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Prenatal exposure to Benzo[a]pyrene affects maternal–fetal outcomes via placental apoptosis

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Prenatal exposure to Benzo[a]pyrene (BaP) has been suggested to increase the risk of adverse pregnancy outcomes. However, the role of placental apoptosis on BaP reproductive toxicity is poorly understood. We conducted a maternal animal model of C57BL/6 wild-type (WT) and transformation-related protein 53 (Trp53) heterozygous knockout (p53KO) mice, as well as a nested case–control study involving 83 women with PB and 82 term birth from a birth cohort on prenatal exposure to BaP and preterm birth (PB). Pregnant WT and p53KO mice were randomly allocated to BaP treatment and control groups, intraperitoneally injected of low (7.8 mg/kg), medium (35 mg/kg), and high (78 mg/kg) doses of 3,4-BaP per day and equal volume of vegetable oil, from gestational day 10.5 until delivery. Results show that high-dose BaP treatment increased the incidence of preterm birth in WT mice. The number of fetal deaths and resorptions increased with increasing doses of BaP exposure in mice. Notably, significant reductions in maternal and birth weights, increases in placental weights, and decrease in the number of livebirths were observed in higher-dose BaP groups in dose-dependent manner. We additionally observed elevated p53-mediated placental apoptosis in higher BaP exposure groups, with altered expression levels of p53 and Bax/Bcl-2. In case–control study, the expression level of MMP2 was increased among women with high BaP exposure and associated with the increased risk of all PB and moderate PB. Our study provides the first evidence of BaP-induced reproductive toxicity and its adverse effects on maternal–fetal outcomes in both animal and population studies.

Keywords Benzo[a]pyrene, Prenatal exposure, Adverse maternal–fetal outcomes, Placental apoptosis, Matrix metalloproteinases

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants generated by combustion of cigarette smoking, fossil fuels, industrial or domestic coal, and during cooking foods[1,2], and belong to group 1 carcinogenic to humans according to the International Agency for Research on Cancer[3]. Studies, including ours[2], have suggested that exposure to PAHs during pregnancy is associated with adverse pregnancy outcomes, including early embryo mortality, stillbirths, intrauterine growth restriction (IUGR), preterm birth (PB), and low birth weight (LBW)[2,4–7]. A plausible mechanism is that PAHs exposure affect embryonic development by destroying nucleotides, as they can directly alter cell function at the molecular level by forming DNA adducts, which result in DNA mutation and transcription factors activation[8]. As a representative marker of exposure to total carcinogenic PAHs, Benzo[a]pyrene (BaP) is systematically distributed to form DNA adducts in numerous tissues after exposure to PAHs. In Chinese birth cohorts, we previously observed an increased risk of PB in relation to interquartile increase in maternal BaP-DNA adducts level (3.56 ± 1.46 μg/gDNA in PB)[2]. Tang et al. have found that high levels of BaP-DNA adduct (0.29 ± 0.13 adducts/10^8 nucleotides) are associated with reduced fetal growth[9]. Moreover, the presence of BaP–DNA adducts (0.32 ± 1.04 adducts/10^8 nucleotides) is adequate to

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alter global genomic DNA methylation in cord blood[10]. A recent Chinese cohort study reported that prenatal exposure to PAHs (mean BaP levels in 55 subjects: 0.75 ng/mL) might increase the risk of LBW by modulating the DNA methylation status of genomic DNA and growth-related genes in the umbilical cord blood[11]. However, the variations in study populations, regions, and the samples and measures used for detection across different studies cannot be ignored, the associations between BaP exposure and adverse maternal–fetal outcomes are still inconclusive. In addition, the mechanisms underlying the associations remain unclear.

Apoptosis is recognized as a critical mechanism underlying cytotoxic effects and teratogenicity[12]. Growing evidence supports its role in fetal development and reproductive health[13]. During embryogenesis, apoptosis is pivotal for cell elimination, which is essential for shaping tissues and body formation. p53 can interact with and regulate various components of the apoptotic signaling pathways, and has been suggested to inhibit the expression of anti-apoptotic proteins, such as Bcl-2 protein family (Bax, Bak)[14,15]. Apoptosis might lead to the activation or increased expression of matrix metallopeptidase (MMP), MMP family is involved in the breakdown of the extracellular matrix in embryonic development and reproduction[16]. On the other hand, when apoptosis is triggered by exposure to exogenous substances such as bacteria, viruses, and parasites, it can disrupt placental and fetal growth, potentially leading to fetal death, organ injury, or subsequent limited sequelae[17]. Therefore, altered expression patterns of MMPs and pro-apoptotic elements may be associated with adverse pregnancy outcomes[18].

Few studies have investigated the role of apoptosis in the association between prenatal BaP exposure and reproductive health. A previous study has shown that BaP disrupts genomic DNA stability and increased cell apoptosis in mouse embryonic stem cells[19]. An important gene in the apoptosis pathway, Bax (Bcl-2-associated X protein) was activated in PAHs-exposed embryos, and embryonic loss was diminished in Bax-deficient mice[20]. After PAHs exposure, Bax were found to be downregulated in aryl hydrocarbon receptor-deficient mouse fetuses, with less placental cell death observed compared to wild-type (WT) fetuses[21]. However, increased proliferative activity in trophoblast and complete inhibition of apoptosis were detected in afterbirths exposed to aromatic hydrocarbons[22]. Consequently, whether apoptosis in placenta is activated or inhibited remains controversial.

