

Appendix E – Summaries of genotoxicity studies

This Appendix reports summaries of genotoxicity studies on BPA identified in the literature (2013 - 2021) and studies considered in the Scientific opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs (EFSA CEF Panel, 2015).

The evaluation of the studies on the main genetic endpoints (gene mutations, structural and numerical chromosomal alterations) is reported in tabular format in Annex L.

E.1. *In vitro* studies

E.1.1. Gene mutation

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 $\mu\text{mole/plate}$ (100 μL of 1 mM solution). No mutagenic effect was observed.

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 $\mu\text{g/plate}$ 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 $\mu\text{g/plate}$ of BPA in each strain, but the increase was statistically significant only in strain TA 102 at 25 $\mu\text{g/plate}$.

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), [sulfonylbis(benzene-4,1-diyl)oxy]diethanol (BP-1) and 4,4'-sulfanediylidiphenol (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 $\mu\text{mol/L}$ to 10 $\mu\text{mol/L}$. 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.

Xin et al., 2015 [RefID 8150]

The study evaluated the cytotoxic, genotoxic and clastogenic activity of BPA (purity 99%)³⁰ in 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 $\mu\text{g/plate}$) 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.

Zemheri and Uguz, 2016 [RefID 9535]

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 $\mu\text{g/plate}$). The results were negative, with and without metabolic activation.

³⁰ Information on BPA purity provided by the study authors on 11 October 2021, upon EFSA request.

Balabanič et al., 2021 [RefID 224-G]

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, 1,000 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 substance was also assessed in HepG2 cells with MTT assay for cell viability and with comet assay at 1, 10, 100 and 1,000 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.

Hu et al., 2021a [RefID 295-G]

This study shows that BPA (purity ≥ 99%) and styrene-7,8-oxide (SO) causes DNA damage and mutations in HEK293T human embryonic immortalised cells. A concentration-dependent increase in the formation of γH2AX foci was induced by a 24 h exposure to BPA (0.1, 1, 100 µM). Multiple clonally amplified cell populations, derived from HEK 293T cells treated with 100 µM BPA for 24 h, were subjected to whole genome sequencing (WGS). To identify mutations that were associated with BPA exposure, genomic changes occurring in BPA-treated cell populations were compared with those occurring in the parental cell line. The frequency of acquired single base substitutions (SBS), double base substitutions (DBS) and small insertions and deletions (InDel) were all increased in BPA-treated cell populations in comparison with untreated cell populations. Analyses of genome-wide point mutations and genomic rearrangements associated with BPA exposure indicate that a subset of SBS- and DBS substitutions occur near or at guanines. The authors conclude that these locations are consistent with BPA preference to form guanosine adducts. The occurrence of other mutational signatures suggests additional mutagenic events occurring at A:T base pairs (TA>CG transitions and TA>GC transversions). Analysis of data from 19 cancer cohorts suggest that tumours of digestive and urinary organs show a relatively high similarity in mutational profiles, with the burden of mutations increasing with age. The authors conclude that BPA (and SO) are relatively mild mutagens and other environmental agents might also potentially generate similar, complex mutational patterns in cancer genomes.

E.1.2. Chromosomal damage**Johnson 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 visualise modifications of the microtubule organising 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 visualise 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.

Tayama et al., 2008

In this study, the authors evaluated the genotoxicity of some environmental oestrogen-like compounds including BPA using sister chromatid exchanges (SCEs), CA and DNA strand breaks (comet) assays in CHO-K1 cell line *in vitro*. For CA and SCEs six concentrations of BPA (purity > 99%) ranging from 0.1 to 0.6 mM were added to CHO-K1 cells for 3 h. Following treatments cells were

further incubated for 27 h in the presence of BrdU until preparation of slides for both SCE and CA from the same culture. For comet assay seven concentrations of BPA ranging from 0.1 to 0.7 mM were added to CHO-K1 for 1 h and following washes of test compound cells were processed for comet assay using a silver-staining method. The method applied for comet analysis was not clearly reported. Statistically significant increase for both SCE and chromosome aberrations were reported at the highest concentrations tested (0.4 and 0.5 mM and 0.5, 0.55 and 0.6 mM, respectively). Significant increases of c-mitotic effects were detected at highest concentrations 0.3–0.6 mM. The positive results were observed only in the presence of severe cytotoxicity. For comet assay significant increases of DNA breakage were only reported at the highest concentration (0.7 mM) for which the cytotoxicity was not adequately evaluated.

Ribeiro-Varandas et al., 2013 [RefID 6189]

The study investigated micronucleus formation in human umbilical vascular endothelial cells (HUVEC) and human colon adenocarcinoma (HT29) cell lines cultured in the presence of BPA (44 nM and 4.4 μ M, i.e. 10 ng/mL and 1 μ g/mL) for 24, 48 or 72 h. Effects on DNA stability and DNA damage repair were concurrently evaluated by means of the TUNEL assay and by the analysis of histone-H3 acetylation on lysine 56 (H3K56ac). At the concentrations applied, BPA did not affect cell viability and proliferation of both cell lines. The TUNEL assay did not detect DSBs or apoptotic cells associated with exposure to BPA. Micronucleus frequency was significantly increased in HUVEC cells (1.1% vs 1.6% and 2.2 % in control, low and high BPA dose, respectively), but not affected in HT29 cells (4.45% vs 4.6% and 4.4%). The transcriptional analysis of expression of genes encoding for proteins involved in chromosome segregation (CDCA8, SGOL2, Aurora A, α -tubulin and γ -tubulin), performed by quantitative real-time PCR, showed small (less than two-fold) increases in the expression of CDCA8 (both cell lines) and SGOL2 (HUVEC cells only). The immunofluorescence analysis of cytoskeleton organisation of HUVEC cells with anti- α -tubulin and anti- γ -tubulin showed several aberrations, such as multipolar spindles and microtubule misalignment, associated with BPA exposure.

Šutiaková et al., 2014 [RefID 7026]

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) 24 h after stimulation by L-phytohemagglutinin and incubated for total 72 h. Micronucleus frequency was determined using the cytokinesis block method, adding 6 μ g/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.

Aghajanzpour-Mir et al., 2016 [RefID 57]

The study evaluated the clastogenic activity of BPA in the MCF-7 human breast cancer cell line and male and female human amniocytes. Cells were exposed 48 h to 0 (control), 0.4, 1, 4, 40 and 100 μ g BPA/mL in the absence of exogenous metabolic activation. Giemsa staining was applied for the identification of structural chromosome aberrations in treated cells (200 per dose). Cytotoxicity was evaluated using the MTT assay. In a preliminary evaluation of cytotoxicity, the IC₅₀ of BPA was 100, 40 and 4 μ g/mL in MCF-7 and ER-negative (male) and ER-positive (female) amniocytes, respectively. Following treatment with BPA a significant increase of cells with chromosome aberrations was observed at 1 μ g/mL (and above) with all cell types, with no clear association with ER expression. The aberrations recorded were almost exclusively fragments in amniocytes (both female and male), and fragments and chromatid breaks and gaps in MCF-7 cells. The increase in cells with aberrations was not clearly concentration related and decreased at the highest doses, possible due to cytotoxicity that was not concurrently evaluated. A few numerical aberrations (both hypoploidy and hyperploidy) were also recorded in amniocytes metaphases; in female amniocytes the decrease of cells with normal chromosome number reached statistical significance at the highest not toxic concentration (4 μ g/mL).

Huang et al., 2018a [RefID 296-G]

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 μ M 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 μ M 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 μ M, respectively, and an increase of the expression of proapoptotic proteins BAX, BID and BAD beginning at 10, 10 and 30 μ M, 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 μ M 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.

Sonavane et al., 2018 [RefID 419-G]

The study investigated BPA's ability to confer resistance to the chemotherapeutic agent camptothecin (CPT). The study reports the results of an experimental study on the high-dose BPA (Sigma-Aldrich) co-exposure effects with the chemotherapeutic agent CPT in mouse embryonic fibroblasts (MEFs). A 24 h and a 48 h treatment of MEFs with a concentration of BPA (150 μ M) did not result in an increase of DNA strand breaks detected by the comet assay and by the analysis of phosphorylated H2AX (γ H2AX) and of chromosomal aberrations. The BPA treated cells showed a reduction in the nuclear volume associated with a DNA compaction detected by 3-D imaging using the nucleic acid fluorescent stain. This effect reduces the accessibility of DNA to topoisomerase-I (Top1). The mechanism of action of CPT is the formation of Top1 covalent complexes inducing DNA-protein cross-links, that result in single-strand and double-strand DNA breaks and chromosomal aberrations in treated cells. A co-exposure of MEFs with CPT and BPA for 24 and 48 h showed a significative reduction of Top1-DNA adducts decreasing the DNA strand breaks and chromosomal damage induced by CPT alone.

