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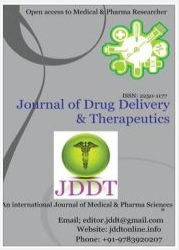

Open Access Research Article

Study on the biological safety of TiO_2 nanoparticles based on the oxidative stress pathway

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ABSTRACT

Objective: To study the effects of TiO_2 nanoparticles in mice and explore the biological safety of TiO_2 nanoparticles. **Methods**: The biological safety of TiO_2 nanoparticles based on the oxidative stress pathway is investigated. One hundred mice were randomly divided into 10 groups. A low medium and high concentration experimental group and negative and positive control groups were investigated for both the intravenous injection and tracheal injection exposure experiments. Mice were observed for any symptoms, and the serum and lung homogenate for activity of SOD and content of MDA in serum and lung tissue were prepared. The scores of the allergic reaction determined the degree of anti-oxidation and the comprehensive experimental data determined the degree of injury in the mice. **Results**: An increase of TiO_2 concentration in the nanomaterials led to a decrease of the content of MDA in serum and lung tissue of mice and the activity of SOD increased, indicating that mice showed oxidative stress in vivo. **Conclusion**: The strong direct exposure to a high concentration of TiO_2 nanoparticles was harmful to the organism.[1]

Key words: Foundation medicine; TiO_2; Nanoparticles; Oxidative stress; Biosafety nanomaterials

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1. INTRODUCTION

The superior properties of nanomaterials have enabled nanotechnology to rapidly become a frontier field in science and technology. Among these materials, titanium dioxide (TiO_2) is a material with great practical prospects, and it has been widely used in industry, agriculture, and other fields [1-3]. The long-term exposure of humans to nanoscale materials in the workplace has increased the probability of its entry into the body [4-5]. Although the research on the safety of titanium dioxide is continuously increasing, the depth and comprehensiveness of this knowledge still need to be improved.

In this study, tail vein and tracheal exposure to various doses of TiO2 nanoparticles were administered to mice. The biosafety of nanoparticle titania, based on the oxidative stress pathway, was determined by observing the allergic reaction in mice and detecting the contents of MDA and SOD in the blood and lung tissues after administration of the TiO_2 nanoparticles.

2. EXPERIMENTAL MATERIALS AND METHODS

2.1 Experimental animals

Mice, KM species, male, weighing 20 ± 2 g.

2.2 Experimental materials

Samples were prepared by mixing 11.5 mg, 5.0 mg and 0.9 mg of titanium oxide nanopowder (20 nm, purchased from EVONIK) with 115 mL, 100 mL and 90 mL of distilled water, respectively. Then, ultrasonication of the samples for one hour resulted in 0.1 mg/mL, 0.05 mg/mL and 0.01 mg/mL titanium dioxide mixed solutions for the tail vein (Group 1 to Group 3). Tween-80 (0.5 mL) was mixed with distilled water (50 mL) to result in a 1% Tween-80 as the fourth group for the positive control group, and the fifth group was the negative control group with distilled water. Titanium dioxide samples of 0.2 mg/kg, 1 mg/kg, and 2 mg/kg were used in the tracheal tube for exposure to the trachea (group 7 to group 9). The test was carried out by using 50 mg, 25 mg and 5 mg of titanium dioxide powder, respectively, and 50 mL of distilled water. Tween-80 (0.5 mL) was mixed with distilled water (50 mL) to make 1% Tween-80 for the positive control as the tenth group. Distilled water was used for the sixth group as the negative control group. An SOD kit and MDA kit were provided by Nanjing Jiancheng Bioengineering Research Institute. A BCA protein concentration determination kit was provided by the Beijing-cable Technology Co. The lung tissue homogenate was prepared according to the BCA Protein Concentration Assay Kit. Three different dilution concentrations were pre-tested. The concentration of protein in each lung tissue sample was determined by choosing the most appropriate dilution factor for the correlation and absorbance.