In light of literature gap regarding the relationship between prenatal exposure to BaP and adverse maternal–fetal outcomes, as well as the role of placental apoptosis in this association, we conducted an intervention study in mice exposed to BaP and a nested case–control study from a birth cohort. We aimed to investigate association between BaP exposure and maternal–fetal outcomes, and the underlying mechanism potentially involving placental apoptosis.

Results

Prenatal exposure to BaP and adverse maternal–fetal outcomes

As presented in Fig. 1, compared to pregnant mice in control group, maternal and birth weights were lower in BaP treatment groups. Higher doses of BaP were associated with decreases in maternal and birth weights as compared to low doses of BaP (Fig. 1D, WT-C vs WT-L: $p = 0.64$, WT-C vs WT-M: $p = 0.24$, WT-C vs WT-H: $p = 0.034$, WT-C vs p53KO-C: $p = 0.06$, p trend = 0.16; Fig. 1C, WT-C vs WT-M: $p = 0.0006$, WT-C vs WT-H: $p = 0.0018$, p trend < 0.0001) (Tables S1, 2). We also observed higher placental weights in BaP-exposed mice as compared to those in non-exposed group (WT-C vs WT-L: $p = 0.028$, WT-C vs WT-H: $p = 0.0072$, p trend = 0.017) (Fig. 1E, Table S3). PB was observed in WT-H group ($n = 2$, $p = 0.14$) at day 11 and 15 of gestation and WT-M group ($n = 1$, $p = 0.32$) at day 13 of gestation (Fig. 1F). There were fewer living fetuses in WT-M (4.50 ± 1.35) and WT-H (1.00 ± 0.78) groups than in WT-C (5.50 ± 1.31) and WT-L (5.38 ± 1.32) groups with no statistical significance. Higher doses of BaP were associated with decreases in number of livebirths as compared to low doses of BaP (WT-C vs WT-M: $p = 0.60$, WT-C vs WT-H: $p = 0.0084$, p trend (between groups) = 0.034). However, we observed only one livebirth in both p53KO-C and p53KO-L groups, corresponding to one of six and one of eight livebirth rates, respectively (WT-C vs p53KO-C: $p = 0.017$) (Fig. 1G, Table S4). As seen in Fig. 1H (Table S5), pregnant mice administered with higher doses of BaP were more likely to have stillbirth and resorbed fetuses (2.00 ± 0.63 in WT-M and 3.25 ± 1.32 in WT-H group), compared with those treated with vegetable oil and low dose of BaP (1.40 ± 0.51 in WT-C and 0.60 ± 0.24 in WT-L group, p trend = 0.12). Those associations between levels of BaP exposure and pregnancy outcomes, such as placental and birth weights, and the number of livebirths, showed significant dose-dependent manners. Additionally, we observed that interaction terms of different dosages and mouse genotype was significantly associated with placental weights ($p = 0.017$, Fig. 1E) and the number of death and resorbed fetuses ($p = 0.016$, Fig. 1H), respectively.

Placental apoptosis in BaP-exposed pregnant mice

As shown in Fig. 2A, the level of BaP-DNA adducts in placenta was increased as BaP doses increased in WT treatment groups (0.0560 ± 0.0258 ng/mL in WT-C, 0.1611 ± 0.0126 ng/mL in WT-L, 0.4832 ± 0.1586 ng/mL in WT-M, 0.1120 ± 0.02810 ng/mL in WT-H; WT-C vs WT-L: $p = 0.06$, WT-C vs WT-M: $p = 0.011$, WT-C vs WT-H: $p = 0.22$, WT-L vs WT-M: $p = 0.046$) in a significant dose-dependent manner among WT-C, WT-L, and WT-M groups (p trend = 0.0058), as well as in p53 treatment group (0.1821 ± 0.0741 ng/mL in p53KO-C, 0.3221 ± 0.0741 ng/mL in p53KO-L; p53KO-C vs p53KO-L: $p = 0.25$) (Table S6). Except for the BaP-DNA adducts level of placentas in WT-H was lower than that in WT-L and WT-M groups (WT-L vs WT-H: $p = 0.48$, WT-M vs WT-H: $p = 0.11$). Cell apoptosis in placentas assessed by TUNEL staining was showed in Fig. 2B and C. We observed TUNEL staining spots only in placentas of BaP-exposed mice, as well as an increase in the number of brown spots was found in higher doses groups (WT-M and WT-H). In p53KO mice, there were significant less TUNEL staining positive spots observed in p53KO-L group than in WT-L group. Figure 2D and E presented the placental protein expression levels of genes in apoptosis pathway. Expression levels of p53 and Baxwere up-regulated in placentas of WT mice after exposed to BaP, while Bcl-2 was not detected (data not shown). However, a slight up-regulation in p53 expression was observed in p53KO mice group and no change in other genes.

