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EFFECTS OF BISPHENOL A AND NICOTINE ON CYTOCHROME P450S IN BRAIN AND LIVER OF PREGNANT RATS

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1. INTRODUCTION

Bisphenol A (BPA) is a chemical produced in very high amounts globally with four million tons estimated to be produced yearly (Matuszczak et al., 2019). This monomer is used to produce polycarbonate thermoplastics. These are usually used in the packaging of drinks and food. It also has a role as an ingredient in water pipes made of polyvinyl chloride. In addition, it is used in the lining of cans, food wrapping sheets, and cigarette filters (Biedermann et al., 2010). Packaging degradation causes BPA to migrate into food and drinks, exposing humans through consumption. (Calafat et al., 2005; Groff, 2010). BPA or its metabolized metabolites can be detected in the bodily fluids of people of all age categories (Birnbaum, 2013; Gerona et al., 2016) including new-borns, pregnant females, and adults (Dhimolea et al., 2014; Schönfelder et al., 2002; Vandenberg et al., 2009). Remarkably, females usually show greater levels of exposure (Calafat et al., 2008).

BPA exposure at very low concentrations has been linked, by several studies, to endocrine and tumorigenic side effects (Kundakovic and Champagne, 2011). Recent research on metabolism and toxicokinetics has revealed that BPA is rapidly absorbed orally. When absorbed, conjugation with glucuronic acid takes place in the liver (Cimmino et al., 2020). BPA was shown to have the propensity to stock up in a variety of human tissues, impairing bodily activities and having negative health consequences (Healy et al., 2015; Mathieu-Denoncourt et al., 2016; Valentino et al., 2016). BPA induces adipogenesis, boosts lipid accumulation in the liver, and disrupts cytokine levels by modifying Peroxisome proliferator-activated receptors. BPA also interferes with thyroid hormone synthesis, secretion, and signaling. BPA acts as an agonist on the estrogen receptors and an antagonist on the androgen receptors due to its anti-androgenic properties (Ahmed and Atlas, 2016; Rotondo and Chiarelli, 2020). By binding to various receptors, altering transcription factors, and generating epigenetic alterations, BPA impacts glucose metabolism, the onset and development of numerous cancers, and immunological function (Provvisiero et al., 2016; Xu et al., 2016). In adult female rats, it was shown that BPA blocked synapse formation which was augmented by beta-estradiol (Wolstenholme et al., 2011). It was also found to affect the methylation of specific genes in adult brains (Santoro et al., 2019).

BPA's health hazards are linked to a number of pathways, including the interaction with several receptors; the aryl-hydrocarbon receptor (AhR), the estrogen receptor (ER), and the constitutive androstane receptor (Kwintkiewicz et al., 2010a; Lee et al., 2003; Moriyama et al., 2002). Several dangerous compounds have an AhR ligand, therefore researchers are paying more attention to the link between its pathway and toxic reactions to contaminants present in the environment. The AhR pathway stimulates the production of XMEs, or xenobiotic metabolic enzymes, especially oxidation enzymes of the Phase I cytochrome P450s. This might affect the metabolic capacities of tissues (Singh et al., 2012). The main method by which BPA is metabolized is via conjugating a phenolic hydroxyl group to glucuronide (Knaak and Sullivan, 1966). It was hypothesized that cytochrome P450-dependent mixed-function oxidases convert some of the BPA supplied to rat hepatic microsomal suspensions to 3-hydroxybisphenol A (3-OH-BPA), and the intermediate is then oxidized to bisphenol-o-quinone (Atkinson and Roy, 1995). Furthermore, it was found that UGT2B1 is the major UDP-glucuronosyltransferase (UGT) isoform that glucuronidates BPA in rat liver microsomes. As a result, the metabolism and toxicity of BPA in animals are intimately related to drug-metabolizing enzymes including CYP and UGT (Yokota et al., 1999).

Cigarette smoking, in addition, is the greatest cause of several diseases in addition to death worldwide. It is regarded as a major health concern, including in pregnant females. The percentage of females who smoke during pregnancy ranges from 30 to 80% in different countries (Lange et al., 2018). Components of tobacco smoking, notably nicotine, are believed to cause physiological changes in the brains of humans, in addition to cardiovascular disorders and cancer (Benowitz, 2008). Nicotine is mainly metabolized in the liver by various enzymes such as UDP-glucuronosyltransfease, and flavin-containing monooxygenase (Benowitz et al., 2009).

There are still many unknowns regarding the changes caused by BPA downstream of nuclear receptors (Cimmino et al., 2020; Wolstenholme et al., 2011). Moreover, there are still significant differences between studies in terms of BPA toxicity outcomes and threshold amounts. The goal of this study is to check how BPA affected CYP450s protein expression levels and those of the activating cascade of the AhR receptor; ARNT and AhRR in adult female rat brain and

liver, in vivo. The selected CYPs were shown to be affected by BPA in other studies (Gilibili et al., 2014; Kwintkiewicz et al., 2010b; Olsvik et al., 2019; Yu et al., 2021; Ziv-Gal et al., 2013). The study also looks at the modulatory effects of nicotine, a common tobacco product component that has been demonstrated to lower CYP19A1 (aromatase) levels of expression and change the production of estrogen (Kanungo et al., 2012).

