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New advanced models (NAMs) for risk assessment of bisphenol A alternatives

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Abstract

The safety of bisphenol A (BPA) due to its adverse effects on the immune system has led to an increasing concern and a significant regulatory shift. The European Food Safety Authority (EFSA) proposed a reduction in the tolerable daily intake (TDI) of BPA in food in their 2023 scientific opinion, highlighting the need for stricter regulations compared to their previous assessment in 2015. This regulatory action has spurred the production of BPA alternatives, raising concerns about their safety due to insufficient toxicological data. Addressing this gap is crucial for ensuring human and environmental health. In this project, multiple genotoxicity endpoints were applied for testing of two regulatory relevant BPA alternatives, bisphenol E (BPE) and bisphenol P (BPP), in different human models: 2D HepG2 liver cells, 3D liver spheroids and primary human peripheral blood lymphocytes. DNA strand breaks and oxidised base lesions were evaluated by the enzyme-modified version of the comet assay, while clastogenicity and aneugenicity were analysed by the in vitro micronucleus assay (OECD TG 487, 2016), together with cytotoxicity. Development of new advanced models (NAMs), as 3D spheroids, are essential for next-generation risk assessment (NGRA) in line with the 3R's to replace, reduce or refine animal experiments. In this aspect, validation and standardisation of NAMs are needed to reach regulatory readiness level and development of OECD Test Guidelines. Therefore, a standardisation and pre-validation of the advanced 3D liver spheroid model was performed by using multiple genotoxicity endpoints and by comparing the obtained results with standard genotoxicity models.

KEYWORDS

BPA alternatives, comet assay, genotoxicity, metabolism, micronucleus test, NAMs, spheroid liver cells

CONTENTS

SUMMARY

This project aimed to develop and validate new advanced models (NAMs) for assessing the risk of bisphenol A (BPA) alternatives. The focus was on addressing data gaps in the genotoxicity profiles of two BPA alternatives BPE and BPP, previously identified as regulatory relevant by PARC, ECHA and EFSA. For the first time, multiple genotoxicity endpoints were evaluated in vitro in liver spheroids, including DNA strand breaks and oxidised base lesions using the enzyme-modified comet assay as well as clastogenicity and aneugenicity assessed by the micronucleus assay, in conjunction with cytotoxicity. The two BPA alternatives, BPE and BPP, along with positive controls, were tested using the standard 2D HepG2 and 3D HepG2 spheroid liver model. The cytotoxicity and genotoxicity of BPA alternatives were also investigated in human peripheral blood lymphocytes in the presence and absence of metabolic activation.

Moreover, to better understand the genotoxic mechanisms associated with BPAs, the liver spheroid model underwent pre-validation against standard genotoxicity models in an experienced laboratory setting. The model was established and validated in both laboratories, confirming its transferability.

The study strictly adhered to EFSA recommendations on New Approach Methodologies (NAMs) in risk assessment, employing only in vitro approaches using cells and experimental systems of human origin. Human lymphocytes, primary non-transformed cells and the human hepatocyte cell line HepG2 cultivated in 3D were employed for the experiments. Despite known limitations in metabolic competence, HepG2 spheroids exhibited liver-specific functionalities, making them a promising model in translational pharmacology and toxicology.

This project significantly contributed to advancing genotoxicity assessment methods for chemicals used as BPA alternatives in combination with NAMs, laying the groundwork for potential OECD Test Guideline development and regulatory readiness.

