Weight of evidence

In this guide

In this guide

Gene mutations in vitro and in vivo

In vitro gene mutation

16. Of the six available studies of the mutagenicity of BPA in bacteria, only one describes the application of the Ames test in a comprehensive battery of Salmonella Typhimurium strains (TA1535, TA97, TA98, 11407 TA100 and TA102) at a range of concentrations up to 5000 µg/plate. It reports negative results both in the presence and absence of metabolic activation (Xin et al., 2015).

17. Three studies reported negative results in TA98 and TA100 (Masuda et al., 2005; Fic et al., 2013; Zemheri and Uguz, 2016). A study shows negative results in TA98, TA100 and TA102 strains (Tiwari et al., 2012). The sixth used the bacterial SOS/umuC assay with a range of concentrations from 1 to 1000 µg/L in presence and absence of S9 mix. It also reported negative results (Balabanič et al., 2021). The CEP Panel concluded that BPA does not induce gene mutations in bacteria.

Summaries of studies

18. Summary of Xin et al study 2015: The study evaluated the cytotoxic, genotoxic and clastogenic activity of BPA (purity 99%) in Chinese hamster ovary cells (CHO) cells and its mutagenicity in the Ames test. The battery of assays applied in CHO cells included the MTT assay for the evaluation of cytotoxicity, and the comet, micronucleus and chromosome aberration tests. In the Ames test, BPA (10-5000 µg/plate) was uniformly negative in all Salmonella Typhimurium strains (TA1535, TA97, TA98, TA100 and TA102), with and without metabolic activation. Exposure of CHO cells to four BPA doses (40, 80, 100 and 120 µM) for 12 and 24 h resulted in a significant decrease in cell viability (at 80 µM and above), which however remained above 50% in all cases; a concentration-related increase of DNA damage was observed in comet assay [increased Olive tail moment (OTM), tail length and % tail DNA, statistically significant at all doses] after 12 and 24 h exposure to BPA; after 24 h treatment, an increase in micronuclei (MN) (statistically significant at 100 and 120 µM) and structural chromosomal aberrations (chromatid breaks and chromosome fragments, statistically significant at 80 µM and above) was also observed.

19. Summary of Masuda et al., 2005: The study evaluated the mutagenicity of BPA in Ames test in the presence or absence of S9-mix. BPA (Tokyo Kasei Kogyo Co., Ltd) was tested on S. Typhimurium strains TA98 and TA100 at the single dose of 0.1 µmole/plate (100 µL of 1 mM solution). No mutagenic effect was observed.

20. Summary of Fic et al., 2013: In this study the mutagenic and genotoxic potential of eight BPA (purity >99%) structural analogues [BPF, BPAF, bisphenol Z (BPZ), BPS, bis(4-hydroxy-3-methylphenyl)propane (DMBPA), 4,4’-sulfonylbis(2-methylphenol) (DMBPS), [sulphonylbis(benzene-4,1-diyloxy)]diethanol (BP-1), and 4,4’-sulphanediyldiphenol (BP-2)] were investigated using the Ames and comet assay. None of these bisphenols were mutagenic in Salmonella Typhimurium strains TA98 and TA100 either in the presence or absence of external S9-mediated metabolic activation (Aroclor 1254-induced male rat liver). Potential

genotoxicity of bisphenols was determined in the HepG2 human hepatoma cell line following 4-h and 24-h exposure to non-cytotoxic concentrations 0.1 µmol/L to 10 µmol/L. In the comet assay, BPA and its analogue BPS induced significant DNA damage only after the 24-h exposure, while analogues DMBPS, BP-1, and BP-2 induced a transient increase in DNA strand breaks observed only after the 4-h exposure. BPF, BPAF, BPZ, and DMBPA did not induce DNA damage.

21. Summary of Zemheri and Uguz, 2016: The study evaluated the mutagenicity of BPA (Merck) in a limited Ames test, using two tester strains (TA98 and TA100) and four dose levels (0.1, 1, 10 and 100 µg/plate). The results were negative, with and without metabolic activation.

22. Summary of Tiwari et al., 2012: The study evaluated the mutagenicity of BPA in Ames test. BPA (purity 99%) was tested at concentrations from 6.25 to 200 µg/plate on different strains of S. Typhimurium (TA 98, TA 100 and TA 102). The mutagenic response was not observed in any of the tester strains at the various concentration of BPA in absence of S9 fractions. A slight increase in the numbers of revertants was observed in the presence of S9 fractions from the 6.25 - 25 µg/plate of BPA in each strain, but the increase was statistically significant only in strain TA 102 at 25 µg/plate.

23. Summary of Balabanič et al., 2021: The study evaluated cytotoxic and genotoxic effects of some endocrine disrupting chemicals (EDCs), including BPA, which have been previously identified in effluents from two paper mills. BPA (Sigma-Aldrich) tested at concentrations of 1, 10, 100, 1000 µg/L with the bacterial SOS/umuC assay in S. Typhimurium TA1535/pSK1002 strain did not induce toxic nor genotoxic effects in the presence or absence of S9 metabolic activation. The compound was also assessed in HepG2 cells with MTT assay for cell viability and with comet assay at 1, 10, 100 and 1000 µg/L for 4 and 24 h. No significant reduction of the viability. A statistically significant concentration-dependent increase of DNA damage, expressed as percent of DNA in tail, was reported starting from 10 µg/L.

In vivo gene mutation

24. No studies on gene mutation assays in mammalian cells following the OECD guidelines were available.

Induction of chromosomal aberrations/micronuclei in vitro and in vivo

In vitro chromosomal aberrations/micronuclei

25. Fifteen in vitro studies of micronuclei (MN) and structural chromosomal aberrations (CA) induction in different cell lines were available for evaluation. Of these, nine were further considered in the assessment, classified as having high (1 study) or limited relevance (8 studies).

26. All showed positive results in both blood cells and established cell lines. In the single study classified as of high relevance, a concentration-dependent increase of MN frequency over a wide range of concentrations (1.5 to 37 µg/ml corresponding to 6.6 µM and 162 µM) was observed in the AHH-1 human lymphoblastoid cell line (Johnson and Parry, 2008). Positive CA results were also reported from cultures of human peripheral lymphocytes in two studies with limited relevance (Santovito et al., 2018; Di Pietro et al., 2020). In one of these (Santovito et al., 2018), MN frequency was also measured. A study of MN in bovine peripheral blood lymphocytes also reported positive findings (Šutiaková et al., 2014).

27. In murine macrophage RAW264.7 cells, positive MN results were associated with an increase in reactive oxygen species (ROS), and a decreased level of antioxidant enzymes (GPx, SOD and CAT. Concomitant phosphorylation of P53 and release of cytochrome C from mitochondria were detected along with increased apoptosis. Pretreatment with N-acetylcysteine (NAC) reduced BPA-induced cytotoxicity, apoptosis and genotoxicity (MN frequency was reduced by 30%). These results indicate that the toxic effect of BPA in macrophages was mainly through the oxidative stress-associated mitochondrial apoptotic pathway (Huang FM et al., 2018).

