Fifth draft statement on the safety of Titanium Dioxide (E171) as a Food Additive- Results

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Results

Evaluation of Sexual Function and Fertility

Male fertility

116. 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

117. 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 and100%).

118. 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

119. 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.

120. 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 on the male and female anogenital distance (AGD) and the retention of nipples in males.

121. 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. A divergence from the required method was examination of balanopreputial gland cleavage instead of examining balanopreputial separation which does not comply with the OECD TG 443 and therefore cannot be considered a measure of puberty in males.

122. Neurofunctional screening: Male and female F1 cohort 2A offspring were tested for auditory startle response between PND 23 and 25, and for a functional observation battery including grip strength evaluation and for quantitative locomotor activity between PND 58 and 64. No differences in the response to an auditory startle stimulus were observed between the control and all the tested doses. Compared to controls, an increase in hindlimb splay was observed in

females, reaching statistical significance at 100 and 1,000 mg/kg bw per day. A statistically significant increase in mean forelimb grip strength was noted at 300 mg/kg bw per day in both males and females.

Developmental Immunotoxicity

123. Effects on developmental immunotoxicity were determined in the F1 cohort 3 animals through an examination of their ability to raise an antibody response to a foreign antigen. Animals were sensitised and the primary IgM antibody response to the sensitising antigen, in this case to keyhole limpet haemocyanin (KLH) antigen, was measured. The ability of the test compound to modulate serum anti-KLH antibody titre was taken as indicative of a developmental immunotoxic effect. A KLH-immunised control group also exposed to a known immunosuppressant (i.e., cyclophosphamide (CY)), resulting in at least 50% inhibition in serum IgM anti-KLH titre, was considered crucial for the verification of assay performance.

124. These data can be considered in combination with additional data related to potential immunotoxic effects. In the F1 cohort 1A animals, the following may also contribute to the general assessment for immunotoxicity: weight and histopathology of the spleen, thymus and lymph nodes, as well as bone marrow histopathology, total and differential peripheral WBC count and splenic lymphocyte subpopulation distribution and the T-cell-dependent anti-KLH response (KLH assay). Determination of serum anti KLH-IgM antibodies was performed in F1 cohort 3 (10/sex per group, PND 53–61) using an enzyme-linked immunosorbent assay (ELISA).

125. The animals were sacrificed 5 days after intravenous bolus injection (tail vein) of KLH, blood was withdrawn and the level of anti-KLH IgM was measured in serum. In addition, satellite animals of F1 (10/sex, PND 55) were immunised with KLH and treated with CY (single administration of 40 mg/kg bw by gavage on the same day of KLH treatment) to provide a positive control (for an inhibition of immune response).

126. A slight, but statistically significant decrease in the antigen specific IgM level was measured at the highest dose tested (1,000 mg/kg bw per day) in males only (– 9%) and without an apparent dose response. In addition, the Panel noted that treatment with CY was not performed at the same time as the rest of F1 cohort 3, without a separate control for the CY response, conducted at the same time.

127. The sensitivity of the test was not demonstrated due to invalid CY positive control results. It was noted that the assay conditions may have not been optimal resulting in an apparent low antibody response to KLH when compared to literature (Gore et al., 2004).

128. It was considered that all tested animals in the study had a weak immunogenic response to KLH that was insufficient to identify a T-cell-dependent immunotoxic effect of E171 therefore no conclusion can be drawn on the effect of E171 on the developing immune system.

129. Assessment of pathology, haematology and splenic lymphocyte subpopulations at necropsy, pathology of lymphoid organs, haematology and lymphocyte subpopulations in the spleen were investigated. The following lymphocyte subpopulations were determined via flow cytometry analysis (FACS): T cells, T helper cells, T suppressor/cytotoxic cells, NK cells and B cells.

130. For the splenic lymphocyte subpopulation analysis, no statistically significant differences were observed in the percentage of T cells, T helper cells, T suppressor/cytotoxic cells, NK cells and B cells of any of the treated groups compared to control in both sexes. The study authors concluded that no test substance-related effect was observed on the proportion of the examined lymphocyte subtypes.

