Journal of Applied Toxicology

WILEY

Journal of Applied Toxicology

RESEARCH ARTICLE OPEN ACCESS

Subchronic Inhalation of TiO_2 Nanoparticles Leads to Deposition in the Lung and Alterations in Erythrocyte Morphology in Mice

Pavel Kulich[1] | Soňa Marvanová[1] | Radim Skoupý[2,3,4] | Miša Škorič[5] | Jan Vysloužil[6] | Omar Šerý[6,7] | Pavel Mikuška[8] | Lukáš Alexa[8] | Pavel Coufalík[8] | Kamil Křůmal[8] | Pavel Moravec[9] | Zbyněk Večeřa[8] | Miroslav Machala[1]

[1]Department of Pharmacology and Toxicology, Veterinary Research Institute, Brno, Czech Republic | [2]Institute of Scientific Instruments, Czech Academy of Sciences, Brno, Czech Republic | [3]Department of Bionanoscience, Delft University of Technology, Delft, The Netherlands | [4]Kavli Institute of Nanoscience, Delft University of Technology, Delft, The Netherlands | [5]Department of Pathological Morphology and Parasitology, Faculty of Veterinary Medicine, University of Veterinary Sciences, Brno, Czech Republic | [6]Laboratory of Neurobiology and Molecular Psychiatry, Department of Biochemistry, Faculty of Science, Masaryk University, Brno, Czech Republic | [7]Laboratory of Neurobiology and Pathological Physiology, Institute of Animal Physiology and Genetics, Czech Academy of Sciences, Brno, Czech Republic | [8]Department of Environmental Analytical Chemistry, Institute of Analytical Chemistry, Czech Academy of Sciences, Brno, Czech Republic | [9]Department of Environmental Engineering, Institute of Chemical Process Fundamentals, Czech Academy of Sciences, Prague, Czech Republic

Correspondence: Soňa Marvanová (sona.marvanova@vri.cz)

Received: 10 December 2024 | **Revised:** 22 January 2025 | **Accepted:** 22 January 2025

Funding: This study was supported by the Ministry of Agriculture of the Czech Republic (project No. RO0523) and by the Czech Science Foundation (project No. 24-10051S).

Keywords: cathodoluminescence | electron microscopy | lung | powder nanobeam diffraction | titanium dioxide nanoparticles

ABSTRACT

TiO_2 nanoparticles (NPs) are extensively used in various applications, highlighting the importance of ongoing research into their effects. This work belongs among rare whole-body inhalation studies investigating the effects of TiO_2 NPs on mice. Unlike previous studies, the concentration of TiO_2 NPs in the inhalation chamber (130.8 μg/m^3) was significantly lower. This 11-week study on mice confirmed in vivo the presence of TiO_2 NPs in lung macrophages and type II pneumocytes including their intracellular localization by using the electron microscopy and the state-of-the-art methods detecting NPs' chemical identity/crystal structure, such as the energy-dispersed X-ray spectroscopy (EDX), cathodoluminescence (CL), and detailed diffraction pattern analysis using powder nanobeam diffraction (PNBD). For the first time in inhalation study in vivo, the alterations in erythrocyte morphology with evidence of echinocytes and stomatocytes, accompanied by iron accumulation in spleen, liver, and kidney, are reported following NP's exposure. Together with the histopathological evidence of hyperaemia in the spleen and kidney, and haemosiderin presence in the spleen, the finding of NPs containing iron might suggest the increased decomposition of damaged erythrocytes. The detection of TiO_2 NPs on erythrocytes through CL analysis confirmed their potential systemic availability. On the contrary, TiO_2 NPs were not confirmed in other organs (spleen, liver, and kidney); Ti was detected only in the kidney near the detection limit.

1 | Introduction

TiO_2 represents poorly soluble low toxicity particles (Driscoll and Borm 2020), noted for its properties as opacity, brightness, hydrophobicity, high refractive index and UV resistance. Therefore, TiO_2 is extensively used in paints, coatings, plastics, papers, cosmetics, pharmaceuticals and in the food industry outside the EU. TiO_2 particle size depends on its application. Fine particles and nanoparticles (NPs; <100 nm), the latter ones also termed ultrafine particles, produced mostly in rutile or anatase form,

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2025 The Author(s). *Journal of Applied Toxicology* published by John Wiley & Sons Ltd.

are frequently present together (Baranowska-Wójcik et al. 2020; IARC 2010; NIOSH 2011; Younes et al. 2021). TiO_2 NPs possess antimicrobial properties and therefore they are used in water treatment technologies, in the textile industry, and also in various biomedical applications such as orthodontics, wound healing, biosensors, and drug delivery systems (Han et al. 2016; Jafari et al. 2020; Kumar et al. 2018; Younis et al. 2022). The widespread use of TiO_2 has raised significant concerns regarding its potential health effects (Zeman et al. 2018). Studies on effects of TiO_2 are monitored by various authorities, and the safety assessment of TiO_2 is being regularly updated. Exposure levels are considered to be low with the possible exception of workers handling large quantities of TiO_2 and thus exposed by inhalation (Boffetta et al. 2004; Fryzek et al. 2003; IARC 2010). Epidemiological studies have not reported any increased risk of cancer in humans after exposure to TiO_2 (Boffetta et al. 2001; Boffetta et al. 2004; Fryzek et al. 2003). However, recent re-analysis of one epidemiological study (Boffetta et al. 2004) taking into account the healthy worker survivor effect concluded that a positive association between cumulative exposure to TiO_2 and lung cancer mortality was observed and that TiO_2 epidemiological data could demonstrate an exposure-effects relationship if analysed appropriately (Guseva Canu et al. 2022). In addition, an incomplete confounder assessment was recently referred as another methodological flaw in previous epidemiological studies (Hansa et al. 2023). Only two in vivo inhalation studies found increased evidence of tumours in rats (Heinrich et al. 1995; Lee, Trochimowicz, and Reinhardt 1985a); one study (Lee, Trochimowicz, and Reinhardt 1985a) only after chronic inhalation of excessive concentration 250-mg/m^3 TiO_2 with conclusion that 'biological relevance of these lung tumours for man is negligible'. The other study (Heinrich et al. 1995) found benign squamous-cell tumour, adenocarcinoma and squamous-cell carcinoma after chronic inhalation of approximately 10-mg/m^3 TiO_2, which is considered to be 'rat lung overload conditions', resulting in the impairment of particle clearance mechanisms in the lung, subsequent chronic inflammation, possibly leading to the carcinogenic effects (General Court 2022). Contrary to them, other studies on rats and other animal species such as mice or hamsters reported no evidence of carcinogenicity (Muhle et al. 1995; Muhle et al. 1989; Thyssen et al. 1978; Yamano et al. 2022). Therefore, TiO_2 is currently classified by the International Agency for Research of Cancer (IARC) as possibly carcinogenic to humans (Group 2B) (IARC 2010). The National Institute for Occupational Safety and Health (NIOSH) has concluded that ultrafine but not fine TiO_2 particulate matter is a potential occupational carcinogen by inhalation (NIOSH 2011). Occupational exposure limits are set at 2.4 mg/m^3 for fine TiO_2 and 0.3 mg/m^3 for ultrafine TiO_2 (NIOSH 2011) reflecting the increased adverse effects of NPs in comparison with fine particles (IARC 2010; Oberdörster 2001; Zhao et al. 2009). The topic of the safety evaluation of TiO_2 is still ongoing and has practical impact on manufacturers and users. Recently, the General Court has annulled the Commission Delegated Regulation (EU) 2020/217 on the labelling of specific substances or mixtures with TiO_2 in powder form as carcinogen Category 2 by inhalation (General Court 2022).

Besides inhalation, another important exposure route to TiO_2 NPs is ingestion. TiO_2 food additive (E 171) contains less than 50% of constituent particles by number with the minimum external dimension <100 nm (Verleysen et al. 2022; Younes et al. 2021). The absorption of ingested TiO_2 is very low; the oral systemic availability is probably not greater than 0.5% (Younes et al. 2021). Dermal exposure is assumed to be insignificant because healthy skin is considered to be an effective barrier to TiO_2 NPs present in sunscreens and cosmetics (IARC 2010; Shi et al. 2013).

The main effect of TiO_2 NPs is the formation of reactive oxygen species (ROS), pulmonary inflammation and alveolar macrophage dysfunction (Shi et al. 2013). The pro-inflammatory effect of TiO_2 NPs is caused by the release of IL-1α and IL-1β after the induction of NLRP3 inflammasome both in vivo and in vitro (Cameron et al. 2022; Sun et al. 2013; Winter et al. 2011; Yazdi et al. 2010). TiO_2 NPs may modulate systemic immune response and increase the levels of reduced glutathione as a defensive response to oxidative stress in mice (Lehotska Mikusova et al. 2023). Oxidative stress was shown to induce insulin resistance after oral administration of TiO_2 NPs and thus increased plasma glucose in mice (Hu et al. 2015). A recent review of both in vivo and in vitro studies concluded that ROS generation, stress of endoplasmic reticulum and inflammatory response might result in glucose homeostasis disruption, but the evidence of this effect of TiO_2 NPs is yet inconsistent (Mohammadparast and Mallard 2023). Concerning genotoxicity, in vivo and in vitro studies from recent years show that TiO_2 particles may induce genotoxic damage even at low realistic doses, but the overall results still remain inconclusive (Carriere, Arnal, and Douki 2020; Shi et al. 2013). Because of the many uncertainties, the European Food Safety Authority (EFSA) panel recently concluded that 'a concern for genotoxicity could not be ruled out' and therefore 'titanium dioxide (E 171) may no longer be used in foods' due to the precautionary decision of the European Commision (EC 14 January 2022; Younes et al. 2021). Due to the different regulatory approach, titanium dioxide is considered safe, and therefore, it is still used as a food additive in other countries as the USA, Canada, Great Britain etc. (Health Canada 2022; COT 2024; FAO/WHO 2023).

Besides immune and systemic responses, poorly soluble low toxicity NPs (including TiO_2 NPs) might exert nonspecific effects as pneumoconiosis and effects on the cardiovascular and/or autonomic nervous system, which might contribute to development of cancer, autoimmune or cardiovascular diseases (Ichihara et al. 2016; Riediker et al. 2019; Yamano et al. 2022).

Conventionally used analytical determination of low concentrations of Ti in tissues by inductively coupled plasma mass spectrometry (ICP-MS) encounters challenges related to detection limits, which complicates obtaining reliable results (Younes et al. 2021). Therefore, there is a need for advanced microscopic and detection methods for the purposes of toxicokinetic studies. Transmission electron microscopy (TEM) and scanning TEM (STEM) may provide information on the NP distribution not only at the tissue level but also at the subcellular level. This may contribute to the elucidation of mechanisms by which NPs exert toxicological effects (Riediker et al. 2019). TEM and STEM coupled with energy-dispersed X-ray spectroscopy (EDX), which is one of the methods of detecting the elemental composition of a sample, have been increasingly used in recent years (Coméra et al. 2020; Elgrabli et al. 2015; Guillard et al. 2020; Heringa et al. 2018; Peters

et al. 2020; Vysloužil et al. 2020). Chemical characterization of particles such as EDX analysis is thought to be essential to avoid the uncertainty on the NPs' identity (Younes et al. 2021). Another detection method gaining significant interest in last years is cathodoluminescence (CL) coupled with STEM (Coenen and Haegel 2017) or detailed diffraction pattern analysis using powder nanobeam diffraction (PNBD), a method originally developed for identification of sparsely distributed crystals (Slouf et al. 2021b).

The aim of this study was to investigate the distribution of TiO_2 NPs in lungs and other murine organs after subchronic continuous inhalation using the state of the art microscopy methods STEM-EDX, STEM-C and STEM-PNBD. As an integral part, histopathology analysis of lung, spleen, liver and kidney was provided. Moreover, special attention was paid to changes of red blood cells morphology. We selected an 11-week exposure period for this study to thoroughly investigate the long-term effects and systemic distribution of TiO_2 NPs, ensuring a comprehensive assessment of both pulmonary and systemic impacts (Zeman et al. 2018).

