Meeting

Annex B to TOX/2023/16 - Review of Efsa Opinion on the Reproductive Toxicity of Titanium Dioxide as a Food Additive

This is a paper for discussion.

This does not represent the views of the Committee and should not be cited.

Introduction

1. The information presented in this annex should be read in conjunction with the main draft statement on the EFSA opinion on the reproductive toxicity of titanium dioxide as a food additive. It contains a summary of the data contained in the relevant literature considered by EFSA to support the opinion and discussion of the categorisation used for papers obtained by the literature search.

Categorisation of Papers

2. The advice elaborated on the nanoscale considerations (NSC) and adaptations related to specific aspects of study design with TiO2 which are adequate for a hazard identification and hazard characterisation of small particles, including nanoparticles.

3. Toxicokinetic and toxicity studies were scored for NSC (dispersion and/or confirmation of internal exposure). The confidence for assessing the toxicological effects of the fraction of small particles, including nanoparticles was as follows:

- Scoring 1 for NSC*: the study is suitable.
- Scoring 2 for NSC: the study has some limitations.
- Scoring 3 for NSC: the relevance of the results cannot be verified.
- Scoring 4 for NSC: the relevance of the results is low.

*Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure).

Table of Additional Papers

Immunotoxicity

Paper	Test System	Exposure	Characterisation of test substance	Result

Urrutia-Ortega et al. (2016)	Mice	Qualitative analysis in tissues, methodology reliable with some limitations.	E 171, particles below and above 100 nm, crystalline form not reported.	Results obtained indicate that E 171 alon was unable to induce tumour formation, but dysplastic alterations were observed in the distal colon with a statistical significant enhancement of tumou formation in CAC + E 171 group vs. CAC group (p 0.01). Some E 171 particles were internalised in colonic cells of the E 171 and CAC + E 171 groups, and both groups showe a decrease in goblet cells in the distal colon. CAC tumour progressio markers including COX2 Ki67 and b-catenin indicated that E 171 exacerbates tumour progression and p65 NF κB, a key regulator of inflammation was also induced by E 171 administration in CAC group. In the E 171 group, despite the absence of tumour formation, a slight statistical significant increase in COX2, Ki67 and b-catenin, and p65 NF-κB were observed. Regarding cytokines in colon tissue, no changes, despite enhanced the p65-NF-κ expression, were found in the E 171 alone and

Talamini et al. (2019)	M:	Quantitative analysis in tissues with methodology	E 171 (35% nano), anatase, 201 nm in suspension	This study aimed to investigate whether TiO is deposited in the digestive system and internal organs and whether there are any molecular and cellular alterations associated with an inflammatory response. Neither overt structural and morphological histological alterations nor significant recruitment of monocytes/macrophage were observed in the stomach and whole intestine in E 171-treated animals. Neithe disruption of crypt structure nor atypical epithelial cell proliferation in colon wa observed. No changes i spleen histology were mentioned. In the liver, increased size of necroinflammatory foci infiltrated with F4/80 monocytes/macrophage was noted three days after the last E 171 dos (n = 4) vs. controls (n = 2), while no changes were observed in stomach or intestine of treated animals. Liver histological findings are not supported by ROS production data or

Riedle et al. (2020)	Mice	Qualitative analysis in tissues, methodology reliable with some limitations.	E 171, anatase, 119 nm (TEM).	Peyer's patches from 18 weeks feeding were examined by confocal microscopy and SEM with EDX analysis. Remaining Peyer's patches were collected and enzymatically digested to form single-cell suspensions for analysis by flow cytometry after immunostaining for CD4 CD45R and CD8a for lymphocytes or CD11b, CD11c and CD8a for myelocytes.