2. MATERIALS AND METHODS

2.1 Animal Experiment

This study gained the authorization of the Institutional Animal Care and Use Committee at the American University of Beirut. The study abided by the US Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training (Council, 2011). Female Sprague-Dawley adult rats, aged 8 weeks and weighing about 250 g (n =24), were placed individually, for 2 days, in a cage with a Sprague-Dawley adult male rat. The existence of vaginal plugs was inspected in the morning to screen for pregnancy. Day 1 was defined as the day the vaginal plug was observed; exposure started on this day. Each conceiving female rat was housed in its cage with a 12:12 hour light/dark cycle. Each pregnant rat was left in its own cage.

2.2 Doses and Groups

Drinking water solutions with concentrations of 0 (control), 0.5, 5, and 50 mg/L of BPA were used to achieve exposure dosages of 0 (control), 50, 500, and 5000 g/kg/day, respectively (≥99%; Sigma-Aldrich, USA) (supplementary table 1). A daily water intake of 9 to 12 ml per 100 g of body weight was used to compute this (Claassen, 1994). Each pregnant rat had its own drinking water bottle. Based on the literature, the dosage interval was selected to address a wide range of dosages (Mendoza-Rodríguez et al., 2011; Merii et al., 2022). The doses used were likewise in line with prior human research' anticipated values in maternal plasma and placenta units (Patisaul et al., 2012; Schönfelder et al., 2002). BPA was prepared in a 1% ethanol solution and then mixed with water (Somm et al., 2009). Nicotine hydrogen tartrate was prepared in water at 50 mg/L (Sigma-Aldrich, USA) to reach a 3000 µg/kg/day exposure dose, imitating tobacco smoking exposure in humans. Water consumption was expected to drop by 30% when nicotine was added to the drinking water. This was caused by the bitter flavor of nicotine and as a result, it was compensated (Schneider et al., 2010). This was also applied with the BPA and nicotine group, and for compensation, the 50 mg/L BPA solution was adjusted to 71 mg/L. (Schneider et al., 2010). A daily nicotine intake of 100 g results in a plasma cotinine concentration of 1 ng/ml (Benowitz, 2008). Dams exposed to nicotine concentrations ranging from 0.3 to 1.2 mg/mL usually achieve daily dosing intervals of 2–7 mg/kg body weight, which translates to nicotine plasma levels of 20–75 ng/mL (Schneider et al., 2010). Two female rats were randomly assigned to each dose group, given free access to food, and dosed ad libitum by the drinking water. In total, there were six dosing groups in the experiment. Dams were sacrificed after 2 weeks of exposure, and brains and livers were removed for analysis.

2.3 Extraction and Quantification of Proteins

Extraction of total proteins from brain tissues was done using the N-PER Neuronal Reagent (Thermo, USA), and those from liver tissues were extracted using the NP-40 solution (150 mM NaCl, 50 mM Tris-HCl pH 8, 0.1 % Triton-X). Afterward, proteins were quantified using the DC Assay (Bio-Rad, USA). The brain and liver tissues were homogenized with their aforementioned extraction solutions, after being rinsed in a phosphate-buffered saline solution. After centrifuging the homogenate, the supernatant was collected. To quantify proteins, dilutions and standards (bovine serum albumin standard, Bio-Rad, USA) were constructed, and absorbance was measured at 750 nm.

2.4 Immunoblotting

The quantified proteins (30 µg) of samples were denatured and run on SDS- PAGE. Afterward, protein transfer to PVDF membranes was performed (Bio-Rad, USA). Red Ponceau solution was used to check for transfer quality. Membrane blocking was then performed using a 5 percent non-fat dairy milk solution in a Tris-Buffered Saline / Tween 20 solution (TBST). The membranes were then incubated with specific, well-established primary antibodies (supplementary table 2) against AhRR (ab108518, Abcam, UK) (Anderson et al., 2019; Silveira et al., 2019), ARNT (sc-17811, Santa Cruz, USA) (Rijo et al., 2021; Zhang et al., 2021), CYP2E1 (MAB3817, Abnova, Taiwan) (Gelboin and Friedman, 1985; Goldfarb et al., 1993), CYP19A1 (ab124776, Abcam, UK) (Molehin et al., 2018; Storman et al., 2018), CYP1A1 (sc-393979, Santa Cruz, USA) (Vyhlídalová et al., 2020; Zhang et al., 2021), and CYP1B1 (ab185954, Abcam, UK) (Yang et al., 2021; Zhang et al., 2020), as per manufacturer's recommendations. Membranes were then washed in TBST and incubated in HRP-conjugated goat antibodies against mouse (1705047, Bio-Rad, USA) or rabbit (1705046, Bio-Rad, USA), as per the manufacturer's recommendations. Clarity Western ECL Substrate was used to detect bands in samples, which were run in duplicates. (Bio-Rad, USA). Analysis of the intensities of bands was performed using the ChemiDoc MP Imaging System (Bio-Rad, USA). As per the manufacturer's guidelines, a specific, well-established antibody against Beta-actin (sc-47778 HRP, Santa Cruz, USA) (Joshi et al., 2021; Yoon et al., 2021) was employed for loading control. Protein expression was determined relative to Beta-actin, and quantitative data for dosed groups were normalized against the data of the control group. All antibodies used were monoclonal, except for AhRR.