28. Finally, two studies in the Chinese hamster ovary (CHO) and V79 cell lines reported positive results (Xin et al., 2015; Yu et al., 2020). Xin and co-workers reported a concentration dependent increase of both MN and CAs in CHO cells in the absence of metabolic activation. In contrast, the BPA-induced increase in MN frequency in V79, reported by Yu and colleagues, apparently required CYP1A1 and CYP1B1 expression.

29. Overall, the significant increases of chromatid and chromosome breaks observed in several studies in vitro indicated that BPA has clastogenic activity also at non-cytotoxic concentrations. Two reports indicated that oxidative stress is implicated in the observed induction of chromosomal damage. In addition, Johnson and Parry (2008) reported the formation of aberrant mitotic spindles, with multiple poles, in cells treated with BPA.

30. In conclusion, the in vitro studies on CA and MN induced by BPA indicated that both clastogenic[1] and aneugenic[2] mechanisms may operate.

Summary of studies

31. John and Parry 2008: In this mechanistic study the aneugenicity of two known spindle poisons model compounds, namely rotenone and BPA, has been investigated following low dose-exposure to mammalian cells, using the cytokinesis blocked micronucleus assay (CBMA) and immunofluorescence methods to visualize modifications of the microtubule organizing centres (MTOCs) of the mitotic spindles. For induction of MN BPA (Sigma-Aldrich) was added over a range of narrowed low concentrations (1.5, 3.1, 6.2, 7.7, 9.2, 10.8, 12.3, 18.5, 24.6, and 37.0 µg/ml) to cultures of human (AHH-1) lymphoblastoid cell line for a complete cell cycle (22-26 h dependent upon any cell cycle delay) in the presence of cytochalasin-B. A minimum of five separate experiments were performed. A concentration-related and statistically significant increase of binucleate-micronucleated cells from 12.3 µg/mL was reported with a clear threshold for induction of MN (NOEL at 10.80 µg/mL and LOEL at 12.3 µg/mL). A NOEL and LOEL for percentage of binucleate cells was also observed at 9.2 µg/mL and 10.8 µg/mL BPA respectively. For mechanistic evaluation of the aneugenic effects of BPA, fluorescently labelled antibodies were used to visualize microtubules (α-tubulin) and MTOCs (γ-tubulin) in V79 culture. BPA in this case was added to V79 cells growing on sterile glass microscope slides placed in Petri dishes at concentrations 4.2, 4.9, 5.6, 7.0, 8.4, 9.8, 11.2 and 14 µg/mL for 20 h (i.e. one cell cycle for V79). Similarly for induction of aberrations in the mitotic machinery a NOEL was observed at 7.0 µg/mL and a LOEL at 8.4 µg/mL BPA in V79 cells. Aberrant mitotic divisions, in the form of multiple spindle poles were detected and it was suggested by the study authors to be the mechanism for the production of chromosome loss into MN.

32. Santovito et al., 2018: In this study the possible induction of chromosomal damage by BPA (Sigma-Aldrich) was tested in human peripheral blood lymphocytes cultures applying the CA assay and the micronucleus test (MN). Cell cultures were exposed to a range of concentrations from 0.01 to 0.20 µg/mL, (including the reference dose established by United States Environmental Protection Agency (US EPA) (0.05 µg/mL), the tolerable daily intake established by European Union (0.01 µg/mL) and the highest concentration of unconjugated BPA found in human serum (0.02 µg/mL)) for 24 h for the chromosomal aberration test and for 48 for the micronucleus test. A statistically significant increase of cells with structural chromosomal aberrations, with a prevalence of chromatid breaks,

was reported starting from 0.05 µg/mL; no numerical aberration was observed. A concentration related increase in MN frequency was detected starting from 0.02 µg/mL in which a four-fold increase with respect to the control level was observed.

33. Di Pietro et al., 2020: The study investigated the effects of BPA exposure on cell proliferation, cell cycle progression and DNA damage in human peripheral blood mononuclear cells (PBMC) and the BPA-induced neurotoxicity in rats exposed to environmental relevant doses of BPA during development. Human PBMC from five unrelated healthy donors (adult males and females) were cultured and treated with BPA (Merck) from 5 nM to 200 µM. The treatment with BPA of unstimulated resting PBMC did not affect cell proliferation (determined by the colorimetric MTT) at all the concentrations tested except for 200 µM for which a marked inhibition of cell proliferation was observed at 24 and 48 h after the treatment. By contrast, in PHA-stimulated cells, BPA caused a pronounced increase of cell growth starting from 10 nM to 100 nM and a concentration-dependent decrease of cell proliferation from 25 to 200 µM. The cell cycle was analyzed by flow cytometry. BPA at 50 nM increased the percentage of cells in S phase of the cell cycle at 24 h and this effect was higher at 48 h with an increase of about 17% of cells in the S phase compared with the control. At 100 µM, BPA induced a significant increase of the percentage of cells in the G0/G1 phase, suggesting that BPA affected cell growth in a non-monotonic way. BPA-treatment at 25, 50 and 100 nM for 48 h induced a significant increase ($p < 0.001$) of both the percentage of aberrant cells (about 20% at 100 nM) and structural aberrations (about 27% at 100 nM) including chromatid and chromosome breaks, rings and fragments. BPA also increased significantly the percentage of highly fragmented metaphases (shattered cells). In PHA-stimulated PBMC treated with BPA (50 nM) for 24 h, γH2AX was significantly increased in CD3+ T lymphocytes and was also detected in a higher proportion of CD8+ T lymphocytes than the CD4+ T lymphocytes and a slight percentage of γH2AX was reported among the B cells. The treatment of PHA-stimulated PBMC with BPA (50 nM) induced p21/Waf1 and PARP1 protein expressions approximately within the same time interval. These findings suggest that BPA could affect the p53-p21/ Waf1 checkpoint and PARP1 levels resulting in DNA damage repair defects. BPA (50 nM) for 24 h modulated the expression of ER-α and ER-β in both sexes inducing or inhibiting its expression in males and in females with effects similar to the variations induced by pharmacological concentrations of E2 (100 nM). The study investigated also the BPA-induced neurotoxicity in terms of DNA damage. After the coupling period, three females/group received BPA (0.1 mg/L), or vehicle (ethanol 0.1 mL/L) in the drinking water during gestation, lactation and weaning of their offspring. Five

females and three males pups from BPA-exposed mothers and five females and three males newborns from vehicle-treated dams were then sacrificed at PND 17. BPA was shown to induce ɣH2AX phosphorylation in cells possessing immune function in the CNS, such as microglia and astrocytes of rat hippocampus. In BPA-exposed rats a marked decreasing trend of ERα expression was found therefore proposing a role for this receptor in the effects induced by BPA.

34. Šutiaková et al., 2014: The study evaluated the genotoxic and cytotoxic effects of BPA (Sigma-Aldrich) on bovine peripheral lymphocytes in vitro. Lymphocyte cultures from two animals were exposed to four different concentrations of BPA (1×10−4, 1×10−5, 1×10−6 and 1×10−7 mol. L−1) 24 h after stimulation by L-phytohemagglutinin, and incubated for total 72 h. Micronucleus frequency was determined using the cytokinesis block method, adding 6 µg/mL cytochalasin B at 44 h. A significant increase in the number of MN ($p = 0.018$) was observed at the highest concentration of BPA; at lower concentrations micronucleus frequency was not significantly different from vehicle (DMSO) control. The nuclear division index (NDI) was not affected by BPA treatment at any concentration level.