Santovito et al., 2018 [RefID 11220]

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 μ g/mL, (including the reference dose established by United States Environmental Protection Agency (US EPA) (0.05 μ g/mL), the tolerable daily intake established by European Union (0.01 μ g/mL) and the highest concentration of unconjugated BPA found in human serum (0.02 μ g/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 μ g/mL; no numerical aberration was observed. A concentration related increase in MN frequency was detected starting from 0.02 μ g/mL in which a four-fold increase with respect to the control level was observed.

Ramos et al., 2019 [RefID 396-G]

The study evaluated the cytotoxic and genotoxic effects of BPA in human laryngeal carcinoma cells (Hep-2) and human lung fibroblasts (MRC-5). BPA (Sigma) was freshly diluted in ethanol and added to the culture media at the concentrations of 0.44 nM, 4.4 nM and 4.4 μ M (0.1 ng/mL, 1 ng/mL, 1 μ g/mL) for 48 h. Cell viability after treatment was evaluated using the resazurin assay. DNA damage and oxidative DNA damage were assessed in cryopreserved cell samples by comet assay (100 cells per slide), with and without the formamidopyrimidine-DNA glycosylase (Fpg). Mitotic abnormalities and MN were evaluated in DAPI stained cells (1000 cells per concentration). Treatments had no effect on cell viability in either cell lines. In comet assays with Hep-2 cells, DNA damage, measured as % DNA in the tail, was significantly increased at the lowest concentration (0.44 nM) and decreased at 4.4 nM and 4.4 μ M; oxidative DNA damage (in presence of Fpg) was significantly decreased at all concentrations. In MRC-5 cells, there was no effect on DNA damage and an increase in oxidative DNA damage at the two highest concentrations. The microscopic analysis of DAPI stained cells showed a significant increased mitotic index (percent of cells undergoing mitotic division) in MRC-5 cells at all BPA

concentrations and in Hep-2 cells at the highest concentration. The percentage of MN were slightly (up to 2.5-fold) but significantly increased in both cell lines at the two highest concentrations.

Di Pietro et al., 2020 [RefID 258-G]

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 μ M. 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 μ M 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 μ M. The cell cycle was analysed 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 μ M, 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, γ H2AX 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 γ H2AX 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 γ H2AX 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 ER α expression was found therefore proposing a role for this receptor in the effects induced by BPA.

Yu et al., 2020 [RefID 475-G]

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 substance with all the enzymes tested: CYP1A1, 1A2, 1B1, 2B6, 2E1 and 3A4. BPA (99.6% analytical purity) tested at 40, 80 and 160 μ M 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 docking was observed only for CYP1A1 and CYP1B1. Following extended exposure (two cell cycles, i.e. 24 h in V79-derived cells) without recovery period, BPA reduced the cell viability at 80 μ M in V79-Mz and V79-hCYP1A1 cells; at lower concentrations, the cell growth was even enhanced (compared with the control). BPA induced MN in V79-hCYP1A1 (24 h treatment) and C3A cells (72 h treatment) from 5 and 2.5 to 80 μ M in a concentration-dependent manner. The increase of MN was completely blocked by 1-aminobenzotriazole (1-ABT CYP inhibitor) and by 7-hydroxyflavone (7-HF selective CYP1A1 inhibitor). Co-exposure of C3A cells to pentachlorophenol (sulfotransferase 1 inhibitor) or ketoconazole (UDP-glucuronosyltransferase 1A inhibitor) potentiated MN induction with thresholds lowered to 0.6 μ M. The use of Centromere Protein B-staining immunofluorescent assay indicated clastogenic effects. BPA tested for 9 h from 10 to 160 μ M in V79-Mz, V79-hCYP1A1 and

C3A cells induced a concentration-dependent increase of γ H2AX foci which were blocked in presence of 1-ABT and 7-HF.

Özgür et al., 2021 [RefID 631-G]

In this study, BPA was tested in a chromosomal aberration assay in cultured human peripheral blood lymphocytes. Cells were exposed for 24 h to BPA, dissolved in DMSO, at the final concentrations of 5, 10 and 20 μ g/mL. No data on chromosome aberrations are reported as, under the conditions of this study, BPA (source and purity not defined) was completely cytotoxic.

E.1.3. DNA damage (by comet assay)

Iso et al., 2006

In this study the effects of BPA and 17 β -estradiol (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 γ H2AX foci measured by immunofluorescence microscopy were increased after treatment with E2 or BPA. Foci of γ H2AX co-localised 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.

Xin et al., 2014 [RefID 8147]

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 μ M 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 μ M (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).

Chen et al., 2016 [RefID 1130]

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 μ M 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 γ H2AX foci detected through the use of immunofluorescence and increased γ H2AX expression evaluated by western blot in BPA treated cells are indicative of DNA double strand breaks. In addition, 50 μ M 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.

Porreca et al., 2016 [RefID 5892]

This study reports on the thyroid-disrupting activity of low-dose BPA (Sigma-Aldrich) on the rat immortalised FRTL-5 thyrocytes cell line using microarray experiments. Exposures to 10⁻⁹ M BPA for 1 and 3 days resulted in increased ROS production. Exposures for 3 and 7 days lead to gene expression changes with transcriptome alterations being quantitatively and qualitatively dependent on the duration of exposure. Cell survival (decreased), cell death (increased), cell cycle (decreased) and cancer (increased) were among the most significant functions predicted deregulated. The response involved many genes belonging to specific pathways mainly related to cell death/proliferation and DNA

repair (genes involved in 'DNA replication, recombination and repair' including 'checkpoint control' functions). Although gene expression levels are only slightly altered, a long-term exposure to BPA (28 days, 10^{-9} M) impaired the cellular response to other stressors (mainly COP9 signalosome). A delayed reduction of UV-C-induced DNA damage was identified by comet assay. This was due to the impairment of p21-Tp53 axis, with BPA-treated cells being more prone to cell death (increased apoptosis and decreased proliferation following UVC damage). The authors propose a possible mechanism by which BPA does not cause direct genetic damage but may exert an indirect genotoxic activity.

Lei et al., 2017 [RefID 3960]

In this study changes in cytotoxicity, genotoxicity, intracellular ROS formation and Ca^{2+} levels induced by BPA were evaluated in MCF-7 cells in comparison with other BPs. The range of BPA (98% purity, Tokyo Chemical Industry) concentrations tested was 0.01–100 μM (24 h exposure). An increase in cell viability was observed at 1 μM , while a significant drop in cell survival (to approximately 30%) was observed at higher concentrations (10–100 μM). In parallel, a significantly increase in LDH release was observed (10–100 μM). BPA (1–25 μM) significantly increased ROS levels in a concentration-dependent manner. At 50 μM however BPA caused death of over 90% cells. BPA exposure resulted in a significantly increase in DNA-damaging effect on MCF-7 cells in the range 10–50 μM . In addition, BPA at 0.0001–1 μM significantly increased intracellular Ca^{2+} level. Finally, the estrogenic and thyroidal hormone effects induced by BPA were also evaluated using the yeast two-hybrid bioassay.

Li et al., 2017b [RefID 4176]

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 h 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.

Mokra et al., 2017 [RefID 5170]

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 $\mu\text{g/mL}$ 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 $\mu\text{g/mL}$, while BPS caused the lowest effect at 10 $\mu\text{g/mL}$ after 4 h of exposure. Statistically significant increases of DNA double strand breaks were induced by BPA at concentrations of 1 and 10 $\mu\text{g/mL}$ after 1 h incubation and at 0.1 and 1 $\mu\text{g/mL}$ 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 $\mu\text{g/mL}$. A significant decrease of the DNA damage was observed at 60 min, but the repair was not complete after 120 min.

Durovcova et al., 2018 [RefID 262-G]

The study evaluated by alkaline comet assay the induction of DNA strand breaks by BPA (Sigma-Aldrich) in cultures of peripheral blood lymphocytes at concentrations of 0.001, 0.1, 2.5 mM. The results show a statistically significant increase only at the first two concentrations tested. The presence of oxidative DNA damage was also shown using modified comet assay with Fpg enzyme: the extent of DNA single strand breaks is higher at the lower concentration. The study reports also a protective effect of BPA on plasmid DNA against iron ions induced single-strand DNA breaks as showed by a test measuring the electrophoretic mobility of pDNA topoisomers (DNA-topology assay).

George and Rupasinghe, 2018 [RefID 277-G]

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, γ H2AX 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 μ M BPA for 24 h). According to the authors this concentration caused 50% of cell viability loss (IC_{50}). However, the data reported indicate high levels of toxicity (90%), with all the results being unreliable at this level of toxicity.

Goncalves et al., 2018 [RefID 11104]

The aim of this study was to evaluate the effects of BPA (Sigma-Aldrich) on the growth, viability and testosterone production of TM3 murine Leydig cells after exposure to BPA for 24 or 48 h (1, 10, 100 μ M). BPA reduced testosterone production, cell viability and cell growth in a concentration-dependent manner. The highest tested concentration of BPA (100 μ M) increased cellular death, as indicated by an increased sub-G1 phase population and a larger number of cells labelled with Hoechst 3342. This concentration of BPA also decreased the number of metabolically active mitochondria as revealed by rhodamine staining. No DNA strand breaks evaluated by alkaline comet assay was detected in the cells after 3 h of BPA exposure at any concentration tested. The authors conclude that BPA is toxic to Leydig TM3 cells and impairs their steroidogenic function.

