Regulatory Toxicology and Pharmacology 62 (2012) 1–6

Contents lists available at SciVerse ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

In vivo genotoxicity study of titanium dioxide nanoparticles using comet assay following intratracheal instillation in rats

Masato Naya [a,*], Norihiro Kobayashi [a], Makoto Ema [a], Sawako Kasamoto [b], Masahito Fukumuro [b], Shigeaki Takami [b], Madoka Nakajima [b], Makoto Hayashi [b], Junko Nakanishi [a]

[a] Research Institute of Science for Safety and Sustainability, National Institute of Advanced Industrial Science and Technology (AIST), 16-1 Onogawa, Tsukuba, Ibaraki 305-8569, Japan
[b] Biosafety Research Center, Foods, Drugs and Pesticides (BSRC), 582-2 Shioshinden, Iwata, Shizuoka 437-1213, Japan

ARTICLE INFO

Article history:
Received 18 October 2011
Available online 17 December 2011

Keywords:
Titanium dioxide
Nanomaterials
Genotoxicity
In vivo

ABSTRACT

Titanium dioxide (TiO_2) is widely used as a white pigment in paints, plastics, inks, paper, creams, cosmetics, drugs and foods. In the present study, the genotoxicity of anatase TiO_2 nanoparticles was evaluated *in vivo* using the comet assay after a single or repeated intratracheal instillation in rats. The nanoparticles were instilled intratracheally at a dosage of 1.0 or 5.0 mg/kg body weight (single instillation group) and 0.2 or 1.0 mg/kg body weight once a week for 5 weeks (repeated instillation group) into male Sprague–Dawley rats. A positive control, ethyl methanesulfonate (EMS) at 500 mg/kg, was administered orally 3 h prior to dissection. Histopathologically, macrophages and neutrophils were detected in the alveolus of the lung in the 1.0 and 5.0 mg/kg TiO_2 groups. In the comet assay, there was no increase in % tail DNA in any of the TiO_2 groups. In the EMS group, there was a significant increase in % tail DNA compared with the negative control group. TiO_2 nanoparticles in the anatase crystal phase are not genotoxic following intratracheal instillation in rats.

1. Introduction

Titanium dioxide (TiO_2) is widely used as a white pigment in paints, plastics, inks, paper, creams, cosmetics, drugs and foods. Based on data published in openly available scientific literature, the genotoxicity of TiO_2 nanoparticles was evaluated in *in vitro* comet assays (single-cell gel electrophoresis), bacterial and mammalian cell mutation tests, chromosomal aberration assays and *in vivo* micronucleus assays. In bacterial gene mutation assays and chromosomal aberration assays of TiO_2 nanoparticles, both negative and positive results have been reported (Lu et al., 1998; Nakagawa et al., 1997; Theogaraj et al., 2007; Wang et al., 2007; Xu et al., 2009). In the *in vitro* micronucleus assays of TiO_2 nanoparticles, negative and positive results were also reported (Gurr et al., 2005; Kang et al., 2008; Linnainmaa et al., 1997; Lu et al., 1998; Rahman et al., 2002; Vevers and Jha, 2008; Wang et al., 2007). A previous study found that TiO_2 nanoparticles generate reactive oxygen species and oxidative stress leading to genotoxicity in mammalian cells (Shukla et al., 2011). Positive results were reported in tests on DNA damage by TiO_2 nanoparticles in studies with *in vitro* comet assays (Bernardeschi et al., 2010; Dunford et al., 1997; Ghosh et al., 2010; Gopalan et al., 2009; Gurr et al., 2005; Karlsson et al., 2009; Kang et al., 2008; Nakagawa et al., 1997; Reeves et al., 2008; Tiano et al., 2010; Turkez, 2011; Vevers and Jha, 2008; Wang et al., 2007), and *in vivo* comet assays (Trouiller et al., 2009). Negative results were reported in tests on DNA damage from TiO_2 nanoparticles in studies with *in vitro* comet assays (Bhattacharya et al., 2009; Hackenberg et al., 2010; Struwe et al., 2007; Tiano et al., 2010), and *in vivo* comet assays (Landsiedel et al., 2010). In genotoxicity testing, an *in vivo* comet assay is useful for follow-up testing of positive *in vitro* findings and for the evaluation of local genotoxicity. To assess the toxicity of nanoparticles and manage their risks, it is important to understand whether nanoparticles are more toxic than micron-sized particles. Therefore, in the present study, well-dispersed TiO_2 nanoparticles of secondary sizes were studied *in vivo* comet assay using lung tissue following an intratracheal instillation to rats.

