Reproductive and Developmental Toxicity - Statement on the safety of Titanium Dioxide (E171) as a Food Additive

In this guide

Studies using E171 or equivalent form of TiO2

EOGRT study (Leuschner, 2020)

120. Following a call for data by EFSA to address several data gaps for TiO2, an extended one generation reproductive toxicity (EOGRT) study was conducted (Leuschner, 2020). Because the data from this study covered a number of endpoints in addition to reproductive and developmental toxicity, i.e. aberrant crypt foci, , immunotoxicity, and neurotoxicity, the study methodology is summarised in the following paragraphs and the results discussed in the relevant endpoint sections below. No other reproductive or developmental toxicity studies of E171 were identified.

121. The COT reviewed the EOGRT study in TOX/2023/16. The EOGRT study section below uses the information provided in that paper.

Methodology

122. The EOGRT study was commissioned by interested business operators to address the data gaps identified in the EFSA 2016 Opinion. The protocol was later amended to include a satellite group per dose at the F0 generation to accommodate the investigation of additional parameters related to the occurrence of titanium dioxide-related induction of aberrant crypt foci (ACF) in the

colon (preneoplastic lesions that had been reported by Bettini et al. (2017)). The study was carried out according to OECD TG 443 and good laboratory practice (GLP) compliance.

123. The test material: Titanium dioxide E171-E, Particle size (ECD); (number measurement, primary particle size) x10 = 0.070 µm x50 = 0.110 µm x90 = 0.180 µm via the diet. The doses used were: Group 1: 0 mg/kg b.w./day; Group 2: 100 mg/kg b.w./day; Group 3: 300 mg/kg b.w./day; 4: 1000 mg/kg b.w./day. Twenty male and 20 female rats from each dose group were evaluated. The concentration of the test item in the diet was adjusted based on the mean group food consumption per sex. The concentration was adjusted weekly using the food consumption values from the previous week.

124. The test item was administered from 10 weeks prior to mating, during mating and until weaning of the F1 and F2 generations. The F1 generation was dosed in the same way as the F0 generation after weaning. Until weaning, the exposure of the F1 pups to the test item was indirectly through the breast milk, however the pups additionally received the test item directly when commencing feeding by themselves during the last week of the lactation period. The duration of dosing depended on the requested endpoints for the different cohorts of the F1 generation. Cohort 1B animals were maintained on treatment beyond PND 90 and bred to obtain an F2 generation. Detailed examination of key developmental endpoints, such as offspring viability, neonatal health, developmental status at birth, and physical and functional development until adulthood, was performed to identify specific target organs in the offspring. Possible endocrine disruptor effects of the test item were also examined.

125. Table 3 describes the parameters considered in the EOGRT study with the corresponding generation, cohort and number of animals assessed for each.

Table 3. Parameters considered in the EOGRT study.

Generation	**F0**	**F0 satellite**	**F1 pups**	**F1 1A**	**F1 1B**	**F1 2A**	**F1 2B**	**F1 3**	**F2**
Number of animals /sex per dose group	20	30	N/A	20	20	10	10	10	N/A

Mortality	X	N/A	X	X	X	X	N/A	X	N/A
Clinical signs	X	N/A	N/A	X	X	X	N/A	X	N/A
Body weight	X	N/A	X	X	X	X	N/A	X	X
Food consumption	X	N/A	N/A	X	X	X	N/A	X	N/A
Water consumption	X	N/A	N/A	N/A	X	N/A	N/A	N/A	N/A
Haematology	X	N/A	N/A	X	N/A	N/A	N/A	N/A	N/A
Clinical biochemistry	X	N/A	N/A	X	N/A	N/A	N/A	N/A	N/A
Lymphocyte typing (spleen)	N/A	N/A	N/A	X	N/A	N/A	N/A	N/A	N/A
Urinalysis	X	N/A	N/A	X	N/A	N/A	N/A	N/A	N/A
Sexual hormone levels	X	N/A	N/A	X	X	X	X	N/A	N/A
Thyroid hormone levels	X	N/A	X	X	N/A	N/A	N/A	N/A	N/A
Sexual maturation	N/A	N/A	N/A	X	X	X	N/A	X	N/A
Oestrous cycle data	N/A	N/A	N/A	X	N/A	N/A	N/A	N/A	N/A
Sperm parameters	X	N/A	N/A	X	N/A	N/A	N/A	N/A	N/A
Necropsy	X	N/A	X	X	X	X	N/A	X	X
Histopathology	X	N/A	N/A	X	N/A	X	X	N/A	N/A