Mokra et al., 2018 [RefID 364-G]

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 μ g/mL for 4 h and 0.001, 0.01 and 0.1 μ g/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 μ g/mL) and to pyrimidines (from 0.1 μ g/mL) was reported after 4 h treatment. After 48 h treatment significant damage to purine was observed from 0.001 μ g/mL and to pyrimidines from 0.01 μ g/mL. Statistically significant differences for DNA damage between 4 and 48 h exposure at the highest concentrations tested (0.01 and 0.1 μ g/mL)

Yuan et al., 2019 [RefID 478-G]

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 h 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).

Kose et al., 2020 [RefID 325-G]

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 μ M for 24 h exposure. The IC_{20} and IC_{50} values, concentrations that causes 20% and 50% of cell viability loss, after a 24 exposure to BPA were 45 and 113.7 μ M. 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 (IC_{20}), 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.

E.2. Other studies

E.2.1. DNA adducts

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-post-labelling 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.

Zhao et al., 2018

The ability of bisphenol A 3,4-quinone (BPAQ) to interact with DNA was investigated by *in vitro* reaction of BPAQ with 2'-deoxyguanosine (dG), calf thymus DNA and MCF-7 cellular DNA. The structures of DNA adducts were characterised by high-resolution MS/MS spectra and tandem MS DNA adducts were characterised by high-resolution MS/MS and tandem MS. The DNA adduct induced by BPAQ with all these substrates was identified as 3-hydroxy-bisphenol A-N7-guanine (3-OH-BPA-N7Gua).

E.2.2. DNA repair

Yin et al., 2014 [RefID 8519]

The study analysed gene expression profiling in ER-negative human embryonic kidney cells (HEK293) following 48 h exposure to 10⁻⁶ M BPA. The comparison of gene expression profiles of BPA (no information on purity or the supplier company) treated and control (DMSO) samples identified 15 differentially expressed genes after BPA treatment, eight upregulated and seven downregulated (while more than 300 genes are differentially expressed in ER-positive cells). A computer-assisted functional analysis indicated the BPA exposure perturbed many unrelated metabolic pathways. BAX, a proapoptotic regulator, was downregulated by BPA treatment, suggesting that low-dose BPA may reduce apoptosis of HEK293 cells. Differentially expressed genes of BPA exposure include upregulation of ERCC5 encoding a DNA endonuclease involved in nucleotide-excision repair. Using an electrochemical method based on the preferential electrochemical oxidation of chemically damaged DNA, no DNA damage was identified in HEK293 cells treated with 10⁻⁶ M BPA for 48 h, while a peak indicating DNA damage was observed in the ER-positive MCF-7 cell line.

Gassman et al., 2015 [RefID 2214]

In this study, DNA damaging effects of BPA were shown to be induced indirectly *in vitro* in mouse fibroblasts through the generation of ROS. A statistically significant increase of intracellular ROS production, measured by DCFH-DA, was observed in mouse fibroblasts (Ku70-deficient cell line) treated *in vitro* with BPA (Sigma-Aldrich) at 150 µM for 1 h. Treatment with BPA increased also the levels of DNA modified bases (5-OH-Cyt and ThyGly) consistent with the increase in ROS. Ku70 cell line, deficient in strand break repair by non-homologous end joining (NHEJ), a back-up repair pathway for BER, was used to investigate the effect of BPA on the BER response to oxidative stress. Ku70-deficient cells were very sensitive to the oxidative damaging agents: treatment with KBrO₃ induced a significant dose-response reduction of cell viability. Co-treatment with BPA partially reversed the KBrO₃-induced cytotoxicity in these cells, associated with the increase in oxidatively induced DNA base lesions, suggesting that BPA may prevent initiation of repair of oxidised base lesions. Examination of γH2AX foci revealed also a significant reduction in DNA damage signalling induced by BPA confirming the possible inhibition of BER repair.

Tran et al., 2020 [RefID 438-G]

The aim of the study was to investigate the effects of BPA in PBMC by using freshly isolated T cell subpopulations of healthy donors for short and long-term BPA treatment. Long term cultures of CD4+

or CD8+ T lymphocytes isolated from blood of male donors were established. Cell cultures were treated with BPA (purity > 99%) from 0.3 to 30 nM for 24 h. A non-monotonic telomerase enzyme inhibition in CD8+T-cells, but not in CD4+T-cells of male donors was observed. The maximum inhibition (30% compared with control) was seen at 0.3 nM BPA. At higher concentrations the effect subsequently weakened. An impact of BPA on DNA repair capacity of CD8+T-cells upon exposure to oxidative stress (treatment with hydrogen peroxide) was also reported. A decreased DNA repair (maximum 45% inhibition at 0.3 nM), measured by comet assay, after BPA treatment compared with the control was observed. DNA repair gene pathway analysis was carried out by using a human DNA repair RT2 Profiler PCR array: no regulation on the expression of 84 DNA repair genes upon BPA exposure could be observed. Long-term treatment (after 35 days of culture) with BPA significantly reduced cell proliferation only at concentration < 30 nM. The maximum inhibition was seen at 3 nM, with a reduction of 25% in comparison with the solvent control. Long-term treatment (49 days of culture) of CD8+T cells with BPA impaired T cell response upon antigen stimulation, as shown by decrease of telomere length and mitochondrial DNA (mtDNA) copy number by 35% and 25%, respectively. At a 10-fold higher or lower concentration, the effect was marginally evident for both parameters. A strong inhibitory effect of intracellular anti-viral interferon- γ expression was evident on day 42 of BPA exposure, compared with the solvent control.

E.2.3. Spindle stability

Campan et al., 2018 [RefID 240-G]

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 μ M and underwent to *in vitro* maturation for 24 h, then the oocytes were extracted. For BPA experiments, a total of 939 oocytes were analysed 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 μ M, 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 μ M 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*.

Kim et al., 2019 [RefID 319-G]

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 localisation 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.

Yang et al., 2020 [RefID 469-G]

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 μ g/mL) of BPA. After treatment oocytes were analysed by immunofluorescence and live cell imaging to investigate the effect of BPA on spindle dynamics. BPA disrupted spindle organisation 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.

E.2.4. γ H2AX

Audebert et al., 2011

In this study, the authors investigated the capability of established human cell lines, ACHN (human kidney adenocarcinoma cells), HepG2 (human hepatocellular carcinoma cells) and LS174T (human epithelial colorectal adenocarcinoma cells) to biotransform BPA and BPF. The potential genotoxicity of BPA and BPF was assessed by the examination of γ H2AX foci. BPA (radiochemical purity: 95%, specific activity: 3.6 GBq/mmol) was purchased from Moravex Biochemicals and Radiochemicals (Brea, CA) was shown to be metabolised by HepG2 and LS174T cell lines. Intestinal cells showed stronger biotransformation capabilities than liver cells, in terms of production of the glucuronide- and the sulfate-conjugates (phase II metabolites). Conversely, ACHN cell line was not able to metabolise BPA. Relevant metabolites were separated and quantified by radio-HPLC. Following treatment with BPA (Sigma-Aldrich, purity > 99%) at concentration-levels of 1, 5, 10, 50 and 100 μ M for 24 h no increases of γ H2AX were observed at any concentration tested in HepG2 and in LS174T cell lines. A concentration related increase of γ H2AX was observed in ACHN cells.

Pfeifer et al., 2015 [RefID 5815]

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 ER α -negative immortalised benign and normal breast epithelial cell lines (MCF10A and 184A1, respectively) and the ER α -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 γ H2AX 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 ER α -negative 184A1 mammary cells. Silencing c-Myc reduced BPA-mediated increase of γ H2AX 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 localised to mitochondria. The authors concluded that low-concentration BPA exerted a c-Myc-dependent genotoxic and mitogenic effects on ER α -negative mammary cells (results reported as tail moment only and a single BPA concentration analysed).

Ganesan and Keating, 2016 [RefID 2141]

The study investigated ovarian DNA damage induced by BPA exposure using an *in vitro* model system. At PND 4, F344 rat ovaries were cultured in medium containing 1% DMSO $\pm$ BPA (from Sigma-Aldrich, 440 μ M) for 1–4 days. Increased expression of γ H2AX foci was observed by immunofluorescence staining in the ovaries evaluated (three ovaries/treatment/time point) at days 1, 2 and 4 of treatment, with a time-related increasing trend. Increased abundance of phosphorylated H2AX and ataxia-telangiectasia mutated (ATM), markers of DNA double-strand breaks, were also detected by western blotting in total ovarian protein homogenates. Expression of DNA repair genes (ATM, Prkdc, Xrcc6, Brca1, Mre11a, Rad50 and Smc1a) assessed by qRT-PCR was also increased ($p < 0.05$) 1 and 2 days after BPA exposure (the 4-day time point was not analysed). Overall, the results of this study indicate that the *in vitro* exposure to BPA can induce DNA double strand breaks that activate a DDR in ovarian cells.

