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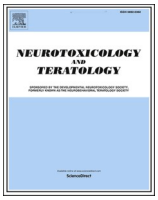

Effect of BPA on CYP450s expression, and nicotine modulation, in fetal rat brain

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ABSTRACT

Human exposure to bisphenol A (BPA) is mainly due to migration from plastic packaging into food and beverages. Studies reported BPA endocrine disruptions through interactions with different nuclear receptors, including the arylhydrocarbon receptor (AhR). AhR mediates xenobiotic responses and regulates expression of drug-metabolizing enzymes (DMEs), including many CYP450s. This study aimed to assess the effects of BPA maternal exposure on CYP450s expression in fetal brain. Sprague-Dawley dams were exposed to BPA concentrations of 0, 0.5, 5, and 50 mg/L in drinking water, individually, and with nicotine. Fetal brains were isolated at gestational days GD14 and GD19, and protein expression was assessed by Western blotting. Results showed a BPA-induced significant decrease in CYP1B1 expression levels at GD14 ($p = 0.001$), and CYP19A1 (aromatase) expression at both mid- and late-stage development ($p < 0.001$). In addition, nicotine individually decreased expression levels of all examined protein targets, significantly for CYP1B1 ($p < 0.001$), CYP19A1 ($p = 0.010$), AhRR ($p = 0.042$), and ARNT ($p < 0.001$), compared to control. When combined with BPA, nicotine suppressive effects were attenuated at both GD14 and GD19. In conclusion, BPA suppresses CYP1B1 and CYP19A1 expression in fetal brain, and attenuates the suppressive effects of nicotine. Observed effects may be mediated by AhR-ARNT independent mechanisms that need further examination.

1. Introduction

Bisphenol A (BPA) is one of the highest volume chemicals produced worldwide, with an estimated global annual production capacity of 4 million tons (Matuszczak et al., 2019). It is the monomer in the production of polycarbonate plastics, commonly used in food, bottled water, and beverages packaging. BPA is also used in the lining of aluminum cans, food wrapping papers, cardboards, cash register receipts, and cigarette filters, and as an additive in polyvinyl chloride (PVC) water pipes (Biedermann et al., 2010). Degradation of plastic packaging leads to migration into packaged water and food and subsequent human exposure by ingestion (Calafat et al., 2005; Groff, 2010). Human exposure to BPA is demonstrated in urine samples from various age groups, including newborns, pregnant women, and adults (Dhimolea et al., 2014; Schönfelder et al., 2002; Vandenberg et al., 2009), with females showing higher levels compared to males (Calafat et al., 2008). Other studies have reported BPA detection in various body fluids and tissues including placental tissue, umbilical cord blood, and amniotic fluid (Ikezuki et al., 2002; Schönfelder et al., 2002).

By escaping conjugation to the estrogen-sequestering protein alpha-fetoprotein, BPA has the capacity to cross the placenta and accumulate in the fetal compartment, particularly in cases of chronic maternal

Abbreviations: BPA, Bisphenol A; AhR, arylhydrocarbon receptor; DMEs, drug-metabolizing enzymes; CYPs, cytochrome P450 enzymes; XMEs, xenobiotic metabolic enzymes; AhRR, aryl hydrocarbon receptor repressor; ARNT, AhR nuclear translocator.

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exposure (Adewale et al., 2009). Fetal accumulation is attributed to its inability to metabolize BPA efficiently (Taylor et al., 2008). Numerous studies have reported endocrine and tumorigenic adverse effects associated with BPA exposure at very low doses (Kundakovic and Champagne, 2011). BPA perinatal exposure, in particular, can alter the development of several organs and organ systems (vom Saal et al., 2007). Animals exposed to low doses of BPA during the perinatal period showed developmental malformations and alterations in the reproductive system, the mammary glands, and the brain (Richter et al., 2007; Vandenberg et al., 2009).

Health risks associated with BPA exposure are attributed to several known and unknown mechanisms, including its interaction with the estrogen receptor (ER), the thyroid hormone receptor, the androgen receptor, the peroxisome proliferator-activated receptor (PPAR), the constitutive androstane receptor, the pregnane X receptor, and the aryl-hydrocarbon receptor (AhR) (Kwintkiewicz et al., 2010; Lee et al., 2003; Moriyama et al., 2002). Because many hazardous substances may consist of an AhR ligand, the relationship between components of the AhR pathway and toxic responses to environmental pollutants is gaining increased interest. The AhR pathway acts as an inducer of xenobiotic metabolic enzymes (XME), particularly the Phase I cytochrome P450 oxidation enzymes, which can impact target tissue metabolic abilities (Singh et al., 2012). However, considerable gaps still exist in understanding BPA-induced effects downstream from nuclear receptors, particularly the AhR pathway during critical stages of fetal development (Nishizawa et al., 2005a). In addition, considerable discrepancies still exist among various studies with respect to BPA toxicity endpoints and threshold dose.

