EFFECT OF TIO_2 NANOFIBRES ON SELECTED BRONCHOALVEOLAR PARAMETERS IN ACUTE AND SUBACUTE PHASE – EXPERIMENTAL STUDY

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SUMMARY

Titanium dioxide nanofibres (TiO_2) were intratracheally instilled in dose of 4 mg/0.2 mL saline solution per animal (Wistar rats). After 48 hours and 14 days the animals were exsanguinated (under i.p. thiopental narcosis), bronchoalveolar lavage (BAL) was perfomed and cells from BAL fluid were isolated. Following inflammatory, cytotoxic and oxidative stress BAL parameters were examined: differential cell count (% of alveolar macrophages (AM), polymorphonuclears and lymphocytes); the viability and phagocytic activity of AM; the proportion of immature cells; the proportion of multinucleated cells; count of AM/mL lavage; count of BAL cells/ mL lavage; the level of ascorbic acid and activity of superoxide dismutase, both in tissue homogenate and in bronchoalveolar lavage fluid. The majority of examined BAL parameters in the acute and subacute phase in our study suggest serious inflammatory and cytotoxic processes in lung after exposure to TiO_2.

Key words: TiO_2 nanofibres, inflammatory and cytotoxic parameters, bronchoalveolar lavage, intratracheal instillation

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INTRODUCTION

According to EPA sources, nanotechnology is defined as "the understanding and control of matter at dimensions of roughly 1–100 nm, where unique physical properties make novel applications possible. As nanoparticles (NP) are therefore considered substances (particles) that are less than 100 nm in size in more than one dimension. They can be spherical, tubular, or irregularly shaped and can exist in fused, aggregated or agglomerated forms" (1).

NP are used in cosmetics and sunscreens, textiles, coatings, in some food and energy technologies as well as in some medical products (probes, imaging techniques etc.) and medicines. Nanotechnology is under active development or already in practical use in several areas:

- In materials science, nanoparticles allow for the making of products with new mechanical properties, including surface friction, wear resistance and adhesion.
- In biology and medicine, nanomaterials are used to improve drug design and targeting. Others are being developed for analytical and instrumental applications.
- Consumer products such as cosmetics, sunscreens, fibres, textiles, dyes, and paints already contain nanoparticles.
- In electronic engineering, nanotechnologies are used for instance to design smaller, faster and less consuming data storage devices.
- Optical devices such as microscopes have also benefited from nanotechnology.
- Moreover, nanotechnology could also be used in reducing environmental pollution but, on the other hand, nanoparticles may also spread and persist in the environment and, therefore, have an impact on the environment.

Nanoparticles can have the same dimensions as some biological molecules and can interact with them. In humans and in other living organisms they may move inside the body, reach the blood and organs such as the liver or heart, and may also cross cell membranes. Insoluble nanoparticles are a greater health concern because they can persist in the body for a long period of time.

The parameters of nanoparticles relevant to health effects are size (smaller particles can be more dangerous), chemical composition, surface characteristics, and shape (1–5).

Non-soluble nanoparticles can remain in the lung, GI-tract or brain for years, they are not well taken up by professional macrophages of the defence system but interact with cells of the epithelium, the interstitial tissue and vascular cells allowing pro-inflammatory reactions of these cells which usually do not see any particles. In addition, NP can bind to proteins or translocate into the circulation and reach secondary target organs like the liver, spleen, kidneys, heart and brain. In some cases (environmental or occupational exposure), they might represent a potential health hazard by inhalation (6–8).

It has been known for a long time that particles cause lung disease (including coal miners' lung cancer and silicosis from quartz particles and asbestos etc.). The latter causes a number of diseases, including mesothelioma, a particularly nasty cancer seen only in people exposed to asbestos, characterised by a long latency period and with a uniformly fatal outcome. So it is clear that the particles do accumulate in the lungs.

The most important class of nanoparticles by far is NP derived from traffic exhaust, which account for 60% of the exposure. These particles have a basic size of tens of nanometers, though they clump into micron sized aggregates, which are very easily respirable.