2 | Material and Methods

2.1 | Production of TiO_2 NPs

TiO_2 NPs were continuously generated in situ via the aerosol route in a hot-wall tube flow reactor (the ceramic work tube of a vertically orientated furnace Carbolite TZF 12/38/850) by thermal decomposition and oxidation of liquid metal organic precursor titanium tetra-iso-propoxide (TTIP) at temperature 751°C in the presence of 20 vol. % of oxygen (purity 99.9995%) (Moravec et al. 2016). TTIP vapours were generated by evaporating its liquid form in a saturator at a temperature of 24°C and released vapours were transported into the flow reactor with a nitrogen stream (purity 99.9995%, 0.85 L/min). Before entering the reactor, it was diluted with another nitrogen stream (purity 99.9995%; flow rate 0.90 L/min). In parallel, a stream of oxygen (purity 99.9996%; flow rate 0.40 L/min) was introduced into the reactor using a silica capillary (I.D. 560 μm, O.D. 730 μm) at a distance of 10 mm after TTIP vapours enter the reactor to oxidize the organic part of the TTIP. At the outlet of the reactor, TiO_2 NPs transported in the nitrogen/oxygen mixture (flow rate 2.15 L/min) were mixed with air (5 L/min). The formed TiO_2 NPs were consecutively diluted using U-HEPA filtrated air (flow rate 20 L/min) resulting in the proper concentration of TiO_2 NPs used for the whole body inhalation experiment. All flow rates were regulated with mass flow controllers (Aalborg GFCS Electronic). The scheme of TiO_2 NPs generation and inhalation is shown in Figure 1.

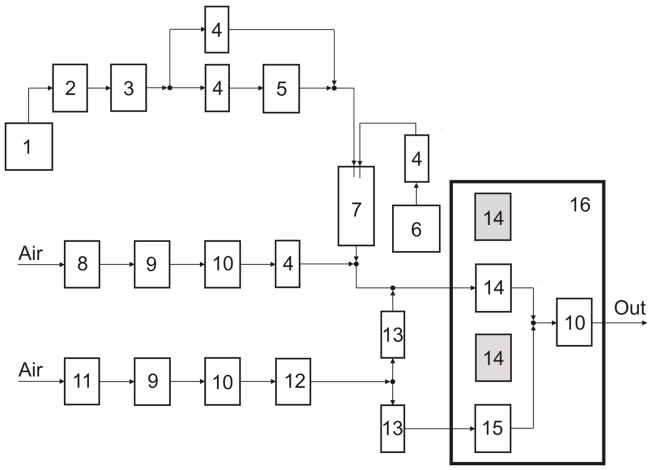

FIGURE 1 | Scheme of TiO_2 NPs generation and inhalation. (1) Liquid nitrogen tank including reducing valve, (2) moisture trap, (3) oxygen trap, (4) mass flow controller, (5) saturator with TTIP, (6) oxygen cylinder including reducing valve, (7) tube flow reactor, (8) air compressor with a dryer, (9) annular diffusion denuder, (10) HEPA filter, (11) air pump, (12) moisture and temperature stabilization, (13) flow meter, (14) inhalation cage, (15) control cage and (16) inhalation chamber.

2.2 | Characterization of Generated TiO_2 NPs

The number concentration and size distribution of TiO_2 NPs (Figure S1A) were measured directly in the inhalation cages using the scanning mobility particle sizer spectrometer (SMPS, model 3936L72, TSI, USA) in the size range of 7.64–229.6 nm.

The SMPS consisted of electrostatic classifier (model 3080, TSI, USA) with long differential mobility analyser (model 3081, TSI, USA) and condensation particle counter (model 3772, TSI, USA). The size distributions were measured continuously in 5 min intervals (i.e. 288 measurements per day) throughout the whole exposure period. The SMPS operates on the principle of isolating a charged particle of a certain diameter according to its mobility in an electric field in the differential mobility analyser, and the particles of the same diameter are then optically counted in the Condensation Particle Counter.

To measure the mass concentration of NPs, generated TiO_2 NPs were sampled on mixed cellulose membrane filters (pore size 0.45 μm, diameter 25 mm, Millipore, Bedford, USA). The filters were weighed before sampling and after sampling of NPs using a microbalance (±1 μg; model M5P, Sartorius, Germany). Filters were equilibrated before weighing for 48 h in air-conditioned room under constant conditions (temperature 20 ± 1°C, relative humidity 50 ± 3%). Mass concentration of generated TiO_2 NPs was calculated by dividing of mass of TiO_2 NPs collected on filter by volume of air sample that was passed through the filter.

TiO_2 NPs for morphology characterization were deposited onto TEM grids using a nanometer aerosol sampler (model 3089, TSI, USA). Size and shape of generated TiO_2 NPs were measured by electron microscopy, details are given below.

2.3 | Exposure of Female Mice to TiO_2 NPs

Adult female mice (ICR line, 6 weeks old, average body weight 24 g) were obtained from Masaryk University (Brno, Czech Republic). Prior to the experiment, animals were allowed to acclimate to laboratory conditions for 1 week. The experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Institute of Analytical Chemistry of the Czech Academy of Sciences (Ministry of Agriculture of the Czech Republic, No. 10031/2013-MZE-17214)

and approved by the Animal Ethics Committee of the Institute of Animal Physiology and Genetics of the Czech Academy of Sciences (No. 081/2010).

The inhalation experiment was carried out in parallel in two whole-body inhalation cages placed inside the inhalation chamber. Adult female mice were exposed to the concentration of TiO_2 NPs of cca 1.51×10^6 particles/cm^3 for 24 h/day, 7 days/week, over period of 11 weeks. Control group was located in another cage without inhalation of NPs. Commercial diet and water were provided ad libitum. A special feeding device (a tube closed at the top, from which the feed falls down into the feeder) was designed to minimize oral ingestion of NPs resulting from contamination of commercial feed granules by adsorption of TiO_2 NPs on their surface. The body weight changes during exposure were not significantly different between the control and exposed group, as checked after 7 weeks of exposure. The exposure is described in detail elsewhere (Lehotska Mikusova et al. 2023).

2.4 | Histopathological Analysis

At the end of the inhalation experiment, mice were anesthetized with diethyl ether. The mice were then euthanized through rapid decapitation, after which blood samples were collected, and the bodies were dissected. Tissue samples from the lung, liver, spleen and kidney were gathered, then fixed in 10% formaldehyde, dehydrated, and processed for paraffin embedding. Tissue sections of thickness 4–5 μm were stained with haematoxylin and eosin. The stained sections were observed with the optical microscope Olympus BX51 (Japan). Throughout the necropsy, careful measures were taken to prevent contamination of the collected samples. The necropsies were performed by OS, ensuring consistency and standardization across all mice, with the same parts of the organs being collected from each experimental mouse.

2.5 | Electron Microscopy

2.5.1 | TEM

Measurement and morphology analysis of TiO_2 NPs was performed by TEM (Philips EM 208 Morgagni, FEI, Czech Republic). TiO_2 NPs were sampled from the inhalation chamber. They were collected by electrostatic precipitation using a Nanometer Aerosol Sampler (model 3089, TSI, USA) on electron microscopy copper grids, coated with formvar and stabilized with evaporated carbon film (300 mesh, Agar Scientific, UK).

The samples of the murine lung, liver, spleen and kidney for the ultrathin section were fixed in 3% glutaraldehyde in cacodylate buffer, post-fixed in 1% OsO_4 solution in cacodylate buffer, dehydrated in 50%, 70%, 90% and 100% acetone and embedded in Epon-Durcupan mixture (Epon 812 Serva, Germany; Durcupan, ACM Fluka, Switzerland). Ultrathin sections (thickness 60–70 nm) were prepared by an ultramicrotome (Leica EM UC7, Austria) and were not contrasted to avoid artefacts (without 2% uranyl acetate and 2% lead citrate). The sections were observed at 80 kV with TEM (Philips EM 208 Morgagni, FEI, Czech Republic).

Energy dispersive X-ray spectra (EDX) were measured by Silicon Lithium Detector Oxford x-MAX 80T, SSD (EDS, Oxford Instruments, UK). The detector was installed on the transmission electron microscope JEOL-2100, 200 kV (JEOL, Japan).

2.5.2 | Scanning Electron Microscopy

Blood samples were centrifuged at 2000 *g*. Supernatant was removed. Erythrocytes were washed three times in Millonig's buffer (Serva, Germany), fixed in glutaraldehyde (3%) in Millonig's buffer, post-fixed in osmium tetroxide (OsO_4 1%) solution in Millonig's buffer. The samples were dehydrated in 50%, 70%, 90% and 100% ethanol and dried in hexamethyldisilazane (HMDS, Sigma-Aldrich, Czech Republic). The samples were then placed on the carbon conductive tabs (Pelco, Ted Pella, USA), which were attached on the cylindrical Hitachi aluminium sample stubs. The samples were coated with Pt/Pd layers using Cressington sputter coater 208HR (Cressington Scientific Instruments, UK). The erythrocytes were observed under the scanning electron microscope Hitachi SU 8010 (Hitachi High Technologies, Japan) at magnification in the range from 500 to 20,000× at 18 kV.

CL was measured by detector MonoCL4 Plus (Gatan, USA), installed on the high-resolution scanning electron microscope Magellan 400L (FEI, Czech Republic). The CL detector is equipped with high-sensitive photomultiplier tube R943-02 (Hamamatsu Photonics K.K., Japan) with a time response of 3 ns and GaAs (Cs) photocathode with spectral range from 160 to 930 nm. The CL images were taken at an acceleration voltage of 8 kV and a probe current of 1.6 nA. The bright field and high angle annular dark field STEM images were captured by the retractable detector STEM3 (FEI, Czech Republic) at the acceleration voltage of 20 kV and the probe current of 50 pA. EDX spectra and maps were recorded by the APOLLO X Silicon Drift Detector (EDAX, USA) at an acceleration voltage of 20 kV and probe current of 6.4 nA. The STEM-PNBD datacube was taken on focused ion beam scanning electron microscope Helios G4 HP (Thermo Fisher Scientific) using T-pix pixel array detector (more information about the detector, detection geometry and data processing can be found in Skoupý et al. 2023).

3 | Results

3.1 | Characterization of TiO_2 NPs

The overall stoichiometry of the TTIP oxidation can be described by the equation (Moravec et al. 2016):

$$Ti(C_3H_7O)_4 + 18\,O_2 \rightarrow TiO_2 + 14\,H_2O + 12\,CO_2$$

To verify that oxidation of TTIP proceeds according to the equation, air with volatile organic compounds (VOCs) potentially

present in the air of the inhalation cage with TiO_2 NPs was sampled to n-heptane by means of a continuous cylindrical wet effluent diffusion denuder (Křůmal et al. 2016), the denuder effluent was analysed for the content of VOCs using a GC–MS, but no VOCs were found, that is, their concentrations, if present, were below the limits of detection. As a result, the mice in this study were not exposed to any contaminants during inhalation of TiO_2 NPs.

The size distribution of TiO_2 NPs, as measured by SMPS during inhalation, is depicted in Figure S1A. The average geometric mean diameter of the NPs was 30.3 nm with a geometric standard deviation of 1.85, and the total number concentration was 1.51×10^6 particles/cm^3. The average mass concentration of TiO_2 NPs was 130.8 μg/m^3. The mass median aerodynamic diameter (MMAD) calculated from SMPS number size distribution was 98.2 nm and the mass geometric diameter was 93.0 nm.

The TiO_2 NPs exhibited an irregular oval shape, as illustrated by TEM (Figure S1B). Size distribution of TiO_2 NPs collected on copper grids was evaluated using TEM and revealed that 98% of NPs was smaller than 50 nm. Size of 75% NPs ranged between 15 and 40 nm (Figure S1C). CL spectrum of pure TiO_2 NPs is shown in Figure S1D.

3.2 | Histopathological Analysis

Following 11 weeks of exposure to TiO_2 NPs, emphysema in the lung parenchyma occurred, and hyperplastic epithelium in the bronchioles was observed. Condensed mucus and macrophages with phagocytized granular material (haemosiderin) were present in the bronchiolar lumen. The alveoli mostly exhibited normal morphology; however, slightly thickened interalveolar septa were also identified. The alveolar epithelium sporadically consisted only of Type II pneumocytes (Figure 2).