Quesnot et al., 2016 [RefID 6033]

The study authors applied an automated *in situ* γ H2AX detection system in metabolically competent HepaRG cells to evaluate the genotoxicity of 10 environmental contaminants, including BPA, after single or long-term repeated *in vitro* exposure (1, 7 or 14 days). Cytotoxicity was determined using the MTT assay. BPA (Sigma-Aldrich) was tested at four concentrations (20, 40, 50 and 60 μ g/mL), selected to avoid excessive toxicity (i.e. with viability > 60%). A significant increase in the H2AX positive cell

index (fold increase over control) was observed after 24h at 40 µg/mL and above, but not after 7 days or 14 days treatments. The incidence of Hoechst-stained MN was also quantified in HepaRG cells exposed to BPA for 1 or 7 days before mitogenic stimulation by EGF for 3 days. Under the test conditions, no significant increase of MN was observed in BPA treated cells, while positive results were obtained in parallel experiments with known genotoxic contaminants (benzo(a)pyrene, AFB1, DMBA).

Liang et al., 2017a [RefID 4246]

The testicular toxicity of BPA (99% purity) was compared with that of BPS, BPAF and tetrabromobisphenol A (TBBPA) on the C18-4 spermatogonial cell line. The authors developed and validated an automated multi-parametric high-content analysis to study nuclear morphology, DNA content, cell cycle progression, DNA synthesis, cytoskeleton integrity and DDRs induced by these compounds. The only marker of DNA damage analysed was the formation of γH2AX foci. BPA showed a small increase at a single dose of 50 µg/mL after 48 and 72h exposure (doses tested: 0.1, 1, 5, 10, 25, 50 µg/mL). The study presents several limitations in the reported methodology.

Mahemuti et al., 2018 [RefID 11171]

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.

Kim et al., 2018b [RefID 11137]

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.

Hercog et al., 2019 [RefID 287-G]

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 substance 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.

Hercog et al., 2020 [RefID 288-G]

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.

Yin et al., 2020 [RefID 474-G]

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 γH2AX foci observed at 72 h make the relevance of these results questionable. No increase in γH2AX used as marker of DNA damage was found up to a dose of 100 mM (70% cell viability).

Nair et al., 2020 [RefID 367-G]

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 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).

Yuan et al., 2021 [RefID 477-G]

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.

Escarda-Castro et al., 2021 [RefID 266-G]

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-localisation 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).

E.2.5. DNA oxidative damage

Barbonetti et al., 2016 [RefID 419]

With the aim of studying whether *in vitro* exposure to BPA affected human sperm integrity, the authors investigated the induction of pro-oxidative/apoptotic mitochondrial dysfunction. The decrease in the mitochondrial membrane potential of motile sperm observed following a 4 h exposure to 300 μ M BPA (Sigma-Aldrich) was associated with increased mitochondrial generation of superoxide anion, caspase-9 and caspase-3 activation and motility decrement. Loss in sperm vitality associated with a complete sperm immobilisation was observed following a 20h exposure to 300 μ M BPA. This was accompanied by a significant increase in the formation of DNA 8-hydroxy-2'-deoxyguanosine. The significance of the increase levels of oxidative DNA damage in these extreme experimental conditions (no viability and no motility) is questionable.

Budiawan et al., 2018 [RefID 757-G]

The formation of the oxidised base 8-oxodG was analysed by reacting dG with BPA with the addition of Fenton's reagent. DNA adduct 8-oxodG was analysed by reverse phase HPLC with a UV/vis light detector at 254 nm. The results indicate that BPA acts as pro-oxidant because of the increase yield of 8-oxodG. The concentration of DNA 8-oxodG increases with increasing pH, temperature and length of incubation time. In the presence of BPA and Fenton's reagent the highest DNA 8-oxodG levels (42.258 ppb) are detected at pH 7.4, 60°C and 12 h incubation time (these are very low levels of DNA adduct formation). The relevance of this short report published in a conference proceeding is limited.

E.2.6. Effects on meiotic cells

Karmakar et al., 2017 [RefID 3388]

The objective of this *in vitro* study was to investigate the proliferation, survivability and apoptotic rate of mouse testicular germ cells (CD-1 and C57-GFP) exposed to BPA (information on purity not reported) and to examine the differential expression of germ cell markers in these cultured cells. Cell viability and proliferation were not affected by low BPA concentrations (0.01, 0.1, 1 and 10 μ M). Germ cell self-renewal and expression of differentiation related marker proteins were also found to be unchanged at these concentrations. In contrast, a significant reduction in survival was observed at 100 μ M (increased apoptosis). When *in vitro* treated BPA germ cells were transplanted into recipient testes, fewer colonies were observed at high BPA concentrations (10 and 100 μ M). A significant frequency of recombination failure during meiosis (production of 'crossover-less' synaptonemal complex in pachytene spermatocytes) was observed in 10 μ M BPA-exposed germ cell transplanted recipient. Experiment on continuous BPA-exposed and 100 μ M BPA-recovered germ cells suggested that spermatogonial stem cells have a larger potential to survive in adverse environment. Finally, in a comparative proteomic analysis several differentially expressed cellular proteins were identified after BPA treatment of germ cell in culture.

E.2.7. Epigenetics

De Felice et al., 2015 [RefID 1462]

The aim of this study was to investigate whether (a) BPA exposure was associated with deregulated expression of microRNAs and (b) these epigenetic effects induced alterations in gene expression able to persist throughout a lifetime. Placenta samples from pregnant women living in a polluted area (40 patients subjected to therapeutic abortion for fetal malformation) and placenta samples from a control group of women living in a non-polluted area (40 pregnant women with a healthy pregnancy) were used for genome-wide miRNA expression profiling using microarray technology. This approach allows identification of miRNAs that were aberrantly expressed in placentas from malformed fetuses. Twelve miRNAs were upregulated and six miRNAs were downregulated in placenta samples from malformed fetuses. BPA was absent in the control group, while was detected only in patients subjected to therapeutic abortion. High levels of BPA were associated with a significantly increased miR-146a expression. The authors conclude that miR-146a, which correlates

with BPA accumulation in the placenta, is a measure of fetal exposure related to fetal malformations. Finally, the use of bioinformatics tools allowed prediction of the target genes of miR-146a and exploring their functions including the downstream pathways. Several pathways involved in immune/inflammatory, cancer and neural diseases were identified.

Karaman et al., 2019 [RefID 269-G]

The aim of this study was to investigate the epigenetic regulation induced by *in vitro* exposure to BPA to reveal a possible role on the progression of prostate cancer. Changes in gene expression of chromatin modifying enzymes, promoter methylation of tumour suppressor genes and histone modifications were studied in the PC-3 human prostate carcinoma cell line. A significant decrease in global levels of 5-methylcytosine and an increase in 5-hydroxymethylcytosine were observed following exposure to 10 μ M of BPA (99% purity, Sigma-Aldrich) for 96 h (in these experimental conditions no changes in cell proliferation were observed). A significant increase in DNA methylation of the *p16* promoter region, with a concomitant decrease in *p16* gene expression, was observed (1 and 10 μ M), while no changes were found for other genes involved in cell cycle control (*Cyclin D2* and *Rassf1*). Significant changes were observed in global histone modifications (H3K9ac, H3K9me3, H3K27me3 and H4K20me3) after 48 and 96 h exposures to BPA (0.1, 1 and 10 μ M). Analysis of the promoter methylation status of 94 tumour suppressor genes identified significant changes in *BCR*, *GSTP1*, *LOX*, *NEUROG1*, *PDLIM4*, *PTGS2*, *PYCARD*, *TIMP3*, *TSC2* and *ZMYDN10* (including the *MGMT* DNA repair protein). The authors conclude that epigenetic signatures such as DNA methylation and histone modifications could be proposed as molecular biomarkers of BPA-induced prostate cancer progression.

E.3. *In vivo* studies

E.3.1. Chromosomal damage

Masuda et al., 2005

The study investigated the systemic genotoxicity elicited in mice from BPA treated with nitrite under acidic conditions to simulate the stomach environment. BPA, purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), was dissolved in DMSO and co-incubated for 1 h at 37°C in buffer (1:100 v:v) containing 100 mM sodium nitrite at pH 3.0–5.0. The reaction mixture was extracted with ethyl acetate, evaporated to dryness, and the residue dissolved in DMSO for the mutagenicity tests: Ames test (reported under *in vitro* studies) and micronucleus test in mouse peripheral blood reticulocytes. For the micronucleus test, samples of nitrite-treated BPA dissolved in DMSO were administered by gastric intubation to male ICR mice (five per experimental group) at a dose of 228 mg/kg body weight (bw); untreated BPA dissolved in DMSO (228 mg/kg bw) served as control. Five microliters of peripheral blood were collected from a tail blood vessel at 24, 48 and 72 h after sample administration. Blood samples were stained on acridine orange and at least 1000 RNA-containing erythrocytes were observed by fluorescence microscopy for micronucleus evaluation. Results obtained indicated that BPA under the condition of the study did not induce a detectable increase of micronucleated reticulocytes in treated mice. Conversely, nitrite-treated BPA significantly increased the frequency of MNRETs compared with vehicle controls and untreated BPA at 48 and 72 h after oral administration.