2.5 Statistical analysis

Expression levels of proteins were designated as mean ± standard error of the mean (SEM). Differences in protein expression across various dose groups were analyzed using Student's *t*-test. Statistical significance was defined as a p-value of less than 0.05.

3. RESULTS

3.1 BPA Effect on Protein Expression in the Brain

BPA decreased the protein levels of CYP1A1, CYP1B1, and CYP2E1 in adult brain tissues after 2 weeks of exposure, compared to the control (figures 1, 2, and 3). This decrease was significant at 0.5 mg/L *(p=0.015)* and 50 mg/L *(p=0.044)* for CYP1A1, and at 5 mg/L *(p=0.003)* for CYP1B1, and at 0.5 mg/L *(p=0.002)* and 5 mg/L *(p=0.029)* for CYP2E1. Aromatase (CYP19A1) exhibited slight non-significant increased expression levels upon exposure to BPA, compared to the control (figure 4). In addition, the repressor (AhRR) showed decreased expression levels after BPA exposure, with respect to the control, which were significant at doses of 5 mg/L *(p<0.001)* and 50 mg/L *(p=0.031)* (figure 5).

3.2 Modulation of nicotine in the brain

Nicotine was able to show a significant decrease in protein expression of CYP1A1 *(p=0.042)* and CYP1B1 *(p=0.007)* in adult brain tissues after 2 weeks of exposure, compared to the control (figures 1 and 2). Also, when combined with BPA, nicotine retained the same effect *(p=0.025 and p=0.010 respectively)* for CYP1A1 and CYP1B1. CYP2E1 protein expression was not significantly changed by nicotine alone or nicotine with BPA, compared to the control (figure 3). Moreover, aromatase only showed a non-significant increase in its expression levels after being exposed to nicotine alone or combined with BPA, compared to the control (figure 4). AhRR showed significantly decreased expression levels after nicotine exposure, with respect to the control *(p=0.044)* (figure 5). When combined with BPA, this decrease was not significant.

3.3 BPA effect on protein expression in the liver

CYP1A1 and CYP1B1 showed no significant change in the expression levels after 2 weeks of exposure to BPA, compared to the control (figures 6 and 7). On the contrary, BPA was able to increase the protein levels of CYP2E1 in adult liver tissues, compared to the control; significantly at 0.5 mg/L *($p<0.001$)* and 50 mg/L *($p=0.002$)* (figure 8). Aromatase showed decreased expression levels after 2 weeks of BPA exposure, which was significant at 5 mg/L *($p=0.0001$)*, compared to the control (figure 9). AhRR protein levels increased non-significantly after BPA exposure, compared to the control (figure 10). ARNT showed a significant decrease at 5 mg/L *($p=0.044$)* after exposure to BPA, compared to the control (figure 11).

3.4 Modulation of nicotine in the liver

Nicotine significantly decreased the protein levels of CYP1A1 *($p=0.017$)* in adult liver tissues after 2 weeks of exposure, compared to the control (figure 6). CYP1B1 and CYP2E1 protein levels were not significantly affected by BPA in the liver (figures 7 and 8). Aromatase and ARNT showed significantly decreased protein levels *($p=0.022$ and $p<0.001$ respectively)* after 2 weeks of BPA exposure, compared to the control (figures 9 and 11). AhRR showed no significant change in the expression levels after exposure to nicotine. Interestingly, when combined with BPA, nicotine was able to change the protein expression levels of most targets with respect to the control (figure 10), but none were significant.

4. DISCUSSION

While BPA exposure has been studied extensively, there are still gaps downstream regarding its mechanism of effect on the brain and liver (Kobayashi et al., 2020; Kourouma et al., 2015). The results of this study indicate that BPA affects protein expression levels of CYP450s in the rat brain and liver of pregnant female rats. The brain and liver are known to express the hemeproteins CYP450s and have a role in the metabolism of endogenous and xenobiotic drugs (Dauchy et al., 2008; Villeneuve and Pichette, 2004). BPA was found to have alarming effects on vital neuronal communication functions in mature vertebrate brains which may contribute to serious neurological defects (Schirmer et al., 2021). Moreover, CYP450s have a role in the pathogenesis of several liver disorders as well as hepatotoxicity (Villeneuve and Pichette, 2004).