Reproductive parameters	X	N/A	X	N/A	X	N/A	N/A	N/A	N/A
Pre-postnatal development	N/A	N/A	X	N/A	X	N/A	N/A	N/A	X
Functional neurotoxicity observations	N/A	N/A	N/A	N/A	N/A	X	N/A	N/A	N/A
Neurohistopathology	N/A	N/A	N/A	N/A	N/A	X	X	N/A	N/A
Lymphocyte typing (spleen) after KLH immunistaion	N/A	N/A	N/A	X	N/A	N/A	N/A	X	N/A
Anti-KLG IgM levels after KLH immunisation	N/A	N/A	N/A	N/A	N/A	N/A	N/A	X	N/A
Aberrant crypt foci (ACF) scoring	N/A	X	N/A	N/A	N/A	N/A	N/A	N/A	N/A

EOGRT: extended one-generation reproductive toxicity; KLH: keyhole limpet haemocyanin; IgM: immunoglobulin M.

This table is taken from EFSA (2021).

Results

Evaluation of Sexual Function and Fertility

Male fertility

126. No statistically significant or dose-related effects on sperm motility, total spermatids/gram testis, percentage of abnormal spermatozoa and male mating index were observed in the F0 generation. The slight decrease in the number of successful matings at doses of 300 and 1,000 mg/kg bw per day appears unrelated to the male partners, as all males that failed to impregnate their females showed normal sperm motility and sperm counts. Only one of the high-dose males was found to have a lower testicular spermatid content (50% of the

group mean), a finding that was also associated with a slightly lower testis weight (85% of the group mean). The number of abnormal sperm was low in all dose groups and remained below 2% in the few males in which abnormal sperm were found.

Female fertility

127. No effects on mean oestrus cycle duration were noted in F0 and F1 (cohort 1B) parental generations and all F0 females in the control, 100, 300 and 1,000 mg/kg bw per day groups mated. In the F1 generation 2 and 3 animals from the mid- and the high-dose groups, respectively, were erroneously removed from the study, before mating had been unequivocally confirmed. All other females mated, except one F1 female in the 100 mg/kg bw per day group. With few exceptions, mating occurred at the first oestrus after the females were housed with males. No effects of treatment were observed. The pregnancy rate was slightly lower in the F0 generation at 300 and 1,000 mg/kg bw per day (100, 96, 92 and 92%). This finding was not confirmed in the F1 generation (100, 95, 94 and 100%).

128. No effects were noted on pregnancy duration, number of implantation sites and post-implantation loss. Although they occurred in the mid-and high-dose groups, three single total litter losses, either from total resorption of all embryos or from death of the litter during or shortly before birth, were not considered to be due to treatment. This is because the two F0 dams had unusually small litters of two pups each, which were stillborn, and the F1 dam showed total resorptions of eight implants at necropsy after failing to litter. Live litter sizes and litter weights were comparable to control values in all dose groups in the F0 and the F1 generation.

Developmental Toxicity

129. Pre-and postnatal lethality and structural abnormalities: No treatment-related pre- or postnatal loss was observed in the F0 and F1 generations. The average litter size at birth in all dose groups was comparable or higher than in the control group and the sex ratio was unaffected. No external or internal abnormalities were detected in F1 and F2 pups at termination.