Clearly, it is the surface area that is important, so nanoparticles cause more inflammation than the same mass of fine respirable particles, in the 2–3 micron range, composed of the same materials.

Inflammation due to the oxidative stress caused by nanoparticles from pollution then leads to a number of different diseases, including cardiovascular disease, asthma, scarring, cancer, and chronic obstructive pulmonary disease.

Pollution nanoparticles could cause cardiovascular disease either through lung inflammation or through the direct effect of blood-borne particles leading to the deterioration of coronary artery disease or increased blood clotting (2, 3, 5).

Titanium dioxide dust, when inhaled, has recently been classified by the International Agency for Research on Cancer (IARC) as an IARC Group 2B carcinogen possibly carcinogenic to humans. The findings of IARC are based on the discovery that high concentrations of pigment-grade (powdered) and ultrafine titanium dioxide dust caused respiratory tract cancer in rats exposed through inhalation and intratracheal instillation. The series of biological events or steps that produce the rat lung cancer (e.g. particle deposition, impaired lung clearance, cell injury, fibrosis, mutations and ultimately cancer) have also been observed in people working in dusty environments. Therefore, the observations of cancer in animals were considered by IARC as relevant to people doing jobs with exposures to titanium dioxide dust. For example, titanium dioxide production workers may be exposed to high dust concentrations during packing, milling, site cleaning and maintenance, if there are insufficient dust control measures in place. However, it should be noted that the human studies conducted so far do not suggest an association between occupational exposure to titanium dioxide and an increased risk for cancer. Safety in the use of nanoparticle sized titanium dioxide, which can penetrate the body and reach internal organs, has been criticized. Studies have also found that titanium dioxide nanoparticles cause genetic damage in mice, suggesting that humans may be at risk of cancer or genetic disorders resulting from the exposure (2, 4, 5, 9, 10).

The aim of the study was to find out how TiO_2 nanofibres influenced the selected parameters of bronchoalveolar lavage (inflammatory, cytotoxic and oxidative stress) after intratracheal instillation in the acute or subacute phase.

MATERIALS AND METHODS

Nanofibres titanium dioxide − TiO_2 [a) prevalence of fiber average size diameter 50 nm (99.7%), prevalence of average size fiber length 500 nm (99.0%) – measured by using a scanning electron microscope and b) chemical composition analysed by X-ray fluorescence spectrometer (%): TiO_2 – 99.5; SiO_2 – 0.01; Al_2O_3 – 0.05; Fe_2O_3 – 0.04; MgO < 0.01; CaO – 0.01; Na_2O – 0.02; SO_3 – < 0.01; P_2O_5 – 0.07; Nb_2O_5 – 0.13; SnO_2 – < 0.01; ZrO_2 – 0.04; Co_2O_3 – < 0.01; Cr_2O_3 – 0.02; NiO < 0.01; CuO < 0.01; SrO < 0.01; ZnO – 0.01; Cl – 0.01] were intratracheally instilled in dose of 4 mg/0.2 mL saline solution per animal (Wistar rats). After

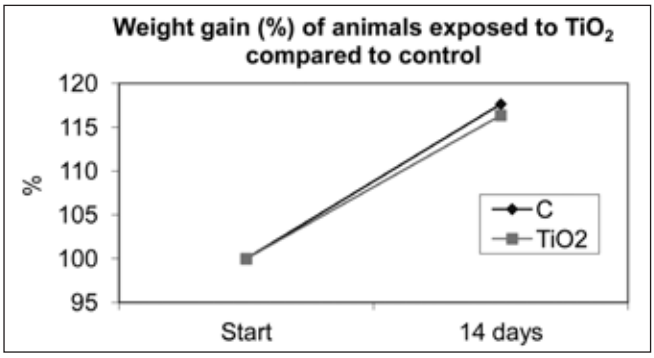

Fig. 1. *Weight gain of experimental animal (comparison of the exposed group with the control group).*

48 hours and 14 days the animals were exsanguinated (under i.p. thiopental narcosis), bronchoalveolar lavage (BAL) was perfomed and cells from BAL fluid were isolated. Then inflammatory and cytotoxic BAL parameters were examined: differential cell count (% of alveolar macrophages – AM, polymorphonuclears and lymphocytes); the viability and phagocytic activity of AM; the proportion of immature cells and proportion of multinucleated cells; count of AM/mL lavage and count of BAL cells/mL lavage; the level of ascorbic acid and activity of superoxide dismutase, both in tissue homogenate and in bronchoalveolar lavage fluid (11).