Our results show that BPA was able to decrease CYP1A1 expression levels after 2 weeks of exposure in the brain. This effect on CYP1A1 by BPA may be mediated through AhR or other transcriptional factors that bind to regulatory regions in the CYP1A1. Phosphorylation modifications in the cytosolic receptor complex could facilitate the observed BPA effects (Kim et al., 2004). In zebrafish, BPA was reported to reduce CYP1A1 expression (Olsvik et al., 2019). On the contrary, increased CYP1A1 protein levels after BPA exposure were reported in human hepatoma (HepG2) cell line (Yu et al., 2021). BPA had no influence on CYP1A1 specific activity, according to another study, excluding the possibility of a role for the estrogen receptor (Jeong et al., 2000). Similarly, this was the case with our results in the liver of the pregnant rats. The results of this study demonstrate that BPA resulted in decreased CYP1B1 protein expression at the level of the brain only. In many organs, BPA was observed to reduce CYP1B1 mRNA and protein expression, which complies with our findings after 2 weeks of exposure in the brain (Gilibili et al., 2014; Ziv-Gal et al., 2013). On the other hand, it was shown that BPA increased CYP1B1 protein expression and levels of radioactive oxygen species in carp spleen lymphocytes (Liu et al., 2020). Our results also show that BPA decreases the expression of CYP2E1 in the brain while increasing it in the liver. It was shown that BPA inhibited the activity of CYP2E1 and other CYPs in a rat hepatic microsome study (Hanioka et al., 2000). On the other hand, bisphenols and especially BPA were able to increase the transcription of CYP2E1 and other CYPs in HepG2 cells (Yu et al., 2021).

The detected decrease of aromatase (CYP19A1) in our study, in the liver after 2 weeks of exposure was in line with previous reports of BPA-induced aromatase suppression in granulosa cells (Kwintkiewicz et al., 2010b). The aromatase inhibition detected, is a cause for concern, resulting in adverse effects on mental health (Gervais et al., 2019). Also, aromatase suppression was associated with Non-alcoholic Fatty Liver Disease (Lee et al., 2019). Aromatase inhibition

has also been linked to serotonergic and catecholaminergic alterations, which have been shown to impact mood and cause cognitive impairment (Kokras et al., 2018). However, in zebrafish, one study concluded that BPA increased the expression levels of aromatase in the embryonic brain (Chunga et al., 2011).

The observed non-significant increase of AhRR expression levels in the liver after BPA exposure was consistent with an in vivo study where BPA was able to enhance expression levels of AhRR and decreased that of AhR in embryos of mice (Nishizawa et al., 2005). On the other hand, our results showed a decrease in AhRR in the brain which was inconsistent with other studies. Following ligand interaction, cytoplasmic AhR penetrates the nucleus and joins ARNT. It then binds to particular DNA-response regions, resulting in the transcription of numerous genes. Furthermore, AhRR can heterodimerize with ARNT to stop the AhR signaling pathway from being activated (Pollenz, 2002; Puga et al., 2009). The results of this study also demonstrated a decrease in the expression of ARNT in the liver, after BPA exposure. This followed a study in which BPA was found to lower ARNT levels in mice ovarian follicles (Ziv-Gal et al., 2013). The AhR/ARNT heterodimer normally binds to ER and stimulates estrogen-responsive gene promoters. This activation, however, may be influenced by the AhR-ligand structure, which may or may not be the situation with BPA (Acconcia et al., 2015). In addition, we were not able to detect AhR protein expression in the tissues of our samples. This was also the case with ARNT in brain tissues. This needs further investigation.

According to our data, nicotine is also a significant suppressor of the expression of some of our targeted proteins in the brain and liver, as well as a modulator of the BPA effect. With some targets, the combined BPA-nicotine impact attenuated that of BPA alone. Moreover, Both BPA and nicotine suppressed the expression of aromatase in the liver. Nicotine has been demonstrated to inhibit aromatase expression by binding to the enzyme active site, resulting in lower estrogen levels and changes in neurophysiology (Biegon et al., 2010). This affects the transformation of androstenedione and testosterone into estrone and estradiol, separately (Zeller and Berger, 1989). Nicotine interferes with placental microsomal CYPs, implying that placental aromatase is inhibited by a change in the role of the P-450 section (Zeller and Berger, 1989).

According to our findings, BPA and other compounds in our environment, such as nicotine and other endocrine-disrupting chemicals (EDCs), may have disruptive effects on health, especially if combined (Vandenberg et al., 2012). One study showed that there are signs that combining anti-androgens at levels that yield minor, statistically insignificant responses individually, produces strong joint effects (Hass et al., 2007). As a result, aggregate exposure to several EDCs could have a major effect on cellular response and gene expression. This was inconsistent with our results since the combination of BPA and nicotine did not have the same effects as either BPA or nicotine alone, especially in the liver.

The exerted effects of BPA and nicotine in the brain and liver gain significant importance due to the vital functions of CYPs. The presence of CYP1A1 in the blood-cerebrospinal fluid barrier allows it to take part in shielding the brain from xenobiotics (Morse et al., 1998). CYP1A1 is also involved in metabolizing arachadonic acid in the brain. This directly affects important processes such as vesicle release and vasodilation of cerebral arteries (Ferguson and Tyndale, 2011). In addition, it was shown that mutations in CYP1B1 caused irregular development Schlemm's canal and the trabecular meshwork in human eyes (Stoilov et al., 1997), and was found to be a major risk factor for congenital glaucoma (Vasiliou and Gonzalez, 2008). Also, the absence of CYP1B1 expression in the perivascular supporting pericytes of the retina may enhance vascular dysfunction and integrity (Palenski et al., 2013). Also, CYP1B1 may play an important role in the development and function of the eye (Choudhary et al., 2007). Furthermore, aromatase Male mice lacking aromatase had severe hepatic steatosis as a result of elevated liver triglyceride and cholesterol levels, which is linked to increased expression of lipid manufacturing enzymes and decreased activity of fatty acid oxidation enzymes (Hewitt et al., 2004; Nemoto et al., 2000).

