Mode of action

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86. BPA did not induce gene mutations in bacteria. All the available in vitro studies on chromosomal damage, classified as of high or limited relevance, reported positive results such as increase of CA or MN frequency, in different cellular systems. The increases of BPA-induced chromatid and chromosome breaks observed in some studies (Xin et al., 2015; Santovito et al., 2018; Di Pietro et al., 2020) in association with the induction of DNA strand breaks, detected by comet assay (Xin et al., 2015) are consistent with a clastogenic activity. Moreover, the potential of BPA to affect the spindle integrity and interfere with the chromosome segregation machinery was demonstrated in some reliable studies. Johnson and Parry (2008) reported the formation of aberrant mitotic spindles, with multiple poles, in V79 cells treated with BPA. Altered cytoskeleton

organization, with multipolar spindles, failure of microtubule attachment to the kinetochore with the concomitant activation of SAC and chromosome misalignment, were also observed in HeLa cells (Kim et al., 2019). Studies on spindle morphology of mouse (Yang et al., 2020) and bovine (Campen et al., 2018) oocytes during in vitro maturation reported a pattern of alterations similar to that observed in permanent cell lines, namely shorter and multipolar spindles, with altered kinetochore-microtubule attachment and chromosome misalignment at M II.

87. The conclusion, based on these in vitro studies, is that BPA may act by both clastogenic and aneugenic mechanisms.

88. The large majority (11 out of 12) of the in vitro studies on comet assay, classified as of limited relevance, reported BPA-induced increases of DNA strand breaks. In some studies, the increase of DNA damage was associated with a parallel increase of ROS and MDA and decrease in antioxidant capacity and in total GSH (Xin et al., 2014; Li XH et al., 2017; Huang FM et al., 2018; Yuan et al., 2019; Kose et al., 2020). A study in macrophages reported also a release of cytochrome c from mitochondria along with increased apoptosis with the indication that the DNA strand breaks could be mainly through the oxidative stress-associated mitochondrial apoptotic pathway (Huang FM et al., 2018). In a study on human PBMC, the application of comet assay with the addition of endonuclease III (Nth) and 8-oxoguanine DNA glycosylase (hOGG1) DNA repair enzymes allowed the detection of oxidative damage to DNA bases (Mokra et al., 2018). Further indication of the role of oxidative damage in induction of DNA strand breaks was provided by the protective effects on DNA damage induced by the pre-treatment with NAC (Xin et al., 2014; Huang FM et al., 2018).

89. In conclusion, the evidence of DNA strand breaks in vitro is in agreement with the ability of BPA to induce clastogenic damage. In addition, the studies on comet assay provide consistent evidence that BPA induces DNA strand breaks most probably related to the induction of oxidative stress.

90. The available in vivo studies for BPA-induced chromosomal damage in somatic cells reported mixed results. No increase of CA and MN frequency was reported after a single administration of BPA to mice in a range of doses inducing toxicity at the bone marrow level (Naik and Vijayalaxmi, 2009). In contrast, in another study in mice, increased MN frequency was detected in the presence of high bone marrow toxicity (Fawzy et al., 2018). Positive results were observed in two rat studies (Tiwari et al., 2012; Panpatil et al., 2020) after repeated dose administration, possibly associated with lipid peroxidation and oxidative stress in

the first study. No induction of hyperploidy or polyploidy was observed in these studies.

91. These results indicate that the in vivo induction of chromosomal damage requires specific conditions such as repeated exposure to BPA.

92. Induction of DNA strand breaks, detected by comet assay in vivo, was observed only after repeated exposure for extensive periods of time up to 8 weeks (Tiwari et al., 2012; Zhou YX et al., 2017; Panpatil et al., 2020). Only one study of high relevance was available on single administration of BPA reporting negative results in multiple mouse organs in a range of doses up to the MTD of 500 mg/kg bw (Sharma et al., 2018). An indication of a possible role of the oxidative stress in inducing DNA strand breaks by BPA was provided by the results of several studies (Abdel-Rahman et al., 2018; Fawzy et al., 2018; Kazmi et al., 2018; Majid et al., 2019; Mohammed et al., 2020) showing the protective effects of natural extracts with antioxidant properties. However, these studies were evaluated as low relevance.

93. Finally, studies on germ cells, carried out by four laboratories in the framework of a collaborative project on aneugenic chemicals, did not provide any evidence of increased frequency of aneuploidy in mouse oocytes and zygotes and in sperm cells following exposure to low BPA doses (Pacchierotti et al., 2008).