The intratracheal instillation method of exposure can be a reliable qualitative screen for assessing the pulmonary toxicity of inhaled particles. This comparison was recently made with different formulations of titanium dioxide particle-types. The results demonstrated that the intratracheal instillation-derived, pulmonary bioassay studies represent an effective preliminary screening tool for inhalation studies with the identical particle-types used in this study (7).

RESULTS

Differential cell count of bronchoalveolar lavage (BAL) − AM, PMNL, Ly: differential cell count is an important indicator of cellular analysis of BAL. Exposure to harmful substances caused the change in the differential number of BAL cells by the proportional increasing of inflammatory cells PMNL and LY and by the reduction of AM (Fig. 2–4).

Reducing the number of macrophages and the viability of AM may result in attenuated clearance of inhaled materials, which

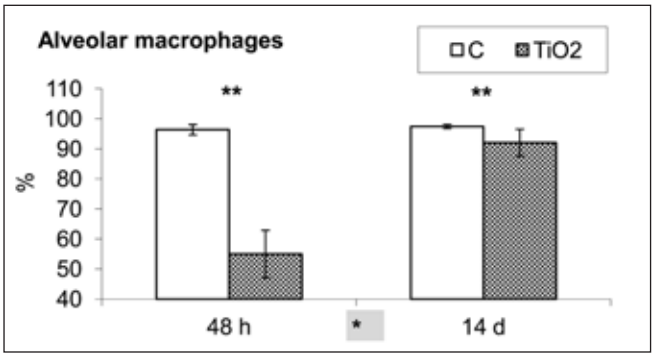

Fig. 2. *Diferential count − proportion of AM*

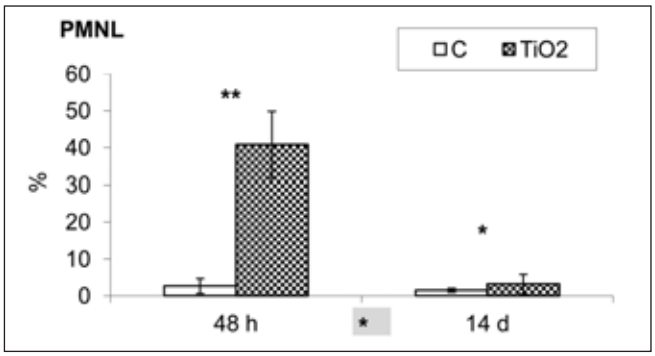

Fig. 3. *Diferential count − proportion of PMNL.*

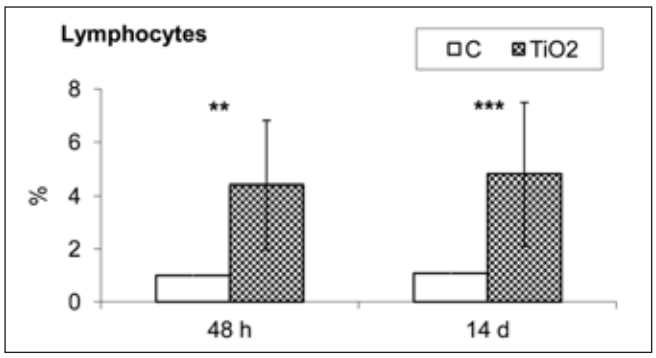

Fig. 4. *Diferential count − proportion of LY.*

may lead to an increase of effective dose of potential pollutants. Significantly increased % phagocytic activity proves the involvement of defence reactions of the organism against harmful substances (Fig. 5, 6).