131. Compared to the animals of F1 cohort 1A, F1 cohort, 3 animals showed a shift in the lymphocyte subpopulation that indicated activation of the immune system by injection of KLH and concluded that increased B-cell proliferation may have led to the production of antigen-specific antibodies. In the F1 cohort 3 animals, no differences in the relative size of the lymphocyte subpopulations were observed between the control group and the E171-treated groups, after immunisation of the animals with KLH.

132. The proposed reason was that the B-cell shift in F1 cohort 3 was caused by KLH immunisation, supported by the fact that there was no such shift found for the positive control animals that were sensitised to KLH and treated with CY. It was also considered that KLH induced an immune reaction, and that this response was influenced by CY as expected; KLH would increase the percentage of splenic B cells and decrease the percentage of T cells.

133. Some findings regarding immunotoxicity and inflammation with E171 as well as neurotoxicity with TiO2 Nanoparticles may be indicative of adverse effects including indications of an induction of ACF with E171.

Developmental Neurotoxicity

134. Male and female offspring were tested for auditory startle response between PND 23and 25, including grip strength evaluation and for quantitative locomotor activity between PND 58 and 64. No differences in the response to an auditory startle stimulus were observed and an increase in hindlimb splay was observed in females, reaching statistical significance at 100 and 1,000 mg/kg bw per day. A statistically significant increase in mean forelimb grip strength was noted at 300 mg/kg bw per day in both males and females.

135. Grip strength and hindlimb splay belong to the same domain of neurological function, however, the increase in hindlimb splay and increase in mean forelimb grip strength are opposed in this case - increases in hindlimb splay indicate muscular weakness but an increase in mean forelimb grip strength may indicate myotonia. No dose response was observed for any of these endpoints or for the two functional measurements, indicating that the likelihood of an association with test substance is low. No other changes in observed including in histopathological findings in brain or in peripheral nerve tissue.

Aberrant Crypt Foci

136. A satellite group of the EOGRT study used doses up to 1,000 mg/kg bw per day and up to this dose did not induce ACF in the colon.

Aberrant Crypt Foci Examination in Satellite F0 Animals (EOGRT Study).

137. Evaluation of ACF in the colon of a satellite group of F0 animals (10/sex per group) treated with 0, 100, 300 and 1,000 mg E171/kg bw per day and terminated after weaning was undertaken. The colon was excised, opened longitudinally and the contents removed by rinsing with a 0.9% NaCl solution. Thereafter, the tissue was divided in parts of a suitable size for fixation by immersion in 5% buffered formalin. A blind examination of these samples stained with 0.5% (w/v) methylene blue in water was performed under a stereomicroscope at 50x magnification for presence of ACF. The Panel noted that the design of the study did not include a positive control group (e.g., treatment with a known gastrointestinal tract tumour initiator such as dimethyl hydrazine (DMH) for the development of ACF.

138. The definition of ACF used was ‘foci containing more than 2 aberrant crypts (ABCs), taken from Shwter et al. (2016). No ACF were found in the colons of the control and the treated groups. A mildly increased morphological variability

(increased size and intensity of the staining of a small portion) of the crypts in the two caudal parts of colon was observed in seven animals (See tables 4 & 5 below). These changes were assessed as inconsistent with the appearance and definition of ACF discussed above. Incidence of these single crypts observed in the mid and high doses was not significantly different from the control. The EFSA Panel agreed with this conclusion.

Table 4: Aberrant Crypt Foci Presence in Satellite F0 Animals:

Aberrant Crypt Foci Present

Dosage Group	Control	Low-Dose	Mid-Dose	High-Dose
Females	1/10	0/10	1/10	2/10
Males	1/10	0/10	1/10	1/10

139. An additional submission of data included photomicrographs of mildly increased variability in crypt morphology from all seven animals. A re-examination was extended to an additional randomly selected nine control animals (4 males and 5 females) and eight high-dose group animals (3 males and 5 females). A mild increased variability in crypt morphology was observed in eight of the nine controls and six of the eight high-dose animals (see Table x).