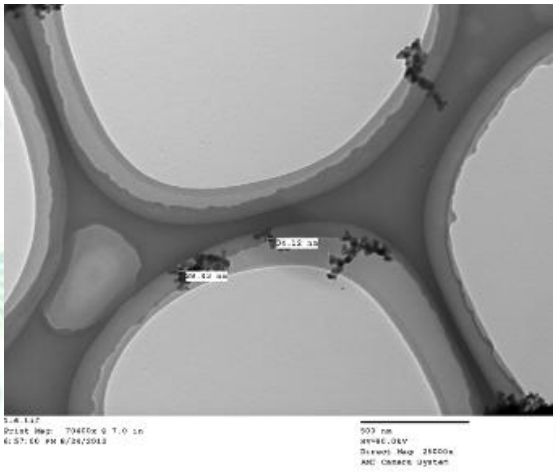

Figure 1: TEM detection results of TiO_2

2.3 Experimental Method

2.3.1 Groups of animals

The mice were divided into 10 groups, with each group including 10 mice.

Tail vein exposure: In the first, second and the third experimental groups, the dose was respectively 0.1 mg/mL, 0.05 mg/mL and 0.01 mg/mL. The fourth group was the positive control group, and the fifth group was the negative control group.

Tracheal exposure: the sixth group was the negative control group. The seventh, eighth and ninth experimental groups had the respective doses of 0.2 mg/kg, 1 mg/kg and 2 mg/kg. The tenth group was the positive control group.

2.3.2 Preparation of experimental drugs

1. Tail vein exposure of the experimental drug preparation (groups 1 to 5) was as follows:

First, the TiO_2 was characterised. The titanium dioxide powder (11.5 mg, 5.0 mg and 0.9 mg) was weighed using an analytical balance, and distilled water (115 mL, 100 mL and 90 mL, respectively) was added to prepare the samples. Ultrasonication of the samples was performed for half-an-hour after mixing. The mixed solutions of 0.1 mg/mL, 0.05 mg/mL and 0.01 mg/mL of titanium dioxide were prepared as the experimental groups. On the other hand, measure the temperature -80 0.5mL and distilled water 50mL,and mixed them. A mixed solution of 1% Tween-80 was prepared as the positive control group and distilled water was used for the negative control group.

2. Tracheal exposure of the experimental drug preparation (groups 6 to 10) was as follows:

Titanium dioxide powder (50 mg, 25 mg, 5 mg) was weighed using an analytical balance, then distilled water (50 mL) was added to prepare the samples. Ultrasonication of the samples was performed for half-an-hour after mixing, and the mixed solutions of 0.2 mg/kg, 1 mg /kg and 2 mg/kg of titanium dioxide were prepared as the experimental groups. On the other hand, measure the temperature -80 1mL and distilled water 50mL, and mixed them. A mixed solution of 2% Tween-80 was prepared as the positive control group and distilled water was used for the negative control group. Other samples were prepared according to the kit instructions.

2.3.3 Administration

1. Intravenous injection into the mice tail was performed with an injection dose of 0.1 mL/10 g. The first, second and third groups were respectively injected with 0.1 mg/mL, 0.05 mg/mL, 0.01 mg/mL of the titanium dioxide solution, the fourth group was injected with 1% Tween-80, and the fifth group was injected with distilled water.

2. Mice were anaesthetized with 1% sodium pentobarbital with a dose of 1 g/mL. After anaesthesia, the trachea was injected with the drug with a dose of 1 μL/g. The seventh, eighth and ninth groups were respectively injected with 0.2 mg/kg, 1 mg/kg, 2 mg/kg of titanium dioxide solution, the twelfth group was injected with 2% Tween-80. The wound was sutured after administration.

2.3.4 Detection of allergic reactions:

The response of the selected experimental group within half-an-hour of administration was scored according to the allergic response score, as shown in Table 1, and the allergic response grade was determined, as shown in Table 2.

2.3.5 Blood biochemical tests:

After 24 hours from administration, 1% sodium pentobarbital was injected into the abdominal cavity with a dose of 0.8 mL/g. Blood was collected from the eyeballs of the mice after anaesthesia, and then centrifuged at 3000 rpm for 15 minutes. The supernatant was extracted, and the contents of SOD and MDA in the serum were determined in strict accordance with the kit instructions.

2.3.6 Detection of lung biochemical tests:

The lung tissue homogenate was prepared after blood sampling. The mouse chest was opened and lung tissue was removed and blot dried after rinsing the remaining blood with ice saline. The ice saline was used to prepare a 10% mixture. The tissue homogenate was mixed using an electric homogenizer and centrifuged to obtain the supernatant, and the contents of SOD and MDA in serum were determined in strict accordance with the kit instructions.

