Perfluorooctane sulfonic acid (PFOS), Hypertensive Disorders of Pregnancy (HDP), and Offspring Health: A Mechanistic Investigation

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This dissertation is dedicated to:

My parents, who never stopped believing in me; My brother, for his constant support; My husband, for his unconditional love and endless support; My daughter, who inspires and fills my heart with joy each day.

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ABSTRACT

Hypertensive disorders of pregnancy (HDP) constitute a major global health challenge, responsible for significant maternal and fetal morbidity and mortality worldwide. Despite medical advances, prevalence of HDP is increasing, imposing substantial burdens on healthcare systems. Perfluorooctane sulfonate (PFOS), a member of the persistent perfluoroalkyl and polyfluoroalkyl substances (PFAS) family, is a ubiquitous environmental contaminant. The widespread use of PFAS in consumer and industrial applications has led to pervasive contamination. While epidemiological links between PFAS exposure and HDP are emerging, the precise mechanisms underlying this association remain poorly understood. This dissertation delves into the mechanisms through which gestational PFOS exposure disrupts maternal vascular adaptations and placental endocrine function, contributing to HDP pathogenesis and potentially influencing long-term offspring health. Pregnant Sprague Dawley rats exposed to varying PFOS doses exhibited dose-dependent increases in maternal blood pressure and reductions in fetal and placental weights. PFOS exposure significantly impaired uterine artery blood (UA) flow. Mechanistically, heightened vasoconstrictor responses to angiotensin II associated with decreased angiotensin type-2 receptor (AT2R) expression in UA and compromised endothelium-dependent vasodilation associated with decreased eNOS expression in UA were observed. Further, this study showed treatment of PFOS-exposed rats with the AT2R agonist Compound 21 (C21) demonstrated improvements in vascular function, uterine blood flow, and fetal growth outcomes providing potential intervention strategy against PFOS-induced HDP. Notably, PFOS exposure significantly altered steroid hormone profiles in maternal plasma associated with sex-specific changes in placental steroidogenic enzyme expression, highlighting placenta as a direct target of PFOS endocrine disruption. In addition, prenatal PFOS exposure led to lower birth

weights and hypertension in adult offspring. Endothelium-dependent vascular relaxation was impaired along with altered vascular eNOS/phospho (Ser1177)-eNOS levels in prenatally exposed PFOS offspring. These findings underscore the potential for enduring cardiovascular risks following in utero PFOS exposure. The findings highlight the disruption of maternal vascular function, placental endocrine signaling, and long-term offspring health as key areas of PFOS toxicity. This work identifies potential targets for therapeutic intervention and underscores the urgency of research aimed at safeguarding maternal and fetal health from the deleterious effects of environmental pollutants.

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Chapter 1

PER- AND POLYFLUOROALKYL SUBSTANCES (PFAS) AND HYPERTENSIVE DISORDERS OF PREGNANCY- INTEGRATION OF EPIDEMIOLOGICAL AND MECHANISTIC EVIDENCE

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ABSTRACT

Background: Hypertensive disorders of pregnancy (HDP) remain a significant global health burden, despite medical advancements. HDP prevalence appears to be rising, leading to increased maternal and fetal complications, mortality, and substantial healthcare costs. The etiology of HDP is complex and multifaceted, influenced by factors like nutrition, obesity, stress, metabolic disorders, and genetics. Emerging evidence suggests environmental pollutants, particularly Per- and polyfluoroalkyl substances (PFAS), may contribute to HDP development.

Objective: This review integrates epidemiological and mechanistic data to explore the intricate relationship between PFAS exposure and HDP.

Epidemiological Evidence: Studies show varying degrees of association between PFAS exposure and HDP, with some demonstrating positive correlations, particularly with preeclampsia. Meta-analyses suggest potential fetal sex-specific differences in these associations.

Mechanistic Insights: Mechanistically, PFAS exposure appears to disrupt vascular hemodynamics, placental development, and critical processes like angiogenesis and sex steroid regulation. Experimental studies reveal alterations in the renin-angiotensin system, trophoblast invasion, oxidative stress, inflammation, and hormonal dysregulation – all of which contribute to HDP pathogenesis. Elucidating these mechanisms is crucial for developing preventive strategies.

Therapeutic Potential: Targeted interventions such as AT2R agonists, caspase inhibitors, and modulation of specific microRNAs show promise in mitigating adverse outcomes associated with PFAS exposure during pregnancy.

Knowledge Gaps and Future Directions: Further research is needed to comprehensively understand the full spectrum of PFAS-induced placental alterations and their long-term

implications for maternal and fetal health. This knowledge will be instrumental in developing effective preventive and therapeutic strategies for HDP in a changing environmental landscape.

1. HYPERTENSIVE DISORDERS OF PREGNANCY (HDP):

HDP are recognized as the primary contributors to maternal mortality and morbidity [1,2]. The spectrum of these disorders includes chronic hypertension, gestational hypertension, preeclampsia/eclampsia, and preeclampsia superimposed on chronic hypertension [2]. HDPs are linked to severe maternal complications, including hepatic rupture, cerebrovascular accident, or renal failure [3,4]. Furthermore, HDPs lead to adverse fetal outcomes, encompassing fetal growth restriction, preterm birth, stillbirth, and neonatal death [3,5,6]. Mothers who survive HDP and their offspring are at an elevated risk of long-term health effects, such as cardiovascular and metabolic disorders [4,7-9]. A systemic meta-analysis review of 43 studies demonstrated that women with HDP, compared to those without HDP, have an odds ratio of 2.28 (95% confidence interval (CI): 1.87, 2.78) for developing cardiovascular disorders and a relative risk of 3.13 (95% CI: 2.51, 3.89) for developing hypertension during the postpartum period [10]. Another systemic review meta-analysis study primarily focused on preeclampsia reported that a history of preeclampsia increased the risk of future heart failure by 4.19 (95% CI: 2.09, 8.38) and the relative risk of cardiovascular death by 2.21 times (95% CI: 1.83, 2.66) [11].

Despite advancements in medical science, the prevalence of HDPs is increasing, affecting approximately 14% of pregnancies in the United States [12], with an estimated 18.1 million HDP cases globally in 2019 [13]. Pregnant women with HDP experience higher rates of hospitalizations during and after pregnancy, leading to increased costs for the US healthcare system. The estimated cost to the US healthcare system for preeclampsia alone is $2.18 billion,

with $1.03 billion allocated for maternal care in the first year postpartum and $1.15 billion for infant care [13]. The cost burden for infants often escalates if they are born at a lower gestational age. As the number of women affected by HDP increases and the strain on the healthcare system continues to grow, it is crucial to expedite our research to prevent further personal and public health losses. HDP is a hyper-heterogeneous syndrome of multifactorial nature, and known risk factors include poor nutrition, obesity, stress, metabolic disorders, and genetic predisposition [1,4]. Recent evidence highlights environmental pollutants, such as Per- and polyfluoroalkyl substances (PFAS), as a significant factor contributing to the risk of HDP [14-17] and fetal growth restriction [18-26]. Although PFAS exposure during pregnancy is believed to play a role in the pathogenesis of HDP, therapeutic options to counter HDP remain limited due to the largely unknown underlying mechanisms. Therefore, this review aims to integrate epidemiological and mechanistic studies to identify the mechanisms involved in HDP.

2. MATERNAL ADAPTATIONS IN NORMAL PREGNANCY:

2.1. CARDIOVASCULAR AND HEMODYNAMIC CHANGES:

Pregnancy is a dynamic process marked by numerous physiological modifications to meet to the escalating needs of the mother and fetus. Among these adaptations, substantial alterations occur in the cardiovascular system, leading to adaptive hemodynamic and vascular changes [27-30]. Heart rate and cardiac output increase without change in myocardial contractility and ejection fraction [27,30,31], accompanied by 50% rise in blood volume [30,31]. Despite these changes, the peripheral vascular resistance (blood pressure) decreases substantially with a more profound effect in uteroplacental circulation, where blood flow increases by 20-fold near-term [30,31]. The uterine vascular adaptations involve an increased sensitivity of endothelial cells to

vasodilators such as nitric oxide (NO), hydrogen sulfide, endothelium-derived hyperpolarizing factor (EDHF), and prostacyclin [32,33]. Moreover, the enzymes producing vasodilators, such as endothelial NO synthase (eNOS) and cystathionine beta-synthase (CBS), also increase during pregnancy [33].]. Alongside increased vasodilation, decreased vascular contractility to vasoconstrictors such as angiotensin II (Ang II), and endothelin 1 also contribute to vascular adaptations [33]. For instance, Ang II, a well-characterized vasoconstrictor, results in decreased vascular resistance and vasodilation during normal pregnancy through angiotensin type 2 receptor (AT2R) mediated signaling. This physiological response ultimately leads to an increase in uterine artery blood flow [34-36]. Insufficient vascular adaptations, characterized by heightened vasoconstriction and attenuated endothelium-mediated relaxation, play a crucial role in the pathogenesis of HDPs [32,37].

2.2. Placentation and Spiral artery remodeling:

In the initial phases of gestation, the advancement of placental development, which includes the differentiation of trophoblast cells and the remodeling of spiral arteries, is vital for a healthy pregnancy [38,39]. Following the implantation of an embryo, trophoblast cells (originating from the embryo's outer layer) differentiate into cytotrophoblasts and syncytiotrophoblasts [38]. Syncytiotrophoblasts form a continuous, specialized epithelial cell layer that covers the entire surface of the villous trees [38]. This layer not only provides structural and biochemical barriers at the feto-maternal interface but also functions as a significant endocrine organ. It produces numerous growth factors and hormones such as human chronic gonadotropin (hCG), placental lactogen, placental growth hormone, and insulin-like growth factors that support and regulate placental and fetal development and growth [40].

Located beneath the syncytiotrophoblasts are the cytotrophoblasts, which continually differentiate into syncytiotrophoblasts and extravillous trophoblast cells (EVTs) [40]. The remodeling of the uterine artery by EVTs is crucial to enhance blood flow to the placenta and fetus [39,41]. This process involves complex interactions between maternal decidual immune cells, such as decidual natural killer (dNK) cells and regulatory T (Treg) cells, and EVTs in the uterine wall [39,41]. EVTs express a specific antigen known as human leukocyte antigen (HLA)-C, which can trigger immune responses from both dNK cells and Treg cells [42]. dNK cells produce a broad array of growth factors, angiogenic factors, and cytokines [43], while Treg cells assist in promoting maternal-fetal immune tolerance [44]. These interactions facilitate the migration, invasion, and replacement of the vascular smooth muscle cells and tunica media of the uterine artery by EVTs, which increases the diameter of the uterine artery [39,41]. This process aids in the conversion of high-resistance/low-capacity arteries to low-resistance/high-capacity arteries [39,41], thereby enhancing the blood flow to the placenta and fetus. Abnormal placentation and dysfunction in spiral artery remodeling could lead to abnormal uteroplacental perfusion, and placental dysfunction also plays a role in the pathogenesis of HDPs [45].

2.3. Angiogenesis in the placenta:

The placenta, a highly vascularized organ, undergoes regulated processes of vasculogenesis (the de novo formation of primitive vessels) and angiogenesis (the sprouting of new capillaries from pre-existing blood vessels), which are orchestrated by angiogenic factors [46]. Angiogenesis in the placenta plays a pivotal role in remodeling and enhancing vasodilation, thereby increasing uterine blood flow [47]. Angiogenic factors such as vascular endothelial growth factor (VEGF) and placental growth factor (PIGF) are produced through the activation of

endothelial cell signaling [48]. VEGF operates through two receptors, VEGF receptor-1 (VEGFR-1, also known as Flt-1) and VEGF receptor-2 (VEGFR-2, also known as KDR/Flk-1) [49]. As gestation progresses and the need for continued expansion in increased blood flow diminishes, the production of antiangiogenic factors such as sFlt-1 increases [50]. sFlt-1, a soluble form of VEGFR-1, binds to circulating VEGF and placental growth factors, thereby inhibiting their angiogenic activities [49]. sFlt-1 has also been shown to decrease trophoblast invasion [51] and induce vasoconstriction and endothelial dysfunction [52]. Numerous studies have implicated an imbalance in angiogenic to antiangiogenic factors as one of the key regulators in the pathogenesis of HDPs, particularly preeclampsia [53-57].

2.4. Sex steroids:

The placenta, a crucial endocrine organ, synthesizes numerous endocrine hormones. Among these, sex steroids such as estrogen and progesterone play a significant role in the remodeling of uterine and placental vasculature [58]. Estrogen is reported to facilitate angiogenesis during the early stage of pregnancy [59]. Progesterone contributes to decidualization at the implantation site [60]. Elevated levels of estrogen and progesterone are associated with lower blood pressure due to their vasodilation properties [61,62]. These hormones can promote vasodilation by inducing an increase in eNOS expression in endothelial cells, thereby enhancing uterine artery blood flow [61-64]. In normal pregnancy, the levels of estrogen and progesterone increase as the pregnancy progresses [65].

However, numerous epidemiological studies have consistently reported higher plasma testosterone levels in pregnancies with preeclampsia compared to normotensive pregnancies [66-79]. Preeclampsia is also associated with elevated levels of the androgen receptor in placental

tissue [67,80,81]. Studies in animal models have demonstrated that mimicking the increase in testosterone, as found in pregnancies with preeclampsia, replicates key features of preeclampsia [82-84], including gestational hypertension, endothelial dysfunction [83,85], exaggerated vasoconstriction to angiotensin II [82,85], placental hypoxia [86], decreased nutrient transport [87,88], and fetal growth restriction [84,89].

3. PFAS:

PFAS constitute a family of chemicals that includes an ever-expanding list of over 10,000 distinct fluorinated substances [90,91]. These compounds possess carbon-fluorine (C-F) bonds in their structure, which confer unique physical and chemical properties such as oleophobicity and hydrophobicity [92-94]. C-F bonds, being the strongest covalent bonds, necessitate substantial energy for their disruption. Consequently, PFAS demonstrates high chemical stability in the presence of oxidants and high-temperature environments, as well as resistance to microbial biodegradation [95-101]. Owing to these distinctive properties, PFAS are extensively utilized in a variety of consumer products, including cookware, carpeting, waterproof outdoor gear, dental floss, food packaging, and cosmetics [102-105]. Furthermore, they were a primary component of aqueous film-forming foam (AFFF) due to its ability to reduce surface tension [106]. AFFF is commonly employed at airports and military bases for firefighting and training purposes, inadvertently leading to PFAS contamination of surface water bodies [106]. Subsequent studies have underscored their global contamination, revealing their presence even in the most remote regions of the world, such as the Arctic and Antarctic [107-111]. This ubiquitous distribution underscores the pervasive nature of PFAS contamination and emphasizes the urgency of addressing its environmental and health implications on a global scale.

3.1 TYPES OF PFAS:

PFAS encompass a broad spectrum of compounds characterized by a carbon-fluorine alkyl chain, referred to as the backbone, and a functional group. These compounds are categorized based on the number of carbon atoms they contain: long-chain compounds (more than 8 carbon atoms) and short-chain compounds (less than 6 carbon atoms) [112]. Furthermore, they are classified as either legacy PFAS or emerging PFAS, depending on the duration of their usage. List of commonly detectable PFAS in human maternal serum and their classification is illustrated in **Table 1**. Historically, PFOA and PFOS, two long-chain compounds, have been the most extensively used and researched members of the PFAS group [113]. These compounds, recognized for their persistence, toxicity, and bioaccumulation potential, have been voluntarily phased out by developed countries since the 2000s. However, despite these efforts, PFOS continues to be detected in most water sources in the US and globally [114,115]. Legacy PFASs have been replaced by emerging compounds, such as PFBA, PFBS, and PFHxA [116,117]. This shift has resulted in an ever-expanding list of over 10,000 PFAS chemicals. While there are no comprehensive toxicity databases for the suite of PFAS, these chemicals share structural similarities, and the health risks associated with one PFAS are expected to apply to others as well. Moreover, these emerging PFAS can revert to the stable PFOS through oxidation pathways during conventional drinking water treatment with chlorine and ozone [95], and by aquatic organisms [96], bacteria [97,98] and earthworms [99]. Consequently, PFOS is considered a representative of this class.

3.2. EXPOSURE ROUTES:

The ubiquitous use of PFAS in industrial applications and everyday products has led to their accumulation in landfills and surface water bodies [107,118-121]. The propensity of PFAS to percolate through unsaturated soil zones enables them to infiltrate groundwater, thereby contaminating potable water sources [122]. Consequently, drinking water has been recognized as a significant vector for human exposure to PFAS [120,123].

In March 2023, the Environmental Protection Agency (EPA) proposed new maximum contamination levels (MCLs) for PFOA and PFOS, both set at 4 ng/L, as part of the National Primary Drinking Water Standards Rule [124]. However, a recent study by the US Geological Survey revealed that over 67% of private well waters and 77% of public water supply tap waters exceeded the proposed MCLs for PFOS [125]. Among the various PFAS, PFOS is frequently detected at high concentrations in drinking water across numerous regions in the USA [126].

PFAS can also migrate from food packaging or contact materials, such as wax paper, pizza boxes, and popcorn packets, into food, resulting in direct human exposure [127-129]. Elevated PFAS concentrations have been reported in populations that consume seafood due to the bioaccumulation of PFAS in fish and other seafood [130-132]. Meat and dairy products have also been found to contain higher PFAS levels, suggesting PFAS uptake into dietary sources such as plants and grain from biosolids [133,134]. PFAS exposure through dermal contact and inhalation is considered less predominant [135].

Given the persistent nature of these chemicals and their widespread use, PFAS has been detected ubiquitously in human blood [136,137], urine [138], breast milk [126], and amniotic fluid [139]. A report from the Agency for Toxic Substances and Disease Registry (ATSDR) states that nearly all individuals in the USA have detectable levels of PFAS in their blood [140]. PFOS concentrations in maternal serum range from 1.7 to 22.8 ng/mL [141,142], while an

increased concentration (2.5 to 83.4 ng/mL) has been detected in pregnant women with preeclampsia [143-149]. However, plasma PFOS levels of more than 1500 ng/mL have been observed in American blood donors [150,151]. Furthermore, serum PFOS concentrations as high as 12800 ng/mL to 31,400 ng/mL were reported in occupational populations and individuals in high-exposure areas [152,153].

