IVIVE-PBPK based New Approach Methodology for addressing early life toxicity induced by Bisphenol A

Mengmei Ni[a,b], Deepika Deepika[b,c], Xiaomeng Li[a], Wei Xiong[a], Lishi Zhang[a], Jinyao Chen[a,*], and Vikas Kumar[b,c,d*]

[a]*West China School of Public Health/West China Fourth Hospital and Healthy Food Evaluation Research Center, Sichuan University, Chengdu, China*

[b]*Environmental Engineering Laboratory, Departament d' Enginyeria Quimica, Universitat Rovira i Virgili, Av. Països Catalans 26, 43007, Tarragona, Catalonia, Spain*

[c]*IISPV, Hospital Universitari Sant Joan de Reus, Universitat Rovira i Virgili, Reus, Spain*

[d]*German Federal Institute for Risk Assessment (BfR), Max-Dohrn-Str. 8-10, 10589 Berlin, Germany*

Abstract

Bisphenol A (BPA) is a known endocrine disruptor mimicking natural estrogens with the potential to affect human health, especially during prenatal and postnatal exposure at or below current acceptable daily intake levels. Different adverse effects of BPA are still under investigation, and multiple mechanisms of action remain unexplored. This may be one of the reasons for the continuously changing tolerable daily intake (TDI) of BPA with the emergence of new adverse health effects over time. In addition, translational modelling through in vitro-in vivo extrapolation (IVIVE) can act as prerequisite bridge for translating in-vitro finding into human risk assessment. The objective of this study was to conduct in-vitro experiments and utilize an IVIVE-pregnancy physiologically based pharmacokinetic (P-PBPK) modeling to investigate developmental neurotoxicity and embryotoxicity in humans. The data obtained from human embryonic stem cells-based assays (study conducted between October 2020-2021) were used for the IVIVE-P-PBPK models to obtain the human equivalent doses (HEDs) which were further extrapolated to reference doses (RfDs). The results showed that simulated mean RfDs (μg/kg/day) derived from the HSD3B1 and NFATC2 gene of embryotoxicity and neurodevelopmental toxicity tests, respectively, were 4.94 and 5.18. The simulated RfDs were close to the temporary-tolerable daily intake (t-TDI) recommended by European Food Safety Authority (EFSA) in 2015 (t-TDI: 4 μg/kg·bw) and higher than the TDI of 2023 (0.2 ng/kg·bw). In conclusion, in-vitro toxicogenomics dose-response data combined with PBPK modeling can become a promising alternativenew approach methodology (NAM) to support decision-making in chemical risk assessment. Based on the simulated RfDs derived from this NAM, the t-TDI set by EFSA in 2015 may be considered a safe exposure limit for mothers and fetuses at the current BPA intake levels in Chinese mothers. This study provided an animal-free new strategy for NAMs based risk assessment by combining toxicogenomics and computational toxicology.

Key words: Bisphenol A; IVIVE, PBPK; Embryotoxicity; Neurotoxicity; human embryonic stem

cells; toxicogenomics

1. Introduction

Bisphenol A (BPA) is a synthetic endocrine disruptor widely used to manufacture polycarbonate plastics and epoxy resins since the early 1950s[1]. It is often used in plastic containers, toys, baby bottles, electrical equipment, medical devices, and much more[2, 3]. With the continuously growing demand for BPA production, the global BPA market is estimated to have a value of 30.62 billion dollars by 2026[4]. BPA is highly prevalent in the environment and has been found in water (surface water, river water, groundwater, wastewater, and freshwater), sediments and soil samples worldwide[5]. A recent study in Mexico showed a BPA concentration of 148–9340 ng/L in water samples used for the irrigation of crops[6]. China has the highest concentration of BPA in lake sediments, ranging from 1.1 to 270 ng/g dry weight[7] and in soil ranging from 72,500 to 198,000 ng/g·dw[8]. Humans can be exposed to BPA via ingestion through food (vegetables, fruits, fish, meat, canned food and infant food) which remains the major source of exposure followed by dermal and inhalation[9, 10].

As an endocrine-disrupting chemical (EDC), BPA can disrupt the hormonal system and also exerts a harmful impact on human health by affecting reproductive, developmental, immunological, and neurological functions[11-14]. BPA is being detected in human urine, blood, amniotic fluid, breast milk and other biological specimens[14-16]. The fetal development stage is known to be the critical period of vulnerability to BPA exposure[17], which may result in adverse birth outcomes[18].

Many countries worldwide have issued strict regulations to limit BPA exposure[19, 20]. Regulatory authorities from Canada and Europe have banned the BPA use in polycarbonate infant feeding bottles[21]. A specific migration limit (SML) of 0.05 mg of BPA per kg of food was established by the E.U. in February 2018 to avoid migration from varnishes or coatings applied to food packaging materials (No 2018/213). The U.S. Food and Drug Administration (FDA) announced that BPA could no longer be used in baby bottles and sippy cups in 2012. Also, the European Food Safety Authority (EFSA) has continuously decreased the tolerable daily intake (TDI) of BPA over the years. In 2006, the temporary-tolerable daily intake (t-TDI) of 50 μg/kg bw/day was set based on the reduced body weight in rats[22] which was reduced to 4 μg/kg bw/day in 2015 based on the increase in relative kidney weight[23]. In the recent scientific opinion of 2023, EFSA established the TDI to 0.2 ng/kg bw based on immune toxicity in mice[24,25]. The continuous reduction in TDI over the years emphasizes the need to fully elucidate the adverse effects of BPA on human health especially in sensitive age-groups. The traditional risk assessment is mainly based on animal testing, which is time-consuming and expensive and is not in accordance with the Three Rs principles (replacement, reduction and refinement)[26]. The other concerns are the uncertainty in dosimetry, extrapolation from animal to human due to different toxicokinetics, and extrapolating high-dose effects in animals to low-dose human exposures[27]. Moreover, the adverse effects found in animal testing may not represent the most sensitive toxic effects for the human population especially the fetus, leading to an underestimation of safety limits. To reduce these uncertainties, in-vitro cell line data translated with in-vitro to in-vivo extrapolation (IVIVE)- physiologically based pharmacokinetic model (PBPK) for humans can be a good alternative. For studying developmental toxicity, embryonic stem cells (ESCs) are considered good models as they can proliferate fast and have the ability to differentiate into any types of somatic cells[28, 29]. In addition, human ESCs avoid bias in interspecies differences and may have better predictability for human risk assessment compared to animal ESCs.

The new approach methodology (NAM) has started gaining popularity to enable translation for

regulatory decision-making after the publication of NRC report on "Toxicity Testing in the 21st Century"[30, 31]. Currently, the IVIVE is being used to quantitatively extrapolate in-vitro experimental results to predict in-vivo biological effects and translate to human equivalent doses (HEDs)[32-34]. IVIVE-PBPK is one of the applications for translation of in-vitro data which falls under NAMs since it can reduce experimental studies, especially testing in animals. There have been few studies in the past utilizing this approach for assessing risk. For instance, Chen et al.[35] used differentially expressed gene (DEG) data from literature of mouse in-vivo and human in-vitro toxicogenomic to convert to reference doses (RfDs) using PBPK model for PFOS chemical. Zhang et al.[36] integrated cell-based assays with a PBPK model combined with the Monte Carlo method to conduct health risk assessment of cadmium exposure. Lin et al.[37] converted ToxCast in vitro estrogen receptor (ER) assays to HEDs of multiple BPs (BPA, BPS, BPF, and BPAF) by a population-based IVIVE extrapolation using PBPK models coupled with Monte Carlo simulations.The objective of this study was to utilize Human ESCs to conduct experiments after BPA exposure for evaluating toxicity in embryos and developing brains. The toxicogenomic data produced in the study was used to obtain dose response through benchmark dose analysis. A Pregnancy PBPK (P-PBPK) model was used to calculate the HED and later RfD to compare with the established regulatory guidelines. This is one of the studies of its kind where human cell-based toxicogenomic data for developmental neurotoxicity (DNT) and embryo toxicity was utilized for risk assessment. Such studies in future may reduce the dependence on animal testing by integrating in-vitro with computational models and reduce inter- and intraspecies variability.