Determination of the protein concentration in lung tissue sample was performed by taking the configured lung tissue homogenate. First, three different dilution pre-experiments were carried out according to the BCA protein concentration determination kit instructions. Then, selection of the correlation and absorbance of the most appropriate dilution factor for the formal experiment to determine the protein concentration of each lung tissue samples was performed.

2.4 Statistical method:

The results were expressed as mean ± standard deviation (± S). SPSS 19.0 software was used to analyse the differences among the groups. Statistical analysis was performed by the t-test, and the difference was statistically significant as P <0.05.

Table 1 Symptoms of allergic reactions

0	Normal	7	Shortness of breath	14	Gait instability
1	Restlessness	8	urination	15	jump
2	Vertical hair	9	Defecation	16	respite
3	Trembling	10	Tears	17	Spasm
4	Scratch the nose	11	Difficulty breathing	18	Rotate
5	sneeze	12	Wheeze	19	Tidal breathing
6	cough	13	Purpura	20	death

Table 2: Systemic sensitisation evaluation criteria

0	-	Allergic reactions were negative
1-4 symptom	+	Allergic reaction weakly positive
5-10 symptom	++	Allergic reactions were positive
11-19 symptom	+++	Allergic reactions strongly positive
20	++++	Allergic reaction strongly positive

3. EXPERIMENTAL RESULTS

3.1 Allergy test results

Allergic sensitivities of the first group to the fifth group were determined according to the degree of hypersensitivity: strong positive (+++), positive (++), weak positive (+) and negative (-) statistics. The results are shown in Table 3. The first, second and fourth groups showed a 100% positive rate, the third group showed a 70% positive rate, and the fifth group showed a 20% positive rate.

Table 3: Comparison of allergic reactions in different groups of mice

Group	Degree of allergic reaction				
	Strong positive +++	Positive ++	Weakly positive +	negative -	Positive rate
First group	3	7			100%
Second Group	2	3	5		100%
Third group		1	6	3	70%
Fourth group	5	3	2		100%
Fifth group			2	8	20%

The first group and the fifth group were tested by Fisher's exact test, with P=0.007, which was statistically significant. The difference between the second and fifth groups was statistically significant (P=0.019) by Fisher's exact test. There was no significant difference between the third group and the fifth group for the

Fisher's exact test (P=0.138). The difference between the fourth and fifth groups was statistically significant (P=0.005).

3.2 Blood biochemical indicators test results

3.2.1 Tracheal exposure test

3.2.1.1 In serum:

Total serum SOD activity = (OD value of the control − determination of OD) ÷ OD value of the control ÷ 50 × dilution of the reaction system × Sample dilution before the test.

MDA content in serum = (OD value − OD value) ÷ (standard OD value − blank OD value) × standard concentration × sample dilution before the test.

3.2.1.2 In lung tissue:

Total lung tissue SOD activity = (OD value of the control − determination of OD) ÷ OD value of the control ÷ 50 × dilution of the reaction system × Sample dilution before the test.

MDA content in lung tissue = (OD value − OD value) ÷ (standard OD value − blank OD value) × standard concentration × sample dilution before the test

3.2.2 Tail vein exposure

1. total SOD activity = (OD value of the control − determination of OD) ÷ OD value of the control ÷ 50 × dilution of the reaction system × sample dilution before the test

2. MDA content = (OD value - OD value) ÷ (standard OD value - blank OD value) × standard concentration × sample dilution before the test.

3.3 Lung biochemical tests

1. The SOD (U / mL) activity of each group was calculated by the absorbance A of the serum measured at 550 nm and the protein concentration of the lung tissue sample using the SOD reagent kit.

Animal tissue homogenate total SOD activity = (OD value of the control − measured OD value) ÷ 50% × (total volume of the reaction sample ÷ sample) ÷ sample protein concentration to be tested.

2. The absorbance of lung tissue measured at 532 nm and the protein concentration of lung tissue and the content of MDA (nmoL/mg protein) in each group were measured by the MDA kit. Results are shown in Table 4.

MDA content in tissues = (OD value − OD value) ÷ (OD value − OD value) × Concentration of standard sample ÷ Sample protein concentration.