130. Growth and sexual development: No treatment-related effects were observed in birth weights and growth of the pups. There were no indications for any androgenic and/or oestrogenic effects from the male and female anogenital distance (AGD) and the retention of nipples in males.

131. The mean age at vaginal opening was comparable between control and treated groups. The statistically significant lower body weight on the day of vaginal opening in cohort 1A at 300 mg/kg bw per day was not considered to be biologically relevant due to the slightly higher litter sizes in all treated groups. There was no effect of treatment on the age at balanopreputial gland cleavage. It was noted that there was a deviation from OECD TG 443 in that balanopreputial gland cleavage, rather than balanopreputial separation, was examined.

Reproductive and developmental toxicity studies using the nanoparticle form of TiO2

Lee et al., (2019)

132. Mated female Sprague–Dawley rats (12 females per group) were treated with TiO2 NPs (nominally 21 nm) daily by gavage at dose levels of 0, 100, 300 and 1,000 mg/kg bw per day from GDs 6 to 19. The physicochemical characterization of TiO2 nanoparticles, included analyses of primary shape, primary size, purity and hydrodynamic size. The majority of the TiO2 nanoparticles had spherical and anatase crystal shapes with a purity of 100%. The mean primary size of the TiO2 NPs was 17.8 ± 5.46 nm. The hydrodynamic size of the TiO2 NPs was 341.5 nm, which indicated that TiO2 NPs were prone to aggregation.

133. In the Lee *et al*., (2019) study, no statistically significant differences were noted in general clinical signs, bodyweight, organ weights (absolute and relative to body weight), or macroscopic findings. There were no significant differences in parameters measured after Caesarean section, fetal external or visceral examinations. The COT considered that no adverse maternal and developmental effects were observed with TiO2 NPs (21 nm) up to 1,000 mg/kg bw per day, the highest dose tested.

Warheit, Boatman and Brown, (2015)

134. Warheit, Boatman and Brown (2015) evaluated the potential maternal or developmental toxicity of six different forms of predominantly NP form of TiO2 in pregnant rats (22 or 23 rats per group). The dose levels for each form of TiO2 were 0, 100, 300, and 1000 mg/kg bw per day. The TiO2 formulations were in sterile water and delivered via oral gavage. This study noted that in a set of companion analyses, one was of the pigment-grade (pg) TiO2 and the other was

of the ultrafine (uf) TiO2 test material (BET (Brunauer-Emmett-Teller) and the surface areas of pg and uf samples ranged from 7-17 m^2/g and 50-82 m^2/g respectively). Each material was evaluated for potential systemic exposure/uptake from the GI tract to the blood for circulation. Studies were performed according to OECD TG 414.

135. In three studies, Warheit, Boatman and Brown (2015) dosed time-mated pregnant Sprague–Dawley, Crl:CD(SD), rats (n=22 per group), daily with TiO2 (uf-1, uf-3, & pg-1) by gavage on Gestational Days 6 to 20. Three additional studies included pregnant Wistar rats (n = 22 – 23 per group) exposed daily to TiO2 (uf-2, and pg-2, pg-3) by gavage from Gestational Days 5 to 19. The dose levels used in the studies were 0, 100, 300 or 1,000 mg/kg bw per day. The dose volume was 5 mL/kg bw per day with the TiO2 specifications detailed below:

i. uf-1: 89% anatase/11% rutile (100% nanomaterial), d50 = 43 nm (by XSDC), d50 = 23 nm (by TEM), irregular shape (TEM).

ii. uf-2: 100% anatase (100% nanomaterial), d50 = 42 nm (by XSDC), d50 = 19 nm (by TEM), irregular shape (TEM).

iii. uf-3: 100% rutile (100% nanomaterial), d50 = 47 nm (by XSDC),d50 = 22 nm (by TEM), rod-like shape (TEM).

iv. pg-1: 100% anatase (27% nanomaterial), d50 = 153 nm (by XSDC), d50 = 120 nm by (TEM), irregular shape (TEM).

v. pg-2: 100% rutile (11% nanomaterial), d50 = 195 nm (by XSDC), d50 = 165 nm (by TEM), irregular shape (TEM).

vi. Pg-3: 100% rutile (26% nanomaterial), d50 = 213 nm (by XSDC), d50 = 132 nm (by TEM), irregular shape (TEM).

