Rats – Oral administration

The study by Tassinari et al. (2014) has already been described in the EFSA opinion on the re-evaluation of E 171 published in 2016 (EFSA ANS Panel, 2016). In the current opinion, the analysis of the data with respect to an estimate of absorption is presented. Three groups of Sprague–Dawley rats (n = 14 per dose group per sex) were dosed by gavage with TiO_2 NPs 1 mg/kg bw per day or 2 mg/kg bw per day or with vehicle for 5 days. On day 6 (24 h after the last treatment), blood samples were taken under anaesthesia and organs were excised after sacrifice. Total Ti concentration was determined in tissues (uterus, ovary, testes, thyroid and adrenals) by inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS), TiO_2 NPs by SEM-EDX in spleen slices and TiO_2 NPs by single particle inductively coupled plasma mass spectrometry (spICP-MS) in spleen homogenates. The limit of detection of ICP-DRC-MS for Ti determination in tissues was 0.009 μg/g. The Ti concentrations in the tissues investigated were not different from the controls with the exception of spleen and ovary in the 2 mg/kg bw dose group. Ti concentrations equal to 0.046 ± 0.008 μg/g (2 mg/kg bw per day) vs. 0.036 ± 0.009 μg/g (control) and 0.28 ± 0.07 μg/g (2 mg/kg bw per day) vs. 0.12 ± 0.04 (control) were measured in spleen (n = 8 animals per group) and in ovary (number of animals not mentioned), respectively. Agglomerates of TiO_2 particles (diameter 200–400 nm) were identified by SEM-EDX in spleen of the 2 mg/kg bw per day dose group. The mass concentration of such TiO_2 particles was quantitatively determined by single-particle ICP-MS and was found to be in agreement with the total Ti concentration determined by ICP-DRC-MS.

The Panel concluded from this study that the absorption of the investigated material is low. Based on Ti determinations, the Panel calculated that 0.001% of the total TiO_2 NP dose was present in the spleen and ovaries.

In the study of Hendrickson et al. (2016), TiO_2 NPs were administered at a dose of 250 mg/kg bw per day to 6 rats (with 6 rats as controls) by gavage administration. After 28 days treatment, the animals were euthanised and blood was obtained from abdominal vein. Lungs, liver, spleen, brain, testicles, small intestine, heart, stomach and kidneys were harvested. Ti was measured by graphite furnace atomic absorption spectroscopy. TiO_2 particles in tissues were detected by TEM and diffraction analysis. After 28 days, the Ti concentration was below the LOD in all tissues from the control group. In treated animals, the Ti concentration was the highest in liver, followed by spleen and small intestine and the lowest in kidney. It was below the level of detection in lungs, brain, testicles, heart and blood.

From these data, the Panel estimated the Ti amount of the body[14] after 28 days of exposure. Body Ti amount was determined to be 3.4 μg. The oral systemic availability can be estimated from the body Ti amount of 3.4 μg divided by cumulative dose of 60 mg (daily Ti dose) × 28 days, which equates to 2.1×10^{-4}% of the cumulative Ti dose. For those samples where Ti was below the LOD, the Ti amount was taken to be 0.5 LOD.

In the study of Ammendolia et al. (2017), male and female Sprague–Dawley rats (n = 10/sex per group) were treated with TiO_2 NPs with doses of 0, 1 or 2 mg/kg bw per day for 5 consecutive days by gavage. TiO_2 NPs were dispersed in water by sonication, performed on a daily basis. Twenty-four hours after the last treatment, the rats were euthanised and small intestine was sampled. After removing any GI digestion residues, the tissue samples were characterised for their Ti levels by ICP-DRC-MS (LOD of 0.009 μg/g).

Ti levels in small intestine tissue (mean ± SD, n = 4) were 0.08 ± 0.02 μg/g in the control, 0.09 ± 0.02 μg/g in the dose group of 1 mg/kg and 0.13 ± 0.03 μg/g in the dose group of 2 mg/kg.

The Panel noted that results for Ti concentration were provided by four animals per dose group.

Based on Ti determinations and subtracting control levels of Ti, the Panel calculated that 0.01% of the total TiO_2 NPs dose was present in the small intestine. As only measurements were made in the small intestine tissue, no estimate for systemic exposure could be made.

In the study of Kreyling et al. (2017b), female Wistar-Kyoto rats received an aqueous [^{48}V]TiO_2 NPs suspension by gavage. Preparation and the procedure to calculate the concentrations are described in Kreyling et al. (2017a). After gavage, rats were kept separately to be able to collect individual urine and faeces. At 1 h, 4 h, 24 h and 7 days, four animals per time point were euthanised by exsanguination under 5% isoflurane anaesthesia. Blood, all organs, tissues and excreta were collected and ^{48}V radioactivities were measured.

[14] The content of Ti in the tissues was calculated by multiplying the measured concentrations with the organ weights (Schoeffner, 1999). In the cases where the concentrations were reported as being below the LOD, the LOD was used. To receive the percentage of dose, the sum of the contents was related to the dose applied.

After gavage, most of the radioactivity was excreted in the faeces. Absorption was calculated as the fraction of the dose that could not be accounted for by the radioactive content of the intestinal tract plus faeces. About 0.6% of the applied dose was absorbed during the first h after gavage; the fraction still present in tissues after 7 days amounted to about 0.05% of the applied dose. The authors noted that the distribution patterns between animals were variable and that several data were below the LOD during the first 4 h. Measurable deposition could be observed only after 4 h in spleen, kidneys, heart and uterus. The retention maximum was reached in spleen, kidneys and heart at 24 h post-dosing. In liver, lung and blood, nanoparticle retention declined from 4 h to 7 days. In brain, uterus and kidneys, the highest concentrations were observed at day 7. The peak concentration in liver and spleen was 12.5% (4 h) and 2.6% (24 h) of the absorbed dose, respectively. According to the authors, due the slow excretion kinetics, accumulation of systemically circulating particles in specific cells and organs is likely to occur in subjects chronically exposed to TiO_2 NPs.

By means of the radiotracer method used, up to 50% of the suspended [^{48}V]TiO_2 NPs dose to be administered was demonstrated to be retained in syringes and cannulas, presumably due to adsorption on plastic surfaces. According to the authors, such effects are likely to occur to a variable extent in *in vivo* studies (depending on the materials used and their handling), are difficult to detect and might be one reason for variations in reported results.

Comparing the biodistribution of [^{48}V]TiO_2 nanoparticles retained after oral administration with that determined after intravenous injection (Kreyling et al., 2017a), the authors conclude that the kinetics patterns are very different and intravenous injection does not appear an adequate surrogate for assessing the biodistribution occurring after oral exposure to TiO_2 NPs.

The Panel considered that this study demonstrates that the systemic availability of orally administered TiO_2 NPs is 0.6%, based on the assumption that ^{48}V is a faithful tracer for TiO_2 NPs. The Panel further considered that this study shows significant differences in distribution between the routes of exposure.

In the study of Hendrickson et al. (2020), the isolated intestinal loop technique was used to administer 50 mg/kg bw TiO_2 NPs to Wistar rats. Three hours after administration the isolated loop was cut out, in addition to the liver and spleen. The presence of particles in tissues was studied by TEM and diffraction analysis. Loose agglomerates were seen with a size of 100 nm and larger. By diffractions analyses, it was confirmed that the particles were TiO_2. TiO_2 NPs were detected on the surface and between the microvilli of the mucosal cells of the small intestine and also in the mucosal tissue. Nanoparticles were detected in the Peyer's patches, both as single nanoparticles and agglomerates of sizes ranging between 20 and 60 nm. In the liver, parenchymal tissue aggregates of TiO_2 NPs (150–200 nm) and up to 300 nm were seen. In the spleen red pulp, single nanoparticles (20–30 nm), agglomerates (up to 100 nm) and conglomerates (up to 800 nm) could be observed.

The Panel noted that this study demonstrates the presence of TiO_2 NPs, either as single particles or as agglomerates of variable size, in intestine, liver and spleen. However, no quantitative data were provided.

In the study of Chen et al. (2020a,b), 4-week-old Sprague–Dawley (n = 6/dose group) were administered daily doses of 0, 2, 10 and 50 mg TiO_2 NPs/kg bw per day for 90 days by oral gavage, using a suspension in distilled water (sonicated and mixed before administration). Blood, liver, stomach, small intestine, colon, spleen, heart, lung, kidneys and testicles were harvested at day 91. Ti was determined in the organs and blood by high-resolution ICP-MS. In spleen and heart, the Ti concentrations were below the LOD (32 ng/g tissue). In blood, liver, intestine, lung, kidney and testicle, the measured concentration of Ti (ng/g tissue) was not statistically different in the treated groups compared with controls. In the colon, the concentration was higher in the 50 mg/kg bw per day group than in the controls and the groups treated with 2 and 10 mg/kg bw per day. The authors considered that the high concentration of Ti in the colon of the animals from the highest dose group was due to TiO_2 nanoparticles attached on the surface of the colonic mucosa tissue and not in mucosa cells. The authors concluded that the absorption of TiO_2 in this study was very low.

The Panel considered that TEM without chemical characterisation of the particles (e.g. by EDX) would lead to uncertainty on the identity of the particles.