On the other hand, cigarette smoking is the leading cause of preventable diseases, disabilities, and death on a global scale, and continues to be a major public health problem, even among pregnant women. A high proportion of women reported maintaining smoking on a daily basis during pregnancy, with a smoking-during pregnancy (SDP) prevalence ranging from 30% in European countries to 80% in the Western Pacific Region (Lange et al., 2018). Besides cardiopulmonary diseases and cancer, tobacco smoke components, particularly nicotine, are thought to produce behavioral and physiological alterations in both the developing and the adult human brain, such as changes in cognition, anxiety, and aggression (Benowitz, 2008). Several lines of evidence suggest that these changes may stem from the effects of nicotine and other tobacco alkaloids on estrogen synthesis via the enzyme CYP19A known as aromatase (Biegon et al., 2012). Nicotine was previously reported to downregulate CYP19A expression and alter estrogen synthesis (Kanungo et al., 2012). Humans may be concurrently exposed to both BPA and nicotine arising from common sources and various products, hence bearing the double burden effect. The current study aimed to assess the effects of BPA, on the protein expression levels of CYP450s and upstream AhR receptor activating cascade in fetal rat brain tissues in vivo, and to examine the modulatory effects of nicotine.

2. Materials and methods

2.1. Experimental animals

The study was approved by the Institutional Animal Care and Use Committee (IACUC) at the American University of Beirut (AUB). Experimental animals were handled in accordance with the US Government Principles for the Utilization and Care of Vertebrate Animals used in Testing, Research and Training (National Research Council Committee, 2011). After acquiring 8-week-old adult female Sprague-Dawley female rats weighing around 200–250 g ($n = 28$), each was placed in a separate cage along with an adult Sprague-Dawley male for two days. The appearance of vaginal plugs was monitored in the morning as a marker of gestation. The day of observation of the vaginal plug was considered gestational day 1 (GD1). Each conceiving dam was placed in a separate cage at standard 12:12 h light and dark cycles.

2.2. Reagents and dose-groups

Dams dosing started on GD1 for all dose-groups; in order to achieve BPA exposure doses of 0, 50, 500, and 5000 μg/kg/day, drinking water solutions of 0 (control), 0.5, 5, and 50 mg/L of BPA (≥99%; Sigma-Aldrich, USA) were prepared based on an estimated average daily water intake of 9–12 mL per 100 g body weight as described (Claassen, 1994). Dosage interval was selected based on the literature to cover a broad range of doses (Mendoza-Rodríguez et al., 2011). This included lower doses and higher doses that were tested in animal models (Desai et al., 2018), and shown to result in a significant increase in serum BPA concentration in dams (Yoshida et al., 2004), and significant BPA concentrations in tissues (Kabuto et al., 2004; Nakajima et al., 2012). The choice of doses was also in line with estimated levels in maternal plasma and placenta units in previous human studies (Patisaul et al., 2012; Schönfelder et al., 2002). BPA was dissolved in 1% ethanol prior to mixing with drinking water (Somm et al., 2009).

In addition, a drinking water solution of 50 mg/L nicotine hydrogen tartrate (Sigma-Aldrich, USA) was prepared to achieve an exposure dose of 3000 μg/kg/day for Dams, mimicking human tobacco smoking, and assuming a 30% drop in water consumption during exposure to nicotine due to its bitter taste (Schneider et al., 2010). For the same reason, when combining BPA with nicotine, a BPA solution of 50 mg/L was adjusted to 71 mg/L. A daily dose of 100 μg of nicotine is equivalent to an estimated plasma cotinine concentration of 1 ng/ml (Benowitz et al., 2009). According to the literature, dams were exposed to a range of 0.3 to 1.2 mg/mL nicotine concentrations in order to achieve a daily dosage interval of 2–7 mg/kg.bw, which is estimated to translate into a range of 20–75 ng/mL nicotine plasma levels (Schneider et al., 2010).