Histopathological analysis of liver sections revealed no significant alterations (data not shown). In the spleen parenchyma, hyperaemia and a relatively large amount of haemosiderin phagocytosed within macrophages were observed (Figure 3A). Other changes were not detected. In the kidney

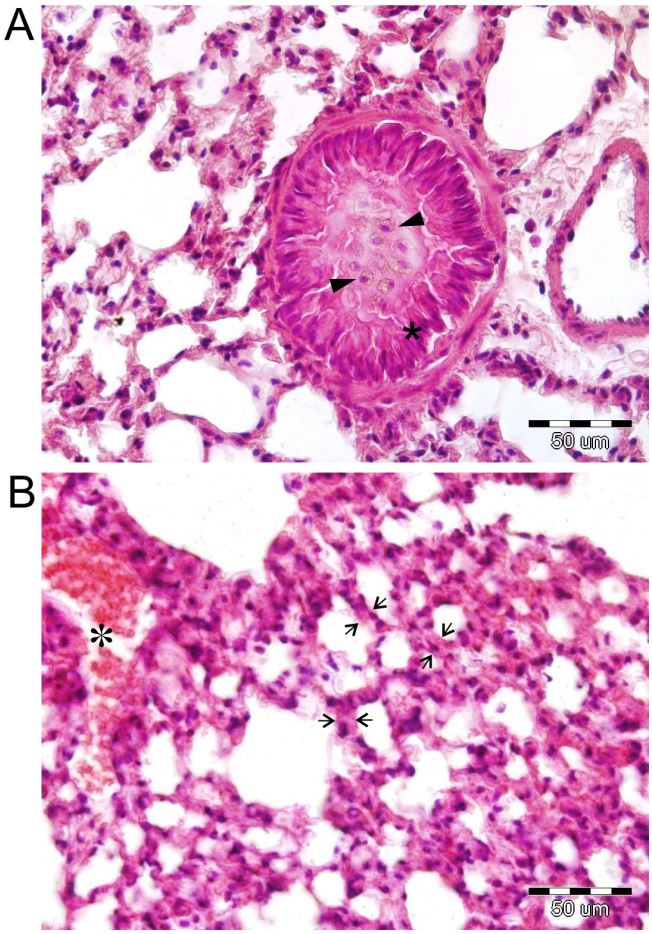

FIGURE 2 | Lung—histopathological analysis, haematoxylin and eosin staining. (A) The bronchiole with hyperplastic epithelium (denoted by asterisk) filled with condensed mucus and macrophages containing phagocytosed granular material (denoted by triangles). Alveoli show normal morphology. (B) Alveoli with slightly thickened interalveolar septa (denoted by arrows). Erythrocytes in the dilated blood vessel on left.

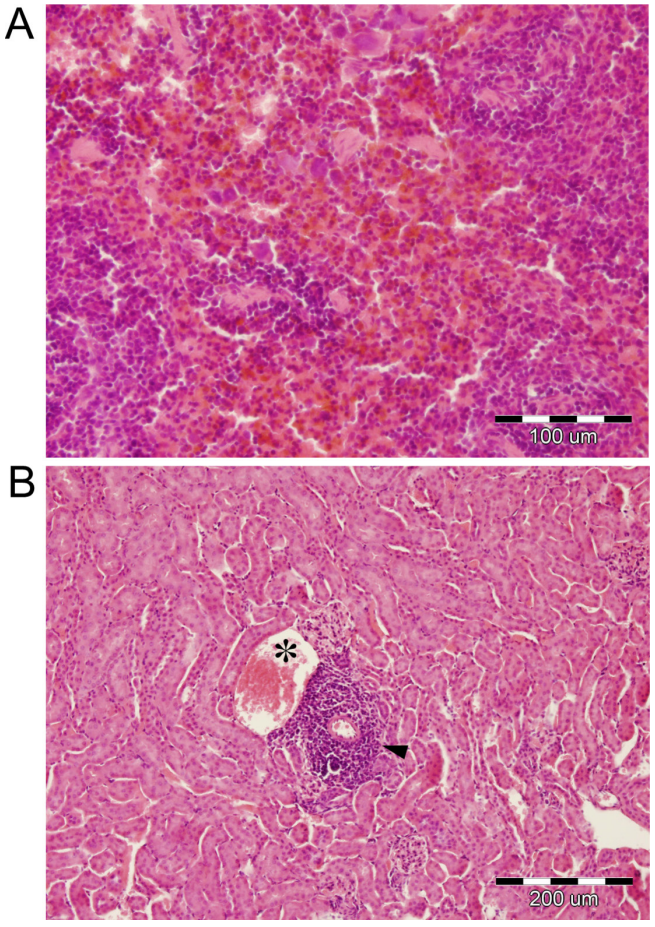

FIGURE 3 | (A) Spleen—histopathological analysis, haematoxylin and eosin staining. Multiple areas of haemosiderosis in parenchyma. Haemosiderin phagocytosed in macrophages (rust-coloured areas). (B) Kidney - histopathological analysis, haematoxylin and eosin staining. Kidney cortex with chronic mononuclear inflammation site (denoted by triangle) around the small blood vessel and in a proximity of the larger blood vessel (denoted by asterisk). The latter one contains blood, which is a sign of hyperaemia.

medulla and cortex, several foci of chronic mononuclear inflammation were identified. Mild hyperaemia was observed (Figure 3B).

3.3 | Electron Microscopy, Elemental Analysis and Powder Diffraction of the TiO_2 NPs in the Lung

TEM analysis revealed TiO_2 NPs clustered within vesicles of type II pneumocytes in ultrathin lung sections (Figure 4A). At higher magnification, TiO_2 NPs and their small aggregates were found in physical interaction with cytoplasmic membrane and several membrane invaginations were observed. In cells, TiO_2 NPs were present predominantly as aggregates inside endosomes, multivesicular bodies or lysosomes. Possible autophagosomes or autophagic compartments could not be distinguished in our TEM micrographs, as cytoplasmic membranes lacked contrast. Sporadically, TiO_2 NPs were present seemingly free in cytoplasm (Figure 4B). TEM images showed TiO_2 NPs also in the structures of Golgi complex and endoplasmic reticulum (Figure 4B). TiO_2 NPs in large vesicles were found in close proximity to the capillary (Figure 4C). In lung macrophages, TiO_2 NPs were found in vesicles as well (Figure 4D).

The elemental composition of the aggregates in larger vesicles of type II pneumocytes was determined by using both EDX and CL analysis, which confirmed the presence of Ti in the dense NP structures (Figure 5). This was further confirmed by PNBD (Slouf et al. 2021a; Slouf et al. 2021b), where a clear match with the simulated TiO_2 spectrum of anatase was found (Figure 6).

3.4 | TiO_2 NPs on Erythrocytes

In samples from mice exposed to TiO_2 NPs, erythrocytes displaying markedly altered morphology were occasionally found.

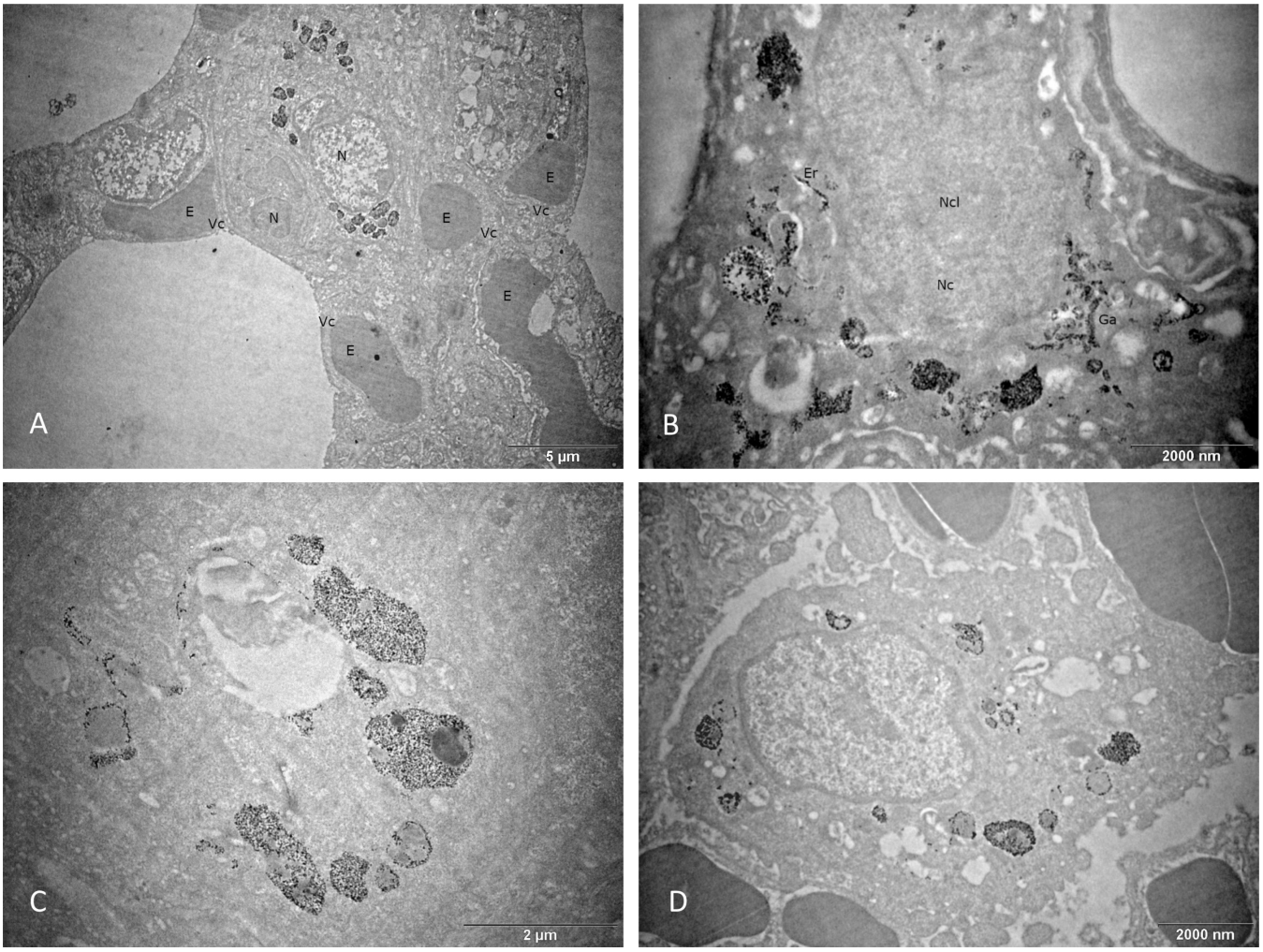

FIGURE 4 | TEM micrograph of TiO_2 NPs deposited in lung cells. (A) TiO_2 NPs deposition in pulmonary alveolus in the type II pneumocytes. N, nucleus; E, erythrocyte; Vc, vas capillare, capillary. (B) Intracellular distribution of TiO_2 NPs in the type II pneumocyte. NPs are present in vesicles, seemingly free in cytoplasm, and in interaction with Golgi apparatus and endoplasmic reticulum. Ga, Golgi apparatus; Er, endoplasmic reticulum; Nc, nucleus; Ncl, nucleolus. (C) Type II pneumocyte with a capillary containing erythrocytes. TiO_2 NPs are aggregated in large vesicles, some of them are in the very close contact with the capillary. TiO_2 NPs are apparent in the endothel of the capillary. (D) A lung macrophage containing TiO_2 NPs aggregated in vesicles.

