMP-12 mRNA expression and protein expression in macrophage and matrix extracellular.

**Keywords:** MMP-12 expression, TiO<sub>2</sub> nanoparticles effects, MMP-12 immunostaining, emphysema

## INTRODUCTION

Nanotechnology implementations, spreaded 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. The characteristic of nanoparticles and their small size can become easily a carriage for linking and transmitting of poisonous chemical pollutants. There are numeral studies showing that NPs and nanotubes can be released into the environment and cause harmful effects to humans and/or living organisms. (Valavanidis and Vlachogianni, 2010) . Titanium dioxide, as well as called titanium (IV) oxide or titania, is the naturally synthesized oxide of titanium, chemical formula TiO<sub>2</sub>. When utilized as a Pigment, it is termed titanium white: Pigment White 6 (PW6), or CI 77891. Generally, it is sourced form rutile, ilmenite, and anatase. It has a huge range of applications, from sunscreen to paint to food coloring. When utilized as a food coloring, it has E number 171 (E171). World

production in 2014 was about 7.5 million tons and the amount is raising (<http://nanopartikel.info/en/nanoinfo/materials/titanium-dioxide>).

Extracellular matrix (ECM) is nearly connected with inflammation and followed by fibrosis. Extracellular matrix precipitation probably counts on synthesis and degeneration of collagen. Degeneration of collagen is controlled by matrix metalloproteinase and their inhibitors (TIMPs); matrix metalloproteinases (MMPs) are a group of zinc and calcium-subordinate endopeptidases that assume a key part in ECM remodeling in the lung (Gill and Parks, 2008). MMPs have the capacity to corrupt the different parts of connective tissue matrixes, particularly coordinated to ECM compounds. However, the MMPs activities are controlled at a few levels incorporating with particular inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) (Morimoto et al., 2011). An instability between MMPs and TIMPs has been caused

metalloproteinase activation or inactivation, and relative levels of MMPs instead of TIMPs may take part on degeneration or synthesis of collagen in the interstitial space. Fibrosis may occur when the balance between these proteins was described due to the infiltration of inflammatory cell, inflammation and increased density of collagen fiber (Gill and Parks, 2008). Among MMPs, macrophage elastase or MMP-12 is released as a 54 kDa proenzyme and is transferred into a 45 and 22 kDa active forms. MMP-12 main function degradation elastin and a broad selection of other substrates, including type IV collagen, laminin, fibronectin, gelatin proteoglycan, heparin and chondroitin sulfates. MMP-12 originally detected in alveolar macrophages of cigarette smokers (Kaspiris et al., 2015).

In the latest few years, nanomaterials component was assumed in many fields, for example, instruments, optics, beautifiers and nourishment, additionally utilized as a part of the medication and diagnostics. This emotional increment of modern generation and utilization of nanomaterials has prompted explore their potential well-being impacts. For sure, the nanotoxicology is a field entrenched. Investigations of the impacts of nanoparticles (NPs) from various industry branches on cells and pathways are rising, and the majority of the natural impacts of NPs appear to be because of their associations with proteins.

## MATERIALS AND METHODS

### Preparation of Nano-TiO<sub>2</sub> solution

Manufactured nano-TiO<sub>2</sub> was purchased from Sigma with characteristic white powder with a mean diameter of 21 nm, and the solutions were prepared as describe in Jasim *et al.* (2017). Briefly, the TiO<sub>2</sub> powder was sterilization then it was scattered into a fluid with 0.9% (w/w) sodium chloride (NaCl) arrangement. For adequately scatter particles, arrangements containing TiO<sub>2</sub> particles were sonicated for 5 min at 30% abundance by Ultrasonicator processor and vortexes before it's utilized as a part of each treatment.

### Animals and treatments

Sixty-three male rats (8weeks age) with an average body weight of 123.87±22.47 g were used. The experiment design as described in Jasim *et al.* (2017) briefly, rats were divided into seven groups included: a control group which treated with 0.9% w/w NaCl solution, experimental groups which treated with (0.5, 1.5, 5, 15, 50, 150) mg/kg of nano-TiO<sub>2</sub>. Intratracheal instillation with nano-TiO<sub>2</sub> solutions was repeated twice a week for 4 weeks. Then rats were sacrificed at the day 4th, a month and 3 months after instillation, then lung was isolated.