Four dams were randomly assigned to each dose-group, given access to food, and dosed through drinking water ad libitum. In total, the experiment consisted of six dose-groups, where dams were sacrificed at two time points, gestational days GD14 and GD19. Two fetuses were collected per dam per time point, and then whole fetal brains were isolated for further analysis (Braidy et al., 2015). The total number of fetal brains analyzed amounted to 48, where for each dose-group two litters, and two fetal brains per litter were included in the analysis.

2.3. Protein extraction and quantification

Proteins were extracted using the cell lysis reagents N-PER Neuronal Protein Extraction Reagent (Thermo, USA), and then quantified using a modified Lowry protein assay, namely DC Protein Assay (Bio-Rad, USA), according to the manufacturer's instructions. Briefly, fetal brain tissues were washed with a phosphate-buffered saline solution, then N-PER reagent was added, and tissues were homogenized. The homogenate was then centrifuged and the supernatant was collected. To quantify proteins, dilutions and standards were prepared, and absorbance was measured at 750 nm.

2.4. Western blotting

Quantified proteins were denatured, migrated by SDS- PAGE, and transferred onto a PVDF membrane (Bio- Rad, USA). Transfer quality was evaluated by a Ponceau solution. Then, membranes were blocked using 5% non-fat dairy milk (NFDM) in Tris-Buffered Saline / Tween 20 (TBST) solution. Membranes were then incubated with specific primary antibodies (described in Supplementary Table 1) with probing dilutions (PD) according to manufacturers' recommendations, against CYP1A1 (PD 1:500) (sc-393,979, Santa Cruz, USA) (Vyhlídalová et al., 2020; Zhang et al., 2021), CYP1B1 (PD 1:2000) (ab185954, Abcam, UK) (Yang et al., 2021; Zhang et al., 2020), CYP2E1 (PD 1:500) (MAB3817, Abnova, Taiwan) (Gelboin and Friedman, 1985; Goldfarb et al., 1993), CYP19A1 (PD 1:2000) (ab124776, Abcam, UK) (Molehin et al., 2018; Storman et al., 2018), AhR (PD 1:100) (sc-133,088, Santa Cruz, USA) (Asai et al., 2018; Paris et al., 2021), ARNT (PD 1:1000) (sc-17,811,

Santa Cruz, USA) (Rijo et al., 2021; Zhang et al., 2021), and AhRR (PD 1:1000) (ab108518, Abcam, UK) (Anderson et al., 2019; Silveira et al., 2019), washed with TBST, then incubated with HRP-conjugated goat anti-rabbit (1,705,046, Bio-Rad, USA) or anti-mouse (1,705,047, Bio-Rad, USA) secondary antibodies. Primary antibody optimal concentrations were determined as per manufacturer's instructions, starting from a recommended concentration and adjusting according to results. Western blotting samples were run in duplicates. Binding bands were detected using an HRP-conjugate secondary detection reagent by ECL Chemiluminescence (Clarity Western ECL Substrate; Bio-Rad, USA). Band intensities were analyzed using a ChemiDoc MP Imaging System and an Image Lab 6.1 software (Bio-Rad, USA) (Fig. 1).

Beta-actin was used as an internal loading control via a specific and well-established antibody (sc-47,778 HRP, Santa Cruz, USA) (Joshi et al., 2021; Yoon et al., 2021) with a a probing dilution of 1:500. Expression levels of targeted proteins in different dose-groups were computed as a ratio normalized to Beta-actin expression levels (Pillai-Kastoori et al., 2020), then illustrated as relative to expression of the target in the control group. More specifically, after lanes were detected, lane borders were adjusted to encompass all the protein in a lane and avoid lane overlapping. Then volume data was background subtracted for each lane using the local background subtraction method, where for each volume the intensities of the pixels in a 1-pixel border around the volume are added together and divided by the total number of border pixels. For accuracy, the subtraction of an equivalent background profile from each lane in the normalization channel was also verified. In addition, empty lanes, those with saturation pixels, and those with poor transfer quality, were excluded from the analysis. Hence, the final computed expression levels were the ratio of the background-adjusted volume of the target protein divided by the background-adjusted reference volume of the housekeeping protein.

In order to control for the quality of quantitative immunoblotting data, we conducted Western Blot normalization control tests as follows: an expression level test was performed to confirm stability of Beta-actin expression bands across the different BPA and nicotine dose-groups. Beta actin showed similar band intensities across the different dose groups. We also conducted a linearity check in order to control for the ability of the loading control to reflect increased sample concentration. A linear range was observed for Beta-actin across a range of loading volumes, where serial dilutions of one sample was checked and resulted in proportional signals across the different loading control dilutions. Tests confirmed that Beta-actin is a suitable internal loading control for our experiments. In addition, positive control protein lysates from adult rat brain and liver tissues, and negative controls, were run for Beta-actin with each batch of samples.