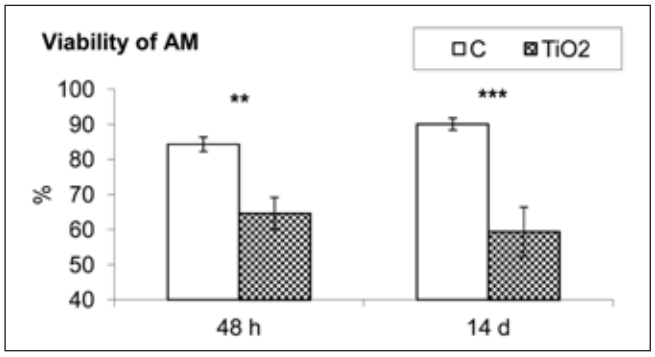

Fig. 5. *Percentage of living cells in BAL.*

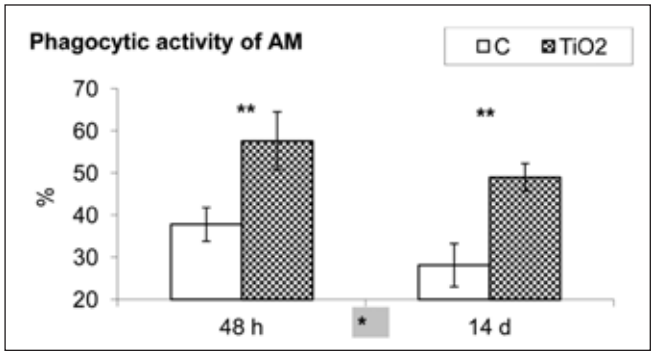

Fig. 6. *Percentage of phagocytizing AM in BAL.*

Increased number of AM immature forms is found in the need of intense inflammatory response. Multinucleated cells are a reflection of rapid mitotic cell division. Exposure to TiO_2 fibers in these intervals represents a greater burden for an organism (Fig. 7).

Multinucleated cells (MNC) were observed at some chronic inflammation processes in the lung. It has been recognized that they may arise by fusion of macrophages engaged in cleaning foreign material from tissue and were named also "foreign body cells". Experimental evidence for this view derives from the finding that if more than one macrophage is attached simultaneously to the same foreign material in vitro, it may result in fusion to multinuclear cells. An increased number of MNC after 14 days exposure to TiO_2 in our experiment has been found (12) (Fig. 8).

An increased number of BAL cells observed after some particle exposure as a result of inflammatory response has been described by numerous authors. We found similar results in our experimental study (Fig. 9–12).