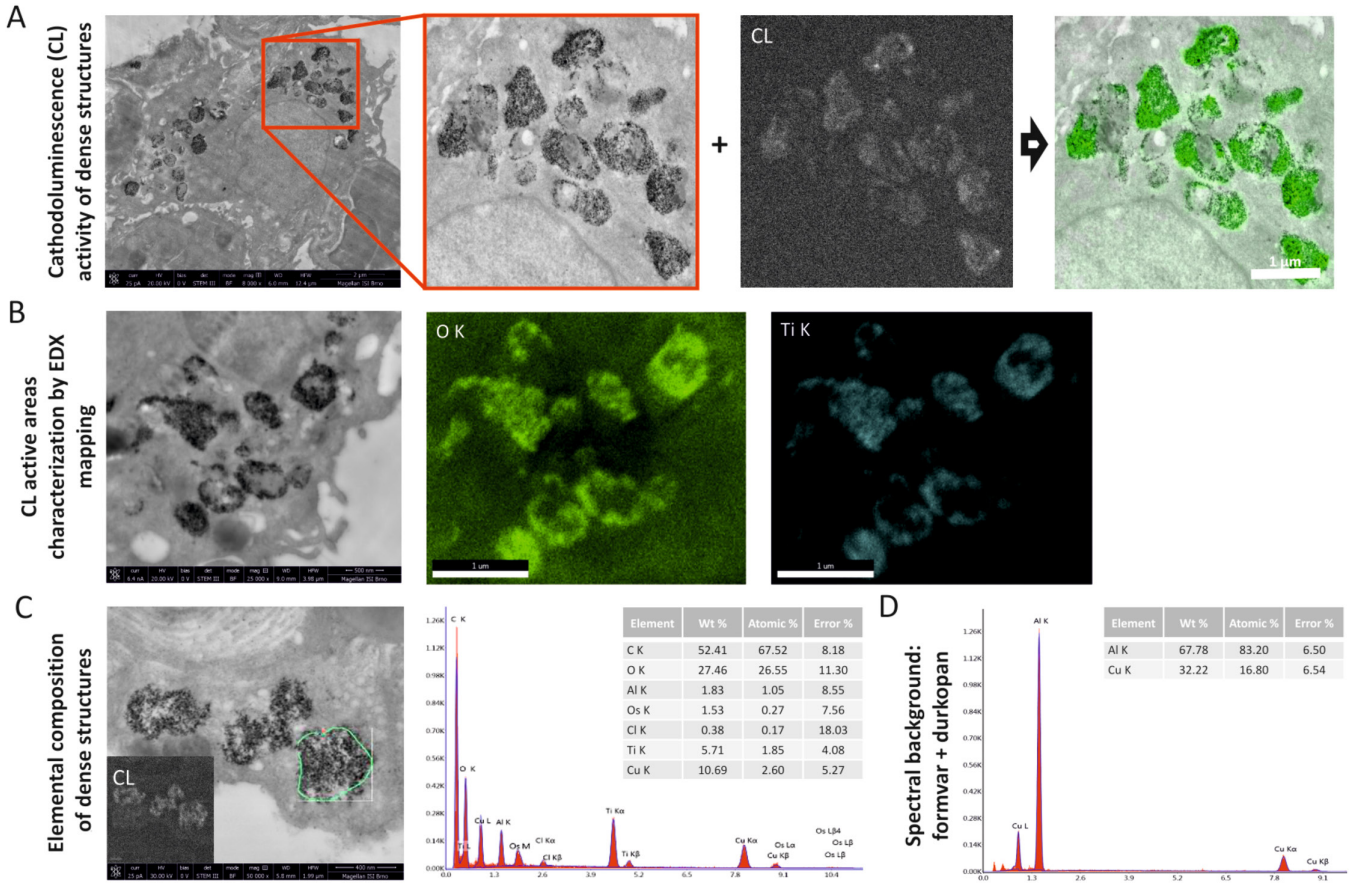

FIGURE 5 | Deposition of TiO_2 NPs in type II pneumocytes. (A) Dense structures consisting of NPs show cathodoluminescence activity. Correlative image composed of bright field STEM and CL image is on the right. (B) The dense structures are mainly formed by titanium and oxygen (estimated by EDX mapping of Kα emission line). (C) Elemental composition (EDX analysis) of one of the structures shows C, O, Os, Cl and Ti. Cu and Al are elements which the TEM grid and sample holder are made from. (D) Elemental composition (EDX analysis) of formvar support film and durkopan resin in an area without a sample. C, O and material of the sample holder (Cu, Al) are recognizable in the spectrum.

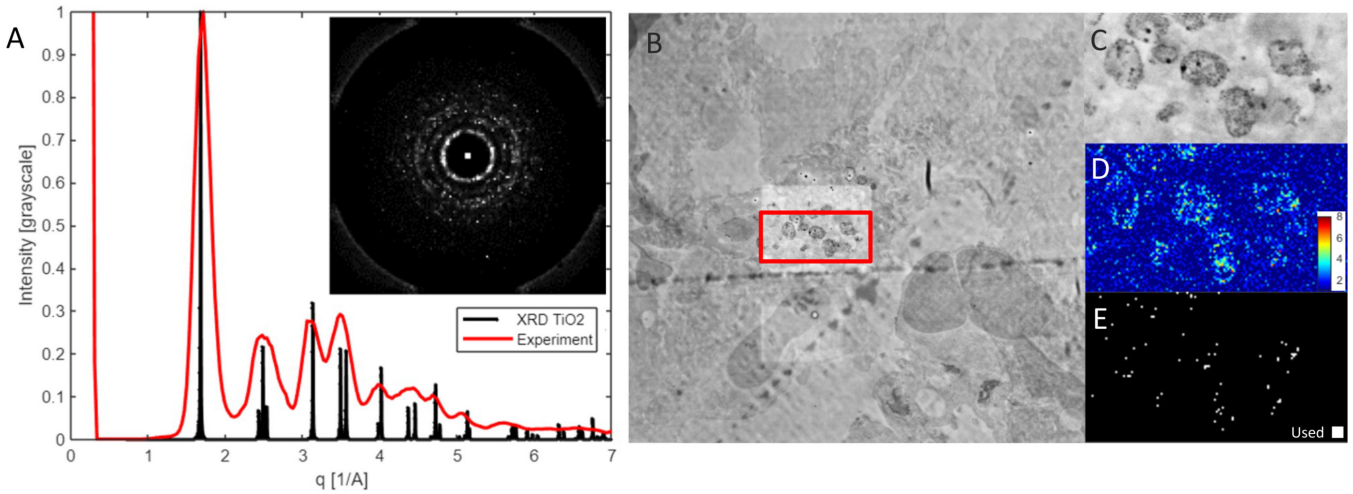

FIGURE 6 | STEM-PNBD analysis (A). Estimated radial profile in red corresponds to X-ray diffraction simulations of TiO_2 in the form of anatase (black). Final 2D powder diffractogram (inlet) was created from 128 points carefully chosen from taken 40,000 point diffractograms. Mapped area is highlighted in BF STEM image (B), magnified in (C), number of detected peaks per pattern in (D) and position of selected diffraction patterns in (E).

These morphological alterations were represented mostly by echinocytes and stomatocytes of various stages (Figure 7). SEM investigation of erythrocytes isolated from peripheral blood revealed NPs attached to their surface (Figure 7A). To determine the elemental composition of NPs attached to erythrocytes, EDX analysis could not be used, as it was difficult to find individual NPs attached to or possibly within the erythrocytes. EDX measurements over larger areas lead to the position-based spectral

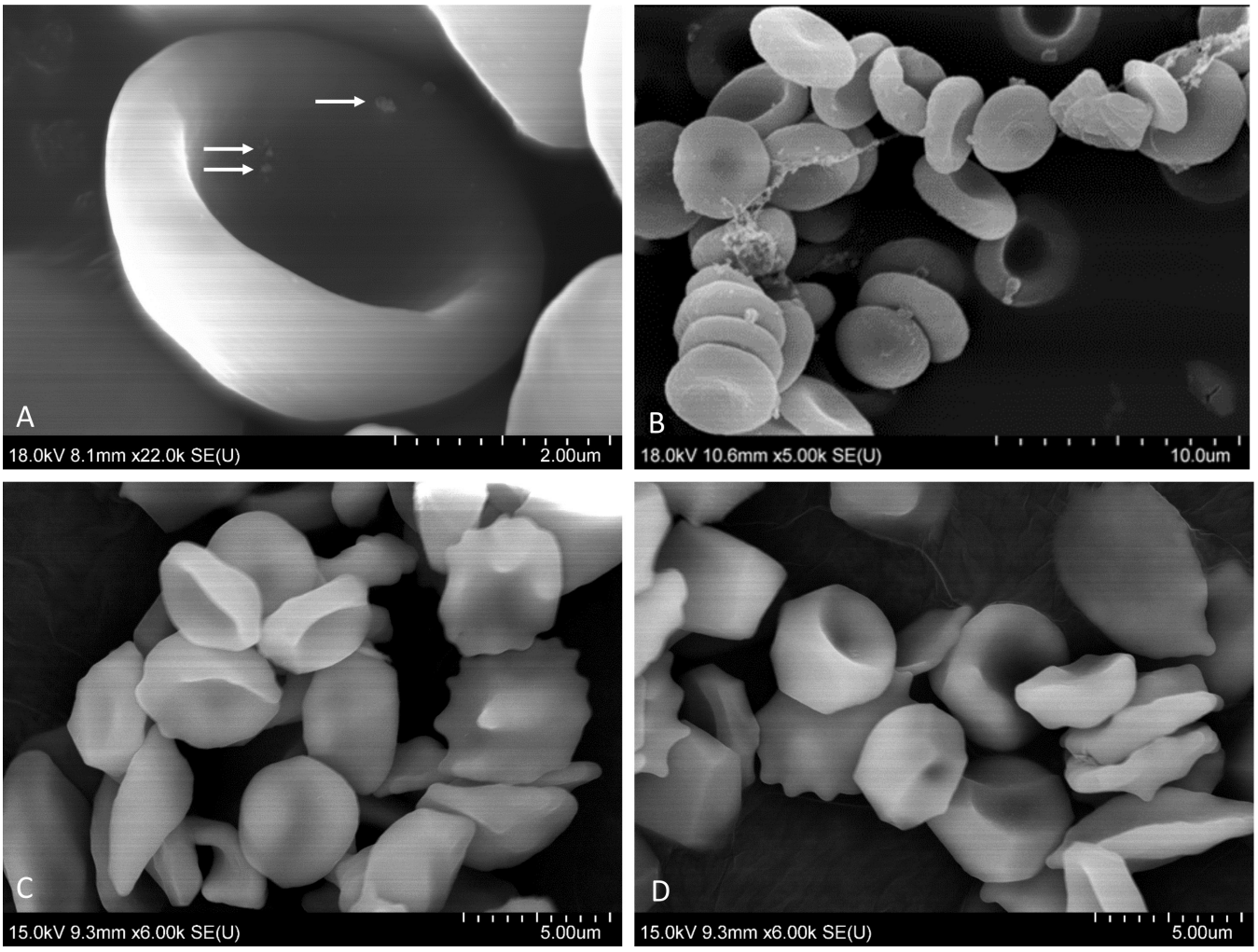

FIGURE 7 | SEM micrographs of erythrocytes. (A) Erythrocyte with TiO_2 NPs (arrows) attached to its surface. (B) Erythrocytes from non-exposed control mice. (C, D) Erythrocytes from mice exposed to TiO_2 NPs with markedly altered morphology including stomatocytes and echinocytes.

averaging and to the drop of TiO_2 concentration under the detection limit. Therefore, CL spectra were measured on the whole sample of erythrocytes attached on carbon conductive tabs. The CL spectrum of exposed erythrocytes is a combination of CL spectra of pure erythrocytes (control) and pure TiO_2 powder. It is clearly visible that peaks around 450 and 500 nm, which are part of TiO_2 spectrum, can be found in the spectrum of exposed erythrocytes, but do not occur in the control erythrocyte spectrum (Figure 8). Therefore, the CL analysis showed the occurrence of TiO_2 NPs on erythrocytes in mice after subchronic inhalation.

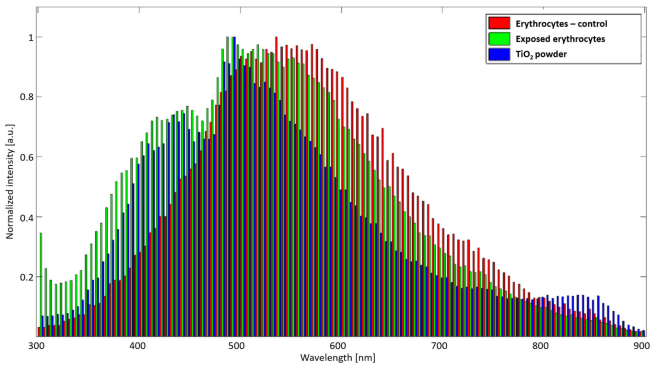

FIGURE 8 | CL spectra comparison of control erythrocytes (red), erythrocytes exposed to TiO_2 NPs (green) and pure TiO_2NPs (blue). Spectrum of erythrocytes exposed to TiO_2 NPs is a combination of both others (visible around the peak at 430 nm). The peak at 850 nm (visible on the spectrum of pure TiO_2 NPs) is caused by a larger cluster of particles. In case of erythrocytes exposed to TiO_2 NPs it is not recognizable. All spectra were captured at acceleration voltage 30 kV, emission current 1.6 nA, step in spectrum 5 nm, dwell time 10 s and wavelength range 300–900 nm.