35. Huang FM et al., 2018: The study reported positive results for induction of DNA strand breaks (evaluated by comet assay) and MN frequency in murine macrophage RAW264.7 cells. Cell cultures were treated at 0, 3, 10, 30, and 50 µM of BPA (Sigma-Aldrich) dissolved in DMSO for 24 h. Concentration-dependent increase of tail length, based on the analysis of 50 cells/slide, and of MN frequency by the evaluation of 1000 binucleated cells per concentration were observed. No positive controls were used. The genotoxic effects were observed starting from 10 µM and were associated with an increase of reactive oxygen species (ROS), measured by Dichlorofluorescein Diacetate Assay (DCFH-DA) and a decrease of antioxidant enzymes, including GPx, SOD and CAT. Concomitant phosphorylation of P53 and release of cyto C from mitochondria into cytosol were reported. A reduced expression of antiapoptotic proteins BCL2 and BCL-XL significant from 10 and 3 µM respectively and an increase of the expression of proapoptotic proteins BAX, BID, and BAD beginning at 10, 10 and 30 µM respectively were observed in a concentration-dependent manner. Increased level of the apoptosis-inducing factor (AIF) in the nucleus and a decrease in the mitochondria was detected. Expression of pro-caspase-3 and pro-caspase-9 is reduced by BPA in a concentration-dependent manner and PARP-1 cleavage was induced by BPA. Pre-treatment of the cell cultures with N-acetylcysteine (NAC), a cysteine precursor of the antioxidant glutathione, at the concentration of 10 µM for 30 min reduced BPA-induced cytotoxicity, apoptosis, and genotoxicity. The

results of this study indicates that the toxic effects induced by BPA in macrophages was mainly through oxidative stress-associated mitochondrial apoptotic pathway.

35. Xin et al 2015: See summary in the in vitro gene mutation section.

36. Yu et al 2020: In this study, induction of MN and double-strand DNA breaks by BPA, BPF, and BPS were investigated in Chinese hamster V79-derived cell lines expressing various human CYP enzymes and a human hepatoma (C3A) (metabolism-proficient) cell line. In a first step a prediction of BPA, BPF, and BPS as potential substrates for several human CYP enzymes, which are commonly involved in the metabolic activation of compounds, was conducted by molecular docking. The results of the analysis showed a similar affinity of the compound with all the enzymes tested: CYP1A1, 1A2, 1B1, 2B6, 2E1, and 3A4. BPA (99.6% analytical purity) tested at 40, 80 and 160 µM for 9 h, followed by 15 h of recovery induced a concentration related increase of MN frequency in V79-hCYP1A1. In V79-hCYP1B1 cells MN were observed only at the two highest concentrations. No induction of MN was reported in V79-Mz, V79-hCYP1A2, V79-hCYP2E1, or V79-hCYP3A4-hOR cells. A consistency with the results of the molecular.

In vivo chromosomal aberrations/micronuclei

37. Eleven in vivo studies addressing BPA-induced MN and structural CA after oral exposure were evaluated. After a screening for the reliability and relevance of the results, six studies from four publications, all ranked as of limited relevance, were selected for further consideration (Table 1). Of these, three studies were considered positive for the induction of MN and CA in the same publication (Tiwari et al., 2012) or of MN (Panpatil et al., 2020) in rats following daily oral BPA administrations for 6 and 28 days, respectively. Tiwari et al. (2012) applied a range of doses from 2.4 µg up to 50 mg/kg bw per day. In a separate publication, the same authors (Tiwari and Vanage, 2017) reported that these experimental conditions were associated with the induction of lipid peroxidation (malonaldehyde, MDA) and oxidative stress (decreased SOD, CAT, GSH) in rat bone marrow and peripheral blood lymphocytes. In Panpatil et al. (2020) the dose range was much lower (50 and 100 µg/kg bw per day). A fourth study tested positive in the mouse bone marrow MN test after the administration of a daily dose of 50 mg/kg bw for 28 days in presence of high level of cytotoxicity (Fawzy et al., 2018). A study by Naik and Vijayalaxmi (2009) reported negative findings in the mouse bone marrow MN test and CAs following a single dose in the range 10

to 100 mg/kg bw.

38. Overall, the available data provided evidence of chromosomal damage after multiple oral administrations but not after single oral administration of BPA.

Table 1. Summary table of test results of MN and CAs in vivo studies.

Test System	Dose	Results	Reference
MN and CA in bone marrow Swiss albino mice 6 animals /group	10, 50 and 100 mg/kg bw, single dose by gavage; 10 mg/kg for 5 days (50mg by gavage	**Negative** No significant decrease of PCE/NCE ratio but significant increase of gaps and C mitoses.	Naik and Vijayalaxmi, 2009
MN in bone marrow Holtzman rats 10 animals /group	2.4µg, 10 µg, 5 mg snf 50 mg/kg bw per day orally for 6 days	**Positive** Dose related increase of CA and MN PCE starting from 10 µg	Tiwari et al., 2012
MN in bone marrow Male Swiss albino mice 10 animals /group	50 mg/kg bw per day orally for 28 days	**Positive** Significant reduction in the ratio of PCE/NCE	Fawzy et al., 2018
MN in bone marrow Male Wistar rats 6 animals / group	50 and 100 µg/kg/bw per day orally for 28 days	**Positive** Dose related increase of MDA in blood and of urinary 8OHdG	Panpatil et al., 2020.

Source: Re-evaluation of the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs, EFSA, (2021)

Summary of studies

40. Tiwari et al., 2012: This study was aimed to assess potential genotoxic effects of BPA (Sigma-Aldrich) in rats (five males and five females per group) following oral administration of test compound once a day for 6 consecutive days at dose-levels of 2.4 µg, 10 µg, 5 mg and 50 mg/kg bw by measuring induction of MN and structural chromosome aberrations in bone marrow cells and primary DNA damage in blood lymphocytes using single cell gel electrophoresis (comet assay). Furthermore, plasma concentrations of 8-hydroxydeoxyguanosine (8-OHdG), lipid peroxidation and glutathione activity were evaluated to assess potential induction of oxidative DNA damage. Results obtained for genotoxicity endpoints show marked dose-related increases of both MN and structural chromosome aberrations in bone marrow cells of male and female rats exposed to BPA. The observed increases achieved statistical significance at dose-levels as low as 10 µg/kg bw per day. Similarly, primary DNA damage evaluated by comet assay, in isolated peripheral blood lymphocytes showed marked and dose-related increases that were statistically significant at dose-levels as low as 10 µg/kg bw per day. Significant increase in plasma concentration of 8-OHdG was detected only at 50 mg/kg bw. A dose-related increase of malonaldehyde and decrease of glutathione were observed in liver.