2. Materials and methods

The experiments were performed at the Biosafety Research Center, Foods, Drugs and Pesticides (BSRC, Shizuoka, Japan) in compliance with the Law Concerning the Protection and Control of Animals (1973), Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (1980) and Guidelines for Animal Experimentation, Biosafety Research Center, Foods, Drugs and Pesticides. The study was performed in accordance with the ethics criteria contained in the bylaws of the Committee of the National Institute of Advanced Industrial Science and Technology (AIST).

* Corresponding author. Fax: +81 29 861 8904.
E-mail address: masato.naya@aist.go.jp (M. Naya).

0273-2300/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.yrtph.2011.12.002

2.1. Chemicals

Anatase TiO_2 nanoparticles (ST-01) 5 nm in diameter were obtained from Ishihara Sangyo Kaisha, Ltd., Osaka, Japan. As a dispersant for the particles, disodium phosphate (DSP, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was applied at 2 mg/mL according to our previous study (Kobayashi et al., 2009). Ethyl methanesulfonate (EMS, Sigma–Aldrich Corporation, USA) was used as the positive control. Dulbecco's phosphate-buffered saline, regular melting agarose and Triton-X were obtained from Sigma–Aldrich Corporation, and low melting agarose was purchased from Lonza Walkersville, Inc., USA. Ethylene diamine tetra acetic acid (EDTA) disodium salt was obtained from DOJINDO LABORATORIES, Japan. Hanks' balanced salt solutions and SYBR® Gold nucleic acid gel stain were purchased from Life Technologies Corporation, USA. Dimethyl sulfoxide (DMSO), tris hydroxymethyl aminomethane and sodium *N*-lauroyl sarcosinate were obtained from Wako Pure Chemical Industries, Ltd., Japan. TE buffer solution (pH 8.0) was obtained from Nippon Gene, Japan.

2.2. Preparation and characterization of particles

In our previous study (Kobayashi et al., 2009), the DSP solution was provided as a good phosphate-buffered vehicle for preparation of the TiO_2 nanoparticles. TiO_2 nanoparticles were dispersed in 2 mg/mL DSP and agitated in an UAM015 agitating bead mill (Kotobuki Industries Co., Ltd., Tokyo, Japan) at 10–12 m/s for 2 h with 15-μm zirconium oxide (ZrO_2) beads. Subsequently, the supernatant was removed by centrifugation at 8000*g* for 1 h. TiO_2 particles in the DSP solution after sample preparation were measured by the dynamic light scattering (DLS) method (Microtrac UPA150; Nikkiso Co., Ltd., Tokyo, Japan), dropped on TEM grid and dried and then observed by transmission electron microscopy (TEM).

2.3. Animals and treatment

Sixty-four male Crl: CD (SD) rats (7 weeks old) were purchased from Charles River Laboratories, Japan, Inc. (Yokohama, Japan). The rats were kept individually in a positive-pressure air-conditioned unit (20–26 °C, 35–75% relative humidity) for animal housing on a 12:12-h light/dark cycle. After a 6-day acclimation, 55 rats were assigned to the study. A standard rodent pellet diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and drinking water were provided *ad libitum*.