3.3. PFAS IN PREGNANT WOMEN:

Exposure to PFAS has been linked to detrimental outcomes in both maternal and fetal health [21,25,143-148,154]. Pregnancy represents a critical developmental window for the mother and fetus, with the fetal period being particularly sensitive. Exposure to environmental factors during intrauterine growth can predispose the fetus to disease development later in life [155]. Recent risk assessments have identified PFOS exposure as a potential reproductive toxicant capable of causing adverse developmental effects during pregnancy [133]. PFAS in pregnant women have been shown to cross the placental barrier and bioaccumulate in the placenta and fetus [156,157].

In a high-risk pregnancy cohort in North Carolina, USA, PFAS levels were analyzed and PFOS, PFHxS, PFHpS, and PFUnA were detected above the reporting limit in 99%, 75%, 55%, and 49% of placentas, respectively [158]. A cross-sectional study in a San Francisco pregnancy cohort (n= 302) detected PFOS and PFHxS in 97% of maternal samples [159]. In another study where PFAS concentrations were measured in pregnant women, PFOS was detected at the highest concentration in maternal serum (median 4.91 ng/ml; range 1.04–16.66 ng/ml), placenta (median 1.24 ng/g, range 0.45–3.87 ng/g), and in fetal tissues (median 0.83 ng/g, range 0.19–12.61 ng/g) [160]. Similarly, in a Spanish study where PFAS levels were analyzed in 1230

pregnant women, PFOS was detected at the highest concentration (6.05 ng/mL), followed by PFOA (2.35ng/mL), PFNA (0.65 ng/mL), and PFHxS (0.58 ng/mL) [161].

The placental transfer efficiency of PFAS, analyzed in 132 paired maternal and cord serum samples, showed a positive correlation between maternal weight and placental transfer efficiency of PFOS [162]. The elimination of PFOA has been shown to be hindered with an increase in testosterone levels [163], as found in preeclamptic pregnancies [66-79]. Given that PFAS has been detected in the blood samples of most pregnant women [160,161], understanding the potential health effects of PFOS exposure during pregnancy has become a pressing concern.

4. Relation between PFAS and HDP:

4.1. Epidemiological Evidence:

In recent years, a growing body of epidemiological studies has suggested a correlation between PFAS exposure and HDP. However, these findings have not been consistent across all studies. To date, six prior studies (reviews and meta-analyses) [14-16,164-166] have investigated the epidemiological evidence to provide a comprehensive overview of the association between PFAS and HDP.

In this section of the review, we aim to succinctly discuss the epidemiological evidence and summarize the associations of PFAS with HDP. A systematic search was conducted in PubMed using the search terms described in **Supplementary Table 1s**. A total of 366 articles were retrieved from PubMed's electronic records. After screening titles and abstracts, five articles without full text, 53 review articles, and 27 irrelevant articles (not pertaining to PFAS) were excluded. Upon reviewing the full texts of the remaining articles, 93 were excluded due to a lack

of data on pregnancy-associated hypertension. Ultimately, 188 studies were categorized into 45 epidemiological studies and 60 experimental studies **(Figure 1)**.

Of the 56 epidemiological studies shortlisted from the PubMed search, 24 studies have validated the association between PFAS and HDP with varying degrees of association (**Table 2**). The remaining 32 studies explored the association of PFAS with placental abnormalities and birth outcomes. Most individual studies have indicated that exposure to PFOA and PFOS are primarily associated with higher odds of developing preeclampsia [145,148,167-170]. Additionally, individual studies examining PFAS mixtures (combinations of PFAS compounds) also showed a positive association with preeclampsia [171], pregnancy-induced hypertension [172], and HDP [172,173]. However, a meta-analysis of PFAS exposure and HDP (including HDP, gestational hypertension, and preeclampsia) did not find any association, except for PFOS exposure [173]. For each log unit increase in PFOS levels, higher odds (odds ratio (OR) =1.41, 95%CI: 1.13, 1.77) of developing HDP were reported [14]. Moreover, studies with stratified analysis have reported sex-specific differences in the association between PFAS and HDP. For instance, the presence of a male fetus among pregnant women showed an association between PFOS as well as PFHxS and gestational hypertension [143]. Another study reported the effect of PFDA and PFUdA on systolic blood pressure only in pregnant women carrying a female fetus [174].

Community based concerns regarding PFAS began to emerge in the United States after 1998, leading to a lawsuit that funded the C8 Health Project. The median plasma levels of PFOA (21.2 ng/mL) and PFOS (13.6 ng/mL) in the C8 Health Project are higher [174] compared to the current geometric mean levels of PFOA (1.42 ng/mL) and PFOS (4.25 ng/mL). A study investigating the association between PFAS and HDP in the C8 Health Project reported a weak

but positive association for preeclampsia with PFOA and PFOS levels [174]. Furthermore, another study [168] using the same C8 Health Project data examined how exposure rankings changed among participants due to variability and epistemic uncertainty in independent exposure parameters. This study reported that the true adjusted odds ratio of HDP might be higher for PFOA. Additionally, an evaluation of the potential impact of mischaracterized exposure concentrations due to geocoding uncertainty on the predicted serum PFOA concentrations observed a 41% increase in the average adjusted odds ratio of preeclampsia occurrence [167].

Epidemiological studies conducted after 2007 have shown a positive association between HDP and pregnancy-induced hypertension in PFNA and PFBS exposure [144,149,175]. For instance, a comprehensive meta-analysis of studies using PFBS showed significantly higher odds (OR=1.27, 95%CI: 1.14, 1.41) of developing HDP [175]. Additionally, studies have shown a significant association between emerging PFASs like PFUdA, and PFDoA with gestational hypertension [174]. However, epidemiological studies examining the association between PFHxS and HDP have yielded inconsistent results [143,174,176]. Studies that found a positive association between PFHxS and HDP [143,176] had higher levels of PFHxS in the plasma compared to the study that found an inverse association [177]. These inconsistencies among studies could arise due to differences in exposure assessment timing, population demographic differences, disease diagnosis methods or criteria used, and analytical and statistical methods.

Placental anomalies are identified as potential contributors to the onset of HDP. Consequently, research has scrutinized the methylation status [178,179], placental development biomarkers [180], and angiogenic markers [181] within the placenta. These investigations have found a significant correlation between placental methylation and maternal plasma levels of persistent organic pollutants [178]. The concentration of PFOS in placental tissue was inversely

related to overall methylation at Long Interspersed Nuclear Element-1 (LINE-1), a surrogate for global DNA methylation, and the mixture of PFASs was inversely associated with the methylation of all CpG loci of LINE-1 and overall methylation of Nuclear Receptor Subfamily 3 Group C Member 1 (NR3C1) [179]. Furthermore, a stratified analysis by newborn sex revealed that PFOA, PFNA, and the PFASs mixture were negatively associated with overall methylation of LINE-1 only in the subgroup with male newborns, and the methylation of all CpG loci of LINE-1 was negatively associated with the ponderal index only in the female newborn subgroup [179]. Placental DNA methylation is linked to changes in vascular function in response to elevated maternal blood pressure [182]. PFAS levels in the placental tissue have been reported to have an inverse relationship with immunoreactivity markers such as Integrin Alpha-1 (ITA1), and Vascular Endothelial-Cadherin (CDH5) [180]. ITA1 and CDH5 are essential molecules for the proper differentiation of placental cells into invasive phenotypes. Aberrant differentiation of placental trophoblast cells may contribute to pregnancy complications. A case-control study that quantified the angiogenic biomarkers and PFAS levels from maternal plasma samples reported a few associations between PFAS and angiogenic markers [181]. For instance, inverse associations were found between MeFOSAA and both soluble Fms-like Tyrosine Kinase-1 (sFLT-1) and the sFLT-1: placental Growth Factor (PLGF) ratio, and an inverse association was found between PFHpA and PFOA and PLGF concentrations [181]. These biomarkers are proposed as predictors of preeclampsia. In conclusion, these epidemiological studies provide robust evidence that PFAS exposure during pregnancy is associated with HDP. Sex-specific differences observed in a few studies underscore the complexities in the relationship between PFAS exposure and HDP. Studies examining placental abnormalities further illuminate potential mechanisms underlying the association between PFAS exposure and HDP. These studies underscore the importance of

investigating the biological mechanisms by which PFAS exposure affects pregnancy outcomes. This research offers valuable insights for the development of targeted interventions and preventive strategies to mitigate the risks associated with PFAS exposure during pregnancy.

4.2. Mechanistic link between PFAS and HDP:

While epidemiological studies substantiate the association between PFAS and HDPs, elucidating the mechanistic relationship between PFAS and HDP is crucial for devising preventive strategies to mitigate adverse maternal and fetal outcomes. In this section, our objective is to unravel this mechanistic link by conducting a comprehensive review of 60 experimental studies retrieved from a PubMed search. The underlying mechanisms of HDP associated with PFAS exposure are depicted in **Figure 2**.

4.2.1. Vascular hemodynamics:

Remarkably, a limited number of experimental studies have investigated the effects of PFAS exposure on vascular and hemodynamic adaptations during pregnancy. Our research group has pioneered in demonstrating that exposure to PFOS in pregnant rats provides compelling evidence for the role of PFOS exposure in the development of HDP. PFOS exposure induced amplified vascular contractile responses to Angiotensin II (Ang II) and attenuated endothelium-dependent vascular relaxation responses [183]. The intensified vascular contraction to Ang II is attributable to increased Angiotensin II Type 1 Receptor (AT1R) protein expression levels and decreased Angiotensin II Type 2 Receptor (AT2R) protein expression levels in the uterine artery. The attenuated endothelium-dependent vascular relaxation responses are due to the reduction of endothelial nitric oxide synthase (eNOS) expression in the uterine artery. Furthermore, our

subsequent investigation unveiled a promising therapeutic avenue. Treatment with an AT2R agonist successfully restored the vascular contractile and relaxation responses in PFOS-exposed dams [184]. Additionally, PFOS exposure in pregnant Sprague Dawley (SD) rats resulted in altered endothelial-mediated vascular relaxation response in offspring in a sex-dependent manner [185] and increased the renal glucocorticoid receptor gene expression [186].

In New Zealand rabbits, exposure to PFBS during pregnancy elicited signs of hypertension and renal injury [188]. Consistently, exposure to a mixture of 10 PFAS compounds also exhibited signs of hypertension and renal injury [187]. RNA sequencing identified the dysregulation of the angiotensinogen (AGT) gene in high-dose PFBS placenta [187], and in placenta exposed to the PFAS mixture [187]. Additionally, studies have shown that PFOS exposure increases aldosterone levels [188], and aldosterone synthase (CYP11B2) gene expression [189,190]. Collectively, these studies suggest that PFAS exposure alters the renin-angiotensin mediated pathways, thereby affecting vascular and endothelial function.

4.2.2. Placentation and spiral artery remodeling:

Numerous studies propose that exposure to PFAS influences placental development via mechanisms such as oxidative stress, inflammation, apoptosis, mitochondrial dysfunction, and epigenetic regulation. For instance, exposure to PFOS, PFOA, and GenX inhibits trophoblast invasion in HTR-8 cells by reducing the expression of chemokines, chemokine receptors, and inflammatory enzymes involved in migration [191]. In contrast, exposure to Perfluorobutane Sulfonate (PFBS) enhances trophoblast invasion and increases Matrix Metalloproteinase 9 (MMP-9) levels in HTR-8 and JEG3 cells through the induction of Inducible Nitric Oxide Synthase (iNOS)/nitric oxide signaling [192]. Whole-genome transcriptomic studies using

human Uterine Artery Endothelial Cells (hUAECs) isolated from pregnant women provide evidence for osmotic stress and cellular stress response due to PFOS exposure [193]. Additionally, PFOS exposure has been shown to upregulate genes involved in oxidative stress [193]. In JEG3 cells, exposure to PFOA and GenX for 24 hours induced disruption of 46 genes (e.g., GPEX1, GPER 1, ABCG2) that participate in pathways critical to proper placental development and function [194]. Moreover, studies have shown that exposure to PFOS, PFOA, and HFPO-DA increases the expression of apoptotic-related genes including BAD, BAX, and cleaved-caspase 3 in placental cells using Bewo and JEG3 cells [195,196] and pregnant mice model [197]. The apoptosis induced by PFOS was further demonstrated by a decrease in pro-caspase3 and anti-apoptosis protein Bcl-2, accompanied by an increase in DNA fragmentation and nuclear condensation in Bewo cells [196]. Blocking apoptosis with the pan-caspase inhibitor Z-VAD-FMK restored the impairment of placental endocrine function caused by PFOS [196]. Potential PFOA alternatives like PFDMO2HpA and PFDMO2OA exposure also revealed significant alterations in the expression of genes involved in inflammation and immunity in the placenta of mice [198].

In silico transcriptomic analysis of cultured human cytotrophoblasts from the second trimester suggests that exposure to PFOS affects pathways mediated by peroxisome proliferator-activated receptor (PPAR), which are associated with lipid metabolism and innate immune response [199]. These pathways are implicated in oxidative stress and inflammation. A comprehensive lipid analysis in a zebrafish model reveals that PFHxS exposure leads to impaired fatty acid β-oxidation, resulting in oxidative stress and inflammation [200]. In a murine pregnancy model, exposure to PFOA decreased the number of dNK cells in the placental decidua [197]. Emerging compounds such as GenX have been found to replicate the effects of legacy

compounds like PFOA and PFOS on placental abnormalities [201]. In Sprague Dawley rats, exposure to GenX resulted in neutrophil infiltration in the decidual zone and congestion in the labyrinth zone. Proteomic analysis of the placenta suggested alterations in the expression of inflammation-related proteins in the Rap1 signaling pathway [202].

Exposure to PFOS in HTR-8 cells has been shown to reduce mitochondrial content and biogenesis, leading to mitochondrial dysfunction [203], which further results in oxidative stress. In JEG3 cells, exposure to PFOS resulted in SLC25A5-mediated mitochondrial damage, generation of reactive oxygen species (ROS), and decreased ATP production. This was accompanied by the activation of p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) signaling pathways [204], leading to apoptosis and cell death. Similarly, in neuronal cells, PFOS exposure has been shown to initiate apoptosis via JNK activation [205]. However, RNA-seq analysis in PFBS-exposed HTR-8 cells revealed dysregulation of HIF-1α target genes, but not the Notch, ERK1/2, AKT, and p38 pathways [206].

Exposure to PFOA in HTR-8 cells has been shown to induce endoplasmic reticulum (ER) stress via ROS-dependent ERK signaling and ATF4-dependent C/EBP homologous protein (CHOP), leading to proliferation and apoptosis of trophoblast cells [207]. Additionally, exposure to PFOS in HTR-8 cells has been shown to induce ROS generation via epigenetic regulation of increased microRNA (miR)-29b, resulting in decreased DNA methyltransferases (DNMT1, DNMT3A, DNMT3B), sirtuins (SIRT1, SIRT3), global DNA methylation, and increased protein lysine acetylation [208]. Knockdown of miR-29b rescued the epigenetic alteration and decreased ROS production [208].

A study using HTR-8 cells and pregnant mice models has demonstrated that exposure to PFOS results in the inhibition of placental cells. This inhibition is mediated through the long

noncoding RNA (lncRNA) maternally expressed gene 3 (MEG3) derived miR-770 and its direct target, pentraxin 3 [209]. MEG3, an imprinted gene located on human chromosome 14q32, encodes the lncRNA [210]. MEG3 has been shown to play a pivotal role in the regulation of vascular smooth muscle cells during the remodeling of the placental spiral artery via the Notch 1 signaling pathway [211]. Another study using a similar HTR-8 cell model revealed that PFOS exposure induces hypomethylation of lncRNA H19, resulting in the inhibition of placental cell growth [212]. H19 is located on human chromosome 11p15.5, proximal to the maternally imprinted insulin-like growth factor (IGF)-2 gene [210]. A decrease in the activity or expression of H19 has been associated with a reduction in trophoblast invasion [213], mediated through the Nodal 1 signaling pathway [211].

These findings not only highlight the detrimental effects of PFAS exposure on trophoblast invasion and gene expression critical for placental health, but also illuminate the complex interplay between PFAS exposure and various signaling pathways, including PPAR-mediated lipid metabolism and MAPK-mediated cellular stress responses. The evidence further suggests potential interventions, such as caspase inhibitors and targeted modulation of specific miRNAs, to mitigate the adverse effects of PFAS exposure on placental function. In summary, these studies underscore the urgent need for further research to fully elucidate the spectrum of PFAS-induced placental alterations and to develop effective strategies for mitigating their harmful consequences on maternal and fetal health.

4.2.3. Angiogenesis:

The EPA ToxCast™ high-throughput screening project has identified PFOS as a potential disruptor of blood vessel formation and remodeling [214]. Studies have reported that PFOS

exposure inhibits angiogenesis in the placenta [215], placental Bewo cells [216] and hUVECs [217]. In Bewo cells, PFOS exposure has been shown to decrease the expression of the potent angiogenic factor PlGF gene [216]. Investigations using hatched zebrafish embryos indicate that PFOS induces vascular injury, characterized by significant reductions in the formation and length of intersegmental and posterior cardinal veins, while simultaneously enhancing the formation of dorsal aorta vessels [218]**.** PFOS and PFOA have been shown to decrease vessel formation in a 3D co-culture model of hUVECs and colon fibroblasts [217]. They also impede cell growth, migration, and angiogenesis in HTR-8 cells [219,220] and JEG-3 cells [220] and reduce placental vascular density [197].

The PPARγ pathway [220] and NOTCH signaling [219] have been implicated in the observed increase in inflammation and impaired angiogenesis. PFOS exposure has been shown to downregulate VEGF receptor 2 signaling in hUVECs, resulting in reduced branching and tip formation *in invitro* [217]. These mechanistic investigations selectively implicate pathways such as PPARγ and NOTCH signaling, and VEGFR2 signaling in the observed anti-angiogenic effects. This underscores the importance of further research to understand the precise mechanisms and potential health implications of PFOS exposure on vascular development.