### Immunohistochemistry of MMP-12

The lung specimens were fixed in 10% buffered formalin for a maximum of 24 h, followed by paraffin embedding. From each block, 5-µm thick sections were cut and mounted on 3-amino-propyltriethoxy-silane coated slides then dewaxed in xylene and rehydrated in graded ethanols according to standard procedures. Endogenous peroxidase activity was blocked by pretreatment of slides with 3% hydrogen peroxidase for 10 min. Rabbit Polyclonal Antibody Matrix Metalloproteinase-12 (ab66157, ABCAM) for the MMP-12. The antigenic epitope was unmasked by antigen retrieval. The most commonly used antigen retrieval is a citrate buffer method. The slides were arranged in a staining container. Cast 300 ml of 10 mM citrate buffer, pH 6.0 into the staining container and incubate it at 95-100°C for 10 min then the staining container was removed to room temperature and the slides were allowed to cool for 20 min. Slides were incubated with the primary antibodies diluted in Tris -buffered solution (50 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.4). 100 µl diluted Sav-HRP conjugates (using the antibody dilution buffer) was applied to the sections on the slides and incubated in a humidified chamber for 30 min (kept protected from light). 100 µl DAB substrate solution (freshly made: 0.05% DAB - 0.015% H<sub>2</sub>O<sub>2</sub> in PBS) was applied to the sections on the slides to reveal the color of antibody staining this take < 5 min to obtain desired color intensity. Slides were counterstained with Mayer's hematoxylin. The MMP-12 immunohistochemistry was evaluated using a semiquantitative immunoreactive score (0 - 6) based on the percentage of positively stained cells (PS) (0-3) points and the intensity of staining (SI) (0-3) points, and shown in the table (1). The total score (TS) was calculated by adding the PS and SI scores, and the mean of the TS was used for the statistical analysis (Poulopoulos et al., 2013).

### Total RNA extraction and cDNA synthesis

A thirty mg specimens of lung tissue took from each animal to use for RNA extraction by RN easy Mini Kit (QIAGEN Hilden, Germany) according to the manufacturer's instruction. Total RNA concentration was measured using a Nano Drop spectrophotometer (Mecasys Co., Korea). Then single strand cDNA was synthesized using Accupower Rocket script RT PreMix (Bioneer, Korea)

### MMP-12 expression by RT-qPCR

Real-time quantitative PCR (qPCR) was performed to evaluate the expression of *MMP-12* mRNA. Gene expression was normalized to that of hypoxanthine

**Table 1.** Immunoreactive score for matrix metalloproteinase immunohistochemistry

score	Percentage of immunopositive cells (PS)	Staining intensity
0	0	Negative
1	<10%	Weak
2	10%-50%	Moderate
3	>50%	Stronge

**Figure 1.** Positive control specimen to specify MMP-12 antibody staining reaction in intestinal section

phosphoribosyltransferase (*HPRT*) as a housekeeping gene. It was expressed stably under the experimental conditions used in this study. Real-time qPCR analysis was performed using AccuPower<sup>R</sup> 2X Greenstar<sup>TM</sup> qPCR master mix (Bioneer, Korea). The following primer sequences were used: *MMP-12*, forward primer: CCCAACACATTTTCGTCTCTCT, reverse primer: GGATTTGTCAAGGATGGGTTT, *HPRT*, forward primer: TTGGAAGGGTGTATTTCCTCAT, reverse primer: ATCCAGCAGGTCAGCAAAGAA (Roulet et al., 2012). at 95 °C for 10 min, followed by 45 cycles at 95 °C for 1 min, 50.9 °C for 1 min and 72 °C for 1 min, melting from 50°C to 95°C for 1sec each temp. for 1 cycle. Relative quantification values were calculated as described previously (Ma et al., 2009).

### Statistical analysis

Statistical analysis of all data was carried out using the ANOVA test with differences at  $p < 0.05$  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) also used.