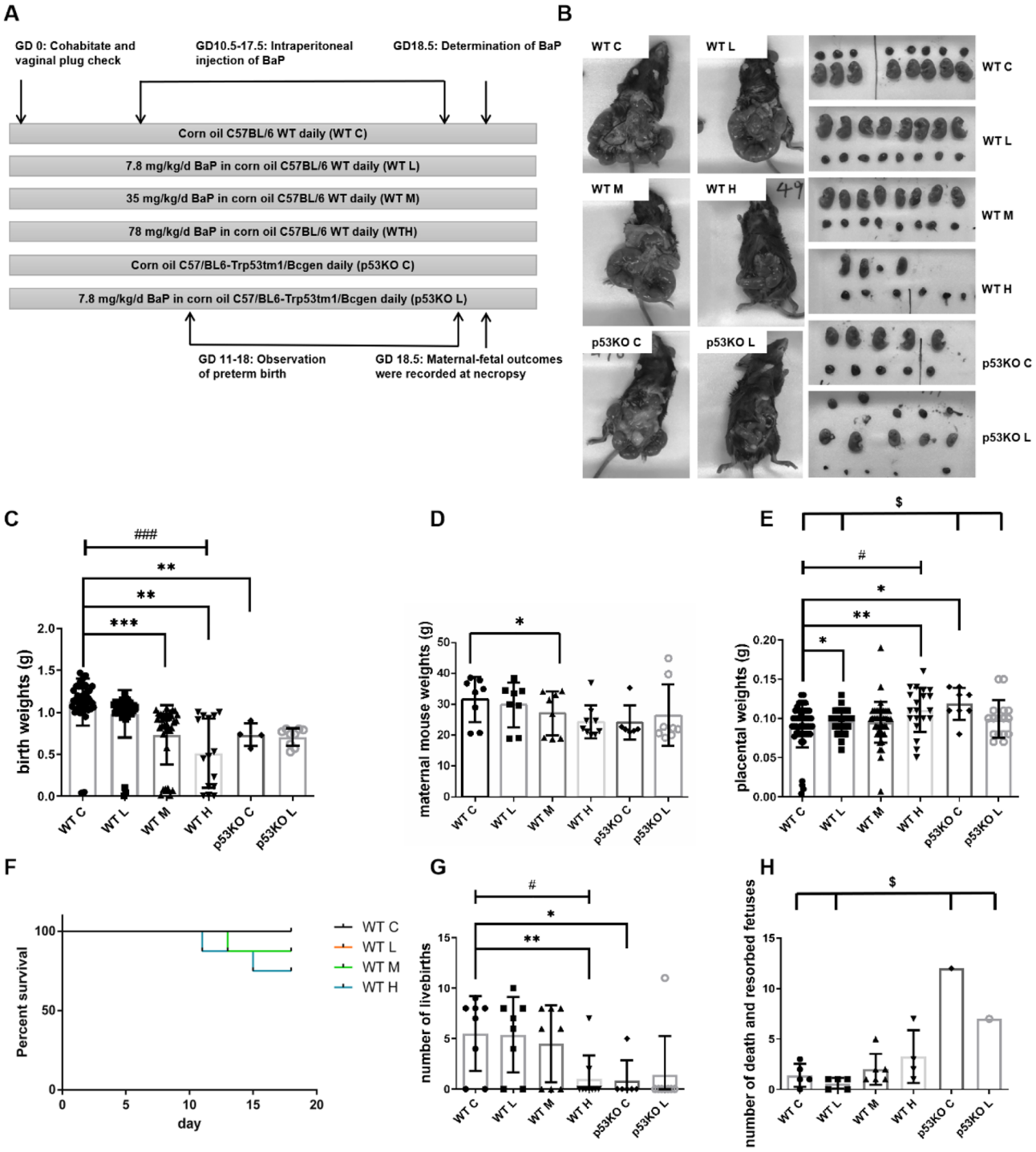

Figure 1. Mice modeling of BaP exposure and observations on maternal–fetal outcomes. (**A**) Schematic diagram of BaP-treated WT and p53 heterozygous knockout pregnant mice in high (78 mg/kg/d), medium (35 mg/kg/d), low (7.8 mg/kg/d) dose, and blank (corn oil) groups. Time points of female mice impregnation, drug injection to pregnant mice, C-section, and observation of maternal–fetal outcomes were presented in chronological order. (**B**) Photographs of pregnant mice and fetuses at autopsy. (**C**) Maternal weights on day 18.5 of gestation/at delivery (Student's *t*-test: $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA: $^{\#\#\#}p$ trend < 0.001). (**D**) Birth weights of maternal mice delivered by C-section (Student's *t*-test, $^{*}p < 0.05$). (**E**) Placental weights (Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$, one-way ANOVA: $^{\#}p$ trend < 0.05, two-way ANOVA: $^{\$}p < 0.05$). (**F**) The occurrence of preterm births observed before full term gestational day. The vertical coordinate represents percentage of the numbers of female mice that gave full term birth at the end of observation. The horizontal coordinate represents the birth date of the premature fetuses. (**G**) Number of livebirths per pregnant mice (Student's *t*-test: $^{*}p < 0.05$, $^{**}p < 0.01$, one-way ANOVA: $^{\#}p$ trend < 0.05). (**H**) Number of still births and resorbed fetuses per pregnant mice that gave birth (two-way ANOVA: $^{\$}p < 0.05$).