94. BPA is genotoxic in vitro inducing chromosomal damage and DNA breaks. However, in vivo the evidence of genotoxic properties of BPA is contradictory. This might depend on multiple mechanisms of action described or proposed for BPA. A major difficulty in the interpretation of these contradictory results is the lack of knowledge on the role of BPA metabolism that could be operational in genotoxic activity. Indeed, the role of the proposed DNA adducts has not been clarified. Other uncertainties include the role of ER receptors in the oxidative stress induced by BPA.

Summary of studies

95. Xin et al., 2015: summary in the in vitro gene mutation section.

96. Santovito et al., 2018: summary in the in vitro chromosomal aberrations/micronuclei section.

97. Di Pietro et al., 2020: summary in the in vitro chromosomal aberrations/micronuclei section.

98. Johnson and Parry (2008): summary in the in vitro chromosomal aberrations/micronuclei section.
99. Kim et al., 2019: In vitro effects of BPA (Sigma-Aldrich) on mitotic progression were examined in HeLa cells exposed to 100 nM BPA for 5 h. Proteins involved in mitotic processes were detected by western blot, live cell imaging, and immunofluorescence staining. Under the applied treatment conditions, BPA was shown to disturb spindle microtubule attachment to the kinetochore, with the concomitant activation of spindle assembly checkpoint (SAC). Spindle attachment failure was attributed to BPA interference with proper localization of microtubule associated proteins, such as HURP to the proximal ends of spindle microtubules, Kif2a to the minus ends of spindle microtubules, and TPX2 on the mitotic spindle. BPA also caused centriole overduplication, with the formation of multipolar spindle.

100. Yang et al., 2020: The effect(s) of exposure to BPA (Sigma-Aldrich) on assembled spindle stability in ovulated oocytes were studied. Mature M II oocytes, recovered from the oviducts of superovulated B6D2F1 mice, were cultured for 4 h in the presence of increasing concentrations (5, 25, and 50 µg/mL) of BPA. After treatment oocytes were analyzed by immunofluorescence and live cell imaging to investigate the effect of BPA on spindle dynamics. BPA disrupted spindle organization in a dose-dependent manner, resulting in significantly shorter spindles with unfocused poles and chromosomes congressed in an abnormally elongated metaphase-like configuration, with increased erroneous kinetochore-microtubule interactions.

101. Campen et al., 2018: The aim of the study was to compare the effects of in vitro exposure to either BPA (Sigma-Aldrich) or BPS on meiotic progression, spindle morphology and chromosome alignment in the bovine oocyte. Bovine ovaries were sourced from an abattoir. Groups of 5–20 cumulus–oocyte complexes (COCs) extracted from the bovine ovaries were treated with BPA or BPS at 10 concentrations between 1 fM and 50 µM and underwent to in vitro maturation for 24 h, then the oocytes were extracted. For BPA experiments, a total of 939 oocytes were analyzed for meiotic stage (including 250 vehicle-only control oocytes), of which a total of 767 were at metaphase II (MII) (including 211 MII oocytes in the control) and were included for analysis of spindle and chromosome configuration. Immunocytochemistry was used to label the chromatin, actin and microtubules in the fixed oocytes. The meiotic stage was assessed using immunofluorescence, and the MII oocytes were further assessed for spindle morphology and chromosome alignment (in all MII oocytes regardless of spindle morphology). No difference in the proportion of bovine oocytes that

reached MII was observed for BPA treatment. Significant effect on spindle morphology ($p < 0.0001$) was induced by BPA treatment at very low concentration (1 fM). Fewer oocytes with bipolar spindles were seen following exposure to BPA at concentrations of 1 fM, 10 fM, 100 fM, 10 pM, 1 nM, 10 nM, 100 nM and 50 µM, compared with the control. There was no effect of BPA on spindle morphology at concentrations of 1 or 100 pM. Increased chromosome misalignments were observed at BPA concentrations of 10 fM, 10 nM and 50 µM of BPA, no effect was detected at any other concentration. The study presents limitations: in the ovaries the effects were evaluated in a specific period of development (namely, the 24 h window of oocyte maturation), without considering potential prior historical exposures in vivo.

102. Xin et al., 2014 : summary in the in vitro comet assay section.
103. Li XH et al., 2017: summary in the in vitro comet assay section.
104. Huang FM et al., 2018: summary in the in vitro chromosomal aberrations/micronuclei section.