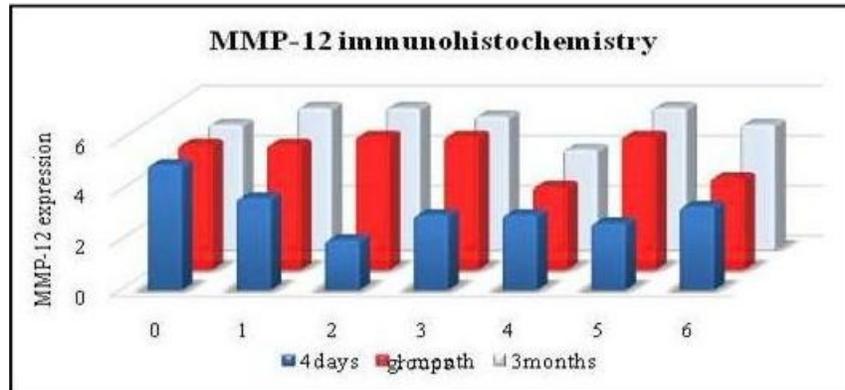
## RESULTS

### Immunohistochemistry

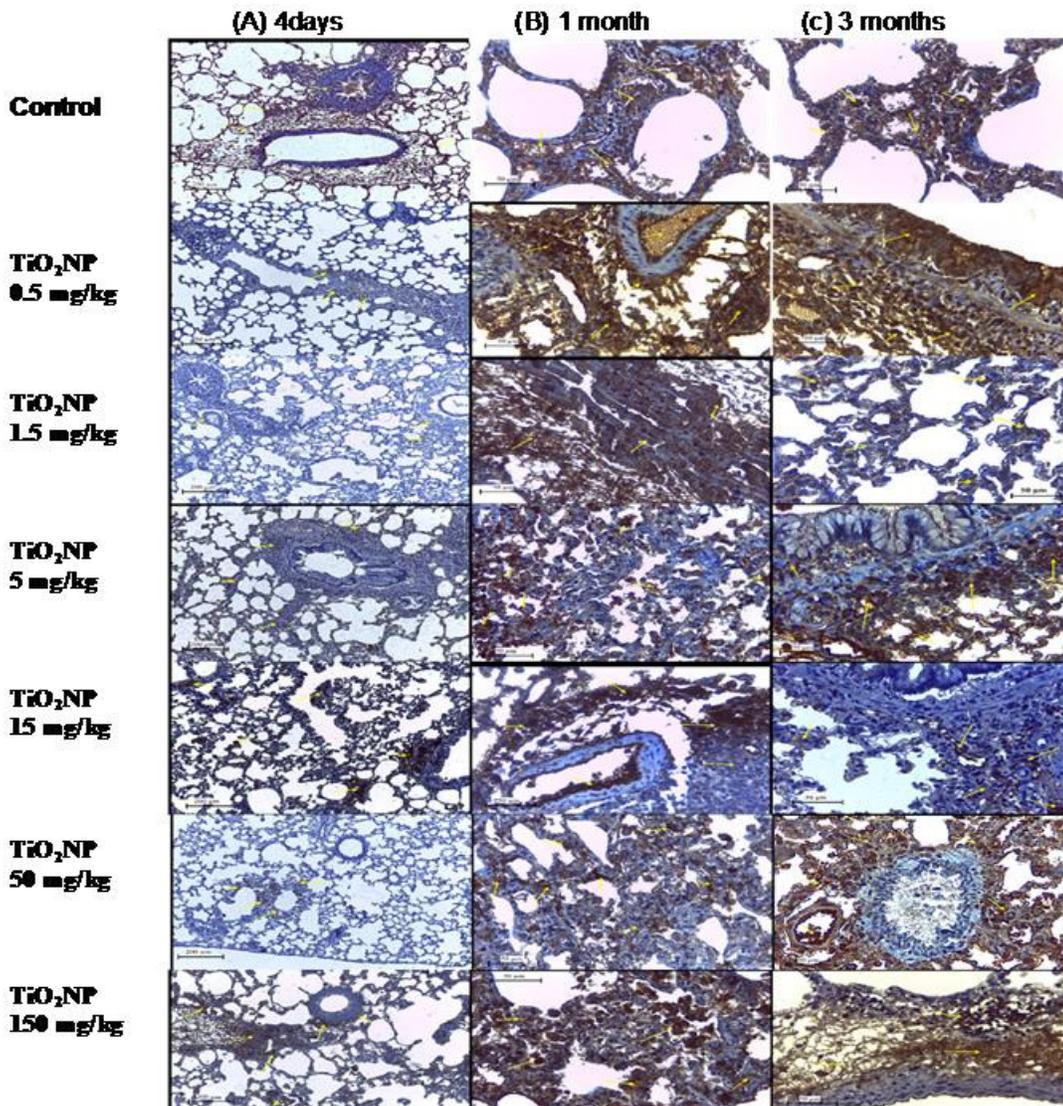
Semiquantitative expression was performed by immunostaining of anti-MMP-12. A positive control confirmed the specific of the staining reaction (Figure 1) in the areas of fragmented, distorted and thickened elastic fiber, in additionally MMP-12 expression in chronic inflammatory cells and most in the macrophage. The results show that anti-MMP-12 was minimal to the negative reaction after 4 days of instillation in treated groups compared with the control group (figure 2,3A). After a month of instillation noticed a significant increase in the immunostaining reaction which was moderate to a strong reaction in all treated groups (figure 2,3B). Also, the same observation was after 3 months of instillation (figure 2,3C).