2.5. Statistical analysis

All protein expression levels were computed as averages ± standard error means (SEMs). Differences in protein expression levels between the various dose-groups were analyzed using linear mixed models (LMM) with litter as random effect and dose-group as fixed effect using the Statistical Software for Data Science (Stata 15. MP) "Mixed" command. Regression coefficients with their respective 95% confidence intervals (CI) were determined. A p-value <0.05 was considered statistically significant.

3. Results

3.1. BPA impact on expression levels at GD14

Expression levels of CYP1A1 were not significantly affected by any of the BPA doses. However, mild decreased levels were observed at 0.5 and 5 mg/L doses (Fig. 2A). Both CYP1B1 and CYP19A1 (aromatase), showed a significant decrease at the highest dose of BPA (50 mg/L) as compared to control ($p = 0.001$) (Fig. 2B and C). Expression levels of AhRR and ARNT did not show any particular expression patterns and were not statistically significant compared to control. On the other hand, expression of both AhR and CYP2E1 were not detected in any of the samples at GD14.

3.2. BPA impact on expression levels at GD19

Only CYP19A1 (aromatase) showed a significant decrease in expression levels at 5 mg/L BPA compared to control ($p = 0.026$) (Fig. 3D). All other tests of targeted proteins showed no statistical significance in any of the dose-groups when compared to control at late-stage development.

3.3. Modulatory effect of nicotine

At both GD14 and GD19, nicotine, individually, generally induced a

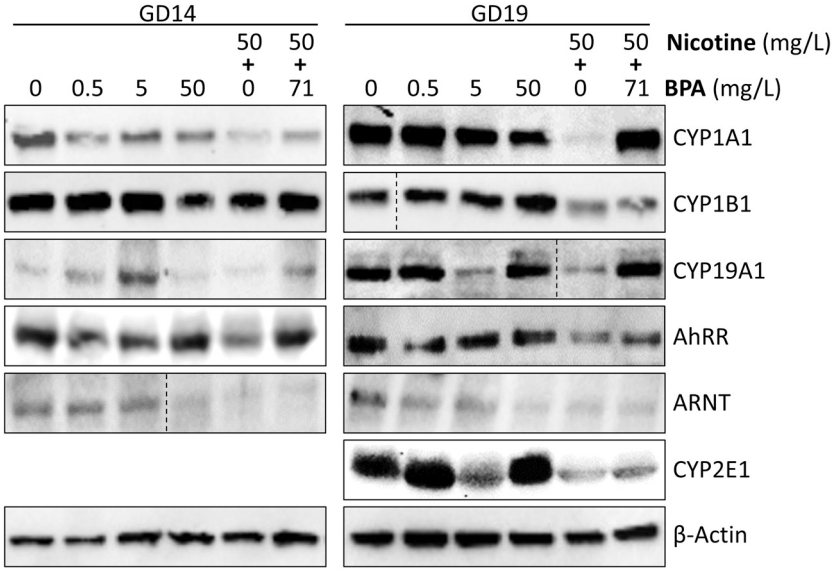

Fig. 1. Western blots of targeted proteins in rat fetal brain tissues prenatally exposed to both nicotine and BPA at (A) GD14 and (B) GD19. Dams were exposed via drinking water ad libitum starting GD1 and until sacrificed at GD14 and GD19 respectively.