2. Methods

A conceptual diagram of this study is shown in Figure 1. The schematics followed for this study are as follows:

1) In vitro studies: Human ESCs (H9 line) were differentiated into two types of cells——neuronal cells and embryonic cells. The equivalent cell dose was calculated using the P-PBPK model, considering exposure in adult women in the Chinese population. Both types of cells were exposed to different concentrations of BPA for 14 days during differentiation. Cell cycle, qPCR tests and trascriptomics were performed for embryonic cells, while apoptosis and PCR array analysis was conducted for neuronal cells. Toxicogenomic dose-response data was collected and used further for modeling.
2) BMD and P-PBPK Modeling: DEGs were identified by integrating and calculating the above experimental results. The BMD approach was used to conduct the dose-response modeling with PCR data of DEGs. The gene BMD_5 was calculated as POD to derive HED. The POD was used as input for the P-PBPK model to reconstruct exposure for deriving HEDs (The detailed calculation is shown in 2.3).
3) Risk assessment: The RfDs were calculated using simulated HEDs and by considering an additional uncertainty factor of 10 (The detailed calculation is shown in 2.4). The calculated RfDs were compared with the TDI of BPA set by EFSA to evaluate the risk for the fetus.

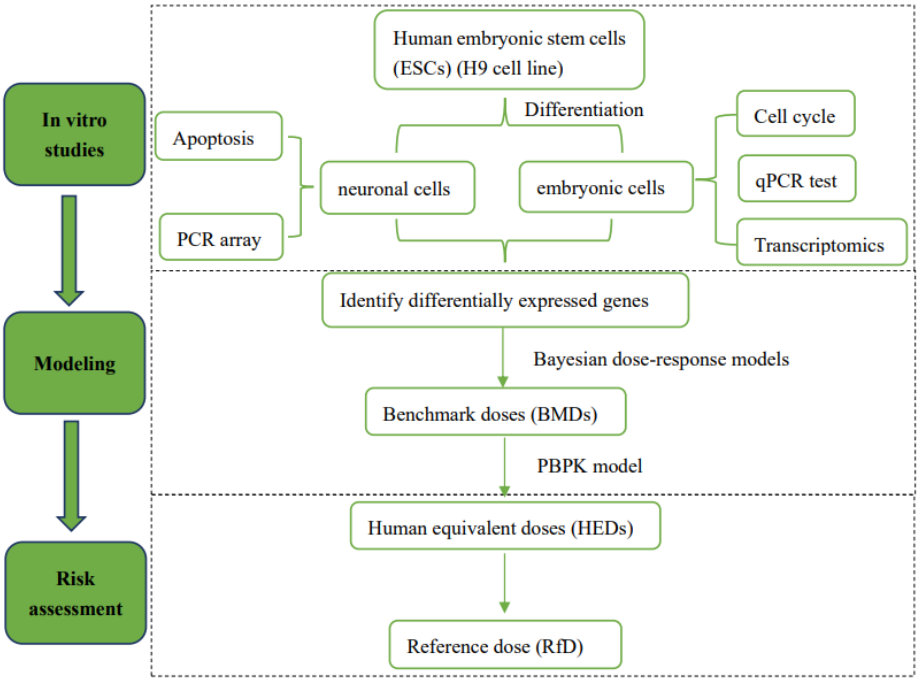

Figure 1. Framework of the study integrating in-vitro data with in-silico model for risk assessment. First, BPA-induced neuronal and embryonic dose-response data were obtained from in vitro studies. Second, the differentially expressed genes were identified and analysed using the Bayesian benchmark dose (BMD) method. The internal dosimetry and the human equivalent doses (HEDs) were subsequently calculated using a published PBPK model. Third, the reference doses (RfDs) for HEDs of different genes associated with BPA exposure were calculated.

In-vitro experiments

2.1.1 Cell culture and treatment

Treatment of H9 cells

Human ESCs (H9 cell line) were obtained from Applied Cell (Shanghai, China). The cells were cultured with matrigel human ESC-qualified Matrix (Corning, Arizona, US) under feeder-independent conditions and grown in mTeSR™ Plus (Stemcell Technologies, Vancouver, Canada) at 37°C and 5% CO_2. The manufacturer's protocol was followed for maintaining, expanding culturing, and passaging the cells. Further details have been provided in (Supplementary file 1).

Differentiation of embryonic cells

The formation and differentiation of embryoid bodies (EBs) were derived from H9 cells, which was based on a previous description[42]. Briefly, single H9 cells were obtained from H9 clones and cultured with the EB formation medium (StemCell Technologies, Inc.) in an Aggrewell™ plate for 2 days. EBs were then collected and placed in an ultra-low adherence 6-well plate with the EB suspension culture medium (knock-out DMEM, supplemented with 20% knockout serum, 1% nonessential amino acids, 2 mM L-glutamine and 0.1mmol/L mercaptoethanol) for 3 days. On day 5, EBs were plated in the EB adherent medium (90% DMEM, 10% fetal calf serum and 4mM L-glutamine) for 11 days. EBs were exposed to BPA for two weeks, starting from the day of EB formation.

Differentiation of neuronal cells

For the generation of neuronal cells, the differentiation process was performed according to

the manufacturer's protocol. A single-cell suspension of H9 cells was collected in AggreWell™800 24-well Plate to generate EBs with STEMdiff™ SMADi Neural Induction. Daily partial (1/2)-medium was changed. After 5 days, EBs were harvested and plated onto matrix-coated wells of a tissue culture-treated 6-well plate. After 7 days, neural rosette selection and replating were conducted to form neuronal precursors. A daily full-medium change with warm (37°C) STEMdiff™ Neural Induction Medium + SMADi was performed for 7 days. Then the neuronal precursors were passaged using STEMdiff™ forebrain neuron maturation medium for neuron maturation. Neurons were exposed to BPA with STEMdiff™ forebrain neuron maturation medium for two weeks, starting from the day of neuron maturation.

2.1.2 Cell viability assay and PBPK modeling for dose setting

For cell viability assay, H9 single cells were plated into a 96-well plate with 3000 cells/well. After 24 hours, cells were exposed to 0, 50, 75, 100, 125, 150, 200, and 250 μM BPA for 7 days, 6 wells for each exposed group. The medium was replaced every other day. On the 7th day, cell viability was measured using the Cell-Counting-Kit 8 test kit (Dojindo, Japan).

The minimum test dose for embryonic (0.05 nM) and nerve cells (0.014 nM) was calculated from a previously developed and validated P-PBPK model. This was done by converting a female oral dose of 218.320 ng/kg bw/day (the oral dose represents the average dietary exposure level of BPA in adult women between 20-50 years old reported in the fifth total dietary study of China[40]) into internal exposure doses during pregnancy. Since the test cells are embryonic and neuronal cells, amniotic fluid and fetus's brain were selected as the target sites for calculating internal exposure doses using total Area under Curve (AUC_{total}) (Supplementary file 2, Figure S1). The pregnancy scenarios were set as gestation day (GD) 56 for embryonic cells (the first trimester was chosen as ESCs only exist in the early stages of embryonic development), and GD 245 (the third trimester was chosen as the fetus´s brain has not developed until the third trimester) for neuronal cells. Further details about the model have been provided in the supplementary file along with major equations (Supplementary file 3). Additional details can be found in the original publication[38]. Other exposure routes were not considered as dietary exposure is the major contributor to BPA exposure in humans. Since the BPA dietary exposure of pregnant women was not available in the fifth Chinese Total diet study (TDS), the dietary exposure of BPA in adult women between 20-50 years old was used. In this model, the height and weight of Chinese adult women were set at 158 cm and 59 kg, respectively[41]. The t-TDI of BPA established by EFSA in 2015 is 4 μg/kg·bw and was also used to obtain the equivalent test dose (1 nM for embryonic cells, 0.256 nM for nerve cells) using the P-PBPK model. These doses were set as the initial concentrations for the embryonic and neurodevelopmental toxicity test. Considering the cell viability assay results and given that embryonic cell is naturally differentiated from H9 cells, the maximum dose of the embryonic cell was set to 30 μM. Similarly, the maximum dose for nerve cells was set at 100 μM. Finally, embryonic cells were treated with 0, 0.05 nM, 1 nM, 100 nM, 10 μM, and 30 μM BPA; for nerve cells, the doses were set as 0, 0.014 nM, 0.256 nM, 50 μM, 75 μM, and 100 μM BPA.