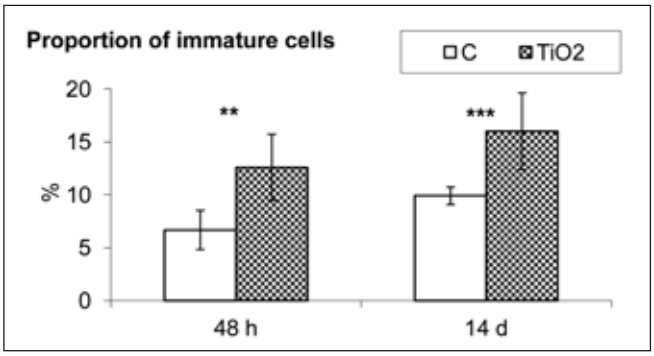

Fig. 7. *Percentage of immature AM in BAL.*

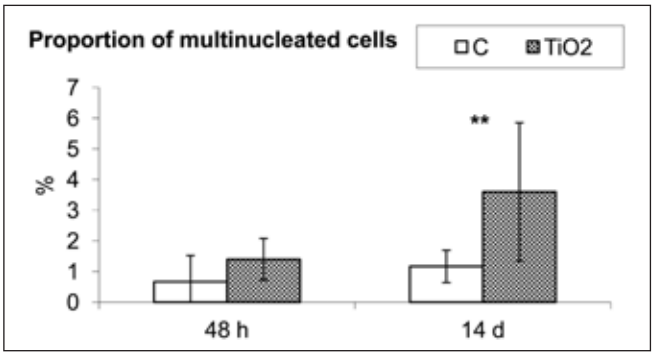

Fig. 8. *Percentage of multinucleated cells in BAL.*

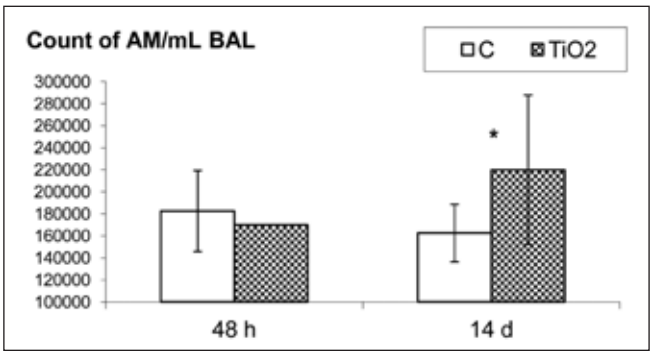

Fig. 9. *Count of AM per mL in BAL.*

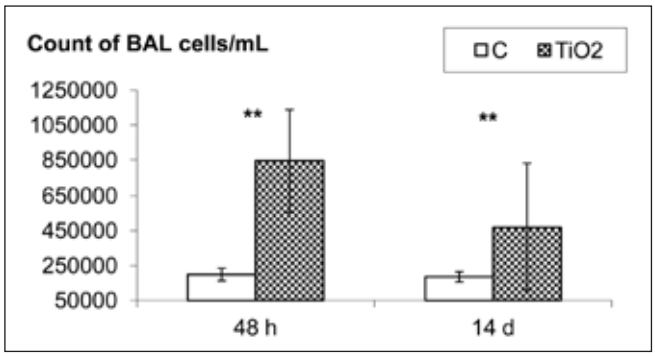

Fig. 10. *Count of BAL cells per mL.*

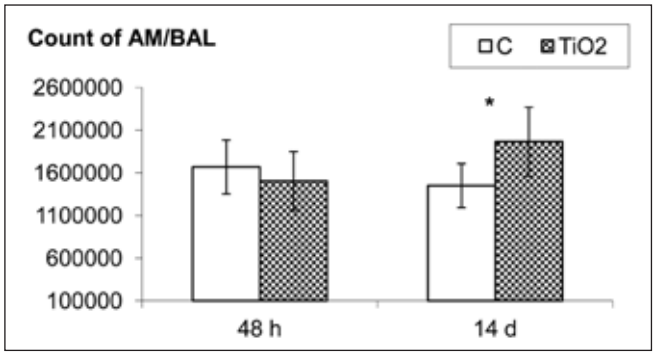

Fig. 11. *Total count of AM in BAL.*

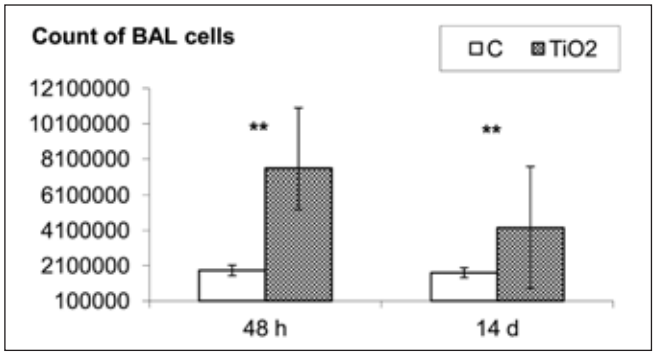

Fig. 12. *Total count of BAL cells.*

An increased level of ascorbic acid (antioxidant) after 14 days of TiO_2 intratracheal instillation represents an escalation of defence against developed oxidative processes (Fig. 13).