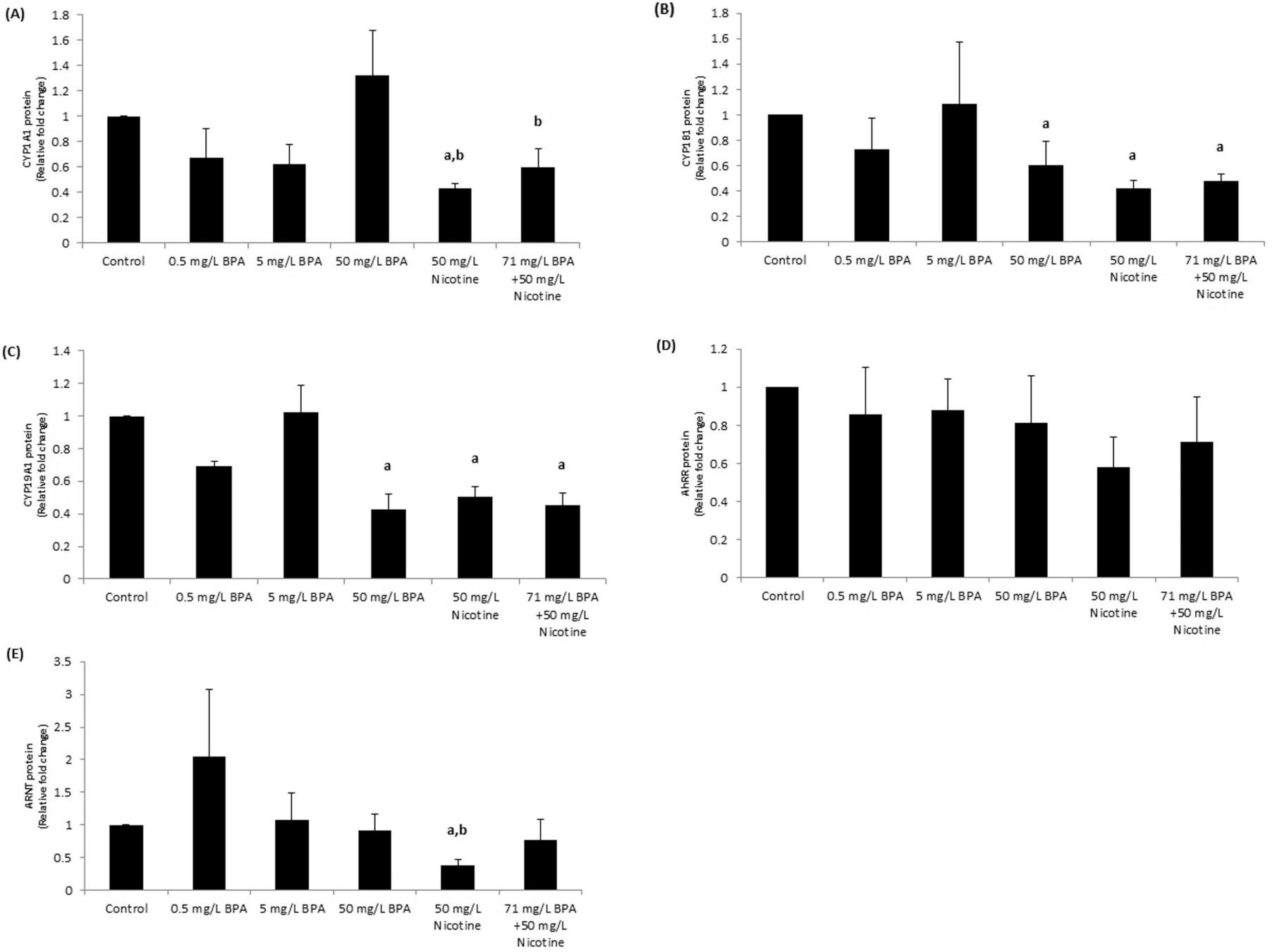

Fig. 2. GD14 protein expression levels of CYP450s, AhRR, and ARNT in rat fetal brain tissues prenatally exposed to both BPA and nicotine. Dams were exposed via drinking water starting GD1 and until sacrificed at GD14. Expression levels are presented as means with standard error, and statistical significance is indicated with an"a" when compared to the control group, and a "b" when compared to the 50 mg/L BPA group, where $p < 0.05$.