2.1.3 Cell cycle and apoptosis assay

The cell cycle assay of embryonic cells was performed using a Cell Cycle Kit (KeyGEN, China, KGA512). After 14 days of BPA exposure, the embryonic cells were harvested and treated according to the manufacturer's protocols. The cells were analyzed using Guava InCyte software (version 2.7, Millipore, USA). Apoptosis of neuronal cells was conducted using Annexin V-FITC/PI Apoptosis Detection Kit (KeyGEN, China, KGA108-1). After 14 days of exposure to BPA, the neuronal cells

were collected and stained according to the manufacturer's procedure. The apoptotic rate was detected by flow cytometry using Guava InCyte (Millipore, USA).

2.1.4 Transcriptomics analysis

After 14-day of exposure, total RNA of the embryonic cells was collected using TRIzol reagent. The quantification, purity and integrity of the RNA were assessed using the Agilent Bioanalyzer system. Then following the manufacturer's instructions of TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, USA), the libraries were constructed. Sequencing and transcriptome analysis were performed by OE Biotech Co., Ltd. (Shanghai, China).

The DESeq (1.20.0) software was used for differential expression analysis. Significant differential expression was defined as a P value < 0.05 and fold change> 2 or < 0.5. In order to demonstrate the expression patterns of genes across groups and samples, hierarchical cluster analysis was performed with DEGs. To understand how DEGs interact and relate, their interactions were incorporated into pathways. On the basis of hypergeometric distribution, Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG)[43] pathway enrichment analysis on DEGs were conducted to identify enriched genes and pathways.

2.1.5 Quantitative real-time PCR test

After 14-day exposure to BPA and dimethyl sulfoxide (DMSO), total RNA was extracted from embryonic and neuronal cells, and cDNA was produced using iScript^{TM} cDNA Synthesis Kit (Bio-Rad, USA) and a cDNA reverse transcription instrument (Bio-Rad, USA) according to the manufacturer's instructions. For nerve cells, the human neurodevelopmental-related gene microarray (Wcgene Biotech, Shanghai, China) was used to detect selected 45 genes encoding the neurodevelopmental system (The genes are listed in Supplementary file 2, Table S1). The Sangon Biotech primer bank (Shanghai, China) and NCBI primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) were used to generate the gene primer sequences (Supplementary file 2, Table S2) of interest genes. qPCR tests were conducted with SsoFast Eva Green Kit (Bio-Rad, USA) and the CFX96 Touch real-time system (Bio-Rad, USA). $2^{-\Delta\Delta Ct}$ method was used to analyse relative target mRNA expressions with GAPDH as an internal standard.

2.2 Bayesian dose-response modeling

Bayesian benchmark dose (BBMD) analysis[44] (https://benchmarkdose.com) was done to conduct the dose-response modeling with PCR data. All types of fitted models were considered, including Exponential 2, Exponential 3, Exponential 4, Exponential 5, Hill, Power, Michaelis-Menten and Linear models. The prior distributions for model parameters were assumed as uniform distributions. Markov chain Mon Carlo (MCMC) sampling was conducted using the default settings in BBMD System, with 1 chain for 30,000 iterations. The warmup percentage was set at 50%. A model-averaged BMD was calculated using continuous data to estimate gene BMDs. A benchmark response (BMR) of 5% (i.e., the dose corresponding to 5% relative change to the control group) was defined in our study. The BBMD system can calculate BMD using a model-averaged method, taking all available models without selection bias. This model-averaged method is also recommended by the EFSA[45]. Chou et al[46] demonstrated the validity of this BBMD method by comparing it with traditional U.S. EPA BMD software. The input data of BBMD website is available in Supplementary file 4.

2.3 Extrapolation of human equivalent dose

The gene BMD_5 values of selected biomarkers were used as the POD to derive the HED. As embryonic and neuronal cells were used to examine the changes in gene expression due to BPA, plasma and brain concentrations were utilized to estimate HEDs. A previously developed and validated P-PBPK model for BPA[38] was applied in this study to calculate the concentration-time profiles for brain and plasma-based IVIVE. The dose metrics of the maximum steady-state plasma and brain concentration (Cmax,ss) were extracted and then applied for reconstructing the exposure utilizing the POD as input with equation 1[34, 46, 47].

$$HED\ (mg/kg/day) = \frac{POD\ (\mu M) \times 1 (mg/kg/day)}{Cmax,ss\ (\mu M)} \qquad \text{eq. 1}$$

For reconstructing exposure or reverse dosimetry, MCMC simulation was performed with 100,000 iterations. Four Markov chains were run with the initial 40000 iterations as a burn-in required for the model to converge from the initial parameter vector and hence, not considered for the convergence, the remaining (60000) were used as output iterations. The convergence of posterior parameters was diagnosed by the potential scale reduction factor ($\hat{R}$). A $\hat{R}$ value of 1.2 or less was considered as the criterion for the possible convergence[48].

2.4 Reference Dose Estimation

The fifth percentile of estimated HEDs was taken as the threshold dose (TD) for each gene. Then the TD was divided by an overall uncertainty factor (10 for interindividual variability) to get the RfD (eq. 2). An uncertainty factor of 10 for intraspecies variability was considered according to the literature to calculate the RfD[35].

$$RfD\ (mg/Kg/day) = \frac{TD(mg/kg/day)}{10} \qquad eq.2$$

2.5 Statistical analysis

Experimental data from cell viability assay, cell cycle, apoptosis, and PCR were analyzed by SPSS software (version 20.0, IBM, USA). The results were shown as mean ± standard deviation and were analyzed by one-way ANOVA. After testing the homogeneity of variance, the LSD test was used to compare the statistical differences between groups. A *P* value less than 0.05 was considered statistically significant. The figures were generated using Rstudio 2022.12.0 and GraphPad Prism 5 (GraphPad Software Inc., USA). Simulations related to PBPK model was done using MCSim under Rstudio (2023.03.0).

3. Results

3.1 Toxicity of BPA on H9-based embryonic/nerve cells

The cytotoxicity test was conducted by exposing H9 cells to BPA at 250, 200, 150, 125, 100, 75, and 50 μM along with the control. After 1 week of treatment, cells treated with 50- 250 μM BPA showed a significant reduction in viability at 150, 200 and 250 μM compared to the control (Supplementary file 2, Figure S2).

The cell cycle of embryonic cells was analyzed after 14 days of treatment. Compared to the control group, the proportion of G0/G1 cells statistically increased in the 0.05 nM and 30 μM groups. There was no significant difference in S-phase cells between the groups. G2 cells in the 1 nM, 100 nM and 10 μM groups were more than that in the control group (Supplementary file 2, Figure S3). There was no significant difference in the apoptosis test of nerve cells (Supplementary file 2, Table

S3).

From the transcriptomics of the embryotoxicity test, the DEGs number between DMSO control group and 10 μM BPA was 359 (q-value<0.05&|log2FC|>1) after 14-day exposures. Of these, 301 genes were up-regulated, and 58 genes were down-regulated among the total genes. KEGG enrichment analysis showed that the DEGs were involved in many pathways (Figure 2), including ovarian steroidogenesis, ECM-receptor interaction, phosphatidylinositol 3-kinase (PI3K)-AKT signaling pathway, etc. GO classification analysis (Figure 3) showed that most DEGs were present in biological processes, rather than cellular components or molecular functions.

Figure 2. KEGG enrichment top 20 from embryo toxicity test. The horizontal axis in the figure represents the enrichment score. The vertical axis represents the top 20 toxicity pathways (with pathway number and name) in KEGG. The larger the bubble, the more differential protein coding genes the pathway contains. The color of the bubble changes from purple, blue, green to red, and the smaller the enrichment p-value, the greater the significance.