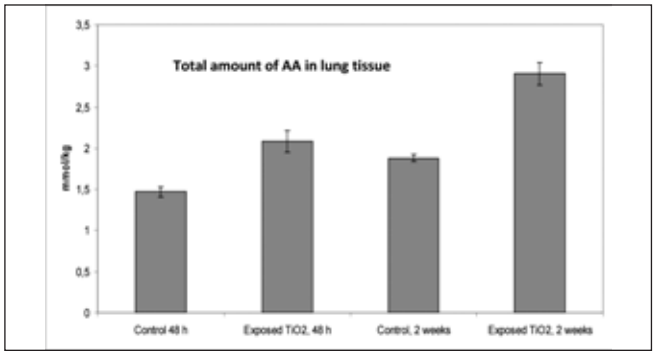

Fig. 13. *Level of AA in lung tissue (mmol/kg).*

A higher percentage of ascorbic acid (AA) and superoxid dismutase levels (SOD) – antioxidant enzyme – is located in the lung tissue (compared with BALF) (Fig. 14, 15). The results were statistically evaluated by the Mann-Whitney Test (mean with 95% confidence interval).

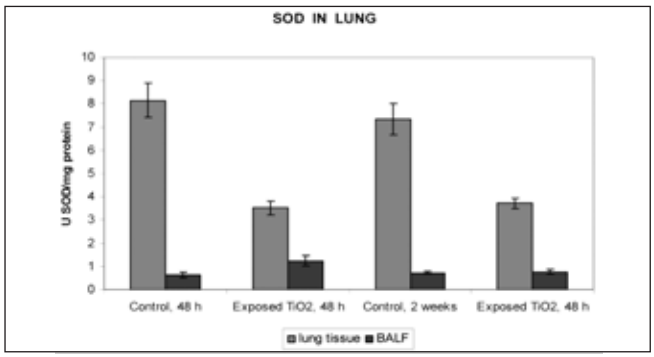

Fig. 14. *Comparison of the amount of SOD (U SOD/mg protein) in the lung tissue and in BAL.*

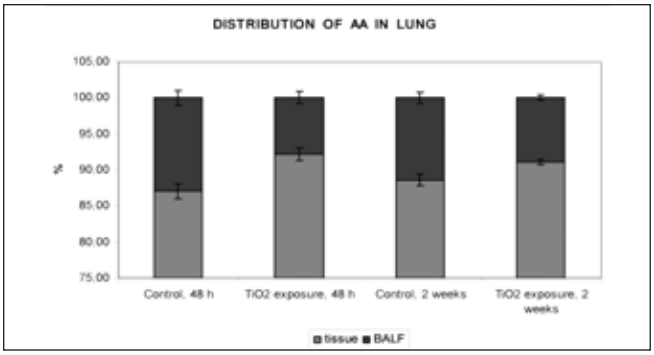

Fig.15. *Comparison of the distribution (%) of AA in the lung tissue and in BAL.*

DISCUSSION

Inhaled nanoparticles can deposit in the lungs and then potentially move to other organs such as the brain, liver, spleen, and possibly the foetus in pregnant women. Some materials could become toxic if they are inhaled in the form of nanoparticles. Inhaled nanoparticles may cause lung inflammation and heart problems. With the exception of airborne particles reaching the lungs, information on the behaviour of nanoparticles in the body is still minimal. Assessment of the health implications of nanoparticles should take into account the fact that age, respiratory tract problems and the presence of other pollutants can modify some of the health effects. Currently, inhalation is the main route of human exposure to nanoparticles (1).

Slowly dissolving and insoluble NP deposited in alveolar region are digested by specialised defence cells – alveolar macrophages which are located in the alveoli. Pulmonary toxicity studies in rats demonstrate that NP produce enhanced inflammatory responses when compared to larger-sized particles of identical chemical composition at equivalent mass concentrations. Inflammation is the common factor that binds together these adverse

effects and the ability of NP to cause inflammation can be seen as an important property. There is the potential for pulmonary inflammation to results in changes in membrane permeability that in turn may impact the potential for particles to distribute beyond the lung. NP have shown significant translocation from the lung to blood (2, 6).