136. No dose-dependent increase in TiO2 was observed in the rat blood at 48 or 72 hours, or in the liver at 72 hours following a single exposure (2000 mg/kg bw TiO2). These results suggested that there was little to no absorption of particles from the GI tract into the blood, which conflicted results from a previous study by Tassinari *et al.*, 2014.

137. Gross necropsy included gross examination of the dam, counting of the number of corpora lutea, implantation sites, resorptions, live and dead fetuses, fetal sex and weight. Fetal pathological external, visceral and skeletal examinations were performed in order to identify any abnormalities. Among all

six forms of TiO2, effects were seen only with uf-1. At 1,000 mg uf-1/kg bw per day, mean fetal sex ratio and the means for male and female fetuses per litter were statistically significantly different from the control group means. The mean number of male fetuses was 7.2 compared with 5.5 male fetuses for the concurrent control group; the test facility historical control group data ranged at that time from 5.2 to 7.4. The mean number of female fetuses was 4.8 compared with 6.7 for the concurrent control group; the test facility historical control group data ranged at that time from 5.8 to 8.3. Mean fetal sex ratio of the 1,000 mg uf-1/kg bw per day group was 60% (males/females) compared with a sex ratio of 46% in the concurrent control group; the test facility historical control group data ranged at that time from 43% to 53%. The authors did not consider the change in sex ratio to be test substance related, because fetal sex is determined shortly after conception and well before the onset of dosing with TiO2 on GD 6.

138. Apart from some incidental changes in body weight and feed intake, no other changes were observed in the dams or the fetuses in these studies. The authors concluded that there were no significant toxicological or developmental effects in females or fetuses at any of the dose levels or compounds tested and considered the NOAEL for each compound to be 1,000 mg/kg bw per day, the highest dose tested.

EFSA review and conclusions on reproductive and developmental toxicity of TiO2

139. EFSA based its review of the development and reproductive toxicity of E171 only on the results of the EOGRT study as no other reliable studies were found in the literature addressing these effects with E171.

140. Male Fertility: The Panel noted that the epidydimal sperm parameters were not evaluated but that this deviation has no effect on the final conclusion of the study. There were no effects on any of the sperm endpoints in the cohort 1A.

141. Female Fertility: The EFSA Panel concluded that there were no indications of effects on general toxicity, thyroid or sex hormone levels, reproductive function and fertility in either male or female rats, no effects on pre- and postnatal development or on neurofunctional endpoints in F1 offspring.

142. Developmental Toxicity: EFSA concluded that “No effects of E 171 on pre- and postnatal development were observed. Data on the attainment of puberty in males (i.e. an appropriate assessment of the timing of the balanopreputial

separation) were missing; however, given the lack of any other treatment-related effects on other parameters, the (FAF) Panel does not consider this to be critical in this case”.

143. EFSA concluded that there were no indications of general toxicity, no effect on thyroid or sex hormone levels, no effect on reproductive function and fertility in either male or female rats, and no effects on pre- and postnatal development in F1 offspring.

144. EFSA concluded that the NOAEL for reproductive and developmental toxicity was 1,000 mg/kg bw per day, the highest dose tested, of a commercially-available E171 in the EOGRT study.

Health Canada review and conclusions on reproductive and developmental toxicity of TiO2

145. Health Canada concluded that in the EOGRT study “There was no evidence of adverse effects on reproduction or development up to the highest dose tested, and a NOAEL of 1,000 mg/kg bw per day can be established for reproductive and developmental effects on the basis of this study. The recent EOGRT study is considered the most reliable study found in the literature that addresses reproductive and developmental effects of E171 exposure”.