### Real-Time qPCR

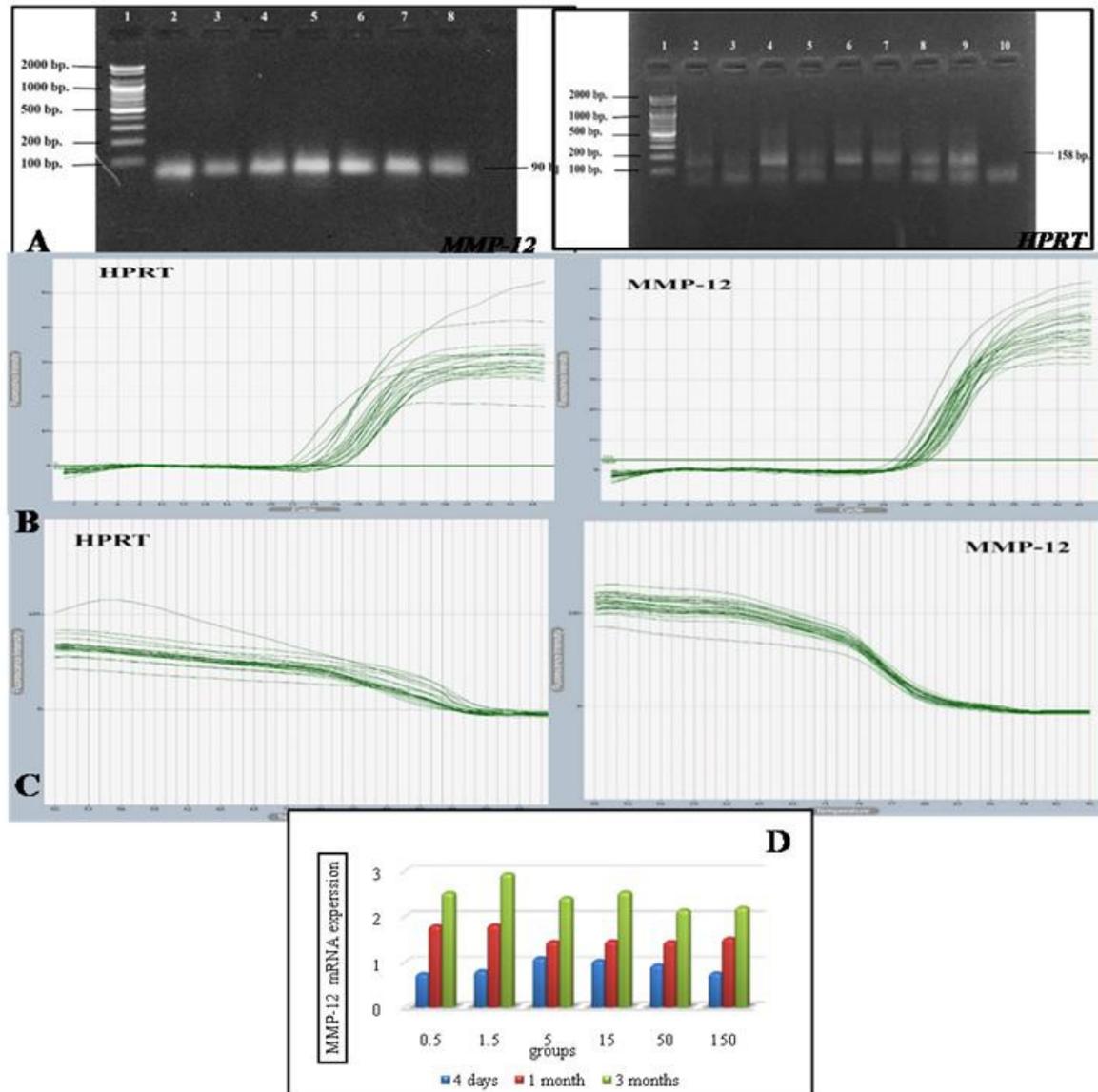
Real-Time quantitative polymerase chain reaction (RT-qPCR) was used to utilize the effect of TiO<sub>2</sub> NP on the MMP-12 mRNA expression. Specificity of RT-qPCR