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.

de Flora et al., 2011

The study evaluated the MN frequency in bone marrow cells and DNA damage by the alkaline comet assay in peripheral blood lymphocytes, in male Sprague-Dawley rats treated with BPA (Sigma-Aldrich) via drinking water for a calculated daily intake of 200 mg/kg bw for 10 consecutive days and sacrificed at the end of treatment. Eight rats were used for each experimental group (control and BPA treated animals). No increase of MN frequency was reported in the bone marrow. No increase of DNA damage, expressed as tail moment, was observed in peripheral lymphocytes.

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.

Gajowik et al., 2013 [RefID 2120]

The study investigated the effects of 2-week exposure to BPA, either alone or in combination with X-rays, on the induction of genotoxic effects in different tissues of female mice. Outbred female mice were treated with BPA (no information on purity) at 5, 10 or 20 mg/kg in drinking water for 2 weeks. The statistically significant increase in DNA damage, evaluated by comet assay was observed in lung only at 5 and 10 mg/kg. Negative results were reported in all the other organs analysed in the same animals (spleen, kidneys, liver and bone marrow). Increase of MN frequency was observed in peripheral blood reticulocytes, but not in the bone marrow after 2 weeks of exposure. No increase was detected after 1 week of treatment. No clear results were reported for the combined exposure BPA and X-rays for both the biomarkers.

Srivastava and Gupta, 2016 [RefID 9611]

The genotoxic effects of repeated oral exposure to BPA in adult male Wistar rats was evaluated. Groups of 10 animals were administered with BPA (Sigma-Aldrich) dissolved in olive oil at three dose levels (5 µg, 50 µg and 100 µg/100 g bw), with and without vitamin E (4 mg/100 g bw), once a day for 90 days and sacrificed on day 91. The positive control group was injected with mitomycin C (3 µg/g bw) 48 h before sacrifice. Bone marrow was collected, and micronucleus frequency determined in polychromatic and normochromatic erythrocytes in bone marrow smears stained with May-Gruenwald and Giemsa. A slight increase of MN in polychromatic (three-fold) and normochromatic (two-fold) erythrocytes was observed comparing the overall incidence of MN in vehicle controls and high dose BPA in PCE (4/2362 vs 12/2456) and NCE (5/9635 vs 11/9712). No statistical analysis was performed. The effect was not observed when animals were co-administered with BPA + vitamin E. No deviation of PCE/NCE ratio was observed.

Fawzy et al., 2018 [RefID 270-G]

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 MNPCs (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 characterised 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.

Panpatil et al., 2020 [RefID 379-G]

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 $\mu\text{g/kg}$ 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: 2,000 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 $\mu\text{g/kg}$. 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 MNPCs 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.

E.3.2. DNA damage on somatic cells

Ulutaş et al., 2011

The authors aimed to assess potential genotoxicity of BPA (purity > 99%) in peripheral blood nucleated cells of rats by comet assay. Groups of six rats were dosed orally for 4 weeks at dose levels of 125 and 250 mg/kg bw. Control group animals (5 animals) were administered orally with corn oil for 4 weeks. At the end of treatment peripheral blood cells were collected via cardiac puncture and stored at 4°C until preparation of slides for comet assay. Authors showed significant increases of both tail length and tail moment for BPA only at the highest dose level (250 mg/kg bw per day) used.

Dobrzyńska and Radzikowska, 2013

This study investigated the effects of BPA (no information on purity) alone or in combination with X-rays on the sperm and induction of DNA strand breaks in somatic and germ cells of mice. Male Pzh: SFIS mice received BPA orally in drinking water for 2 weeks. Levels in drinking water were designed to achieve BPA intakes of 0, 5, 10, 20 or 40 mg/kg bw per day. Two additional groups received either 5 or 10 mg BPA/kg bw per day via drinking water in combination with daily radiation doses of 0.05 Gy or 0.10 Gy of X-rays. For comet assay animals were sacrificed 24 h after the last treatment and DNA tail moment was used to assess the levels of DNA breakage induced in cells isolated from liver, spleen, bone marrow, lungs and kidneys. Results obtained indicate that BPA induced statistically significant increases of DNA tail moment in bone marrow, spleen, kidney and lung cells at any of the dose-levels assayed. No DNA breakage was detected in liver cells.

Zhou et al., 2017c [RefID 9083]

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 5,000 $\mu\text{g/kg}$ 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 analysed. 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.

Abdel-Rahman et al., 2018 [RefID 199-G]

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 administered 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 CYP450 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' vacuolisation 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.

Kazmi et al., 2018 [RefID 315-G]

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, vacuolisation, 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.

Elhamalawy et al., 2018 [RefID 510-G]

The study was aimed to evaluate the protective effects of sesame oil (SO) against BPA-induced hepatotoxicity in mice. Groups of male Swiss albino mice ($n = 10$) were given BPA (from Sigma-Aldrich, dissolved in ethanol and diluted in corn oil) by gavage at 50 mg/kg bw, with/without SO (1 ml/kg bw), once a day for 28 successive days. SO was administered through three regimens (before, with or after treatment with BPA). Liver DNA damage was evaluated by alkaline comet assay in 50 nuclei per experimental group. Mean tail length, tail moment and % tail DNA were significantly increased ($p < 0.05$) in liver of BPA treated mice. The effect was partially alleviated by SO administration at all the three regimens.

Sharma et al., 2018 [RefID 662-G]

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 sulfonate, 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 $\mu\text{mol/L}$ for 1h. BPA 3 $\mu\text{mol/L}$ 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.

Amin, 2019 [RefID 216-G]

The aim of the study was to evaluate the cardiotoxicity of BPA and to assess copeptin as a cardiotoxic diagnostic and prognostic biomarker. Three groups of Wistar rats were treated subcutaneously (SC) once daily (6 days/week) for 4 weeks: group I, naive group received regular diet and water; group II, vehicle group administered corn oil; and group III received BPA (Sigma-Aldrich) daily (30 mg/kg bw per day SC). Rats were sacrificed immediately after the last administration: blood samples were collected for estimating serum copeptin levels and the hearts were subjected to histological, immunohistochemical and electron microscopic examination. Heart cells were isolated and processed for comet assay. A statistically significant increase of % of DNA in tail was detected in BPA treated animals with respect to controls (6.88 vs 1.67). BPA induced a significant increase in mean values of serum copeptin level and histopathological changes with dilated congested blood vessels and extensive collagen fibre deposition in the myocardium. Light microscopic examination revealed focal disruption of cardiomyocytes with some nuclear changes, such as karyolysis and pyknosis and sarcoplasmic vacuolisation. The mitochondria appeared swollen and deranged with different sizes and shapes.

Majid et al., 2019 [RefID 354-G]

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 *Ipomoea 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 *Ipomoea batatas* significantly reduced the gonadotoxicity induced by BPA, the DNA damage and restored the levels of antioxidant enzymes.

Mohammed et al., 2020 [RefID 363-G]

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.

Zhang et al., 2020 [RefID 485-G]

The study investigated the long-term neurotoxicity of maternal exposure to BPA in mouse offspring. Pregnant mice (F0) were orally dosed with BPA (from Sigma-Aldrich) dissolved in tea oil at 0.5, 50, 5000 µg/kg bw per day until weaning. Then, the first generation (F1) of mice were used to generate the F2. DNA damage in brain cells was evaluated by comet assay in eight male and eight female mice from both F1 and F2. DNA damage, expressed as AU, was slightly (less than 2-fold) increased in the F1 male mice at the lowest dose and in females at the intermediate dose. No effect of BPA exposure was observed in the F2 mice.

E.3.3. DNA damage on germ cells

Ullah et al., 2019 [RefID 443-G]

The study evaluated the effect of subchronic exposure to BPA (Santa Cruz Biotechnology) and the analogues bisphenol B, F and S, on DNA integrity of rat spermatozoa. Sprague-Dawley rats (7 per group) received daily administrations of bisphenols by gavage in 0.1% ethanol solution at 5, 25 and 50 mg/kg bw per day. Animals were sacrificed on day 29th and DNA damage in spermatozoa from the cauda epididymis evaluated using a modified neutral comet assay scoring 100 cells per animal. Both tail moment and % tail DNA were significantly ($p < 0.05$) increased in the BPA 50 mg/kg bw per day group compared with vehicle controls, while no significant difference with controls was observed in the BPA 5 and 25 mg/kg bw per day groups. Comet assay was also performed on rats sperm cells treated *in vitro* with BPA 0, 1, 10 and 100 µg/L for 2h. A statistically significant increase in % tail DNA was observed at the highest concentration tested. Under the same treatment conditions statistically significant increases in SOD, TBARS and total ROS were observed at BPA 100 µg/L.