1 | INTRODUCTION

Bisphenol A (BPA) is an organic chemical compound belonging to the phenol group. It is commonly used as a monomer in the production of polycarbonate plastic and epoxy resins. BPA has a wide range of applications in both food and non-food industries due to its desirable properties, such as durability and clarity. BPA is extensively used in the manufacturing of food containers, which include reusable beverage bottles, tableware such as plates and mugs, and storage containers (Harris & Lindsay, 2008; vom Saal & Barrett, 2007). One significant concern associated with BPA is its potential to migrate from food contact materials into the food they contain. This migration can occur under certain conditions, such as high temperatures or prolonged storage times, leading to BPA exposure in humans (Rochester & Diamanti-Kandarakis, 2013). The human population encounters BPA through various routes and sources, with diet identified as the main source of exposure. Consuming canned food has been highlighted as a major factor contributing to BPA exposure. BPA is well-known for its endocrine-disrupting properties, which may cause potential toxicological effects on the reproductive, nervous and immune systems (EFSA CEP Panel, 2023; Ben-Jonathan et al., 2009; Diamanti-Kandarakis et al., 2009). This has led to regulations and development of BPA alternatives. Given the rising production of BPA alternatives, developed to replace BPA due to its concerned effects on human health, and the incomplete toxicological profiles of these compounds, there is an urgent need for comprehensive studies to assess their potential risks. PARC (Partnership for the Assessment of Risks from Chemicals) has initiated a project (Task 5.1) focused on evaluating the genotoxicity of BPA alternatives. This project aligns with the EFSA data package, aiming to complement existing genotoxicity datasets and enhance the robustness of genotoxicity risk assessments. The genotoxicity of BPA alternatives is considered a key event in their hazard identification and characterisation, serving as a critical 'gate-keeper' in the regulatory framework (PARC, 2022).

Evaluating the genotoxicity of chemical substances is most effectively done using models that closely mimic human tissue characteristics. While animal testing and 2D cell cultures have provided valuable insights into how humans respond to chemicals, their relevance is limited due to several factors. In 2D assays, cells grow in monolayers, which differs from the 3D geometries of in vivo tissues. This difference leads to altered biochemistries, non-physiological microenvironments, rapid proliferation and loss of differentiation. Moreover, 2D cultures may exhibit abnormal gene expression profiles. Conversely, animal models may not accurately predict human toxic responses due to disparities in physiological, genetic and metabolic systems between species. These differences underscore the need for developing NAMs, including 3D human tissue models. Three-dimensional (3D) cell culture assays facilitate direct cell–cell contacts and incorporate an extracellular matrix (ECM), making them more representative of in situ cellular responses. Thus, such advanced models offer a promising approach to better understand the potential genotoxic effects of chemical substances.

For this project, we selected the comet assay and the micronucleus assay to evaluate in vitro the genotoxicity of BPA alternatives, BPE and BPP. The comet assay was chosen for its sensitivity in detecting DNA strand breaks and oxidised base lesions, making it valuable for identifying potential DNA damage at an early stage (Tice & Strauss, 2000). The micronucleus assay, on the other hand, provides insights into clastogenicity (chromosome breakage) and aneugenicity (chromosome missegregation), which are crucial for understanding the broader genotoxic impact (Fenech, 2007). The inclusion of the comet assay allowed us to compare its results with those from the micronucleus assay. The comet assay is particularly useful as a screening method to predict genotoxicity and can provide preliminary insights before more detailed analyses are conducted.

The liver plays a critical role in the metabolism and detoxification of xenobiotic agents, including BPA and its alternatives. Hepatocytes, or liver cells, are involved in the biotransformation of chemicals through enzymatic activities mediated by cytochrome P450 (CYP450) enzymes (Guengerich, 2008). This makes liver models essential for assessing the potential toxicity of chemical compounds. Primary hepatocytes, which exhibit liver-specific functions, are highly relevant for such studies but are expensive and have limited longevity in culture. As an alternative, immortalised cell lines like HepG2, derived from human liver cancer, are frequently used (Khetani & Elsayed, 2013). HepG2 cells are cost-effective and provide a reliable model for early safety assessments due to their high availability, unlimited growth potential and reproducibility of results. Primary human peripheral blood lymphocytes were also included in this study due to their importance in assessing genotoxicity. Lymphocytes, being a key component of the immune system, are sensitive to genetic damage and can reflect the potential effects of chemical exposure on human health (Harrison & Smith, 2004). Their use provides insights into how chemicals might affect human immune cells and contributes to a more comprehensive understanding of genotoxic risks.