**Figure 2.** Immunohistochemistry analysis showing the change in MMP-12 expression between treated groups compared with control group during the entire interval of study. groups 0-6 (0.5 ,1.5, 5, 15, 50, 150) mg/kg B.W. respectively.



**Figure 3.** Moderate immunostaining of MMP-12 in lung tissue of control and treated groups. A: after 4 days, B: after a month, C: after 3 months. MMP-12 was selectively stained in the extracellular matrix and in the macrophage (arrows).



**Figure 4.** Analysis RT-qPCR results. (A) 2% Gel electrophoresis (at 60 volts for 45 min) of RT-qPCR products of *HPRT* and *MMP-12* in all groups: lane 1:100bp DNA molecular ladder, lanes 2-9: treated groups with TiO<sub>2</sub> NP showing the genes bands and lane 10: NC: negative control. (B) Quantification of *HPRT* and *MMP-12* mRNA level by real-time quantitative PCR (RT-qPCR), (C) Melting point for *MMP-12* gene. (D) Effect of TiO<sub>2</sub> NP on the mRNA expression of *HPRT* and *MMP-12* in the lung after 4 days, a month and 3 months post- instillation.

**Table 2.** Effect of TiO<sub>2</sub> NP on the amplification of *MMP-12* mRNA by RT-qPCR analysis after 4days, a month and 3 months post-instillation.

		TiO <sub>2</sub> NP (mg/kg BW)						
<i>Genes</i>		0	0.5	1.5	5	15	50	150
4days	<i>MMP-12</i>							
	<b>Ct</b>	32.055	32.112	32.196	32.002	32.198	32.420	32.469
	<b>Relative Copies Ratio of MMP-12/HPRT</b>	1.26E+3	1.42E+3	3.41E+3	1.05E+3	1.75E+3	3.00E+3	3.45E+3
		0.002±0.0003	0.002±0.0002	0.002±0.001	0.002±0.001	0.002±0.001	0.002±0.0005	0.002±0.001
1month								
<i>MMP-12</i>	<b>Ct</b>	29.072	28.229	29.602	28.963	28.792	29.379	28.459

Table 2. Continue

	<b>Relative Copies</b>	1.26E+3	1.417E+3	3.412E+3	1.052E+3	1.746E+3	3.003E+3	3.451E+3
	<b>Ratio of MMP12/HPRT</b>	0.013±0.004	0.024±0.012	0.019±0.018	0.016±0.009	0.019±0.007	0.016±0.008	0.019±0.006
3months <i>MMP-12</i>	<b>Ct</b>	25.242	25.389	25.068	24.759	25.169	24.191	24.691
	<b>Relative Copies</b>	2.09E+5	2.37E+6	1.39E+5	7.82E+4	3.05E+5	4.12E+4	1.31E+5
	<b>Ratio of MMP-12/HPRT</b>	0.189±0.078	0.542±0.408	0.529±0.232	0.451±0.171	0.507±0.319	0.433±0.262	0.388±0.218

Values present mean ± Sd, (n=3)

<sup>a</sup> The mean difference is significant ( $p < 0.05$ )

product was archived by gel electrophoresis, which resulted in a single band with size 90 bp. In addition, a lightcycler melting curve was performed to detect the melting temperature for each gene (figure 4A-C). The result showed the decrease in *MMP-12* expression but with no significant ( $p > 0.05$ ) compared with the control group at 4 days post-instillation. While it was up-regulated with no significant difference ( $p > 0.05$ ) after a month and 3 months post-instillation. Figure (4D), Table (2)