4.2.4. Sex steroids:

Docking analysis has indicated that PFAS can bind to the steroid binding site of 3β-hydroxysteroid dehydrogenase (HSD3B) [221]. Exposure to PFOS in pregnant Sprague Dawley rats has been reported to increase the plasma concentrations of progesterone, aldosterone, corticosterone, and testosterone, while decreasing the levels of plasma estradiol, hyperglycosylated chronic gonadotropin (hCG-H), and prolactin [188]. This study hypothesized

that sex-specific dysregulation of enzymes involved in hormone biosynthesis and metabolism in the junctional zone of the placenta may contribute to the observed alterations in maternal hormone levels following PFOS exposure during pregnancy [188]. In male rats, exposure to PFHpA resulted in an increase in luteinizing hormone (LH) through negative feedback following the downregulation of steroidogenic enzymes and inhibition of testosterone production in individual Leydig cells [222]. PFOS exposure in primary human placental cytotrophoblasts isolated from term placenta was found to suppress aromatase levels and decrease estradiol, hCG, and progesterone levels [196]. However, exposure to a leachate containing a mixture of pollutants from a solid waste dumpsite in African catfish resulted in an increase in aromatase levels [223]. This increase could be attributed to exposure to a mixture of compounds, including phthalates and PFASs.

Exposure to sodium OBS, a replacement for PFOS, in pregnant mice increased the androgen levels in F0 generation mice and altered the expression of endocrine-related genes in male mice of the F1 generation [224]. PFBS exposure was found to increase cortisol concentrations in zebrafish larvae during both acute direct exposure [225] and prenatal exposure [226]. PFOS exposure in pregnant rats has been reported to increase the activity of 11β-hydroxysteroid dehydrogenase 2 in the placenta, thereby increasing fetal serum cortisol levels [227]. Gene expression profiling of the placenta from PFOS-exposed dams revealed significant downregulation in 45 genes, which are important for extracellular matrix, ion/protein binding, signal transduction, structural constituents, and transport functions in the placenta [227].

Exposure to PFOA and PFOS in pregnant mice resulted in a significant decrease in mRNA levels of placental prolactin family genes [228,229]. It is speculated that the inhibitory effects of PFOA on prolactin family genes may be secondary to decreased trophoblast differentiation

and/or increased trophoblast cell death [228,229]. PFOS exposure in placental Bewo cells has been shown to decrease the production of chronic gonadotropin subunit 7 and human chronic gonadotropin (hCG) [216].

While studies have shown that PFAS exposure can alter thyroid hormone regulation and have implications in preeclampsia [230,231], the focus of this review is on sex steroid hormone regulation in the placenta. In conclusion, the studies discussed above highlight the complex effects of PFAS exposure on endocrine regulation within the placenta, shedding light on mechanisms underlying altered hormone levels during pregnancy, which may lead to adverse maternal outcomes.

5. SUMMARY:

Epidemiological investigations have consistently demonstrated a correlation between elevated PFAS concentrations in maternal subjects and an increased probability of gestational hypertension and preeclampsia. However, the specific PFAS compounds involved and the characteristics of these associations exhibit variability across different studies. For example, while certain studies indicate a positive correlation between exposure to PFOS, PFOA, PFNA, and PFBA and HDP, others present mixed outcomes or even negative correlations in areas with high contamination.

Mechanistic studies provide insights into the potential pathways through which PFAS may induce HDP. Exposure to PFAS has been demonstrated to interfere with the formation and remodeling of blood vessels, induce vascular damage, and inhibit angiogenesis in various cellular and animal models. Moreover, PFAS exposure has been associated with dysregulation of placental function, increased oxidative stress and inflammation, alterations in gene expression

linked to endothelial dysfunction, and impacts on fetal growth and vascular function in animal studies.

Furthermore, exposure to PFAS during gestation has been shown to modify renin-angiotensin mediated pathways, which could potentially contribute to the development of hypertension and renal damage.

TABLES:

Table 1. Commonly detected PFAS in maternal serum and their classification

PFAS	Acronym	Chain Length	Legacy or Emerging
4:2 Fluorotelomer Sulfonic Acid	4:2 FTSA	6	Legacy
Perfluoro(2-((6-chlorohexyl) oxy) ethanesulfonic acid)	9CL-PF3ONS	6	Legacy
Perfluorohexanesulphonic acid	PFHxS	6	Legacy
Perfluorohexanoic acid	PFHxA	6	Legacy
Perfluoroheptanoic acid	PFHpA	7	Legacy
Perfluorooctanesulfonic acid	PFOS	8	Legacy
Perfluorooctanoic acid	PFOA	8	Legacy
Perfluorooctanesulfonamide	PFOSA	8	Legacy
2-(N-Methylperfluorooctanesulfonamido) acetic acid	N-MEFOSAA	8	Legacy
Perfluorononanoic acid	PFNA	9	Legacy
Perfluorodecanoic acid	PFDA	10	Legacy
Perfluorobutanesulfonic acid	PFBS	4	Legacy and/or emerging
Perfluorobutanoic acid	PFBA	4	Legacy and/or emerging
Perfluoroheptanesulfonic acid	PFHpS	7	Emerging
Perfluorohexadecanoic acid	PFHxDA	15	Emerging
perfluoroundecanoic acid	PFUNDA	11	Emerging
Hexafluoropropylene oxide dimer acid (GenX)	HFPO-DA	6	Emerging
Perfluoro-2,5-dimethyl-3,6-dioxo-heptanoic acid	PFDMO2HpA	7	Emerging
Perfluoro-2,5-dimethyl-3,6-dioxo-octanoic acid	PFDMO2OA	7	Emerging

Table 2. Epidemiological studies of the relationship between PFAS and HDP

Demographics Study period	**PFAS levels**	**Adverse outcome studied**	**Reference**
Sweden 1952-1993	Median range (ng/mL) PFOA- 1.81–2.83 PFOS-9.36–12.9 PFNA-0.38–0.47 PFHxS-0.45–0.66	Low exposure quartile- positive association to Preeclampsia	[232]
Australia 1986-2018	Not clear	PFAS- positive association with pregnancy induced hypertension	[172]
Sweden 1995-2013	Median (ng/mL) PFOS-169 PFHxS-129 PFOA-9	No association	[233]
USA 1990-2004	Median (ng/mL) PFOA- 7.7	Weak association with growth restriction	[234]
USA 1999-2002	Median (ng/mL) PFOS-25.6 PFOA-5.9 PFHxS-2.5 PFNA-0.7	PFOA, PFOS- association with increased DBP PFOA, PFOS, PFHxS- higher odds of gestational hypertension but not preeclampsia	[176]
USA 1999-2006 C8 Health project data	Plasma levels (ng/mL) PFOA-21.2 PFOS-13.6	PFOA and PFOS-positive association with preeclampsia	[148,167,168]
Norway 2003-2004	Plasma levels Median (ng/mL) PFOA-2.25 PFOS-13.03 PFHxS-0.60 PFNA-0.39 PFDA-0.09	PFOS- positive association to High density lipids	[146]
Norway 2003-2007	Plasma levels median(ng/mL) PFOA-2.78 PFOS-12.87 PFHxS-0.69 PFNA-0.54	PFOS- week association to preeclampsia	[145]

	PFDA-0.10		
USA 2005- 2006	Predicted serum levels (ng/mL) PFOA- 1.5 to 3 times higher than background 4 ng/mL	PFOA- positive association with preeclampsia	[169,170]
Sweden 2007-2010	Geometric mean (ng/mL) PFOS-5.34 PFOA-1.61 PFHxS-1.32 PFNA-0.54 PFDA-0.26	PFOS, PFNA- Positive association with preeclampsia	[170]
Canada 2008-2011	Geometric mean (ng/mL) PFOA-1.65 PFOS-4.56 PFHxS- 1.02	PFHxS- positive association with preeclampsia PFOA, PFOS- positive association with increased DBP PFOA- male fetus- Positive association with gestational hypertension	[143]
Canada 2009-2012	Geometric mean (ng/mL) PFOA-2.24 PFOS-3.46 PFNA-0.80 PFHxS-1.05	PFNA- strong association with pregnancy induced hypertension	[175]
Denmark 2010-2012	Median (ng/mL) PFOS- 7.50 PFOA-1.68 PFHxS-0.36 PFNA-0.64 PFDA-0.29	Higher PFAS levels -Positive association with higher SBP and DBP	[235]
China 2011-2012	Median (ng/mL) PFOA-6.98 PFOS-2.38 PFNA-0.64 PFDA-0.36 PFHxS-0.16 PFBS-0.047	PFBS- Positive association with preeclampsia	[149]

USA 2013-2015	Predicted serum levels (ng/mL) PFOA- 1.7 PFOS-3.8 PFHxS-4.4	Weak positive association with PFAS levels in drinking water and HDP	[173]
USA 2014-2018	Geometric mean (ng/mL) Branched PFOS-0.3 Linear PFOS-1.1	No association with hypertension	[159]
China 2013-2016	Median (ng/mL) PFOS-9.36 PFOA-11.85 PFHxS-0.54 PFNA-1.69 PFDA-1.69	No association	[236]
USA 2015-2018	In placenta (median ng/g) PFOS- 0.480 PFHxS-0.06 ng/g PFHpS- 0.09 ng/g	No association	[158]
China 2015-2021	Geometric mean (ng/mL) PFOA-2.258 PFOS-1.270 PFNA-0.613 PFDA-0.428 PFHxS-0.054	PFOS, PFNA, PFBS- Positive association with HDP PFHxS- Inverse association with HDP	[177]
China 2016-2018	Geometric mean (ng/mL) PFOA-11.59 PFOS-8.00 PFHxS-5.42 PFNA-2.25	PFOS, PFDA, PFUdA, and PFDoA- Inverse association with gestational hypertension	[174]
China 2020-2021	Median (ng/mL) PFOS-3.78 PFOA- 3.63 6:2Cl PFESA-2.81 PFUnDA-1.18	PFAS mixture- positive association with preeclampsia	[171]

Table 1s. PubMed Search terms.

Database	Search strategies	Result
PubMed	(Perfluoroalkyl[Title/Abstract] OR PFASs[Title/Abstract] OR PFAS[Title/Abstract] OR Polyfluorinated[Title/Abstract] OR PFHxA[Title/Abstract] OR PFHpA[Title/Abstract] OR PFOA[Title/Abstract] OR PFNA[Title/Abstract] OR PFDA[Title/Abstract] OR PFUnDA[Title/Abstract] OR PFDoDA[Title/Abstract] OR PFTrDA[Title/Abstract] OR PFBS[Title/Abstract] OR PFHxS[Title/Abstract] OR PFOS[Title/Abstract] OR GenX[Title/Abstract] OR MeFOSAA[Title/Abstract] OR EtFOSAA[Title/Abstract] OR PFCA[Title/Abstract]) AND (placenta[Title/abstract] OR placental insufficiency[Title/abstract] OR Trophoblast[Title/abstract] OR hypertensive disorders of pregnancy[Title/Abstract] OR HDP[Title/Abstract] OR Pregnancy-induced hypertension[Title/Abstract] OR PIH[Title/Abstract] OR gestational hypertension[Title/Abstract] OR GH[Title/Abstract] OR preeclampsia[Title/Abstract] OR PE[Title/Abstract] OR eclampsia[Title/Abstract] OR Hypertension[Title/Abstract] OR Hypertensive[Title/Abstract] OR Fetal growth restriction[Title/abstract] OR FGR[Title/abstract] OR Intrauterine growth restriction[Title/abstract] OR IUGR[Title/abstract] OR placenta[Title/abstract] OR placental insufficiency[Title/abstract] OR Trophoblast[Title/abstract] OR Birth outcome[Title/abstract] OR Low birth weight[Title/abstract] OR Low fetal weight[Title/abstract] OR Small for gestational age[Title/abstract] OR Angiogenesis[Title/Abstract])	**366 articles**

FIGURES:

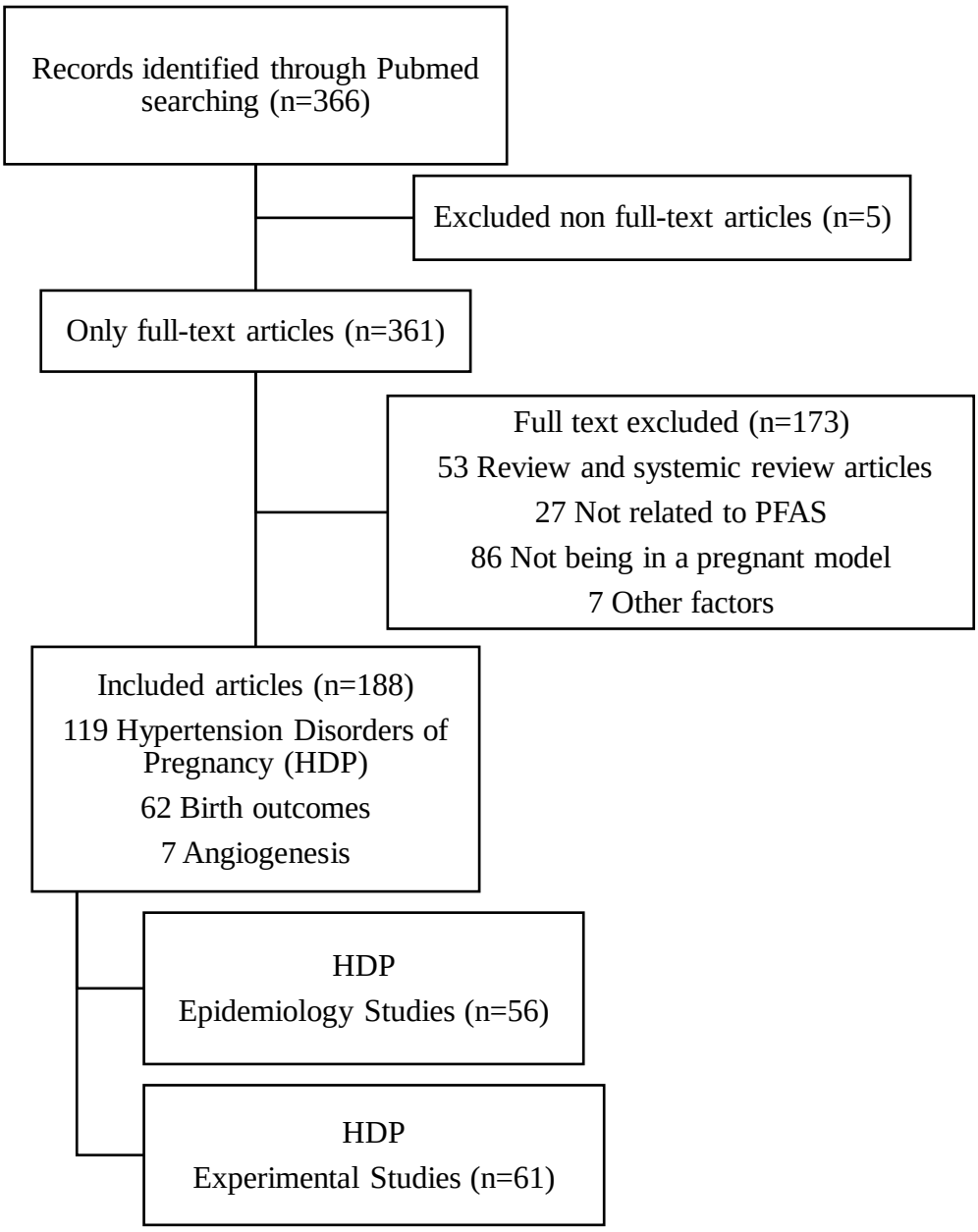

Figure 1. Literature Screening Process Flowchart.

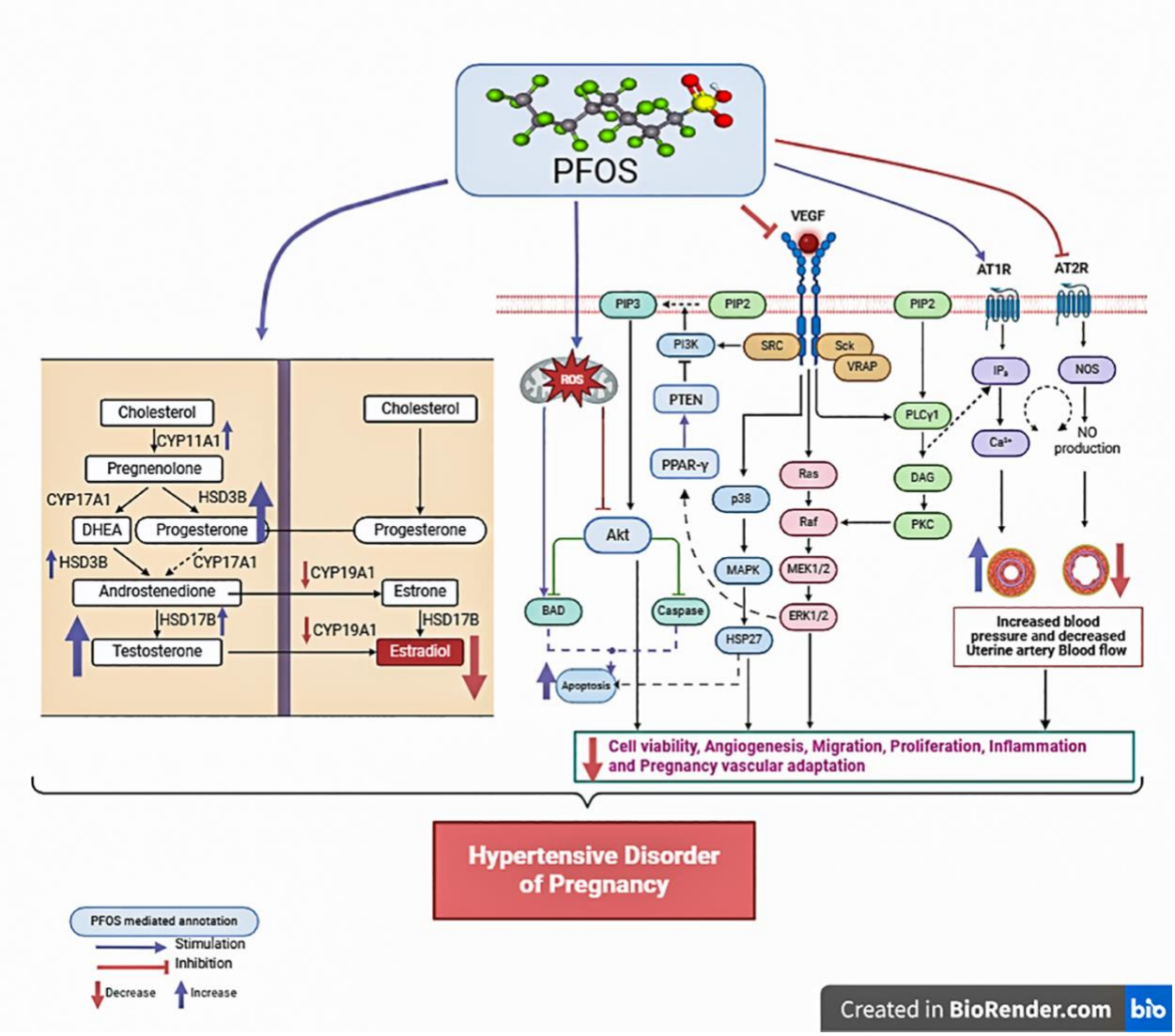

Figure 2. Mechanistic Model of PFAS-Induced Disruptions in Hypertensive Disorders of Pregnancy (HDP) - Integrating Published and Novel Findings. Created by Bio Render.