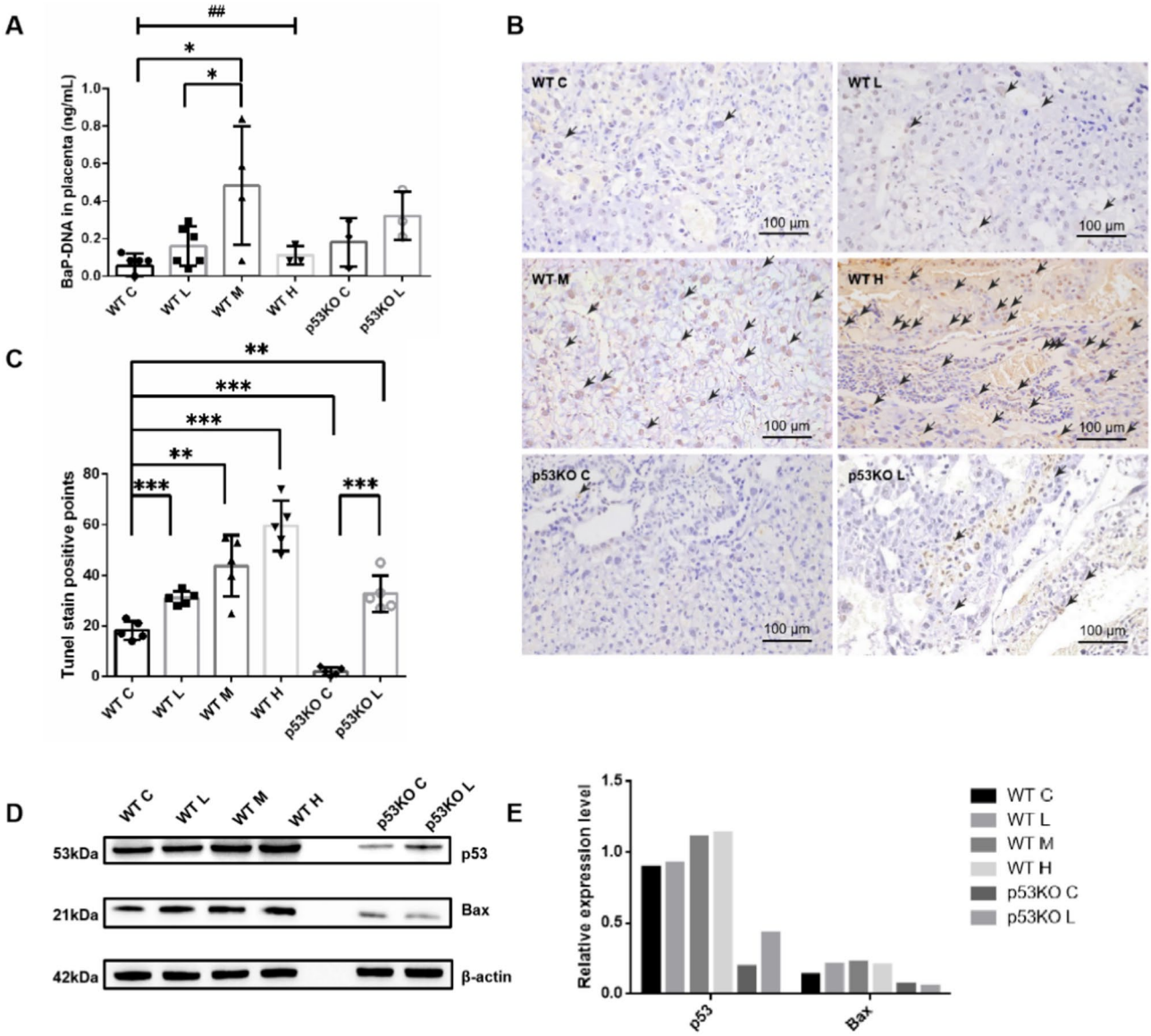

Figure 2. Apoptosis in placental cells. (**A**) Levels of BaP-DNA adducts in placentas. The vertical coordinate represents the levels of BaP-DNA adducts in placentas dissected from the abdominal cavity of pregnant mice (Student's *t*-test: $^{*}p < 0.05$, one-way ANOVA: $^{\#\#}p$ trend < 0.01). (**B**) TUNEL immunohistochemical staining of placental apoptosis. The arrow points to the DNA break signal observed under the microscope at 10X magnification. (**C**) The number of positive spots by TUNEL in (**B**) (Student's *t*-test: $^{**}p < 0.01$, $^{***}p < 0.001$). (**D**) Protein relative expression levels of apoptosis genes in placenta. The expression of β-actin was regarded as internal standard. (**E**) Relative quantitative level of protein by Western blot in (**D**).

Associations between BaP exposure, MMP2, and preterm birth in pregnant women

In case–control study, we observed that the expression levels of placental MMP2 were higher among pregnant women with high BaP exposure levels (9.49 ± 17.93 ng/ml), compared to those with low BaP levels (8.24 ± 12.12 ng/ml) (Table 1). Table 2 showed that, after adjustment for BaP-DNA adducts level and other important confounding factors, there were slight increased risks of PB and moderate PB observed as per unit increase in expression of placental MMP2.