This project aimed to address regulatory data gaps in the genotoxicity assessment of BPA alternatives by utilising advanced in vitro models and next-generation risk assessment (NGRA) methodologies. By incorporating both 3D liver models and primary human lymphocytes, we ensured a thorough evaluation of potential genotoxic effects, aligning with the recent EFSA scientific report on NAMs that emphasises the need for human-relevant models that better mimic in situ conditions.

2 | DATA AND METHODOLOGIES

This project focused on investigating the genotoxicity of BPA alternatives, specifically testing up to nine concentrations of BPE and BPP (1.25, 2.5, 5, 10, 20, 40, 60, 80 and 100 μM), using advanced 3D in vitro models that better mimic the in vivo conditions than traditional models. The results were obtained from experiments on human lymphocytes and HepG2 cells cultured in 2D as well as 3D spheroids. Data were derived from cytotoxicity analyses as well as different genotoxicity assessment methods, the comet assay to analyse DNA strand breaks and the micronucleus assay to evaluate chromosomal damage.

2.1 | Methodologies

2.1.1 | Comet assay in HepG2 liver cells (Figure 1)

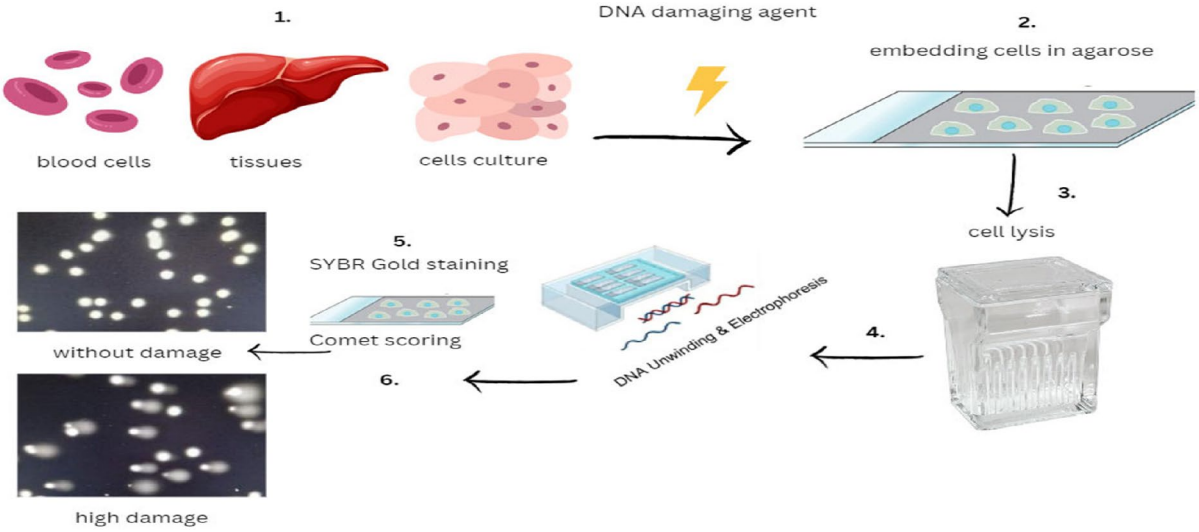

FIGURE 1 Scheme of the comet assay procedure. (1) Isolate tissues or cells to create a single-cell suspension for DNA damage study (2) Embed single cells in agarose gels. (3) Lyse the cells to remove membranes and cellular material, leaving nucleoids. (4) Conduct alkaline electrophoresis to form single-cell comets. (5) Stain and visualise comets using fluorescence microscopy. (6) Score comets and perform data analysis.

The comet assay is an effective tool for studying DNA damage in various eukaryotic cells (Collins et al., 2023; Yamani et al., 2022). Due to various protocol modifications, this method allows for accurate assessment of DNA damage while maintaining high sensitivity and specificity (Figure 1). Its wide application and validation by different research and regulatory institutions underscore the importance of this method in genotoxicity studies. Due to their ready availability, immortalised hepatic cells have been extensively utilised in comet assay-based genotoxicity testing (Cowie et al., 2015). Advanced 3D mini-tissue and mini-organ models provide a more accurate simulation of cellular organisation and function than traditional two-dimensional cell cultures grown in monolayers. By using human-derived cells, these advanced in vitro models can potentially offer a more representative reflection of human biology compared to conventional animal models used in vivo (Štampar et al., 2019).