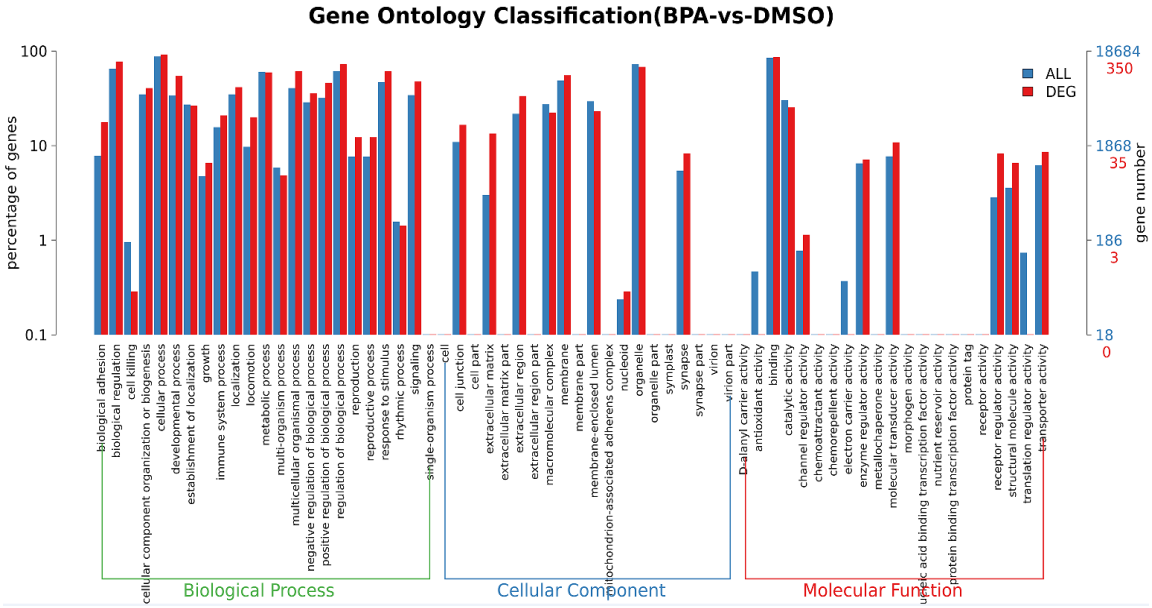

Figure 3. Gene ontology classification in embryo toxicity test. Blue represents GO entries enriched with all genes, red represents GO entries enriched with differential genes. The horizontal axis represents the entry name, and the vertical axis represents the number (on right) and percentage of genes (on left) corresponding to the entry.

3.2 $BMDL_5$ estimation based on in-vitro data

The dose-response relationship between exposure concentrations and responses of gene expressions was examined using BBMD analysis. Genes that showed a significant difference in the PCR test were included in BBMD analysis (Table 1). The Benchmark does lower confidence limit ($BMDL_5$) values for G protein-coupled estrogen receptor 1 (GPER1) and cytochrome P450 family 11 subfamily A member 1 (CYP11A1) were above 100 nM. The $BMDL_5$ value of hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (HSD3B1) was the lowest (7.24 nM). For the neurodevelopmental toxicity test, the selection of genes in the PCR array was based on previous bioinformatics analysis conducted by our group[49] (ESR1, ESR2, MAPK1, MAPK3, GRIN2B, GRIN1, GRIA1, ARAF, and RPS6KA2), related literature[50, 51] (EGR3, FABP5, PTN, POMC, NTRK2, NFATC2, NFAT5), and AOP 12 (https://aopwiki.org/aops/12#graphical_representation) (GRIN2A, GRIN3A, GRIN2D, RCAN1, BDNF and BCL2 were chosen according to the MIE and cellular effects of AOP 12). Then genes mentioned above were selected for the analysis of variance and differential genes were further analysed using BBMD analysis. The $BMDL_5$ values of ARAF, BDNF, GRIN2A, GRIN3A and POMC are above 25000 nM. While the $BMDL_5$ values of EGR3, GRIA1, NFATC2 and NFAT5 are below 500 nM.

Table 1. HEDs in embryo and neurodevelopmental toxicity test

Embryo toxicity test				
Gene	$BMDL_{5th}$/nM	POD/μM	Cplasma-fetus/μM	HED (mg/kg/day)
GPER1	102.830	0.103	1.473	0.070
CGA	8.200	0.008	0.128	0.064
CYP19A1	12.870	0.013	0.200	0.064
CYP11A1	119.870	0.120	1.695	0.071
CDKN1C	63.220	0.063	0.939	0.067
CACNA1C	29.650	0.030	0.454	0.065

HSD3B1	7.240	0.007	0.113	0.064
Neurodevelopmental toxicity test				
Gene	$BMDL_{5th}$/nM	POD/μM	Cbrain-fetus/μM	HED (mg/kg/day)
ARAF	55153.430	55.153	232.703	0.237
BCL2	2021.400	2.021	27.411	0.074
BDNF	39962.170	39.962	188.396	0.212
EGR3	436.480	0.436	7.348	0.059
FABP5	3560.690	3.561	42.571	0.084
GRIA1	364.640	0.365	6.275	0.058
GRIN2A	25477.300	25.477	143.313	0.178
GRIN2D	1534.430	1.534	21.832	0.070
GRIN3A	36673.380	36.673	178.491	0.205
RPS6KA2	8623.050	8.623	76.863	0.112
RCAN1	6809.130	6.809	66.418	0.103
PTN	2164.240	2.164	28.969	0.075
POMC	48627.900	48.628	213.895	0.227
NTRK2	589.290	0.589	9.562	0.062
NFATC2	155.590	0.156	2.967	0.052
NFAT5	381.830	0.382	6.534	0.058

3.3 Extrapolation of HEDs based on P-PBPK model

The reverse dosimetry using a P-PBPK model was used to calculate P2.5, P5, P50 and P97.5 of the $C_{max,ss}$ after the exposure, and HEDs using equation 1, as shown in Figure 4A, B. For plasma, the $C_{max,ss}$ values (mean) ranged from 0.11 (HSD3B1) to 1.70 (CYP11A1) μM. The $C_{max,ss}$ values (mean) calculated from the brain were higher than those of plasma. The minimum $C_{max,ss}$ for the fetus's brain was 2.97 μM (NFATC2) and the maximum was 232.70 μM (ARAF). The plasma $C_{max,ss}$-derived HEDs (mean) were comparable, ranging from 0.064 (CGA, glycoprotein hormones, alpha polypeptide) to 0.071 (CYP11A1) mg/kg/day. The brain $C_{max,ss}$-derived HEDs had wider ranges (0.052-0.24) (mg/kg/day) compared to the plasma $C_{max,ss}$-derived HEDs (0.065-0.071) (mg/kg/day).

