OXFORD Toxicology Research

Toxicology Research, 2024, **13**, 1–10
https://doi.org/10.1093/toxres/tfae127
Paper

Comparative in silico and in vitro evaluation of possible toxic effects of bisphenol derivatives in HepG2 cells

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Introduction : Bisphenols are widely used in the production of polycarbonate plastics and resin coatings. Bisphenol A (BPA) is suggested to cause a wide range of unwanted effects and “low dose toxicity”. With the search for alternative substances to BPA, the use of other bisphenol derivatives namely bisphenol F (BPF) and bisphenol S (BPS) has increased.

Methods : In the current study, we aimed to evaluate the in silico predicted inhibitory concentration 50s (pIC50s) of bisphenol derivatives on immune and apoptotic markers and DNA damage on HepG2 cells. Moreover, apoptotic, genotoxic and immunotoxic effects of BPA, BPF and BPS were determined comparatively. Effects of bisphenols on apoptosis were evaluated by detecting different caspase activities. The genotoxic effects of bisphenols were evaluated by measuring the levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) and 8-oxoguanine glycosylase (OGG1). To determine the immunotoxic effect of bisphenol derivatives, the levels of interleukin 4 (IL-4) and interleukin 10 (IL-10), transforming growth factor beta (TGF-β) and tumor necrosis factor-alpha (TNF-α), which are known to be expressed by HepG2 cells, were measured. Results: In silico data indicate that all of the bisphenols may cause alterations in immune and apoptotic markers as well as DNA damage at low doses. İn vitro data revealed that all bisphenol derivatives could affect immune markers at inhibitory concentration 30s (IC_{30}s). In addition, BPF and BPS may also have apoptotic immunotoxic effects.

Conclusion : Both in silico and in vivo research are needed further to examine the toxic effects of alternative bisphenol derivatives.

Key words: bisphenols; HepG2; genotoxicity; apoptosis; DNA base damage; immunotoxicity.

Introduction

Bisphenols are abundant environmental pollutants, some of which are suspected to be endocrine disruptors. The most produced and most widely used derivative of these chemicals is bisphenol A (BPA, 4,4′-isopropylidenediphenol). BPA is a high-volume industrial chemical with a global consumption of approximately 7.7 million tons in 2015. Its production is estimated to increase to 10.6 million tons by 2022.[1,2] BPA is mostly used in the production of transparent, durable, and rigid polycarbonate plastics (e.g. water bottles, feeding bottles, food storage containers). Moreover, it is used as an inner lining material in metal food containers and cans to prevent the contact with the metal surface. BPA is also present in dental materials.[3,4]

Various studies indicate that BPA may lead to a wide variety of toxic effects, including reproduction and development disorders, carcinogenesis, thyroid hormone disorders, metabolic diseases, diabetes and obesity.[3,5–8] BPA may cause agonistic or antagonistic effects on many hormone receptors (especially on estrogen receptors). Moreover, this particular bisphenol derivative may lead to epigenetic changes, disrupt cell-signaling pathways and cause neurodevelopmental problems, especially during growth and development.[3,6,9,10] It has been determined that these changes are accompanied by both cellular and adaptive immunomodulatory effects. The modulation of immune system components by certain environmental chemicals may cause immune system-related diseases or early and severe outcome of diseases related to immune system, such as multiple sclerosis, type 1 diabetes, asthma, allergies or breast cancer.[5,11,12]

The negative effects of BPA on health attracted the attention of different regulatory agencies and the use of BPA in consumer products is tightly controlled and limited today.[13–15] Considering the potential toxic effects of BPA, today BPA-free products are widely chosen by consumers. Other bisphenol alternatives, particularly bisphenol F (BPF, 4,4′-dihydroxydiphenyl-methane) and bisphenol S (BPS, 4,4′-sulfonylbisphenol), are now preferred over BPA.[2,16–18]

Bisphenol F is frequently used as an alternative to BPA in construction materials with high durability, epoxy resins, industrial coatings and water pipes.[19,20] BPS is also used as a BPA-alternative in epoxy resins and inner lining of cans. In addition, BPS is widely available present in thermal papers, tickets, fast food boxes, brominated flame-retardants, luggage tags, flyers and

Received on 23 May 2024; revised on 2 July 2024; accepted on 6 August 2024

newspaper material in various industrial products due to its high temperature stability and resistance to sunlight.[20,21]

Although the use of BPF and BPS increases day by day, very little is known about the toxicity mechanisms of these alternative bisphenols.[22,23] A few in vivo and in vitro studies have shown that exposure to BPF may lead to endocrine disruption and cause genotoxicity.[16,22–29] Although BPS is more resistant to leaking from plastic material compared to BPA, it can still pose a potential risk. It is stated that BPS may also have estrogen-like effects.[19,29,30]

Concerning all the available data, this study aimed to examine in vitro possible apoptotic, genotoxic and immunotoxic effects of bisphenol derivatives in HepG2 cell line. As liver is crucial for xenobiotic metabolism and detoxification processes, HepG2 cells can reflect how bisphenol derivatives affect hepatic caspase activities, levels of 8-hydroxy-desoxyguanosine (8-OHdG), 8-oxoguanine glycosylase (OGG1), interleukin 4 (IL-4), interleukin 10 (IL-10), transforming growth factor beta 1 (TGF-β1) and tumor necrosis factor alpha (TNF-α). Moreover, In this study, Half-maximal inhibitory concentration50 (IC_{50}) values of BPA, BPF and BPS on biological targets (IL-4, IL-10, TGF-β1, TNF-α, 8-OHdG, OGG1, caspase 3, caspase 8, and caspase 9) were investigated with machine learning models.

Materials and methods

Dataset

All activity data of the biological targets examined in this study were achieved from the ChEMBL database.