Pinget et al. (2019)	Mice	Internal exposure not examined	E 171, anatase, 30–300 nm (SEM).	Five to six male C67Bl/6JAusb mice were exposed by drinking water to E 171 (0, 2, 10 50 mg/kg bw per day) for 4 weeks. Histologica analysis of the gut revealed a reduction of colonic crypt length. An increase in colon macrophages and CD8 cells were observed by FACS analysis in cell suspensions prepared from colon, and increased mRNA encoding for IL-10, TNF-α and IL-6 was detected by RT-PCR in RNA extracted form colon tissue.

Bettini et al. (2017)	Rats	Qualitative measurement in tissues, methodology reliable with some limitations.	1) E 171, anatase, 20–340 nm (118 nm) (TEM); 44.7% particles 100 nm; 2) TiO2 NPs (NM-105), anatase/rutile, 15–24 nm.	Titanium was detected in the immune cells of Peyer's patches. Effects were not noted in the spleen, but in Peyer's patches dendritic cell percentage were increased, as measured by flow cytometry of cells isolated from tissu samples. This effect wa transient, as it was observed 7 days after exposure, but not at 10 days. The percentage o regulatory T cells and T helper (Th) cells were significantly decreased at both time points in E 171 exposed animals. Stimulation of immune cells isolated from Peyer's patches showed a decrease in T-helper (Th)-1 IFN-γ secretion, while splenic Th1/Th17 inflammatory responses sharply increased, as measured in cells taken from exposed rats, stimulated in vitro with anti CD3/CD28 antibodies. Regarding the effects of TiO2 NP, similar to E 171 an increase in the percentage of dendritic cells in Peyer's patches was observed with no decrease in the percentage of Tregs. Stimulation of immune cells isolated from

Blevins etal. (2019)	Rats Six-week-old male Wistar Han	Internal exposure not examined	E 171, anatase, 110–115 nm (SEM), 36% particles 100 nm.	Collectively, these results suggest that E 171 consumption does not alter T-cell-mediate mechanisms of immune control, either promotin inflammatory CD4+T helper cell activation or in reducing the percentage of anti-inflammatory Treg cells Regarding the effects o cytokines, data presented suggest that dietary E 171 does not induce inflammation peripherally or in the GI tract at both time point A modest increase in th relative spleen weight i 22.4 mg E 171/kg bw pe day + DMH compared t not initiated animals, ar increase in IL-17A in colon (22.4 mg E 171/k bw per day + DMH) and IL-12p70 in plasma (3.5 mg E 171/kg bw per day + DMH), with no dose-related effects, were observed. There were n changes in spleen cellularity across any of the treated groups. No changes were observed in the percentage of CD103+ DC, CD4+ T helper cells or total or activated Treg in peripheral blood, spleen or Peyer's patches in animals exposed to E

Han et al. (2020a)	Sprague–Dawley rats	Quantitative analysis in tissues; methodology not reliable.	E 171, anatase, 150 nm (DLS).	There were no changes in the weight of immun organs or their histolog The proportion of lymphocytes slightly decreased (by 9%) in male but not female rat administered the highe E 171 dose, without an apparent dose response relationship. According to the study authors, th level of GM-CSF was reduced by 41% in females only at the highest dose of 1,000 mg/kg bw per day. In males there was also a decrement in GM-CSF level (approximately by 30%), that was slightly less pronounced than in females, and due to a higher variability in the controls, did not gain statistical significance. reduction in the levels o IgM was observed in both sexes at the highest dose tested, i.e by 12% in females and by 9% in males, but there were no clear dos response relationships. Transcriptomics also showed that immune response-related microRNAs were most strongly affected by E 171 exposure, which may support the effect observed at the high dose.