## DISCUSSION

Immunohistochemistry of *MMP-12* antibody showed weak to negative stain after 4 days from instillation then gradually increased after a month and 3 months post-instillation. *MMP12* is a matrix metalloproteinase with elastolytic ability, that is secreted by activated macrophages and has been involved in emphysema pathogenesis in mouse models of chronic obstructive pulmonary disease (COPD) and asthma via different independent mechanisms including (i) proteolytic inactivation of antiproteases such as  $\alpha 1$ -antitrypsin, (ii) degradation of the extracellular matrix, and (iii) proteolytic activation of pro-inflammatory cytokines including TNF $\alpha$  (Zheng et al., 2000; Churg and Wright 2005). Interestingly, *MMP12* is also a signature gene of alternatively activated macrophages (AAM) and the plurality of genes that were diversely upregulated in the lungs from mice (Wagner et al., 2016). Alveolar macrophages are known the provenance of both *MMP-9* and *MMP-12* (Demedts et al., 2005). Increased in cytokines secretion which detected by ELISA led to increasing *MMP-12* in macrophage and in the extracellular matrix. Cytokines are known regulators of *MMP* expression (Greenlee et al., 2007).

Different particles, such Paris Metro or atmospheric particulate matter (PM 10), could stimulate *MMP-12* expression in macrophages (Kobayashi et al., 2009). Cobos-Correa et al. (2009) study provided the first confirmation of an increase in *MMP-12* expression by manufactured NPs, both at the mRNA and protein levels.

The increase in *MMP-12* expression after a single exposure CB NPs could have remarkable pathophysiological consequences because this protease plays a crucial role in emphysema and COPD (Hunnunghak et al., 2009). Oxidative stress, revealed by the increased HO-1 expression, could explain *MMP-12* creation after CB NPs administration (Lavigne and Eppihimer, 2005) while TiO<sub>2</sub> NP on *MMP-12* expression is independent of oxidative stress or IL-1 $\beta$  induction (Roulet et al., 2012).

While *MMP-12* expression which demonstrated by RT-qPCR was downregulated at 4 days post-instillation then gradually increased in a month and 3 months post-instillation comparing with the control group due to the effect of TiO<sub>2</sub> NP.

TiO<sub>2</sub> NPs stimulated inflammation or protease expression but, rather, potentiated *MMP-12* mRNA induction by elastase, potentiated toluene di-isocyanate induced allergic inflammation in mice without inducing inflammation (Hussain et al., 2011). However, the relation of the potentiation of elastase-induced *MMP-12* mRNA expression by TiO<sub>2</sub> is questionable, because it was unable to detect a parallel augmentation in protein expression. Moreover, this phenomenon was noticed in the absence of a parallel increase in HO-1 and IL-1 $\beta$  expression, which suggests that the effect of TiO<sub>2</sub> NPs on *MMP-12* expression is independent of oxidative stress or IL-1 $\beta$  induction (Roulet et al., 2012). The physiological role of *MMP-12*-mediated proteolysis remains poorly understood and may be bind to remodeling of connective tissue during growth and development, and migration of macrophages into tissues (Gaggar et al., 2011).

The regulation of *MMP* expression in diseases of airway obstruction is multifactorial because many regulators including allergen challenge, smoke exposure, activation of inflammatory cells, and the underlying inflammation have been implicated (Greenlee et al., 2007; Churg et al., 2012). The mechanism of *MMP* activation is essentially the same for each family member via the cleavage of a 10-kD proenzyme peptide. In vivo activation of *MMP* may occur through several mechanisms. *MMP* can auto-catalyze the cleavage of the

propeptide, and other proteases, such as NE, MMP-7, and cathepsins, will also activate MMP. Recent studies have investigated the role of reactive oxygen species in MMP activation (Russell et al., 2002).

## CONCLUSION

TiO<sub>2</sub> NP induce many alterations in lung structure after intra-tracheal instillation TiO<sub>2</sub> NP affected on MMP-12 expression in lung detected by immunohistochemistry after 4 days from instillation while it was raised after a month and 3 months from instillation, also TiO<sub>2</sub> affected on MMPs expression which was decreased the expression of *MMP-12* in the lung.

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