41. Panpatil et al., 2020: The study evaluated the protective action of turmeric acid on the genotoxic effects of BPA in Wistar rats. Six groups of six animals were administered with BPA (Sigma-Aldrich) at 0, 50 and 100 µg/kg by oral gavage for a period of 4 weeks: three groups were fed with a normal diet, the others with a diet containing 3% turmeric. At the end of the experiment the animals were sacrificed. Urine was collected 24 h before the sacrifice. 8-OHdG was measured in urine using an ELISA kit. DNA damage by comet assay was evaluated in blood, liver and kidney: 50 cells per slide were counted twice. Micronucleus assay was applied in bone marrow: 2000 PCE were evaluated. A weak but statistically significant and dose related increase of tail length was observed in liver. In kidney an increase of DNA damage was observed only at the dose of 50 µg/kg. A dose related increase of 8-OHdG in urine and of the concentration of MDA in blood serum was observed. A dose related increase of MNPCE was reported associated with a low decrease of the PCE/NCE ratio. A significant decrease of the genotoxic effects was observed in animal fed with diet with turmeric.

42. Tiwari and Vanage, 2013: This study investigated the induction by BPA of dominant lethal mutations in the different stages of spermatogenesis in the rat. Furthermore, the induction of DNA damage by BPA in epididymal sperm was investigated. Holtzman male rats (7 per group) were treated by oral gavage with BPA (Sigma Chemical Co.) dissolved in ethyl alcohol and diluted in sesame oil, at dose-levels of 10 µg/kg bw and 5 mg/kg bw once a day for 6 consecutive days. Negative controls were treated with vehicle. Each treated male was mated with two females per week over a period of eight weeks. The mated females were then sacrificed on the day 15th of their gestation and uterine content examined. DNA damage in epididymal sperm was evaluated by alkaline comet assay in sperm samples from treated males (4 animals per group) sacrificed after completion of the mating phase. In the dominant lethal study, a significant decrease in total implants/female and live implants/female, with a concurrent significant increase in the number of resorbed embryos per female, was observed during the fourth week and sixth week in females mated with males treated with 5 mg BPA/kg bw, suggesting the induction of post-implantation loss due to dominant lethal mutations in mid-spermatids and spermatocytes. No significant change was observed in the pre-implantation and post-implantation losses in pregnant female mated with males exposed to 10 µg/kg bw of BPA. In the comet assay with epididymal sperm, a significant increase in comet parameters (tail length, tail moment and % tail DNA) was observed in rats treated with 5 mg/kg bw compared with control.

43. Fawzy et al., 2018: The study was conducted to evaluate the protective action of pumpkin seed oil (PSO) against adverse effects induced by BPA. BPA (Sigma-Aldrich) was administered orally to male Swiss albino mice at 50 mg/kg bw once a day for 28 days. PSO was administered at 1 mL/kg bw either before, with or after treatment of BPA, for 28 days. Seven groups of animals (n = 10) were treated: group 1 (control); group 2 (vehicle); group 3 (PSO); group 4 (BPA); group 5 (PSO before BPA); group 6 (PSO with BPA) and group 7 (PSO after BPA). DNA damage was evaluated by comet assay in liver and testes. Fifty randomly selected nuclei per experimental group were analysed. MN frequencies were evaluated in bone marrow. Two thousand polychromatic erythrocytes (PCE) were scored per animal. A significant ($p<0.05$) increase of tail DNA % in liver and testes of BPA-treated group with respect to controls (19.93 ± 0.68 vs 13.15 ± 0.22 and 23.56 ± 0.45 vs 15.00 ± 0.50) was observed. A significant increase of MNPCEs (66.40 ± 9.94 vs 10.40 ± 2.96) and a decrease in the ratio of PCE/NCE were also detected. The histopathological examination revealed hepatocyte vacuolar degeneration with many necrotic cells. A defective spermatogenesis was also observed characterized by severe necrosis and loss of the spermatogonial layers

with multiple spermatid giant cells formation in most of the seminiferous tubules and a congestion of the interstitial blood vessels. The treatment with PSO reduced the genotoxic effects induced by BPA. PSO before BPA treatment was the best regimen in the alleviation of the adverse effects.

44. Naik and Vijayalaxmi, 2009: This study evaluated potential genotoxic effects of BPA by induction of chromosomal aberrations and MN in bone marrow cells of Swiss albino mice. To assess for potential interference of BPA with mitotic spindle apparatus, induction of c-mitoses was also performed. BPA (Loba Chemie, Mumbai, India) was administered orally in a 2% acacia gum suspension at dose-levels of 10, 50 and 100 mg/kg bw to groups of three male and three female mice, as single acute dose. Cumulative dose-level experiments were also performed at the lowest (10 mg/kg bw) dose-level for five consecutive days. In single treatment schedule, sampling of bone marrow was performed at 6, 24, 48 and 72 h from beginning of treatment for both micronucleus and chromosome aberration assays. In cumulative treatment schedule, bone marrow was sampled in both assays 24 h after the last administration of BPA. For induction of c-mitoses, the same dose levels used for micronucleus and chromosome aberration assays were applied as single dose and sampling of bone marrow was performed at 2, 6, 12, 24, 48 and 72 h. Results showed that no significant increases of chromosomal aberrations or MN were induced at any dose-level and sampling time used. Conversely, significant increases in the frequencies of gaps were observed in all dose-levels assayed at the 48 and 72 h sampling time and at the two higher dose-levels (50 and 100 mg/kg bw) at the 24 h sampling time. The significant increases of achromatic lesions (gaps) are not considered relevant for clastogenicity. In addition, BPA also induced c-mitotic effects through increases of mitotic indices and decrease in anaphase for both higher dose-levels at 24, 48 and 72 h sampling times.

Comet Assay

In vitro comet assay

45. Twenty-two in vitro studies using a comet assay in different cell lines were available for evaluation. Twelve were classified as of limited relevance and further considered in the assessment. Most cell lines used in these studies were of human origin from blood, mammary gland and prostate. Rodent cell lines from rat, mouse and hamster and one cell line from monkey were also considered.

46. Eleven of the 12 studies reported positive results. Three studies on HepG2 cell line yielded both positive (Li XH et al., 2017); Balabanič et al., 2021) and negative (Fic et al., 2013) results. In a non-tumorigenic human prostatic cell line, BPA induced a significant increase in DNA strand breaks paralleled by a decrease in total GSH, antioxidant capacity, glutathione peroxidase 1 (GPx1) and SOD activity and an increase in glutathione reductase (Kose et al., 2020). Positive results were also reported in CHO cells (Xin et al., 2015). Positive results were reported from two studies in which human PBMC were analysed by both alkaline and neutral comet assays (Mokra et al., 2017). Evidence of oxidative damage to DNA bases was provided by the addition of endonuclease III (Nth) and 8-oxoguanine DNA glycosylase (hOGG1) DNA repair enzymes (Mokra et al., 2018). DNA strand breaks induction by BPA was associated with increased ROS, MDA and reduced SOD activity in HepG2 (Li XH et al., 2017). In murine macrophage RAW264.7 cells, positive DNA strand breaks were associated with an increase in ROS and decreased level of antioxidant enzymes (Huang FM et al., 2018). In Marc-145 rhesus monkey embryo renal epithelial cells, DNA strand breaks induction was associated with increased ROS and TBARS and decrease in GSH and SOD activity (Yuan et al., 2019).