The experimental design was decided in accordance with the standard protocol "International Validation of the *In Vivo* Rodent Alkaline Comet Assay for the Detection of Genotoxic Carcinogens" issued by the Japanese Center for the Validation of Alternative Methods (JaCVAM). For clarifying the relationship between inflammatory response and positive findings of comet assay, the dosage was selected to induce lung inflammation or not. Based on the results of the dose-finding test and our previous study (Kobayashi et al., 2009), 5.0 mg/kg TiO_2 were used for the high dosage group which expected to induce lung inflammation, and 1.0 mg/kg were used for the low dosage group which expected to induce non-inflammation in a single instillation study. In a repeated (intermittent) instillation study, the dosage of 1.0 or 0.2 mg/kg body weight once a week for 5 weeks was selected because these dosage were expected to induce sub-acute lung inflammation or not. TiO_2 nanoparticles were dispersed in 2 mg/mL DSP and instilled in a volume of 1.0 mL/kg body weight. As a negative control, 2 mg/mL DSP was instilled intratracheally by single or repeated administration in a similar manner. EMS was used for a positive control. In our pilot study, intratracheal instillation of EMS did not shown fine results, the other side, single oral administration of EMS shown fine results in the lung epithelial comet assay. Therefore, 500 mg/kg EMS was administered orally once 3 h before sacrifice in both single and repeated study. In the single instillation group, rats were anesthetized and sacrificed 3 or 24 h after the treatment, while in the repeated instillation group, rats were anesthetized and sacrificed 3 h after the last treatment. Five rats per group, except the 0.2 mg/kg TiO_2 repeated instillation group in which one rat died, were used for each time point. The lungs were excised immediately after sacrifice. The left lobe was used for the histopathological examination, and the right lobe, for the comet assay.

2.4. Histopathological examination

The left lobes of the lungs were fixed in 10% neutral buffered formalin. All fixed tissues were routinely processed, embedded in paraffin, sectioned at 3 μm, and stained with hematoxylin and eosin (H&E) for light microscopic examination. The slides scored double blind.

2.5. Comet assay

The comet assay was conducted in accordance with the standard protocol "International Validation of the *In Vivo* Rodent Alkaline Comet Assay for the Detection of Genotoxic Carcinogens" issued by the JaCVAM, as follows:

The right lobes were washed out with homogenizing buffer (Hanks' balanced salt solution containing 25 mmol/L EDTA-2Na and 10% v/v DMSO) and homogenized in about 5 mL of the homogenizing buffer using a Downs homogenizer. Cell suspensions were chilled on ice for about 5 min and centrifuged at 800 rpm for 5 min. After the supernatant was removed, the cells were re-suspended in homogenizing buffer. The 10 μL of the single cell suspension was mixed with 90 μL of 0.5% low-melting agarose gel, and 90 μL of the mixture was placed on a slide pre-coated with 1.0% agarose gel and covered with non-coated superfrosted glass. After solidification, the non-coated slide was removed, and 90 μL of low melting agarose was added again. Two slides were prepared from each rat. The slides were transferred to lysing solution (2.5 mol/L NaCl, 100 mmol/L EDTA-2Na, 10 mmol/L, pH 10 Tris buffer, 10 vol.% DMSO and 1 vol.% Triton X-100) for at least one night at about 4 °C in the dark. They were next placed in a submarine-type electrophoresis chamber (BIO CRAFT Co., Ltd., Tokyo, Japan) and covered with chilled electrophoresis buffer (pH > 13) for 20 min to allow DNA to unwind. Electrophoresis was then conducted at a constant voltage of 0.7 V/cm (25 V) (current at the start: 300 mA) for 20 min. The slides were transferred into neutralization buffer and left to stand for about 10 min. Subsequently, they were dehydrated with ethanol. Finally, the slides were air-dried and stored at room temperature until scoring. The slides were stained with SYBR® Gold nucleic acid gel stain diluted 5000-fold with TE buffer. The migration of DNA in cells was examined using a fluorescence microscope (Olympus Corporation, Tokyo, Japan) with IB excitation [excitation filter (BP470–490) and auxiliary absorbing filters (BA515IF)]. The final magnification was 200×. Images were taken with a CCD camera (Allied Vision Technologies GmbH, Stadtroda, Germany) attached to the microscope and analyzed using a Comet assay analyzer (Comet Assay IV system, Perceptive Instruments Ltd., Suffolk, UK). The parameter used to measure DNA damage in the cells was the percentage of DNA in the tail [% tail DNA]. Images of 100 (50 × 2) cells per rat were analyzed. The mean % tail DNA value (mean value for 100 cells) of each group was calculated.

2.6. Statistical analysis

Data for the TiO_2 groups and negative control group were analyzed using the Dunnett multiple comparison test (two-sided,