Discussion

Our study supported that maternal exposure to high levels of BaP was associated with the occurrence of adverse maternal–fetal outcomes in mice. We found a significant decrease in maternal weight and an increased incidence of preterm birth after high dose (78 mg/kg/day) of BaP treatment for 7 days in WT mice. The number of fetal deaths and resorptions increased as the dose of BaP exposure increased. Especially, significant decreases in birth weight, increase in placental weight, and the number of livebirths were observed in higher doses BaP

BaP-DNA adducts (μg/gDNA)	MMP2 (ng/mL)		
	Mean	SD	P
≤ 3.21[a]: low level	8.24	12.12	0.60
> 3.21[a]: high level	9.49	17.93	

Table 1. Test for difference in MMP2 concentrations between pregnant women with high and low BaP exposure levels in population study (N = 165). [a]Based on the published results in reference #2.

Logistic regression	Crude OR (95% CI)	Adjusted OR (95% CI)[a]
All Preterm, cases = 83 and controls = 82		
BaP-DNA adducts	1.858 (1.000, 3.450)	2.085 (1.058–4.108)
MMP2	0.994 (0.973, 1.014)	1.001 (0.978–1.026)
Moderate preterm, cases = 78 and controls = 82		
BaP-DNA adducts	0.951 (1.039, 3.665)	2.178 (1.089–4.353)
MMP2	0.995 (0.975, 1.015)	1.004 (0.980–1.029)

Table 2. Associations between BaP exposure levels, MMP2 concentrations and risk of preterm birth in population study (N = 165). *OR* odds ratio, *CI* confident interval. [a]Adjusted for maternal age, education, family income per month, maternal BMI, passive smoking, parity, C-section, newborn gender, activity, employment during pregnancy, and supplementation status.

groups with significant dose–response relationships. Additionally, elevated p53-mediated placental apoptosis and MMP2 expression were observed in higher BaP exposure groups in pregnant mice and women, respectively.

Population studies including ours have suggested that PAHs exposure during pregnancy may increase the risk of adverse pregnancy outcomes, such as PB and LBW[1,2,4,5,23]. Five previous studies in vivo have investigated the reproductive toxicity of BaP exposure on adverse pregnancy outcomes, including oocyte meiotic arrest and fertilization failure[24], changes in endometrium receptivity[25]; endometrial cell apoptosis[26], defect in embryo implantation and pregnancy maintenance[27]; and recurrent pregnancy loss[28]. However, the causal effect of prenatal BaP exposure on maternal–fetal outcomes in vivo, and whether this relationship is mediated by placenta apoptosis, remains unclear. By conducting a mouse model of BaP intervention during early pregnancy, our study prospectively observed adverse pregnancy conditions in mice after high-level of BaP exposure. The gestational period in mice is shorter than in humans, with mice typically giving birth 18–21 days after conception, compared to the 9–10 months human gestational period. The specific dosing window from 10.5 to 17.5 days in mice aligns with a critical period in human development, corresponding to the late first trimester to delivery. Consistent with previous evidence from population studies, we observed adverse pregnancy outcomes at a dose of 35 mg/kg BaP and more severe outcomes at a dose of 78 mg/kg, including a decrease in livebirths, an increase in stillbirths and resorbed fetuses, and the occurrence of PB. Additionally, we observed reduced maternal and birth weights with increasing doses of BaP exposure in a significant dose–response relationship, suggesting an important role of BaP toxicity in fetal development.

Previous population study has demonstrated that BaP can be biologically transformed to BPDE across placenta barrier and accumulated as BaP-DNA adducts, resulting in abnormal murine fetuses[28]. Consistently, we observed BaP-DNA adducts accumulated in pregnant women's whole blood and in mice's placentas (Table 1, Fig. 2A), suggesting that the reproductive toxicity of BaP via the placenta might be initiated[10,11]. The induction of placental apoptosis by BaP might be attributed to oxidative stress, systemic inflammation, DNA damage, endocrine disruption[29]. An in vitro study has shown that BaP can interfere with cell growth and genomic DNA stability during mouse embryonic development and increase cell apoptosis[19]. Placental apoptosis has been reported to be accompanied by more macrophage infiltration into the placenta in patients with preeclampsia after nicotine exposure[30], a high placenta/birth weight ratio has also been suggested to associated with adverse maternal–fetal outcomes[31], which may be a possible reason for the increase in placental weight observed in our study. However, the variation of placental inflammatory factors after BaP exposure and whether cell apoptosis may trigger placental aseptic inflammation remained unclear. We speculated that BaP exposure may affect the placenta by activating the p53 induced apoptosis pathway. BaP may cause apoptosis by increasing Bax activity and Bax/Bcl-2 ratio in trophoblast cells and endometrial[25,28,32]. Our study identified the apoptotic signaling proteins in animal models of BaP exposure, and provided evidence that p53-induced apoptosis might be triggered by the upregulation of Bax (Figs. 2D,E and 3), similar results was documented at RNA level by Detmar et al.[20].

