

EOGRT Study - Review of EFSA Opinion

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This is a paper for discussion.

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

161. The Extended One Generation Reproductive Toxicity (EOGRT) study was commissioned by interested business operators to address the data gaps identified in 2016. The protocol was later amended to accommodate the investigation of additional parameters related to the occurrence and titanium dioxide-related induction of aberrant crypt foci (ACF) in the colon (preneoplastic lesions that had been reported by Bettini et al. (2017). Methodology: Test

Material, Doses, Administration of Treatment.

162. 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.

163. 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 20 male and 20 female rats 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.

164. The test item was administered in graduated doses to several groups of males and females prior to, during and after mating until weaning of the F1 and F2 Generation. 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.

Evaluation of Sexual Function and Fertility

Male fertility:

165. An overview of the results for male fertility parameters is reported in Annex A. 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.

166. The Panel noted that the epididymal 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.

Female fertility:

167. An overview of results for female fertility parameters is reported in Annex A. 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%). As this finding was not confirmed in the F1 generation (100, 95, 94 and 100%) the Panel considered it as incidental and not treatment related.

168. 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.

169. 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.

170. The EOGRT study with E 171 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 TiO₂NP < 30 nm up to the highest dose tested of 100 mg/kg bw per day.

Developmental Toxicity

171. 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.

172. Growth and sexual development: An overview of the results related to growth and sexual development for the F1 and F2 generations is included in Annex A. 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.

173. 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.

174. 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.

175. To check whether the significant differences in grip strength and hindlimb splay could be due to systematic bias in group testing order, the testing order was checked. 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.

176. 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.

177. Furthermore, there were no notable histopathological findings in brain or in peripheral nerve (sciatic). 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 E 171 had no adverse effects on neurofunctional endpoints in F1 cohort 2A offspring at the doses used.

178. EFSA conclusions on developmental toxicity results of the EOGRT study: 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. The Panel did not consider this to be critical in this case.

Developmental Immunotoxicity

179. 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 are sensitised and the primary IgM antibody response to the sensitising antigen, in this case to keyhole limpethaemocyanin (KLH) antigen, is measured. The ability of the test compound to modulate serum anti-KLH antibody titre is 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, is considered crucial for the verification of assay performance.

180. These data can be considered in combination with additional data related to potential immunotoxic effects. In the F1 cohort 1A animals, the

following may 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. 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).

181. 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).

182. 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.

183. 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).

184. 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 E 171 therefore no conclusion can be drawn on the effect of E 171 on the developing immune system. The Panel agreed with the conclusion of the study authors.

185. 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. The Panel noted that haematology, spleen weight and histopathology of lymphoid organs in animals from F1 cohort 1A did not indicate any dose-related effects.

186. 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. The Panel agreed with the study author conclusion that the splenic lymphocyte subpopulations in this cohort were not affected. However, the Panel considered that an isolated observation in F1 cohort 1A is not sufficient to conclude on immunotoxicity.

187. 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 E 171-treated groups, after immunisation of the animals with KLH.

188. 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.

189. 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 E 171 on sensitisation to KLH.

190. 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 E 171.

Immunotoxicity Summary

191. A marginal but statistically significant decrease in antigen-induced IgM levels (9%) in males of the F1 Cohort 3 only was noted, with no apparent dose-response.

192. The Panel noted that there were methodological shortcomings in the design of this part of the EOGRT study. Therefore, the Panel could not conclude on immunotoxicity.

193. Some findings regarding immunotoxicity and inflammation with E 171 as well as neurotoxicity with TiO₂ Nanoparticles may be indicative of adverse effects including indications of an induction of ACF with E 171.

Neurotoxicity

194. EOGRT Study - Male and female offspring were tested for auditory startle response between PND 23 and 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.

195. 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.

196. The Panel considered that the effects on grip strength and hindlimb splay were not treatment-related but that quantitative information on peripheral nerves was not available. Overall, the Panel considered that E 171 had no adverse effects on neurofunctional endpoints at the doses used.

197. No neurotoxicity studies performed with E 171 were identified from the published literature that were considered sufficiently reliable. Some papers were identified noting effects of TiO₂ NP <30 nm but these are not discussed further in this paper.

Aberrant Crypt Foci

198. 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.