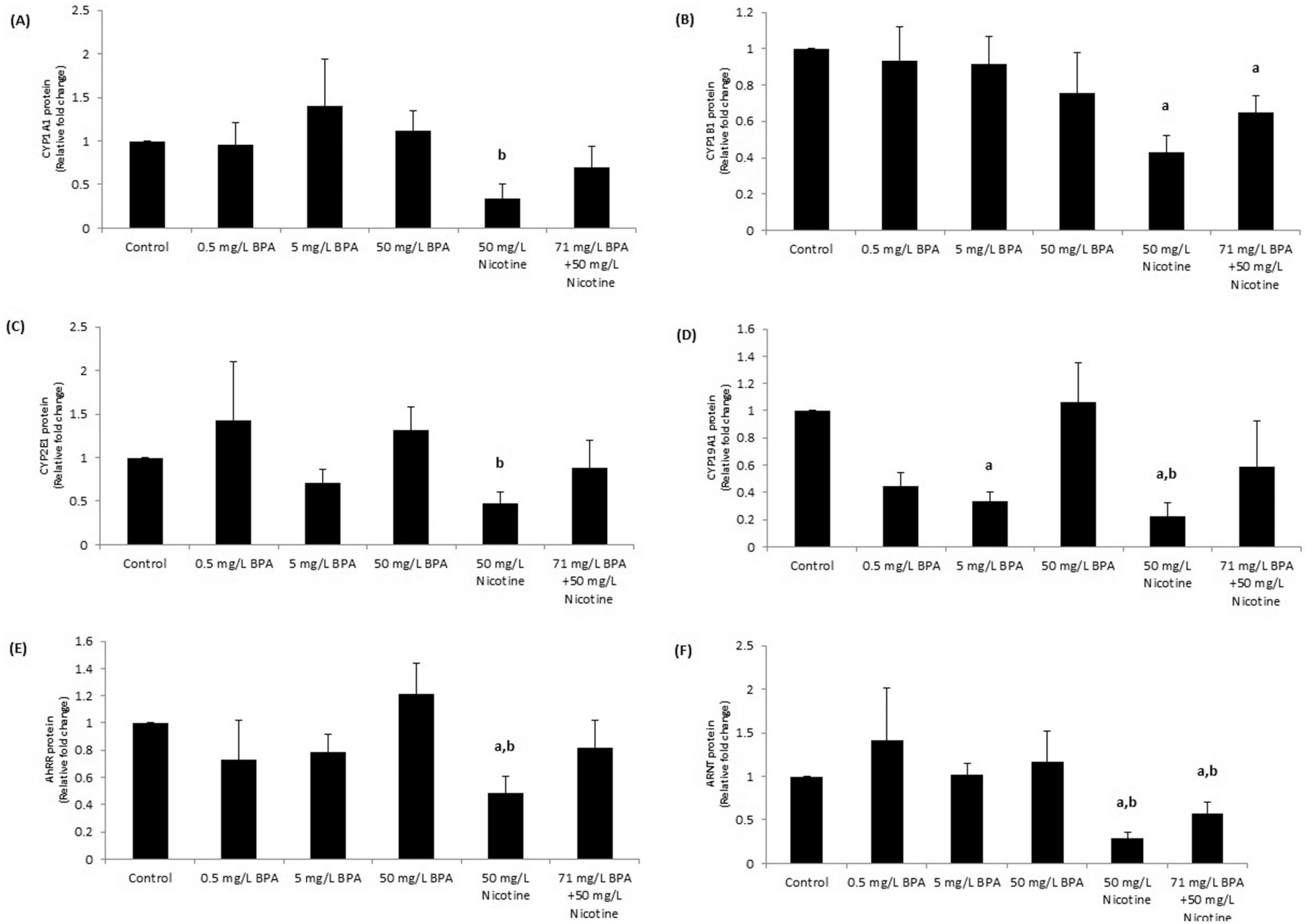

Fig. 3. GD19 protein expression levels of CYP450s, AhRR, and ARNT in rat fetal brain tissues prenatally exposed to both BPA and nicotine. Dams were exposed via drinking water ad libitum starting GD1 and until sacrificed at GD19. Expression levels are presented as means with standard error, and statistical significance is indicated with an"a" when compared to the control group, and a "b" when compared to the 50 mg/L BPA group, where $p < 0.05$.

sharper decrease in expression levels of CYP450s and components of the AhRR-ARNT pathway in fetal rat brain tissues compared to BPA-dosed animals and control (Fig. 2 and Fig. 3).

At GD14, nicotine alone was able to significantly decrease the expression levels of CYP1A1 ($p = 0.018$), CYP1B1 ($p < 0.001$), CYP19A1 ($p < 0.001$), and ARNT ($p = 0.033$) compared to control (Fig. 2); only AhRR did not show a significant decrease compared to control ($p = 0.08$). In addition, nicotine induced a significant decrease in the expression of CYP1A1 ($p = 0.005$) and ARNT ($p = 0.001$) when compared to the highest BPA dose (50 mg/L) (Fig. 2A and E). When nicotine was combined with BPA, both CYP1B1 ($p < 0.001$) and CYP19A1 ($p < 0.001$) were significantly decreased when compared to control. Moreover, the combination of nicotine and BPA resulted in a significant decrease in the expression levels of CYP1A1 compared to highest BPA dose ($p = 0.032$) (Fig. 2A).