A B

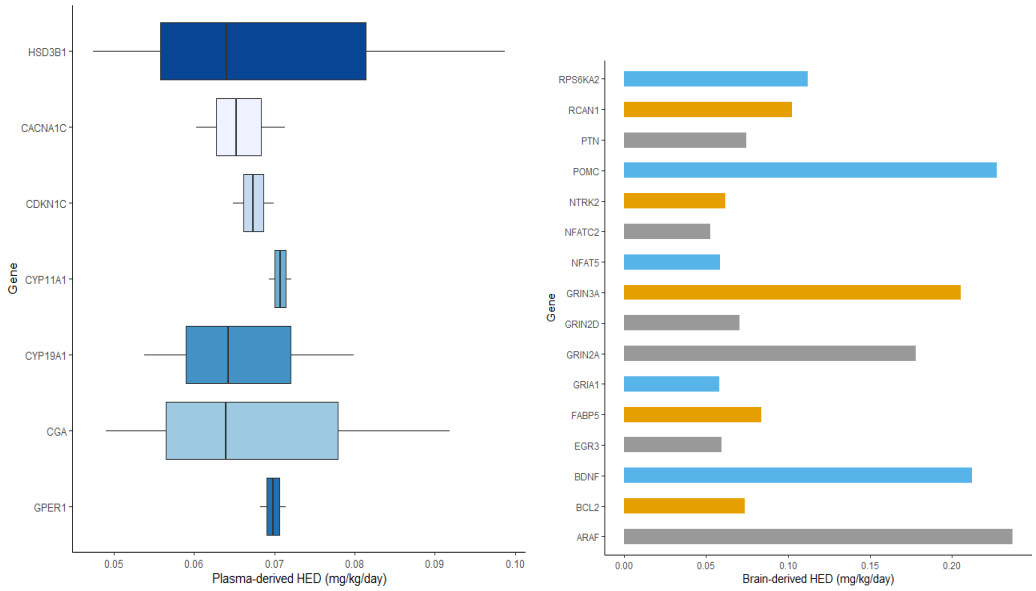

Figure 4. HEDs estimation using Markov Chain Monte Carlo simulations. A: the P2.5, P50 and P97.5 of the plasma Cmax,ss-derived HEDs. B: the P50 of the fetus brain Cmax,ss-derived HEDs.

3.4 Convergence achieved during reverse dosimetry

The convergence for MCMC simulation done to reconstruct the exposure was checked to increase the credibility of results. The well-mixed Markov chains probabilistic density plots (Supplementary file 2, Figure S4 and S5) for the mean (μ_a) and standard deviation (σ_a) of informative parameter "a" showed convergence in all simulations. A scale reduction factor ($\hat{R}$)≤1.00 was achieved for all simulations and ESS tails were above 100 per chain, indicating convergence.

3.5 Risk characterization

The RfDs were calculated (eq.2) as the TD (The fifth percentile of HEDs) divided by 10 (uncertainty factor)[35]. This uncertainty factor was considered to address the pharmacokinetic variability in ADME (absorption, distribution, metabolism and excretion) profile when moving from in-vitro cell lines to human risk assessment. The RfDs derived from the plasma concentration ranged from 4.94 to 6.96 µg/kg·bw per day, which are narrower than the mean RfDs estimated with brain dosimetry (5.18-23.70 µg/ kg·bw per day) (Supplementary file 2, Table S4). The RfDs are much lower than an EFSA (2006) t-TDI of 50 µg/ kg·bw per day for BPA but close to the EFSA t-TDI updated in 2015 (4 µg/kg·bw per day). The EFSA Panel applied a total uncertainty factor of 150 to establish a t-TDI of 4 µg/kg bw based on the mean relative kidney weight effect in mice. As EFSA has drastically reduced the TDI to 0.2 ng/kg·bw per day in the 2023 scientific opinion report[25], the t-TDI of 2015 is approximately 50 times higher compared to 2023 (TDI).

4. Discussion

In the present study, Bayesian dose-response analysis of human in-vitro toxicogenomic data was combined with PBPK modeling to assess the risk of BPA in humans. Human ESCs were used to perform in-vitro experiments to reduce the uncertainty associated with extrapolation between different species. The H9 cell line is differentiated naturally into various types of cells and also differentiated specifically into neurons to conduct the developmental and neurodevelopmental toxicity test. Given the time required for differentiation and to be comparable between the two developmental tests, the same frequency of exposure was set by us for the in-vitro experiment. The

dose setting in this study was based on the oral exposure of the Chinese population and a long-term (14-day) exposure of human ESCs was done to mimic long-term human exposure.

Transcriptomic analysis was done for embryotoxicity, and genes were selected based on AOP12, previous experiments conducted by our group[49] and some literature[50, 51]. Transcriptomics analysis for BPA showed that the DEGs were enriched in the following pathways: ovarian steroidogenesis, protein digestion and absorption, ECM-receptor interaction, PI3K-Akt signaling pathway, MAPK signaling pathway and Ras signaling pathway. Many of these pathways, particularly the last three, are involved in one or another step of the cell cycle like transcription, translation, proliferation, survival, growth, differentiation etc. pointing towards important processes of embryo development[52]. The ovarian steroidogenesis pathway plays a significant role in uterine function, and the establishment and maintenance of pregnancy[53]. These pathways may help elucidate the mechanism and explore the mode of action of BPA's embryotoxicity. However, further studies can be done to explore in detail. Further PCR test of the selected DEGs in the transcriptomics analysis revealed that the genes which showed statistical significance were mostly related to different kinds of hormones (steroid hormones: HSD3B1, glycoprotein hormones: CGA). The gene (CDKNIC) involving cell cycle also showed statistically significant among different dose groups. Combining the results of the top 20 KEGG pathways from transcriptomics analysis and cell cycle assay, ovarian steroidogenesis pathway and cell growth were found to be two major events happening after BPA exposure. Both these events may be contributing towards embryotoxicity due to BPA exposure as evidenced by altered cell cycle and decrease viability (Fig S1, S2). In the neurodevelopmental toxicity test, genes involving NMDAR (GRIN2A, GRIN3A, GRIN2D), calcineurin A (RCAN1), cell injury/death (BCL2) and encoding a member of the nerve growth factor family of proteins (BDNF) were statistically significant. The proteins associated with these genes also appear to be a part of the neurodegenerative process in an endorsed AOP (AOP12, Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development leads to neurodegeneration with impairment in learning and memory in aging, https://aopwiki.org/aops/12). This suggests that BPA may play an important role in inducing developmental neurotoxicity, but further studies are needed to reach a conclusion. Multiple epidemiological studies have also provided evidence with early BPA exposure and neurodevelopmental disorders like attention deficit/hyperactive disorder and autism spectrum disorder[54]. We also used in-vitro data along with in-silico models to estimate POD, HED and RfD for human risk assessment.

Bayesian BMD is considered as a preferred method for dose-response evaluation[55] to investigate the relationship between intracellular concentrations and the toxicogenomic response to obtain POD. The genes with the lowest BMDL for embryotoxicity and neurodevelopmental toxicity test were HSD3B1 and NFATC2, respectively. The protein encoded by HSD3B1 is an enzyme that can catalyze and produce all classes of steroid hormones[56]. The nuclear factor of activated T cells 2 (NFATC2) is a member of the nuclear factor of the activated T cells (NFAT) family and plays a central role in inducing gene transcription during the immune response[57].

The calculated BMD_5 was used as input for the P-PBPK-IVIVE approach to predict the HED which was then extrapolated to calculated the RfD. Lin et al[37] conducted a population-based IVIVE using PBPK models coupled with Monte Carlo simulations to convert ToxCast in vitro ER assays of BPA, BPS, BPF and BPAF to HEDs. The HED for the ER gene transcription was 0.40 mg/kg/day for BPA, which is much more higher than the HEDs calculated in the present study. The reason behind this is the different endpoints considered to derive HEDs. Even though the disruption of the ER is a major concern for risk assessment[58] the toxicity mechanism of BPA has not been fully

elucidated. Further research is necessary to explore more biological processes and sensitive endpoints to fully understand the toxicity mechanism of BPA and obtain more accurate HEDs. The RfD was calculated based on the DEGs which may be involved directly or indirectly in the BPA-induced toxicity. The result shows that the DEGs for the embryo and neurodevelopment are different, but the minimum RfDs derived from the plasma and brain dosimetry are similar. However, the maximum RfD derived from the brain dosimetry is three times that derived from plasma dosimetry, which explains the variation in sensitivities of different genes. For RfD analysis, NFATC2 also shows the lowest value among all the selected genes like BMDL.