5. CONCLUSION

Nicotine may moderate the disruptive effect of BPA on the expression levels of CYP450 enzymes in the brain and liver of pregnant rats. These actions have a major impact on xenobiotic metabolism in the brain and liver, particularly in pregnant women. At the molecular level, AhR-ARNT dependent or independent pathways could be at the root of the observed results, which has to be investigated further. Also, protein activities, as well as other BPA targets in the brain and liver, should be investigated, as should the effects on organ structure and function. Further investigations into the effect of BPA on AhR performed, as well as on epigenetics. This could be considered as a limitation to this study.

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Competing Interest

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

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APPENDICES

Figures

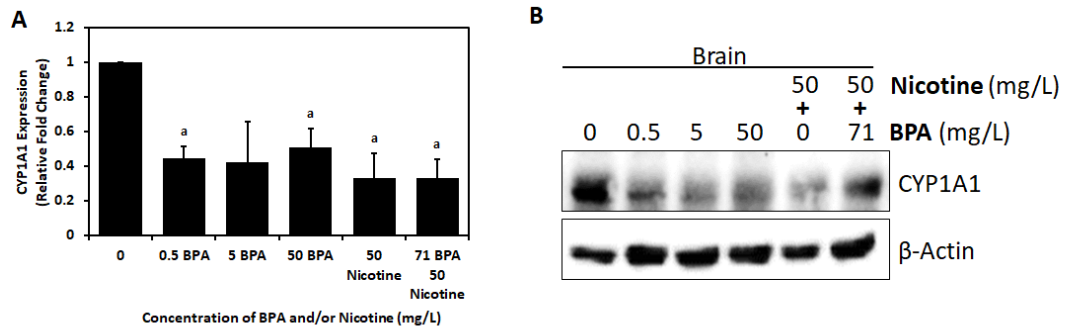

Fig.1: (A) CYP1A1 protein expression levels in rat brain subjected to BPA, nicotine, and BPA combined with nicotine. For two weeks, rats were exposed to the substance through their drinking water. Expression levels are expressed as mean ± SEM, and statistical significance is denoted by the letter 'a' when compared to the control group, with a p value of less than 0.05. (B) CYP1A1 western blots in pregnant rat brain tissues exposed to the respective substances.

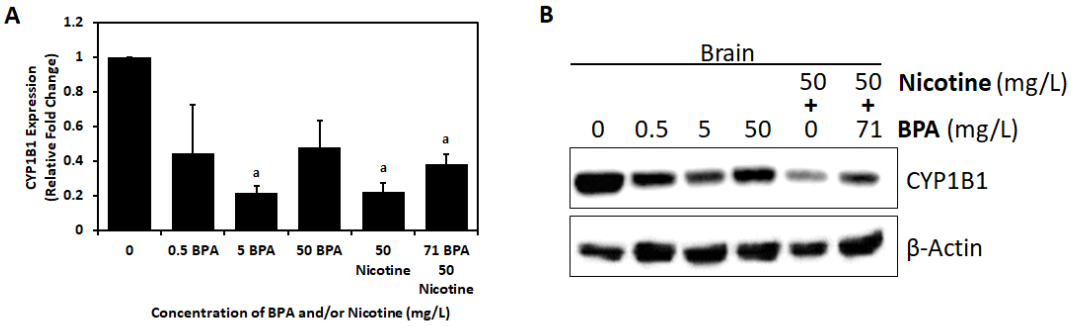

Fig.2: (A) CYP1B1 protein expression levels in rat brain subjected to BPA, nicotine, and BPA combined with nicotine. For two weeks, rats were exposed to the substance through their drinking water. Expression levels are expressed as mean ± SEM, and statistical significance is denoted by the letter 'a' when compared to the control group, with a p value of less than 0.05. (B) CYP1B1 western blots in pregnant rat brain tissues exposed to the respective substances.

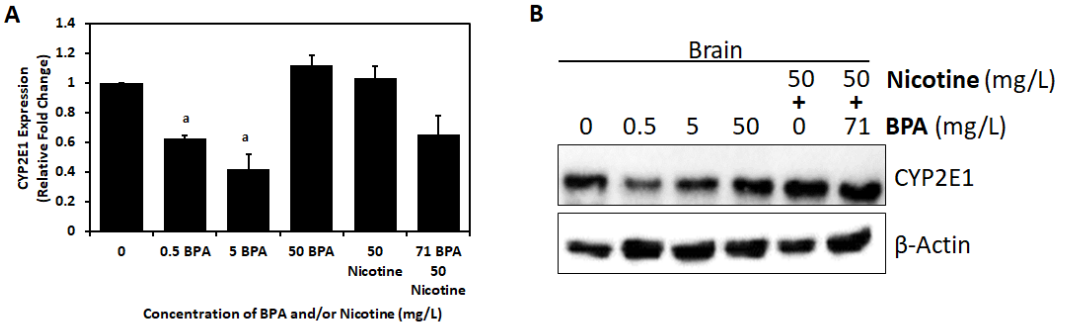

Fig.3: (A) CYP2E1 protein expression levels in rat brain subjected to BPA, nicotine, and BPA combined with nicotine. For two weeks, rats were exposed to the substance through their drinking water. Expression levels are expressed as mean ± SEM, and statistical significance is denoted by the letter 'a' when compared to the control group, with a p value of less than 0.05. (B) CYP2E1 western blots in pregnant rat brain tissues exposed to the respective substances.