Zhang et al., 2019a [RefID 486-G]

The study investigated the effects of maternal exposure to BPA on testicular development in offspring males. Pregnant Kunming mice were randomly divided into seven groups with 20 mice in each group. One group served as control, the others received BPA (Sigma, USA) in drinking water at 0.05, 0.5, 5, 10, 20 and 50 mg/kg bw per day, for 40 days from gestation day 0 to lactation day 21. F1 male mice were sacrificed at weaning (PND21). Testicular DNA damage was evaluated by comet assay and expressed as OTM. The results obtained showed that the testicular germ cell damages of group D, group E, group F and group G (5, 10, 20 and 50 mg/kg bw per day, respectively) were significantly higher than that of the control group ($p < 0.05$). No experimental data are shown.

Pan et al., 2020 [RefID 377-G]

The study evaluated the effect of BPA on DNA integrity and protamination of mouse sperm cell. Newborn male mice were subcutaneously injected with BPA (from Sigma-Aldrich, 0.1 and 5 mg/kg bw, $n = 15$) or corn oil (control group, $n = 20$) daily from PND 1 until 35. At PND 70, epididymis caudal spermatozoa and testes were collected. A flow cytometry sperm chromatin structure assay (SCSA) was performed to evaluate DNA fragmentation of sperm cells. DNA fragmentation was expressed as DNA Fragmentation Index (DFI), calculated as the ratio between sperm cells with strands DNA. Apoptosis of spermatogenic cells (spermatogonia, spermatocytes, round spermatids and elongated spermatids) was determined by the TUNEL method. Exposure to BPA was associated with a significant ($p < 0.01$) and dose-related increase of the DFI in sperm cells and apoptosis in spermatogenic cells ($p < 0.05$).

Sahu et al., 2020 [RefID 405-G]

The study investigated BPA toxicity in sperm cells of juvenile Sprague-Dawley rats. BPA (Sigma-Aldrich) was administered by gavage daily for 5 days per week for 8 consecutive weeks to 4 rat groups (7 animals/group, of which only 4 used for genotoxicity assessment): (i) Control (normal feed and water); (ii) BPA (100 mg/kg bw per day); (iii) control fed with a zinc deficient diet (ZDD); (iv) BPA + ZDD. Sperm DNA damage was evaluated by the comet and Halo assays, apoptosis in testes cells was quantified by TUNEL assay and testicular levels of 8-OHdG were determined by immunohistochemistry. All comet assay parameters (tail length, OTM and % DNA in the tail) and the nuclear diffusion factor in the halo assay, were slightly but not significantly increased in testes cells of BPA treated rats compared with controls. When associated with a ZDD, BPA exposure resulted in a significant ($p < 0.05$) effect in both assays. TUNEL-positive cells and percent of 8-OHdG positive areas in testicular tissue were also slightly but non significantly increased in BPA treated rats fed with standard diet, while the effect was statistically significant in rats receiving a ZDD.

Zahra et al., 2020 [RefID 481-G]

The study aimed to evaluate the protective potential of methanol extract of the Apocynaceae plant *Vincetoxicum arnotianum* (VAM) on BPA induced testicular toxicity in male SD rat. BPA (source and purity not specified) diluted in 10% DMSO was injected intraperitoneally at 25 mg/kg on alternate days for 30 days, with or without the oral co-administration of VAM extracts (150 and 300 mg/kg bw) to groups of seven rats. Sperm DNA damage was evaluated by comet and DNA ladder assays. Subchronic administration of BPA was associated with a significant ($p < 0.01$) increase of all comet parameters compared with vehicle controls. The effect was attenuated by co-administration of VAM extract in a dose-related manner. Electrophoresis on agarose gel showed extensive DNA fragmentation in testes of BPA treated rats.

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 μ g/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 8 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 μ g/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.

E.3.4. Effects on meiosis**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 aneugenecity in female germ cells, M II oocytes were harvested 17 h after induced superovulation, and cytogenetically analysed 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 analysed 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 hybridised with fluorochrome-labelled DNA probes for chromosomes 8, X and Y and 10000 sperm per animal were analysed 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.

Liu et al., 2014a [RefID 4378]

The study authors exposed 9-week-old male Wistar rats to BPA (Sigma-Aldrich) by gavage at 20 µg/kg bw per day for 60 consecutive days to coincide with one cycle of spermatogenesis. At the end of treatment testes were removed for the analysis of the staging of the seminiferous epithelium and microscopic analysis of meiotic chromosomal spreads. The formation of DNA double strand breaks in meiotic cells was assessed by immunofluorescence staining with anti-γH2AX antibodies. Immunostaining for synaptonemal complex protein 3 (SCP3), ATM kinase and pCHK1 was also performed on the spread nuclei. The assessment of the relative frequency of the 14 stages of the cycle of the seminiferous epithelium showed significant increases in stage VII and decreases in stages VIII and XIV, indicating an impairment of spermatogenesis following BPA exposure. Meiotic recombination, initiated by programmed double-strand breaks (DSBs) and marked by γH2AX expression, was also impaired in BPA-exposed rats. BPA exposure resulted in meiotic DSBs accumulation in pachytene spermatocytes. These cells exhibited chromosomal abnormalities, including asynapsis, end-to-end associations and interrupted regions of SCP3 staining and increased activation of ATM. The authors concluded that BPA exposure at an environmentally relevant dose (20 µg/kg bw per day) induced meiotic abnormalities in adult male rats, with inhibition of spermiogenesis and disruption of spermatogenesis. BPA exposure resulted in the delayed initiation of meiosis in the early meiotic stage and chromosomal abnormalities and meiotic DSBs accumulation in the late meiotic stage, which subsequently activated the ATM DNA damage checkpoint kinase.

Vrooman et al., 2015 [RefID 7521]

The aim of the study was to test the effect of estrogenic chemicals on meiotic chromosome dynamics during neonatal development when the first cells initiate meiosis. Outbred CD-1 and inbred C57BL/6J (B6) newborn male mice were given single oral doses of 20 or 500 ng/g per day BPA (supplied by NIEHS), ethinyl estradiol (EE) as positive control 0.25 ng/g per day or vehicle from 1–12 days post-partum (dpp). In total, six to 12 males (one to three males per litter from at least three litters) for each exposure group at 20 dpp at 12 weeks or 1 year of age (CD-1 only) were analysed to assess the effects on synapsis and recombination. Immunostaining surface spread preparations of meiotic cells with antibodies for SYCP3 (a component of the synaptonemal complex) and MLH1 (DNA mismatch repair protein that localises to the large majority of sites of meiotic exchange) was applied to evaluate the synapsis and recombination foci. Perturbations in synapsis were not observed in either strain, but MLH1 foci were significantly reduced in BPA and EE exposed CD-1 males. Mean MLH1 counts for juvenile CD-1 males were 22.27 ± 0.12 , 21.50 ± 0.10 , 21.87 ± 0.10 and 20.85 ± 0.10 for placebo, 20 ng BPA, 500 ng BPA and 0.25 ng EE-exposed, respectively ($p < 0.0001$). No differences were detected in B6 males with any exposure. Similar reductions were observed in CD-1 males at 12 weeks and at 1 year. Insensitivity of B6 males to oestrogen was considered as a reflection of genetic differences. The study shows that a brief exposure of newborn male mice to exogenous oestrogen reduces the meiotic recombination increasing the incidence of meiotic errors and affecting permanently the spermatogenesis in the adult. The effect induced by BPA is similar to the positive control, no dose-response was observed for the BPA doses tested.

Zhang et al., 2017b [RefID 8813]

The study investigated the protective role of melatonin against the adverse effects induced by BPA on the female reproductive system in mice. Three groups of female ICR mice were used: a control group, a BPA group treated daily with oral doses of BPA (supplier company and purity not reported) at 100 µg/kg bw and a group treated with BPA+melatonin (administered daily at 15 or 30 mg/kg bw) for 7 days preceding oocyte collection and analysis. The number of animals in each group was not reported. Oocytes were immunostained with anti- α -tubulin-FITC antibody to observe the spindle morphologies and counterstained with Hoechst to visualise the chromosome alignment. The treatment with BPA was shown to disrupt normal spindle assembly (70% of oocytes had disrupted spindles) chromosome alignment (80% of oocytes exhibited misaligned chromosomes). Metaphase I oocytes were briefly chilled to induce the depolymerisation of microtubules that are not attached to kinetochores and then immunostained with CREST to detect kinetochores, with anti- α -tubulin-FITC antibody to visualise the microtubules and counterstained with Hoechst to observe the chromosomes. An increased frequency of kinetochores without attachment by microtubules was observed in BPA-exposed oocytes ($76.1 \pm 6.1\%$ $n = 99$ vs $19.7 \pm 1.8\%$, $n = 92$). A statistically significant higher frequency of aneuploid oocytes in M II was also observed in BPA-exposed oocytes ($42.6 \pm 1.8\%$, $n = 94$ vs $3.7 \pm 3.7\%$, $n = 82$). In BPA-exposed oocytes, the fluorescence intensity of ROS was increased compared with controls (153.8 ± 7.7 , $n = 138$ vs 70.9 ± 3.1 , $n = 96$). An increase of early apoptosis was shown by the annexin-V signal. In addition, BPA-exposed eggs had significantly reduced fertilisation rates ($41.1 \pm 1.5\%$, $n = 65$ vs $87.2 \pm 0.9\%$, $n = 72$). The melatonin administration significantly improved the oocyte quality via reduction of ROS levels and inhibition of apoptosis.