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Chapter 2

MATERNAL PFOS EXPOSURE DURING RAT PREGNANCY CAUSES HYPERSENSITIVITY TO ANGIOTENSIN II AND ATTENUATION OF ENDOTHELIUM-DEPENDENT VASODILATION IN THE UTERINE ARTERIES

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ABSTRACT

Epidemiological studies show a strong association between environmental exposure to perfluorooctane sulfonic acid (PFOS) and preeclampsia and fetal growth restriction; however, the underlying mechanisms are unclear. We tested the hypothesis that gestational PFOS exposure leads to pregnancy complications via alterations in uterine vascular endothelium-independent angiotensin II-related mechanisms and endothelium-derived factors such as nitric oxide. Pregnant Sprague Dawley rats were exposed to PFOS 0.005, 0.05, 0.5, 5, 10, and 50 μg/mL through drinking water from gestational day 4 to 20, and dams with PFOS 50 μg/mL were used to assess mechanisms. PFOS exposure dose-dependently increased maternal blood pressure but decreased fetal weights. Uterine artery blood flow was lower and resistance index was higher in the PFOS dams. In PFOS dams, uterine artery contractile responses to angiotensin II were significantly greater, whereas contractile responses to K^+ depolarization and phenylephrine were unaffected. Plasma angiotensin II levels were not significantly different between control and PFOS dams; however, PFOS exposure significantly increased AGTR1 and decreased AGTR2 protein levels in uterine arteries. Endothelium-dependent relaxation response to acetylcholine was significantly reduced with decreased endothelial nitric oxide synthase expression in the uterine arteries of PFOS dams. Left ventricular hypertrophy and fibrosis were observed, along with increased ejection fraction and fractional shortening in PFOS dams. These results suggest that elevated maternal PFOS levels decrease uterine blood flow and increase vascular resistance via heightened angiotensin II-mediated vasoconstriction and impaired endothelium-dependent vasodilation, which provides a molecular mechanism linking elevated maternal PFOS levels with gestational hypertension and fetal growth restriction.

1. INTRODUCTION

The cardiovascular system undergoes significant physiological adaptations during pregnancy. Heart rate and cardiac output increase significantly without changes in myocardial contractility and ejection fraction [1]. Peripheral vascular resistance decreases substantially with a more profound effect in uteroplacental circulation, where blood flow increases by 20-fold near-term [2, 3]. The uterine vascular adaptations are accomplished by decreased vascular contractility to adrenergic agonists and angiotensin II (Ang II) and enhanced endothelium-dependent relaxation responses [4]. These cardiovascular changes are mechanisms by which the body adapts to metabolic demands of the mother and fetus to ensure a successful pregnancy. Insufficient hemodynamic changes result in maternal and fetal morbidity, as seen in intrauterine growth restriction [5]. These growth-restricted babies not only are at a higher risk of perinatal and childhood morbidity and mortality, but also, as adults, tend to develop chronic conditions such as hypertension, dyslipidemia, and diabetes mellitus [6-10]. The pathogenesis of fetal growth restriction is incompletely understood [11], and effective prevention recommendations and treatment options are currently limited.

Known risk factors for fetal growth restriction include poor maternal nutrition, smoking, hypertension, preeclampsia, diabetes mellitus, chronic renal disease, and fetal genetic abnormalities [12, 13]. New evidence shows a relationship between prenatal exposure to environmental pollutants and reduced birth weight [14]. More specifically, prenatal exposure to perfluorooctane sulfonic acid (PFOS), an environmental pollutant, has attracted significant attention for its fetal growth inhibitory properties [15-23]. PFOS is a pervasive and recalcitrant compound that has emerged in environmental systems, including air, food, and drinking-water resources because of its extensive use in consumer and industrial applications related to the

automotive, aviation, and aerospace industries, as well as airports, electronics, military activities, imaging, stain removers, leather production, textile water-repellant fabric coatings, and non-stick cookware [24, 25]. PFOS is also widely used in aqueous film-forming foam; a fire-suppression system integrated into fire-fighting vehicles and fire-training facilities [26]. PFOS contains a strong carbon-fluorine bond that makes it resistant to degradation and highly persistent in all environmental compartments [27].

Drinking water is the main source of PFOS exposure in humans. PFOS is well absorbed in the gastrointestinal tract [28], minimally metabolized [29], poorly eliminated [30, 31] and mainly accumulated in plasma [32]. In humans, the half-lives of PFOS range from 3.3 to 6.9 years [33, 34]. PFOS concentrations in maternal serum range from 1.7 to 22.8 ng/mL [35, 36], while an increased concentration (2.5 to 83.4 ng/mL) has been detected in pregnant women with preeclampsia [37-43]. However, plasma PFOS levels of more than 1500 ng/mL have been observed in American blood donors [44, 45]. Furthermore, serum PFOS concentrations as high as 12800 ng/mL to 31,400 ng/mL were reported in occupational populations and people in high-exposure areas [46, 47].

Of concern is that PFOS is increasingly detected in the serum of pregnant women [37-43] and associated with multiple adverse maternal outcomes, including pregnancy-induced hypertension, preeclampsia [37-42, 48, 49], and reduced birth weight [15-23]. Based on a systematic review of the literature and meta-analysis, the shift in birth weight associated with PFOS exposure has been estimated to be −3.22 g birth weight per 1 ng/mL increase in serum PFOS [95% confidence interval (CI): −5.11, −1.33] [50]. Experimental studies in pregnant rats [51-57] and mice [55, 56, 58, 59] confirm that PFOS induces fetal growth restriction. Furthermore, offspring born to PFOS-exposed mothers developed hypertension and glucose

intolerance during their adult lives [51, 52, 54]. Owing to both PFOS's adverse health effects during pregnancy and associated developmental toxicities, the United States Environmental Protection Agency (EPA) and European Food Safety Authority have recognized PFOS as a contaminant of concern [60, 61].

Although evidence shows that PFOS causes fetal growth restriction and developmental abnormalities, the underlying mechanisms by which PFOS exerts these adverse effects are not known. Theoretically, maternal PFOS could cross the placenta [62] and directly affect fetal growth. Alternatively, PFOS could reduce fetal growth by impairing maternal cardiovascular adaptations. The latter is possible because ***in vitro*** studies show that PFOS induces aberrations in endothelial permeability [63] and triggers tight junction opening through the PI3K pathway [64]. In human umbilical vein endothelial cells, PFOS inhibited angiogenesis by reducing cellular sprouting through diminished vascular endothelial growth factor receptor 2 signaling [65]. In addition, high-throughput screening by the EPA ToxCast™ project has identified PFOS as a chemical that could disrupt blood vessel formation and remodeling [66]. Furthermore, in mice, PFOS disrupted crosstalk between endothelial cells and astrocytes [67]. In nonpregnant rats, PFOS triggered inflammation, cardiac fibrosis, and myocardial hypertrophy [68]. A nested case-control study noted a growing association between PFOS and congenital heart defects [69].

However, despite well-documented effects of PFOS on cardiac and endothelial cell function and available circumstantial evidence of PFOS's association with fetal growth restriction, no research has examined the possible effects of PFOS on maternal cardiovascular function. In the present study, we hypothesized that, in pregnant rats, PFOS exposure would impair maternal cardiovascular function by exacerbating vascular contraction and decreasing vascular relaxation responses. We tested this hypothesis by exposing pregnant rats to PFOS

through drinking water and investigated whether (1) cardiac function and systemic arterial pressure are altered in PFOS–exposed compared with control dams; (2) uterine arterial blood flow is altered in PFOS–exposed dams compared with control dams; (3) vascular contraction, particularly in the uterine artery, would be more pronounced in PFOS-exposed dams than in control dams; and (4) endothelium-dependent vascular relaxation in the uterine artery would be impaired in PFOS-exposed dams than in control dams.

2. Materials and Methods

2.1. Animals

All animal procedures were carried out as per the US National Institutes of Health guidelines (NIH Publication No. 85–23, revised 1996) with approval by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison. Twelve-week-old timed-pregnant Sprague-Dawley rats (positive plug = gestation day (GD) 1) were obtained on GD 3 from Envigo Laboratories (Indianapolis, IN) and housed individually in a room with controlled temperature and a 12:12-h light-dark cycle. The rats were randomly divided into seven groups, exposed either to standard drinking water with no detectable levels of PFOS (i.e., the control group) or drinking water containing PFOS potassium (CAS #2795-39-3, Sigma Aldrich, St. Louis, MO) at 0.005, 0.05, 0.5, 5, 10, and 50 μg/mL from GD 4 to GD 20 (i.e., the treatment groups). Experimenters and technicians were blinded to the identity of treatment groups throughout the experiments. PFOS doses were selected based on past studies to cover the full spectrum of human exposure [53-57]. PFOS 0.05 μg/mL is a critical dose at which no detrimental effects are observed (i.e., no observable effect level (NOEL), and EPA used this dose to establish tolerable current human-lifetime health-advisory level of 70 parts per trillion (ppt)

PFOS in drinking water [70]. The highest PFOS exposure groups (10 and 50 μg/mL) correspond to serum PFOS concentrations observed in occupational populations and high-exposure areas [55] and have been commonly used in previous studies where developmental metabolic and cardiovascular deficits were observed [51, 54]. Rats were fed with a standard breeder chow (D15092401; Research Diets, New Brunswick, NJ) *ad libitum*. On GD 20, blood-pressure measurements, uterine artery ultrasounds, and echocardiography were performed. Following these procedures, rats were sacrificed to collect plasma and measure fetal and placental weights. The uterine arteries were collected for vascular reactivity studies and RNA and protein isolation. The left ventricles were also collected for histopathological analysis.

2.2. Blood Pressure

At GD 20, blood pressures were measured using a non-invasive tail-cuff method (Kent Scientific, Torrington, CT) as described previously [71, 72]. Briefly, rats were acclimated to the restraint warming chamber for 15 min between 8:00 to 10:00 AM for two days. On the day of blood-pressure measurements, rats were placed on the restraint warming chamber preset at 30^{o}C and allowed to rest for 10 min to dilate the peripheral blood vessels and stimulate blood flow to the tail. An occlusion cuff and a volume pressure-recording cuff were applied to the base of the tail. The cuff was programmed to inflate and deflate automatically within 90 seconds. Blood pressure was recorded and analyzed with Kent Scientific software. Results from the first five inflation cycles were used for acclimation, and the average obtained from the subsequent five cycles was taken as the individual mean blood pressure for the given rat.

2.3. Uterine Artery Ultrasound

Rats were anesthetized with 2% isoflurane in oxygen and placed on a heated platform. Uterine arteries were examined with a 30-MHz transducer and Vevo 2100 ultrasound system (Visual Sonics, Toronto, ON, Canada) [73]. Briefly, velocities of the main uterine arteries were recorded below the bladder and at the level where the main uterine artery branches from the internal iliac artery. Peak systolic velocity (PSV) and end-diastolic velocity (EDV), the area under the peak velocity-time curve, and the R-R interval were measured from three consecutive cardiac cycles, and the results were averaged. To determine blood-flow velocity distribution, we used the following formula: $F = ½ MV\pi (D/2)2$ (where MV = mean peak velocity over the cardiac cycle [cm/s], D = diameter [cm], and F = blood flow [mL/min]). Uterine artery resistance index (RI = [PSV – EDV]/PSV) and pulsatility index (PI = [PSV – EDV]/MV) were calculated.

2.4. Ex-vivo Vascular Reactivity Studies

The main uterine arteries that branched from the internal iliac artery were removed and dissected free of adherent connective tissues. Arterial ring segments (2 mm in length) were mounted with tungsten wires on a wire myograph (Danish Myo Techniques, Aarhus, Denmark) to record isometric tension. Arterial rings were immersed in Krebs physiological solution (KPS) (37°C, aerated with a 95% O_2 /5% CO_2 gas mixture, pH 7.4) composed of NaCl, 118 mM; KCl, 4.7 mM; CaCl2, 2.5 mM; MgSO4, 1.2 mM; KH2PO4, 1.2 mM; NaHCO3, 25 mM; and glucose, 11.1 mM. The rings were allowed to equilibrate in KPS for 1 hour at a resting tension. After stabilization, the rings were normalized with a normalization software package (Myodata; Danish Myotechnology). For endothelium-intact arterial rings, extreme care was taken to avoid injury to the endothelium. For endothelium-denuded arterial rings, the endothelium was removed by gently rubbing the ring interior with tungsten wire. Endothelium removal was verified by the

absence of relaxation to acetylcholine (ACh) in arterial rings precontracted by a submaximal concentration of phenylephrine (PE).

2.4.1. Assessment of Vascular Contractile Responses

The arterial rings were exposed to 80mM of potassium chloride (KCl) until reproducible depolarization-induced contractions were achieved. After the second round of washing and equilibration with KPS, vascular contractile responses to cumulative doses of PE (10^{-9} to $3x10^{-5}$ M) and Ang II (10^{-11} to 10^{-7} M) were determined.

2.4.2. Assessment of Vascular Relaxation Responses

Endothelium-dependent relaxation was measured by using ACh (10^{-9} to 10^{-5} M)-induced relaxation in PE-precontracted arteries. Endothelium-independent relaxation was measured with sodium nitroprusside (SNP) (10–9 to 10–6 M) in PE-precontracted endothelium-denuded arteries. The PE concentration that produced 80% of the maximal response (pEC_{80}) was used for precontraction.

2.5. Plasma Angiotensin II Levels

Plasma Ang II concentrations were measured using an enzyme immunoassay kit (Enzo Life Sciences, Farmingdale, NY, USA) per the manufacturer's instructions. The detection range was 3.9–10,000 pg/ml. A total of 50 μl of plasma in duplicate was used for this assay.

2.6. RNA Isolation and Quantitative Real-time Polymerase Chain Reaction

Total RNA was extracted from uterine arteries with the RNeasy mini kit (QIAGEN, Valencia, CA). Nanodrop spectrophotometer (ThermoFisher Scientific, Newark, DE) was used to determine RNA concentration and integrity. One microgram of total RNA was reverse transcribed to cDNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). After dilution, cDNA equivalent to 100 ng of RNA was amplified using quantitative real-time reverse transcription-polymerase chain reactions (qRT-PCR), with FAM (Invitrogen; Thermo Scientific, Grand Island, NY) serving as the fluorophore in a CFX96 real-time thermal cycler (Bio-Rad). PCR conditions for the TaqMan Gene Expression Assay were 2 min at 50°C and 10 min at 95°C for one cycle, and then 15 seconds at 95°C and 1 min at 60°C for 50 cycles. Results were calculated based on the $2^{-\Delta\Delta CT}$ method and expressed as fold change regarding the gene of interest in PFOS dams versus control dams. All reactions were performed in duplicate, and β-actin was used as an internal control. TaqMan assays were carried out in 10 μL volumes at a final concentration of 250 nM TaqMan probe and 900 nM of each primer. *Agtr1a* (Rn02758772_S1), *Agtr1b* (Rn02132799_S1), *Agtr2* (Rn00560677_s1), endothelial nitric oxide synthase (eNOS) (Rn02132634_S1), and β-actin (Rn00667869_m1) assays were obtained from ThermoFisher Scientific, Newark, DE.

2.7. Western Blotting

Uterine arteries were homogenized in ice-cold radioimmunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA) containing a protease inhibitor tablet (Roche, Indianapolis, IN) and phosphatase inhibitor cocktail-2 and -3 (Sigma). Tissue lysates were centrifuged (14,000 g for 10 min at 4°C), and the supernatant was used to measure protein concentration with the Pierce BCA protein assay kit (Thermo Scientific, Waltham, MA). The

supernatant was re-suspended in the NuPAGE® sample buffer and reducing agent (Invitrogen; Thermo Scientific, Waltham, MA). Proteins (30 μg) alongside Precision Plus Standard (Kaleidoscope, Bio-Rad) were loaded in wells on 4% to 12% gradient NuPAGE® Bis-Tris Gels (Invitrogen) at 100 V for 2 hours at room temperature and then transferred onto Immobilon-P membranes (Millipore Inc, Billerica, MA) with a mini-Blot Module (Invitrogen) at 20 V for 1 hour. The membranes were blocked with 5% skim milk for 1 hour and then incubated overnight at 4°C with primary antibodies. The primary antibodies were AGTR1 (rabbit polyclonal, SAB2100073, 1:1000; Sigma, Burlington, MA), AGTR2 (rabbit monoclonal, ab92445, 1:1000; Abcam, Cambridge, MA), eNOS (rabbit monoclonal, #32027, Cell Signaling Technologies, Danvers, MA), and β-actin (rabbit monoclonal, #4070, 1:5000; Cell Signaling Technologies). After washing, the membranes were incubated with secondary antibodies (anti-rabbit conjugated with horseradish peroxidase) for 1 hour and detected with Pierce enhanced chemiluminescence detection kits (Thermo Scientific, Waltham, MA). Densitometric measurement was performed using Image J software. Results were normalized and expressed as ratios of the intensity of a specific band to that of β-actin.