3.4 Experimental oxidative stress levelling

Table 4 Comparison of allergic reactions in different groups of mice (first to fifth groups of mice as the test items)

Group	Number of mice	SOD（u/ml）	MDA(nmol/ml)
First group（0.1mg/ml）	10	113.79±26.97	10.68±3.80
Second Group (0.05mg/ml)	10	123.40±28.59	9.55±2.18
The third group (0.01mg/ml)	10	168.40±30.17	9.32±1.66
The fourth group (Negative control group)	10	88.63±37.11	10.75±3.36
Fifth group（Positive control group）	10	173.62±39.32	9.34±2.44
P		P<0.05	P>0.05

Table 5 Comparison of allergic reactions in different groups of mice
(first to fifth groups of mice as the test items)

Group	Number of mice	SOD（u/ml）	MDA(nmol/mgprot)
Six Group (Negative control group)	10	254.17±41.83	7.70±1.03
Seventh group（Low concentration group）	10	241.02±38.01	7.52±1.84
Eight group（Medium concentration group）	10	200.76±29.91	8.57±1.32
The ninth group（High concentration group）	10	179.38±33.07	9.192±1.47
Tenth group（Positive control group）	10	196.64±29.77	10.61±3.04
P		P＜0.05	P＜0.05

To summarise the data analysis and results for the oxidative damage induced by TiO_2 in mice, the SOD activity of serum in the first, second and third groups were significantly different from those in the fourth group (positive control group) and the fifth group (negative control group), P <0.05.

In addition, for the tracheal exposure test, the activity of SOD decreased, MDA content increased, and the oxidative stress of the body was increased. According to the single factor variance analysis (P <0.05), the serum SOD activity of the sixth (negative control group) and the tenth groups (positive control group) and those of the seventh, eighth and ninth groups were significantly difference.

Based on the above data, it was found that the presence of titanium dioxide had a certain effect on the free radical scavenging and detoxification of lung tissue, and the oxidative and antioxidant levels of the organism were stimulated by medium and high concentration exposure with a greater degree of damage to the body in the oxidative stress state (P <0.05). The SOD activity in the lung tissue of the seventh, eighth and ninth groups was significantly higher than that in the negative control group (sixth group) and positive control group (tenth group) from the single factor analysis of variance difference.

In addition, the data analysis in the case of titanium dioxide poisoning showed the lung tissue cells by free radical attack for the ninth group significantly enhanced the body lipid peroxidation rate and intensity (P <0.05). The content of MDA in the lung tissue of the seventh, eighth and ninth groups were compared with the sixth group (negative control group) and the tenth group (positive control group) and showed a significant difference.

4. DISCUSSION

Nanotechnology is one of the three main pillars of technology in the 21st century, and is a hot topic of global debate. With the development of nanotechnology, nano-sized materials with a small scale, special structure and physicochemical properties have been applied in a variety of fields. Currently, more than 1,000 consumer products, identified as nanotechnology-dependent, are being used in the market, so experts are increasingly aware of the significance of a comprehensive assessment of nanomaterial safety and understanding the biological effects. However, with the wide application of nanomaterials, people are increasingly exposed to nanomaterials that may enter the body through various ways, such as: breathing, skin, injection, and being ingested. Many nanomaterials are not toxic by themselves, but can become toxic by altering their physical and chemical properties, such as particle size, shape, surface modification and the charge, composition, stability, solubility, and so forth. Zhang and co-workers have reported that iron oxide nanoparticles (IONPs) with a size of 10 mm predominantly accumulate in the liver whereas IONPs with a size of 40 mm were more concentrated in the spleen while the smaller IONPs (10 mm) were more likely to alter the expression level of a gene [8]. Compared with ZnO nanoparticles, rod-like ZnO NPs are more likely to result in toxic effects [9].

TiO_2 NPs have been shown to become part of the toxic substances carrier to effectively increase the biological toxicity of the original Cd [10]. Therefore, it is very important to study the safety of nanomaterials and establish a safety detection system for nanomaterials.