Artificial intelligence

In this study, machine learning methods were used. The python library lazypredict was used to determine the appropriate machine learning algorithm. The Lazypredict library was installed on 2022 September 20 using pip, the standard python installer, as described in the documentation. The Lazy Predict version used is 0.2.12, released on 2022 February 6. The Lazy Predict library is available at https://pypi.org/project/lazypredict/. For each target, 42 different algorithms were created. The algorithms were trained with 80% selected molecules randomly. Predicted IC_{50} (pIC_{50}) values from the prepared data set and tested with the data were not used in the training.

Chemicals, kits and reagents

Bisphenol A, BPF, BPS, trypsin/EDTA, phosphate buffered saline (PBS) powder, trypsin–EDTA, cell lysis buffer and protease inhibitor cocktail were purchased from Sigma-Aldrich (Mannheim, Germany). Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's Phosphate Buffered Saline (DPBS), fetal bovine serum (FBS), and penicillin G/streptomycin were purchased from Biowest (Riverside, MO). Commercial caspase 3/8/9 kit was purchased from Abcam (Cambridge, England). 8-OHdG kit and OGG1 kit were purchased from MyBiosource (San Diego, CA). Ouick-DNA Minirep kit was purchased from Zymo Research (Irvine, CA). Legend Max Interleukin 4 (IL-4) kit was purchased from Biolegend (San Diego, CA). IL-10, TGF-β and TNF-α kits were purchased from BT LAB (Shanghai, China). Protein determination kit was purchased from Cayman (Ann Arbor, MI).

Cell line

The human hepatoma cell line (HepG2 cells, with passage number up to 18-20) was used for experiments. HepG2 Cell line was obtained from American Type Cell Collection (ATCC; HB-8065™) (Rockville, MD). The Declaration of Helsinki was not needed as study was performed on a commercially available cell line.

Cells were grown in 10% FBS and 1% penicillin/streptomycin in DMEM and incubated at 37 °C and 5% CO_2. Cell medium has been changed 2-3 times a week depending on the proliferation of cells. After the cells reached 95% confluency, they were exposed to BPA, BPF and BPS.

Experimental groups

The inhibitory concentration 30 (IC_{30}) were obtained from our previous study.[31] 3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed to determine the cytotoxic effects of BPA, BPF, and BPS. 10^4 HepG2 cells were seeded per well in 96-well plates and incubated overnight. On the second day, cells were treated with various concentrations of BPA (25–800 μM), BPF (25–600 μM), and BPS (10–600 μM) in the cell medium for 24 h. Cell viability was calculated as previously described in the literature.[31] The values were normalized and presented as the percentage difference of controls. The experiments were repeated three times on different days, with two replicates each day. The mean of all the experiments was calculated. The cells were exposed to BPA, BPF and BPS at IC_{30} doses.

1) **Control group**: Control HepG2 cells.
2) **BPA group:** HepG2 cells exposed to IC_{30} dose (397.27 μM) of BPA for 24 h.
3) **BPF group:** HepG2 cells exposed to IC_{30} dose (371.89 μM) of BPF for 24 h.
4) **BPS group:** HepG2 cells exposed to IC_{30} dose (191.52 μM) of BPS for 24 h.

Preparation of bisphenol analogues

All stock solutions were prepared freshly. For all the bisphenol derivatives, a stock of 1 mg/mL was prepared in 0.01% ethanol. Later, the stock solution of each bisphenol derivative was diluted to the IC_{30} dose with medium.

Cell lysis

Cells were washed with 4 mL Dulbecco's Phosphate-Buffered Saline (DPBS) twice. After washing, cells were trypsinized and the cell suspension was transferred at falcon tubes. Tubes were centrifuged at 1,200 rpm for 5 min and supernatants were discarded. The cell pellets were lysed with 400 μL cell lysis buffer which contained 1% protease inhibitor cocktail. The lysates were aliquoted and stored at −80°C until experiments were performed.

Determination of caspase levels involved in extrinsic and intrinsic apoptosis pathways

The activity of caspase 8, which is involved in the extrinsic apoptotic pathway, caspase 3 which are active in both intrinsic and extrinsic pathways, and caspase 9, which is involved in the intrinsic caspase pathway, were determined by using a commercial spectrofluorometric caspase 3/8/9 kit that used appropriate substrates.

Genotoxicity

DNA isolation

A commercial DNA isolation kit which uses Zymo-Spin Column technology was used to isolate total DNA (e.g. genomic, mitochondrial, viral) from cell lysates. DNA from HepG2 cells was isolated by using the elution column, wash buffers and genomic lysis buffer.

DNA base damage

Levels of 8-OHdG, one of the most common guanine damages, were determined spectrophotometrically with a commercial kit following DNA isolation. This assay employs the competitive inhibition enzyme immunoassay.

DNA repair enzyme levels

8-oxoguanine glycosylase (OGG1) levels, the most important enzyme in the base excision repair (BER) repair pathway, were determined spectrophotometrically with a commercial human-specific ELISA kit. This kit uses the quantitative sandwich ELISA principle.

Measurement of immune system parameters

To evaluate immune system parameters, measurements of IL-4, IL-10, TGF-β and TNF-α levels were conducted in the cell culture media of experimental groups. These parameters were measured with commercial ELISA kits.

Total protein determination

The total protein levels in cell lysates and cell culture supernatants were measured with a commercial kit that employs Bradford method.[32]

Statistical analysis

Statistical Package for Social Sciences Program (SPSS) 17.0 (Chicago, IL) was used for statistical analysis. The differences among the groups were evaluated with Kruskal–Wallis one-way analysis of variance, followed by Mann–Whitney U test. Results were expressed as mean ± standard deviation (SD). *P* values < 0.05 were considered as statistically significant.

