

## EXPERIMENTAL STUDY

The effects of Nano titanium dioxide (TiO<sub>2</sub>NPs) on lung tissueMohammadi F<sup>1</sup>, Sadeghi L<sup>2</sup>, Mohammadi A<sup>3</sup>, Tanwir F<sup>4</sup>, Yousefi Babadi V<sup>5</sup>, Izadnejad M<sup>6</sup>

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**Abstract**

**OBJECTIVES:** The application of nanoparticles is widely spread in all aspects of modern life because of their unique features such as small size and high surface area. High surface area can be very reactive and produce reactive oxygen species (ROS). These nanoparticles can damage human and animal cells by increasing oxidative stress mechanism. Titanium dioxide nanoparticles (TiO<sub>2</sub>NPs) are among the top five nanoparticles used in consumer products, paints, and pharmaceutical preparations. TiO<sub>2</sub> NPs have various capabilities such as robust oxidation, biocompatibility and photocatalytic properties. They are frequently used in a wide range of sciences, including pharmaceuticals, cosmetics, medicine and engineering. The ever increasing industrial and consumer applications of TiO<sub>2</sub>NPs raise concern over the possible risk association with their environmental exposure.

**METHODS:** This study investigates the effects of TiO<sub>2</sub>NP on lung tissue by intraperitoneal injection to rats at different doses (15, 30, 60 and 70 mg/kg).

**RESULTS:** Our results showed that intraperitoneal injection of TiO<sub>2</sub>NP creates capillary congestion and hemorrhage in alveolar wall, granulomas in lung parenchyma, and hemosiderin depositions in blood vessels adjacent to bronchioles without any inflammation. The pulmonary side effects could be due to the production of ROS post TiO<sub>2</sub>NP exposure (Tab. 1, Fig. 5, Ref. 27). Text in PDF [www.elis.sk](http://www.elis.sk).

**KEY WORDS:** titanium dioxide nanoparticle, lung tissue, reactive oxygen species, alveolar wall, inflammation.

**Introduction**

Based on their physical and chemical properties, special shape, size and ratio of surface area to volume, the nanoparticles have a unique potential for being applied in biology, medicine and industry. One of the most useful features of nanoparticles is high surface area that causes its widespread application in medical sciences and production of nano-based drugs as a cure for some of incurable disease such as cancer (1).

Published literature showed that rather than large-sized particles, nano-sized particles have a relatively greater toxicity because they are highly reactive and therefore cause oxidative stress in humans and animals. Not only can nanoparticles pass through the cell membrane easily, they even pass through blood-brain and blood-testes barriers (2). Consequently they affect all organs of the body (3). Nanoparticles can enter the bloodstream and rapidly reach the organs (brain, heart, kidneys and lungs) by blood circulation (2). Previous researches confirmed that the exposure to nanoparticles creates malignant pulmonary damage in laboratory animals (4).

Nowadays, titanium dioxide nanoparticles are found in compositions of a variety of products such as self-cleaning glass and tiles as well as in air and water filters (5, 6). The heavy usage of TiO<sub>2</sub>NPs in various retail and industrial products has increased the risk of its potential environmental release (7). The estimated world-wide production of TiO<sub>2</sub>NPs is 10,000 tons/year as for 2011–2014, and by 2025 it is expected to have increased to 2.5 million metric tons/year. The direct evidence of environmental release of TiO<sub>2</sub>NPs from the domestic products has also been documented (8). Therefore, the widespread production and application of these particles attach significance to the investigation of damage they can incur in humans and animals. Numerous laboratories worldwide are studying the harmful effects of particles on humans and animals. There is evidence showing free radical generation by TiO<sub>2</sub>NPs under various environmental conditions, and its cytotoxicity has also been evaluated (9, 10). Oxidative stress such as generation of reactive oxygen species (ROS) and their detrimental effects are considered to be the main mechanism for TiO<sub>2</sub>NP toxicity and these agents can damage any cells (11, 12).