2.1.2 | Establishing the micronucleus assay in liver spheroids for testing BPA alternatives

The cytokinesis-block micronucleus assay (CBMN) is a method used to detect and quantify chromosomal damage in binucleated cells (Fenech, 2007). Micronuclei form from acentric chromosomal fragments or whole chromosomes that are not incorporated into the daughter nuclei during cell division. This method is valued for its sensitivity, simplicity and ability to provide clear insights into both clastogenic and aneugenic effects. This assay utilises cytochalasin B to inhibit actin filament polymerisation and the formation of contractile microfilaments, thereby halting cytokinesis and resulting in the formation of binucleated cells with micronuclei in their cytoplasm (Figure 2).

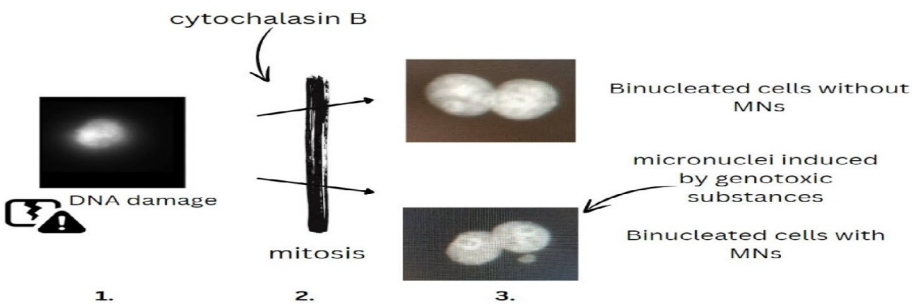

FIGURE 2 Cytokinesis-block micronucleus assay. (1) Nucleus with DNA damage. (2) Cytokinesis is inhibited using cytochalasin B. (3) The frequency of micronuclei (MN) is assessed in binucleated cells only. Top section – control binucleated cells without MN, bottom section – binucleated cells with one MN visible in the cytoplasm.

Optimal 3D assays with representative human cell lines can provide suitable conditions for genotoxicity testing (Ume-Kulsoom Shah et al., 2018; Yamani et al., 2022).

2.1.3 | Evaluating the genotoxicity of BPA alternatives in human lymphocytes

2.1.3.1 | *In vitro micronucleus test*

To assess the potential genotoxicity and cytotoxicity of BPA alternatives (BPE and BPP), the in vitro micronucleus test was performed in human lymphocytes exposed to the test items in the presence and absence of metabolic activation. The metabolic activation was provided by rat liver homogenate (S9 fraction). It contains both phase I and phase II metabolic enzymes, including the crucial cytochrome P450 (CYP) enzymes. These enzymes play a key role in the detoxification and bioactivation of various compounds (Kohn & Durham, 1993).

The added value of the present project was the comparison between the results obtained in metabolically competent human cells (HepG2) and in human peripheral blood lymphocytes grown in the presence of exogenous metabolic activation.

2.1.3.2 | *In vitro chromosomal aberration test*

The chromosomal aberration test was applied in human peripheral blood lymphocytes to assess the potential of BPE to induce structural chromosome damage. The cells treated with the substance applying a continuous exposure were analysed under a microscope to detect chromosomal aberrations, such as breaks, exchanges, chromosomal fragments (OECD TG 473, 2016a, 2016b). In accordance with the 'EFSA Guidance on the Assessment of Aneugenicity' (EFSA Scientific Committee, 2021), the comparison of the results obtained with the micronucleus test and the chromosome aberration assay could give insights on the mode of action of a genotoxic compound, clarifying whether the genotoxic effect is due to clastogenic or aneugenic activity. Since aneugenicity is a thresholded mechanism, identifying such an effect has a significant impact on risk assessment of the selected compounds.

3 | ASSESSMENT

3.1 | Comet assay in HepG2 liver cells

In this study, the cytotoxicity and genotoxicity of BPA, BPE and BPP were assessed using the comet assay in HepG2 cell cultures. The tests were conducted over 3 and 24 hours in a 2D model and over 24 h in a 3D model. Three independent experiments were performed, and six different concentrations were tested for each compound (5, 12.5, 25, 50, 75 and 100 μM).