105. Yuan et al., 2019: summary in the in vitro comet assay section.

106. Kose et al., 2020: summary in the in vitro comet assay section.

107. Mokra et al., 2018: summary in the in vitro comet assay section.

108. Naik and Vijayalaxmi, 2009: summary in the in vitro chromosomal aberrations/micronuclei section.

109. Fawzy et al., 2018: summary in the in vitro chromosomal aberrations/micronuclei section.

110. Tiwari et al., 2012: Summary in the in vitro gene mutation section.

111. Panpatil et al., 2020: summary in the in vivo chromosomal aberrations/micronuclei section.

112. Zhou YX et al., 2017: summary in the in vitro comet assay section.

113. Sharma et al., 2018: summary in the in vivo comet assay section.

114. Abdel-Rahman et al., 2018: The study evaluated the protective action of lycopene (LYC), an antioxidant agent, on the toxic effects of BPA (Sigma-Aldrich). Four groups of seven Wistar rats were treated daily for 30 days via gavage: the first group (controls) received corn oil, the second group was given lycopene at a dose of 10 mg/kg bw, the third group was given BPA at 10 mg/kg bw, the fourth

group was administrated both BPA and LYC at the 10 mg/kg. Rats were sacrificed immediately after the last administration. Liver was frozen at -80 °C. Single-cell suspensions for comet assay were prepared from frozen livers. No positive controls were used. The comet method applied was not reported. A significant ($p < 0.05$) increase of tail DNA % in liver of BPA-treated group with respect to controls (25.05 vs 6.68) was observed. Higher activities ($p < 0.05$) of liver enzymes (serum ALT, alkaline phosphatase (ALP) and GGT and lower levels of total protein and albumin than control rats were detected in serum. Antioxidant enzymes (GPx, SOD and CYPR450 activities) significantly ($p < 0.05$) decreased while MDA level significantly increased in liver of BPA treated animals. Caspase-3 protein in liver of BPA-treated rats is overexpressed. Histopathological analyses showed deleterious hepatic changes ranging from hepatocytes' vacuolization and eccentric nuclei to focal necrosis and fibrosis. LYC administration reduced the cytotoxic effects of BPA on hepatic tissue, through improving the liver function biomarkers and oxidant-antioxidant state as well as DNA damage around the control values.

115. Kazmi et al., 2018: The study evaluated the protective role of Quercus dilatate extracts against BPA (no information on purity) induced hepatotoxicity. Ten groups of SD rats (7 animals/group) were considered, including untreated control group and a group receiving the vehicle. The distilled water-acetone (QDDAE) and methanol-ethyl acetate (QDMEtE) extracts were administered in high (300 mg/kg bw) or low (150 mg/kg bw) doses to SD rats, intraperitoneally injected with BPA (25 mg/kg bw). A group of rats was treated only with BPA. Rats were sacrificed after 4 weeks of treatment and blood and liver were collected. The comet method applied was not sufficiently detailed. An increase of DNA strand breaks in hepatocytes was reported for animals treated with BPA alone. However, the results reported using the different parameters (tail length, % of DNA in tail, tail moment) are not consistent. The % of DNA in tail is 28.35 ± 1.2 in BPA treated animals vs 0.01 ± 0.005 in controls. The value of % of DNA in tail in controls is extremely low with respect to the data reported in the scientific literature. Significant reduction in haemoglobin level, red blood cells and platelet count, whereas elevated levels of white blood cells and erythrocyte sedimentation rate (ESR) were observed in the BPA treated group. Administration of BPA significantly ($p < 0.05$) decreased the endogenous antioxidant enzyme (CAT,GPx, superoxide dismutase (SOD) and GSH) levels compared with control group. In addition, in the BPA treated group, H_2O_2, nitrite and TBARS levels in the hepatic tissue were found to be higher when compared with controls. Histopathological examination of BPA treated animals revealed intense hepatic cytoplasm inflammation, centrilobular necrosis, cellular hypertrophy, fatty degeneration, vacuolization,

steatosis and distortion of portal vein. A dose dependent hepatoprotective activity was exhibited by both the extracts of Quercus dilatate in different extent for the parameters analysed.