The RfD of the most sensitive gene related to neurotoxicity (NFATC2, 5.2 μg/kg/day) was close to the EFSA's 2015 t-TDI. However, the current derived RfDs are below the estimated dietary exposures of BPA in European and Chinese populations. Dietary exposures to BPA in European populations were estimated on average to 81 μg/kg·bw/d for children, 55 μg/kg·bw/d for adolescents and 42 μg/kg·bw/d for adults between 45 and 65 years old[22]. According to the fifth Chinese TDS, the mean dietary exposure to BPA was 200.955 ng/kg·bw/d for adults above 19 years old[40]. In recent EFSA's scientific opinion of BPA[25], EFSA established a TDI of 0.2 ng/kg·bw based on the effect on Th17 cells in mice. This means that simulated RfD is more than 1000 times higher than TDI. However, there may be some uncertainty in calculating the RfD. For instance, consideration of uncertainty factor of 10 to account for undefined variability like genetic polymorphism, individual differences etc. Currently, there is no established method for interspecies extrapolating toxicogenomic data as there are no unified standards to use toxicogenomic data to estimate RfDs. Another limitation can be that during dosimetry IVIVE, we considered Cmax but instead AUC can be taken as standard. This is an ongoing discussion among experts and needs further reviewing before coming to concrete decisions. While doing in-vitro studies, we assumed that the applied exposure is the actual concentration in the cell, but this may not hold true. Some compounds may bind to plastic or degrade, reducing the overall active concentration. This requires further investigations and some in-vitro PBPK model can be developed to reduce the uncertainty. However, this study is a starting point to extrapolate in-vitro toxicogenomic data by in-silico rather than animal experiments, which is a step towards NAM. This NAM is not limited to the type of contaminants as long as there is a PBPK model and in-vitro data for the target contaminant.

However, there are some limitations of this study. We considered dietary exposure for adult female and not for pregnant females which can be one of the uncertainties in the prediction. The BMD analysis took the PCR data to conduct the dose-response evaluation. However, a gene is usually involved in several pathways, which makes it ambiguous to determine the most significant one. And gene expression changes may not lead to a clear adverse toxicological effect. Thus, the association between gene expression changes and adverse outcomes is not well understood and explained and using toxicogenomic data for risk assessment is still difficult. Nevertheless, the genes selected in our study were based on the PCR test, transcriptomics analysis and previous bioinformatic analysis, which reduced the uncertain causality. Another major limitation is that the in-vitro study done for short-time interval may not represent human long-term exposure. This is an open question to be resolved by the scientific community. The exposure pathway simulated in this study is oral exposure. However, dermal and inhalation exposure can also be considered when exposure data will become available. The P-PBPK model for fetus did not consider gender differences due to limited data availability for such a population. However, the model can be further extended if required. The prediction results of risk assessment for pregnant women can be different for other age groups as pregnant women and fetuses have different physiology. However, the reference dose calculated for pregnant females can be extended for other age-groups using the life-

stage PBPK model.

5. Conclusion

We conducted human-embryonic-cell-based in-vitro experiments and then integrated toxicogenomic dose-response data and IVIVE-PBPK for the human health risk assessment of BPA. The in-vitro study suggests that steroid hormones might be one of the factor contributing to BPA´s embryotoxicity while the immune response-related gene was found to be the most sensitive in the neurodevelopmental toxicity test. The RfDs were derived from DEGs showing significant differences in the embryotoxicity/neurotoxicity test. The simulated RfD for the most sensitive gene was closer to the EU guidance value (t-TDI: 4 μg/kg·bw) recommended by EFSA in 2015. However, these are results from specific in-vitro cell lines and there are some uncertainties which may be present while considering simulated RfD for any regulatory purpose. The simulated RfD is considerably high compared to the EFSA TDI established in 2023 for a different endpoint (immunotoxicity). This study has also shown the application of an IVIVE-PBPK-based NAM which can be applied to other chemicals to conduct risk assessments and improve public health decisions.

Acknowledgement

The first author is partially supported by the grant CSC #202106240169 from the P. R. China. Translational modelling, data analysis and manuscript writing have partially been supported by the funding from the European Union's Horizon 2020 research and innovation program under grant agreement No. 101057014 (PARC).

Conflict of interests

The authors declare no competing financial interest.

Data Availability

The code used in the work is available at InSilicoVida/Toxicogenomic-in-BPA-with-PBPK: IVIVE-P-PBPK for extrapolating risk from in-vitro to human health especially for DNT (github.com). The data generated from in-vitro study like transcriptomic data is provided as per OECD reporting framework. The raw sequencing data, mapped data, and data for visualization of the RNA-Seq analyses of the embryonic stem cell with exposure to 10 μM BPA for 14 days transcriptome data have been deposited in the Bioproject at the National Center for Biotechnology Information (NCBI) under accession number PRJNA998583.

Abbreviations

Abbreviations	Definition
AUC	Area under the curve
BBMD	Bayesian benchmark dose
BMD	Benchmark dose
BMDL	Benchmark does lower confidence limit
BMR	Benchmark response
BPA	Bisphenol A
Cmax,ss	Maximum steady-state concentration
CYP11A1	Cytochrome P450 family 11 subfamily A member 1

DEGs	Differentially expressed genes
DMSO	Dimethyl sulfoxide
DNT	Developmental neurotoxicity
EBs	Embryonic Bodies
EDC	Endocrine-disrupting chemical
EFSA	European Food Safety Authority
ER	Estrogen receptor
ESCs	Embryonic stem cells
FDA	Food and Drug Administration
GD	Gestation day
GO	Gene Ontology
GPER1	G protein-coupled estrogen receptor 1
HEDs	Human equivalent doses
HSD3B1	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1
IVIVE	In vitro-in vivo extrapolation
KEGG	Kyoto Encyclopedia of Genes and Genomes
MCMC	Markov chain Monte Carlo
NAM	New approach methodology
NFAT	Nuclear factor of the activated T cells
NMDARs	N-methyl-D-aspartate receptors
PI3K	Phosphatidylinositol 3-kinase
POD	Point of departure
P-PBPK	Pregnancy physiologically based pharmacokinetic model
RfD	Reference dose
SML	Specific migration limit
TD	Threshold dose
TDI	Tolerable daily intake
t-TDI	Temporary-tolerable daily intake
TDS	Total diet study

References

1. Matuszczak, E.; Komarowska, M. D.; Debek, W.; Hermanowicz, A., The Impact of Bisphenol A on Fertility, Reproductive System, and Development: A Review of the Literature. *International journal of endocrinology* 2019, *2019*, 4068717.
2. Gimeno, P.; Spinau, C.; Lassu, N.; Maggio, A. F.; Brenier, C.; Lempereur, L., Identification and quantification of bisphenol A and bisphenol B in polyvinylchloride and polycarbonate medical devices by gas chromatography with mass spectrometry. *Journal of separation science* 2015, *38* (21), 3727-34.
3. Dursun, E.; Fron-Chabouis, H.; Attal, J. P.; Raskin, A., Bisphenol A Release: Survey of the Composition of Dental Composite Resins. *The open dentistry journal* 2016, *10*, 446-453.
4. Hahladakis, J. N.; Iacovidou, E.; Gerassimidou, S., An overview of the occurrence, fate, and