Human

In the study of Heringa et al. (2018), Ti was measured using ICP-HR-MS in liver and spleen from 15 deceased human subjects (nine women and six men) who had donated their bodies for research and educational purposes. The LOD of the method was 10 ng/g tissue. The size of Ti-containing particles in the organs was quantified using spICP-MS, and the presence of TiO_2 particles in the tissues was verified

by SEM-EDX. In 8 of the 15 liver samples and in 1 of the spleen samples, the total Ti concentration was below the LOD. The average concentration in samples where Ti could be determined was 40 ng/g in the liver and 80 ng/g in the spleen. The average particle size was 86–426 nm in the liver and 88–445 nm in the spleen, with the lower size being the lower size limit of detection. According to the authors, almost all Ti was present as particles, as the concentration of the particle-Ti and the concentration of total-Ti in the organs measured by an independent procedure was overlapping. The amount of Ti in the liver of females (including only the females in which concentrations were above LOD) was 56.0 ± 37 μg and that in males (including only the males in which concentrations were above LOD) was 72 ± 25 μg, calculated by the Panel.[15] The amount of Ti in spleen (including only the females in which concentrations were above LOD) was 12 ± 17 μg in females and that in males (including only the males in which concentrations were above LOD) of 5.5 ± 4 μg. Summing the amounts in liver and spleen for both genders gives amounts of 83 ± 51 μg. The mean exposure to E 171 in the age group most representative of the human subjects is 200–2,800 μg/kg bw per day (non-brand loyal scenario in the elderly; EFSA ANS Panel, 2016), corresponding to ~ 8.4 and 117 mg Ti per day. Assuming that the majority of body Ti is present in liver and spleen and that all body Ti is derived from dietary E 171, the Panel estimated that the oral systemic availability of E 171 in human would be below 1% at the most.

The Panel noted that nanoparticles of TiO_2 can be present in liver and spleen in humans at low concentrations. Complete data (i.e. concentration in liver and spleen) were available only for a limited subset of the investigated subjects (50% of the subjects). Since the subjects were between 79 and 104 years of age, the Panel considered that steady-state levels of TiO_2 had been reached. From the comparison of the Ti body burden with the mean exposure in the non-brand loyal scenario of exposure towards TiO_2 in the elderly, the Panel concluded that the absorption of TiO_2 as food additive in humans under normal life conditions would be low.

In the study of Peters et al. (2020), Ti content was measured using ICP-HR-MS in liver from 15 deceased human subjects, eight women and seven men, aged 64–97 years, who had donated their bodies for research and educational purposes. Further measurements in jejunum and ileum from seven women and five men were done. The LOD of the method was 0.01 μg/g tissue. The presence of nanoparticles in the tissues was verified by SEM-EDX. In four liver samples, in two spleen samples and one kidney sample out of 15 samples, the total Ti concentration was below the LOD. In one jejunum sample out of 12 samples, the total Ti concentration was below the LOD. The average concentration in the liver samples where Ti could be quantified was 0.03 μg/g, that in the spleen samples was 0.06 μg/g, that in kidney 0.08 μg/g, that in jejunum 0.34 μg/g and that in ileum 0.43 μg/g. The particle sizes, measured by spICP-MS, ranged between 50 and 500 nm in the different tissues, with 50 nm being the lower size detection limit. The TiO_2 particle concentrations were considered by the authors to represent about 80% of the total Ti concentrations, showing that most of the Ti in these organs consisted of particulate material and that Ti content can be seen as a good surrogate for the presence of particles. For samples with concentrations above the LOD, the content in spleen amounted to 78 ± 5 μg in females and to 11 ± 13 μg in males. Using the same criterion (samples > LOD), the content in kidney amounted to 33 ± 34 μg in females and to 16 ± 13 μg in males. Summing up the content in liver, spleen and kidney for both genders, an average amount of 105 ± 83 μg is obtained. The high content of 262 ± 185 μg as the sum of the content in jejunum and ileum, obtained using the same calculation approach, is notable.

The Panel noted that Ti can be present in particulate form in liver, spleen, kidney, jejunum and ileum in humans at low concentrations.

Conclusions

Overall, the Panel noted that the toxicokinetics of E 171 was addressed in three studies in mice and in two studies in humans.

In two studies in mice, the data enabled the derivation of estimates of internal exposure at 0.01% (Coméra et al., 2020) and 0.1% (Talamini et al., 2019) of the external dose, respectively. However, these estimates are based on Ti concentrations measured in a limited number of organs. Although it is uncertain to what extent TiO_2 distributes to other organs, the Panel's estimates have always included the Ti amount in the liver, which accounted for about 12.5% of the Ti amount in the body (Kreyling et al., 2017b). The underestimation in body burden and absorption is therefore unlikely to be more than 5-fold. The Panel noted that in mice, TiO_2 can be taken up from the small intestine by the

[15] The content of titanium in the liver and the spleen was calculated by multiplying the measured concentrations with the organ weights for men (liver 1800 g, spleen 150 g) and for women (liver 1400 g, spleen 130 g) (Valentin, 2002.)

paracellular pathway and by endocytosis. Furthermore, in two of the studies (Coméra et al., 2020; Riedle et al., 2020), uptake of TiO_2 particles was demonstrated into M-cells of the Peyer's patches, whereby the quantitative contribution to the systemic exposure seems to be low.

In humans, the Panel considered that after oral administration of 100 mg E 171, Ti concentration in blood increased ca. 5- to 10-fold from 6 to 10 h post-dosing (Pele et al., 2015), demonstrating some oral systemic availability. TiO_2 particles were found in human placenta in low concentrations (Guillard et al., 2020), indicating that TiO_2 is systemically available after ingestion and also can distribute to the placenta. In an *ex vivo* human placenta model, particles were transferred and the size distribution of the particles was similar to the E 171 present in the perfusate. The Panel noted that the extent of transfer across placental membranes was small.

The Panel noted that materials other than E 171, mainly TiO_2 NPs, were investigated in rats and humans.

In rats, two intravenous studies (Disdier et al., 2015; Kreyling et al., 2017a) demonstrated long half-lives and, hence the potential for accumulation. Together with data from an intravenous study (Geraets et al. (2014) already addressed in the EFSA opinion on the re-evaluation of E 171 (EFSA ANS Panel, 2016), half-lives of 83 days (for liver) and of 450 days (for whole body) were estimated and accumulation factors between 135 and 450. Based on these data, the steady state would be reached between 1.5 and 5 years.

Out of five oral rat studies, one provided an estimate for oral systemic availability of 0.0002% based on a limited number of organs (Hendrickson et al., 2016) and another study provided an estimate of 0.6% (Kreyling et al., 2017b). The Panel noted that in a study employing the model of isolated loop technique, the authors could provide data indicating the presence of TiO_2 NPs either as single particles or as smaller and larger agglomerates in intestine, liver and spleen (Hendrickson et al., 2020). The other studies did not give data suitable to quantify absorption and/or accumulation. The Panel considered that in two studies analysing tissues from deceased subjects, deposition of Ti-containing nanoparticles was observed in liver, spleen and kidney as well as in intestine. After quantification of the Ti amount in the organs and comparison with the estimated mean daily intake of E 171, the Panel concluded that the oral systemic availability of TiO_2 NP ingested from a number of sources, including dietary exposure to E 171, would be low (less than 1% by mass).

In summary, the Panel considered that E 171 has a low oral systemic availability, probably not greater than 0.5%. It may pass the placenta and may be transferred to the fetus.

Furthermore, the Panel considered that rat studies with TiO_2 NPs, consisting of nanoparticles with primary particle sizes between 7 and 90 nm, showed long half-lives (roughly 200–450 days), a potential for accumulation (accumulation factor of 290 to 450) and long time to reach steady state (3–5 years) (Geraets et al., 2014; Disdier et al., 2015). The oral systemic availability of these materials was low (most probably < 1%) but higher than for E 171. In tissues from deceased subjects, TiO_2 particles were identified in liver and spleen, the low Ti amount of the investigated organs indicating low oral systemic availability of TiO_2 ingested from a number of sources, including dietary exposure to E 171.

4.1.2. Toxicity studies

Toxicity studies considered sufficiently reliable with respect to their internal validity (reliability 1 and reliability 2 according to Appendix C) are described in Appendix H, including details of the appraisals of their suitability for investigating the toxicological effects of nanoparticles.

The score for the assessment of suitability of the studies to investigate the toxicity of nanoparticles has been performed according to Appendix E and is independent from the score on internal validity.

General and organ toxicity

E 171

Mice

Talamini et al. (2019) (scoring 1 for NSC) examined the effect of exposing mice (n = 4) to 5 mg E 171/kg bw per day, 3 days per week, for 3 weeks (nine treatments in 21 days, providing an average daily dose of 2 mg E 171/kg bw). The exposure did not affect body weight, feed intake or organ weights. Some inflammatory biomarkers were changed in some of the tissues examined (stomach, gut, liver). Total area of 'necroinflammatory' foci in the liver of exposed mice (n = 4) was greater as compared to the area in the controls (n = 2). The Panel noted that this study was limited to one dose

group, that the histological examination included a small number of mice and that the increase in 'necroinflammatory' area in the E 171 exposed mice was not accompanied by additional endpoints indicative of evidence for liver injury.

Rats

Talbot et al. (2018) (scoring 1 for NSC) examined the effect of exposing rats to E 171 (0, 0.1 and 10 mg/kg bw per day) for 60 days, with a focus on GIT microbial production of short-chain fatty acids (SCFAs) and mucin O-glycosylation. The Panel considered that the results indicated no effects of E 171 on SCFA production and mucus barrier in the gut at the highest dose tested of 10 mg/kg bw per day.

Han et al. (2020a) (scoring 2 for NSC) examined the effects of E 171 (0, 10, 100 or 1,000 mg/kg bw per day) for 90 days in rats. Although some changes were reported by the authors (6–10% higher feed intake of high-dose males, an 8% decrease in relative lymphocyte count in low- and high-dose males, respectively), overall, the Panel considered that gavage administration of E 171 at doses up to 1,000 mg/kg bw per day to rats for up to 90 days did not induce any adverse effects on clinical appearance, survival, body weight, feed intake, haematology, clinical chemistry, urinalysis, organ weights, or gross and microscopical pathology.