47. DNA strand breaks induction in mouse embryonic fibroblast cell line (NIH3T3) is associated with elevated ROS and a modest increase in DNA 8-hydroxy-2′-deoxyguanosine (8-OHdG) at the highest concentration tested (Chen et al., 2016). In rat INS-1 insulinoma cells DNA strand breaks and ROS level increased in parallel along with the induction of DNA damage-associated proteins (p53 and p-Chk2). At the highest concentration of 100 µM, pre-treatment with NAC reduced the number of induced DNA strand breaks by two-fold (Xin et al., 2014). Finally, ER-positive MCF-7 cells were more sensitive than ER-negative MDA-MB-231 cells to BPA-induced DNA damage, as measured by comet assay (Iso et al., 2006).

48. The available in vitro studies provided evidence that BPA induces DNA strand breaks most likely related to the induction of oxidative stress.

Summary of studies

49. Li XH et al., 2017: The study investigated the cytotoxic effects and oxidative stress induced by BPA (Sigma-Aldrich) alone and in combination with dibutyl phthalate (DBP) or cadmium (Cd) in vitro in HepG2 cells. The cell cultures were exposed for a period of 6 h to a range of concentrations of the single substances ensuring a cell viability above 50%. BPA tested from 10-8 to 10-4 mol/L for 6

hours induced a concentration dependent increase of reactive oxygen species (ROS), measured by DCFH-DA, and malondialdehyde (MDA) level and a decreased activity of SOD. An increase of DNA strand breaks (up to eight- fold with respect to the control value) applying the comet assay, was detected after BPA treatment at 10-8, 10-7, 10-6 mol/L for 24 h without a clear concentration response. The co-exposure treatments (BPA and DBP or BPA and Cd) showed higher ROS and MDA levels and lower SOD activity than the mono-exposure treatments. The combined treatments with BPA and Cd had stronger DNA damage effect.

50. Balabanič et al., 2021: See summary in the in vitro gene mutation section.

51. Fic et al., 2013: See summary in the in vitro gene mutation section.

52. Kose et al., 2020: This study investigated the relative toxicity, potential oxidative stress and genotoxicity induced by BPA (>99% purity), BPS and BPF on the RWPE-1 non-tumorigenic prostatic cell line. RWPE-1 cells were incubated with BPA at concentrations of 50–600 µM for 24 h exposure. The IC20 and IC50 values, concentrations that causes 20 and 50% of cell viability loss, after a 24 exposure to BPA were 45 and 113.7 µM. BPA induced significant decreases in the activities of glutathione peroxidase (GPx1) and SOD, an increase in glutathione reductase and total GSH and a decrease in total antioxidant capacity. At a single concentration (IC20), BPA produced significantly higher levels of DNA damage vs the control both in the standard (2.5-fold increase) and Fpg-modified comet assays. No changes in the mRNA levels of p53 and the OGG1, Ape-1, DNA polymerase β base excision repair (BER) proteins were induced by BPA. The single exception was a small decrease in the expression levels of MYH expression.

53. Xin et al study 2015: See summary in the in vitro gene mutation section.

54. Mokra et al., 2017: The study reported concentration-related induction of DNA single and double strand breaks (detected with alkaline and neutral comet assay) by BPA (Sigma-Aldrich) and its analogues, BPS, BPF and BPAF in human peripheral blood mononuclear cells (PBMC) treated in the concentrations ranging from 0.01 to 10 µg/mL after 1 and 4 h treatment. No significant decrease of cell viability, evaluated using calcein-AM/PI stains, was observed at the concentrations tested for DNA damage. After 1 h incubation, BPA caused statistically significant increase in DNA strand breaks at 0.1 mg/mL. The highest effects were induced by BPA and BPAF, which produced single strand breaks starting from 0.01 µg/mL, while BPS caused the lowest effect at 10 µg/mL after 4 h of exposure. Statistically significant increases of DNA double strand breaks were induced by BPA at concentrations of 1 µg/mL and 10 µg/mL after 1 h incubation and at 0.1 µg/mL

and 1 µg/mL after 4 h incubation. The strongest effect was observed with BPAF. DNA repair was also evaluated at different times (30, 60 and 120 min) after the treatment with BPA at 10 µg/mL. A significant decrease of the DNA damage was observed at 60 min, but the repair was not complete after 120 min.

55. Mokra et al., 2018: The study reported that BPA (Sigma-Aldrich) and its analogues, BPS, BPF and BPAF caused oxidative DNA damage to purine and pyrimidines in human peripheral blood mononuclear cells (PBMC) treated at concentrations of 0.01, 0.1 and 1 µg/mL for 4 h and 0.001, 0.01 and 0.1 µg/mL for 48 h. BPA was dissolved in ethanol. No significant decrease of cell viability, evaluated using calcein-AM/PI stains, was observed at the concentrations tested. DNA damage was detected with alkaline comet assay coupled with repair enzyme endonuclease III (Nth) and 8-oxoguanine DNA glycosylase (hOGG1). Statistically significant and concentration related oxidative damage to purines (from 0.01 µg/mL) and to pyrimidines (from 0.1 µg/mL) was reported after 4 h treatment. After 48 h treatment significant damage to purine was observed from 0.001 µg/mL and to pyrimidines from 0.01 µg/mL. Statistically significant differences for DNA damage between 4 h and 48 h exposure at the highest concentrations tested (0.01 and 0.1 µg/mL).

56. Huang FM et al., 2018: See summary in the in vitro chromosomal aberrations/micronuclei section.

57. Yuan et al., 2019: In this study, markers of oxidative stress and DNA damage were evaluated in Marc-145 rhesus monkey embryo renal epithelial cells exposed to BPA (Sigma-Aldrich, purity > 99%) in the range 10-1, 10-2, 10-3, 10-4, 10-5 and 10-6 M (24 hr exposure). The results showed that BPA induced a concentration-dependent decrease in cell viability (from 20% at the lowest concentration up to almost 80% at the highest concentration), in SOD activity and GSH level. Concomitant concentration-dependent increases in apoptosis, lactate dehydrogenase (LDH) activity, ROS and thiobarbituric acid reactive substances content were observed. BPA also induced a concentration-dependent increase in DNA strand breaks by comet assay in the range of concentrations measured (10-3 -to 10-6 M).

58. Chen et al., 2016: The study investigated the cytotoxic and genotoxic effects induced by BPA alone and in combination with cadmium (Cd) in vitro in mouse embryonic fibroblast cell line (NIH3T3). The treatment of the cell cultures with BPA (Sigma-Aldrich) at 2, 10 and 50 µM was shown to induce, only at the highest concentration tested, a decrease in the cell viability and an increase of the oxidative damage as reactive oxygen species (ROS), measured by DCFH-DA

and as 8-OHdG. Significant increase of DNA strand breaks was also detected as tail DNA% and tail moment by comet assay. Higher number of γH2AX foci detected through the use of immunofluorescence and increased γH2AX expression evaluated by western blot in BPA treated cells are indicative of DNA double strand breaks. In addition, 50 μM BPA treatment did significantly decrease the percentage of cells in G1 phase and increased the percentage of cells in G2 phase but not in S phase. Pre-treatment of cells with Cd was observed to aggravate BPA- induced cytotoxicity, and increase ROS production, DNA damage, G2 phase arrest, total TUNEL positive cells and cleaved-PARP expression levels.