Results

Data set and artificial intelligence

In this database, we removed repeated data taken from IC_{50} data for each goal. The more accurate data belong to the molecules to be established fingerprint a machine learning model to PubChem Simplified Molecular Input Line Entry System (SMILES) in which IC_{50} data were converted to pIC_{50} ($-\log(IC_{50})$). The number of molecules that can be used in artificial intelligence training for IL-4, IL-10, and 8-OHdG targets is zero. For this reason, studies on these targets could not be carried out. The number of molecules taken from the database and used to train the artificial intelligence is shown in Table 1. At the same time, the number of molecules in the database used for training in OGG1 and Caspase 9 targets is low. For optimization of results, the results of these two targets should be supported by in vitro results.

Table 1. Number of molecules obtained for each biological target and used for training purposes in ML.

Biological Target	Number of molecules in the database	Number of molecules used in artificial intelligence training
TGF-β1	1681	1135
TNF-α	1139	952
OGG1	44	21
Caspase 3	2450	2155
Caspase 8	444	417
Caspase 9	51	48
IL-4	0	0
IL-10	0	0
8-OHdG	2	0

Considering the R^2 values and prediction times generated by the models using the test data, it was determined that the Random Forest Regression algorithm was suitable for the 6 targets to be examined. As an example, the R^2 values of all models trained for the TNF-α target are given in Fig. 1, and the time required to construct these models is given in Fig. 2.

OGG1 training set experimental and calculated pIC_{50} values are shown in Fig. 5 and Table 4. R^2 value was found to be 0.86. For caspase 3, 8 and 9 training set experimental and calculated pIC_{50} values are shown in Fig. 6 and Tables 5, 6, and 7, respectively. The R^2 values were found to be 0.95, 0.93 and 0.82, respectively.

TGF-β1 training set experimental and calculated pIC_{50} values are given in Fig. 3 and Table 2. R^2 value was found to be 0.89. The TNF-α training set experimental and calculated pIC_{50} values are given in Fig. 4 and Table 3. R^2 value was found to be 0.90.

Determination of caspase 3, 8 and 9 activities

Caspase 3, 8 and 9 levels are given in Fig. 7. When the caspase 3 activities of the groups were compared, the caspase 3 activities of the BPF (~15%) and BPS (~18%) groups were found to be significantly higher than the control and BPA groups ($P < 0.05$, all).

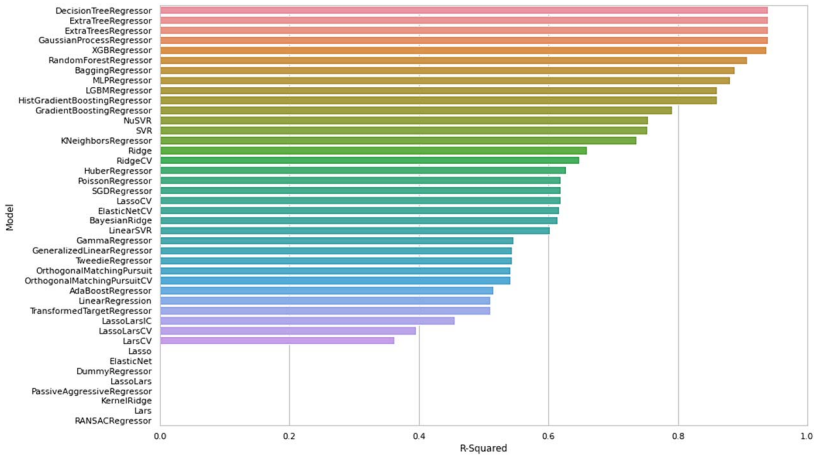

Fig. 1. Accuracy data for models created for the TNF-α target. TNF-α: Tumor necrosis factor-α.

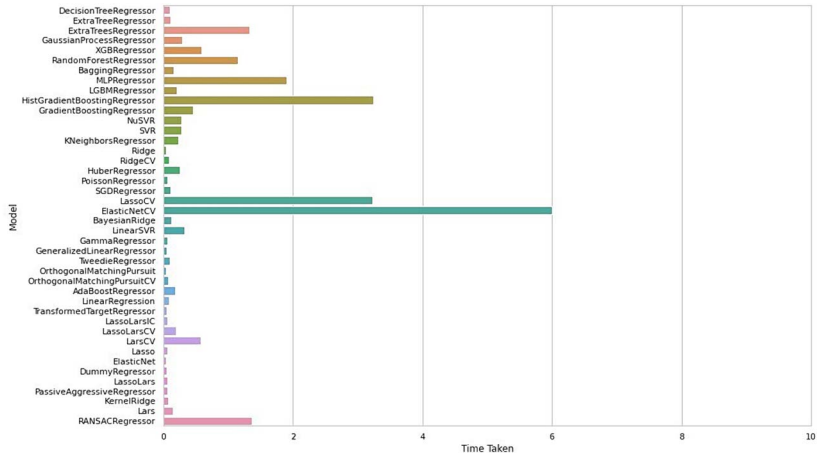

Fig. 2. Prediction times for models created for the TNF-α target. TNF-α: Tumor necrosis factor-α.

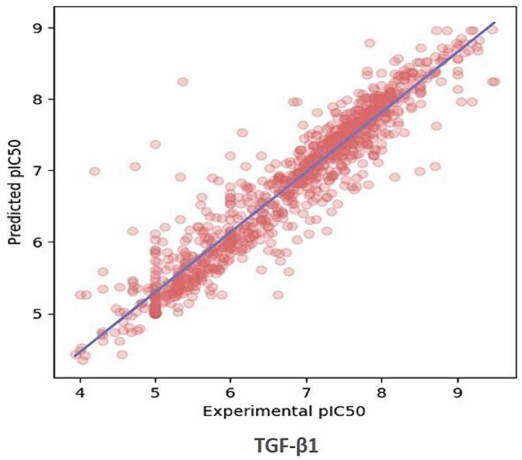

Fig. 3. TGF-β1 training set experimental and calculated pIC50 values. R2 = 0.89 TGF-β1: Transforming growth factor beta 1.