TiO2 or TiO2 NPs	Test System	Exposure	Characterisation of test substance	Result
Mohamed (2015)	Male Swiss Webster mice, aged 10–12 weeks	Quantitative measurement in tissues, methodology with important flaws	TiO2 NPs, rutile/anatase (77/22%), 47 nm (TEM).	According to the authors, routine histopathology showed dose-dependent effects in the stomach. At 5 mg/kg, submucosal oedema was noted afte 24 h that developed int ulcerations and mucosa necrosis after one and two weeks, respectively After exposure to 50 mg/kg bw per day, submucosal vasculitis, massively degenerated glands and both mucos and submucosal necros was evident after 24 h, days and 14 days, respectively. Submucosal congested blood vessels, focal areas of leucocytic cell infiltrations and necroti glands with mononuclea cell infiltrations (i.e. the highest grade of damage) were seen wit TiO2 NPs at 500 mg/kg bw per day at all time points.

Li et al. (2019)	Male C57BL/6 mice (8 weeks old)	Quantitative analysis in tissues, methodology with important flaws.	(1) TiO2 NPs, anatase, 25 nm; (2) TiO2 NPs, anatase, 50 nm; (3) TiO2 NPs, anatase, 80 nm. Purity not reported for none of them.	Results indicate that short-term ingestion of TiO2 NPs (25 nm) (1 mg/kg bw per day for 7 days) led to colonic epithelial injury, reduce expression levels of tigh junction proteins and reduced thickness of th 'luminal mucus layer'. This was associated wit altered gut microbiota composition, with reduction in number of Bifidobacterium compared with controls Regarding immunological organs, histological findings were not reported.

Hashem et al. (2020)	Adult male Wistar rats	Internal exposure not examined.	TiO2 (from Sigma, no information on constituent particle size distribution nor crystalline form).	At the end of treatment the body weight of low- and high-dose groups was 25% lower than tha of the control group and there was a statistically significantly dose-dependent leucopenia and thrombocytopenia, as well as eosinophilia and neutrophilia . Statistically significantly dose-dependent spleen alterations were observed that included lymphoid necrosis, whit pulp expansion and increased numbers of macrophages. A marke increase in $CD4^+$ and $CD8^+$ immunolabelling was noted in the spleen In addition, IgG and IgM measured by ELISA wer statistically significantly elevated in TiO2-treate rats. Phagocytic activity measured by a modifie colorimetric nitro blue tetrazolium assay as well as lysozyme expression and nitric oxide levels measured by ELISA were significantly reduced following TiO2 exposure Lymphocyte proliferatio in response to PHA, measured by a lymphocyte

TiO2 NPs 30 nm	Test System	Exposure	Characterisation of test substance	Result

Yu et al. (2016)	CD-1 (ICR) female mice	Internal exposure not examined.	TiO2 NPs, anatase, 5–6 nm	Inflammatory lesions and tissue damage were seen histopathologically and were more pronounced at the mid and high doses. It was not reported whether the histopathology was performed blinded, but the results were corroborated with objective measures. The expression of NF-κB, and of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IFN-γ expression were increased in a dose-dependent fashion (statistically significant increase up to 1.8-fold compared with the control); expression of the NF-κB inhibitor I-κB was decreased in a dose-dependent fashion (statistically significant decrease up to 1.55-fold compared with the control), as evidenced by western blotting.

Li et al. (2018)	Male C57BL/6 mice, 8 weeks old	quantitative measurement in tissues, methodology with important flaws.	: 1) TiO2 NPs, anatase, 20 nm (in water, DLS); 2) TiO2 NPs, rutile, edged with corners morphology (SEM), 15 nm (in water, DLS).	There were no effects o body weight. Whereas particles were observed in the spleen, examination of H&E-stained samples of the spleen revealed no histopathological changes. No histopathological changes were seen recorded in other tissue examined (the lung, jejunum, kidney, liver o brain). In colon, the increased length of villi was increased and irregularly arranged epithelial cells were reported irregularly arranged after exposure to TiO2 NPs. Rutile NPs had a more pronounced influence o the gut microbiota than anatase NPs. The most influenced phylum was Proteobacteria, which was significantly increased by rutile NPs but not by anatase NPs At the genus level, Rhodococcus was enriched by rutile NPs, Prevotella was significantly decreased by both the TiO2 NPs.