At GD19, nicotine induced a decreased expression in all targets. More specifically, nicotine individually induced a significant decrease in the expression levels of CYP1B1 ($p < 0.001$), CYP19A1 ($p = 0.010$), AhRR ($p = 0.042$), and ARNT ($p < 0.001$), compared the control (Fig. 3). In addition, this decrease was significant for CYP1A1 ($p = 0.028$), CYP2E1 ($p = 0.001$), CYP19A1 ($p = 0.003$), AhRR ($p = 0.013$), and ARNT ($p < 0.001$) when compared to the highest BPA dose (50 mg/L) (Fig. 3). However, when combined with BPA, the suppressive effects of nicotine were generally attenuated. Nicotine in combination with BPA significantly decreased the levels of CYP1B1 ($p = 0.023$) compared to control. Moreover, this combination also induced a significant decrease in the levels of ARNT compared to control ($p = 0.004$), and to the highest BPA dose-group ($p = 0.047$) (Fig. 3F). Detailed statistical analysis is described in Supplementary Table 2.

4. Discussion

Although BPA adverse effects have been examined in numerous studies, extensive gaps still persist downstream targeted nuclear receptors, especially during fetal development (Nishizawa et al., 2005a). Our results show that BPA may potentially alter protein expression levels of some CYP450s in fetal rat brain, particularly at high doses. CYP450s are hemeproteins involved in metabolizing both endogenous substances and xenobiotics, and many isozymes are known to be expressed in the brain (Dauchy et al., 2008; Dutheil et al., 2008). Their role in the central nervous system is gaining attention as well as their effect on neuronal survival in brain disorders (Ghosh et al., 2016). Our results show a BPA-induced decrease in CYP1B1 expression levels during early-stage development. This is consistent with the literature where BPA was found to decrease CYP1B1 mRNA and protein expression levels in several tissues (Gilibili et al., 2014; Ziv-Gal et al., 2013). CYP1B1 is thought to play a role in carcinogenesis (Alsubait et al., 2020). Its overexpression was reported in different types of cancer including breast, colon, lung, esophagus, skin, lymph node, brain, and testicular cancer (Murray et al., 1997), and is thought to promote invasion and inhibit apoptosis (Kwon et al., 2016). In contrast, our results showed that BPA has no significant effect on the expression of all other examined CYPP450s including the ubiquitous CYP1A1. Our findings are consistent with an older in vitro study that did not find any impact for BPA on CYP1A1 specific activity, and which eliminated the possibility of an estrogen-receptor mediated role (Jeong et al., 2000).

In addition, our observations of a CYP19A1 (aromatase) suppression are also consistent with the literature (Xu et al., 2019). In a recent study, BPA-exposed human cells during early pregnancy showed alterations in placenta formation and embryonic development due to its action on CYP19A1 protein levels (Xu et al., 2019). Our results are also consistent with another report on BPA-induced downregulation of aromatase expression in human fetal osteoblastic cells (Watanabe et al., 2012). The observed inhibition of aromatase is considered an adverse effect, which could result in various embryonic disruptions during prenatal development (Tiboni et al., 2008). Aromatase was previously found to mediate neuronal development in the neocortex during embryogenesis (Martínez-Cerdeño et al., 2006), and trigger sexual differentiation in mammalian embryos (Naftolin et al., 2001). At the same time, aromatase is expressed in radial glial cells in zebrafish, which are progenitors of the developing and adult brains cells (Diotel et al., 2010). For instance, during brain sexual differentiation, aromatase suppression reduced anogenital distance and sexual incompetence in males (Gerardin et al., 2008). Aromatase inhibition was also found to be associated with serotonergic and catecholaminergic changes, hence affecting mood (Kokras et al., 2018). According to our results, the possibility of a BPA effect on aromatase following a non-monotonic dose-response cannot be ruled out; however, the lack of statistical significance, in addition to the wide confidence interval observed for the 50 mg/L dose-group, preclude us from making a clear conclusion. In contrast, BPA was found to increase the expression of aromatase in zebrafish embryonic brain (Chunga et al., 2011). These species differences in outcomes warrant further studies that could better clarify the mechanism of action. Due to emerging evidence of possible sex dimorphisms in the brain, particularly in the ability of estrogen to influence cognition and synaptic plasticity, it would be important to control for fetal gender in the analysis (Gillies and McArthur, 2010). This could be considered a limitation in our study.