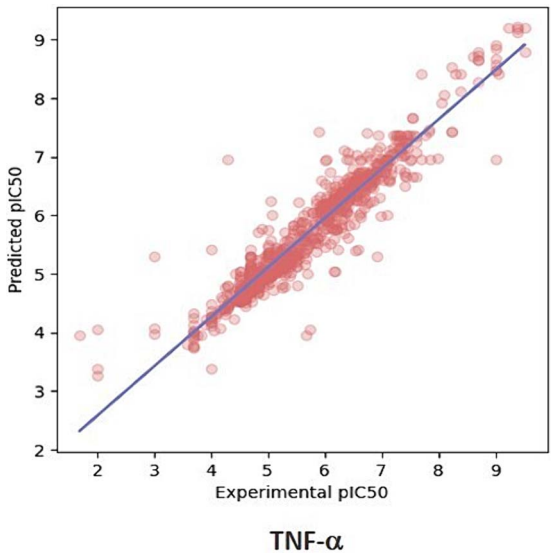

Fig. 4. TNF-α training set experimental and calculated pIC50 values. R2 = 0.90 TNF-α: Tumor necrosis factor-α.

Table 2. Predicted pIC_{50} and IC_{50} values of TGF-β1.

Molecule	Predicted pIC_{50}	Predicted IC_{50} (nM)
BPA	5.813	1538
BPF	5.7205	1903
BPS	5.7101	1949

Table 3. Predicted pIC_{50} and IC_{50} values of TNF-α.

Molecule	Predicted pIC_{50}	Predicted IC_{50} (nM)
BPA	4.8986	12629
BPF	5.1634	6864
BPS	5.2052	6234

When the caspase 8 activities of the groups were compared, the caspase 8 activities of the BPF (~15%) and BPS (~16%) groups were found to be significantly higher than the control and BPA groups ($P < 0.05$, all).

When the caspase 9 activities of the groups were compared, it was determined that the caspase 9 activities of the BPF and BPS groups were significantly higher than the BPA group ($P < 0.05$). Caspase 9 activities of the BPF and BPS groups were found to be approximately 12% and 11% higher than the control group. However, the difference was not statistically significant.

DNA base damage and repair

8-OHdG and OGG1 levels are given in Fig. 8. When the 8-OHdG levels of the groups were compared, the 8-OHdG levels of the BPA (33%) and BPS groups were not different from the control group ($P > 0.05$, both). However, there was a decrease in the BPF (62%) group ($P < 0.05$). When OGG-1 levels were evaluated, there were no significant difference between the study groups.

Interleukin 4, interleukin 10, transforming growth factor beta 1 and tumor necrosis factor-α levels

Interleukin 4, IL-10 TGF-β1 and TNF-α levels are given in Fig. 9. Interleukin 4 levels of the groups treated with BPA (31%), BPF (29.5%) and BPS (20%) were significantly lower than the control ($P < 0.05$, all).

Interleukin 10 levels of the groups treated with BPA (31%) and BPF (39%) were significantly lower than the control ($P < 0.05$). An insignificant decrease (18%) was observed in the BPS group compared to the control group ($P > 0.05$).

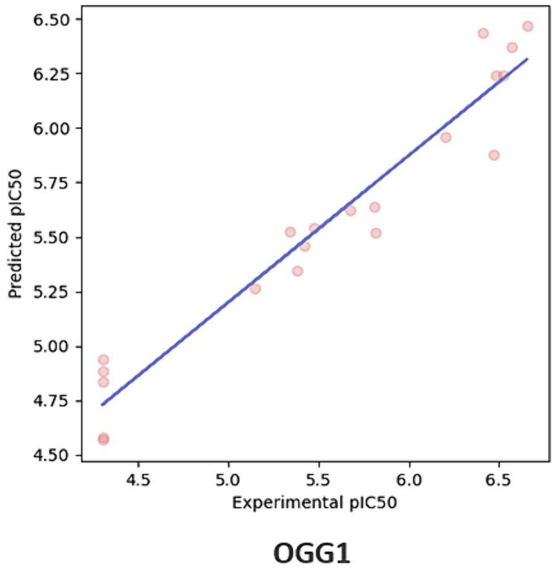

Fig. 5. OGG1 training set experimental and calculated pIC50 values. R2 = 0.86 OGG1: 8-oxoguanine glycosylase.

Table 4. Predicted pIC_{50} and IC_{50} values of OGG1.

Molecule	Predicted pIC_{50}	Predicted IC_{50} (nM)
BPA	4.9897	10240
BPF	4.7171	19182
BPS	4.7270	18749

When the TGF-β1 levels of the study groups were measured, it was found that there was a significant increase in the BPS administered group compared to the control (63%), BPA (77%) and BPF (65%) groups ($P < 0.05$, all).

When the TNF-α levels of the study groups were compared, we observed a significant increase in the BPS administered group compared to the control (61%), BPA (~3-fold) and BPF (88%) groups ($P < 0.05$). Moreover, TNF-α levels in BPA group was 45% lower than control ($P < 0.05$).