3.5 | TiO_2 NPs in Spleen, Liver and Kidney

In distal organs such as the spleen, liver and kidney, NPs were present to a significantly lesser extent compared with the lung. In spleen, liver and kidney, STEM-EDX analysis of NPs mostly revealed the presence of iron in the form of ferritin and haemosiderin particles (Figures S2, S3 and S4). Titanium was determined only rarely above the detection limit in kidney (Figure 9), but not in liver and spleen. This rare occurrence of titanium near the limit of detection does not represent sufficient evidence for the confirmation of TiO_2 NPs in kidney. Neither STEM-CL

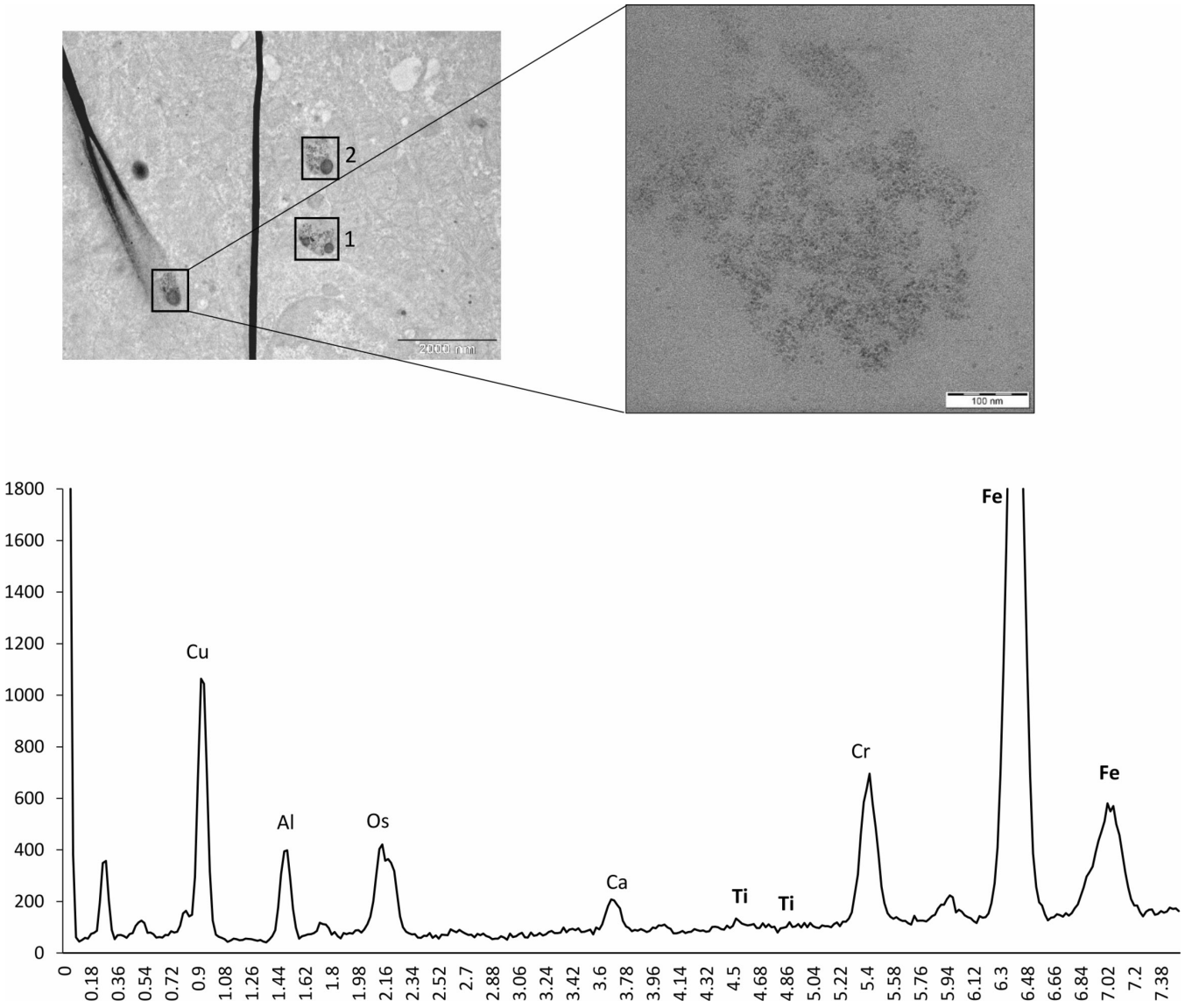

FIGURE 9 | EDX analysis of NPs enclosed in a vesicle in kidney. NPs consisted of iron. Titanium was found near the limit of detection (LOD 0.04 wt%). TEM micrographs were taken on different microscopes; therefore, the region with defects on the slices was chosen intentionally for better orientation. Selected area of vesicle no. 1 exerted very similar EDX spectrum with Ti detected close to the LOD. EDX analysis of selected area of vesicle no. 2 did not show Ti presence, but Fe was detected.

revealed any titanium in the samples from distal organs (data not shown). Therefore, it can be concluded that no significant accumulation of TiO_2 NPs was identified in liver, spleen and kidney.

4 | Discussion

The presented work belongs among sporadic whole-body inhalation studies on TiO_2 NPs (Bermudez et al. 2004; Grassian et al. 2007; Heinrich et al. 1995; Lee, Trochimowicz, and Reinhardt 1985a; Lee, Trochimowicz, and Reinhardt 1985b; Muhle et al. 1995; Yamano et al. 2022; Yamano et al. 2022; Yu et al. 2015). In contrast to these previous studies, the concentration of TiO_2 NPs in the inhalation chamber in our study (130.8 μg/m^3) was markedly lower, resembling a realistic exposure scenario in occupational settings (NIOSH 2011). The conditions of the TiO_2 NPs generation and of the inhalation exposure were the same as in a recent study focused on immunomodulatory effects (Lehotska Mikusova et al. 2023), only with different duration of exposure (here 11 weeks instead of 7 weeks). Exposure for 11 weeks (24/7) has already been used in our previous studies (Dumková et al. 2020; Rossner et al. 2019; Smutná et al. 2022) and represents subchronic exposure as defined in the Integrated Risk Information System Glossary (US EPA).

This study focuses on the identification of TiO_2 NPs in the lungs and other distal organs in mice by using electron microscopy and methods detecting NPs' chemical identity/crystal structure. As a novelty, alterations in red blood cell morphology resulting from inhalation exposure to TiO_2 NPs were found for the first time in inhalation study in vivo. These alterations might be connected with histopathological changes observed in lungs and distal organs.

Majority of TiO_2 NPs deposited in the airways of the respiratory tract is phagocytized by alveolar macrophages and may be cleared by expiration of sputum or by gastrointestinal tract (Shi et al. 2013). However, evidence suggests that NPs may be cleared less effectively by alveolar macrophages compared with fine particles; thus, the retention of NPs in lungs and the possibility of their uptake by endothelial cells is increased (Geiser et al. 2008; Geiser and Kreyling 2010; Oberdörster 2001). A small fraction of TiO_2 NPs is transported from the lung tissue into systemic circulation (Gaté et al. 2017; Mühlfeld et al. 2007; Pujalté et al. 2017; Riediker et al. 2019; Shi et al. 2013). TiO_2 NPs can be then distributed from systemic circulation to various organs and tissues as mesenteric lymph nodes, liver, spleen, kidney, heart, reproductive organs, brain and placenta (Fabian et al. 2008; Gaté et al. 2017; Geiser and Kreyling 2010; Pujalté et al. 2017; Shi et al. 2013; Younes et al. 2021).

In the present study, TiO_2 NPs were observed predominantly in the lungs. TiO_2 NPs were found in macrophages and in type II pneumocytes mostly in vesicular structures such as endosomes, lysosomes, multivesicular bodies, autophagosomes or autophagic compartments. This is in line with another in vivo inhalation study on mice (Yu et al. 2015), in vitro studies on various NPs and various cell lines (Konczol et al. 2011; Lammel et al. 2019; Lojk et al. 2015; Martin et al. 2022; Mühlfeld et al. 2007) or studies on airborne particulate matter (Gualtieri et al. 2009; Reibman et al. 2002), suggesting that the uptake and intracellular fate of TiO_2 NPs is directed by the endo-lysosomal pathway. However, certain TiO_2 NPs appeared not to be enclosed in vesicles, but seemingly free in the cytoplasm, which could be explained rather by possible endosomal escape than by direct penetration through cytoplasmic membrane (Chu et al. 2014; Lammel et al. 2019). The accumulation of indigestible material in lysosomes may result in lysosomal overload, which may cause the blockage of autophagic flux. The autophagy and lysosomal dysfunctions were suggested to be emerging mechanisms of nanomaterial toxicity, which may manifest in a number of pathologies in humans (Cohignac et al. 2014; Meijer and Codogno 2009; Ravikumar et al. 2010; Stern, Adiseshaiah, and Crist 2012). TiO_2 NPs sporadically observed in the structures of the Golgi complex and endoplasmic reticulum in type II pneumocytes in this in vivo study might be transported there from early endosomes by retrograde transport, a way common for certain bacterial and plant toxins. It is less common but possible also for NPs (Iversen, Skotland, and Sandvig 2011; Lord and Roberts 1998; Sandvig et al. 2013; Skotland, Iversen, and Sandvig 2021), which have been observed in endoplasmic reticulum in several in vitro studies (Luo et al. 2013; Yan et al. 2016; Zhang et al. 2016).

In this study, the confirmation that the observed NPs in the lung are indeed TiO_2 NPs was provided by both EDX analysis and PNBD, a method specially designed for crystal identification (Skoupý et al. 2023; Slouf et al. 2021b). As an alternative method to confirm the NP identity, we successfully used CL, a method applied in the past primarily for the characterization of semiconductors, minerals, and ceramics, but recently applied also in nanophotonics, plasmonics and in imaging of nanostructures including TiO_2 (Barberio et al. 2012; Coenen and Haegel 2017; Keevend, Coenen, and Herrmann 2020; Plugaru 2008). Therefore, this in vivo study corroborates the findings of previous in vitro studies regarding the presence of TiO_2 NPs in pulmonary cells at the ultrastructural level. To our knowledge, electron microscopy identification of TiO_2 NPs in lung intracellular structures has been published previously only in one whole-body inhalation in vivo study on mice (Yu et al. 2015).

A vast majority of NPs having been deposited in the lungs is taken up by macrophages and removed by common clearance processes, but a small fraction can reach the bloodstream, thus become systemically available and reach other organs and tissues (Gaté et al. 2017; Krug 2014; Krug and Wick 2011; Pujalté et al. 2017). Interactions between NPs and blood cells are still poorly understood, and little is known about the effects of NPs on the morphology, structure and function of erythrocytes, the most abundant cells in the circulatory system (de la Harpe et al. 2019; Tian et al. 2021). In vitro studies showed that NPs may interact with the erythrocyte membrane and exert haemolytic and agglutinating effects on erythrocytes. Several in vitro studies reported that various NPs including Ag, Au, SiO_2 and TiO_2 NPs induced changes in erythrocyte morphology from discocytes to echinocytes and stomatocytes (Asharani et al. 2010; Avsievich et al. 2019; Bian et al. 2021; Ghosh, Chakraborty, and Mukherjee 2013; He, Liu, and Du 2014; Li et al. 2008; Solarska-Ściuk et al. 2021). Recently, prothrombotic effect of TiO_2 NPs via the procoagulant activity of erythrocytes was detected both in vitro on human isolated erythrocytes and in vivo in rats after intravenous injection (Bian et al. 2021). Such in vivo confirmation of effects or mechanisms detected in vitro is ultimately essential, yet currently still rare (Rennick, Johnston, and Parton 2021). In the presented study, we show to our best knowledge for the first time in inhalation study in vivo that TiO_2 NPs caused the changes in erythrocyte morphology in mice after 11 weeks of inhaling low concentration of TiO_2 NPs.

We also confirmed the presence of TiO_2 NPs on erythrocytes using cathodoluminescence coupled with SEM. The SEM-CL use provided us the advantage of the ability to detect TiO_2 collectively on a number of erythrocytes. Previously, the finding of erythrocytes with attached TiO_2 NPs in the in vivo samples was rather difficult so their detection by SEM–EDX was barely possible. Such in vivo confirmation of TiO_2 NP on erythrocytes might be an evidence of their systemic availability.