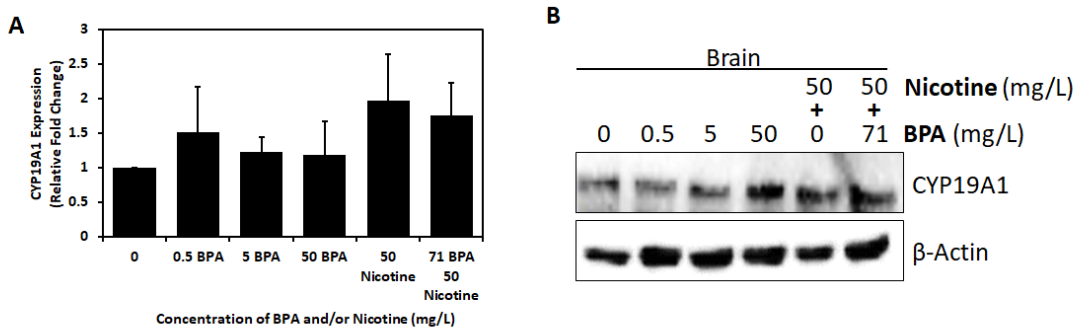

Fig.4: (A) CYP19A1 protein expression levels in rat brain subjected to BPA, nicotine, and BPA combined with nicotine. For two weeks, rats were exposed to the substance through their drinking water. Expression levels are expressed as mean ± SEM, and statistical significance is denoted by the letter 'a' when compared to the control group, with a p value of less than 0.05. (B) CYP19A1 western blots in pregnant rat brain tissues exposed to the respective substances.

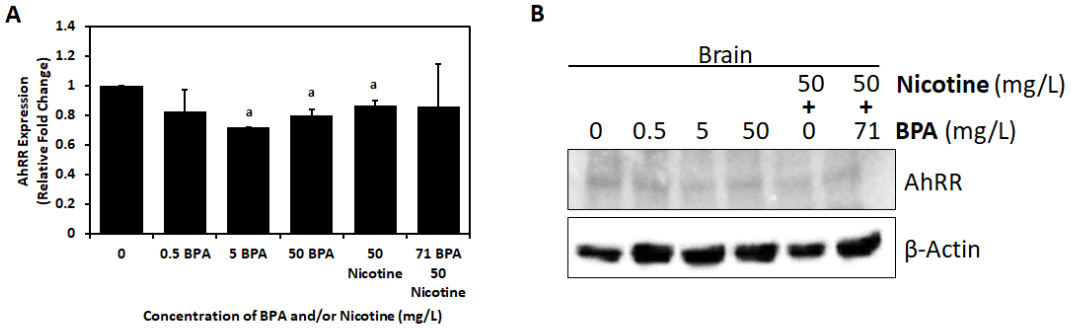

Fig.5: (A) AhRR protein expression levels in rat brain subjected to BPA, nicotine, and BPA combined with nicotine. For two weeks, rats were exposed to the substance through their drinking water. Expression levels are expressed as mean ± SEM, and statistical significance is denoted by the letter 'a' when compared to the control group, with a p value of less than 0.05. (B) AhRR western blots in pregnant rat brain tissues exposed to the respective substances.

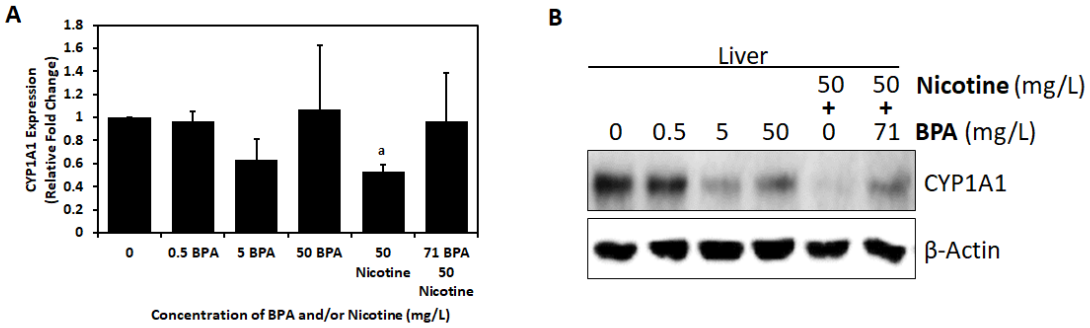

Fig.6: (A) CYP1A1 protein expression levels in rat liver subjected to BPA, nicotine, and BPA combined with nicotine. For two weeks, rats were exposed to the substance through their drinking water. Expression levels are expressed as mean ± SEM, and statistical significance is denoted by the letter 'a' when compared to the control group, with a p value of less than 0.05. (B) CYP1A1 western blots in pregnant rat liver tissues exposed to the respective substances.