Discussion

Bisphenol derivatives are the most abundant chemicals. They are present in many products used in human life.[33] Studies on the toxic effects of BPA, the most widely used bisphenol derivative, suggest that it has oxidant, genotoxic, epigenotoxic and immunotoxic effects.[34] However, there is limited data on the mechanisms of toxicity of other bisphenol derivatives, including BPF and BPS. Therefore, there is still need to identify their mechanisms of action and whether they lead to apoptotic, genotoxic and immunotoxic effects. Moreover, as in vitro and in vivo data are missing, there is need for in silico studies in order to understand the toxic doses of bisphenol derivatives on the components of immune system and on apoptotic markers. We have used HepG2 cells as an in vitro model in order to evaluate the possible hepatotoxic effects of bisphenol derivatives comparatively.

Our data from in silico studies clearly show that BPA, BPF and BPS can affect different immune system components as well as apoptotic markers and DNA damage. In our previous study on HepG2 cells, we have observed that the IC_{50} values of BPA, BPF and BPS were 623.30, 611.72 and 428.8 μg/mL, respectively.[31] The predicted IC_{50} values for TGF-β1 for BPA, BPF and BPS were 1,538, 1,903 and 1,949 nM, respectively. In addition, the predicted IC_{50} values for TNF-α for BPA, BPF and BPS were 12,629, 6,864 and 6,234 nM, respectively. These findings suggest that these compounds can affect immune response before they cause cell death. The predicted IC_{50} values for OGG1 for BPA, BPF and BPS were 10,240, 19,182 and 18,749 nM. This suggests that at even lower concentrations than they cause cytotoxicity, they can affect the levels of OGG1 which is an essential enzyme in DNA base repair. On the other hand, our in silico findings suggest that the apoptotic markers, namely caspases are also affected by exposure to bisphenol derivatives at lower doses. All these findings may suggest that the lower dose effect of bisphenols. Typical human exposures to endocrine disrupting chemicals (EDCs) like bisphenols occur at low doses from residues on food, personal care products, household detergents, lawn care products, food packaging, and other sources. Biomonitoring studies of environmentally exposed individuals showed that serum, plasma or urine levels of EDCs are in the ppm, ppt and ppb range. These levels refer to the circulating levels of EDCs, or the amount of chemical that is excreted by urine. However, these levels alone does not provide information about the actual exposure levels that is required to lead to these circulating concentrations. The term "low dose" is used widely in the field of environmental health science and indicates that low doses of chemicals may also cause alterations in different organ systems, particularly in endocrine system. However, there are currently no agreed-upon standards for a "low-dose cut-off point". In 2002, National Toxicology Program (NTP) defined low-dose cut-off as "doses in the range of typical human exposures or doses below those tested in traditional toxicological assessments".[35] Until the last decades, toxicologists have mainly concentrated on high dose effects like occupational exposures. However, we can suggest that any epidemiology study examining environmentally exposed individuals would be defined as a low-dose study because such studies are conducted on the general population. Occupational exposures are often considered "high dose" and thus are examined separately. Scientists should now begin to investigate the toxicokinetic properties of a chemical at low doses as at these doses the metabolic response of an organism toward a chemical may be different than high doses and low dose in vivo studies may be a better reflection of the blood concentrations in the general human population.[36,37] The "rules" of hormones are obeyed by EDCs.[38–42]

i) Hormones act at low levels and EDCs like bisphenols are suggested to show different toxic effects at low levels.
ii) Hormones have diverse integrations with different the systems and have roles from conception through aging and bisphenols, particularly BPA also affects many processes from in utero development to the late life.
iii) Hormones act via different cytoplasmic or nuclear receptors as well as EDCs, like bisphenols.
iv) Hormones may have non-linear or even non-monotonic dose–response curves and BPA is also suggested to have non-linear dose response in vivo.
v) The effects of hormones depend on age. Thus, it is suggested that effects of EDCs are more pronounced in early life than in late life.

Considering all the data in literature and the in silico data generated in this work, we can suggest that bisphenols show significant effects on immune parameters, DNA repair and apoptotic markers long before they cause cytotoxicity. Our findings also suggest the low dose effect of these chemicals.

The increase in caspase 8 activity mainly indicate the activation of extrinsic caspase pathway while activation of caspase 9

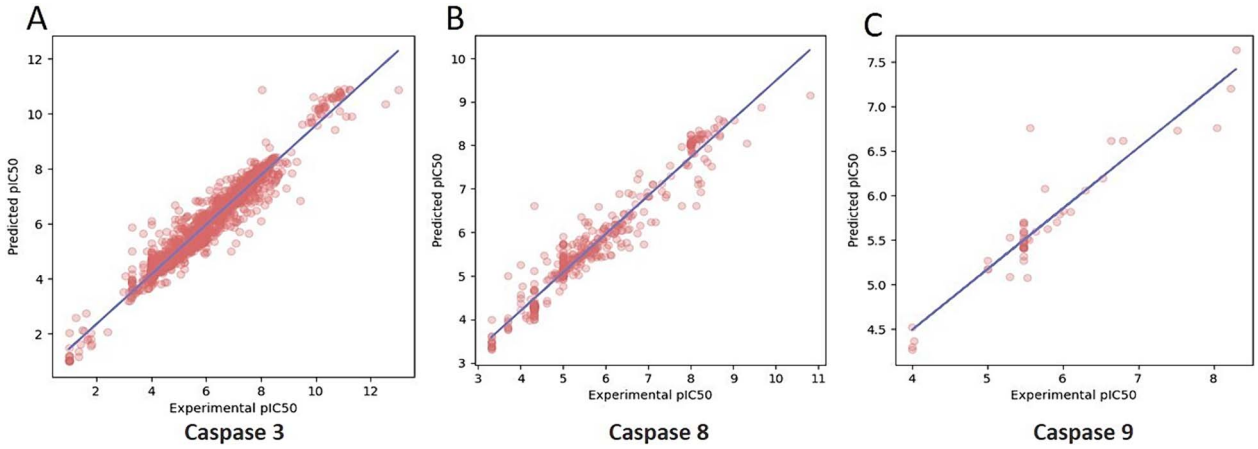

Fig. 6. Caspase 3, caspase 8 and caspase 9 training set experimental and calculated pIC50 values. A) Caspase 3 training set experimental and calculated pIC50 values. B) Caspase 8 training set experimental and calculated pIC50 values. C) Caspase 9 training set experimental and calculated pIC50 values. R2 = 0.95 for caspase 3; R2 = 0.93 for caspase 8 and R2 = 0.82 for caspase 9.