The Panel considered that E 171 had no adverse effects in a mouse study (Talamini et al., 2019) on parameters regarded as indicative of systemic toxicity (body weight, feed intake, organ weights or morphology of the examined tissues) given by gavage at a mean daily dose of 2 mg/kg be per day for 21 days. The Panel considered that E 171 was dispersed and administered such that exposure to small particles and NPs present in E 171 would have taken place. The Panel considered the reported enlargement of 'necroinflammatory' liver foci in the exposed mice deserved attention. However, the Panel could not conclude on the association of this finding with exposure to E 171, due to very limited number of livers examined. The Panel noted the absence of additional endpoints indicative of evidence for liver injury and the fact that these reported changes can variably occur as a background pathology in murine liver. In the rat, no signs of systemic toxicity were seen after gavage administration for 90 days at doses up amounting to 1,000 mg E 171/kg bw per day; the study had limitations for assessing the toxicological effects of the fraction of nanoparticles (Han et al., 2020a). E 171 did not affect caecal microbial production of SCFA in the caecum or mucus barrier in small and large intestine after 60 days of exposure via gavage at the highest dose tested of 10 mg/kg bw per day (Talbot et al., 2018).

TiO_2 NPs or TiO_2 containing a fraction of NPs

Mice

No studies available.

Rats

Vasantharaja et al. (2015) (scoring 2 for NSC) examined the effect of exposing rats to TiO_2 NPs (0, 50 and 100 mg/kg bw per day) by gavage for 14 days on clinical chemistry parameters. Although some changes were reported by the authors, the Panel considered that the only major change was a decrease in serum triacylglycerols to 64% of the control levels in both treatment groups.

El-Din et al. (2019) (scoring 4 for NSC) examined the effect of exposing rats to TiO_2 NPs (0, 1,200 mg/kg bw per day) by gavage for 90 days on morphology of the heart ventricles. The authors reported histological changes in the myocardium of the treated group. However, due to the limited information reported, the Panel was not able to conclude on the relationship between the reported histopathological changes and treatment with TiO_2 NPs.

Warheit et al. (2015a) (scoring 4 for NSC) examined the effect of exposing rats by gavage to two types of TiO_2 (11% nanoparticles) at a single, unrealistically high-dose level of 24,000 mg/kg bw per day for 29 days. Microscopic evaluation revealed the presence of the test substance in intestinal lymphoid tissue, considered by the authors as not adverse. The study examined all endpoints incorporated in an OECD TG 407 study. The Panel considered that no treatment-related adverse effects on any of the endpoints were found; however, the Panel further noted that the scoring for NSC was 4 and there was no demonstration of internal exposure.

The Panel considered that there were no adverse effects on clinical chemistry parameters in rats exposed by gavage to TiO_2 NPs at doses up to 100 mg/kg bw per day for 14 days (Vasantharaja et al., 2015). A potential adverse effect on the heart was reported after subchronic (90-day) exposure to 1,200 mg TiO_2 NPs/kg bw per day, but due to limited reporting no conclusion could be drawn (El-Din et al., 2019). Gavage administration of 24,000 mg TiO_2 (11% NPs)/kg bw per day for 29 days, without

demonstrating of internal exposure, had no adverse effects on the endpoints required by the OECD TG 407 testing guideline (Warheit et al., 2015a).

TiO_2 NPs < 30 nm

Mice

Hu et al. (2015) examined the effects of gavage administration of TiO_2 NPs (26 nm) (0, 64 and 320 mg/kg bw per day for 14 weeks) on the hormonal control of glycaemia in male CD-1 mice. The authors reported that treated mice had increased fasting blood glucose levels from weeks 10. Impaired glucose tolerance was observed (without showing a dose response), but no changes in blood insulin or lipids could be detected. The Panel considered that the oral administration of TiO_2 NPs at both doses, 64 and 320 mg/kg bw per day led to increases in fasting state plasma glucose, and also to increases in glucose levels in a glucose tolerance test without showing a dose response and without differences in plasma insulin levels, indicating inconsistency between the measured outcomes.

Yu et al. (2016) examined the effects of gavage administration of TiO_2 NPs (5–6 nm) (0, 2.5, 5 and 10 mg/kg bw for 90 consecutive days) in female CD-1(ICR) mice on the heart. Body weight gain was statistically significantly decreased in a dose-dependent manner to 30.3% in all the TiO_2 NPs groups. Based on the limited reporting, the Panel was not able to conclude on the decreased body weight gain and the relationship between the reported histological changes and treatment with TiO_2 NPs.

Another study (Hong et al., 2016) examined the effects of gavage administration of TiO_2 NPs (5–6 nm) (0, 2.5, 5 and 10 mg/kg bw for 90 consecutive days) in male CD-1(ICR) mice on the liver. A dose-dependent decrease in body weight gain was observed at all tested doses (approx. 5%, 5% and 7% decrease in BW gain compared to control at 2.5, 5 and 10 mg/kg bw per day, respectively), with statistically significant differences at the two highest doses. Relative liver weights increased by ~ 10–15% compared to control; however, absolute liver weights were unchanged. Histological alterations of the liver (lymphocyte infiltration and necrobiosis) were reported. Changes in the liver expression of inflammation-related proteins were also found. The Panel noted that the histopathological data in the liver were not accompanied by any other confirmatory investigations (e.g. clinical chemistry) and considered the effects reported in this study as likely an hepatic inflammatory response to TiO_2 NPs (5–6 nm).

Yang et al. (2017) examined the effects of gavage administration of TiO_2 NPs (21 nm) (0, 250 and 500 mg/kg bw for 14 consecutive days) in male C57BL/6 mice on the liver. The only noteworthy effect was a 3-fold increase of serum bilirubin (both total and indirect) at the highest dose, in the absence of inflammation, apoptosis, necrosis and molecular defects in bilirubin metabolism. The Panel noted structural changes in hepatocytes which were not quantified. However, these increases occurred in the absence of any changes in relative liver weight, changes in other serum markers for liver injury or quantitative histopathological changes in the liver. Changes in the hepatic expression of selected genes were considered as either incidental or adaptive, but not evidence of adversity.

Rats

Chen et al. (2015a) (scoring 2 for NSC) examined the effect of exposing rats to TiO_2 NPs (24 nm) (0, 2, 10 and 50 mg/kg bw per day) with and without glucose (1.8 g/kg bw per day) for 30 and 90 days. Some haematological parameters were changed; however, the Panel considered that these were of no toxicological significance. The authors reported 'oedema, fatty degeneration and necrosis' in livers of the high-dose group in the absence of serum enzyme activities reflecting liver injury. The Panel considered there were limitations in the reporting of histopathological changes in the liver and in the absence of changes in serum enzyme activities reflective of liver injury considered effects on the liver as not adverse.

Chen et al. (2015b) (scoring 1 for NSC) examined the effect of exposing rats to TiO_2 NPs (24 nm) (0, 2, 10 and 50 mg/kg bw per day) with a focus on investigating effects on the cardiovascular system. Although some changes were reported by the authors, the Panel considered that gavage administration of TiO_2 NP (24 nm) in doses up to 50 mg/kg bw per day to rats for up to 90 days did not induce any treatment-related effects.

Grissa et al. (2015) (scoring 2 for NSC) administered TiO_2 NPs (5–12 nm) in distilled water by daily gavage at doses of 0, 50, 100 or 200 mg/kg bw to rats for 60 days. The Panel considered the reported haematological changes to be of no toxicological significance.

Chen et al. (2020a) (scoring 2 for NSC) daily gavaged TiO_2 NPs (29 nm) at doses of 0, 2, 10 or 50 mg TiO_2 NPs/kg bw per day for 90 days to male rats. Starting from week 8, the 10 and 50 mg/kg bw

per day groups showed a decrease in body weight gain of up to about 15%, with no effect on food intake. Serum levels of triglycerides (TGs) in the 10 and 50 mg/kg bw per day groups were statistically significantly lower than in the control group while serum total cholesterol, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol (LDL-C) were not affected. The Panel considered that, while the change in body weight gain may be adverse, other reported changes were of no toxicological significance.

Chen et al. (2020b) (scoring 2 for NSC) examined the effect of exposing rats to TiO_2 NPs (24 nm) (0, 2, 10 and 50 mg/kg bw per day) with and without glucose (1.8 g/kg bw per day) for 90 days. Effects were seen with TiO_2 NPs (24 nm) on blood glucose levels, blood glycoproteins (glycated haemoglobin (HbA1c); glycated serum protein (GSP)), blood insulin, C-peptide and an oral glucose tolerance test (OGTT; performed at day 90). The Panel considered the changes in blood glucose, HbA1c, GSP, insulin, C-peptide, glucagon and glucose tolerance as either not test substance related or irrelevant for the safety evaluation of E 171.

Grissa et al. (2017) (scoring 3 for NSC) gave male rats control vehicle or 100 mg TiO_2 NPs (5–10 nm)/kg bw per day by gavage for 8 weeks. The TiO_2 NP-treated group had a statistically significantly decreased body weight gain and serum cholesterol, glucose and TG concentrations were statistically significantly higher. The authors reported significant changes in plasma oxidative stress markers and an increase in plasma interleukin-6 (IL-6) compared to control. The Panel noted the changes in glucose levels which are potentially adverse, and considered that the changes in cholesterol and TG are of unclear toxicological relevance.

Hassanein and El-Amir (2017) (scoring 3 for NSC) administered a single dose level of 150 mg/kg bw TiO_2 NPs (21 nm) in 1% Tween 80 daily by gavage. The control group received 1% Tween 80. Based on the many flaws in the study reporting (e.g. descriptions of 'histopathological' lesions are unclear, some of the findings are not histopathological lesions, the number of lesions per organ and the number of animals with any lesions are not clearly stated), the Panel was not able to draw any conclusions.