However, this study did not find TiO_2 NPs in other organs, including the spleen and liver. In kidney, Ti was detected by EDX analysis in several vesicles only close to the limit of detection (0.04 wt%). This might result from the fact that rather low concentrations of TiO_2 NPs were intentionally used in this inhalation study. In these distal organs, NP structures found by TEM in various types of vesicles, in clusters or freely in cytoplasm were shown by EDX analysis to contain iron. Ultrastructurally, these NPs correspond with storage forms of iron—ferritin and haemosiderin (Iancu 1992, 2011). Together with the histopathological evidence of hyperaemia in the spleen and kidney and haemosiderin presence in the spleen, the finding of NPs containing iron might suggest the increased decomposition of damaged erythrocytes. The excess of iron is then stored in the form of haemosiderin, a water-insoluble protein storing iron in the conditions of iron overload, whereas in normal conditions, the excess of iron is stored in the form of the water-soluble

protein ferritin (Harrison and Arosio 1996; Saito 2014). This is in accordance with in vitro studies reporting the haemolytic effects of TiO_2 NPs (Bian et al. 2021; Ghosh, Chakraborty, and Mukherjee 2013; Li et al. 2008). The chemical analysis of titanium content (not published here) by using ICP-MS revealed high titanium amounts only in the lungs, while in other organs (including blood) titanium concentrations were below the limits of detection. Nevertheless, it should be noted that the sensitivity of the method used was low, as we did not have a more sensitive method available in the course of the study. In fact, there is a lack of in vivo inhalation studies reporting the distribution of TiO_2 NPs into the distal organs, as the studies are mostly focused on the effects in the lung (Bermudez et al. 2004; Grassian et al. 2007; Heinrich et al. 1995; Muhle et al. 1989; Yamano et al. 2022; Yamano et al. 2022; Yu et al. 2015). To our knowledge, only one whole-body chronic inhalation study on rats exists reporting transmigration of TiO_2 particles (mass median diameter 1.5 μm, geometric diameter 0.4 μm) to lymph nodes, spleen and liver, but with no tissue reaction (Lee, Trochimowicz, and Reinhardt 1985b). Two nose-only inhalation studies (exposure 6 h, resp. 4 weeks) on rats detected titanium using ICP-MS in various distal organs, thus confirming in vivo the systemic circulation of a small amount of the inhaled TiO_2 NPs (Gaté et al. 2017; Pujalté et al. 2017). Other in vivo studies analysed tissue distribution after intravenous or intraperitoneal injection of TiO_2 NPs (Disdier et al. 2015; Elgrabli et al. 2015; Fabian et al. 2008; Valentini et al. 2019), an administration suitable for nanomedicine research.

Indeed, few animal inhalation studies exist on TiO_2 NPs, as they demand special maintenance of a controlled aerosol environment in comparison with administration by instillation or injection, thus are more elaborate and costly, but closer to reality. Although in vitro studies provide valuable information on the mechanisms of NP effects and fate in biological systems, in vivo studies are necessary to confirm whether these mechanisms are relevant in living organisms, especially during such a complex process as inhalation (Krug 2014; Rennick, Johnston, and Parton 2021).

5 | Conclusion

In summary, this 11-week study on mice confirmed in vivo the presence of TiO_2 NPs in lung macrophages and type II pneumocytes including their intracellular localization and their identification by EDX, CL and PNBD analysis. For the first time using the inhalation exposure route, this in vivo study reports the changes in erythrocyte morphology after subchronic inhalation of TiO_2 NPs in low concentration. CL analysis detected the presence of TiO_2 NPs on erythrocytes, thus suggesting their potential systemic availability. CL analysis appeared to be useful method for titanium detection on erythrocytes, when EDX and chemical analysis were not efficient or enough sensitive. The study did not prove the presence of TiO_2 NPs in distal organs such as the spleen, liver and kidney. Ti was found only in kidney near the limit of detection. Sporadic, but increased occurrence of iron NPs in these organs in the form of haemosiderin compared with control mice might result from haemolysis of the damaged erythrocytes. These findings suggest the suitability of determining the NP elemental composition in order to avoid the misinterpretation of the results.

Author Contributions

Pavel Kulich: conceptualization, formal analysis, investigation, methodology, visualization, writing the original draft, writing review and editing. **Soňa Marvanová**: formal analysis, investigation, visualization, writing the original draft, writing review and editing. **Radim Skoupý**: formal analysis, investigation, methodology, visualization, writing the original draft, writing review and editing. **Miša Škorič**: formal analysis, investigation, methodology, visualization, writing the original draft, writing review and editing. **Jan Vysloužil**: formal analysis, investigation. **Omar Šerý**: conceptualization, funding acquisition, investigation, writing the original draft, writing review and editing. **Pavel Mikuška**: conceptualization, funding acquisition, investigation, methodology, writing the original draft, writing review and editing. **Lukáš Alexa**, **Pavel Coufalík**, **Kamil Křůmal** and **Pavel Moravec**: investigation, formal analysis, methodology. **Zbyněk Večeřa**: conceptualization, investigation, methodology, supervision, writing the original draft. **Miroslav Machala**: conceptualization, funding acquisition, methodology, project administration, supervision, writing the original draft, writing review and editing.

Acknowledgements

The authors would like to thank to Jana Stráská from the Czech Advanced Technology and Research Institute (CATRIN) at Palacký University (Olomouc, Czech Republic) and Andrej Rusnák from the Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences (Bratislava, Slovak Republic) for EDX analysis. Open access publishing facilitated by Vyzkumny Ustav Veterinarniho Lekarstvi, as part of the Wiley - CzechELib agreement.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

Asharani, P. V., S. Sethu, S. Vadukumpully, et al. 2010. "Investigations on the Structural Damage in Human Erythrocytes Exposed to Silver, Gold, and Platinum Nanoparticles." *Advanced Functional Materials* 20: 1233–1242. https://doi.org/10.1002/adfm.200901846.

Avsievich, T., A. Popov, A. Bykov, and I. Meglinski. 2019. "Mutual Interaction of red Blood Cells Influenced by Nanoparticles." *Scientific Reports* 9, no. 1: 5147. https://doi.org/10.1038/s41598-019-41643-x.

Baranowska-Wójcik, E., D. Szwajgier, P. Oleszczuk, and A. Winiarska-Mieczan. 2020. "Effects of Titanium Dioxide Nanoparticles Exposure on Human Health—A Review." *Biological Trace Element Research* 193, no. 1: 118–129. https://doi.org/10.1007/s12011-019-01706-6.

Barberio, M., P. Barone, V. Pingitore, and A. Bonanno. 2012. "Optical Properties of TiO_2 Anatase—Carbon Nanotubes Composites Studied by Cathodoluminescence Spectroscopy." *Superlattices and Microstructures* 51, no. 1: 177–183. https://doi.org/10.1016/j.spmi.2011.11.011.

Bermudez, E., J. B. Mangum, B. A. Wong, et al. 2004. "Pulmonary Responses of Mice, Rats, and Hamsters to Subchronic Inhalation of Ultrafine Titanium Dioxide Particles." *Toxicological Sciences* 77, no. 2: 347–357. https://doi.org/10.1093/toxsci/kfh019.

Bian, Y., H. Y. Chung, O. N. Bae, K. M. Lim, J. H. Chung, and J. Pi. 2021. "Titanium Dioxide Nanoparticles Enhance Thrombosis Through Triggering the Phosphatidylserine Exposure and Procoagulant Activation of red Blood Cells." *Particle and Fibre Toxicology* 18, no. 1: 28. https://doi.org/10.1186/s12989-021-00422-1.

Boffetta, P., V. Gaborieau, L. Nadon, M. F. Parent, E. Weiderpass, and J. Siemiatycki. 2001. "Exposure to Titanium Dioxide and Risk of Lung Cancer in a Population-Based Study From Montreal." *Scandinavian Journal of Work, Environment & Health* 27, no. 4: 227–232. https://doi.org/10.5271/sjweh.609.

Boffetta, P., A. Soutar, J. W. Cherrie, et al. 2004. "Mortality Among Workers Employed in the Titanium Dioxide Production Industry in Europe." *Cancer Causes & Control* 15, no. 7: 697–706. https://doi.org/10.1023/b:Caco.0000036188.23970.22.

Cameron, S. J., J. Sheng, F. Hosseinian, and W. G. Willmore. 2022. "Nanoparticle Effects on Stress Response Pathways and Nanoparticle–Protein Interactions." *International Journal of Molecular Sciences* 23, no. 14: 7962. https://doi.org/10.3390/ijms23147962.

Carriere, M., M. E. Arnal, and T. Douki. 2020. "TiO_2 Genotoxicity: An Update of the Results Published Over the Last Six Years." *Mutation Research, Genetic Toxicology and Environmental Mutagenesis* 854-855: 503198. https://doi.org/10.1016/j.mrgentox.2020.503198.

Coenen, T., and N. M. Haegel. 2017. "Cathodoluminescence for the 21st Century: Learning More From Light." *Applied Physics Reviews* 4: 031103. https://doi.org/10.1063/1.4985767.

Cohignac, V., M. J. Landry, J. Boczkowski, and S. Lanone. 2014. "Autophagy as a Possible Underlying Mechanism of Nanomaterial Toxicity." *Nanomaterials (Basel)* 4, no. 3: 548–582. https://doi.org/10.3390/nano4030548.

Coméra, C., C. Cartier, E. Gaultier, et al. 2020. "Jejunal Villus Absorption and Paracellular Tight Junction Permeability Are Major Routes for Early Intestinal Uptake of Food-Grade TiO_2 Particles: An in Vivo and Ex Vivo Study in Mice." *Particle and Fibre Toxicology* 17, no. 1: 26. https://doi.org/10.1186/s12989-020-00357-z.

Commission Regulation (EU) 2022/63 Amending Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council as Regards the Food Additive Titanium Dioxide (E 171) (14 January 2022).

COT. (2024) Statement on the Safety of Titanium Dioxide (E171) as a Food Additive. COT/2024/05, Last Updated: 02 October 2024. Committee on Toxicity, Food Standards Agency.

de la Harpe, K. M., P. P. D. Kondiah, Y. E. Choonara, T. Marimuthu, L. C. du Toit, and V. Pillay. 2019. "The Hemocompatibility of Nanoparticles: A Review of Cell–Nanoparticle Interactions and Hemostasis." *Cells* 8, no. 10: 1209. https://doi.org/10.3390/cells8101209.

Disdier, C., J. Devoy, A. Cosnefroy, et al. 2015. "Tissue Biodistribution of Intravenously Administrated Titanium Dioxide Nanoparticles Revealed Blood–Brain Barrier Clearance and Brain Inflammation in rat." *Particle and Fibre Toxicology* 12: 27. https://doi.org/10.1186/s12989-015-0102-8.

Driscoll, K. E., and P. J. A. Borm. 2020. "Expert Workshop on the Hazards and Risks of Poorly Soluble Low Toxicity Particles." *Inhalation Toxicology* 32, no. 2: 53–62. https://doi.org/10.1080/08958378.2020.1735581.

Dumková, J., T. Smutná, L. Vrlíková, et al. 2020. "A Clearance Period After Soluble Lead Nanoparticle Inhalation Did Not Ameliorate the Negative Effects on Target Tissues due to Decreased Immune Response." *International Journal of Molecular Sciences* 21, no. 22: 8738. https://doi.org/10.3390/ijms21228738.

Elgrabli, D., R. Beaudouin, N. Jbilou, et al. 2015. "Biodistribution and Clearance of TiO_2 Nanoparticles in Rats After Intravenous Injection." *PLoS ONE* 10, no. 4: e0124490. https://doi.org/10.1371/journal.pone.0124490.

Fabian, E., R. Landsiedel, L. Ma-Hock, K. Wiench, W. Wohlleben, and B. van Ravenzwaay. 2008. "Tissue Distribution and Toxicity of Intravenously Administered Titanium Dioxide Nanoparticles in Rats." *Archives of Toxicology* 82, no. 3: 151–157. https://doi.org/10.1007/s00204-007-0253-y.

FAO/WHO. 2023, Ninety-Seventh Meeting (Safety Evaluation of Certain Food Additives) 31 October–9 November 2023, Summary and Conclusions. The Joint FAO/WHO Expert Committee on Food Additives (JECFA).

Fryzek, J. P., B. Chadda, D. Marano, et al. 2003. "A Cohort Mortality Study Among Titanium Dioxide Manufacturing Workers in the United States." *Journal of Occupational and Environmental Medicine* 45, no. 4: 400–409. https://doi.org/10.1097/01.jom.0000058338.05741.45.