The results indicated that there were no changes in cytotoxicity with increasing concentrations of BPA and BPE after both 3 and 24 h of exposure. Therefore, BPA and BPE were found to be non-cytotoxic under our experimental conditions. BPP reduced cell viability at concentrations higher than 50 μM after 3 h of exposure and at 25 μM after 24 h of exposure. This indicated that BPP exhibited concentration- and time-dependent cytotoxicity under our experimental conditions. BPA exhibited the lowest genotoxic effect after 3 h of exposure compared to BPE and BPP. BPE and BPP induced greater DNA damage at higher concentrations, but the data from this test suggested that the damage was moderate compared to the positive control. The results suggested that BPA, BPE and BPP could induce oxidative DNA damage in 2D HepG2 cells after 24 h of exposure, with BPE and BPP showing similar trends to BPA. However, the lack of a significant increase in the Lys signal indicated that, while oxidative damage was evident, direct DNA strand breaks could not be as prominent at the tested concentrations. For BPP, the cytotoxic effect at higher concentrations limited the possibility to fully assess its genotoxicity, highlighting the need for further studies to better understand its potential risks. The results suggested that BPA and BPP could induce oxidative DNA damage in 3D HepG2 cells after 24 h of exposure, with both BPA and BPP showing similar trends, and a higher frequency of damages observed at elevated concentrations. For BPE, no clear differences were observed between oxidative damage and direct DNA strand breaks. However, the lack of a significant increase in the Lys signal indicated that, despite the presence of oxidative damage, direct DNA strand breaks could not be as pronounced at the tested concentrations.

3.2 | Establishing the micronucleus assay in liver spheroids for testing BPA alternatives

In the frame of this collaborative project, a protocol was established to apply the CBMN assay to 3D liver spheroid cells during the hosting period of the fellow at ISS, making advantage of the complementary background experiences of the two involved institutes. Preliminary results from a single experiment with 3D HepG2 spheroids showed a slight concentration-related increase in micronucleus frequency both with BPE and BPP. Notably, in the case of BPP, cytotoxicity was evident at concentrations of 40 μM and above.

3.3 | Evaluating the genotoxicity of BPE and BPP in human lymphocytes

To evaluate the potential of BPE and BPP to induce structural and numerical chromosomal damage in mammalian cells, an in vitro micronucleus test was conducted in accordance with OECD TG 487 (2016a, 2016b) using human peripheral blood

lymphocytes. The experiments were performed applying a short treatment in the presence and absence of metabolic activation (3 + 21 h of recovery) and a continuous treatment in the absence of metabolic activation (24 h). The negative control cultures showed a frequency of micronuclei within the historical values of the laboratory; positive controls (i.e. mitomycin C and cyclophosphamide in the absence and presence of metabolic activation, respectively) induced a statistically significant increase of micronuclei, demonstrating the sensitivity of the test system and the efficacy of the metabolic activation (S9-mix).

Nine concentrations of BPE were selected for the analysis of micronuclei in binucleated cells, based on the results of a preliminary cytotoxicity test (range from 1.25 to 100 μM). Cytotoxicity up to 40% was detected at the highest concentration tested of BPE (100 μM). A statistically significant increase in the frequency of micronuclei was observed starting from 20 μM ($p < 0.05$) after continuous treatment in the absence of metabolic activation. No induction of micronuclei was observed after short treatment with BPE both in the absence and presence of metabolic activation. The positive results, reproducible in two independent experiments, indicate that BPE may induce chromosomal damage in mammalian cells under the experimental conditions applied in the study.

Nine concentrations of BPP were tested in a preliminary concentration-finding test (i.e. 1.25, 2.5, 5, 10, 20, 40, 60, 80, 100 μM). Visible precipitate was observed at 60 μM and above. No severe cytotoxicity was detected up to the top concentration tested. Precipitation limited the range of BPP concentrations selected for the analysis of micronuclei. A statistically significant increase in the frequency of micronuclei was induced by BPP at 5 μM and above in any experimental condition. These preliminary results, obtained in a single experiment, suggest that BPP may induce chromosomal damage in the experimental conditions applied in this study.