59. Xin et al., 2014: The aim of this study was to assess how BPA can influence the function of pancreatic islets. To measure DNA damage, rat INS-1 insulinoma cells were exposed to different concentrations of BPA (Sigma-Aldrich, 99% purity) (0, 25, 50, 100 μM for 24 h) and analysed by the single-cell gel electrophoresis (comet assay). To investigate the possible mechanism of DNA damage induced by BPA, p53 and p-Chk2 levels were also analysed by western blotting together with measurements of intracellular ROS and glutathione (GSH). The results show that BPA caused an increase in DNA strand-breaks at 50 and 100 μM (as measured by tail moment, tail length and tail DNA %). The authors state that these experimental conditions did not cause any significant toxicity (90% survival; no data provided). Pre-treatment with NAC decreased to half the number of DNA strand breaks induced at the highest dose. A significant increase in intracellular ROS, which was decreased by NAC pre-treatment, was also observed. A significant reduction in the level of GSH levels was observed at all BPA concentrations. Finally, expression of DNA damage-associated proteins (p53 and p-Chk2) was significantly increased by BPA exposure (all concentrations).

60. Iso et al., 2006: In this study the effects of BPA and 17β-oestradiol (E2) on DNA damage was analysed in ER-positive MCF-7 cells by comet assay. One thousand higher concentrations of BPA (Wako Pure Chemicals Industries, Ltd.) were needed to induce the same levels of effects of E2. Levels of γH2AX foci measured by immunofluorescence microscopy were increased after treatment with E2 or BPA. Foci of γH2AX co-localized with the Bloom helicase, an enzyme involved in the repair of DSBs. In comparison with MCF-7 cells, DNA damage was not as severe in the ER-negative MDA-MB-231 cells. In addition, the ER antagonist ICI182780 blocked E2 and BPA genotoxic effects on MCF-7 cells. These results together suggest that BPA causes genotoxicity ER dependently in the same way as E2.

In vivo comet assay

61. In the current assessment only 5 of 21 in vivo comet assay studies of DNA strand breaks induction by BPA were classified as of high (one study) or limited relevance and have been considered for evaluation. Among the five oral studies selected, three were positive and two were negative. A single study of high relevance reported negative results in multiple mouse organs (liver, kidney, testes, urinary bladder, colon and lungs) after single treatment at three doses up to the MTD of 500 mg/kg bw (Sharma et al., 2018). Negative results were also reported in rats exposed to 200 mg/kg bw per day orally for 10 days (De Flora et al., 2011). In contrast, dose-related increases in DNA strand breaks were reported at doses greater than 10 µg/kg bw in rats treated for 6 days with a range of doses between 2.4 µg and 50 mg/kg bw per day (Tiwari et al., 2012). A weak and dose-dependent increase in liver DNA strand breaks was observed at 50 and 100 mg/kg bw per day, whereas the increase in kidney was limited to 50 µg/kg bw (Panpatil et al., 2020). Finally, in a study on BPA neurotoxicity, a significant increase of strand breaks in brain cells was observed after treatment in a range of doses from 0.5 to 5000 µg/kg bw per day for 8 weeks (Zhou YX et al., 2017).

62. Overall, the comet assays provided only limited evidence of DNA damage following multiple administrations of BPA, but not following single dose administrations.

Table 3. Summary table of test results of Comet in vivo studies.

Test system	Dose	Results	Reference
Comet assay in liver, kidney testes, urinary bladder, colon and lungs			
CD-1 male mice 5 animals/group	125, 250 and 500 (MTD) mg/kg bw Single dose by gavage	**Negative**	Sharma et al., 2018
Comet assay in liver, kidney, testes, urinary bladder, colon and lungs			

Spraguely Dawley rats 8 animals/group	200 mg/kg bw per day orally for 10 days	**Negative**	De Flora et al., 2011
Holtzman rats 10 animals/group	2.4 µg, 10 µg, 5mg and 50 mg/kg per day orally for 6 days	**Positive** Dose-related increase starting from 10 µg/kg	Tiwari et al., 2012
Comet assay in liver and kidney			
Male Wistar rats (WNIN) 6 animals/ group	50 100 µg/kg orally for 4 weeks	**Positive** Weak dose-related in liver, only at 50 µg/kg in kidney	Panpatil et al., 2020
Comet assay in brain cells			
KM male mice 11 animals/group	0.5, 50 and 5000 µg/kg bw per day Orally for 8 weeks	**Positive**	Zhou YX et al., 2017

Source: Re-evaluation of the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs, EFSA, (2021)

Summary of studies

63. Sharma et al., 2018: The in vivo genotoxic potential of BPA in mouse organs was investigated using the alkaline comet assay. Male CD-1 mice (5 per group) were administered by gavage with BPA (Sigma-Aldrich) suspensions in corn oil prepared by ultrasonication at three dose levels (125, 250 and 500 mg/kg bw), twice 24 h apart. Ethyl methane sulphonate, given once by gavage at 300 mg/kg bw, served as positive control. Animals were sacrificed 3 h after the last treatment and DNA damage investigated by a commercial kit for comet assay in

liver, kidney, testes, urinary bladder, colon and lungs cells. For each mouse, 200 cells were analysed (100 per gel) using an automatic comet assay scoring imaging system. Median values for each tissue from each animal were used, and the mean of the median values was evaluated in a statistical analysis. The results of comet assay did not show BPA related effects in any tissue, except for the testes, in which an increased level of DNA strand breaks ($p < 0.01$ compared with control group) was observed at the lowest dose; however, no dose response relationship was observed as the effects at the medium and highest doses were at the same level as the control group. A modified alkaline comet assay was conducted on human sperm cells treated with BPA 0, 1, 1.5, 2 and 3 µmol/L for 1h. BPA 3 µmol/L reduced cell viability to 60%, therefore it was the highest concentration tested. Ethyl methanesulfonate (EMS) was used as positive control. In total, 600 cells were scored for each concentration. No increase in % tail DNA was observed compared with the negative control.

64. De Flora et al., 2011: The ability of BPA to form DNA adducts was investigated in two human prostatic cell lines: PNT1a non tumorigenic epithelial cells and PC3 cells androgen-independent prostate cancer cells originated from bone metastasis of prostatic carcinoma. PNT1a and PC3 cells were treated with BPA (Sigma-Aldrich), dissolved in ethanol at a concentration corresponding to the IC50 (200 µM for PNT1a and 250 µM for PC3) for 24 h. PNT1a cells were also treated at a concentration of 1 nM, for 2 months. Significant levels of DNA adducts were detected by 32P-postlabeling technique in prostate cell lines treated with high-concentration of BPA for 24 h (4.2-fold increase over controls) in PNT1a cells and a 2.7-fold increase over controls in PC3 cells) and in a lower extent in PNT1a cells treated at low-concentration for 2 months.