Gaté, L., C. Disdier, F. Cosnier, et al. 2017. "Biopersistence and Translocation to Extrapulmonary Organs of Titanium Dioxide Nanoparticles After Subacute Inhalation Exposure to Aerosol in Adult and Elderly Rats." *Toxicology Letters* 265: 61–69. https://doi.org/10.1016/j.toxlet.2016.11.009.

Geiser, M., M. Casaulta, B. Kupferschmid, H. Schulz, M. Semmler-Behnke, and W. Kreyling. 2008. "The Role of Macrophages in the Clearance of Inhaled Ultrafine Titanium Dioxide Particles." *American Journal of Respiratory Cell and Molecular Biology* 38, no. 3: 371–376. https://doi.org/10.1165/rcmb.2007-0138OC.

Geiser, M., and W. G. Kreyling. 2010. "Deposition and Biokinetics of Inhaled Nanoparticles." *Particle and Fibre Toxicology* 7: 2. https://doi.org/10.1186/1743-8977-7-2.

General Court. 2022. "Judgment of the General Court in Joined Cases T-279/20 and T-288/20 and in case T-283/20." CWS Powder Coatings GmbH and Others v Commission (23 November 2022).

Ghosh, M., A. Chakraborty, and A. Mukherjee. 2013. "Cytotoxic, Genotoxic and the Hemolytic Effect of Titanium Dioxide (TiO_2) Nanoparticles on Human Erythrocyte and Lymphocyte Cells in Vitro." *Journal of Applied Toxicology* 33, no. 10: 1097–1110. https://doi.org/10.1002/jat.2863.

Grassian, V. H., P. T. O'Shaughnessy, A. Adamcakova-Dodd, J. M. Pettibone, and P. S. Thorne. 2007. "Inhalation Exposure Study of Titanium Dioxide Nanoparticles With a Primary Particle Size of 2 to 5 nm." *Environmental Health Perspectives* 115, no. 3: 397–402. https://doi.org/10.1289/ehp.9469.

Gualtieri, M., P. Mantecca, V. Corvaja, et al. 2009. "Winter Fine Particulate Matter From Milan Induces Morphological and Functional Alterations in Human Pulmonary Epithelial Cells (A549)." *Toxicology Letters* 188, no. 1: 52–62. https://doi.org/10.1016/j.toxlet.2009.03.003.

Guillard, A., E. Gaultier, C. Cartier, et al. 2020. "Basal Ti Level in the Human Placenta and Meconium and Evidence of a Materno-Foetal Transfer of Food-Grade TiO(2) Nanoparticles in an ex Vivo Placental Perfusion Model." *Particle and Fibre Toxicology* 17, no. 1: 51. https://doi.org/10.1186/s12989-020-00381-z.

Guseva Canu, I., A. Gaillen-Guedy, A. Antilla, et al. 2022. "Lung cancer Mortality in the European Cohort of Titanium Dioxide Workers: A Reanalysis of the Exposure–Response Relationship." *Occupational and Environmental Medicine* 79: 637–640. https://doi.org/10.1136/oemed-2021-108030.

Han, C., J. Lalley, D. Namboodiri, K. Cromer, and M. N. Nadagouda. 2016. "Titanium Dioxide-Based Antibacterial Surfaces for Water Treatment." *Current Opinion in Chemical Engineering* 11: 46–51. https://doi.org/10.1016/J.COCHE.2015.11.007.

Hansa, J., H. Merzenich, L. Cascant Ortolano, S. J. Klug, M. Blettner, and E. Gianicolo. 2023. "Health Risks of Titanium Dioxide (TiO_2) Dust Exposure in Occupational Settings—A Scoping Review." *International Journal of Hygiene and Environmental Health* 252: 114212. https://doi.org/10.1016/j.ijheh.2023.114212.

Harrison, P. M., and P. Arosio. 1996. "The Ferritins: Molecular Properties, iron Storage Function and Cellular Regulation." *Biochimica*

et Biophysica Acta 1275, no. 3: 161–203. https://doi.org/10.1016/0005-2728(96)00022-9.

He, Z., J. Liu, and L. Du. 2014. "The Unexpected Effect of PEGylated Gold Nanoparticles on the Primary Function of Erythrocytes." *Nanoscale* 6, no. 15: 9017–9024. https://doi.org/10.1039/c4nr01857e.

Health Canada. 2022. *State of the Science of Titanium Dioxide (TiO2) as a Food Additive*. Health Canada: Food Directorate.

Heinrich, U., R. Fuhst, S. Rittinghausen, et al. 1995. "Chronic Inhalation Exposure of Wistar Rats and two Different Strains of Mice to Diesel Engine Exhaust, Carbon Black, and Titanium Dioxide." *Inhalation Toxicology* 7, no. 4: 533–556. https://doi.org/10.3109/08958379509015211.

Heringa, M. B., R. J. B. Peters, R. Bleys, et al. 2018. "Detection of Titanium Particles in Human Liver and Spleen and Possible Health Implications." *Particle and Fibre Toxicology* 15, no. 1: 15. https://doi.org/10.1186/s12989-018-0251-7.

Hu, H., Q. Guo, C. Wang, et al. 2015. "Titanium Dioxide Nanoparticles Increase Plasma Glucose via Reactive Oxygen Species-Induced Insulin Resistance in Mice." *Journal of Applied Toxicology* 35, no. 10: 1122–1132. https://doi.org/10.1002/jat.3150.

Chu, Z., S. Zhang, B. Zhang, et al. 2014. "Unambiguous Observation of Shape Effects on Cellular Fate of Nanoparticles." *Scientific Reports* 4: 4495. https://doi.org/10.1038/srep04495.

Iancu, T. C. 1992. "Ferritin and Hemosiderin in Pathological Tissues." *Electron Microscopy Reviews* 5, no. 2: 209–229. https://doi.org/10.1016/0892-0354(92)90011-e.

Iancu, T. C. 2011. "Ultrastructural Aspects of iron Storage, Transport and Metabolism." *Journal of Neural Transmission (Vienna)* 118, no. 3: 329–335. https://doi.org/10.1007/s00702-011-0588-7.

IARC. 2010. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Carbon Black, Titanium Dioxide, and Talc*. Vol. 93. Lyon, France: International Agency for Research on Cancer.

Ichihara, S., W. Li, S. Omura, et al. 2016. "Exposure Assessment and Heart Rate Variability Monitoring in Workers Handling Titanium Dioxide Particles: A Pilot Study." *Journal of Nanoparticle Research* 18: 52. https://doi.org/10.1007/s11051-016-3340-2.

Iversen, T.-G., T. Skotland, and K. Sandvig. 2011. "Endocytosis and Intracellular Transport of Nanoparticles: Present Knowledge and Need for Future Studies." *Nano Today* 6, no. 2: 176–185. https://doi.org/10.1016/j.nantod.2011.02.003.

Jafari, S., B. Mahyad, H. Hashemzadeh, S. Janfaza, T. Gholikhani, and L. Tayebi. 2020. "Biomedical Applications of TiO_2 Nanostructures: Recent Advances." *International Journal of Nanomedicine* 15: 3447–3470. https://doi.org/10.2147/ijn.S249441.

Keevend, K., T. Coenen, and I. K. Herrmann. 2020. "Correlative Cathodoluminescence Electron Microscopy Bioimaging: Towards Single Protein Labelling With Ultrastructural Context." *Nanoscale* 12, no. 29: 15588–15603. https://doi.org/10.1039/d0nr02563a.

Konczol, M., S. Ebeling, E. Goldenberg, et al. 2011. "Cytotoxicity and Genotoxicity of Size-Fractionated iron Oxide (Magnetite) in A549 Human Lung Epithelial Cells: Role of ROS, JNK, and NF-kappaB." *Chemical Research in Toxicology* 24, no. 9: 1460–1475. https://doi.org/10.1021/tx200051s.

Krug, H. F. 2014. "Nanosafety research—are we on the right track?" *Angewandte Chemie (International Ed. in English)* 53, no. 46: 12304–12319. https://doi.org/10.1002/anie.201403367.

Krug, H. F., and P. Wick. 2011. "Nanotoxicology: An Interdisciplinary Challenge." *Angewandte Chemie (International Ed. in English)* 50, no. 6: 1260–1278. https://doi.org/10.1002/anie.201001037.

Křůmal, K., P. Mikuška, K. Večeřová, O. Urban, E. Pallozzi, and Z. Večeřa. 2016. "Wet Effluent Diffusion Denuder: The Tool for Determination of Monoterpenes in Forest." *Talanta* 153: 260–267. https://doi.org/10.1016/j.talanta.2016.03.032.

Kumar, N., N. S. Chauhan, A. Mittal, and S. Sharma. 2018. "TiO_2 and Its Composites as Promising Biomaterials: A Review." *Biometals* 31, no. 2: 147–159. https://doi.org/10.1007/s10534-018-0078-6.

Lammel, T., A. Mackevica, B. R. Johansson, and J. Sturve. 2019. "Endocytosis, Intracellular Fate, Accumulation, and Agglomeration of Titanium Dioxide (TiO_2) Nanoparticles in the Rainbow Trout Liver Cell Line RTL-W1." *Environmental Science and Pollution Research International* 26, no. 15: 15354–15372. https://doi.org/10.1007/s11356-019-04856-1.

Lee, K. P., H. J. Trochimowicz, and C. F. Reinhardt. 1985a. "Pulmonary Response of Rats Exposed to Titanium Dioxide (TiO_2) by Inhalation for two Years." *Toxicology and Applied Pharmacology* 79, no. 2: 179–192. https://doi.org/10.1016/0041-008x(85)90339-4.

Lee, K. P., H. J. Trochimowicz, and C. F. Reinhardt. 1985b. "Transmigration of Titanium Dioxide (TiO_2) Particles in Rats After Inhalation Exposure." *Experimental and Molecular Pathology* 42, no. 3: 331–343. https://doi.org/10.1016/0014-4800(85)90083-8.

Lehotska Mikusova, M., M. Busova, J. Tulinska, et al. 2023. "Titanium Dioxide Nanoparticles Modulate Systemic Immune Response and Increase Levels of Reduced Glutathione in Mice After Seven-Week Inhalation." *Nanomaterials* 13, no. 4: 767 Retrieved from https://www.mdpi.com/2079-4991/13/4/767.

Li, S. Q., R. R. Zhu, H. Zhu, et al. 2008. "Nanotoxicity of TiO_2 Nanoparticles to Erythrocyte in Vitro." *Food and Chemical Toxicology* 46, no. 12: 3626–3631. https://doi.org/10.1016/j.fct.2008.09.012.

Lojk, J., V. B. Bregar, M. Rajh, et al. 2015. "Cell Type-Specific Response to High Intracellular Loading of Polyacrylic Acid-Coated Magnetic Nanoparticles." *International Journal of Nanomedicine* 10: 1449–1462. https://doi.org/10.2147/ijn.S76134.

Lord, J. M., and L. M. Roberts. 1998. "Retrograde Transport: Going Against the Flow." *Current Biology* 8, no. 2: R56–R58. https://doi.org/10.1016/s0960-9822(98)70034-x.

Luo, Y. H., S. B. Wu, Y. H. Wei, et al. 2013. "Cadmium-Based Quantum Dot Induced Autophagy Formation for Cell Survival via Oxidative Stress." *Chemical Research in Toxicology* 26, no. 5: 662–673. https://doi.org/10.1021/tx300455k.

Martin, N., B. Wassmur, A. Baun, and T. Lammel. 2022. "Availability and Effects of n-TiO_2 and PCB77 in Fish in Vitro Models of the Intestinal Barrier and Liver Under Single- and/or Co-Exposure Scenarios." *Aquatic Toxicology* 253: 106343. https://doi.org/10.1016/j.aquatox.2022.106343.

Meijer, A. J., and P. Codogno. 2009. "Autophagy: Regulation and Role in Disease." *Critical Reviews in Clinical Laboratory Sciences* 46, no. 4: 210–240. https://doi.org/10.1080/10408360903044068.

Mohammadparast, V., and B. L. Mallard. 2023. "The Effect and Underlying Mechanisms of Titanium Dioxide Nanoparticles on Glucose Homeostasis: A Literature Review." *Journal of Applied Toxicology* 43, no. 1: 22–31. https://doi.org/10.1002/jat.4318.