Table 5. Predicted pIC_{50} and IC_{50} values of caspase 3.

Molecule	Predicted pIC_{50}	Predicted IC_{50} (nM)
BPA	4.6376	23035
BPF	4.6187	24060
BPS	4.5816	26205

Table 6. Predicted pIC_{50} and IC_{50} values of caspase 8.

Molecule	Predicted pIC_{50}	Predicted IC_{50} (nM)
BPA	4.6915	20343
BPF	4.7052	19710
BPS	4.7454	17970

Table 7. Predicted pIC_{50} and IC_{50} values of caspase 9.

Molecule	Predicted pIC_{50}	Predicted IC_{50} (nM)
BPA	6.6056	248
BPF	7.0542	88
BPS	7.0993	80

shows the induction of intrinsic caspase pathway [34]. Mocra et al. (2015) studied the apoptotic potential of BPA and its analogs in human peripheral blood mononuclear cells (PBMCs) and found that BPA caused the activation of caspase 3, caspase 8 and caspase 9.[43] Similarly, Huang et al.[44] observed increases in caspase 3, caspase 8, and caspase 9 activities after BPA exposure in human macrophages.[44] In PBMCs, BPA also caused the activation of caspase 3 and caspase 9.[44,45] In one of our previous studies, we have observed that that fetal and neonatal exposure of rats to BPA and di(2-ethylhexyl)phthalate (DEHP) could lead to significant increases in caspase 3 and caspase 8 levels in testicular tissue.[46] In two recent studies, Harnett et al. determined that BPA (1-10 μM), BPS (1–100 μM) and BPAF (3×10^{-4}-30 μM) can cause apoptosis in rat and human stem cells.[47,48] In our study, we observed that caspase 3, caspase 8 and caspase 9 activities of the BPF and BPS groups were found to be significantly higher than the control and BPA groups. These findings indicate that BPF and BPS can activate both extrinsic and intrinsic caspase pathways, the importance of which must be elucidated with further studies.

Biomacromolecular damage, especially DNA damage caused by BPA, is one of the mechanisms underlying its apoptotic property.[49] Pietro et al.[50] demonstrated that BPA causes chromosome fragmentation by affecting DNA damage checkpoints in human PBMCs.[50] On the other hand, other bisphenol derivatives, such as BPS, may also cause DNA damage. In human bronchial epithelial cells, BPA and BPS administration caused significant DNA damage.[51] In a study with a macrophage cell line (RAW264.7), bisphenol-A-glycidyldimethacrylate (BisGMA), a bisphenol derivate, exhibited genotoxicity by causing a dose-dependent increase in the numbers of micronuclei and DNA strand breaks.[52] Exposure of human PBMCs to BPA analogues like BPS, BPF and BPAF at a dose of 1 ng/mL, also led to genotoxicity and caused oxidative DNA damage.[45] Our results showed that the 8-OHdG levels of the BPA and BPS groups were not different from the control group ($P > 0.05$). However, there was a decrease in the BPF (62%) group ($P < 0.05$). The decrease in 8-OHdG levels in BPF group can be explained, in part, by the higher levels of OGG1 in BPF-exposed HepG2 cells though the increase in OGG-1 levels was only ~8% higher than control ($P < 0.05$). In a study by Kose et al. (2020), RWPE-1 cells were used as a model to compare cytotoxicity, oxidative stress-causing potential and genotoxicity of BPA, BPF and BPS. BPS produced significantly higher levels of DNA damage vs. the control in the standard and modified Comet assay. DNA repair proteins (OGG1, Ape-1, and MyH) involved in the base excision repair pathway, as well as p53 protein levels were down-regulated in all of the bisphenol-exposed groups. The researchers suggested that BPA alternatives led to alterations in the expressions of DNA repair enzymes.[22] The inconsistence of the results obtained in these studies and our study may arise from the different types of cell lines as well as from the different applied doses. As being hepatic cells, HepG2 cells have a higher rate of DNA repair compared to prostate cells or hematopoietic cells. On the other hand, as the current experiments are conducted after 24 h of exposure, the repair was achieved. However, there is need to observe the changes in DNA base damage after both shorter and longer periods of exposure in HepG2 cells.

Interleukin 4 is a key regulatory cytokine in humoral and adaptive immunity. It induces differentiation of naive helper T cells (Th_0 cells) to Th_2 cells, stimulates activated B cell and T cell proliferation, and differentiates B cells into plasma cells.[53] Overproduction of IL-4 is associated with allergies.[54] On the other hand, IL-10 is a cytokine with multiple effects in