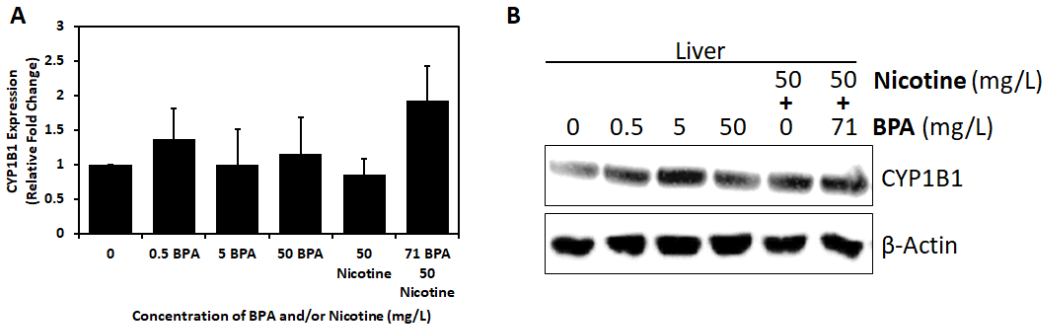

Fig.7: (A) CYP1B1 protein expression levels in rat liver subjected to BPA, nicotine, and BPA combined with nicotine. For two weeks, rats were exposed to the substance through their drinking water. Expression levels are expressed as mean ± SEM, and statistical significance is denoted by the letter 'a' when compared to the control group, with a p value of less than 0.05. (B) CYP1B1 western blots in pregnant rat liver tissues exposed to the respective substances.

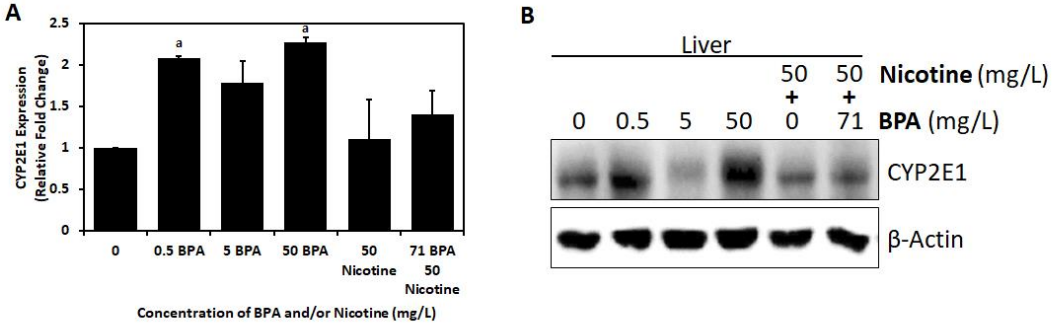

Fig.8: (A) CYP2E1 protein expression levels in rat liver subjected to BPA, nicotine, and BPA combined with nicotine. For two weeks, rats were exposed to the substance through their drinking water. Expression levels are expressed as mean ± SEM, and statistical significance is denoted by the letter 'a' when compared to the control group, with a p value of less than 0.05. (B) CYP2E1 western blots in pregnant rat liver tissues exposed to the respective substances.

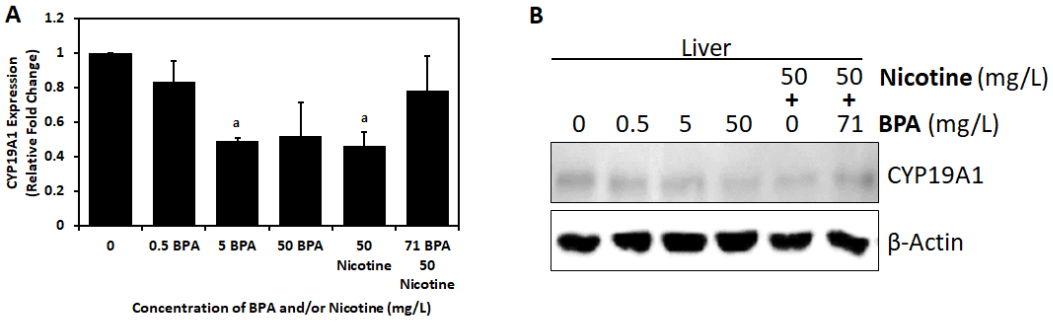

Fig.9: (A) CYP19A1 protein expression levels in rat liver subjected to BPA, nicotine, and BPA combined with nicotine. For two weeks, rats were exposed to the substance through their drinking water. Expression levels are expressed as mean ± SEM, and statistical significance is denoted by the letter 'a' when compared to the control group, with a p value of less than 0.05. (B) CYP19A1 western blots in pregnant rat liver tissues exposed to the respective substances.