Apoptosis has been linked to adverse pregnancy outcomes such as preeclampsia, IUGR, and PB[32,33]. To initiate apoptosis, cellular stress or injury signals are activated by the release of pro-apoptotic proteins BH3, which can be bound and isolated by pro-survival proteins such as Bcl-2 protein family. When these pro-survival proteins are saturated or absent, Bax and/or Bak (Bcl-2 antagonist/killer) can be activated and cause mitochondrial outer membrane permeabilization, resulting in the release Cytochrome C from the mitochondrial intermembrane space.[34]. The Bcl-2, Bax and Bak were considered as a three-way apoptotic switch as their co-regulation of apoptotic mechanisms[14]. Cells were considered apoptosis-tolerant when Bcl2/Bax was ≥ 50%, and conversely

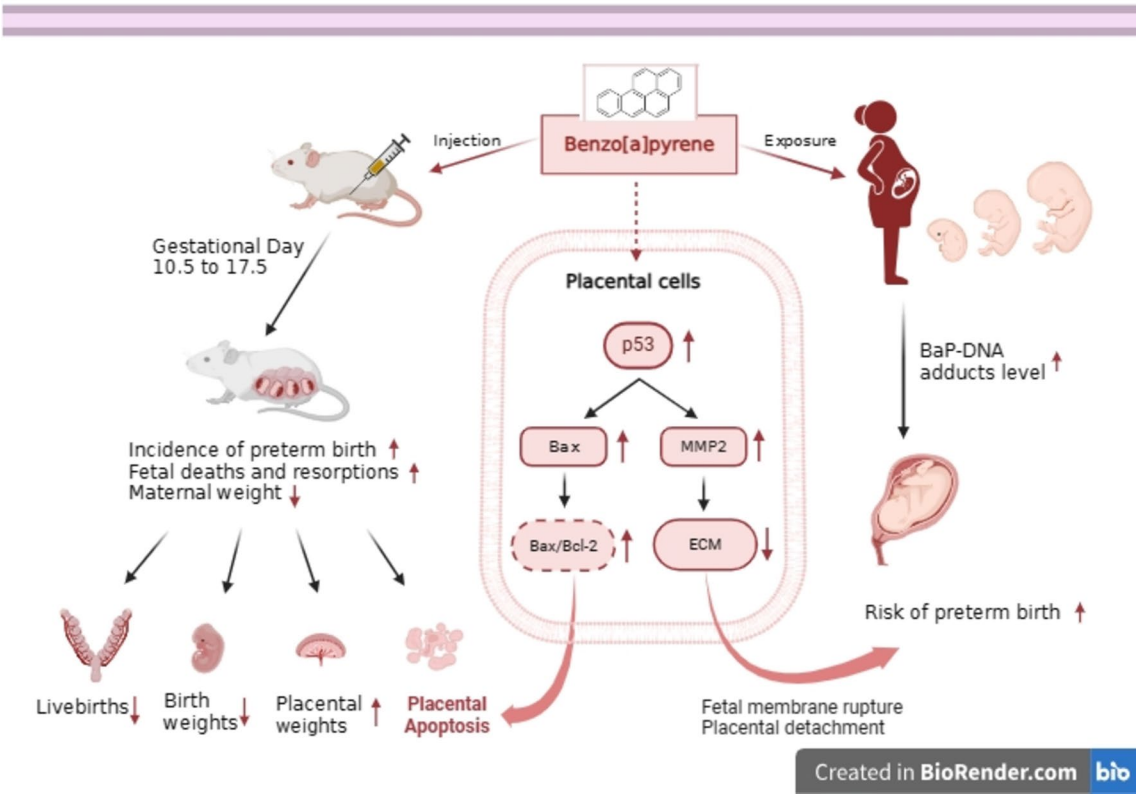

Figure 3. The plausible mechanism underlying BaP exposure and adverse maternal–fetal outcomes via placental apoptosis.

the smaller the Bcl2/Bax ratio the more active the apoptosis[14]. In our western blot results (Fig. 2D and E), a up-regulated expression of Bax in placentas, which means an up-regulation of Bax/Bcl-2, were observed. A slight increase in expression level of placental p53 was observed in p53KO mice after BaP exposure, but the changes in the expression level of Bax were less pronounced, indicating that BaP induced placenta apoptosis in mice model via the p53-Bax signaling pathway (Fig. 3). To the best knowledge, no study has reported the detection of the protein p53 in the placenta, including ours. However, it has been suggested that upregulated p53 induced transcription and expression of the MMP2 gene, resulting in elevated MMP2 enzyme expression. Accordingly, MMP2 induces extensive extracellular matrix (ECM) degradation in the fetal membranes and placenta, leading to rupture of the fetal membranes and placental abruption[15], inducing preterm labor[35] (Fig. 3). Further study regarding this underlying mechanism was warranted.