Heo et al. (2020) (scoring 3 for NSC) performed a repeated-dose 28-day and a repeated-dose 90-day study in rats. TiO_2 NPs (21 nm) in sodium phosphate buffer were administered by gavage at 0, 250, 500 and 1,000 mg/kg bw per day. No statistically significant treatment-related differences with respect to body weight gain, food and water intake were observed. No mortality or clinical signs were detected during the exposure period of 28 and 90 days. No effects were detected in a functional observation battery in the last week of the 90-day study. Ophthalmoscopic examination and urinalysis did not show statistically significant differences between the groups. Changes – circulating neutrophils and lymphocytes, blood urea nitrogen and blood Na – occurred without a clear dose response. No abnormal gross findings were found at necropsy in treated animals. Changes in some organ weights were considered unrelated to the treatment. On histopathological examination, differences between the control group and the 1,000 mg/kg bw per day group were found. The Panel considered that the reported changes were within the historical control normal range and therefore of no toxicological significance.

Concluding remarks

In mice, no adverse effects were observed up to 1,000 mg E 171/kg bw per day, the highest dose tested, in a 90-day study (Han et al., 2020a; scoring 2 for NSC). In rats, toxicity studies with TiO_2 NPs or TiO_2 containing a fraction of nanoparticles, having different duration (14–90 days), no adverse effects were observed up to the highest dose tested (100 mg/kg bw per day, Vasantharaja et al. (2015) and scored 2 for NSC). Overall, no adverse effects associated with general toxicity were observed in rats orally exposed to E 171, TiO_2 NPs or TiO_2 containing nanoparticles.

In mice orally exposed to TiO_2 NPs < 30 nm for up to 90 days, some effects were reported, which by their nature could be adverse. However, mild hyperbilirubinaemia was not accompanied by any changes in liver enzymes (Yang et al., 2017); the effect size of increased fasting glycaemia and impaired glucose tolerance (Hu et al., 2015) was not of toxicological relevance and not accompanied by changes in insulin or other changes in lipid metabolism and therefore was not of toxicological relevance. Histopathological changes were reported in the heart (Yu et al., 2016); however, these findings were not supported by incidences and severity scores. Histopathological findings indicating inflammation were reported in the liver, but investigations to confirm hepatic injury were not performed (Hong et al., 2016).

In rats orally exposed to TiO_2 NPs < 30 nm, inconsistent and/or unexplained sex differences in some parameters were reported (e.g. hypobilirubinaemia in females (Chen et al. (2015a); heart rate

and blood pressure changes in females (Chen et al. (2015b); leucocyte changes in females (Heo et al., 2020); higher absolute pituitary weights in males (Heo et al., 2020); lower blood insulin levels in females, lower C-peptide levels in males and differences in blood concentrations compared to controls in a glucose tolerance test in males (Chen et al., 2020b). The Panel considered that the TG changes reported in several studies were likely incidental study findings since the reductions were seen in only one sex and without a clear dose response (Chen et al., 2015b), lacked a clear dose response (Vasantharaja et al., 2015) or increased in a single dose study (Grissa et al., 2015)

The Panel considered that the effects reported in mouse studies with TiO_2 NPs < 30 nm could be associated with accumulation of NPs in various tissues whereas inconsistent findings in rats were considered incidental.

Reproductive and developmental toxicity studies

E 171

No studies performed with E 171 and considered with reliability 1 and 2 have been identified in the literature search.

TiO_2/TiO_2 NPs

Mice

No studies available.

Rats

From an oral prenatal developmental toxicity study in rats with five different TiO_2 materials, TiO_2 NPs or TiO_2 containing a fraction of nanoparticles (Warheit et al., 2015b) (scoring 4 for NSC), no maternal and developmental effects were observed up to 1,000 mg TiO_2 NPs/kg bw per day (the highest dose tested), when administered from gestation days (GDs) 6 to 15.

TiO_2 NPs < 30 nm

Mice

Karimipour et al. (2018) (scoring 2 for NSC) examined the effects of oral administration of TiO_2 NPs (10–25 nm) at 100 mg/kg bw per day for 5 weeks on the histology of ovaries, oestrogen and malondialdehyde (MDA) serum levels (7 animals/group), fertility (10 animals/group) and IVF rates (10 animals/group) in female mice. Impairment of female fertility at the only dose tested was observed.

Khorsandi et al. (2016) (scoring 2 for NSC) examined the effects of oral administration of TiO_2 NPs on testicular parameters in young adult male NMRI mice at doses of 0, 75, 100 and 300 mg/kg bw per day (8 animals/group) for 35 days. Dose-dependent decreases in testis weight occurred from a dose of 100 mg/kg bw per day. At higher doses, additional testicular parameters were affected. The Panel considered that TiO_2 NPs (size unknown) from 100 mg/kg bw per day had an effect on testis weight.

Khorsandi et al. (2017) (scoring 2 for NSC) administered TiO_2 NPs (20–30 nm) by oral gavage at 300 mg/kg bw per day to eight young adult male NMRI mice for 35 days. The authors reported significant decreases in testis weight, circulating and testicular testosterone, testicular catalase (CAT) and superoxide dismutase (SOD) concentrations, sperm counts and sperm motility. Significant increases were found in the percentage of abnormal or degenerative spermatogenic tubules, germ cell apoptosis, testicular MDA concentration and in the percentage of sperm with abnormal morphology. The Panel considered that testicular toxicity was observed with TiO_2 NPs (20–30 nm) at 300 mg/kg bw per day, the only dose tested.

Karimi et al. (2019) (scoring 2 for NSC) treated eight 6- to 8-week-old male NMRI mice daily by gavage with 50 mg TiO_2 NPs (< 30 nm)/kg bw per day for 35 days. TiO_2 NPs significantly reduced testis weight accompanied by reduced serum testosterone, reduced seminiferous tubule diameter and epithelium height and reduced the maturity of the germinal epithelium. Also, adverse findings were observed in reduced sperm counts, increased sperm abnormalities and reduced sperm motility. The Panel noted that 50 mg TiO_2 NPs/kg bw per day, the only dose tested, resulted in adverse effects on the testis.

Lu et al. (2020) (scoring 4 for NSC) treated four groups of 15 male ICR mice, age 6–8 weeks daily by gavage with TiO_2 NPs (7 nm) at doses of 0, 10, 50 or 100 mg/kg bw per day for 30 days. The authors report tight junction damage in the blood–testis barrier (BTB) at 50 and 100 mg/kg bw, though the

histopathological pictures provided are hard to interpret. Serum testosterone was 50% decreased at the two highest doses tested. Sperm motility was dose-relatedly reduced, accompanied by increased sperm malformation rates. The Panel considered that TiO_2 NPs (7 nm), at 50 or 100 mg/kg bw per day, resulted in a dose-related reduction of sperm motility and increased sperm malformations, accompanied by histological observations in the testis, changes in BTB-related protein levels, changes in MAPK-related mRNA levels and reduced circulating testosterone concentrations.

Rats

Lee et al. (2019) (scoring 3 for NSC) treated mated female Sprague–Dawley rats (12 females per group) with TiO_2 NPs (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. There were no statistically significant differences in general clinical signs, body weight, organ weights (absolute and relative to body weight), macroscopic findings. Caesarean section parameters and fetal external and visceral examinations did not reveal any statistically significant differences. The Panel considered that no adverse maternal and developmental effects were reported with TiO_2 NPs (21 nm) up to 1,000 mg/kg bw per day, the highest dose tested.

Concluding remarks

No reproductive or developmental toxicity studies performed with E 171 and considered sufficiently reliable with respect to their internal validity (see Appendix C) have been identified from the published literature.

No maternal and developmental effects were observed up to 1,000 mg/kg bw per day, the highest dose tested, in a single rat developmental toxicity study with five different TiO_2 materials, TiO_2 NPs or TiO_2 containing a fraction of nanoparticles (Warheit et al., 2015a) (scoring 4 for NSC).

In mice, the effects of TiO_2 NPs < 30 nm on the testis (decreased weight, decreased seminiferous tubule diameter, germ cell apoptosis) and sperm (decreased sperm counts and motility, increased percentage of abnormal spermatozoa) were observed in three studies (Khorsandi et al., 2016, 2017; Karimi et al., 2019) at doses ranging from 50 to 300 TiO_2 NPs/kg bw per day. The lowest dose at which the effects were observed was 50 mg TiO_2 NPs/kg bw per day (Karimi et al., 2019). In a mouse study by Lu et al. (2020), no effects were observed at the lowest dose tested, 10 mg/kg bw per day (scoring 4 for NSC). In rats, administration of TiO_2 NPs (21 nm) did not show effects at any dose level in a developmental toxicity study up to 1,000 mg/kg bw per day (Lee et al., 2019, scoring 3 for NSC))

Neurotoxicity and neurodevelopmental toxicity studies

E 171

No studies performed with E 171 and considered with reliability 1 and 2 have been identified.

TiO_2 NPs

Pregnant Wistar rats (n = 6/group) were administrated TiO_2 NPs (< 100 nm) by gavage at 0 or 100 mg/kg bw per day from GD 2 to 21 (gestation group) or from postnatal days (PND) 2 to 21 (lactation group). In offspring (PND 1 in gestation group, PND 22 in lactation group), increased hippocampal apoptosis and reduced hippocampal neurogenesis after both gestational and lactational exposure (Ebrahimzadeh et al., 2017; scoring 3 for NSC) were observed.