2.8. ECHOCARDIOGRAPHY

Rats were anesthetized with 2% isoflurane in oxygen and placed on a heated platform. Transthoracic M-mode, B-mode, and pulsed Doppler echocardiography featuring an MS250 probe with a frequency of 13 to 24 MHz and 30-μm resolution capable of capturing 240 frames per second (Vevo 3100; VisualSonics, Toronto, Ontario, Canada) were used to measure heart function, as reported previously [74]. The aortic diameter was measured just distal to the aortic valve. Heart rate was determined from at least three consecutive intervals from the pulsed-wave

Doppler tracings of the left ventricular (LV) outflow tract. End diastolic and systolic LV diameter and anterior and posterior wall (LVAW and LVPW, respectively) thickness were measured from M-mode images obtained in a parasternal long-axis view using the leading-edge-to-leading-edge convention. LV posterior wall thickening (PWT %) was measured as 100* [(LVPW systole – LVPW diastole)/LVPW diastole]. All parameters were measured over at least three consecutive cardiac cycles and then averaged. The same person obtained all the echocardiography images and measurements. Vevo 3100 software was used calculate the cardiac parameters. Left ventricular fractional shortening was calculated as [(LV diameter diastole – LV diameter systole)/LV diameter diastole] x 100; ejection fraction was calculated as [(7.0/(2.4 + LV diameter diastole)(LV diameter diastole)^3 – (7.0/(2.4 + LV diameter systole)(LV diameter systole)^3/(7.0/(2.4 + LV diameter diastole)(LV diameter diastole)^3 x 100)]; and LV mass was calculated as [1.05 x ((posterior wall diastole + anterior wall diastole + LV diameter diastole)^3 – (LV diameter diastole)^3)]. Isovolumic relaxation time (IVRT) was measured as the time from the aortic valve closing to the opening of the mitral valve from pulsed-wave Doppler tracings of the LV outflow tract and mitral inflow region. Mitral E/V is the ratio of the E-wave value to the A-wave value.

2.9. HISTOPATHOLOGIC ANALYSIS

Left ventricular tissues were fixed and embedded in an optimal cutting temperature compound, cut into 5-μm sections, and stained with Hematoxylin, Eosin, and Masson trichrome as described previously [75]. Image J software was used to measure the length of the LV wall and fibrosis areas within sections. Color-based threshold was used to identify blue-stained areas from each section.

2.10. Statistical Analysis

Statistical analyses were done using GraphPad Prism (GraphPad Software, San Diego, CA). Data were presented as the mean ± SEM. Comparisons between multiple groups were performed with one-way ANOVA, and between two groups were done with an unpaired Student t-test. Cumulative concentration-response curves were analyzed by using a four-parameter sigmoid curve. Contraction responses to PE were calculated as a percent of its maximal contraction. Relaxant responses to ACh and SNP were calculated as percent relaxation of the PE-induced contraction. The normality and homogeneity of the variances were analyzed with the one-sample Kolmogorov–Smirnov test and Anderson-Darling test, respectively. When the data were not normally distributed, a non-parametric Kruskal–Wallis test, with Dunn's multiple comparisons, was used. Differences with a P-value of less than 0.05 were considered statistically significant.

3. Results

3.1. Fetal and Placental Weights and Maternal Blood Pressure

The fetal and placental weights of pregnant dams exposed to PFOS from GD 4 to GD 20 are shown in **Figures 1A and B**. The fetal weight significantly decreased ($P < 0.0003$) by 11.2, 13.6, 15.5, and 16.5% in the dams dosed with 0.5, 5, 10, and 50 µg/ml PFOS, respectively. Significant decreases ($P < 0.03$) in placental weights were observed in the 10 and 50 µg/ml PFOS-exposed group, whereas no significant differences were observed between the other PFOS groups and control group. Consistent with a previous report, there were no significant differences

between the PFOS and control groups regarding the fetoplacental weight ratio, number of living fetuses, food intake, and water intake [51].

As shown in **Figure 1C**, mean arterial blood pressure increased significantly ($P < 0.005$) in dams exposed to 0.5, 5, 10, and 50 µg/ml PFOS; however, the 0.005 and 0.05 PFOS treatment groups did not differ significantly from the control group.

3.2. Uterine Arterial Blood Flow

Because higher doses of PFOS were associated with increased maternal blood pressure, we further focused on the 50 µg/ml group to determine the underlying mechanisms of blood pressure increase. Using transcutaneous micro-ultrasound, we measured uterine arterial blood flow, which was significantly lower by 43% ($P < 0.02$) in 50 µg/ml PFOS dams than in the controls (**Figure 2A**). Resistance ($P < 0.001$) and pulsatility ($P < 0.006$) indices were significantly greater in 50 µg/ml PFOS dams than in controls (**Figures 2B and C**). These results indicate that PFOS exposure decreases uterine arterial blood flow and increases uterine vascular resistance.

3.3. Uterine Arterial Contractile Response

Figure 3 shows the effect of 50 µg/ml PFOS exposure on vascular contractile responses to 80 mM KCl (depolarization-induced contraction), PE (a measure of α_1-adrenoceptor-induced contraction), and Ang II (a measure of Ang II receptor-induced contraction) in endothelium-denuded uterine arteries. PFOS exposure did not alter KCl- and PE-induced contractile responses (**Figures 3A and B**). However, 50 µg/ml PFOS exposure exaggerated the Ang II-induced contractile responses with a leftward shift in dose-response curves ($pD2 = 8.8 \pm 0.05$ vs. $8.4 \pm$

0.02 in controls) and an increase in maximal responses (E_{max} = 126.87 ± 8.12% vs. 107.39 ± 2.34% in controls) (**Figure 3C**).

3.4. Plasma Ang II Levels and Ang II Receptor Expression

Plasma Ang II levels were not significantly different between control (3.01 ± 0.7 ng/ml) and 50 µg/ml PFOS exposed dams (2.99 ± 0.4 ng/ml). We next determined whether Ang II receptor levels correlated with exacerbated Ang II-induced contractile responses in 50 µg/ml PFOS exposed dams. Rodents possess two Agtr1 receptor isoforms at the mRNA level, designated *Agtr1a* and *Agtr1b*. We used qRT-PCR to measure the mRNA levels of *Agtr1a* and *Agtr1b* in uterine arteries. The expression of *Agtr1b* mRNA in uterine arteries in the control dams was comparable to that in the 50 µg/ml PFOS dams (**Figure 4B**). However, in uterine arteries of the 50 µg/ml PFOS dams, the expression of *Agtr1a* mRNA was 1.5-fold higher ($P < 0.001$) than in uterine arteries of the controls, and the expression of *Agtr2* mRNA was 30% lower ($P < 0.004$) than in uterine arteries of the controls (**Figure 4A and C**).

As shown in Figure 4D, Western blotting revealed that AGTR1 protein levels were significantly higher by 42% ($P < 0.02$), while AGTR2 protein levels were significantly lower by 72% ($P < 0.02$) in uterine arteries of the 50 µg/ml PFOS dams than in controls (**Figure 4D**).

3.5. Endothelium-dependent Relaxation

In endothelium intact uterine arteries, ACh-induced relaxation was significantly lower ($P < 0.0003$) in 50 µg/ml PFOS dams (pD_2 = 6.13 ± 0.09) than in the controls (pD_2 = 7.03 ± 0.12; **Figure 5A**). In addition, maximal vascular relaxation to ACh was significantly lower ($P < 0.009$) in the 50 µg/ml PFOS dams (E_{max}: 70.89% ± 4.71%) than in controls (E_{max}: 88.97% ± 2.52%;

Figure 5A). Endothelium-independent vascular relaxation to SNP in uterine arteries was not significantly different between the groups (**Figure 5B**).

3.6. Endothelial Nitric Oxide Synthase Expression

To determine whether the reduced ACh-induced relaxation might be due to reduced eNOS expression in the uterine artery, we measured the eNOS mRNA and protein expression. In uterine arteries of the 50 µg/ml PFOS dams, eNOS mRNA expression was lower by 30% ($P < 0.01$), and eNOS protein expression was lower by 67% ($P < 0.02$) than in controls (**Figures 6A and B**).

3.7. Echocardiography and Cardiac Function

Table 1 presents cardiac echocardiographic data. No differences in heart rate and cardiac output were observed between the 50 µg/ml PFOS and control dams. Systolic measurements such as stroke volume, ejection fraction, and fractional shortening were significantly higher ($P < 0.04$) in the PFOS dams than in the controls. In contrast, diastolic measurements like IVRT and the mitral inflow E/A ratio were similar for the PFOS dams and controls (**Table 1**). Morphological parameters, including LVAW and LVPW thickness at the end of systole, were 18% and 10% higher ($P < 0.01$) in the 50 µg/ml PFOS dams than in the controls. Also, LV mass and posterior wall thickness (PWT %) were significantly higher ($P < 0.01$) in the PFOS dams than in controls. Likewise, hematoxylin and eosin staining revealed that LV wall thickness was significantly greater ($P < 0.0001$) in 50 µg/ml PFOS dams than in controls (**Figure 7A**). Trichrome-Masson staining of hearts revealed that fibrosis in the 50 µg/ml PFOS dams was greater ($P < 0.03$) than in controls (**Figure 7B**).

4. Discussion

Human [15-23] and animal studies [51-56, 58, 59] suggest that maternal PFOS levels are associated with reduced fetal weight; however, the mechanisms underlying this growth-restricted phenotype are unknown. The major findings in our current study are that elevated PFOS levels in pregnant rats led to hypertension and cardiac hypertrophy. We also found that elevated PFOS was associated with uteroplacental vascular dysfunction, as evidenced by an elevated uterine artery resistance index and reduced blood flow. Furthermore, in the uterine artery, PFOS exposure did not affect KCl- and α_1-adrenoceptor-mediated contractions but concentration-dependently increased Ang II-induced contractions with an associated increase in AGTR1 expression and decrease in AGTR2 expression. Also, in the uterine artery, PFOS exposure did not affect endothelium-independent relaxations mediated by SNP, whereas the ACh-induced, endothelium-dependent relaxation was attenuated with a correlated decrease in eNOS expression. Therefore, we suggest that an increase in vascular AGTR1- and Ang II-stimulated responses and a decrease in eNOS and endothelium-dependent relaxation responses may induce maternal cardiovascular dysfunction and fetal growth restriction in pregnant rats exposed to elevated PFOS levels.

Previous studies have suggested that a strong relationship exists between low birth weight and maternal malnutrition [76]. However, low birth weight due to maternal malnutrition represents only a small percentage of low-birth-weight cases in the Western world because of good perinatal care. Thus, low birth weight in well-nourished populations is likely caused by factors other than maternal undernutrition. Maternal PFOS exposure is one potential explanation for these low-birth-weight cases because the percentage of pregnant women exposed to PFOS in the West is rapidly increasing [77-84]. In the present study, PFOS exposure was associated with

fetal growth restriction in pregnant rats, which is consistent with previous reports in rats [51-57] and mice [55, 56, 58, 59]. Also, our finding is in line with observations in humans, where a strong association between maternal PFOS exposure and low birth weight has been reported [15-23]. Consistent with previous findings, our results suggest that PFOS exposure does not affect maternal food and water intake, suggesting that the observed links between PFOS and fetal growth are not due to altered nutritional intake.

Blood pressures in the PFOS-exposed dams were higher than in controls, consistent with previous findings in which elevated PFOS levels in pregnant women were associated with elevated mean blood pressure [37-42, 48, 49]. These observations suggest that PFOS exposure could impair mechanisms controlling gestational blood pressure.

Maternal vascular adaptations, specifically in uterine circulation with a low resistance to blood flow, an enhanced vasodilator response, and a blunted vasoconstrictor response, are essential for fetal growth and survival [85]. The finding that elevated levels of PFOS decreased uterine arterial blood flow and increased uterine artery resistance index suggests that PFOS can induce aberrations in uterine circulation and increase peripheral vascular resistance, which could contribute to the observed blood pressure increase and fetal growth restriction. This finding is consistent with previous research findings that reduced uterine blood flow, and inadequate placental perfusion are associated with an increased clinical incidence of preeclampsia and fetal intrauterine growth restriction [86, 87].

In the search for the possible mechanisms involved in PFOS-associated decreases in uterine blood flow, we found that PFOS caused alterations in uterine vascular function. In the present study, Ang II-induced contractions but not KCl- and PE-induced contractions were more pronounced in the endothelium-denuded uterine arteries of PFOS dams than in controls

suggesting that PFOS may exert its influence on vascular smooth-muscle contractile responses in an agonist-specific manner rather than at common intracellular signaling pathways. Increased contractile response to Ang II can occur owing to altered vascular remodeling. The finding that KCl-induced depolarization-mediated uterine vascular contractions were similar between the PFOS group and control group suggests that the exaggerated Ang II-induced contractions in the PFOS groups were likely not due to changes in vascular-wall thickness or remodeling. The increased Ang II-mediated vascular contractions could reflect changes in the expression of Ang II receptor subtypes. Ang II stimulates two receptor subtypes: AGTR1 and AGTR2. The activation of AGTR1 mediates vasoconstriction [88], whereas AGTR2 opposes this effect and promotes vasodilation [89]. Consistent with enhanced Ang II-induced contractions, the expression of AGTR1, which mediates vasoconstriction, was increased, and AGTR2, which mediates vasodilation, was decreased in the uterine arteries of PFOS dams. Thus, it appears that PFOS induces upregulation of vasoconstrictive AGTR1, allowing it to emerge as a predominant receptor in uterine arteries, and Ang II may mediate most of its effect through this receptor. However, we uncovered no evidence of a significant link between plasma Ang II levels and PFOS exposure, further suggesting that exaggerated Ang II-mediated vasoconstriction is not due to an increase in circulating Ang II levels but may be related to the increased AGTR1-to-AGTR2 ratio. Therefore, the question arises about how PFOS provokes dysregulation in Ang II receptor expressions. The finding that PFOS seems to alter AGTR1 and AGTR2 at the mRNA level suggests that PFOS induces alteration in their transcriptional mechanisms. Further studies should examine the possible transcriptional mechanisms that PFOS targets to cause alterations in Ang II receptor expressions. Also, it would be interesting to determine if modulation of Ang II function

in vivo in PFOS exposure dams using AGTR1 antagonists or AGTR2 agonists could restore normal vascular contractile responses and uterine blood flow.

This study also shows that PFOS exposure attenuates ACh-induced relaxations in uterine arteries. Nitric oxide (NO) is a vital vasodilator released from endothelial cells [90], and significant increases in endothelial NO production have been observed during pregnancy [91]. Thus, PFOS-induced inhibition of ACh-induced relaxation could be due to a decrease in the synthesis and release of NO from endothelial cells or to a change in the sensitivity of vascular smooth muscle to relaxation by NO. The observation that relaxation of endothelium-denuded uterine arteries to SNP did not differ between PFOS-treated and control dams provides evidence that PFOS does not affect the sensitivity of vascular smooth muscle to relaxation. This suggests that reduced ACh-induced relaxation in PFOS-treated dams is likely due to decreased synthesis and/or release of NO from endothelial cells. The precise mechanism by which PFOS could inhibit endothelial NO synthesis is unclear, but the mechanism could be related to changes in either the amount or activity levels of eNOS. Our finding that eNOS mRNA and protein levels were lower in the uterine arteries of the PFOS groups than in controls supports the assertion that PFOS may decrease NO production by downregulation of eNOS transcription. Many upstream factors play a role in regulating eNOS expression during pregnancy. An important possibility is that decreased eNOS may be a consequence of reduced production and activity of other factors that cause pregnancy-associated vascular adaptations, such as vascular endothelial growth factor (VEGF) [92, 93] and placental growth factor (PGF) [94]. For example, PFOS has downregulated VEGF signaling in endothelial cells [65]. Thus, whether PFOS impairs eNOS expression and endothelial dysfunction via a VEGF-dependent mechanism remains to be elucidated.

In the current study, we used echocardiography to assess cardiac function and found that PFOS exposure during pregnancy leads to LV hypertrophy and systolic dysfunction, as evidenced by increases in wall thickness, LV mass, stroke volume, ejection fraction, and fractional shortening. These findings are consistent with previous reports wherein PFOS exposure in male rats increased heart weight and inflammatory response [68]. Whether these cardiac effects are due to the direct action of PFOS on the heart or are secondary to an increase in peripheral vascular resistance is not known. Because PFOS exposure did not alter heart rate, the cardiac deficits may not be likely due to increased adrenergic activity. Previous studies show that PFOS induces mitochondrial dysfunction and damage in cardiomyocytes [95, 96]. This suggests that PFOS has the potential to directly damage cardiac structure and function. In addition, Ang II infusion in pregnant rats has been shown to cause LV hypertrophy and increase ejection fraction and blood pressure via the AGTR1-mediated pathway [97]. It remains to be determined whether PFOS-induces cardiac dysfunction by dysregulating the Ang II receptor expression and hyperactivation of cardiac Ang II signaling similar to that in the uterine arteries. Furthermore, in the present study, PFOS exposure leads to cardiac fibrosis. This observation, along with previous reports of PFOS association with congenital heart defects [69, 98], suggests that perinatal PFOS exposure can impact long-term cardiac dysfunction in both the mother and their offspring. Although PFOS exposure did not affect diastolic function in the present study, this information should be interpreted with caution because diagnosing diastolic function in rats could be misleading because of rapid heart rates [99]. Further studies with cardiac pressure catheterization are needed to confirm PFOS effects on diastolic dysfunction. It is known that oxidative stress and inflammation impair uterine vascular and cardiac dysfunction [4, 100, 101], and PFOS has the potential to induce oxidative stress and inflammation [63, 68]. Thus, PFOS-promoted oxidative

stress and inflammatory signaling may contribute to impaired cardiovascular function; however, further studies are needed to confirm this notion.