One of the recent studies showed that inhalation of TiO<sub>2</sub>NPs in diameter of 12–220 nm causes acute inflammation in rats' lung tissue. Previous results showed that smaller TiO<sub>2</sub>NPs induce greater inflammation with the same mass dose; the difference may be due to different particle surface areas or particle number (13). Fewer investigations have looked into other modes of toxicity such as attachment of NPs to the organism surface (14, 15) and its accumulation in the digestive tract<sup>16</sup> leading to physical impairment of organism behavior or health. So far, only a few studies have been done regarding the effects of TiO<sub>2</sub>NPs on the lung tissue.

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**Tab. 1. Physical parameters of TiO<sub>2</sub>NPs used in present study. These features are important in chemical and biological function of TiO<sub>2</sub> nanoparticles.**

Color	White
Morphology	Spherical
Crystalline phase	8/78% anatase, 2/21% rutile
Specific surface area	100–150 M <sup>2</sup> /g
Density	3.84 gcc
Size	18 nm
Purity	99.986 %

Our study investigated the effects of different doses (15, 30, 60 and 70 mg/kg) of intraperitoneal injection of TiO<sub>2</sub>NPs to rats on reactive oxygen species (ROS) production and pulmonary damage. Our results showed that an increase in ROS production enhances abnormalities such as hemorrhage in lung tissue of rats.

## Material and methods

Nano titanium dioxide (TiO<sub>2</sub>NPs) prepared by neutrino company (Spanish) XRD provide nano TiO<sub>2</sub> in crystalline phase with 18 nm diameter. Purification of TiO<sub>2</sub>NPs was determined as 99.986 % by ICP-MS. Table 1 summarizes features of TiO<sub>2</sub>NPs used in present study.

### Animals

It is an experimental study carried out on animals. We used adult male Wistar rats weighing 200–250, aged two months on average. The rats were housed in groups under standard lighting conditions with free access to water and food. Humidity and temperature (22 ± 1 °C) were controlled in ventilated cages on a 12-hour day/night cycle. Experimental animals were randomly divided into 6 groups (8 rats in each group). First control group kept on usual water and food. Second control group was a placebo group. The rats from placebo group were injected with 1 ml distilled water every other day intraperitoneally for the equivalent of shock that was incurred by intraperitoneal injection. Other groups, 3rd to 6th, were injected with 1 ml TiO<sub>2</sub>NPs in 15, 30, 60 and 70 mg/kg doses while the injection was repeated every other day intraperitoneally. This continued until day 21 (injection repeated 10 times). TiO<sub>2</sub>NPs was resolved in physiological serum in 20 min by sonication method producing a stable suspension.

### Reactive oxygen species

Measurement of ROS was based on the methods of Wang and Joseph with a minor modification (17). Lung tissue homogenates were prepared and diluted in ice-cold buffer to obtain a concentration of 5 mg tissue/ml. The homogenates were then pipetted into 24-well plates (0.45 ml/well) and allowed to warm to room temperature (21 °C) for 5 min. Then, 5 ml of DCFH-DA (10 mM final concentration) was added to each well and the plates were pre-incubated for 15 min at room temperature to allow the DCFH-DA to incorporate into any membrane-bound vesicles and the diacetate group to be cleaved by esterases. After the pre-incubation, 50 ml of the appropriate concentration of Fe<sup>2+</sup> was added into the wells.

After 30 min (at 37 °C), the conversion of DCFH to the fluorescent product DCF was measured using a Perkin Elmer luminescence spectrometer LS 55 with excitation at 485 nm and emission at 530 nm. Excitation and emission slit were both set at 5nm. Background fluorescence (conversion of DCFH to DCF in the absence of homogenate) was corrected by the inclusion of parallel blanks. The interference of NPs alone was subtracted from the results. ROS production was quantified from the DCF standard curve and results were expressed as pmol DCF formed/mg protein/min. The protein concentration did not differ between groups and was determined by Bradford method in homogenates (18).