Zhang et al. (2020)	C57BL/6J mice, aged 7 weeks	Internal exposure not examined.	TiO2 NPs, 21 nm (TEM), crystalline form and purity unknown.	The results show that oral exposure to TiO2 NPs resulted in significantly changed richness and composition of the gut microbiota. No changes in parameters indicating inflammation (IL-6 and IL-1β) in either intestine or brain were observed.

Chen et al. (2015a)	Four-week-old healthy Sprague–Dawley rats	Internal exposure not examined.	TiO2 NPs, anatase 24 nm (TEM).	Regarding the effects o spleen and white blood cells in animals exposed to TiO2 NPs alone, no significant histopathological changes were observed in the spleen in all groups. On the contrary increases in white blood cells parameters (white blood cell counts and granulocytes) were observed in female rats after exposure to TiO2 NPs 50 mg/kg bw per day for 90 days and among male rats exposed to TiO2 NPs 50 mg/kg bw per day for 3 (white blood cells counts, lymphocytes, monocytes absolute numbers and in the percentage of lymphocytes and granulocytes) and 90 days (percentage of monocytes); and a decrease in the while blood cells at 90 days in rats exposed to 10 mg/kg bw per day.

Chen et al. (2019)	Three-week-old male Sprague–Dawley rats	Internal exposure not examined.	TiO2 NPs, anatase, 29 nm (SEM).	Histopathologically, reduced numbers of goblet cells were found as a result of exposure, as well as inflammatory infiltration, while in serum increased IL-6 expression was observed.
Grissa et al. (2020)	Male Wistar rats	Internal exposure: quantitative in tissues; methodology with important flaws.	TiO2 NPs, anatase, 5–12 nm (TEM, XRD).	A statistically significant dose-related increase in the level of NO in 100 and 200 mg/kg bw per day TiO2 NPs groups was observed together with a statistically significant increase in brain TNF-α in 200 mg/kg bw per day TiO2 NPs group. The increase was dose-related for both parameters.

Reproductive Toxicity Studies - TiO2 NPs or TiO2 containing a fraction of nanoparticles.

Paper	Test System	Exposure	Characterisation of test substanc

						In three studies, time-mated pregnant Sprague–Dawley, Crl:CD(SD), rats (n=22/group) were daily exposed to TiO2(uf-1, uf-3 and pg-1) by gavage on GDs 6–20. In three additional studies, pregnant Wistar

1) anatase/rutile (89/11%)(uf-1), d50=43 nm (XSDC), d50=23 nm (TE irregular;

2) anatase (100% nano) (uf-2),Safety assessment of the food additive titanium dioxide (E 171)www.efsa.europa.eu/efsajournal EFSA Journal 2021;19(5):6585d50=4 nm (XSDC), d50=19 nm (TEM)

Khorsandi et al. (2016)	Mice	Internal exposure not examined.	TiO2 NPs 30 nm

Khorsandi et al. (2017)	Mice	Internal exposure not examined.	TiO2 NPs, 20–30 nm (AFM), crystallin form unknown

Karimipour et al. (2018)	Mice	Internal exposure not examined.	TiO2 NPs, anatase, 10–25 nm

Karimi et al. (2019)	6- to 8-week-old male NMRI mice	Internal exposure not examined.	TiO2 NPs, 68 nm (DLS; no direct information on constituent particle si thePanel considered the majority of constituent particles to be below 30 nm), crystalline form and purity unknown

Lu et al. (2020)	ICR mice, age 6–8 weeks	Internal exposure not examined.	TiO2 NPs, anatase, 7 nm (TEM)

Lee et al. (2019)	Sprague–Dawley rats (12 females per group	Internal exposure examined: quantitative analysis in blood/tissues; methodology reliable with some limitations.	TiO2 NPs (the Panel noted that from description of the tested material, it corresponds to P25 (15–24 nm))