In summary, this study presents compelling evidence that PFOS exposure disrupts maternal cardiovascular adaptations by increasing arterial pressure, uterine vascular resistance, systolic dysfunction, and LV hypertrophy. The present study demonstrates for the first time that PFOS may very well have adverse effects on vascular reactivity of uterine arteries in pregnancy, with a selective increase in Ang II-mediated contractions and decreased endothelium-dependent relaxations. Although it is not clear at present whether increased vasoconstriction and inhibition of endothelium-dependent relaxation is a major reason for reduced uterine blood flow and fetal growth restriction observed with PFOS exposure during pregnancy, our study's findings point to a potential mechanism. Strategies that target excessive PFOS action in uterine circulation could have important therapeutic potential in treating pregnancies complicated by fetal growth restriction.

FIGURES:

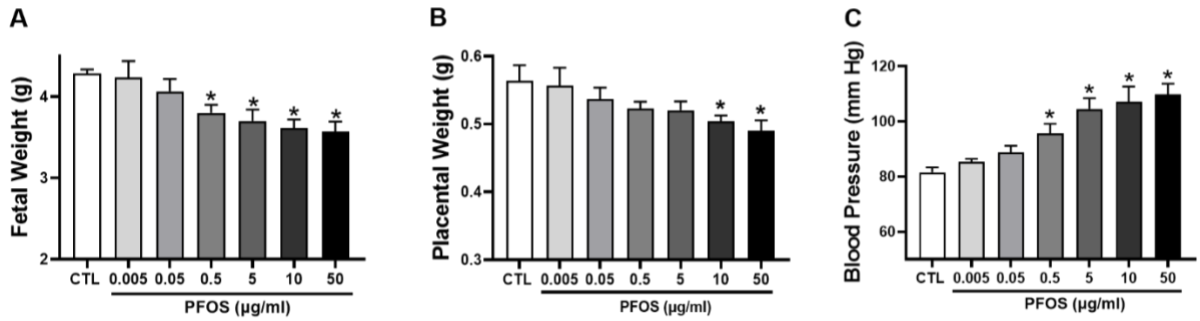

Figure 1. Effect of prenatal PFOS exposure on (A) fetal weights, (B) placental weights, and (C) mean arterial blood pressure measured using a non-invasive CODA system on gestation day 20. PFOS was administered through drinking water from gestation day 4 to 20 at 0.005, 0.05, 0.5, 5, 10, 50 µg/ml. Controls received drinking water with no detectable levels of PFOS. Data are expressed as mean ± SEM of 6 to 8 dams in each group. *p < 0.05 vs controls.

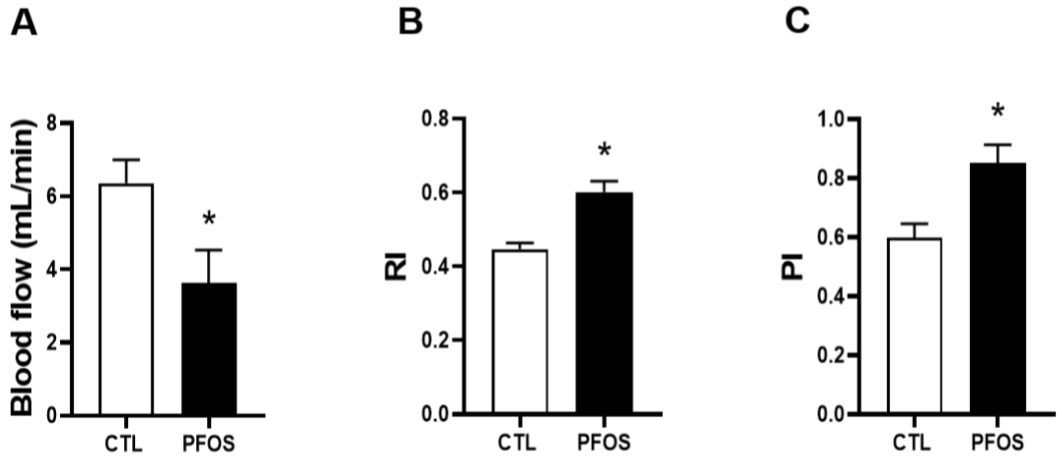

Figure 2. Effect of prenatal PFOS exposure on uterine hemodynamics. (A) Uterine artery blood flow, (B) resistance index, and (C) pulsatility index were measured using micro-ultrasound on gestation day 20 in control and PFOS 50 µg/ml dams. Data are expressed as mean ± SEM of 6 dams in each group. *p < 0.05 vs controls.

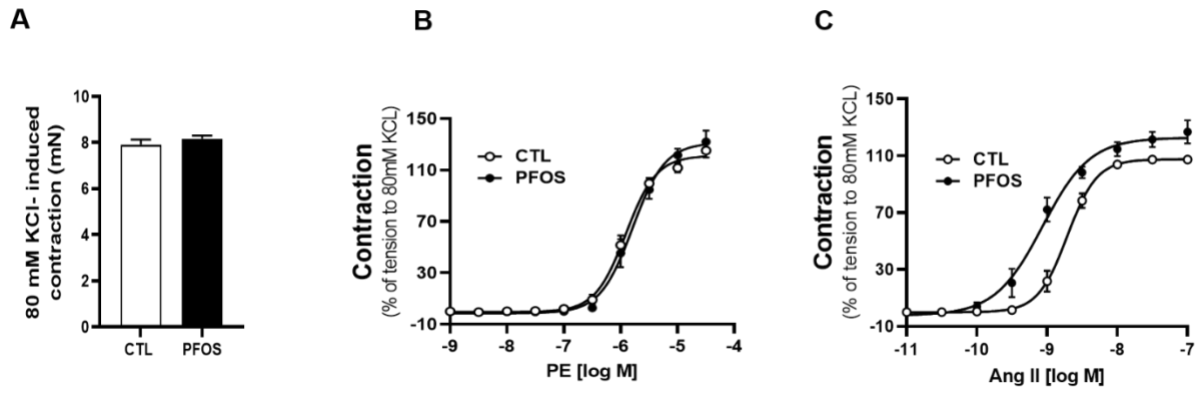

Figure 3. Effect of prenatal PFOS exposure on uterine artery responses to contractile agonists. Contractile responses were taken in endothelium-denuded uterine arteries to (A) potassium chloride (KCl), (B) cumulative doses of phenylephrine (PE), and (C) cumulative doses of angiotensin II (Ang II). Data are expressed as mean ± SEM of 5 to 8 dams in each group.

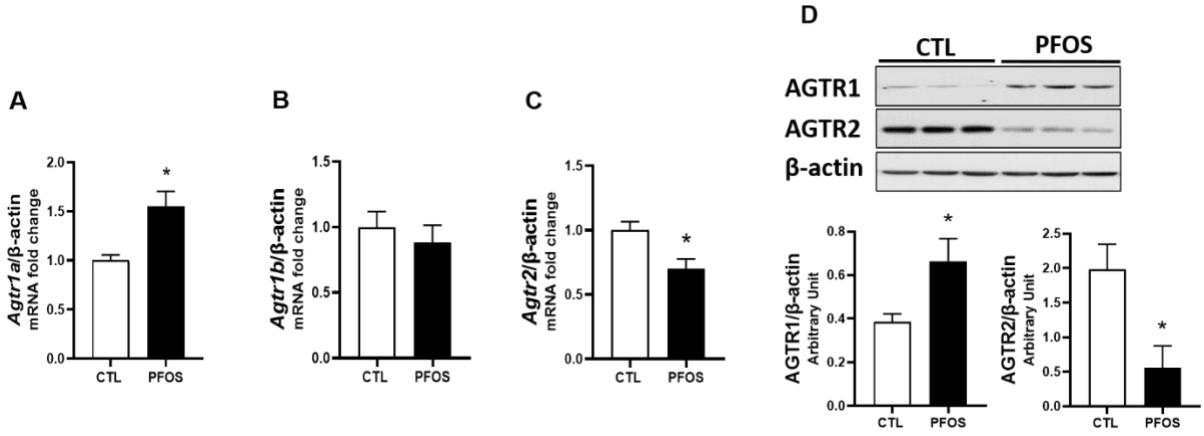

Figure 4. Effect of prenatal PFOS exposure on mRNA and protein expression of Ang II receptors in uterine arteries. (A) *Agtr1a*, (B) *Agtr1b*, (C) *Agtr2* mRNA expression were measured using real-time reverse transcriptase PCR and normalized relative to β-actin levels. (D) AGTR1 and AGTR2 protein levels were measured by Western blotting. Representative blots are shown on the top and densitometric values are shown at the bottom. Data are expressed as mean ± SEM of 5 to 6 dams in each group. *$p < 0.05$ vs controls.

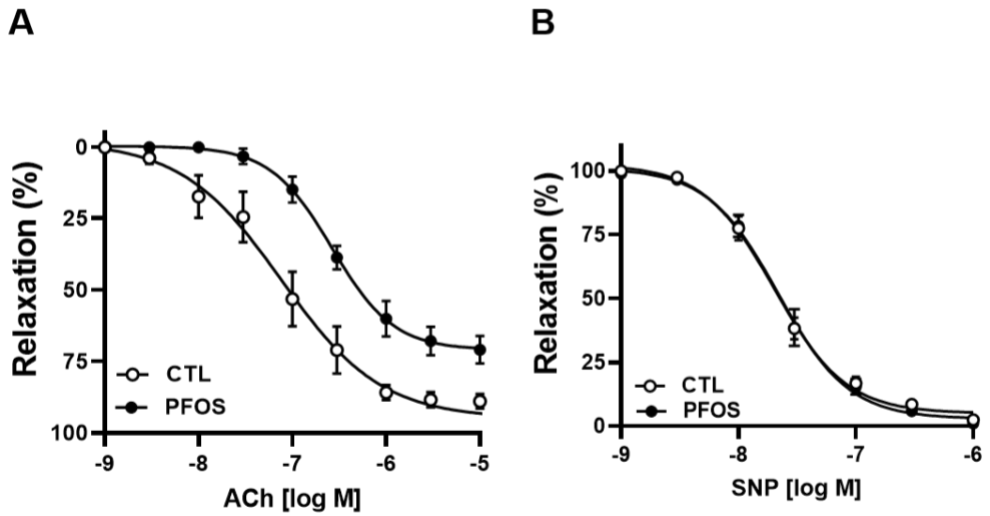

Figure 5. Effect of prenatal PFOS exposure on vascular relaxation in uterine arteries. Arterial rings were precontracted with submaximal PE and examined for relaxation to cumulative concentrations of (A) acetylcholine (ACh), (B) sodium nitroprusside (SNP). Data are expressed as mean ± SEM of 5-6 dams in each group.

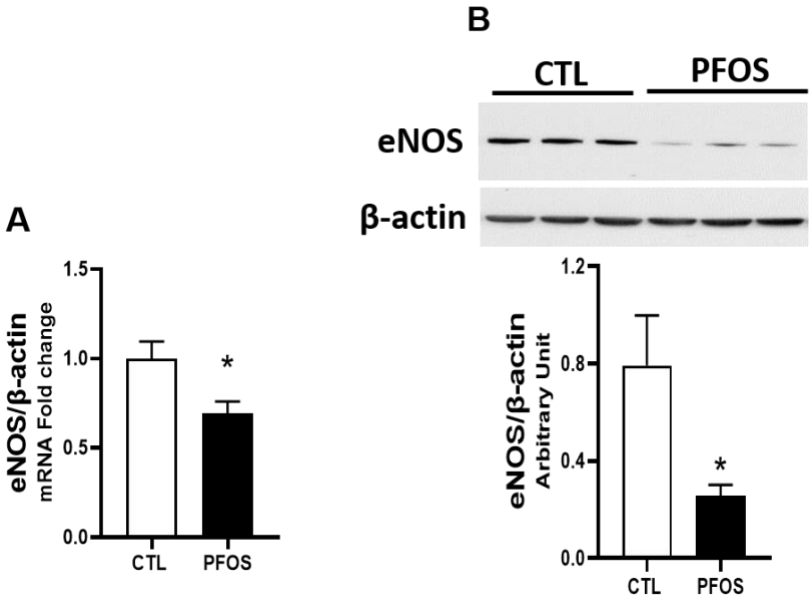

Figure 6. Effect of prenatal PFOS exposure on eNOS mRNA and protein expression in uterine arteries. (A) eNOS mRNA expression was measured using real-time reverse transcriptase PCR

and normalized relative to β-actin levels. (B) eNOS protein expression was measured by Western blotting. Representative blots are shown on the top and densitometric values are shown at the bottom. Data are expressed as mean ± SEM of 5 to 6 dams in each group. *p < 0.05 vs controls.

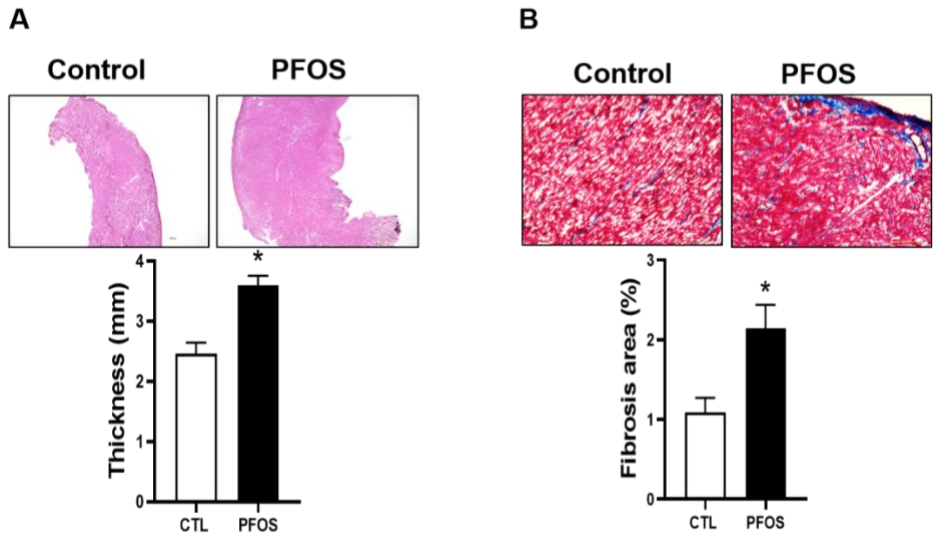

Figure 7. Effect of prenatal PFOS exposure on cardiac structure. (A) Left ventricular wall thickness were measured following hematoxylin and eosin staining. (B) intracardiac collagen deposition (blue staining) was quantified after Masson trichrome staining. Photomicrographs are representative images of at least three animals from each group. Data are expressed as mean ± SEM. *p < 0.05 vs controls.

TABLES:

Table 1. Hemodynamic and echocardiography parameters

Parameters	units	Controls	PFOS dams
Heart rate	BPM	357.5 ± 8.4	361.6 ± 7.2
Volume_s	μL	102.8 ± 7.5	92.3 ± 6.2
Volume_d	μL	255.6 ±10.7	264.5 ± 10.1
Cardiac output	mL/min	54.9 ± 2.4	59.5 ± 1.8
Stroke volume	μL	152.8 ± 5.5	171.9 ± 5.1*
Ejection fraction	%	60.0 ± 1.8	65.2 ± 1.3*
Fractional shortening	%	33.0 ± 1.3	36.8 ± 1.0*
LV mass	mg	488.8 ± 21.5	575.1 ± 30.0*
LVAWs	mm	1.5 ± 0.03	1.7 ± 0.1*
LVAWd	mm	1.1 ± 0.04	1.2 ± 0.1
LVPWs	mm	1.5 ± 0.1	1.7 ± 0.1*
LVPWd	mm	1.2 ± 0.02	1.2 ± 0.04
PWT	%	28.5 ± 3.0	42.6 ± 3.3*
IVRT	ms	26.7 ± 1.0	24.7 ± 0.5
Mitral inflow E/V	ratio	1.2 ± 0.02	1.1 ± 0.03

Values are mean ± SEM. s, end of systole; d, end of diastole; LV, left ventricle; LVAWs and LVAWd, left ventricle anterior wall at the end of systole and diastole; LVPWs and LVPWd, left ventricle posterior wall at the end of systole and diastole; PWT, posterior wall thickness; IVRT, isovolumic relaxation time; E/V, mitral inflow E-wave/A-wave ratio. *$P < 0.05$

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Chapter 3

RESTORING ANGIOTENSIN TYPE 2 RECEPTOR FUNCTION REVERSES VASCULAR HYPER-REACTIVITY AND HYPERTENSION IN PREGNANCY

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ABSTRACT

Perfluorooctane sulfonic acid (PFOS) exposure during pregnancy induces hypertension with decreased vasodilatory angiotensin type-2 receptor (AT2R) expression and impaired vascular reactivity and fetal weights. We hypothesized that AT2R activation restores the AT1R/AT2R balance and reverses gestational hypertension by improving vascular mechanisms. Pregnant Sprague-Dawley rats were exposed to PFOS through drinking water (50 μg/mL) from gestation day (GD) 4─20. Controls received drinking water with no detectable PFOS. Control and PFOS-exposed rats were treated with AT2R agonist Compound 21 (C21; 0.3 mg/kg/day, SC) from GD 15─20. In PFOS dams, blood pressure was higher, blood flow in the uterine artery was reduced, and C21 reversed these to control levels. C21 mitigated the heightened contraction response to Ang II and enhanced endothelium-dependent vasorelaxation in uterine arteries of PFOS dams. The observed vascular effects of C21 were correlated with reduced AT1R levels and increased AT2R and eNOS protein levels. C21 also increased plasma bradykinin production in PFOS dams and attenuated the fetoplacental growth restriction. These data suggest that C21 improves the PFOS-induced maternal vascular dysfunction and blood flow to the fetoplacental unit, providing preclinical evidence to support that AT2R activation may be an important target for preventing or treating PFOS-induced adverse maternal and fetal outcomes.