Moravec, P., J. Schwarz, P. Vodička, and M. Koštejn. 2016. "Study of TiO_2 Nanoparticle Generation for Follow-Up Inhalation Experiments With Laboratory Animals." *Aerosol Science and Technology* 50, no. 10: 1068–1076. https://doi.org/10.1080/02786826.2016.1224803.

Muhle, H., B. Kittel, H. Ernst, U. Mohr, and R. Mermelstein. 1995. "Neoplastic Lung Lesions in Rat After Chronic Exposure to Crystalline Silica." *Scandinavian Journal of Work, Environment & Health* 21, no. Suppl 2: 27–29.

Muhle, H., R. Mermelstein, C. Dasenbrock, et al. 1989. "Lung Response to Test Toner Upon 2-Year Inhalation Exposure in Rats." *Experimental Pathology* 37, no. 1–4: 239–242. https://doi.org/10.1016/s0232-1513(89)80059-3.

Mühlfeld, C., M. Geiser, N. Kapp, P. Gehr, and B. Rothen-Rutishauser. 2007. "Re-Evaluation of Pulmonary Titanium Dioxide Nanoparticle Distribution Using the "Relative Deposition Index": Evidence for Clearance Through Microvasculature." *Particle and Fibre Toxicology* 4: 7. https://doi.org/10.1186/1743-8977-4-7.

NIOSH. 2011. *Occupational Exposure to Titanium Dioxide*. Vol. 63. Cincinnati, OH, USA: National Institute for Occupational Safety and Health.

Oberdörster, G. 2001. "Pulmonary Effects of Inhaled Ultrafine Particles." *International Archives of Occupational and Environmental Health* 74, no. 1: 1–8. https://doi.org/10.1007/s004200000185.

Peters, R. J. B., A. G. Oomen, G. van Bemmel, et al. 2020. "Silicon Dioxide and Titanium Dioxide Particles Found in Human Tissues." *Nanotoxicology* 14, no. 3: 420–432. https://doi.org/10.1080/17435390.2020.1718232.

Plugaru, R. 2008. "Optical Properties of Nanocrystalline Titanium Oxide." *Thin Solid Films* 516, no. 22: 8179–8183. https://doi.org/10.1016/j.tsf.2008.04.039.

Pujalté, I., D. Dieme, S. Haddad, A. M. Serventi, and M. Bouchard. 2017. "Toxicokinetics of Titanium Dioxide (TiO_2) Nanoparticles After Inhalation in Rats." *Toxicology Letters* 265: 77–85. https://doi.org/10.1016/j.toxlet.2016.11.014.

Ravikumar, B., S. Sarkar, J. E. Davies, et al. 2010. "Regulation of Mammalian Autophagy in Physiology and Pathophysiology." *Physiological Reviews* 90, no. 4: 1383–1435. https://doi.org/10.1152/physrev.00030.2009.

Reibman, J., Y. Hsu, L. C. Chen, et al. 2002. "Size Fractions of Ambient Particulate Matter Induce Granulocyte Macrophage Colony-Stimulating Factor in Human Bronchial Epithelial Cells by Mitogen-Activated Protein Kinase Pathways." *American Journal of Respiratory Cell and Molecular Biology* 27, no. 4: 455–462. https://doi.org/10.1165/rcmb.2001-0005OC.

Rennick, J. J., A. P. R. Johnston, and R. G. Parton. 2021. "Key Principles and Methods for Studying the Endocytosis of Biological and Nanoparticle Therapeutics." *Nature Nanotechnology* 16, no. 3: 266–276. https://doi.org/10.1038/s41565-021-00858-8.

Riediker, M., D. Zink, W. Kreyling, et al. 2019. "Particle Toxicology and Health—Where Are We?" *Particle and Fibre Toxicology* 16, no. 1: 19. https://doi.org/10.1186/s12989-019-0302-8.

Rossner, P., K. Vrbova, S. Strapacova, et al. 2019. "Inhalation of ZnO Nanoparticles: Splice Junction Expression and Alternative Splicing in Mice." *Toxicological Sciences* 168, no. 1: 190–200. https://doi.org/10.1093/toxsci/kfy288.

Saito, H. 2014. "Metabolism of iron Stores." *Nagoya Journal of Medical Science* 76, no. 3–4: 235–254.

Sandvig, K., T. Skotland, B. van Deurs, and T. I. Klokk. 2013. "Retrograde Transport of Protein Toxins Through the Golgi Apparatus." *Histochemistry and Cell Biology* 140, no. 3: 317–326. https://doi.org/10.1007/s00418-013-1111-z.

Shi, H., R. Magaye, V. Castranova, and J. Zhao. 2013. "Titanium Dioxide Nanoparticles: A Review of Current Toxicological Data." *Particle and Fibre Toxicology* 10: 15. https://doi.org/10.1186/1743-8977-10-15.

Skotland, T., T.-G. Iversen, and K. Sandvig. 2021. "Cellular Uptake of Nanoparticles: Involvement of Caveolae?" *Precision Nanomedicine* 4: 782–786. https://doi.org/10.33218/001c.22201.

Skoupý, R., D. B. Boltje, M. Slouf, et al. 2023. "Robust Local Thickness Estimation of sub-Micrometer Specimen by 4D-STEM." *Small Methods* 7: e2300258. https://doi.org/10.1002/smtd.202300258.

Slouf, M., R. Skoupy, E. Pavlova, and V. Krzyzanek. 2021a. "High Resolution Powder electron Diffraction in Scanning electron Microscopy." *Materials (Basel)* 14, no. 24: 7550. https://doi.org/10.3390/ma14247550.

Slouf, M., R. Skoupy, E. Pavlova, and V. Krzyzanek. 2021b. "Powder Nano-Beam Diffraction in Scanning Electron Microscope: Fast and Simple Method for Analysis of Nanoparticle Crystal Structure." *Nanomaterials (Basel)* 11, no. 4: 962. https://doi.org/10.3390/nano11040962.

Smutná, T., J. Dumková, D. Kristeková, et al. 2022. "Macrophage-Mediated Tissue Response Evoked by Subchronic Inhalation of Lead Oxide Nanoparticles Is Associated With the Alteration of Phospholipases C and Cholesterol Transporters." *Particle and Fibre Toxicology* 19, no. 1: 52. https://doi.org/10.1186/s12989-022-00494-7.

Solarska-Ściuk, K., K. Adach, S. Cyboran-Mikołajczyk, et al. 2021. "Are Biogenic and Pyrogenic Mesoporous SiO(2) Nanoparticles Safe for Normal Cells?" *Molecules* 26, no. 5: 1427. https://doi.org/10.3390/molecules26051427.

Stern, S. T., P. P. Adiseshaiah, and R. M. Crist. 2012. "Autophagy and Lysosomal Dysfunction as Emerging Mechanisms of Nanomaterial Toxicity." *Particle and Fibre Toxicology* 9: 20. https://doi.org/10.1186/1743-8977-9-20.

Sun, B., X. Wang, Z. Ji, R. Li, and T. Xia. 2013. "NLRP3 Inflammasome Activation Induced by Engineered Nanomaterials." *Small* 9, no. 9–10: 1595–1607. https://doi.org/10.1002/smll.201201962.

Thyssen, J., G. Kimmerle, S. Dickhaus, E. Emminger, and U. Mohr. 1978. "Inhalation Studies With Polyurethane Foam Dust in Relation to Respiratory Tract Carcinogenesis." *Journal of Environmental Pathology and Toxicology* 1, no. 4: 501–508.

Tian, Y., Z. Tian, Y. Dong, X. Wang, and L. Zhan. 2021. "Current Advances in Nanomaterials Affecting Morphology, Structure, and Function of Erythrocytes." *RSC Advances* 11: 6958–6971.

Valentini, X., P. Rugira, A. Frau, et al. 2019. "Hepatic and Renal Toxicity Induced by TiO_2 Nanoparticles in Rats: A Morphological and Metabonomic Study." *Journal of Toxicology* 2019: 5767012. https://doi.org/10.1155/2019/5767012.

Verleysen, E., F. Brassinne, F. Van Steen, et al. 2022. "Towards a Generic Protocol for Measuring the Constituent Particle Size Distribution of E171 in Food by Electron Microscopy." *Food Control* 132: 108492. https://doi.org/10.1016/j.foodcont.2021.108492.

Vysloužil, J., P. Kulich, T. Zeman, et al. 2020. "Subchronic Continuous Inhalation Exposure to Zinc Oxide Nanoparticles Induces Pulmonary Cell Response in Mice." *Journal of Trace Elements in Medicine and Biology* 61: 126511. https://doi.org/10.1016/j.jtemb.2020.126511.

Winter, M., H.-D. Beer, V. Hornung, U. Krämer, R. P. F. Schins, and I. Förster. 2011. "Activation of the Inflammasome by Amorphous Silica and TiO_2 Nanoparticles in Murine Dendritic Cells." *Nanotoxicology* 5, no. 3: 326–340. https://doi.org/10.3109/17435390.2010.506957.

Yamano, S., Y. Goto, T. Takeda, et al. 2022. "Pulmonary Dust Foci as Rat Pneumoconiosis Lesion Induced by Titanium Dioxide Nanoparticles in 13-Week Inhalation Study." *Particle and Fibre Toxicology* 19, no. 1: 58. https://doi.org/10.1186/s12989-022-00498-3.

Yamano, S., T. Takeda, Y. Goto, et al. 2022. "No Evidence for Carcinogenicity of Titanium Dioxide Nanoparticles in 26-Week Inhalation Study in rasH2 Mouse Model." *Scientific Reports* 12, no. 1: 14969. https://doi.org/10.1038/s41598-022-19139-y.

Yan, M., Y. Zhang, H. Qin, et al. 2016. "Cytotoxicity of CdTe Quantum Dots in Human Umbilical Vein Endothelial Cells: The Involvement of Cellular Uptake and Induction of Pro-Apoptotic Endoplasmic Reticulum Stress." *International Journal of Nanomedicine* 11: 529–542. https://doi.org/10.2147/ijn.S93591.

Yazdi, A. S., G. Guarda, N. Riteau, et al. 2010. "Nanoparticles Activate the NLR Pyrin Domain Containing 3 (Nlrp3) Inflammasome and Cause Pulmonary Inflammation Through Release of IL-1α and IL-1β."

Proceedings of the National Academy of Sciences of the United States of America 107, no. 45: 19449–19454. https://doi.org/10.1073/pnas.1008155107.

Younes, M., G. Aquilina, L. Castle, et al. 2021. "EFSA FAF Panel. Safety Assessment of Titanium Dioxide (E171) as a Food Additive." *EFSA Journal* 19, no. 5: e06585. https://doi.org/10.2903/j.efsa.2021.6585.

Younis, A. B., Y. Haddad, L. Kosaristanova, and K. Smerkova. 2022. "Titanium Dioxide Nanoparticles: Recent Progress in Antimicrobial Applications." *Wiley Interdisciplinary Reviews. Nanomedicine and Nanobiotechnology* 15: e1860. https://doi.org/10.1002/wnan.1860.

Yu, K. N., J. H. Sung, S. Lee, et al. 2015. "Inhalation of Titanium Dioxide Induces Endoplasmic Reticulum Stress-Mediated Autophagy and Inflammation in Mice." *Food and Chemical Toxicology* 85: 106–113. https://doi.org/10.1016/j.fct.2015.08.001.

Zeman, T., E. W. Loh, D. Čierný, and O. Šerý. 2018. "Penetration, Distribution and Brain Toxicity of Titanium Nanoparticles in Rodents' Body: A Review." *IET Nanobiotechnology* 12, no. 6: 695–700. https://doi.org/10.1049/iet-nbt.2017.0109.

Zhang, X., H. Zhang, X. Liang, et al. 2016. "Iron Oxide Nanoparticles Induce Autophagosome Accumulation Through Multiple Mechanisms: Lysosome Impairment, Mitochondrial Damage, and ER Stress." *Molecular Pharmaceutics* 13, no. 7: 2578–2587. https://doi.org/10.1021/acs.molpharmaceut.6b00405.

Zhao, J., L. Bowman, X. Zhang, et al. 2009. "Titanium Dioxide (TiO_2) Nanoparticles Induce JB6 Cell Apoptosis Through Activation of the Caspase-8/Bid and Mitochondrial Pathways." *Journal of Toxicology and Environmental Health. Part A* 72, no. 19: 1141–1149. https://doi.org/10.1080/15287390903091764.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.