65. Tiwari et al., 2012: See summary in the in vitro gene mutation section.

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

67. Zhou YX et al., 2017: The study investigated the neurotoxicity of low-dose exposure to BPA in a mouse model, examining brain cell damage and the effects of learning and memory ability after 8 weeks exposure to BPA at 0.5, 50 and 5000 µg/kg bw (daily dose, by gavage). The comet assay was used to detect brain cell damage. At the end of treatment 11 mice per group were sacrificed and brain processed for comet assay. Forty cells from each brain were analyzed. Based on tail DNA percentage, the damage level was divided into five grades, from 0 (undamaged) to 4 (maximum damage). The results obtained indicated that with increasing exposure concentrations the fraction of damaged cells (all types)

increased significantly from 23.0% in the control group to 47.3%, 66.6% and 72.5% in the low-, medium and high exposed groups, respectively. Also, the severity of DNA damage, expressed as arbitrary units (AUs), increased with AUs of 0.28 in the control to AUs of 0.59, 0.96 and 1.28 in the low-, medium and high-exposed groups, respectively.

Other studies

Induction of γH2AX foci

68. Several studies have investigated the induction of γH2AX foci (generally regarded as a marker of DNA DSBs) following BPA treatment (Iso et al., 2006; Pfeifer et al., 2015; George and Rupasinghe, 2018; Kim et al., 2018b; Mahemuti et al., 2018; Hercog et al., 2019; Hercog et al., 2020; Nair et al., 2020; Yin et al., 2020; Escarda-Castro et al., 2021; Yuan et al., 2021).

69. Iso et al. (2006) reported increased levels of γH2AX foci after treatment with 17β-E2 or BPA in ER- positive MCF-7 cells (1000x higher concentrations of BPA were needed to induce the same levels of effects as E2). Induction was less severe in ER-negative MDA-MB-231 cells and the ER antagonist ICI182780 blocked BPA-induced γH2AX focus formation in MCF-7 cells. Taken together, these findings indicate that BPA-induced genotoxicity is ER-dependent.

70. The effects of low-dose BPA were studied in the ERα-negative MCF10A and in 184A1 normal breast epithelial cell lines and the ERα-positive MCF7 and MDA-MB-231 human breast epithelial adenocarcinomas. Low doses (10 and 100 nM) induced DSBs as measured by γH2AX foci in all cell lines and increased the level of c-Myc and of the cell-cycle regulatory proteins cyclins D1 and E and E2F1. Silencing c-Myc reduced BPA-induced γ-H2AX foci and abolished BPA-mediated mitochondrial ROS production. BPA also induced proliferation in ERα-negative mammary cells. The authors conclude that low-dose BPA exerts a c-Myc–dependent genotoxicity and mitogenicity in ERα-negative mammary cells (Pfeifer et al., 2015).

Summary of studies (in order of mention)

71. Iso et al., 2006: See summary in the in vitro comet assay section.

72. Pfeifer et al., 2015: The objective of this study was to investigate the effects of low-dose BPA (Sigma-Aldrich) in mammary gland cells. The human cell lines used in the study are the ERα-negative immortalized benign and normal breast

epithelial cell lines (MCF10A and 184A1, respectively) and the ERα-positive MCF7 and MDA-MB-231 cell lines originate from human breast epithelial adenocarcinomas. Low concentrations BPA (10 and 100 nM) induced double strand breaks (DSBs) as measured by γH2AX foci in all cell lines. Both MCF10A and MCF7 cells had also a greater number of ATM-pS1981–positive nuclei after 24 h treatment compared with the control. Low-concentration BPA significantly increased the level of c-Myc protein and other cell-cycle regulatory proteins (cyclin D1, cyclin E and E2F1) and induced proliferation in parallel in ERα-negative 184A1 mammary cells. Silencing c-Myc reduced BPA-mediated increase of γH2AX suggesting that c-Myc plays an essential role in BPA-induced DNA damage. The increased level of DNA double strand breaks induced by BPA exposure in 184A1 cells was also confirmed in a neutral comet assay and was found to be reduced by c-Myc silencing. Similarly, silencing c-Myc abolished BPA-mediated ROS production, which was localized to mitochondria. The authors concluded that low-concentration BPA exerted a c-Myc–dependent genotoxic and mitogenic effects on ERα-negative mammary cells (results reported as tail moment only and a single BPA concentration analysed).

73. George and Rupasinghe, 2018: This study investigated the relative toxicity of BPA (Sigma-Aldrich) and BPS on human bronchial epithelial cells (BEAS-2B). The tested endpoints included cytotoxicity, induction of ROS, DNA fragmentation, γH2AX foci and DNA tail damage. To evaluate mechanism of cell death the DDR and activation of caspase-3 were also investigated. In all the assays a single concentration and a single time of exposure were used (200 μM BPA for 24 h). According to the authors this concentration caused 50% of cell viability loss (IC50). However, the data reported indicate high levels of toxicity (90%), with all the results being unreliable at this level of toxicity.

74. Kim et al., 2018: BPA (> 99% purity, Sigma-Aldrich) promoted cell proliferation in undifferentiated and differentiated human hepatocyte cell lines (HepG2 and NKNT-3, respectively) at submicromolar concentrations (0.3-5 μM for 24 h). The proliferative effects of BPA disappeared at concentrations higher than 5 μM (cell viability decreased at concentrations higher than 10 μM). Exposure to BPA in the submicromolar range induced DNA damage in both cell lines as shown by a dose-dependent increase in phosphorylation of histone H2AX (γH2AX), p53 activation and induction of cyclin B1. Increased levels of γH2AX were also observed in liver tissue of juvenile rats (PND 9) orally exposed to a relatively low dose of BPA (0.5 mg/kg for 90 days). At a higher BPA dose (250 mg/kg) no increase in hepatocyte proliferation or cyclin B1 was observed. BPA promoted ROS generation as measured by DCF-DA-enhanced fluorescence in HepG2 cells.

Increased levels of ROS were suggested to play a role in BPA-induced proliferation and DNA damage as shown by the partial reversion of both processes upon pre-treatment with NAC.

75. Mahemuti et al., 2018: The aim of this study was to investigate the key molecular pathways involved in the developmental effects of BPA on human fetal lung and their potential implications in the link between pre-natal exposure to BPA and increased sensitivity to childhood respiratory diseases. Global gene expression profiles and pathway analysis was performed in cultured HFLF exposed to non-cytotoxic concentrations of BPA (0.01, 1 and 100 µM BPA for 24 h, 99% purity, Sigma-Aldrich). Molecular pathways and gene networks were affected by 100, but not 0.01 and 1 µM BPA. These changes were confirmed at both gene and protein levels. The pathways affected by BPA included the cell cycle control of chromosome replication and a decreased DDR. BPA increased DNA DSBs as shown by phosphorylation of H2AX and activated ATM signalling (increased phosphorylation of p53). This resulted in increased cell cycle arrest at G1 phase, senescence and autophagy, and decreased cell proliferation in HFLF. Finally, BPA increased cellular ROS level and activated Nrf2-regulated stress response and xenobiotic detoxification pathways. The authors suggest that pre-natal exposure to BPA may affect fetal lung development and maturation, thereby affecting susceptibility to childhood respiratory diseases.