Kandeil et al. (2019) (scoring 3 for NSC) dosed adult male albino rats (n = 20/group) by gavage with TiO_2 NPs (90 nm, range 40–140 nm) at 0 or 500 mg/kg bw per day for 14 days. Adverse effects in CNS possibly related to oxidative stress were observed at the single dose tested of 500 mg/kg bw per day.

The Panel concluded that these data show that oral TiO_2 NPs administered to rats during embryofetal and early postnatal development reduced hippocampal neurogenesis at 100 mg/kg bw per day, and that oral administration to adult rats produced adverse effects in the brain consistent with oxidative stress at 500 mg/kg bw per day.

TiO_2 NPs < 30 nm

A number of studies have reported adverse effects on both adult and developing mouse and rat brain after oral dosing with TiO_2 NPs < 30 nm.

Adult mice

The most sensitive endpoint observed in adult mice was reduced volume of the hippocampus, and the polymorph layer of the dentate gyrus, and reduced density and total number of dentate gyrus

granular cells at all doses tested (in males dosed for 35 days) (Rahnama et al., 2020; scoring 4 for NSC). The mice in this study (n = 20/group) were dosed by gavage with TiO_2 NPs (21 nm) at 0, 2.5, 5 or 10 mg/kg bw per day.

Other studies using adult mice and a single dose of TiO_2 NPs < 30 nm also reported adverse effects on the brain after oral dosing.

Zhang et al. (2020) (scoring 3 for NSC) dosed young adult male mice (probably n = 15/group) by oral gavage with TiO_2 NPs (21 nm) at 0 or 150 mg/kg bw per day for 30 days. Treatment had no effect on body weight or histopathology of gut or brain, but significantly decreased the richness and evenness of gut microbiota, elevated gut HuC/D and TuJ1 and markedly reduced serotonergic markers Sstr1 and Sstr2 in gut but not in cerebral cortex, suggesting an effect on the enteric nervous system. However, gut–brain peptides secreted by endocrine cells and enteric neurons, and also inflammatory cytokines, were not affected by treatment. In the open field test, centre field activity was statistically significantly reduced by the treatment, consistent with anxiety-like behaviour, but MWM learning and spatial memory were unaffected. The Panel considered that TiO_2 NPs (21 nm) at 150 mg/kg bw per day, the only dose tested, altered gut microbiota, without pathological changes in small intestine and brain.

The Panel noted that the most sensitive endpoint in adult mice was reduced volume of hippocampus and dentate gyrus granular layer, and density and number of dentate gyrus granular cells observed with TiO_2 NPs (21 nm) at 2.5 mg/kg bw per day, the lowest dose tested, in males dosed for 35 days (Rahnama et al., 2020).

Adult rats

In adult rats, the most sensitive endpoint was reduced brain cholinesterase activity (about 35–50%) and increased brain Na, K-ATPase activity (about 2-fold), observed with TiO_2 NPs (21 nm) at all doses tested, in female albino rats dosed for 14 days, as reported by Canli et al. (2020) (scoring 4 for NSC). In this study, rats (n = 6/group) were dosed by gavage with TiO_2 NPs (21 nm) at 0, 0.5, 5 or 50 mg/kg bw per day.

Grissa et al. (2016) (scoring 3 for NSC) reported reduced brain cholinesterase activity at 100 and 200 mg/kg bw per day, and reduced plasma cholinesterase activity at all doses tested, in male rats dosed for 60 days. In this study, rats were dosed by gavage with TiO_2 NPs (5–10 nm) at 0, 50, 100 or 200 mg/kg bw per day. Brain cholinesterase activity was not affected at 50 mg/kg bw per day, in contrast to the significant reduction in brain cholinesterase activity at 0.5 mg/kg bw per day reported by Canli et al. (2020). This apparent 200-fold difference in potency adds to uncertainty; possible contributory factors include differences in test substance dispersion and internal exposure between the two studies. The Panel noted that the reduced brain cholinesterase activity reported by Grissa et al. (2016) was not dose related (similar effect at 100 and 200 mg/kg bw per day doses, about 50% reduction), but plasma cholinesterase activity was dose-dependently reduced at all doses (about 35% at 50 mg/kg bw per day, 50% at 100 and 200 mg/kg bw per day). The Panel noted that the method used to determine cholinesterase activity by Grissa et al. (2016) probably measures both acetylcholinesterase (AChE) and butyryl ChE (BChE) activity. In any case, TiO_2 NPs reduced brain cholinesterase activity.

Grissa et al. (2020) (scoring 2 for NSC), using a similar study design (strain age and dosing regimen) as Grissa et al. (2016) and with TiO_2 NPs (5–12 nm), reported reduced SOD and CAT, and increased NO and tumour necrosis factor-alpha (TNF-α) in brain frontal cortex at all doses tested (50, 100 and 200 mg/kg bw per day).

Other studies using adult rats and a single dose of TiO_2 NPs also reported adverse effects on the brain after oral dosing.

Hassanein and El-Amir (2017) (scoring 3 for NSC) dosed adult male Sprague–Dawley rats (n = 10/group) by gavage with TiO_2 NPs (21 nm) at 0 or 150 mg/kg bw per day for 6 weeks. Increased total leucocyte, lymphocyte and neutrophil counts, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lipid peroxidation (LPO), TNF-α and liver DNA damage by Comet assay, and decreased glutathione (GSH), and histopathological alterations in liver, brain, lung, kidney, heart and testes were reported at the only dose tested. However, with respect to neurotoxicity, the Panel noted that the tissue fixation method was not optimal for histology of brain tissue (non-perfused formaldehyde fixation), therefore the Panel considered that artefactual brain findings could not be excluded.

The Panel noted that the most sensitive endpoint in adult rats was reduced (dose related) brain cholinesterase activity and increased brain Na/K-ATPase activity, observed at 0.5 mg/kg bw per day (in

females dosed for 14 days), the lowest of three doses tested, reported by Canli et al. (2020) with TiO_2 NPs (21 nm). However, Grissa et al. (2016) reported reduced brain cholinesterase activity at 100 but not 50 mg/kg bw per day (in males dosed for 60 days with TiO_2 NPs (5–10 nm)). This apparent 200-fold difference in potency adds to uncertainty.

Mice developmental

In pre- and perinatal mice, the study by Zhou et al. (2017) (scoring 2 for NSC) reported inhibited dendritic outgrowth, increased autophagy and oxidative stress and reduced mitochondrial function, in *ex vivo* hippocampal CA1 neurons of the offspring of CD-1 with TiO_2 NPs (6–7 nm) at all doses tested. In this study, mice (n = 6/group) were dosed by gavage with TiO_2 NPs (6–7 nm) at 0, 1, 2 or 3 mg/kg bw per day from GD 7 to PND 21.

The Panel noted that the only available study on developing mice (Zhou et al., 2017) reported inhibited dendritic outgrowth, increased autophagy and oxidative stress and reduced mitochondrial function, in *ex vivo* hippocampal neurons of the offspring with TiO_2 NPs (6 = 7 nm) at all doses tested (1, 2 or 3 mg/kg bw per day).

Rats developmental

In pre- and perinatal rats, offspring passive avoidance behaviour was altered after maternal dosing during lactation with TiO_2 NPs (10 nm) at 100 mg/kg bw per day (the only dose tested) (Mohammadipour et al., 2016) (scoring 3 for NSC). The same dose level increased offspring hippocampal apoptosis and reduced offspring hippocampal neurogenesis after maternal dosing during both gestation and lactation (Ebrahimzadeh et al., 2017; scoring 3 for NSC).

The Panel concluded that gestational and/or lactational maternal rat exposure to TiO_2 NPs (10 nm) at 100 mg/kg bw per day altered passive avoidance behaviour, increased hippocampal apoptosis and reduced hippocampal neurogenesis in the offspring.

The Panel noted that the effects on brain structure and function reported in Mohammadipour et al. (2016) and Ebrahimzadeh et al. (2017) are mutually plausible, given that passive avoidance behaviour is related to hippocampal functioning (Eagle et al., 2016; Anacker et al., 2016).

Concluding remarks

No studies performed with E 171 and considered sufficiently reliable with respect to their internal validity (see Appendix C) have been identified from the published literature.

Oral TiO_2 NPs dosed in rats during embryofetal and early postnatal development reduced hippocampal neurogenesis at 100 mg/kg bw per day exposure (Ebrahimzadeh et al., 2017), and dosed in adult rats produced adverse effects in the brain consistent with oxidative stress at 500 mg/kg bw per day (Kandeil et al., 2019). Both studies scored 3 for NSC.

After oral dosing with TiO_2 NPs < 30 nm, adverse effects in both adult and developing mouse and rat brain were reported. Most of these effects are possibly related to oxidative stress. In mice, the Panel noted that the reduced volume of the polymorph layer of the hippocampal dentate gyrus and reduced density and number of dentate gyrus granular neurons reported by Rahnama et al. (2020) with TiO_2 NPs (21 nm), is consistent with the behavioural effects reported by Zhang et al. (2020) also with TiO_2 NPs (21 nm), i.e. increased open field anxiety-like behaviour and unaffected spatial learning and memory. Ventral dentate gyrus is associated with anxiety behaviour, CA regions with spatial learning/memory (Eagle et al., 2016; Anacker et al., 2018). In adults rats, the most sensitive endpoint in the evaluated studies was reduced brain cholinesterase activity and increased brain Na/K-ATPase activity with TiO_2 NPs (21 nm) at a dose of 0.5 mg/kg bw per day in females dosed for 14 days (Canli et al., 2020).