human risks of the bisphenol-A present in plastic materials, components, and products. *Integrated environmental assessment and management* **2023,** *19* (1), 45-62.
5. Torres-García, J. L.; Ahuactzin-Pérez, M.; Fernández, F. J.; Cortés-Espinosa, D. V., Bisphenol A in the environment and recent advances in biodegradation by fungi. *Chemosphere* **2022,** *303* (Pt 1), 134940.
6. Lesser, L. E.; Mora, A.; Moreau, C.; Mahlknecht, J.; Hernández-Antonio, A.; Ramírez, A. I.; Barrios-Piña, H., Survey of 218 organic contaminants in groundwater derived from the world's largest untreated wastewater irrigation system: Mezquital Valley, Mexico. *Chemosphere* **2018,** *198*, 510-521.
7. Dan, L.; Wu, S.; Xu, H.; Zhang, Q.; Zhang, S.; Shi, L.; Yao, C.; Liu, Y.; Cheng, J., Distribution and bioaccumulation of endocrine disrupting chemicals in water, sediment and fishes in a shallow Chinese freshwater lake: Implications for ecological and human health risks. *Ecotoxicology and environmental safety* **2017,** *140*, 222-229.
8. Sun, Q.; Wang, Y.; Li, Y.; Ashfaq, M.; Dai, L.; Xie, X.; Yu, C. P., Fate and mass balance of bisphenol analogues in wastewater treatment plants in Xiamen City, China. *Environmental pollution (Barking, Essex : 1987)* **2017,** *225*, 542-549.
9. Geens, T.; Aerts, D.; Berthot, C.; Bourguignon, J. P.; Goeyens, L.; Lecomte, P.; Maghuin-Rogister, G.; Pironnet, A. M.; Pussemier, L.; Scippo, M. L.; Van Loco, J.; Covaci, A., A review of dietary and non-dietary exposure to bisphenol-A. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* **2012,** *50* (10), 3725-40.
10. Ribeiro, E.; Ladeira, C.; Viegas, S., Occupational Exposure to Bisphenol A (BPA): A Reality That Still Needs to Be Unveiled. *Toxics* **2017,** *5* (3).
11. Sabuz Vidal, O.; Deepika, D.; Schuhmacher, M.; Kumar, V., EDC-induced mechanisms of immunotoxicity: a systematic review. *Critical reviews in toxicology* **2021,** *51* (7), 634-652.
12. Ni, M.; Li, X.; Zhang, L.; Kumar, V.; Chen, J., Bibliometric Analysis of the Toxicity of Bisphenol A. *International journal of environmental research and public health* **2022,** *19* (13).
13. Ma, Y.; Liu, H.; Wu, J.; Yuan, L.; Wang, Y.; Du, X.; Wang, R.; Marwa, P. W.; Petlulu, P.; Chen, X.; Zhang, H., The adverse health effects of bisphenol A and related toxicity mechanisms. *Environmental research* **2019,** *176*, 108575.
14. Vandenberg, L. N.; Chahoud, I.; Heindel, J. J.; Padmanabhan, V.; Paumgartten, F. J.; Schoenfelder, G., Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol A. *Environmental health perspectives* **2010,** *118* (8), 1055-70.
15. Barraza, L., A new approach for regulating bisphenol A for the protection of the public's health. *The Journal of law, medicine & ethics : a journal of the American Society of Law, Medicine & Ethics* **2013,** *41 Suppl 1*, 9-12.
16. Wiraagni, I. A.; Mohd, M. A.; Bin Abd Rashid, R.; Haron, D., Validation of a simple extraction procedure for bisphenol A identification from human plasma. *PloS one* **2019,** *14* (10), e0221774.
17. Liu, J.; Yu, P.; Qian, W.; Li, Y.; Zhao, J.; Huan, F.; Wang, J.; Xiao, H., Perinatal bisphenol A exposure and adult glucose homeostasis: identifying critical windows of exposure. *PloS one* **2013,** *8* (5), e64143.
18. Namat, A.; Xia, W.; Xiong, C.; Xu, S.; Wu, C.; Wang, A.; Li, Y.; Wu, Y.; Li, J., Association of BPA exposure during pregnancy with risk of preterm birth and changes in gestational age: A meta-analysis and systematic review. *Ecotoxicology and environmental safety* **2021,** *220*, 112400.
19. Chen, D.; Kannan, K.; Tan, H.; Zheng, Z.; Feng, Y. L.; Wu, Y.; Widelka, M., Bisphenol

Analogues Other Than BPA: Environmental Occurrence, Human Exposure, and Toxicity-A Review. *Environmental science & technology* **2016,** *50* (11), 5438-53.
20. Liao, C.; Kannan, K., Concentrations and profiles of bisphenol A and other bisphenol analogues in foodstuffs from the United States and their implications for human exposure. *Journal of agricultural and food chemistry* **2013,** *61* (19), 4655-62.
21. Baluka, S. A.; Rumbeiha, W. K., Bisphenol A and food safety: Lessons from developed to developing countries. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* **2016,** *92*, 58-63.
22. Journal, E. F. S. A. J. E., Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) related to 2, 2-bis (4-hydroxyphenyl) propane bis (2, 3-epoxypropyl) ether (Bisphenol A diglycidyl ether, BADGE). REF. No 13510 and 39700. **2004,** *2* (7), 86.
23. Authority, E. J. E. J., Scientific opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs. **2015,** *13* (1).
24. Luo, S.; Li, Y.; Li, Y.; Zhu, Q.; Jiang, J.; Wu, C.; Shen, T., Gestational and lactational exposure to low-dose bisphenol A increases Th17 cells in mice offspring. *Environmental toxicology and pharmacology* **2016,** *47*, 149-158.
25. Lambré, C.; Barat Baviera, J. M.; Bolognesi, C.; Chesson, A.; Cocconcelli, P. S.; Crebelli, R.; Gott, D. M.; Grob, K.; Lampi, E.; Mengelers, M.; Mortensen, A.; Rivière, G.; Silano Until December, V.; Steffensen, I. L.; Tlustos, C.; Vernis, L.; Zorn, H.; Batke, M.; Bignami, M.; Corsini, E.; FitzGerald, R.; Gundert-Remy, U.; Halldorsson, T.; Hart, A.; Ntzani, E.; Scanziani, E.; Schroeder, H.; Ulbrich, B.; Waalkens-Berendsen, D.; Woelfle, D.; Al Harraq, Z.; Baert, K.; Carfì, M.; Castoldi, A. F.; Croera, C.; Van Loveren, H., Re-evaluation of the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs. *EFSA journal. European Food Safety Authority* **2023,** *21* (4), e06857.
26. Laroche, C.; Aggarwal, M.; Bender, H.; Benndorf, P.; Birk, B.; Crozier, J.; Dal Negro, G.; De Gaetano, F.; Desaintes, C.; Gardner, I.; Hubesch, B.; Irizar, A.; John, D.; Kumar, V.; Lostia, A.; Manou, I.; Monshouwer, M.; Müller, B. P.; Paini, A.; Reid, K.; Rowan, T.; Sachana, M.; Schutte, K.; Stirling, C.; Taalman, R.; van Aerts, L.; Weissenhorn, R.; Sauer, U. G., Finding synergies for 3Rs - Toxicokinetics and read-across: Report from an EPAA partners' Forum. *Regulatory toxicology and pharmacology : RTP* **2018,** *99*, 5-21.
27. Deepika, D.; Sharma, R. P.; Schuhmacher, M.; Kumar, V. J. C. R. i. T., An integrative translational framework for chemical induced neurotoxicity–a systematic review. **2020,** *50* (5), 424-438.
28. Martin, G. R., Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proceedings of the National Academy of Sciences of the United States of America* **1981,** *78* (12), 7634-8.
29. Seiler, A. E.; Spielmann, H., The validated embryonic stem cell test to predict embryotoxicity in vitro. *Nature protocols* **2011,** *6* (7), 961-78.
30. Krewski, D.; Acosta, D., Jr.; Andersen, M.; Anderson, H.; Bailar, J. C., 3rd; Boekelheide, K.; Brent, R.; Charnley, G.; Cheung, V. G.; Green, S., Jr.; Kelsey, K. T.; Kerkvliet, N. I.; Li, A. A.; McCray, L.; Meyer, O.; Patterson, R. D.; Pennie, W.; Scala, R. A.; Solomon, G. M.; Stephens, M.; Yager, J.; Zeise, L., Toxicity testing in the 21st century: a vision and a strategy. *Journal of toxicology and environmental health. Part B, Critical reviews* **2010,** *13* (2-4), 51-138.
31. Council, N. R., *Toxicity testing in the 21st century: a vision and a strategy*. National Academies