On the other hand, our findings show no significant effect of BPA on the expression levels of AhRR and ARNT in the embryonic brain. The AhRR protein normally represses the AhR signal transduction by competing with AhR for the ARNT, as stimulated by the receptor/translocator heterodimer, hence regulating AhR through a negative feedback mechanism (Evans et al., 2008). The AhR/ARNT heterodimer normally associates with ER and activates estrogen-responsive gene promoters. Although the AhR-induced activation of ER may be dependent on the AhR-ligand structure, however, it may not be necessarily occurring with BPA (Acconcia et al., 2015). This requires further examination, particularly that in this study, we did not detect AhR protein expression in embryonic brains samples. Other studies that did detect AhR expression, examined either different tissues or different species (Hsu et al., 2019; Nishizawa et al., 2005a; Nishizawa et al., 2005b; Williams et al., 2014; Ziv-Gal et al., 2013).

In parallel, our results establish nicotine as a strong suppressor of CYP450s expression in fetal brain, and a strong modulator of BPA effects. In addition, while both BPA and nicotine suppressed aromatase expression individually, BPA attenuated the suppressive effects of nicotine. Nicotine was previously shown to decrease brain aromatase by binding to the enzyme active site, causing a drop in estrogen levels and altering neurophysiology (Biegon et al., 2010). Moreover, nicotine-induced aromatase suppression, was reported to reduce local conversion of androgens to estrogens during brain differentiation (von Ziegler et al., 1991). At the same time, BPA and other ubiquitous substances, including nicotine and other endocrine disruptive chemicals (EDCs) in the environment were previously found to have the ability to exert a synergistic effect when combined (Vandenberg et al., 2012). This is contrary to our findings where the combined BPA-nicotine effect on protein expression is midway for most protein targets compared to that of individual chemicals, both at mid- and late- developmental stage. Hence, in cases of a concomitant exposure, BPA seems to be compensating for nicotine suppression during development. This finding becomes particularly important given that tobacco smoking is associated with higher urinary BPA metabolite levels (Braun et al., 2011), and that cigarette filters were established to be an important source of BPA since the early 1980s (Jackson and Darnell, 1985).

In the final analysis, the ability of BPA and nicotine to decrease the expression levels of aromatase will have a significant effect on the brain, given the role of aromatase in brain development, mainly dendritic branching, neuronal proliferation and migration, brain plasticity, and apoptosis (Azcoitia et al., 2011; Boon et al., 2010). Aromatase is thought to play a role in cognitive function skills development, particularly reading, speech, and language (Rosenfeld et al., 2018). For instance, mice deficient in aromatase showed increased cortical neuronal density

and cortical heterotopias, while deficient embryonic rats showed reduced dendritic growth in the hippocampus (Anthoni et al., 2012). Being an estrogen synthase, the suppression of aromatase will decrease estrogen biosynthesis in the brain. Therefore, estrogen receptors that function as transcriptional factors in the brain will be inactivated, hence decreasing expression of several neurotransmitters such as glutamate, acetylcholine, serotonin and noradrenaline, and ultimately leading to cognitive impairment (Luine, 2014). Consequently, human exposure to BPA and nicotine during embryogenesis may affect cognitive abilities of the newborn in learning and using written and spoken language later in life. This hypothesis is worth exploring in future study designs.

5. Conclusion

In summary, prenatal exposure to BPA is capable of disrupting protein expression of CYP450 enzymes in fetal brain, and these disruptions are modulated by nicotine. The observed effects have a definite impact on xenobiotics metabolism in the brain, and on expression and synthesis of endogenous substances crucial for fetal brain development and function. At the mechanistic level, AhR-ARNT independent mechanisms may underlie the observed results, although this is still unclear and requires further examination. Future investigations should attempt to quantify dosage at the fetal level, identify and control for fetal gender in the analysis when possible, and look at the functional implications and long-term effects of the reported observations in the F1 generation. Protein activity, and implications on the organ structure and function, particularly cognitive abilities, should also be examined.

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CRediT authorship contribution statement

Mohammad H. Merii: Formal analysis, Methodology, Writing – original draft. **Manal M. Fardoun:** Methodology, Data curation, Writing – review & editing. **Khalil El-Asmar:** Formal analysis, Writing – original draft. **Mahmoud I. Khalil:** Writing – review & editing. **Ali Eid:** Validation, Resources, Writing – review & editing. **Hassan R. Dhaini:** Conceptualization, Supervision, Resources, Methodology, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no competing interests that could influence the work reported in this article.

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