Horan et al., 2018 [RefID 291-G]³¹

In a comparative study on the meiotic effects in mice of BPA and its analogues, oral doses of 20 ng/g BPA (purity > 99%) or placebo (vehicle only) control were administered on 14 and 15 days post coitum (dpc) to coincide with the time of meiotic entry in the fetal ovary. Variation in meiotic recombination was measured by the number of MLH1 foci in pachytene stage meiocytes. By comparison with unexposed female fetuses, BPA induced a significant increase in mean MLH1 counts ($p < 0.01$), indicating increased levels of meiotic recombination in developing oocytes as a result of maternal BPA exposure. According to the study authors, the subtle changes in meiotic recombination induced are compatible with continued oocyte survival, but increase the frequency of aneuploid eggs and embryos produced by the adult female. In contrast, in male neonatal exposure to BPA causes a permanent reduction in recombination levels spermatocytes, with an increase in the frequency of spermatocytes with at least one synaptonemal complex lacking an MLH1 focus. Low recombination rates were also considered deleterious because spermatocytes with homologues that failed to undergo recombination faced death due to the actions of the spindle assembly checkpoint mechanism that causes arrest of cells with unpartnered chromosomes at metaphase. Further data are presented in this work on the effects on male meiotic recombination in the progeny of 129S1/SvlmJ males (3 animals) inadvertently exposed to BPA released by PC cages, showing that the reduction of meiotic recombination rates persists for several generation (up to F3).

E.4. Other studies**E.4.1. DNA adducts****Izzotti et al., 2009**

The study evaluated the formation of DNA adducts in female CD-1 mice receiving BPA (from Sigma Chemical Co.) in their drinking water (200 mg/kg bw) for 8 consecutive days by the ³²P post-labelling method. Results obtained indicated that administration of BPA, under the experimental conditions of the study, resulted in the formation of bulky DNA adducts (two major DNA adduct) in the liver (3.4 fold increase over control level) as well as in the target mammary cells (4.7 fold increase over control level). The level of DNA adducts in the liver of BPA-treated mice, as evaluated in three replicate analyses, was $5.88 \pm 0.29/10^8$ nucleotides (means $\pm$ SE of the data obtained in five mice) vs. 1.71 ± 0.14 in controls ($p < 0.001$). The level of DNA adducts in the pooled mammary cells of BPA-treated mice was $4.97 \pm 0.61/10^8$ nucleotides (means $\pm$ SE of three replicate analyses) vs.

³¹ Information on BPA purity provided by the study authors on 4 November 2021, upon EFSA request.

1.05 ± 0.13 in controls ($p < 0.001$). DNA adducts were not chemically characterised, but the authors noted that DNA adducts formed in liver and mammary cells had the same chromatographic mobility as those formed *in vitro* by the reaction of BPA with calf thymus DNA in the presence of S9 mix.

E.4.2. γ H2AX

Chianese et al., 2018 [RefID 249-G]

The study investigated the effects of chronic exposure to the low dose of BPA, from fetal period to sexual maturation, on post-natal testis development. Wistar rats were used. BPA (Sigma Aldrich) at 0.1mg/L BPA was orally administered to dams or weaned offspring *via* drinking water. The daily dose of 10 μ g/kg bw was calculated based on daily drinking consumption (the BPA dose used was lower than or within the reference limit for humans, currently considered 'safe' by EFSA and by EPA). To analyse the possible effects of BPA on the first round of spermatogenesis, the male newborns were sacrificed at 17 PND (late infantile), 45 PND (pubertal) or 60 PND (young adult), randomly choosing a total of five animals/treatment/time point from different litters. Serum levels of BPA did not differ between exposed and control groups. The cytoarchitecture of the seminiferous epithelium was impaired in BPA-exposed animals due to low expression and scattered localisation of connexin 43 (Cx43) and zonula occludens 1 (ZO-1), well-known markers of the BTB. DNA breaks were detected by immunofluorescence for γ H2AX in spermatocytes and in round spermatids. A statistically significant increase of the expression rate of the crossover-associated protein *Mlh1*, a marker of DNA mismatch repair system and of *Rad51*, which encodes DNA repair protein (detected by quantitative real-time RT-PCR) was observed in all BPA-exposed rats. Massive and diffuse ROS production was observed in the testis of BPA-exposed animals. CAT and SOD significantly decreased at both 45 and 60 PND in BPA-exposed animals, compared with controls. Oxidative stress at DNA level induced by BPA was determined by 8-OHdG immunostaining. Immunofluorescence for 8-OHdG revealed diffused DNA damage in the germinal epithelium of treated animals. TUNEL signal was detected in the germinal compartment at basal levels and in Sertoli cells in BPA-exposed animals at 45 and 60 PND. A significantly higher fraction of apoptotic cells/tubule was detected in BPA-exposed animals. A different pattern of *sirt1* mRNA expression was observed in BPA-treated rats with respect to the controls during the first round of spermatogenesis at 17, 45 and 60 PND with highest expression levels observed at 45 PND and lowest expression rate observed at 60 PND. SIRT1 has a recognised role in spermatogenesis in the regulation of the hypothalamus-pituitary-gonad (HPG) axis. *SIRT1* impairment may affect the progression of spermatogenesis towards late meiotic and post-meiotic stages, and the maturation of spermatozoa.

Yang et al., 2021 [RefID 471-G]

The aim of this study was to investigate the mechanisms underlying BPA-aggravated atherosclerosis. Four-week-old male *Ldlr*^{-/-} C57BL/6 mice were administered 250 mg/L BPA (Sigma-Aldrich) via drinking water for 30 weeks with or without a western diet and/or resveratrol (RESV) for 12 weeks. The results indicate that chronic BPA exposure significantly aggravated atherosclerosis, enhanced the production of inflammatory cytokines and promoted macrophage infiltration into plaque areas. Peritoneal macrophages isolated from BPA-exposed mice exhibited a more pro-inflammatory phenotype in response to cholesterol crystal treatment than those from control mice. In comparison with the control group no change in the levels of DNA breaks (as measured by tail moment or tail DNA % in comet assays) was found in macrophages isolated from BPA-treated mice (data are not reported). The authors conclude that the DNA repair capacity of BPA-exposed macrophages, as measured by the kinetics of reduction in the number of DNA breaks following methyl methanesulfonate treatment, are reduced in comparison to the control group. However, the significance of a delay in DNA strand joining is uncertain and no quantitative evaluation of the data is presented. The overall significance of these contradictory results is uncertain.

E.4.3. DNA oxidative damage

Esplugas et al., 2018 [RefID 267-G]

The aim of the study is to investigate the renal and hepatic effects induced by a co-exposure to low doses of ionising radiation and BPA. Sixty male mice (C57BL/6J) were randomly assigned to six experimental groups ($n = 10$) at PND 10 and received a single subcutaneous dose of 0.9% saline solution, BPA (Sigma-Aldrich) (25 μ g/kg bw), caesium at two different doses or combined BPA and

caesium. Urine (24 h) and blood were collected after 2 months. No increase of 8-OHdG in liver and kidney was observed in BPA treated mice. Following exposure to BPA the mRNA expression of CYP1A2 in liver decreased in respect to that in the control group. Limitations: only a single subcutaneous dose was applied; the analyses were carried out after 2 months. The study has a low relevance for the genotoxicity evaluation.

Franklyn et al., 2020 [RefID 521-G]

The study investigated the oxidative damage induced *in vivo* in rats by the subchronic administration of BPA, alone or in combination with chromium (VI), through the determination of 8-OHdG adducts in urine. Groups of five young adult rats received water (10 ml/kg bw per day) and served as controls, or BPA (from Sigma-Aldrich) (dissolved in DMSO and water at 2 mg/kg bw per day) or BPA (4 mg/kg bw per day) together with potassium dichromate (1.28 µg/kg bw per day) by gavage for 28 consecutive days. Urine was collected every week and the formation of 8-OHdG analysed using LC-MS/MS with reverse-phase chromatography. The formation of 8-OHdG was detected in urine samples from BPA treated rats from week 1 onwards, with a slight increasing trend. The lowest and highest 8-OHdG concentrations were equal to 27.597 and 31.683 ng/mL, respectively. 8-OHdG level in urine of control animals (only shown in a graph) was apparently zero. 8-OHdG levels in urine of rats treated with BPA and CrVI were higher than in the BPA group only at week 4. The authors concluded that CrVI had a synergistic effect on the formation of 8-OHdG by BPA, but the lack of a groups treated with CR VI alone, and the different BPA levels administered without (2 mg/kg bw per day) and with Cr VI (4 mg/kg bw per day) prevent a firm conclusion on this aspect. This study did not follow a validated test procedure for which an OECD TG is available. No positive control was used. The study has a low relevance for the genotoxicity assessment due to the experimental limitations.