76. Hercog et al., 2019: With the aim of comparing the toxicological profiles of possibly safer analogues of BPA, the authors investigated the cytotoxic/genotoxic effects of BPS, BPF and BPAF and their mixtures in human hepatocellular carcinoma HepG2 cells. Single exposure to BPA (99% analytical purity, Sigma-Aldrich) did not induce any significant changes in cell viability at the tested concentrations (2.5, 5, 10, 20 µg/mL for 24 or 72 h). Induction of a significant increase in DNA double strand breaks, as determined by γH2AX assay, was observed only at the highest dose (20 µg/mL for 72 h). BPA (tested at the 10 µg/mL concentration) induced changes in the expression of some genes involved in the xenobiotic metabolism (CYP1A1, UGT1A1, but not GST1), response to oxidative stress (GCLC but not GPX1, GSR, SOD1, CAT), while no changes were observed in any of the genes involved in the DDR (TP53, MDM2, CDKN1A, GADD45A, CHK1, ERCC4). Similar results were obtained when cells were exposed to BPA as a single compound or in mixtures with its analogues at concentrations relevant for human exposure (10 ng/mL). The relevance of these changes is of uncertain biological significance.

77. Hercog et al., 2020: In a follow-up study by Hercog et al. (2020) the genotoxic effects induced by co-exposure of the cyanotoxin cylindrospermopsin (CYN)(0.5 µg/mL) and BPA (Sigma-Aldrich), BPS and BPF(10 µg/mL, 24 and 72 h exposure) were investigated on HepG2 cells using the same techniques and experimental conditions of Hercog et al. (2019). The results obtained with BPA confirm the previously published observations, but the relevance of these changes remains of uncertain biological significance.

78. Nair et al., 2020: The effects of BPA (Sigma-Aldrich) as a single agent, or in combination with 4-tert-octylphenol (OP) and hexabromocyclododecane (HBCD), were studied in the HME1 mammary epithelial cells and in the MCF7 breast cancer cell line. Following a 2-month exposure to a low non-toxic BPA concentration (0.0043 nM), increased levels of DNA damage were evidenced by upregulation in both cell lines of phosphorylated DNA damage markers (γ-H2AX, pCHK1, pCHK2, p-P53). Disruption of the cell cycle was observed both after short exposures (24 h and 48 h, G2/M arrest) as well as after the 2-month exposure treatment (G1 and S phase increases). BPA increased cellular invasiveness through collagen. Methylation changes were investigated by Methylation Specific Multiplex-Ligation Dependent Probe Amplification (MS-MLPA) using a panel of 24 tumour suppressor genes (all hypomethylated) and identified hypermethylation of TIMP3, CHFR, ESR1, IGSF4 in MCF7 cells and CDH13 and GSTP1 genes in HME1 cells. Finally, BPA induced phosphorylation of six protein kinases in HME1 cells (EGFR, CREB, STAT6, c-Jun, STAT3, HSP60) and increased levels of several other proteins involved in potential oncogenic pathways (HSP27, AMPKα1, FAK, p53, GSK-3α/β, and P70S6).

79. Yin et al., 2020: The scope of the study was developing a novel in vitro three-dimensional testicular cell co-culture mouse model that enables the classification of reproductive toxic substances. BPA (99%, Sigma-Aldrich) as well as BPS, TBBPA, and BPAF were used as model compounds. A concentration-dependent increase in BPA toxicity was found in the range 2.5 - 400 µM following 24, 48 and 72 h exposures. The large variations in the number of gH2AX foci observed at 72 h make the relevance of these results questionable. No increase in gH2AX used as marker of DNA damage was found up to a dose of 100 mM (70% cell viability).

80. Escarda-Castro et al., 2021: The ability of BPA to induce genotoxic and epigenetic changes was investigated before and during cardiomyocyte differentiation in H9c2 rat myoblasts exposed to 10 and 30 µM BPA (92% and 73% of cell viability, respectively). Exposure to BPA (no information on purity or the

supplier company) before differentiation repressed the expression of the Hand2 and Gata4 heart transcription factors and three genes belonging to the myosin heavy chain family (Myh1, Myh3, and Myh8), whereas exposure after the 5 days of differentiation reduced the expression of cardiac-specific Tnnt2, Myom2, Sln, and Atp2a1 genes. BPA did not induce ROS and did not increase DNA 8-oxodG levels (as measured by immunostaining) in either myoblasts or cardiomyocytes. After BPA exposure the percentage of DNA repair foci formed by co-localization of the γH2AX and 53BP1 proteins increased in a concentration-dependent manner in myoblasts (from 44% in the control group to 61% and 86% at 10 and 30 µM BPA, respectively), with no increase in MN. Repair foci also increased in cardiomyocytes (from 45% in the control group to 59% and 72% at 10 and 30 µM BPA, respectively). A small increase (up to 13%) in MN was also reported only in cardiomyocytes treated with 10 µM BPA. A decrease in the epigenetic markers H3K9ac and H3K27ac was also reported. The authors concluded from these in vitro data that BPA interferes with the process of cardiomyocyte differentiation. However, the reliability and significance of the data on BPA-induced DNA damage is questioned by several negative factors (high background levels of DNA repair foci, lack of information on methods for micronucleus assays and the small increase of MN over high background).

81. Yuan et al., 2021: This study investigated the combinatorial toxicity of BPA (≥ 99.8% purity), decabrominated diphenyl ether and acrylamide to HepG2 cells. Increased number of γH2AX foci were induced in HepG2 by a 24h exposure to a single BPA dose that induced 25% toxicity. The majority of the data (ROS measurements, Ca2+ flux, DNA damage, Caspase-3 and decreased mitochondrial membrane potential) refers to additive/synergistic effects induced by varying combinations of contaminants. The authors conclude that BPA induced an increase in γH2AX fluorescence and in the number of γH2AX foci/nucleus. However, this conclusion is not fully supported by the data presented.

Changes in gene expression and DNA methylation

82. Changes in DNA methylation have been investigated in several studies (De Felice et al, 2015; Porreca et al., 2016; Karmakar et al., 2017; Karaman et al., 2019).

83. No specific discussion on DNA repair or DDR genes is reported in these publications.

84. None of the information present in these studies is relevant for the clarification of the genotoxic potential of BPA.

Studies in humans

85. Overall, human studies are not considered to provide additional relevant information for the evaluation of BPA genotoxicity

[1] A clastogen is a mutagenic agent that disturbs normal DNA related processes or directly causes DNA strand breakages, thus causing the deletion, insertion, or rearrangement of entire chromosome sections. These processes are a form of mutagenesis which if left unrepaired, or improperly repaired, can lead to cancer.

[2] An aneugen is a substance that causes a daughter cell to have an abnormal number of chromosomes or aneuploidy.