The Panel noted that inhibition of cholinesterase activity by nanoparticles other than TiO_2, both metal and plastic, has been reported in a number of species (Prüst et al., 2020). Since oxidative stress-related inflammation is generally associated with increased and not decreased cholinesterase activity (Corrêa Mde et al., 2008; Vaknine and Soreq, 2020), it is unclear whether there is a link between TiO_2-induced oxidative stress and TiO_2-induced decrease in cholinesterase activity.

Overall for neurotoxicity, adverse effects were seen with TiO_2 NPs < 30 nm. In mice, Zhou et al. (2017; scoring 3 for NSC), reported adverse effects (i.e. inhibited dendritic outgrowth, increased autophagy and oxidative stress and reduced mitochondrial function) in *ex vivo* hippocampal neurons of weanling mice after dosing TiO_2 NPs (6–7 nm) during gestation and early lactation at a dose of 1 mg/kg bw per day, the lowest dose tested. In adult female rats (Canli et al., 2020; scoring 3 for NSC), adverse effects (reduced brain cholinesterase, and increased brain Na/K-ATPase activity) were observed with TiO_2 NPs (21 nm) at 0.5 mg/kg bw per day, the lowest of three doses tested, in a 14-day study.

Inflammation and immunotoxicity studies

E 171

Mice

No adverse effects in the mouse study (Riedle et al., 2020; scoring 1 for NSC) were observed up to the highest dose tested (100 mg/kg per day). Pinget et al. (2019), scoring 2 for NSC, found a reduction of colonic crypt length, in addition to an increase in colon macrophages and CD8 cells in IL-10, TNF-α and IL-6 mRNA at doses of 10 and 50 mg/kg bw per day. Other studies in mice only included one dose where increased inflammatory parameters were observed at 5 mg/kg bw per day (Urrutia-Ortega et al., 2016; scoring 1 for NSC) or 2 mg/kg bw per day (Talamini et al., 2019; scoring 1 for NSC). In Urrutia-Ortega et al. (2016) study, it was also observed that 5 mg/kg bw per day alone had no effect on tumour formation but could potentiate intestinal tumour formation in mice exposed to azoxymethane/dextran sulfate sodium.

Rats

In Han et al. (2020a) study (scoring 2 for NSC), rats were exposed to E 171 at 10, 100 or 1,000 mg/kg bw per day per 90-day, a statistically significantly decreased granulocyte-macrophage colony-stimulating factor (GM-CSF) plasma level of approximately 40% was observed at the highest dose. Whereas it is difficult to predict an adverse effect from such an intermediate endpoint, GM-CSF is involved in haemopoiesis which may explain the modest but statistically significant decrease in immunoglobulin (Ig) M level (~ 10%).

In Bettini et al. (2017) study (scoring 1 for NSC), only one dose of E 171 was tested (10 mg/kg bw per day) and increased inflammatory parameters were observed. These results were not confirmed in another study (Blevins et al., 2019; scoring 3 for NSC), as no effects were observed up to 267 mg E 171/kg bw per day, the highest dose tested. However, the Panel noted that Blevins et al. (2019) study was scored 3 for NSC.

The Panel noted that the reported effects of E 171 on the immune system were variable. Certain studies with E 171 did not find an effect (Blevins et al., 2019; Riedle et al., 2020), while others did, showing especially changes in parameters indicating inflammatory processes (Urrutia-Ortega et al., 2016; Talamini et al., 2019).

TiO_2 or TiO_2 NPs

Two mouse studies performed with TiO_2 NPs (Mohamed, 2015; Li et al., 2019) were scored 2 for NSC and a rat study (Hashem et al., 2020) was scored 3.

Mice

Both mice studies were short-term studies, 5 days (Mohamed, 2015) and 7 days (Li et al., 2019). In Mohamed (2015), TiO_2 NPs (47 nm) at doses of 5, 50 or 500 mg/kg bw per day were administered and this resulted in an inflammatory response in the stomach, which was already evident at the lowest dose tested, i.e. 5 mg/kg per day. In Li et al. (2019) study, no relevant effects were noted on the histology of the spleen with TiO_2 NPs (25, 50 or 80 nm) at 1 mg/kg bw per day, the only dose tested.

Rats

In rats exposed to TiO_2 at 20 or 40 mg/kg bw per day for 90 days, changes in haematological and immunological parameters were noted at the lowest dose tested, i.e. 20 mg/kg per day (Hashem et al., 2020).

TiO_2 NPs < 30 nm

Mice

In a 90-day study (Yu et al., 2016) (scoring 2 for NSC), animals were exposed to TiO_2 NPs (5–6 nm) at 2.5, 5 or 10 mg/kg bw per day. The Panel noted inflammatory mediators at all doses tested and corroborated by histopathological lesions.

In Li et al. (2018) (scoring 4 for NSC), a single dose of TiO_2 NPs (20 nm) at 100 mg/kg bw per day for 28 days was tested. Zhang et al. (2020) (scoring 4 for NSC) administered TiO_2 NPs (21 nm) at 150 mg/kg bw per day for 30 days. Both studies were focused on the effects of TiO_2 NPs on the microbiota. No change in spleen histology or in local or systemic inflammatory parameters was observed by Li et al. (2018).

Rats

In a rat study (Chen et al., 2015a) (scoring 2 for NSC) animals were exposed to TiO_2 NPs (24 nm) at 2, 10 or 50 mg/kg bw per day. An increase in leucocytes was observed which may suggest an inflammatory response at the highest dose tested. In another study in rats by the same group (Chen et al., 2019), scoring for NSC 2, histopathologically, reduced numbers of goblet cells were found as a result of exposure to 50 mg/kg bw per day, as well as inflammatory infiltration, while in serum increased IL-6 expression was observed.

In Grissa et al. (2020) study (scoring 2 for NSC), rats were exposed to TiO_2 NPs (5–12 nm) at 50, 100 or 200 mg/kg bw per day. The Panel noted changes in inflammatory markers at 100 and 200 mg/kg bw per day.

Concluding remarks

The Panel concludes that these studies indicate immune dysregulatory activity of E 171, evidenced by several immune-related and inflammatory markers. These effects were not observed up 50 mg E 171/kg bw per day. In three single dose level studies with E 171, effects were noted at lower doses, i.e. 2, 5 and 10 mg/kg bw per day.

Effects of E 171 may, at least in part, stem from the activity of the fraction of the smaller TiO_2 particles, as studies with these particles also indicate inflammatory effects of exposure to TiO_2 NPs (5–6 nm) at 2.5 mg/kg per day.

Aberrant crypt foci

This endpoint was specifically requested to be investigated in the EOGRT study (see Section 4.2.6) as part of the follow-up to the re-evaluation of E 171 (EFSA ANS Panel, 2018). Therefore, studies reporting information on aberrant crypt foci (ACF) were collected from the literature search.

In total, there were two studies investigating this endpoint (Bettini et al., 2017; Blevins et al., 2019).

The study by Bettini et al. (2017) (scoring 1 for NSC) was previously reviewed by the ANS Panel (EFSA ANS Panel, 2018) and it has also been evaluated in the current assessment (Appendix H – immunotoxicity). The Panel considered that E 171 per se at a dose of 10 mg/kg bw per day may induce development of ACF in male rats. The Panel also noted that E 171 at a dose of 10 mg/kg bw per day increased the number of ACF initiated by a genotoxic carcinogen.

From the Blevins et al. (2019) (scoring 3 for NSC), the Panel noted that no changes in the number of ACF and aberrant crypts (ABC) were observed due to E 171 exposure alone, while the number of ACF increased not statistically significantly in the colon samples from animals exposed to E 171 and initiated with a carcinogen (dimethylhydrazine (DMH)) relative to a group exposed to the carcinogen alone. However, limitations in the pathological examination of ABC and ACF (sampled colon area limited; technical issues related to fixation) preclude a conclusion on potential for ABC and ACF formation. Dietary E 171, with or without treatment with DMH, had no effect on the length of the colonic glands examined or the number of goblet cells/unit.

The Panel considered that E 171 may induce ACF in male rats at a dose of 10 mg/kg bw per day.

Gut microbiota

A number of studies, considered to be reliable by the Panel, have examined or included analyses of GIT microbiota changes in response to oral exposure to E 171 (Pinget et al., 2019), TiO_2 NPs (Li et al., 2019; Yan et al., 2020) and TiO_2 NPs < 30 nm (Li et al., 2018; Chen et al., 2019, 2020a; Zhang et al., 2020; Zhao et al., 2020). Detailed description of references Li et al. (2018), Pinget et al. (2019), Chen et al. (2019), Li et al. (2019), Zhang et al. (2020) and Chen et al. (2020a), investigating also other relevant endpoints, are available in Appendix H.

E 171

Mice

Mice were administered E 171 via drinking water at doses of 0, 2, 10 and 50 mg E 171/kg bw per day for 3 weeks (Pinget et al., 2019) (scoring 2 for NSC) followed by examination of microbiota populations in faecal samples or the small intestine through 16S rRNA sequencing. Exposure to E 171 had minimal impact on their compositions but altered the release of bacterial metabolites.

Rats

No studies have been identified.

TiO_2 or TiO_2 NPs

Mice

In a 7-day study, mice were administered by gavage with three types of TiO_2 NPs (25 nm, 50 nm and 80 nm) at a dose of 1 mg/kg bw per day (Li et al., 2019; scoring 2 for NSC). The microbiota of the distal colonic content was analysed. According to the authors, observed effects in the colon (epithelial injury, suppressed expression levels of tight junction proteins and reduced thickness of the luminal mucus layer) were associated with altered gut microbiota composition.

In a 28-day study, mice were administered via gavage with TiO_2 (250 nm) or TiO_2 NPs (25 nm) at doses of 10, 40 and 160 mg/kg bw per day (Yan et al., 2020; scoring 2 for NSC). Colon tissues were isolated under aseptic conditions and contents were excised for 16S rRNA analysis. The results showed that exposure to TiO_2 or to TiO_2 NPs led to changes in the composition of the gut microbiota, especially the microbiota associated with mucus.