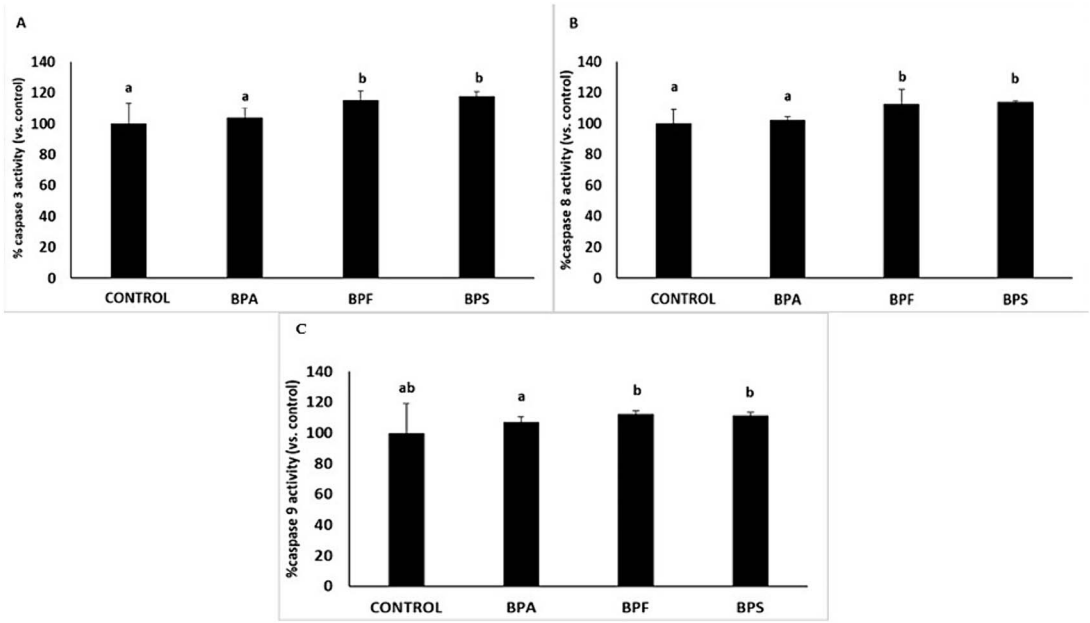

Figure 7. Caspase 3, 8 and 9 activities. A) Caspase 3 activities in study groups. B) Caspase 8 activities in study groups. C) Caspase 9 activities in study groups. Caspase activities of the study groups. The caspase activities of amount of the control cells was assumed as 100% and the other cells caspase activities were calculated as % compared to the control. a.b. Bars that do not carry the same superscripts are statistically different from each other ($P < 0.05$).

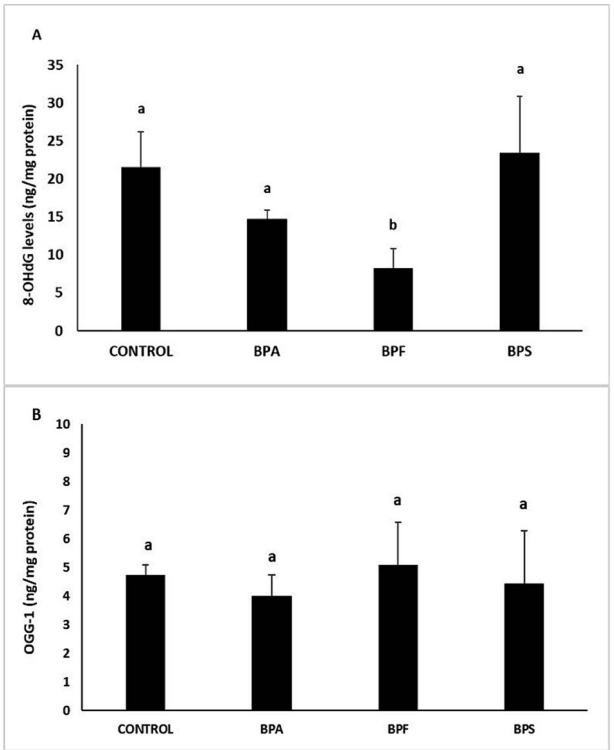

Fig. 8. 8-hydroxy-2′-deoxyguanosine and -oxoguanine glycosylase levels. A) 8-OHdG levels in the study groups. B) OGG-1 levels in the study groups. a.b. Bars that do not carry the same superscripts are statistically different from each other. ($P < 0.05$).
8-OHdG:8-hydroxy-2′-deoxyguanosine; OGG1: 8-oxoguanine glycosylase.

immunoregulation as well as in inflammation. It downregulates the expression of Th_1 cytokines, major histocompatibility complex (MHC) class II antigens, and co-stimulatory molecules on macrophages.[53,54] On the other hand, IL-10 enhances B cell survival, proliferation, and antibody production. IL-10 can block nuclear factor kappa B (NF-κB) activity, and is involved in the regulation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway.[55] It was suggested that IL-4 enhances IL-10 production in Th_1 cells.[56] TNF-α, an adipokine and a cytokine, is produced by a wide variety of immune system cells. In liver, this cytokine stimulates the acute phase response, leading to an increase in C-reactive protein (CRP) and a number of other mediators. It also induces insulin resistance by promoting serine-phosphorylation of insulin receptor substrate-1 (IRS-1), which impairs insulin signaling.[57] The cytokines IL-4 and IL-10 act on monocytes to suppress the release of pro-inflammatory cytokines, including TNF-α and to influence the release of sTNF-R.[58] TGF-β1 is a secreted protein that performs many cellular functions, including the control of cell growth, cell proliferation, cell differentiation and apoptosis. Some T cells (e.g. regulatory T cells) release TGF-β1 to inhibit the actions of other T cells. TGF-β1 inhibits proliferation, stimulates apoptosis of B cells and controls the expression of antibody, transferrin and MHC class II proteins on immature and mature B cells. A combination of TGF-β and IL-10, but not single cytokine, is required to suppress B cell activation induced by toll-like receptor (TLR) stimulation.[59]