### Histological examination

One day after the last injection, the rats were anesthetized, and the lung tissue was separated and immersed in 10% formalin. Thin slices were prepared from paraffin blocks of the preserved tissues (5 micrometer) and then stained with hematoxylin–eosin. The slices were observed through a light microscope.

### Statistics

The data were analyzed using analysis of variance (ANOVA). In case of significant ANOVA, post-hoc analysis was performed using Tukey's test. For rats that received 15, 30, 60 and 70 mg/kg doses, litter was used as statistical measure. Values are expressed as mean ± standard error (SE).

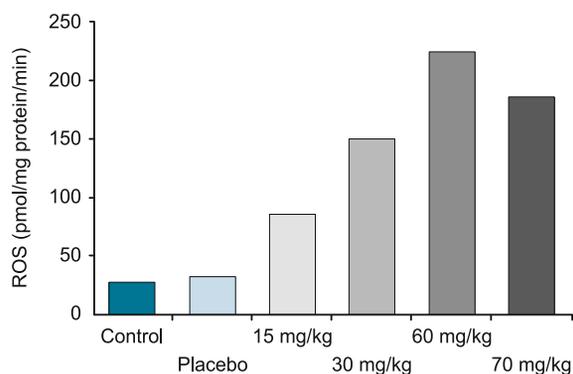
## Results

### Lung weights

Lung weights of rats were increased with an increase in age on the study (i.e., increased per time period following instillation). Lung weights in high dose TiO<sub>2</sub>NP-exposed rats were slightly increased rather than those of controls at weeks 1 and 3 and substantially increased at 3 months (data not shown).

### Oxidative stress analysis

One of the confirmed damaging effects of nanoparticles in the body lies in oxidative stress. Reactive oxygen species (ROS) can be produced in the whole body and hurt all kinds of cells. Therefore we try to assess this parameter in the lungs of treated



**Fig. 1. ROS quantification after TiO<sub>2</sub>NPs injection. Results showed significant increase of ROS in treated animal's lung dose dependent.**

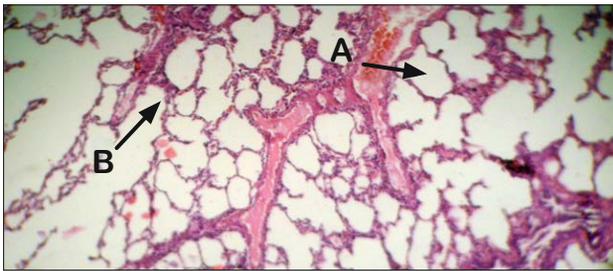


Fig. 2. Histological studies on lung tissue showed healthy lung tissue in control and placebo rats. (A) referred to alveoli space and (B) referred to alveoli wall (x100).

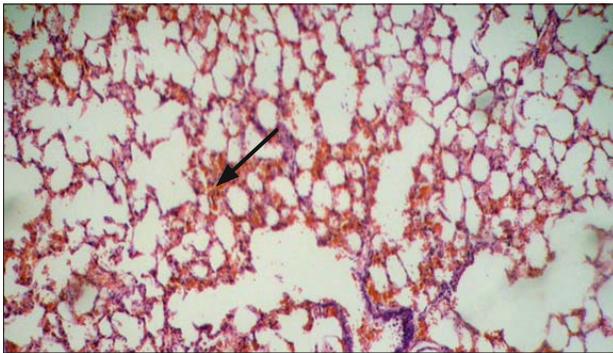


Fig. 3. Histological studies on lung tissue showed unhealthy lung tissue in rats that was administrated by 30 mg/kg dose of TiO<sub>2</sub>NPs. Flash, shows hemorrhage in alveolar wall (x100).