TiO_2 NPs < 30 nm

Mice

Li et al. (2018) (scoring 4 for NSC) performed an analysis of gut microbiota through sampling of faeces and performing 16S rRNA sequencing. Animals were administered via gavage with two types of TiO_2 NPs (20 nm anatase with near spherical shape and 15 nm rutile with edged with corners morphology) at a dose of 0 or 100 mg/kg bw per day for 28 days. The results showed that gut microbiota diversity was not affected by the treatments but it shifted their composition in a time-dependent manner, with rutile NPs having a more pronounced influence than anatase NPs.

In Zhang et al. (2020) (scoring 4 for NSC), animals were administered by gavage with TiO_2 NPs (21 nm) at a dose of 0 or 150 mg/kg bw per day for 30 days. According to the authors, the results showed that oral exposure to TiO_2 NPs decreased the 'richness and evenness' of gut microbiota.

Rats

Rats were administered via gavage with TiO_2 NPs (29 nm) at doses of 0, 2, 10, 50 mg/kg bw per day for 30 days (Chen et al., 2019) (scoring 2 for NSC). Changes in the gut microbiota and gut-associated metabolism were analysed through 16S rRNA sequencing and faeces metabolomics, respectively. The results showed that exposure to TiO_2 NPs led to structural and compositional changes in the gut microbiota over time and to alteration of metabolites in faeces.

In a 90-day study (Chen et al., 2020a) (scoring 2 for NSC), animals were administered by gavage with TiO_2 NPs (29 nm) at doses of 0, 2, 10 or 50 mg/kg bw per day. SCFAs in rat stool samples were analysed by targeted metabolomics. The results showed no change in the SCFAs content after exposure to TiO_2 NPs.

In Zhao et al. (2020) (scoring 1 for NSC), weaning rats were administered via gavage with TiO_2 NPs (25 nm) at doses of 0, 1, 50 and 100 mg/kg bw per day for 14 days. Faeces samples were collected and were used for microbiota analysis. The results indicated that the gut microbiota composition was altered by TiO_2 NPs treatment.

Concluding remarks

The Panel noted that changes in GIT microbiota in response to exposure to other substances used as food additives were observed (Swidsinski et al., 2009a,b; Renz et al., 2012; Merga et al., 2014; Cani and Everard, 2015; Chassaing et al., 2015, 2017; Romano-Keeler and Weitkamp, 2015; Lecomte et al., 2016; Nejrup et al., 2017; Holder and Chassaing, 2018; Jiang et al., 2018; Viennois and Chassaing, 2018; Marion-Letellier et al., 2019). However, there is currently no consensus on when changes in GIT microbiota should be considered adverse.[16] Accordingly, the Panel was unable to come to any conclusion regarding the effects of E 171 on GIT microbiota and related effects on health.

[16] EFSA has launched a thematic Art. 36 grant to review the state of the art and critically appraise the evidence, technologies and models (*in vitro*/*in silico*/*in vivo*) available. This thematic grant should yield a roadmap to advance research to address risk assessment needs to account for effects on/by gut microbiota in humans and domestic animals (https://www.efsa.europa.eu/sites/default/files/documents/art36/gpefsaenco202002/Call%20for%20Proposals_Thematic%20Grant.pdf

4.2. Data submitted to the call for data from the European Commission as follow-up of the re-evaluation of E 171

As mentioned in Section 3, an EOGRT study together with complementary reports (Documentation provided to EFSA No 1, 2, 3,4, 5 and 6) and additional clarifications (Documentation provided to EFSA No 11–13, 16–18) were submitted to EFSA.

The tested material corresponds to a commercial brand of E 171 for which data were submitted during the assessment of the amendment of the EU specifications for E 171 (sample E) (EFSA FAF Panel, 2019). Physico-chemical characterisation by two other laboratories (Documentation provided to EFSA No 11; Verleysen et al., 2020) confirmed that the test substance, after applying sample dispersion protocols, consists almost exclusively of near-spherical constituent particles with a median diameter in the order of 100 nm that are often agglomerated (i.e. 50% of the individual particles are at the nanoscale). The crystalline form of this E 171 is anatase.

E 171 was administered via the diet.[17] The concentration was adjusted weekly using the food consumption values from the previous week to maintain a constant dose level in relation to the animals' body weights. The actual E 171 intake for each test week was reported in mg kg body weight per day and calculated from the relative food consumption per day and the nominal E 171 concentration that was used in that test week. The titanium levels in the control and test diets were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES). The background level of titanium in the diet was 17 mg Ti/kg diet (mean value) and ranged 11–31 mg Ti/kg diet.

The EOGRT study was performed in male and female rats according to OECD TG 443 and good laboratory practice (GLP) compliance. In the F0 generation, E 171 was administered in the diet at doses of 0, 100, 300 or 1,000 mg/kg bw per day from 10 weeks prior to mating until weaning of the F1 generation. The F1 generation received these diets from weaning until PND 4 or 8 of the F2 generation. The F2 generation was exposed through the milk until the termination of the study on PND 4 or PND 8. Duration of dosing depended on the endpoints under evaluation in the different cohorts, with the longest duration of treatment up to 18 weeks. Endpoints included were consistent with those specified in OECD TG 443 guidance in order to ensure the investigation of reproductive and developmental toxicity of E 171 (except for balanopreputial separation, as noted below in the section 'Growth and sexual development'). Table 2 describes parameters considered in the EOGRT study with the corresponding generation/cohort/number of animals for each.

Table 2: 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 group	20	30		20	20	10	10	10	
Mortality	X		X	X	X	X		X	
Clinical signs	X			X	X	X		X	
Body weight	X		X	X	X	X		X	X
Food consumption	X			X	X	X		X	
Water consumption	X				X				
Haematology	X			X					
Clinical biochemistry	X			X					
Lymphocyte typing (spleen)				X					
Urinalysis	X			X					
Sexual hormone levels	X			X	X	X	X		
Thyroid hormone levels	X		X	X					
Sexual maturation				X	X	X		X	
Oestrous cycle data				X					
Sperm parameters	X			X					
Necropsy	X		X	X	X	X		X	X

[17] The appropriate amount of E 171 and diet (ssniff® R-Z V1320) were mixed with an impact mill to produce a premix. The premix was further added to diet, mixed with a mixer for 15 min and the mixture was freshly prepared once a week.

Generation	F0	F0 satellite	F1 pups	F1 1A	F1 1B	F1 2A	F1 2B	F1 3	F2
Histopathology	X			X		X	X		
Reproductive parameters	X		X		X				
Pre-postnatal development			X		X				X
Functional neurotoxicity observations						X			
Neurohistopathology						X	X		
Lymphocyte typing (spleen) after KLH immunisation				X				X	
Anti-KLH IgM levels after KLH immunisation								X	
Aberrant crypt foci (ACF) scoring		X							

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

The scoring for NSC (dispersion and/or confirmation of internal exposure), according to Appendix E (high doses only, with no considerations on dispersion, and insufficient confirmation of exposure to the fraction of small particles) was 4.

4.2.1. Assessment of internal exposure

The Panel noted that the basal diet contained an amount of Ti equivalent to approx. 1.4 mg TiO_2/kg bw per day and that there were variable measurable levels of Ti in blood and urine from control animals. The Panel considered that there were small dose-related increases in Ti concentrations in blood and urine in E 171-treated animals. In particular, the Ti blood concentration in cohort F2 at PND 4–7 was increased after correction for background. This is consistent with exposure via placenta and possibly milk, and thus, the dams must have taken up TiO_2 as well. Data on Ti concentration in urine in parental animals may be not accurate due to possible contact with Ti-containing faeces. The Panel considered that at least a small fraction of the Ti in the mothers' diet was absorbed. Further information is available on Appendix I.

4.2.2. Clinical observation, body weight and food consumption

In the F0 parental animals, no premature death or changes in general behaviour or external appearance were noted in any treatment group of either sex. Pale faeces were noted at 300 and 1,000 mg E 171/kg bw per day in both males and females. No difference in food consumption, body weight or body weight gain between control and treatment groups was observed in males and females during pre-mating, gestation and lactation. A slight but statistically significant increase in water consumption was recorded in females at 100 mg E 171/kg bw per day. Considering the lack of dose–response relationship, the temporary nature and the small magnitude, this effect was considered incidental and not-treatment-related.

In the F1 generation cohort 1A animals, no premature deaths or changes in general behaviour or external appearance were noted in any treatment group of either sex. Pale faeces were noted at 1,000 mg E 171/kg bw per day in both males and females. A slight decrease in body weight was noted in males at 300 mg E 171/kg bw per day, reaching statistical significance on test days 36 and 64. Body weight gain was not affected at any other dose. Considering the lack of a dose–response relationship, its temporary nature and small magnitude, the decrease in body weight was considered incidental and not treatment-related. No other effects on body weight, body weight gain and food consumption were observed in males and females at any dose.

In the F1 generation cohort 1B animals, no premature deaths or changes in general behaviour or external appearance were noted in any treatment group of either sex. Pale faeces were noted at 1,000 mg E 171/kg bw per day in both males and females. A slight but statistically significant decrease in body weight was noted in males at 300 mg E 171/kg bw per day, on five non-consecutive testing days. Body weight gain was not affected at any dose. Considering the lack of dose–response relationship, the temporary nature and the small magnitude, this effect was considered incidental and not treatment-related. No other effects on body weight, body weight gain, food and water consumption were observed in males and females at any dose.