According to our results, IL-4 levels of the groups treated with BPA, BPF and BPS were significantly lower than the control group. IL-10 levels of the groups treated with BPA and BPF were significantly lower than the control and an insignificant decrease was observed in the BPS group compared to the control group ($P > 0.05$). When the TGF-β1 levels of the study groups were measured, it was found that there was a significant increase in the BPS administered group compared to the control, BPA and BPF groups ($P < 0.05$). In addition, we observed a significant increase in TNF-α levels in the BPS group compared to the control, BPA and BPF groups ($P < 0.05$). There are limited studies about the immunotoxic effects of BPA derivatives. In a study with unexplained recurrent spontaneous abortion women, no statistical correlation

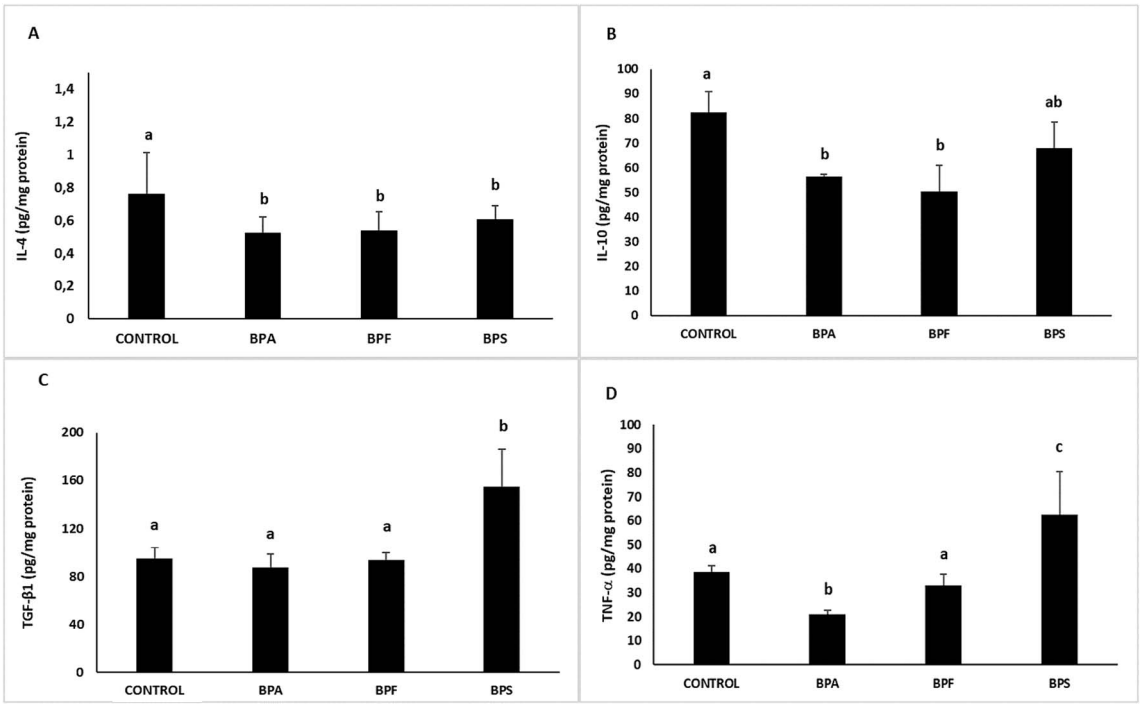

Fig. 9. Interleukin 4, interleukin 10, transforming growth factor beta 1 and tumor necrosis factor-α levels. a.b. Bars that do not carry the same superscripts are statistically different from each other. ($P < 0.05$). A. IL-4 levels in the study groups. B. IL-10 levels in the study groups. C) TGF-β1 levels in the study groups. D) TNF-α levels in the study groups. a.b.c Bars that do not carry the same superscripts are statistically different from each other. ($P < 0.05$). IL-10: Interleukin 10; IL-4: Interleukin 4; TGF-β1: Transforming growth factor beta 1; TNF-α: Tumor necrosis factor-α.

between BPS and biomarkers of immune system (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13), TNF-α, TGF-β and interferon gamma (IFN-γ) was observed.[60] It was suggested that BPA exposure enhances TNF-α and IL-6 expression but inhibited TGF-β and IL-10.[61] BPA in cultured human endometrial stromal cells (ESCs) induced expression of inflammatory genes e.g. TNF-α, IL-6, and IL-1β. In addition, BPA-treated ESCs released TNF-α and IL-6 significantly.[62] BPA exposure led to significant reduction in the IL-6 secretion in lipopolysaccharide (LPS)-stimulated RAW264.7 cell cultures.[63] We can suggest that the changes in the cytokine levels as well as in TGF-β1 may cause alterations in the immune response of liver and complex immunotoxic effects of bisphenols may lead to different pathological conditions yet to be identified.

Conclusion

In conclusion, we can suggest that bisphenol derivatives used as alternatives to BPA also showed similar or higher toxic effects on HepG2 cells. Considering that BPA analogs such as BPF and BPS have a similar chemical structure to BPA, it is not surprising that these compounds have similar toxic effects and mechanisms of actions with BPA. Therefore, the discovery of safer BPA analogues is crucial as both BPF and BPS cause apoptotic and immunotoxic effects. In addition, especially considering the limited number of studies evaluating the immunotoxic effects of BPA analogues, in vivo and human studies evaluating the immunotoxic effects of these compounds are needed.

Author contributions

Aylin Balci-Ozyurt and Pınar Erkekoglu participated in conceptualization, methodology, investigation, formal analysis, original draft preparation, manuscript review, and editing. Anıl Yirun, Deniz Arca Cakır, İbrahim Ozcelik, and Gizem Ozkemahli participated in conceptualization, investigation, formal analysis. Pınar Erkekoglu, Merve Bacanli, Suna Sabuncuoglu, Nursen Basaran participated in conceptualization and supervision. All authors read and approved the final manuscript.

Conflict of interest statement

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

This study was supported by Hacettepe University Scientific Research Unit Research Project [THD-2020-18518].

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