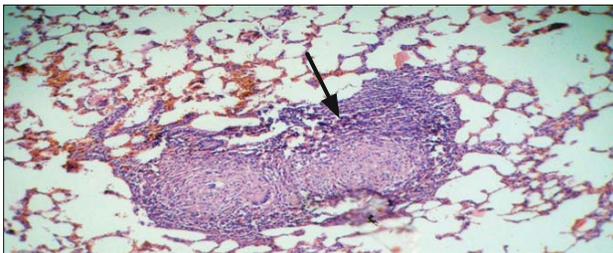


Fig. 4. Histological studies on lung tissue showed unhealthy lung tissue in rats that was administrated 60 mg/kg dose of TiO<sub>2</sub> NPs. Flash referred to granule in parenchymal tissue of lung (x100).

animals by standard method. ROS concentration in the lowest dose (15 mg/kg) is  $80.23 \pm 4.8\%$  which shows a 3-fold increase versus control. The ROS assay showed that free radicals increased dose-dependently. However, the ROS amount reduced at 70 mg/kg dose ( $190.25 \pm 5.0\%$ ) was statistically significant as to ROS at previous concentration of 60 mg/kg ( $230.17 \pm 5.4\%$ ,  $p < 0.05$ ). The notable decrease in ROS can be attributed to the loss of reactivity of NPs probably due to aggregation. It is interesting to note that stress caused by intraperitoneal injection brought about a 0.16-fold increase in ROS in rats (Fig. 1).

#### Histological studies

Histological studies of stained lung tissue were done by light microscope and some pictures were prepared. Figure 2 shows an image of tissue sections of control and placebo groups that look

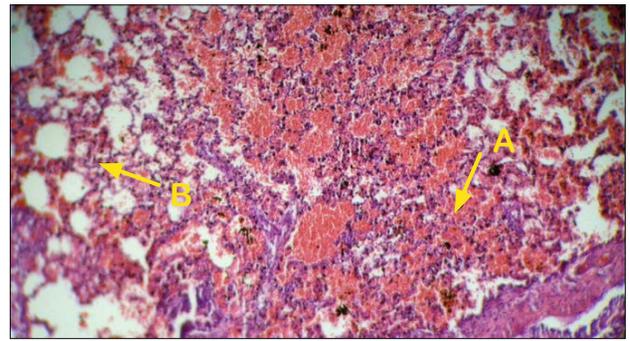


Fig. 5. Showed unhealthy lung tissue in rats that was administrated by 70 mg/kg dose of TiO<sub>2</sub>NPs. Histological studies on lung tissue of this group showed severe damage by nanoparticle on animal lung compared to low doses. Hemorrhage in alveoli space is clear in this figure. (A) referred to hemorrhage in lumen of some alveoli and (B) referred to hemorrhage in alveolar wall (x100).

perfectly normal without any bleeding in the alveolar wall of lungs. But injection of TiO<sub>2</sub>NPs in 30 mg/kg dose causes chronic alveoli wall capillary congestion with focal hemorrhage in this area (alveoli wall) (Fig. 3).

The group 5 administered with 60 mg/kg dose of TiO<sub>2</sub> showed bleeding in border of air space (alveoli wall) and is marked with a number of granulomas in the lung parenchyma (Fig. 4). Injection of 70 mg/kg dose of TiO<sub>2</sub>NPs was accompanied by more severe symptoms such as hemorrhage in alveoli walls and hemosiderin depositions in the vicinity of bronchioles, blood vessels and lymph follicle in the lungs, clearly shown in Figure 5.

Dissection of rats also showed accumulations of aggregate nanoparticles in the pancreas, kidney, liver, and testes, especially at high doses. This accumulation changed the nanoparticles' biological features, inhibited the infiltration of blood vessels and reduced the oxidative damage.