Table 5: Aberrant Crypt Foci Presence in the Re-Examination of Satellite F0 Animals.

Aberrant Crypt Foci Present

Dosage Group	Control	High-Dose
Females	4/5	3/3

Males	4/4	3/5

EFSA review and conclusions

140. The EOGRT study with E171 did not indicate adverse effects up to a dose of 1,000 mg/kg bw per day. Also, no effects were seen in studies retrieved from the literature with TiO2 NP < 30 nm up to the highest dose tested of 100 mg/kg bw per day.

141. EFSA Opinion of the EOGRT Study: The Panel considered that there was no systematic bias in group testing order and that this was therefore not a plausible explanation for the observed group differences. Grip strength and hindlimb splay belong to the same domain of neurological function, i.e. motor function and/or sensory–motor coordination. However, the effects observed (i.e. increase in hindlimbs play and increase in mean forelimb grip strength) seem to point in opposite directions when it comes to muscle strength. In particular, an increase in hindlimb splay can be interpreted as muscular weakness whereas an increase in mean forelimb grip strength could be indicative of myotonia. The Panel noted that the effects observed were not correlated to any other changes (e.g. alterations in muscle tone, righting reflex, gait, wire manoeuvre, posture). No dose response was observed for any of these endpoints or for the two functional measurements, indicating that the likelihood of an association with test substance is low. No other changes in the functional observation battery measurements or locomotor activity were noted. There were no notable histopathological findings in brain or in peripheral sciatic nerve. Based on all the above considerations, the Panel considered that the effects on grip strength and hindlimb splay were not treatment-related. However, the Panel noted that quantitative information on peripheral nerves was not available. Overall, the Panel considered that E171 at these doses had no adverse effects on neurofunctional endpoints in F1 cohort 2A offspring. EFSA conclusions on developmental toxicity results of the EOGRT study: No effects of E171 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. The Panel did not consider this to be critical in this case.

142. The conclusion was that the immune response was affected by CY but was not adversely affected by the TiO2 test substance. The Panel did not agree with the conclusion of the study authors that a shift to B cells by KLH was

substantiated. The Panel considered that it is incorrect to compare the groups of F1 cohort 1A and of F1 cohort 3 because the groups of animals of F1 cohort 3 had a different age than that of the animals in F1 cohort 1A at the time of sacrifice (PND 87–96 vs. PND 53–61, respectively). In addition, the FACS analyses on the splenic cell suspensions were not all performed in the same round of analysis but were performed separately, while it is known that this may have influenced staining and subsequent quantification. The authors suggested that even if the positive CY control did not perform as expected, the data still indicate there is no effect of E171 on sensitisation to KLH.

143. It is worth noting that the EFSA Panel did not agree with this conclusion and overall considered that the data did not allow to conclude on developmental immunotoxicity with respect to E171.

COT review and conclusions

144. The Committee agreed that the EOGRT (Extended One Generation Reproductive Toxicology) study was very detailed and well conducted. There was no evidence of reproductive or developmental toxicity. An extensive range of endpoints had been considered in the study and it was noted that there was some small evidence of focal effects on the testes and sperm abnormalities in the sperm production in one treated rat, however, these changes were not statistically different to the control group.

145. Overall, it was agreed that there was no evidence that titanium dioxide caused reproductive or developmental toxicity at the doses tested.

Aberrant Crypt Foci (ACF) as a potential biomarker for carcinogenicity

146. ACF are a potential biomarker for colorectal cancer. Genetic changes in the malignant transformation process of colorectal mucosa lead to either inactivation or activation of specific target genes. A number of biomarkers associated with genetic changes have been identified for early detection of colorectal cancer including identification of ACF as an early pre-invasive lesion and its relationship to the development of cancer (Cheng and Lai, 2003). The number of ACF were found to be significantly higher in patients with colon cancer compared to those without, but have been found in healthy patients (Health Canada, 2022). However, most ACF do not appear to develop into neoplasms and there is

evidence to suggest that dysplastic changes in ACF are a better predictor of cancer (Clapper et al 2020). Most preclinical studies do not distinguish between hyperplastic and dysplastic changes in ACF.