Press: 2007.
32. Rotroff, D. M.; Wetmore, B. A.; Dix, D. J.; Ferguson, S. S.; Clewell, H. J.; Houck, K. A.; Lecluyse, E. L.; Andersen, M. E.; Judson, R. S.; Smith, C. M.; Sochaski, M. A.; Kavlock, R. J.; Boellmann, F.; Martin, M. T.; Reif, D. M.; Wambaugh, J. F.; Thomas, R. S., Incorporating human dosimetry and exposure into high-throughput in vitro toxicity screening. *Toxicological sciences : an official journal of the Society of Toxicology* **2010,** *117* (2), 348-58.
33. Wetmore, B. A., Quantitative in vitro-to-in vivo extrapolation in a high-throughput environment. *Toxicology* **2015,** *332*, 94-101.
34. Wetmore, B. A.; Wambaugh, J. F.; Ferguson, S. S.; Sochaski, M. A.; Rotroff, D. M.; Freeman, K.; Clewell, H. J., 3rd; Dix, D. J.; Andersen, M. E.; Houck, K. A.; Allen, B.; Judson, R. S.; Singh, R.; Kavlock, R. J.; Richard, A. M.; Thomas, R. S., Integration of dosimetry, exposure, and high-throughput screening data in chemical toxicity assessment. *Toxicological sciences : an official journal of the Society of Toxicology* **2012,** *125* (1), 157-74.
35. Chen, Q.; Chou, W. C.; Lin, Z., Integration of Toxicogenomics and Physiologically Based Pharmacokinetic Modeling in Human Health Risk Assessment of Perfluorooctane Sulfonate. *Environmental science & technology* **2022,** *56* (6), 3623-3633.
36. Zhang, Y.; Liu, Z.; Wang, Z.; Gao, H.; Wang, Y.; Cui, M.; Peng, H.; Xiao, Y.; Jin, Y.; Yu, D.; Chen, W.; Wang, Q., Health risk assessment of cadmium exposure by integration of an in silico physiologically based toxicokinetic model and in vitro tests. *Journal of hazardous materials* **2023,** *443* (Pt A), 130191.
37. Lin, Y. J.; Lin, Z., In vitro-in silico-based probabilistic risk assessment of combined exposure to bisphenol A and its analogues by integrating ToxCast high-throughput in vitro assays with in vitro to in vivo extrapolation (IVIVE) via physiologically based pharmacokinetic (PBPK) modeling. *Journal of hazardous materials* **2020,** *399*, 122856.
38. Sharma, R. P.; Schuhmacher, M.; Kumar, V., The development of a pregnancy PBPK Model for Bisphenol A and its evaluation with the available biomonitoring data. *The Science of the total environment* **2018,** *624*, 55-68.
39. Martínez, M.; González, N.; Martí, A.; Marquès, M.; Rovira, J.; Kumar, V.; Nadal, M., Human biomonitoring of bisphenol A along pregnancy: An exposure reconstruction of the EXHES-Spain cohort. *Environmental research* **2021,** *196*, 110941.
40. Yao, K.; Zhang, J.; Yin, J.; Zhao, Y.; Shen, J.; Jiang, H.; Shao, B., Bisphenol A and Its Analogues in Chinese Total Diets: Contaminated Levels and Risk Assessment. *Oxidative medicine and cellular longevity* **2020,** *2020*, 8822321.
41. Commission, C. N. H., Report on nutrition and chronic-diseases of Chinese residents. **2020**.
42. Fang, H.; Zhi, Y.; Yu, Z.; Lynch, R. A.; Jia, X. J. F. C., The embryonic toxicity evaluation of deoxynivalenol (DON) by murine embryonic stem cell test and human embryonic stem cell test models. **2018,** *86*, 234-240.
43. Kanehisa, M.; Araki, M.; Goto, S.; Hattori, M.; Hirakawa, M.; Itoh, M.; Katayama, T.; Kawashima, S.; Okuda, S.; Tokimatsu, T.; Yamanishi, Y., KEGG for linking genomes to life and the environment. *Nucleic acids research* **2008,** *36* (Database issue), D480-4.
44. Shao, K.; Shapiro, A. J., A Web-Based System for Bayesian Benchmark Dose Estimation. *Environmental health perspectives* **2018,** *126* (1), 017002.
45. Committee, E. S.; Hardy, A.; Benford, D.; Halldorsson, T.; Jeger, M. J.; Knutsen, K. H.; More, S.; Mortensen, A.; Naegeli, H.; Noteborn, H. J. E. J., Update: use of the benchmark dose approach

in risk assessment. **2017,** *15* (1), e04658.
46. Chou, W. C.; Lin, Z., Probabilistic human health risk assessment of perfluorooctane sulfonate (PFOS) by integrating in vitro, in vivo toxicity, and human epidemiological studies using a Bayesian-based dose-response assessment coupled with physiologically based pharmacokinetic (PBPK) modeling approach. *Environment international* **2020,** *137*, 105581.
47. Bell, S. M.; Chang, X.; Wambaugh, J. F.; Allen, D. G.; Bartels, M.; Brouwer, K. L. R.; Casey, W. M.; Choksi, N.; Ferguson, S. S.; Fraczkiewicz, G.; Jarabek, A. M.; Ke, A.; Lumen, A.; Lynn, S. G.; Paini, A.; Price, P. S.; Ring, C.; Simon, T. W.; Sipes, N. S.; Sprankle, C. S.; Strickland, J.; Troutman, J.; Wetmore, B. A.; Kleinstreuer, N. C., In vitro to in vivo extrapolation for high throughput prioritization and decision making. *Toxicology in vitro : an international journal published in association with BIBRA* **2018,** *47*, 213-227.
48. Gelman, S. A., Psychological essentialism in children. *Trends in cognitive sciences* **2004,** *8* (9), 404-9.
49. Li, X.; Ni, M.; Yang, Z.; Chen, X.; Zhang, L.; Chen, J., Bioinformatics analysis and quantitative weight of evidence assessment to map the potential mode of actions of bisphenol A. *Environmental pollution (Barking, Essex : 1987)* **2021,** *273*, 116469.
50. Li, J.; Fu, K. Z.; Vemula, S.; Le, X. C.; Li, X. F., Studying developmental neurotoxic effects of bisphenol A (BPA) using embryonic stem cells. *Journal of environmental sciences (China)* **2015,** *36*, 173-7.
51. Huang, B.; Jiang, C.; Luo, J.; Cui, Y.; Qin, L.; Liu, J., Maternal exposure to bisphenol A may increase the risks of Parkinson's disease through down-regulation of fetal IGF-1 expression. *Medical hypotheses* **2014,** *82* (3), 245-9.
52. Rossant, J.; Tam, P. P. L., Early human embryonic development: Blastocyst formation to gastrulation. *Developmental cell* **2022,** *57* (2), 152-165.
53. Stocco, D. M.; Wang, X.; Jo, Y.; Manna, P. R., Multiple signaling pathways regulating steroidogenesis and steroidogenic acute regulatory protein expression: more complicated than we thought. *Molecular endocrinology (Baltimore, Md.)* **2005,** *19* (11), 2647-59.
54. Welch, C.; Mulligan, K., Does Bisphenol A Confer Risk of Neurodevelopmental Disorders? What We Have Learned from Developmental Neurotoxicity Studies in Animal Models. *International journal of molecular sciences* **2022,** *23* (5).
55. Davis, J. A.; Gift, J. S.; Zhao, Q. J. J. T.; pharmacology, a., Introduction to benchmark dose methods and US EPA's benchmark dose software (BMDS) version 2.1. 1. **2011,** *254* (2), 181-191.
56. Dickson, A.; Yutuc, E.; Thornton, C. A.; Dunford, J. E.; Oppermann, U.; Wang, Y.; Griffiths, W. J., HSD3B1 is an oxysterol 3β-hydroxysteroid dehydrogenase in human placenta. *Open biology* **2023,** *13* (5), 220313.
57. Jakobi, M.; Kiefer, A.; Mirzakhani, H.; Rauh, M.; Zimmermann, T.; Xepapadaki, P.; Stanic, B.; Akdis, M.; Papadopoulos, N. G.; Raby, B. A.; Weiss, S. T.; Finotto, S., Role of nuclear factor of activated T cells 2 (NFATc2) in allergic asthma. *Immunity, inflammation and disease* **2020,** *8* (4), 704-712.
58. Miller, M. M.; McMullen, P. D.; Andersen, M. E.; Clewell, R. A., Multiple receptors shape the estrogen response pathway and are critical considerations for the future of in vitro-based risk assessment efforts. *Critical reviews in toxicology* **2017,** *47* (7), 564-580.