FSANZ review and conclusions on reproductive and developmental toxicity of TiO2

146. FSANZ noted the 2016 EFSA request for an additional developmental and reproductive toxicity study in the form of the subsequent EORGT study. FSANZ stated that the EORGT study (Leuschner, 2020) using food-grade TiO2 showed no evidence of developmental or reproductive toxicity with an upper test dosage and NOAEL of 1,000 mg/kg bw/day.

147. FSANZ noted an additional study (food-grade TiO2 in the diet for 7 weeks and by oral gavage for 10 weeks, 4 per group) which described germ cell sloughing in mouse testes in animals receiving the highest dose of 1,379 mg/kg bw per day in the diet and 5 mg/kg bw er day by gavage (Rodríguez-Escamilla et al., 2019). However, these changes were not observed in the follow-up study, with 9 mice per group and administration for 16 weeks. FSANZ also noted the lack of histopathological changes observed in the testes in three additional studies with

up to 1,000 mg/kg bw/day (Leuschner, 2020; Han et al., 2021; and Blevins et al., 2019).

JECFA 2024 review and conclusions on reproductive and developmental toxicity of TiO2

148. JECFA reviewed the EOGRT study (Leuschner, 2020) and noted that the EOGRT study had not been able to establish whether there were effects on developmental immunotoxicity resulting from exposure to E171 due to a weak immune response against the administered keyhole limpet haemocyanin (KLH) antigen, and that the study authors stated that this part of the study will be repeated. The Committee concluded that, apart from the lack of conclusive results on developmental immunotoxicity, the NOAEL for the study was 1,000 mg/kg bw per day, the highest dose tested.

149. JECFA reviewed the developmental toxicity study in rats by Warheit, Boatman and Brown (2015) and noted that the NOAELs for both maternal and developmental toxicity were 1,000 mg/kg bw per day, the highest dose tested in all the studies.

COT review and conclusions on reproductive and developmental toxicity of TiO2

150. The COT considered that the EOGRT study was carried out according to the relevant scientific guidelines with no obvious deficiencies. No other reliable studies with food-grade TiO2 were identified in the literature. The Committee concluded that no toxicological effects of TiO2 on the reproductive and developmental system were observed in the EOGRT study. A range of endpoints, including body weight, haematology, urinalysis, sex hormone levels, thyroid hormone levels, circadian rhythm and sperm parameters, were investigated, in both the F0 and F1 generations.

151. It was noted that there was a suggestion of focal effects on the testes and epididymides in one male in the highest dose group, which were considered to be spontaneous and not test item-related, and abnormalities in sperm. Data from the EOGRT study showed test item-related differences for the examined absolute and relative testis weights between the control and the treatment groups (low = 100 mg, intermediate = 300 mg or high = 1,000 mg TiO 2 E171/kg bw per day). These slight differences included a small but statistically

significantly reduced absolute right testes weight at the intermediate and the high dose level. These were only noted for the right testes, not the left testes and it was concluded that changes were spontaneous and not of toxicological relevance. The COT agreed with the authors' conclusions. Overall, no adverse effects on reproductive and developmental toxicity were observed, up to the highest dose tested.

152. The best overall quality additional studies TiO2, which used forms other than food grade, were determined by the COT to be those by Warheit, Boatman and Brown (2015) and & Lee *et al.*, (2019). The COT considered the 1,000 mg/kg bw per day NOAEL identified in the EOGRT study may reflect low absorption. Other studies indicate NOAELs consistent with that from the EOGRT study. The COT noted variations in methods and endpoints used to characterise TiO2 among several of the studies and as a consequence the physicochemical differences between the test items were unclear.

153. The COT concluded that there was no good evidence that TiO2 was reprotoxic.