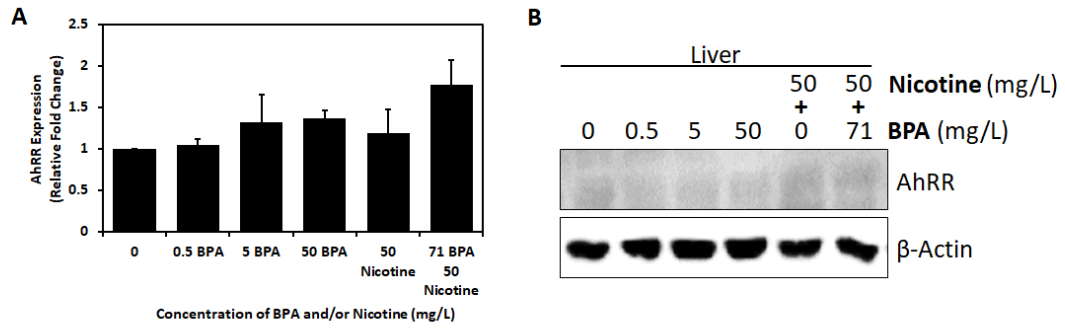

Fig.10: (A) AhRR protein expression levels in rat liver subjected to BPA, nicotine, and BPA combined with nicotine. For two weeks, rats were exposed to the substance through their drinking water. Expression levels are expressed as mean ± SEM, and statistical significance is denoted by the letter 'a' when compared to the control group, with a p value of less than 0.05. (B) AhRR western blots in pregnant rat liver tissues exposed to the respective substances.

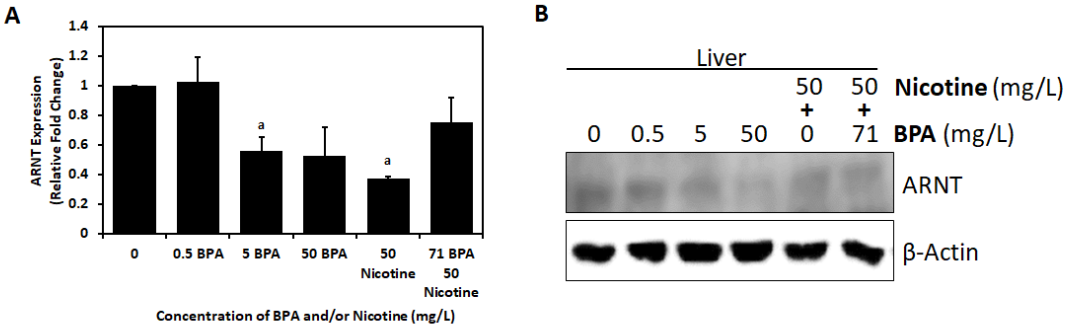

Fig.11: (A) ARNT protein expression levels in rat liver subjected to BPA, nicotine, and BPA combined with nicotine. For two weeks, rats were exposed to the substance through their drinking water. Expression levels are expressed as mean ± SEM, and statistical significance is denoted by the letter 'a' when compared to the control group, with a p value of less than 0.05. (B) ARNT western blots in pregnant rat liver tissues exposed to the respective substances.

Supplementary Tables

Supplementary Table 1: Details of the animal groups.

Group	Treatment	Concentration	Dose
1 (Control)	_	_	_
2	BPA	0.5 mg/L Drinking Water	50 µg/kg/day
3	BPA	5 mg/L Drinking Water	500 µg/kg/day
4	BPA	50 mg/L Drinking Water	5000 µg/kg/day
5	Nicotine	50 mg/L Drinking Water	3000 µg/kg/day
6	Nicotine and BPA	50 mg/L Nicotine + 71 mg/L BPA Drinking Water	Nicotine at 3000 µg/kg/day & BPA at 5000 µg/kg/day

Supplementary Table 2: Details of the antibodies used.

Antibody	Supplier	Part Number	Species	Reactivity	Used Dilution	Recommended Dilution	Type	References
CYP1A1	Santa Cruz	sc-393979	Mouse	Rat, Human	1:500	1:100 – 1:1000	Monoclonal	(Vyhlídalová et al., 2020; Zhang et al., 2021)
CYP1B1	Abcam	ab185954	Rabbit	Rat, Human	1:2000	1:1000 – 1:10000	Monoclonal	(Yang et al., 2021; Zhang et al., 2020)
CYP2E1	Abnova	MAB3817	Mouse	Rat, Human	1:500	1:1000	Monoclonal	(Gelboin and Friedman, 1985; Goldfarb et al., 1993)
CYP19A1	Abcam	ab124776	Rabbit	Rat, Human	1:2000	1:1000 – 1:10000	Monoclonal	(Molehin et al., 2018; Storman et al., 2018)
AhR	Santa Cruz	sc-133088	Mouse	Rat, Human	1:100	1:100 – 1:1000	Monoclonal	(Asai et al., 2018; Paris et al., 2021)
ARNT	Santa Cruz	sc-17811	Mouse	Rat, Human	1:1000	1:200 – 1:2000	Monoclonal	(Rijo et al., 2021; Zhang et al., 2021)
AhRR	Abcam	ab108518	Rabbit	Rat, Human	1:1000	1:1000 – 1:2000	Polyclonal	(Anderson et al., 2019; Silveira et al., 2019)