116. Majid et al., 2019: The study evaluated the protective role of sweet potato (Ipomoea batatas L. Lam.) against BPA-induced testicular toxicity. Sixteen groups of seven Male SD rats were established, including controls, animals treated with the vehicle, with ethyl acetate and methanol extracts from tuber and aerial part of Ipomoea batatas, with BPA (Merck KGaA) and with BPA and different extracts of Ipomea batatas. The BPA group received 50 mg/kg bw dissolved in 10% DMSO, injected intraperitoneal on alternate days for 21 days. The rats were sacrificed 24 h after the last treatment. Comet assay was applied to evaluate the DNA damage. An average 50–100 cells were analysed in each sample for comet parameters (head length, comet length, tail moment, tail length, and amount of DNA in head) of gonadal cell's nuclei. A statistically significant increase of % DNA in tail (3 folds with respect to the control value) was reported in the group of rats treated with BPA. Endogenous antioxidant enzymes were measured in supernatant from the testicular homogenates: BPA decreased the levels of peroxidases (POD), CAT, SOD. BPA induced also gonadotoxicity measured as size and weight of testes and epididymis, concentration and quality of sperms. The treatment with extracts of Ipomea batatas significantly reduced the gonadotoxicity induced by BPA, the DNA damage and restored the levels of antioxidant enzymes.

117. Mohammed et al., 2020: The study evaluated the protective role of ginger extract (GE) against BPA-induced toxic effects on thyroid. Four groups of 20 male albino rats were treated orally with BPA (Sigma–Aldrich), GE or both once a day for 35 days as follow: Control group: 0.1 ml/rat of corn oil; BPA group: 200 mg/kg bw per day (1/20 of the oral LD50); GE group: ginger extract 250 mg/kg bw; BPA + GE group: ginger extract followed by BPA after 1 h with the same doses as the other groups. The animals were sacrificed 24 h after the last administration. DNA damage was evaluated by comet assay. A statistically significant increase of DNA damage expressed as tail % DNA, tail length and tail moment were shown in thyroid follicular cells of animals treated with BPA. A concurrent increase of MDA and a decrease of GSH, and SOD were also observed. Adverse effects on the thyroid gland were reported with a significant decrease in serum levels of T3 and T4 accompanied by a significantly increase in serum TSH level. A decrease of Nrf-2 mRNA relative expression and protein concentration and of HO-1 mRNA expression in the BPA-induced thyroid injured rats were also described. The histopathological analysis revealed an alteration of the thyroid gland follicles most of which containing scanty colloid secretion and some others atrophied. The

treatment with GE significantly reduced the genotoxic damage and the alteration of thyroid hormones regulating genes.

118. Pacchierotti et al., 2008: The study evaluated the potential aneugenic effects of BPA on mouse male and female germ cells and bone marrow cells following acute, subacute or subchronic oral exposure. For experiments with acute and subacute exposure, female C57BL/6 mice were treated by gavage with BPA (from Sigma-Aldrich) dissolved in corn oil once with 0.2 and 20 mg/kg bw, or with seven daily administrations of 0.04 mg/kg bw. In subchronic experiments, mice received BPA in drinking water at 0.5 mg/L for 7 weeks. The dose levels tested for subacute effects in bone marrow and male germ cells were 0.002, 0.02 and 0.2 mg/kg bw for 6 days. For the assessment of aneugenicity in female germ cells, M II oocytes were harvested 17 h after induced superovulation, and cytogenetically analyzed after C-banding. The percentages of metaphase I-arrested oocytes, polyploid oocytes and oocytes that had undergone Premature Centromere Separation (PCS) or Premature Anaphase II (PA) were calculated. To evaluate the aneugenic effects of BPA upon the second meiotic division, zygote metaphases were prepared from superovulated females mated with untreated C57Bl/6 males. Zygote metaphases were prepared, C-banded and cytogenetically analyzed for the occurrence of polyploidy and hyperploidy. Experiments on male germ cells were performed with 102/ElxC3H/El)F1 males. Epididymal sperms were collected and hybridized with fluocrochrome-labelled DNA probes for chromosomes 8, X and Y and 10,000 sperm per animal were analyzed to evaluate the incidence of hyperhaploid (X88, Y88, XY8) and diploid (XY88, XX88, YY88) sperm cells. Micronucleus test was performed with four groups of five (102/ElxC3H/El)F1 male mice treated with 0, 0.002, 0.02 or 0.2 mg/kg BPA by gavage on 2 consecutive days and sacrificed 24 h after the second administration. In total, 2000 PCE from two slides were scored per animal for the presence of MN. No significant induction of hyperploidy or polyploidy was observed in oocytes and zygotes at any treatment condition. The only detectable effect was a significant increase of M II oocytes with prematurely separated chromatids after chronic exposure; this effect, however, had no consequence upon the fidelity of chromosome segregation, as demonstrated by the normal chromosome constitution of zygotes under the same exposure condition. Similarly, with male mice no induction of hyperploidy and polyploidy was shown in epididymal sperm after six daily oral BPA doses, and no induction of MN in PCE.