Our study prospectively conducted a pregnant mice model by different doses of BaP intervention from early pregnancy to delivery, as well as a case–control study on association between prenatal exposure to BaP and risk of PB. The exposure routes in animal study (injection) and population study (inhalation and ingestion) were different, the potential health impacts might not be comparable because of the differences in study design (intervention/observation), study population (animal/human), and exposure assessment methods. However, we observed occurrence of several adverse pregnancy outcomes significantly associated with increased levels of BaP in vivo, which replicated and confirmed the findings in our population study that higher levels of BaP-DNA adduct increase the risk of preterm birth[2]. This study was also the first to suggest that these associations might be mediated by placental apoptosis in mice. The elevated expression of placental MMP2 was found in pregnant women with higher BaP exposure, though p value was not significant. Moreover, this finding from our study cannot provide evidence of intrauterine fetal development with higher BaP exposure, and expression of the protein bcl-2 was not detected in vivo, further studies are warranted. We observed only one pregnant mouse in each p53KO group delivered livebirth due to Trp53 playing a key role in labor and the absence of Trp53 in the uterus causing increased incidence of PB. Together with the reproductive toxicity of high doses of BaP, the fetuses and placentas of p53KO mice exposed to high levels of BaP were more likely to abnormalities and be absorbed during pregnancy as we observed, resulting in no collection at autopsy. Though we employed the low dose of BaP exposure in p53KO mice only, the p53KO mice model provided valuable information to study the activities in apoptotic pathway genes and proteins in placentas. In both WT and p53KO mice, we identified dysregulated expression of related genes and proteins, as well as the activities of BaP-induced placental apoptosis, which have been implicated in adverse pregnancy outcomes.

In conclusion, our study supports the hypothesis that high levels of maternal exposure to BaP was significantly associated with an increased incidence of adverse maternal–fetal outcomes, including fetal death and resorption, reduced maternal and fetal weights, elevated placental weights, and a decrease in livebirths, which replicates and confirmed the observations in our population study. With observations on dysregulation of the signaling

pathway genes and their proteins, it is also noteworthy that there are signs of apoptosis in the placentas of mice and pregnant women exposed to BaP. Our study provide evidence for further investigation on BaP reproductive toxicity and the potential causative links between BaP exposure and adverse pregnancy outcomes. Our findings have important public health implications and are relevant to identify policy recommendation for environment management (e.g. wearing a face mask in downtown, healthier cooking method for foods, etc.) regarding pregnant women and newborns. Future transcriptomics and proteomics studies are warranted to investigate the molecular networks of BaP reproductive toxicity in maternal, placental, and fetal outcomes.

Methods

Experimental animals

The animal study was conducted with the approval by the Institutional Animal Care and Use Committee of Peking Union Medical College Hospital (No. XHDW-2020-00), and all procedures adhered to the Guidelines of the Care and Use of Laboratory Animals issued by Chinese Council on Animal Research and were reported in accordance with ARRIVE guidelines. C57BL/6 and transformation-related protein 53 (Trp53) heterozygous knockout (p53KO) (C57BL/6-*Trp53*^{tm1Bcgen}, BCG-DIS-0001) virgin female mice, aged 6 weeks, were obtained from Biocytogen Pharmaceuticals (Beijing) Co., Ltd. As shown in Fig. 1A, these female mice were cohabited with C57BL/6 male mice and randomly group-housed in separate cages. Gestational age was determined based on the presence of a vaginal plug, with the morning of detection being designated as embryonic day 0.5 (d 0.5) of gestation. Each cage housed five mice and was provided 12 h of light daily and breeding feeds. Daily observations were performed, abnormalities including nutritional hair, skin and mucous membrane, mental behavior, and convulsive reflex were recorded.

Animal grouping and BaP treatment

Female mice were mated with male mice in the afternoon, a vaginal plug was checked in the early morning of the next day and designated as GD0.5. Conception status of female mice was then confirmed based on their weight gain and abdominal bloating around 10 days post-mating (GD10) by an experienced animal experimenter. According to the preliminary experiment and previous animal studies with BaP treatment[21,36,37], 3,4-BaP (B802767, MACKLIN) was dissolved in vegetable oil with volume of 5 mL/kg for administration to pregnant mice. The pregnant mice were randomly allocated to the BaP treatment or control groups. The treatment group received intraperitoneal injection by stepped multiple dosing method of 3,4-BaP at low (7.8 mg/kg), medium (35 mg/kg), and high (78 mg/kg) doses, while the control group received an equivalent volume of vegetable oil, from gestational day 10.5 to day 17.5 (Fig. 1A) (the delivery date of mice is approximately on GD 18–21). Five to ten biological replicates per group were recommended based on previous literature. To enhance the reliability of the results while minimizing unnecessary animal sacrifice, there were 8 biological replicates per group[36], except for the WT-H group allocated 9 mice and p53KO C group allocated 6 mice. Our animal study included 63 mice in total.

Previous literature[38] observed significant decreases in embryonic implantation and pregnant rate in p53-/- female mice. Additionally, based on our pre-experiment, p53KO mice were intolerant to medium and high doses of BaP. Thus, there were two groups left for p53KO mice, which were p53KO low (p53KO-L) and p53KO-control (p53KO-C), and four groups for wild type mice, which were wild type low (WT-L), wild type medium (WT-M), wild type high (WT-H), and wild type control (WT-C). Maternal weight and the occurrence of PB was weighted and observed once a day. Pregnant mice were sacrificed by cervical dislocation and dissected on gestational day of 18.5. Data on the number of livebirth and stillbirth, as well as the maternal, fetal, and placental weights were accordingly recorded (Fig. 1A and B). To ensure the blindness of the experiment, the mouse breeder recorded all data using the ear-labeled numbers without involving the corresponding grouping.