Trouiller et al. also found higher concentrations of inflammation markers and oxidative stress in the mice, that had been exposed to titanium dioxide nanoparticles, which led the researchers to suggest that the toxicity of particles could be caused by their ability to elicit an inflammatory response (13). Oxidative stress belongs to such important factors of the mechanism of action, that has significantly contributed to understanding of many epidemiological studies, that have demonstrated a correlation between health parameters, e.g. mortality or hospitalization due to pulmonary diseases, and concentration of particulate matter in the ambient air (14).

According to the mentioned authors: "The novel principle is that titanium itself is chemically inert. However, when the particles become progressively smaller, their surface, in turn, becomes progressively bigger and in the interaction of this surface with the environment, oxidative stress is induced."

Further human studies are needed in order to truly understand the health effects of titanium dioxide nanoparticles. "It could be that a certain portion of spontaneous cancers are due to this exposure. And some people could be more sensitive to nanoparticles exposure than the others" (13). In the recent years, new toxicity data on the adverse pulmonary effects of exposure to TiO_2 indicated the need for revised risk management recommendations including recommended exposure limits (RELs) for fine (defined as primary particle diameters >100 nm) and ultrafine or nanoscale (defined as primary particle diameters <100 nm) TiO_2. Results from experimental animal studies show persistent pulmonary inflammation and lung tumors for both fine and ultrafine TiO_2 in which the dose-response correlated best with particle surface area (15, 16).

The lung is often a major target organ for the toxic effects of many atmospheric pollutants, both gaseous and particulate in nature. The lung responds continually to chemical and physical stimuli. Not all the responses evoked by substances are injurious. Many responses and changes in the lung are not clearly definable as the lung disease or damage, but can be discerned by bronchoalveolar lavage (BAL). In many cases, the cellular constituents obtained in the lavage provide a good indication of lung injury. Examination of the number and the type of cells obtained via BAL as well as their viability and state of activation enables us to understand the extent of the harmful effects caused to the lung by inhaled noxious substances (17, 18). According to Dziedzic et al. long-term recruitment of PMN might be an important factor in prediction of lung metaplastic processes (17). AM play a significant role in the mechanism, which regulates the response to noxious substance exposure. Besides their phagocytic nature, AM are also important immuno-regulatory cells involved in the defence mechanisms as well as in the pathogenesis of numerous lung diseases. Activated AM release various cytokines, reactive oxygen intermediates and other mediators of the inflammatory reaction (17, 19, 20).

AM constitute important and frequently utilized tools for examination of cytotoxicity. AM are predominant cells present in BAL and changes in their number or function are the factors determining lung injury and characterizing the pathogenesis of such a response. During the migration of monocytes into tissues, come to a further difference until they become multifunctional tissue macrophages. They may at this stage be regarded as "immature macrophages". During the immune process, the number of "immature macrophages" significantly increases. Decreases in the macrophage number, the viability or the phagocytic capacity may result in an impaired clearance of the inhaled materials (17, 18, 20–21).

Oxidant stress placed on lung tissue after exposure to nanoparticles yield superoxide anion, which can then be converted to hydrogen peroxide. In the presence of iron, a constituent of tobacco smoke, the Haber-Weiss reaction may then result in formation of hydroxyl radicals that can cause DNA strand breaks. The release of hydrogen peroxide by activated macrophages and lipid peroxidation through reactive aldehydes may place additional oxidant stress in the lung and thus eventually result in malignant transformation of the epithelia lining the airways (2, 18).

Most of examined BAL parameters after intratracheal instillation of TiO_2 in the acute and subacute phase showed statistically significant changes, suggesting ongoing inflammatory, cytotoxic processes and oxidative "burning" in lung tissue. We expect that results from longer exposure clarify further, whether those processes will continue to damage the lung tissue or regeneration occurs after elimination of TiO_2 from the lungs. In our study, the majority of examined BAL parameters in the acute and subacute phase suggest serious inflammatory and cytotoxic processes in the lung after exposure to TiO_2.

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