#### Discussion

Nanoparticles have very specific chemical and physical properties as to their size, shape and ratio of high surface area to volume. These properties facilitate their medical and biological applications. After injection of nanoparticles, they are rapidly distributed throughout the entire body by circulation and reach all organs and tissues (19). Before the nanoparticles start being applied in medicine, their effects on environment, biocompatibility and toxic effects on human and animals should be assessed. As a result of their small size, these particles have high surface area so they are highly reactive and this is one important reason for their toxic effects (20). Due to their optical, electrical and catalytic properties, TiO<sub>2</sub>NPs have very important applications in various industries including industrial pigments, sun blockers, bioremediation, air and water filtration and also cancer therapy (21). As a result of this widespread use of TiO<sub>2</sub>NPs, humans and animals are exposed to this material.

Our study was aimed at investigation of TiO<sub>2</sub> intraperitoneal injection effects on ROS production and pulmonary damaging

effects. The lungs were chosen because they are organs that are mostly susceptible to ROS production (22).

We have examined the generation of ROS in crude lung homogenates using the probe 2',9'-dichlorofluorescein (DCF), which is a fluorescent product of dichlorodihydrofluorescein (DCFH). Rather than analyzing the products of oxidative degradation, this method assesses directly the production of reactive oxygen species. Therefore is a standard method for ROS measurement.

The most important effect of nanoparticles in the lungs is inflammation because of their oxidative effects. Recent studies confirmed that rather than particles with 250 nm diameter, smaller nanoparticles with 20 nm diameter cause severe inflammation (23). Other studies showed lung inflammation to be accompanied by the presence of granulated neutrophils, while the amount of granulated neutrophils in alveolar fluid directly relates to the surface area of nanoparticles. However in some cases, the nature of nanoparticles is also involved (24). It is quite interesting that our results did not show any inflammation.

The role of nanoparticles in the mechanisms leading to inflammation lies in their direct effect on alveolar macrophages, changes in phagocytosis and cytoskeletal changes. Nanoparticles can also activate neutrophil, macrophage and epithelial cells, which can produce reactive oxygen agent or ROS (25). Our results showed that ROS production is dose-dependent and related to lung injuries. ROS can also attach to the epithelial cells in vessel wall. Therefore, as our study results showed, the damage resulting from ROS may be incurring blood vessels injuries leading to hemorrhage in alveoli wall and alveolar space. Although the oxidative stress induced by NPs appeared to be the major causative factor, the contribution of other factors cannot be overlooked. Frohlich et al (2009) suggested a possibility of oxidative stress being independent of cytotoxicity mechanisms of NPs (26).

In 2006, Chen and coworkers exposed Wistar rats to TiO<sub>2</sub>NPs by intra-tracheal injection with a dose of 0.1–0.5 mg/kg. Their results showed TiO<sub>2</sub>NPs to cause pulmonary emphysema, lung tissue macrophage accumulation, extensive disruption of alveolar walls, type II pneumonia, and epithelial cell apoptosis (27). Oxidative stress accompanied by inflammation is in most of reported cases, but in our present study the increase in ROS did not lead to inflammation. This might be because of the difference in natural features of used NPs, administration method, size, shape and source of NPs.

## Conclusion

Our results showed that an intraperitoneal injection of TiO<sub>2</sub>NPs creates capillary congestion in alveolar wall, hemorrhage in the alveolar space and wall, presence of granulomas in the lung parenchyma, and hemosiderin depositions in blood vessels adjacent to bronchioles. All of these signs showed an unhealthy condition in the lung tissue that may have been caused by ROS that significantly increased in animals treated by TiO<sub>2</sub>NPs. The comparison of figures shows this unhealthy condition worsens by increasing the nanoparticle administration dose and is accompanied by an increase in ROS concentration with the exception of the highest

dose. Interestingly our study does not show any macrophage accumulation in the tissue.

Results of toxicology studies about nanoparticles vary because the biological activity of nanoparticles is severely affected by their size, shape, purity and source, and the latter properties differ from study to study.

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