1. INTRODUCTION

Hypertensive disorders in pregnancy (HDPs) encompass pre-pregnancy (chronic) or pregnancy-associated hypertension and represent prevalent pregnancy complications in the United States. The incidence of HDPs has been on the rise, affecting approximately 15% of women during their reproductive years [1]. HDPs are significantly linked to severe maternal

complications, including heart attack and stroke [2], and remain a primary cause of pregnancy-related mortality in the United States [3]. Moreover, mothers who survive HDPs and their offspring face an elevated risk of enduring long-term health complications, including the development of cardiovascular and metabolic diseases [4-11]. Despite the severe threat HDPs pose to both maternal and fetal health, the underlying mechanism remains unclear. It is proposed that endothelial dysfunction, resulting in inadequate hemodynamic alterations, plays a role in the pathogenesis of HDPs [12]. Due to this uncertain pathogenesis, the available treatment options for HDPs are presently limited.

Known risk factors for HDPs include advanced age at first pregnancy and increasing prevalence of obesity and other cardiometabolic risk factors [2,13]. In recent years, exposure to environmental pollutants, such as perfluorooctane sulfonate (PFOS) during pregnancy, has been linked to unfavorable maternal outcomes, including gestational hypertension, preeclampsia, and fetal growth restriction [14-21]. PFOS is a member of the perfluoroalkyl substances (PFAS) family, comprising approximately 5000 synthetic compounds widely utilized in diverse commercial products and manufacturing processes due to their resistance to extreme temperatures, degradation, and nonstick properties [22,23]. Owing to their extensive usage and stability, these chemicals have become pervasive in the environment and human populations [24]. Despite efforts to phase out PFOS production and reduce exposure, PFOS continues to be detected in most water sources in the US and globally [25,26]. PFOS exposure in humans primarily occurs through drinking water and diet, but it can also arise from sources like house dust, air, cleaning products, and consumer goods [27]. The human half-life of PFOS ranges from 3.3 to 6.9 years [28,29]. PFOS is minimally metabolized [30], poorly eliminated [31,32], and exhibits bioaccumulation in blood and various tissues [33].

PFOS is a reproductive toxicant and also adversely affects cardiovascular function [34-36]. The health-related costs of PFAS exposure were estimated to be $37-59 billion annually in the United States [37]. Studies indicate that PFOS induces vascular injury with significant reductions in the formation and length of intersegmental and posterior cardinal veins while enhancing dorsal aorta vessel formation in hatched zebrafish embryos [38]**.** Furthermore, PFOS exerts proinflammatory effects on human umbilical vein endothelial cells with alterations in actin filament remodeling, increased generation of reactive oxygen species, and disruptions in adhesion junction integrity and endothelial permeability barriers [39-41]. PFOS was shown to decrease vessel formation in a 3D model of human umbilical vein endothelial cells and colon fibroblast co-culture [42] and impedes cell growth, migration, and angiogenesis in human HTR-8/SVneo and JEG-3 cells [43]. Recent studies in pregnant rats demonstrated that PFOS exposure negatively impacts endothelial cell function by reducing endothelial nitric oxide synthase (eNOS) expression and impairing endothelium-dependent vascular relaxation [44]. Additionally, PFOS induces gestational hypertension by causing hypersensitivity and exaggerated vascular contractile responses to angiotensin II (Ang II) [44]. These findings collectively indicate that PFOS affects vascular and endothelial function both *in vivo* and *in vitro*. Identifying a specific PFOS-affected mechanism holds promise for developing preventative and therapeutic strategies. The renin-angiotensin system (RAS) plays a crucial role in regulating blood pressure and blood flow to the uteroplacental unit during gestation [45,46]. Angiotensin II (Ang II), the primary effector of the RAS, exerts its effects through two main receptors, namely AT1R and AT2R. AT1R mediates vasoconstriction and hypertensive effects, while AT2R promotes vasodilation and enhances blood flow [47]. Clinical and experimental research has indicated that increased AT1R levels and decreased AT2R protein levels are associated with the development of

preeclampsia [48,49]. Interestingly, restoring AT2R expression and function in both preeclampsia patients and animal models has been shown to prevent pathological outcomes [48]. Recently, our study demonstrated that exposure to PFOS during pregnancy leads to gestational vascular dysfunction, characterized by reduced AT2R protein expression and increased AT1R levels [44]. Although the dysregulation of AT1R and AT2R expression and function could contribute to gestational hypertension and preeclampsia, the specific role of AT2R in regulating vascular function in the setting of exposure to environmental pollutants like PFOS remains unclear. Also, whether AT2R activation could correct the imbalance in AT1R/AT2R expression in PFOS-exposed dams is unclear. The present study was designed to test the hypothesis that AT2R activation with Compound 21 (C21) restores the AT1R/AT2R balance and reverses gestational hypertension by improving vascular function and vascular contraction and relaxation mechanisms.

2. Results

2.1. Blood Pressure and uterine artery blood flow in pregnant rats

PFOS dams exhibited elevated systolic, diastolic, and mean blood pressure, and administration of AT2R agonist C21 prevented PFOS-induced increase in blood pressure (**Fig 1**; $P \leq .05$; $n = 6$). However, C21 did not significantly impact blood pressure in the control group (**Fig 1**; $P \leq .05$; $n = 6$).

Furthermore, PFOS dams demonstrated a significant reduction in uterine artery blood flow, accompanied by increased resistance and pulsatility indices compared to the control group (**Fig 2**; $P \leq .05$; $n = 6$). Administration of C21 significantly restored uterine artery blood flow and normalized resistance and pulsatility indices to control levels. No significant uterine artery

hemodynamic effects were observed with C21 treatment in the control group (**Fig 2**; P ≤ .05; n = 6).

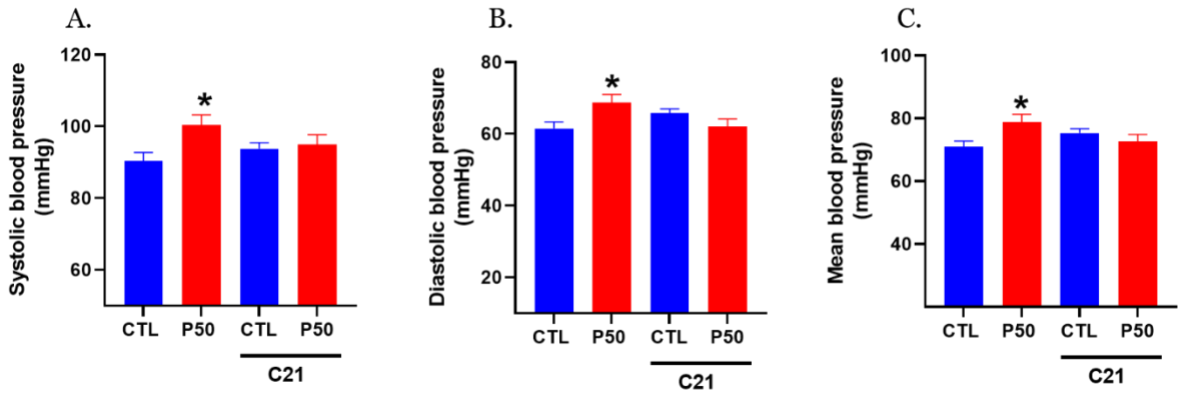

Figure 1. Effect of AT2R agonist C21 treatment on maternal blood pressure. Pregnant rats were exposed to PFOS via drinking water (50 μg/ml) from gestation day 4 to 20. Controls received PFOS-free drinking water. Both control and PFOS-exposed groups were treated with AT2R agonist C21 from GD 15 to 20. On GD 20, (A) systolic, (B) diastolic, and (C) mean arterial blood pressure were measured noninvasively using the CODA system. Data are presented as means ± SEM of 6 rats per group. *$P \leq 0.05$ vs. Control.

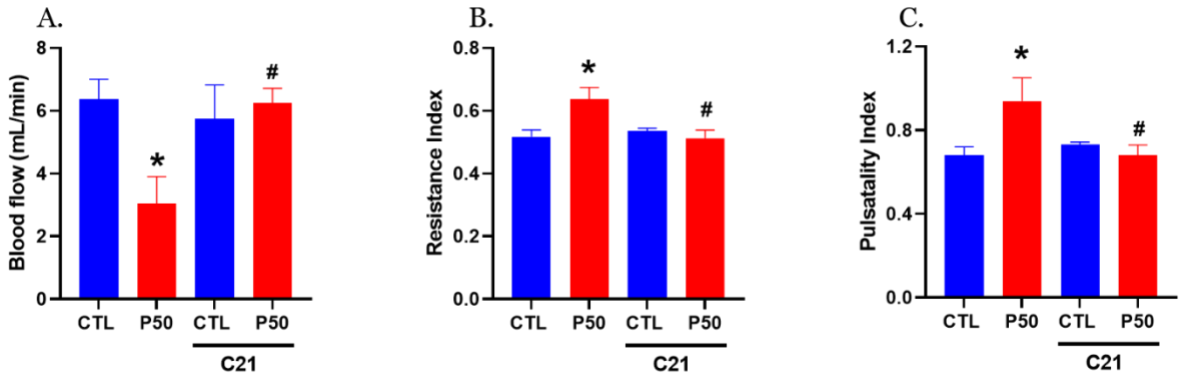

Figure 2. Effect of AT2R agonist C21 treatment on uterine artery hemodynamics. (A) Uterine artery blood flow, (B) resistance index, and (C) pulsatility index were measured using a 30-MHz transducer and Vevo 2100 micro-ultrasound on GD 20 in Control and PFOS dams with

and without C21. Data are expressed as means ± SEM of 6 rats per group. $^{*}P \leq 0.05$ vs. Control. $^{\#}P \leq 0.05$ vs PFOS without C21.

2.2. Vasoconstrictor Response

PFOS dams exhibited greater Ang II-induced contractile responses in endothelium-denuded uterine arteries, characterized by increased sensitivity compared to controls (**Fig 3** and **Table 1**; P ≤ .05; n = 6). However, administration of C21 significantly attenuated the PFOS-induced exaggerated Ang II contraction (**Fig 3** and **Table 1**; P ≤ .05; n = 6). C21 did not elicit any significant effects on Ang II vasoconstriction in controls (**Fig 3** and **Table 1**; n = 6).

The vascular contractile responses to KCl (80 mM), a determinant of depolarization-induced contraction, were similar in PFOS and control dams (**Fig 4**; n = 6). C21 treatment did not induce significant alterations in the KCl-induced contraction in both PFOS and control dams (**Fig 4**; n=6).

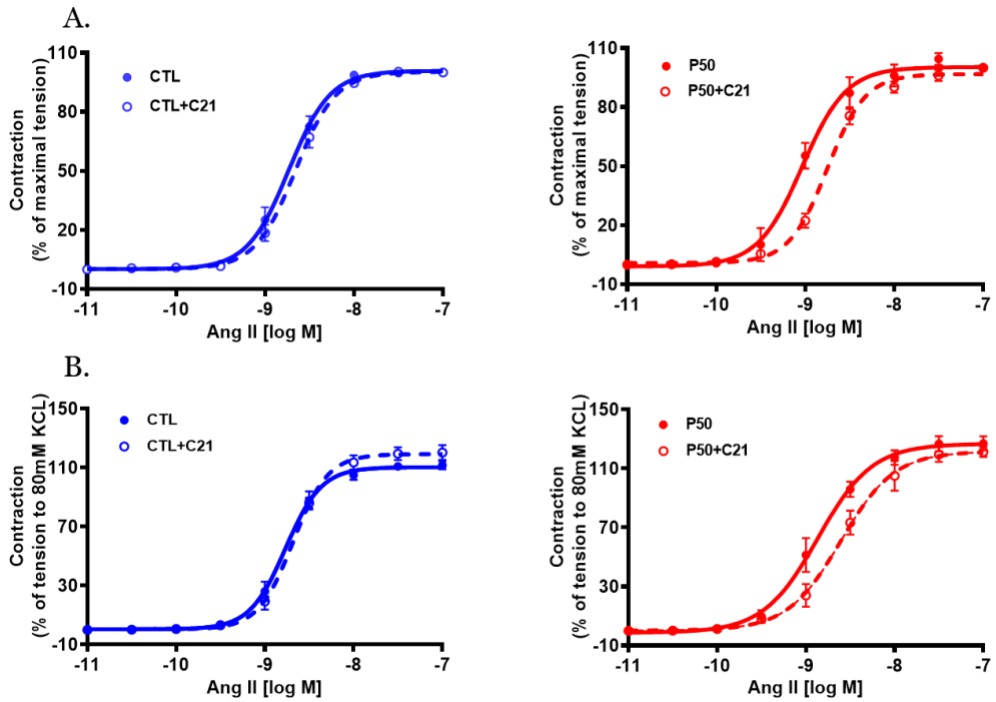

Figure 3. Effect of AT2R agonist C21 treatment on Angiotensin II (Ang II)-mediated uterine artery contractile responses. On gestation day 20, uterine artery rings were isolated from pregnant rats exposed to control conditions and PFOS, both with and without C21 treatment. Vascular contractile responses to cumulative Ang II additions were measured and presented as (A) percentage of maximal contraction and (B) percentage of contraction induced by 80 mM KCl. The data represent means ± SEM of 6 rats per group.

Table 1. Vascular function in Control and PFOS dams with and without C21.

Variable	**Control**	**Control+C21**	**PFOS**	**PFOS+C21**
Ang II pD_2	8.73±0.03	8.65±0.02	9.03±0.05*	8.75±0.02#
Ang II E_{max}	111.76±2.73	120.33±5.09	127.14±4.84	121.39±3.44
ACh pD_2	7.12±0.11	7.26±0.06	6.58±0.07*	7.04±0.12#
ACh E_{max}	98.68±0.73	93.50±2.20	70.90±4.72*	85.01±3.72#
SNP pD_2	6.80±0.06	6.82±0.05	6.84±0.07	6.75±0.03
SNP E_{max}	97.45±4.13	97.79±4.12	96.45±4.64	97.89±4.19

*pD2 (negative log molar concentration that produces 50 % effect) is presented as –log [mol/l] and Emax (maximal responses) is presented as percent of maximal contraction or relaxation. All abbreviations are defined in the text. *P ≤ 0.05 vs. Control. #P ≤ 0.05 vs. PFOS without C21.*

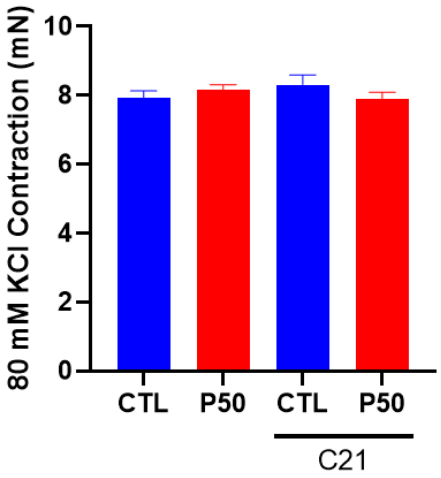

Figure 4. Effect of AT2R agonist C21 treatment on depolarization-induced uterine artery contractile responses to potassium chloride (KCl). On gestation day 20, contractile responses to 80mM KCl were assessed in endothelium-denuded uterine arteries from pregnant rats exposed to control conditions and PFOS, both with and without C21 treatment. The data are presented as means ± SEM of 6 rats per group.

2.3. Vasodilator Response

Acetylcholine (ACh)-induced relaxation was significantly reduced in endothelium-intact uterine arteries with decreased ACh sensitivity and maximal response in PFOS dams than in controls (**Fig 5A** and **Table 1**; $P \leq .05$; n=6). However, C21 restored the decreased ACh relaxation in PFOS dams by increasing ACh sensitivity and maximal relaxation (**Fig 5A** and **Table 1**; $P \leq .05$; n=6). No significant changes in ACh-induced relaxation responses were observed in the control group following C21 treatment **Fig 5A** and **Table 1**; n=6).

The nitric oxide (NO) donor Sodium nitroprusside (SNP) induced concentration-dependent relaxation that was equally potent in control and PFOS dams, with and without C21 treatment (**Fig 5B** and **Table 1**; n=6).

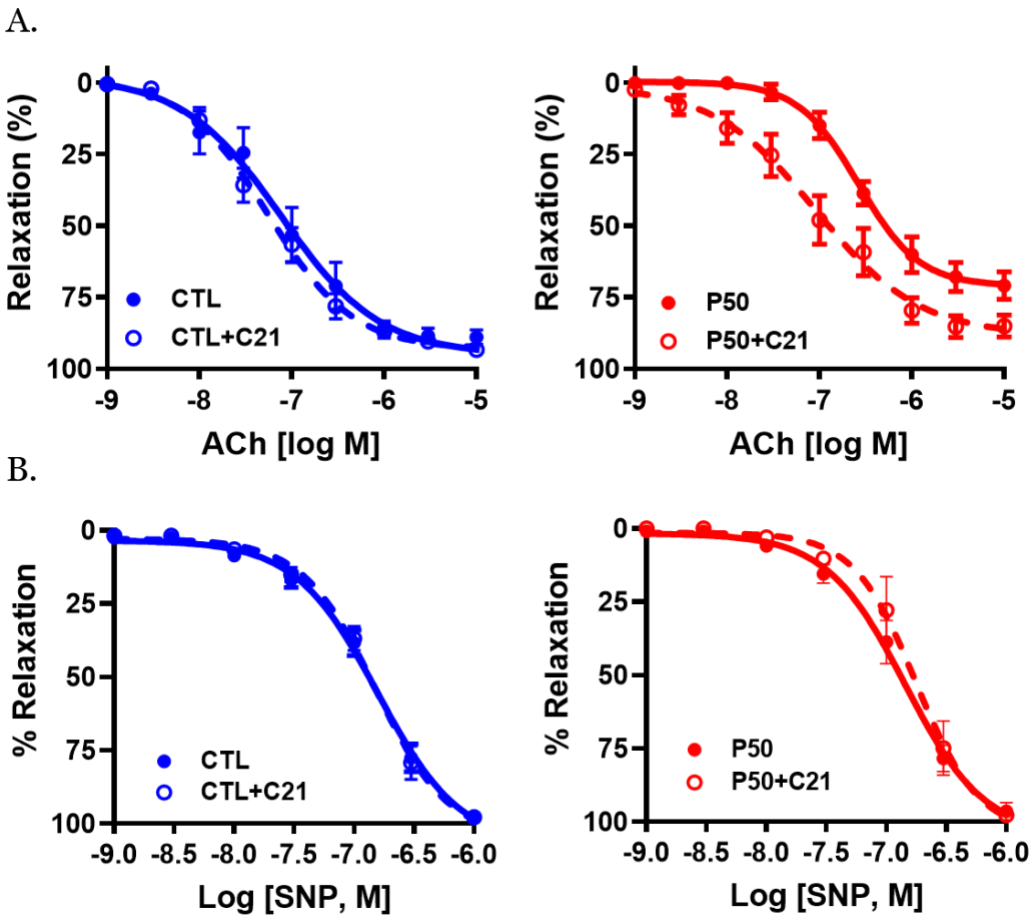

Figure 5. Effect of AT2R agonist C21 treatment on endothelium-dependent vascular relaxation responses. On gestation day 20, uterine artery rings from pregnant rats exposed to control conditions and PFOS, both with and without C21 treatment, were pre-contracted using submaximal phenylephrine. Subsequently, the relaxation responses to cumulative concentrations of (A) acetylcholine (ACh) and (B) sodium nitroprusside (SNP) were examined. The data are presented as means ± SEM of 6 rats per group.