An in vitro chromosomal aberration assay was performed to evaluate the potential of BPE to induce structural chromosomal damage in human lymphocytes. Nine concentrations of BPE were selected for the chromosome aberration test, based on the results of the in vitro micronucleus test (range from 1.25 to 100 μM). No increase in the frequency of structural chromosomal aberrations was observed at any tested concentrations of BPE compared to the negative control cultures. These negative results indicate that BPE does not induce structural chromosomal damage under the experimental conditions applied in this study.

4 | CONCLUSIONS

4.1 | Conclusions from the laboratory results

BPE induced chromosomal damage in an in vitro micronucleus test in human peripheral blood lymphocytes, while negative results were observed in the in vitro chromosomal aberration assay. In this study, an approach for the identification of aneugenic effects alternative to the characterisation of micronucleus content by centromeric staining was applied. The approach was based on the comparison between the results obtained, in the same experimental system, with the micronucleus test (detecting both structural and numerical aberrations) and the chromosome aberration test (measuring only structural aberrations). Negative results in the chromosome aberration assay associated with a positive micronucleus test are considered indicative of an increased frequency of micronuclei due to numerical chromosomal changes more than structural DNA damage, in accordance with the EFSA Guidance on the assessment of aneuploidy (2021). Therefore, the comparison between the results obtained in these two in vitro assays suggests that BPE mainly induces numerical chromosome damage.

Preliminary results showed that BPP induced chromosomal damage in an in vitro micronucleus test in human peripheral blood lymphocytes. However, further experiments are needed to confirm these preliminary data.

The absence of significant genotoxic effects across the tested BPE and BPP concentrations in the 3D spheroid model suggests that, under the conditions tested, BPE and BPP may have a limited impact on DNA strand breaks in this specific human liver model, in agreement with the negative results of the chromosomal aberration test. In alternative, this finding aligns with the general understanding that 3D spheroids mimic in vivo conditions, potentially indicating a lower genotoxic risk of BPA in more physiologically relevant models compared to 2D cultures or less complex systems. It is crucial to confirm these preliminary findings and perform additional experiments with other models and endpoints to draw comprehensive conclusions about BPA alternatives genotoxic potential.

4.2 | Conclusions from the participation in the fellowship programme

The EU-FORA programme offered the fellow an opportunity to gain general information on different topics related to food safety risk assessment by attending the EU-FORA dedicated training modules. These common training modules complemented the 'learning by doing' fellowship realised within the consortium between the two article 36 organisations, i.e. NILU and ISS.

In addition, at NILU (sending organisation), the fellow increased her scientific capability through the project course by presenting the project results at the series of Institute seminars and at the Oslo Science Parc and University of Oslo PhD and Postdoc Breakfast Club. The fellow had constant opportunity to increase her knowledge in regulatory science by interacting with senior scientists in the team at the Health Effects Laboratory at NILU, members of national and international

food safety authorities. Through the monthly scientific meetings held at the Health Effects Laboratory she continuously increased her scientific knowledge. Additionally, to scientific knowledge she was trained with support from Communication department at NILU in outreach skills, e.g. how to present scientific results to public.

The EU-FORA programme gave also the opportunity to the fellow to maximise the acquisition of scientific knowledge on genotoxicity risk assessment at the hosting institute ISS, through exchanges and discussion with the supervisor and researchers dealing with different aspects of the risk assessment. The scientific training was guaranteed by the multidisciplinary aspects of the hosting Unit of Mechanisms, Biomarkers and Models of the Department of Environment and Health. Besides, the fellow at ISS had the possibility of exchanges related to the different techniques and experimental aspects touched within the project.

The collaborative nature of the programme was supported by a continuous exchange of information between the two institutes. A shared folder gathering the results was created and web meetings, involving the fellow, supervisors and other members of the two institutes participating to the study, were organised to monitor the progress of the experiments.

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