Currently, studies on the biosafety of TiO_2 and the impact on the surrounding environment are gradually emerging, and the safety problems have been shown to mostly arise from oxidative stress or the generation of free radicals. Oxidative stress or free radicals can cause lipid peroxidation damage, and cause cell membrane damage, thereby resulting in cell apoptosis or death. The results show that, owing to the nanomaterials exhibiting a small size effect and huge surface area, nanomaterials are able to penetrate the cell tissue and exhibit a strong oxidation and catalytic capacity. Therefore, this study first considers the mechanism of oxidative stress and its role in the relevant principles of the organism. MDA is one of the most representative products of lipid peroxidation, and its content can predict the rate and intensity of lipid peroxidation, but also indirectly reflects the free radicals attack on the body cells and degree of damage. Additionally, SOD is involved in the intracellular free radical scavenging detoxification process, which can convert free radicals into H_2O_2. For oxidation in the body, SOD is an active substance that can be eliminated from the body by the metabolism of free radicals and other harmful substances generated by the body through oxygen free radical attack and will inevitably lead to increased peroxide which will then consume the excessive SOD. Hence, its activity can reflect the body's oxidative stress and the strength of the antioxidant capacity. In this study, we found that the MDA content in the experimental group increased significantly and the activity of SOD decreased significantly with an increase of the concentration of nano-TiO_2. The oxidative and antioxidant levels of the nanostructured materials were greatly impaired and the free radicals were attacked. Additionally, lipid peroxidation increased significantly. Therefore, it is possible to explore the principle and method to establish the biosafety of the nanometre titanium dioxide material through the oxidative stress pathway.

Our research on the biosafety of titanium dioxide nanomaterials is based on the principle of the oxidative stress experiment. Through the different routes of administration by tail vein and tracheal exposure, we set up different concentrations of the experimental groups and the negative and positive control groups. We analysed the data obtained by collecting SOD, MDA in serum and lung tissue (in vivo) and by comparing the different hypersensitive reactions (in vitro). In the tail vein administration experiment, the reaction rate and reaction degree of the allergy test showed that the higher the concentration, the higher the reaction degree and the higher the reaction rate. The SOD activity of the experimental group decreased, and the greater the SOD activity decreased, the greater the degree of its oxidative stress. In the experiment of tracheal exposure, the content of MDA in the serum of the high concentration group (group 9) was significantly higher than that of the control group ($P < 0.05$), indicating that the concentration of titanium dioxide could cause the cells to be attacked by free radicals. The activity of SOD in groups 8 and 9 was significantly lower than that in group 7, but there was no significant difference in the seventh group, which showed that the removal of free radicals and the detoxification process of lung cells had a certain effect under the condition of titanium dioxide exposure. The oxidative and antioxidant levels of the organism were damaged to a greater extent under the medium and high concentrations of exposure, so that the body was in an oxidative stress state. In the case of titanium dioxide exposure, lung tissue cells underwent free radical attack, and the ninth group resulted in the significantly enhanced the body lipid peroxidation rate and intensity. The above data show that the ninth group of lung tissue MDA was significantly increased compared with the seventh and eight groups, but there was no significant difference in the trend, indicating that in the case of titanium dioxide poisoning, lung tissue cells undergo free radical attacks, and for group 9, the rate and intensity of lipid peroxidation were significantly enhanced. In summary, the higher doses of titanium dioxide nanomaterial exposure will cause more serious oxidative damage to the body, resulting in a more severe oxidative stress response of the body, indicating that

direct exposure to higher doses of nanomaterials of titanium dioxide will result in a more severe toxicity.

The study for the establishment of a nanomaterial safety system is of great significance. We have chosen a method which has a wide range of applications to titanium dioxide nanomaterials for research to obtain a safety system for nanomaterials. In a series of experiments, it was concluded that the higher the dose of nanomaterial titania from a direct exposure route would result in a more serious toxicity to the organism, thus laying a solid foundation for the future exploration of the safety system of TiO_2 nanomaterials. However, in this experiment, a threshold of toxic concentration of TiO_2 nanomaterials was not obtained, and it is expected that this will be determined from future experiments, enriching the relevant conclusions of the establishment of the safety system of TiO_2 nanomaterials.

5. CONCLUSION

Higher doses of a nano-titanium dioxide aqueous solution will cause more serious oxidative damage in mice, resulting in a more severe oxidative stress response, indicating that direct exposure to higher doses of nano-titanium dioxide produces a greater toxicity.

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