In the F1 generation cohorts 2A and 2B animals, no premature deaths or changes in general behaviour or external appearance were noted in any treatment group of either sex. Pale faeces were noted at 1,000 mg E 171/kg bw per day in both males and females. No effects on body weight or body weight gain were noted. Transient statistically significant but inconsistent changes in food consumption were noted in both sexes at all doses, but considering the lack of dose–response relationship, their temporary nature and small magnitude, these effects were considered incidental and not treatment-related.

4.2.3. Clinical pathology

Haematology, biochemical analysis and urinalysis were performed on F0 and F1 cohort 1A generations.

Haematology – F0 generation

The only statistically significant haematological changes observed were in low-dose (100 mg/kg bw per day) males for haemoglobin concentration and for the haematocrit value. These differences were marginal and not dose dependent. The study authors, therefore, concluded that there were no test substance-related differences for the examined haematological parameters. The Panel agreed with this conclusion.

Haematology – F1 generation cohort 1 A

The only change observed was the absolute differential count of neutrophilic granulocytes, which was lower in all treated males compared to the control group; the difference attained statistical significance for low and high doses. The study authors considered this as an incidental finding, because a dose response relationship was lacking, the values were in the range of the historical control data from the laboratory, no statistically significant difference was recorded for the relative neutrophilic granulocyte count, and no differences for these endpoints were seen in females. The Panel noted that the total white blood cells (WBC) count was slightly but not statistically significantly lower in all treated males but the change was not dose related. Overall, the Panel agreed with the evaluation of the study authors.

Clinical biochemistry – F0

The only changes observed were decreases in serum LDL-C levels in males, statistically significant only at 100 and 1,000 mg/kg bw per day; serum alkaline phosphatase levels increased in males, statistically significantly at 1,000 mg/kg bw per day and serum chloride levels decreased in females, statistically significantly only at 300 mg/kg bw per day.

Regarding the LDL-C levels in males, the decreases were considered by the study author to be incidental as no dose–response relationship was evident. Additionally, no decreases were observed in females, in contrast, LDL-C levels were statistically significantly increased at the low- and the high-dose levels. The Panel agreed with this conclusion.

The moderate increase in alkaline phosphatase was considered by the authors as incidental. The Panel noted that there were no increases in serum AST and LDH and that serum ALT activity was only slightly increased to a level that was of no toxicologically relevance. Overall, the Panel agreed with the conclusion of the study authors.

The slight decrease in chloride level in females was considered by the authors as incidental, as no dose–response relationship was noted. The Panel agreed with this conclusion.

Clinical biochemistry – F1 cohort 1A

An increase in serum sodium/potassium ratio was noted in females at all doses, reaching statistical significance at 300 mg/kg bw per day.

The author considered this increase as incidental since the difference with respect to the control was only small, no dose–response relationship was noted and no changes were noted for the male animals. Furthermore, the observed changes were in the normal range of the historical control data of the laboratory, and no changes were seen in the serum sodium levels. No other test substance-related changes were observed. The Panel agreed with this conclusion.

Urinalysis – F0

Regarding the examined urinalysis parameters, no test substance-related differences were noted between the control group and all treatment groups in the F0 generation.

Urinalysis – F1 generation cohort 1A

The only changes observed were a statistically significant increase in pH value at all doses in females and an increase in urinary volume at all doses in females, reaching statistical significance at 1,000 mg/kg bw per day.

The author considered this increase as incidental since no dose–response relationship was noted. In addition, all values were in the range of the historical control data of the laboratory. The Panel agreed with the study authors that there were no treatment-related effects.

4.2.4. Endocrine function

Thyroid hormones (T4, T3 and TSH)

Thyroid hormones (T4, T3 and thyroid-stimulating hormone (TSH)) were measured in the F0 generation at necropsy in treatment week 20, in the F1 Pups (at PND 4 and PND 22) and in F1 cohort 1A (at PND 87–92).

In addition, in order to investigate the 24-h circadian variations in hormone levels, an analysis of the variation in T4, T3 and TSH levels were conducted in satellite animals from the F0 control and high-dose groups. Blood from the animals (five animals for each time point) was taken at 2-h intervals pre-dosing (treatment day 14/15) and after 10 weeks of dosing (treatment day 84/85).

F0 generation

According to the authors, there were no test substance-related differences in T4, T3 and TSH levels between the control group and the treatment groups (100, 300 or 1,000 mg E 171/kg bw per day) in either male or female F0 animals.

A slight but statistically significant increase in T3 level was noted in females at the high-dose level (+ 11% compared to control, $p \leq 0.05$). There were no other significant differences in T3, T4 and TSH this or any other dose group.

The mean and individual values from the animals in the control and the three treatment groups were within the same range of values observed in a satellite group monitored for circadian variations of hormones over a 24-h period. The study authors considered the observed change to be incidental and not related to the test substance.

The Panel considered that there were no treatment-related effects.

Satellite – Circadian cycles of thyroid hormones levels

Thyroid hormones are subject to substantial variation during the day, and overall no test substance-related effect was noted between the F0 control and high-dose groups for the examined time point after 10 weeks of treatment.

The Panel agrees with the study authors that there was no treatment-related effect on the circadian variation of thyroid hormones in the F0 high-dose group.

F1 Pups

According to the study authors, there were no test substance-related differences in T4 on PND 4 and T4, T3 and TSH on PND 22) between the control and treatment groups (100, 300 or 1,000 mg E 171/kg bw per day) in either male or female F1 pups.

The Panel agrees with the conclusions of the study authors that there were no treatment-related effects on thyroid hormone levels.

F1 generation cohort 1 A

According to the study authors, there were no test substance-related differences in T3, T4 and TSH at sacrifice at about PND 90 between the control and treatment groups (100, 300 or 1,000 mg E 171/kg bw per day) in either male or female F1 cohort 1A animals.

The study authors reported statistically significantly lower mean T3 levels in low-dose females (–13.2%) compared to controls. The finding was considered incidental as there was no dose relationship. Moreover, all mean and individual values were within the normal range of values associated with the circadian variation. The Panel agrees with the study authors.

Mean TSH was higher than controls in females in all three treatment groups (+ 58.5%, + 120.0% and + 120.4% for 100, 300 and 1,000 mg/kg bw per day, respectively, compared to control), but the increases did not reach statistical significance. According to the authors, lack of statistical significance was due to the variability of the individual values. Furthermore, no dose–response relationship was observed between the intermediate and the high-dose groups.

Although a treatment-related effect on F1 serum TSH levels cannot be excluded, the Panel considered that the observed effect was more likely incidental than a treatment-related effect. This was because the higher mean serum TSH in treated the F1A (cohort 1A, PND 90) was not statistically significant, was within the normal control range and there were no concordant changes in T3/T4 (T3 was slightly but significantly decreased by 13% at 100 mg/kg only, with no significant changes in T4). In addition, there were no group differences in TSH in the F1 pups at weaning (PND 22).

Sex hormones

Fasting serum oestradiol, oestrone and testosterone levels were measured in parental F0 at sacrifice (study week 20), in F1 cohort 1A (PND 86–96), cohort 1B (PND 120–136), cohort 2A (PND 84–90) and cohort 2B (PND 21–23).

In addition, in order to investigate the 24-h circadian variations in hormone levels, oestradiol and testosterone levels were examined in satellite F0 animals from the control and the high-dose groups. Blood was withdrawn at 2-h intervals for 24 h pre-dosing (treatment day 14/15) and after 10 weeks of dosing (treatment day 84/85) (n = 5/time point).

F0 generation

According to the authors, there were no test substance-related differences in serum oestradiol, oestrone and testosterone levels between the control and the treatment groups (100, 300 or 1,000 mg E 171/kg bw per day) in either males or females.

Mean oestrone levels were higher in F0 males at the mid dose (+ 33.5% compared to control, $p \leq 0.01$) and high dose (+47.4% compared to control, not statistically significant).

The study authors considered that these differences were not test substance-related, because the individual oestrone values were in the same range as controls or only marginally higher for the mid dose (control range: 5.8–13.5 pg/mL vs. mid dose: 8.7–14.0 pg/mL).

The authors further noted a single high oestrone level of 36.4 pg/mL for one animal in the high-dose group and considered it as incidental.

The Panel agreed with the study authors, that there were no treatment-related effects on serum oestradiol, oestrone and testosterone levels in F0.

Satellite F0 animals – Circadian cycles of sexual hormone levels

Sex hormones are subject to substantial circadian variation and overall, no test substance-related effects were noted between the control and high-dose groups for any of the examined time points after 10 weeks of treatment.

The Panel agreed with the study authors that there were no treatment-related effects on the circadian range of serum oestradiol and testosterone levels in F0 animals.

F1 generation cohort 1A

The study authors reported no test substance-related differences in serum oestradiol, oestrone and testosterone levels between control and treatment groups (100, 300 or 1,000 mg E 171/kg bw per day) in either male or female F1 cohort 1A animals at sacrifice on about PND 90.

An increase in oestrone levels in males was noted, reaching statistical significance at the high-dose level (+ 14.4%, + 45.0% and + 51.6% compared to control for 100, 300 and 1,000 mg/kg bw per day, respectively). The study authors considered this finding as incidental because the measured values in the mid- and high-dose groups were in the same range as those in the cohort 2A control and low-dose groups of animals of comparable age (about PND 90). The finding was therefore attributed to the inherent variability in the oestrone concentration and not related to the test substance.

The Panel agreed with the study authors' conclusions and additionally noted that the significantly higher mean serum oestrone level seen in 1,000 mg/kg bw per day males of the F1 cohort 1A was an isolated finding, not seen in the cohort 1B; there were no test substance-related effects on serum oestradiol, oestrone and testosterone in F1 cohort 1A.