E.5. Epidemiological studies

Lv et al., 2017b [RefID 4702]

The study investigated the contribution of BPA exposure via dermal contact route to human body burden and the relationship between BPA exposure level and oxidative DNA damage. Six male students living at the university were recruited and required to simulate the cashiers' work in handling the thermal receipts for 8 h/day for a period of 5 days. The volunteers were provided the same foods and drinks during the experimental periods to control the variations of BPA exposure levels from dietary and dust ingestion. Urine was collected for 5 days before the simulation, during the experimental period and for 2 days after the simulation. BPA and 8-hydroxy-2'-deoxyguanosine (8-OHdG) concentrations were determined by high performance liquid chromatography/tandem spectrometer (LC/MS/MS). BPA concentrations was shown to increase four times during the simulation period compared with those before the experimental period. A similar trend was observed also for 8-OHdG. No significant decrease of both the parameters was reported in the post-experimental period. It was estimated that the contribution ratio of BPA via dermal contact to urinary BPA was 70.9%. BPA levels for individuals showed a similar trend with a large interindividual variability. A weak correlation, although statistically significant, was observed between urinary BPA and urinary levels of 8-OHdG ($R^2 = 0.237$, $p < 0.001$). The study presents several limitations such as the low number of the subjects, the short post-experimental period, and does not allow any clear conclusion to be drawn.

Huang et al., 2017cc [RefID 2926]

In this study the association between pre-natal exposure to nonylphenol (NP) and BPA and inflammation biomarkers was investigated in 241 mother-fetus pairs. Third-trimester urinary NP and BPA levels, and urinary biomarkers of oxidative/nitrative stress, were simultaneously measured in 233 urine samples. The biomarkers included products of oxidatively and nitratively damaged DNA (8-hydroxy-2'-deoxyguanosine (8-OHdG) and 8-nitroguanine (8-NO₂Gua)), as well as products of lipid peroxidation (8-isoprostaglandin F_{2α} (8-isoPF_{2α}) and 4-hydroxy-2-nonenal-mercapturic acid (HNE-MA)), analysed by HPLC electrospray ionisation (ESI)-MS/MS. Inflammatory biomarkers (IL-6, TNF-α and C reactive protein) were analysed in maternal and umbilical cord plasma samples by immunoassays, and the antioxidant GPx by a commercial kit. Maternal urinary NP and BPA levels were detectable in 99.2% and 82.2%, respectively. The geometric mean of the NP and BPA levels were 3.99 and 2.24 µg/g creatinine, respectively. After controlling for covariates, NP was significantly associated with increases in 8-NO₂Gua and 8-OHdG and decreases in TNF-α in the pregnant women. BPA was significantly associated with increased maternal 8-isoPF_{2α} levels and decreased maternal and cord

blood GPx levels. This study did not follow an internationally agreed guideline, not available for the effects evaluated, but it was adequately performed and reported.

Radwan et al., 2018 [RefID 390-G]

The study examined the associations between urinary BPA concentration and male fertility in 315 men under 45 years of age with normal sperm concentration, attending a male reproductive health clinic for diagnostic purposes. Participants provided urine and semen samples on the same day of their clinic visit. BPA urinary concentrations were measured using gas chromatography coupled with tandem mass spectrometry. Semen parameters (sperm concentration, motility and morphology) were analysed by standard procedures. Sperm DNA damage was assessed by a flow cytometry-based sperm chromatin structure assay (SCSA). Sperm aneuploidy was evaluated by multicolour FISH analysis using DNA probes specific for chromosomes 13, 18, 21, X, Y; based on the information on six types of chromosome disomies, total disomy rates were calculated. A multiple linear regression analysis identified a positive association between the urinary concentrations of BPA in the 25th to 50th percentile and total sperm sex chromosome disomy ($p = 0.004$), but not with the higher quartiles (50th to 75th and $> 75^{\text{th}}$). When modelled as continuous variable, urinary BPA concentration was associated with increased sperm sex chromosome disomy ($p = 0.01$). Such statistically significant relation was not noticed in case of sperm total chromosome disomy. Urinary concentration of BPA was also associated with increased percentage of immature sperms ($p = 0.018$) and decrease sperm motility ($p = 0.03$). No statistically significant association was observed between urinary BPA and sperm DFI. This study did not follow an internationally agreed guideline, not available for the effects evaluated, but it is adequately planned and reported. As acknowledged by the study authors the findings reported need to be confirmed in future studies. The lack of association between sperm disomy and BPA concentration in the higher quartiles is noticed.

Omran et al., 2018 [RefID 373-G]

The aim of the study is to evaluate a possible role of BPA exposure to the infertility in men. This case-control study involved 50 infertile patients and 50 matched controls. Sperm concentration, morphology and motility were evaluated to estimate the semen quality. Urinary BPA levels were measured by high-performance liquid chromatography. Sperm DNA damage was determined by alkaline comet assay. Oxidative stress (total antioxidant activity and MDA levels) was also determined. BPA concentrations were similar in urine samples from all infertile patients and fertile controls, with median values of 24.2 $\mu\text{g/L}$ and 20.9 $\mu\text{g/L}$, respectively. BPA levels were negatively associated with semen quality and antioxidant levels. The parameters of comet assay were statistically significant higher in infertile patients with respect to the controls (mean tail DNA % was approximately three-folds). The correlations between BPA levels and the comet parameter were analysed: weak but statistically significant correlations were reported for tail DNA % in the control group and in the total cases (Spearman's correlation coefficient: 0.500 (p value < 0.001) and 0.199 (p value < 0.05), respectively). The study presents some limitations: number of subjects recruited, the lack of the analysis of possible confounding factors.

Rocha et al., 2018 [RefID 402-G]

In this study, concentrations of 40 EDCs, including BPA, were determined in urine samples collected from 300 Brazilian children of ages 6–14 years. Oxidative DNA damage was evaluated from the urinary concentrations of 8-hydroxy-2'-deoxyguanosine (8-OHdG). BPA was the major substance found in 98% of the samples analysed at concentrations ranging from $< \text{LOD}$ to 35.9 ng/mL with a geometric mean of 1.74 ng/mL. Statistically significant correlation based on conventional univariate statistics was found between log-transformed urinary concentrations of 8OHdG and BPA. The study has low relevance for risk assessment due to the limitations in identifying the association in a scenario with multiple exposures.

Kiwitt-Cárdenas et al., 2021 [RefID 321-G]

In this cross-sectional study the association between urinary BPA concentrations and human sperm DNA fragmentation was investigated. The study was conducted with 158 healthy university students (18–23 years) that provided urine and semen samples on a single day. Urinary BPA concentrations were measured by dispersive liquid–liquid microextraction and ultrahigh performance liquid chromatography with tandem mass spectrometry detection. Sperm DNA fragmentation was analysed by a Sperm Chromatin Dispersion (SCD) test, which measures halos of DNA loop dispersion after

sequential incubation in acid and lysis solution under fluorescence microscopy, using a commercial kit. A SDF (sperm DNA fragmentation) index was calculated as the percentage of sperm with fragmented DNA divided by the total sperm analysed (300 for each sample). No association was found between urinary BPA concentrations and SDF index in the total study population. A significant positive association with urinary BPA levels was only observed in the subgroup of men with SDF index > 30% (59 subjects). This study used a non-standards method to evaluate sperm DNA fragmentation that is not validated for regulatory purpose.

E.5.1. Polymorphisms in DNA repair genes

Kim and Hong, 2020 [RefID 564-G]

This study investigated the possible role of nine polymorphisms in three DNA repair genes (poly (ADP-ribose) polymerase family member 4 (*PARP4*); X-ray repair cross complementing 3 (*XRCC3*); RAD51 recombinase (*RAD51*)), on the relationship between BPA exposure and liver abnormalities in an elderly population. In total, 502 older adults without liver disease or renal failure aged 60 or over were included in the study. Urinary BPA, malonaldehyde (MDA) and cotinine levels (to monitor tobacco exposure) were measured. The serum levels of ALT, AST and γ -GTP enzymes were used to detect liver abnormalities. LDL cholesterol levels were also measured. A significant association between BPA levels and liver abnormality was found only in elders with the *PARP4* G-C-G haplotype, *XRCC3* G-A-G haplotype or *RAD51* T-A A haplotype (OR = 2.16 for *PARP4*; OR = 1.57 for *XRCC3*; OR = 1.43 for *RAD51*). Particularly, *PARP4* and *XRCC3* showed significant interactions with BPA exposure in relation to liver abnormality ($p < 0.05$ for both genes). These results suggest that *PARP4*, *XRCC3* and *RAD51* gene polymorphisms may have modification effects on the relationship between BPA exposure and liver abnormality.