BaP-DNA adducts in placenta

The placentas from each pregnant mouse were collected and washed three times with pre-cold PBS buffer. Genomic DNA of placental tissue was extracted according to the instruction of tissue DNA extraction kit (DP304, TIANGEN). After concentration determination by micro-nucleic acid quantitation instrument, the level of BaP-DNA adducts was measured by Benzo(a)pyrene diolepoxide (BPDE) DNA Adducts ELISA kit (STA-357, Cell Biolabs).

TUNEL staining

The placentas from the mice were fixed with 10% neutral formalin and routinely embedded and sectioned. The apoptosis of placental tissue was assessed by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay kit-HRP-DAB (C1091, Beyotime), which using terminal deoxynucleotidyl transferase (TdT) to catalyze the incorporation of deoxynucleotides at the free 3'-hydroxyl ends of fragmented DNA. The deoxynucleotides were then labeled with streptavidin-HRP and detected using DAB to generate a brown color. Photos were captured using microscope (ZEISS Imager.A2).

Western blotting

Placentas were weighed and lysed with an appropriate volume of Lysis buffer (including protease inhibitor). Total proteins were extracted from placentas by mechanical homogenization method (Minute total protein extraction kit, SD-001/SN-002, Invent biotechnologies). Protein concentration was measured by BCA method, and 30-50 μg protein was separated on SDS-PAGE gel and transferred. Antibodies including anti-p53 (AF1162, Beyotime), anti-Bax (AF1270, Beyotime), anti-Bcl-2 (AF6285, Beyotime) were assessed to represent the expression levels of

apoptosis related protein. Anti-β-actin (AF5003, Beyotime) was used to detect internal reference. Images were captured using chemiluminescence analyzer (Tanon 5200).

A nested case–control study

Building upon an existing birth cohort in Taiyuan, China, a nested case–control study was conducted to investigate the relationship between BaP exposure, the expression of apoptosis-related proteins in placenta, and the risk of preterm birth. Details of the study population were described in previous publications[2]. In brief, PB and moderate PB were defined as delivery prior to 37 and between 28 and 32 completed weeks of gestation, respectively. Term birth (control) was defined as delivery at 37 or more completed weeks of gestation. Pregnant women who had maternal blood samples available, who gave singleton live births without birth defects, and who had no chronic hypertension or cardiovascular diseases were included in our study. The estimated sample size with 0.80 power to detect the association of OR = 2.0 was 108 for each group. Of 110 PB randomly selected in this population who met the above criterion, we also excluded medically indicated PB (N = 27) to investigate the association between BaP exposure and PB without considering the effect of maternal complications. Controls from the same population were frequency matched to the cases by age (± 1 years), residence, and season of conception (spring: March to May, summer: June to August, autumn: September to November, winter: December to February). Thus, the final sample size was 83 cases and 82 controls (term births) with additional exclusion of one subject with inadequate amount of DNA isolated. The expression levels of protein p53 and MMP2 in placenta sample were measured according to the protocol of the Ray Biotech Porcine p53 and MMP2 ELISA Kits.

Statistical analysis

Data from animal study were presented as the mean ± standard deviation (SD), plotted by GraphPad Prism 8 and Biorender (https://app.biorender.com), and analyzed by the Student's *t*-test (two groups), ANOVA (four groups), and log-rank test. In population study, BaP-DNA Adduct levels were treated categorically as low vs. high by a cutoff of 3.21 μg/gDNA (the mean level among controls was a cutoff point) maternal exposure. Descriptive analyses of MMP2 levels between high and low BaP-DNA adduct level groups were performed by using the Student's *t*-test. Multivariable unconditional logistic regression was used to examine the relationships between maternal BaP-DNA adducts and risk of PB. Confounding variables included maternal age (≤ 30, ≥ 30 years), education level (< college, ≥ college), family monthly income per capita (< 3000, ≥ 3000RMB), employment status during pregnancy (yes, no), pre-pregnancy body mass index (BMI) (≤ 18.5, 18.5–24, ≥ 24), parity (primiparous, multiparous), C-section (yes, no), newborn's gender (female, male), passive smoking during pregnancy (yes, no), activities during pregnancy (yes, no), and supplementation status (ever, never). All statistical analyses were performed using SAS software, version 9.4 (SAS Institute, Inc., Cary, North Carolina). A $P < 0.05$ was considered statistically significant.

Ethics approval and consent to participate

All study procedures were approved by the Human Investigation Committee of Shanxi Medical University and the Institutional Animal Care and Use Committee of Peking Union Medical College Hospital (No. XHDW-2020-00).

Data availability

Data is provided within the manuscript or supplementary information files.

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Author contributions

NZ and TS designed the study, JC and NZ conducted the study and investigation process. JC and TS managed and maintained the data. TS and NZ contributed to statistical analysis and prepared figures, NZ and TS wrote the main manuscript text. NZ, TS, JL, LM, and NM reviewed and edited the final manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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