2.4. Ang II receptors and eNOS protein levels

As shown in **Fig 6**, uterine arteries from PFOS dams exhibited increased AT1R protein levels, while AT2R and eNOS protein levels were reduced compared to controls (**Fig 6**; P ≤ .05; n=6). In contrast, C21 administration to PFOS dams decreased AT1R protein levels and increased AT2R and eNOS protein levels, but it did not significantly affect the control group (**Fig 6**; P ≤ .05; n=6).

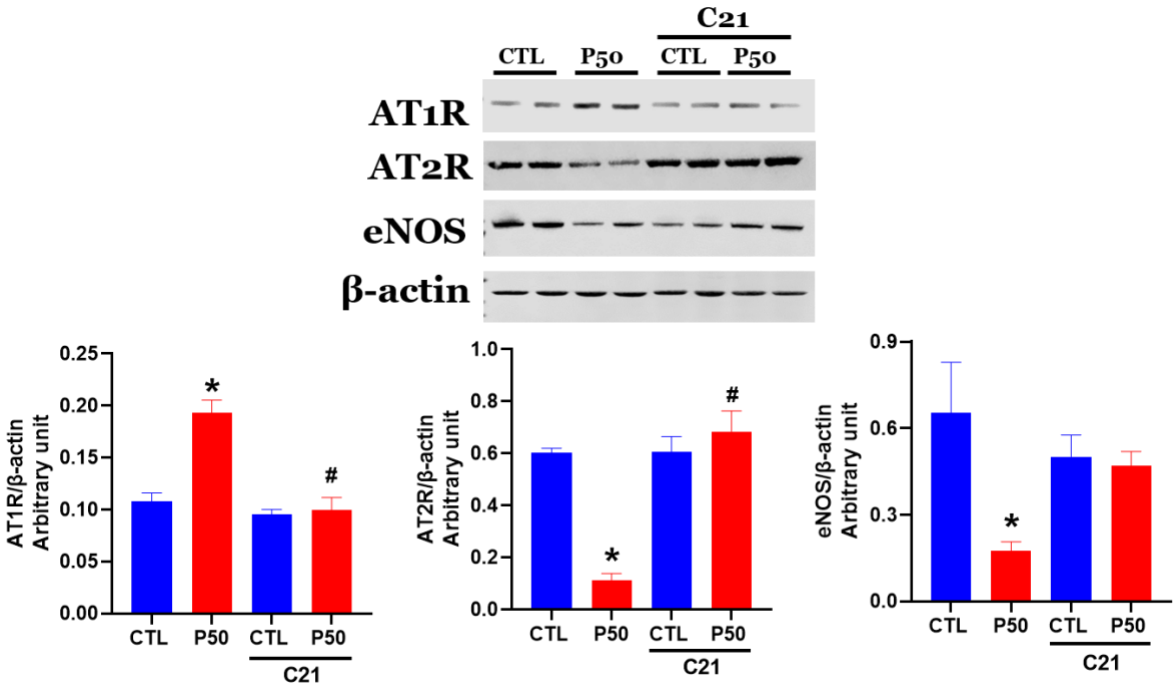

Figure 6. Effect of AT2R agonist C21 treatment on protein expression of Ang II receptors and eNOS in the uterine arteries. Protein expression of AT1R, AT2R, and eNOS in GD 20 uterine artery samples from pregnant rats exposed to control conditions and PFOS, both with and without C21 treatment, were analyzed using Western blotting. The top panel displays representative blots for AT1R, AT2R, eNOS, and β-actin, while the bottom panel shows normalized densitometry data. Data are expressed as means ± SEM of 6 rats per group. ∗P ≤ 0.05 vs. Control. #P ≤ 0.05 vs PFOS without C21.

2.5. Plasma bradykinin levels

As shown in **Fig 7A**, PFOS dams had a significant reduction in plasma bradykinin levels compared to controls ($P \leq .05$; n=6). Conversely, C21 administration ameliorated the PFOS-induced decline in bradykinin levels. The plasma levels of bradykinin remained unaffected in control dams with C21 treatment (**Fig 7A and B**; $P \leq .05$; n=6).

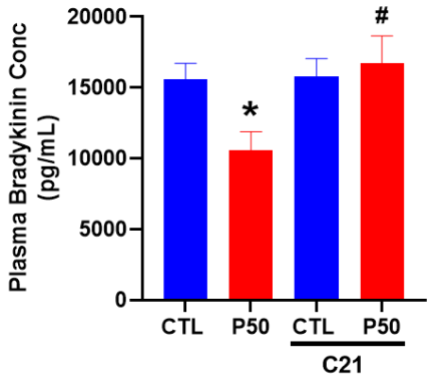

Figure 7. Effect of AT_2R agonist C21 on plasma bradykinin levels. On gestation day 20, blood was collected from pregnant rats exposed to control conditions and PFOS, both with and without C21 treatment, via cardiac puncture following CO_2 inhalation. Bradykinin levels were assessed using an ELISA kit. The data are presented as means ± SEM of 6 rats per group. ∗P ≤ 0.05 vs. Control. #P ≤ 0.05 vs PFOS without C21.

2.6. Placental and fetal weight

As shown in **Fig 8A and B**, elevated maternal PFOS resulted in placental and fetal growth restriction. However, treatment with C21 significantly mitigated the adverse effects of PFOS by rescuing the placental and fetal weight (**Fig 8A and B**; $P \leq .05$; n=6). C21 administration did not significantly impact the placental and fetal weights of control dams (**Fig**

8B and C; P ≤ .05; n=6). Furthermore, C21 treatment did not induce any alteration in the litter size of control and PFOS dams (**Table 2**).

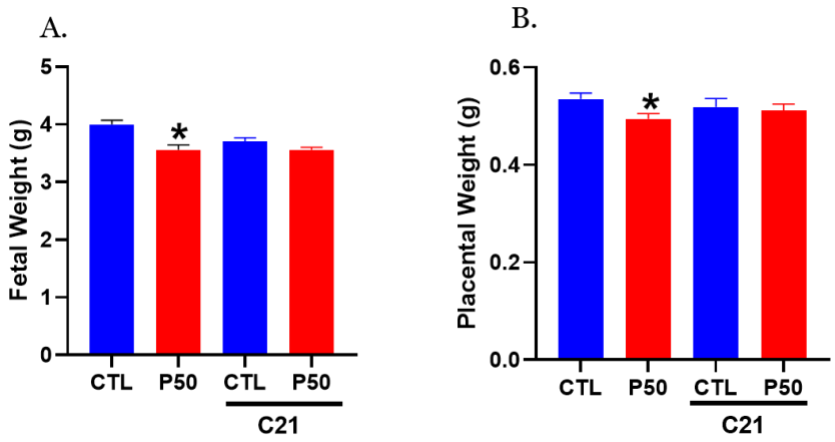

Figure 8. Effect of AT_2R agonist C21 on fetal and placental weights. On gestation day 20, fetal and placental weights were measured in pregnant rats exposed to control conditions and PFOS, both with and without C21 treatment. Placental and fetal weights were averaged for each dam, and each dot represents the mean data per dam/litter. The data are presented as means ± SEM of 6 rats per group. ∗P ≤ 0.05 vs. Control.

Table 2. Litter size and fetal sex-ratio in control and PFOS dams with and without C21.

	Control	**PFOS**	**Control+C21**	**PFOS+C21**
Litter size	12.3 ± 2.6	11.9 ± 1.8	13.4 ± 0.37	13.4 ± 0.91
Sex ratio (percent males per litter)	47 ± 3.9 %	50 ± 4.5 %	48 ± 4.6 %	44 ± 4.2 %

3. Discussion

The major findings of the current study are as follows: (1) Administration of the AT2R agonist C21 to PFOS-exposed dams effectively mitigated PFOS-induced hypertension in pregnant rats by improving uterine artery blood flow and attenuating Ang II-mediated vascular contraction. Furthermore, C21 administration enhanced endothelial-dependent vascular relaxation in PFOS-exposed dams. (2) The suppression of Ang II-mediated vascular contraction by C21 in PFOS-exposed dams was correlated with decreased AT1R receptors and increased AT2R receptors within the uterine artery. (3) The enhanced endothelial-dependent relaxation response observed in C21-treated PFOS dams was associated with elevated eNOS expression in the uterine artery with increased plasma bradykinin levels. (4) C21 treatment also improved feto-placental growth in PFOS-exposed dams, likely attributed to improved vasodilation and enhanced uterine artery blood flow. These findings represent a significant and novel contribution, suggesting that activation of the AT2R receptor by C21 attenuates PFOS-induced hypertension, enhances endothelial-mediated vascular function, and improves feto-placental growth in pregnant rats exposed to PFOS.

Emerging evidence substantiates a link between maternal exposure to PFOS and various detrimental maternal outcomes, including gestational hypertension, preeclampsia [14-21], and decreased birth weight [50-58]. Furthermore, our recent study revealed that PFOS exposure dose-dependently increased mean arterial pressure in pregnant Sprague Dawley rats [44]**.** In the present study, we observed elevated blood pressure in pregnant rats exposed to PFOS, consistent with previous studies [44,59]. However, the administration of the AT2R agonist C21 to PFOS-exposed dams effectively prevented the blood pressure increase, suggesting that C21 may have the ability to attenuate PFOS-induced hypertension. This finding aligns with earlier studies

wherein C21 demonstrated the restoration of blood pressure in various hypertensive models, including testosterone-induced hypertension in pregnant Sprague Dawley rats [48], Ang II-induced hypertension in non-pregnant female Sprague Dawley rats [60], and salt-induced hypertension in male obese Zucker rats [61]. Nevertheless, our study represents the first to demonstrate the potential of AT2R activation by C21 in alleviating hypertension caused by environmental chemical exposure, such as PFOS.

During pregnancy, maternal vascular adaptations are crucial in increasing uterine artery blood flow to meet the metabolic demands of the developing placenta and fetus [62,63]. In the present study, exposure to PFOS decreased uterine artery blood flow and increased resistance and pulsatility indices on GD20, which aligns with our recent investigation [44]. Reduced uterine artery blood flow and increased vascular resistance have been associated with adverse outcomes such as preeclampsia and fetal growth restriction [64,65]. In our study, administration of C21 to PFOS-exposed dams improved uterine artery blood flow and restored the resistance and pulsatility indices. The potential of C21 to enhance blood flow has also been observed in other studies, wherein C21 increased uterine blood flow in hyperandrogenic pregnant rats [48] and enhanced renal blood flow in male and female ten-week-old Sprague Dawley rats [66]. Uterine vascular remodeling and placental angiogenesis are critical processes involved in establishing a "low resistance, high capacitance vessel" capable of augmenting uterine blood flow [67,68]. Notably, significant changes occur in uterine spiral and placental arteries, including increased branching, diameter, and total area, which contribute to enhanced uterine blood flow [69]. Exposure to PFOS is reported to inhibit angiogenesis in the placenta [70] and in human umbilical vein endothelial cells [42] and reduce placental vascular density [71]. In contrast, C21 has been shown to induce angiogenesis and upregulate multiple angiogenic proteins [72]. Therefore, it is

plausible to suggest that C21 treatment in PFOS-exposed dams may improve angiogenesis and enhance placental vascularization, ultimately increasing uterine artery blood flow. However, further investigations are necessary to elucidate the specific role of C21 in vascular remodeling during PFOS exposure.

We investigated uterine artery function to elucidate the potential vascular mechanisms underlying the observed decrease in blood pressure and increase in uterine artery blood flow associated with C21 treatment in PFOS-exposed dams. The increased blood pressure observed in PFOS dams was accompanied by enhanced vasoconstriction in response to Ang II, resembling the Ang II hyperreactivity observed in hypertensive pregnancies [44]. However, administration of C21 to PFOS dams in our study effectively reversed the exaggerated vasoconstriction in response to Ang II, restoring it to levels comparable to those observed in Control pregnant rats supporting the involvement of AT2R activation in restoring vascular function in PFOS-exposed dams. Importantly, the response to KCl, which induces contraction through membrane depolarization, did not differ significantly between endothelium-denuded uterine arteries with and without C21 treatment. This suggests that the attenuated Ang II vasoconstriction observed in PFOS dams treated with C21 is more likely attributed to changes in Ang II receptors rather than generalized nonreceptor-mediated alterations, such as hypertrophy or hyperplasia of vascular smooth muscle cells. Supporting this notion, uterine arteries from PFOS dams exhibited increased protein abundance of AT1R and decreased protein abundance of AT2R, while C21 treatment successfully restored the balance of angiotensin receptors in the uterine arteries of PFOS-exposed dams. The exact mechanism by which AT2R activation decreases AT1R abundance is still not fully understood, but several animal studies suggest the involvement of a complex cross-regulatory mechanism between AT1R and AT2R [48,73-75]. For instance, in both

in vitro and *in vivo* investigations, it has been observed that AT2R stimulation has the capacity to influence AT1R expression, suggesting the existence of intricate cross-regulatory mechanisms between these receptors. Specifically, AT2R stimulation or overexpression has been found to inhibit AT1R expression and signaling [74,75]. In AT2R knockout mice, AT1R expression was elevated in vascular tissues compared to control mice [75]. Moreover, introducing the AT2R gene into rat vascular smooth muscle cells resulted in the inhibition of AT1R-mediated tyrosine phosphorylation of signal transducers and activators (STAT) [74]. Conversely, blocking AT1R led to an upregulation of AT2R expression in vascular smooth muscle cells, suggesting a reciprocal regulation by AT1R [76]. Furthermore, in endothelial cells transfected with the AT2R promoter, AT1R stimulation attenuated AT2R expression [77]. Therefore, the attenuation of exaggerated Ang II vasoconstriction in PFOS dams treated with C21 may be due to the decreased abundance of vasoconstrictive AT1R receptors in uterine arteries of C21-treated PFOS dams.

In order to investigate the effects of C21 on endothelial function, we assessed the endothelium-dependent relaxation response to ACh. Notably, ACh-induced relaxation was reduced in the uterine arteries of PFOS dams, indicating impaired endothelial Control of vascular tone, which is consistent with a previous study [44]**.** Intriguingly, C21 treatment enhanced ACh-induced relaxation in the uterine arteries of PFOS dams while exerting minimal effects in control dams. These findings suggest that the vascular relaxation responses to ACh are preserved in the presence of C21 treatment. Importantly, the relaxation response to the NO donor SNP did not differ significantly between the C21-treated PFOS dams and PFOS dams, indicating that the observed differences were not related to the vasodilatory capacity of smooth muscle but rather to endothelial function. Consistently, previous studies have demonstrated that C21 improves

endothelium-dependent NO-mediated relaxations in spontaneously hypertensive and hyperandrogenic rats [48,78]. These results suggest that AT2R activation may enhance NO synthesis by endothelial cells. This concept is supported by the observation that the levels of eNOS protein were increased in the uterine arteries of C21-treated PFOS dams. Although the exact mechanism by which C21 triggers eNOS expression is not fully understood, previous reports have suggested that C21 can directly stimulate eNOS expression in placental arteries [48]. Moreover, in a mouse model of diet-induced obesity, C21 preserved eNOS levels through PKA/p-eNOS and AKT/p-eNOS signaling pathways [79]**.** It was interesting to note that PFOS exposure decreased plasma bradykinin levels. The precise mechanism by which PFOS reduces bradykinin levels remains unclear. However, activation of AT2R increased bradykinin production, which is consistent with previous reports [48,79]**.** As bradykinin is known to induce vasodilation through increased production of NO, prostacyclin, and endothelium-derived hyperpolarizing factors, the improvement in vasodilation and uterine artery blood flow observed in the C21-treated PFOS dams may also be attributed to the increase in bradykinin levels [80-82]. Therefore, the present study provides evidence supporting the role of AT2R activation in preserving endothelium-dependent vasodilation in PFOS-exposed dams.

The association between PFOS exposure and adverse outcomes, including fetal growth restriction and low birth weight, has been consistently observed in both human [50-58] and animal studies [59,83-90]. In our present study, treatment with C21 significantly improved the weights of both the placenta and fetus in PFOS-exposed dams. These findings suggest that the beneficial effect of C21 treatment in PFOS dams may be attributed, at least in part, to the improvement in vascular function and enhanced uterine artery blood flow. Studies show that

PFOS exposure decreases transplacental glucose and amino acid transport to the fetus [91]. It would be interesting to examine if AT2R activation improves nutrient availability in the fetus.

In summary, this study concurs with the previous report that PFOS exposure during pregnancy disrupts endothelial function, resulting in hypertension in rats by suppressing AT2R-mediated vasodilation [44]. This study provides new information that activation of AT2R using a pharmacological agonist (C21) in PFOS-exposed dams restores the balance of Ang II receptors, leading to optimal blood pressure, enhanced uterine artery blood flow, reduced Ang II vasoconstriction, improved endothelial-mediated relaxation, and enhanced feto-placental growth. It is important to note that the present findings specifically highlight the mitigatory effect of C21 on vascular hemodynamics in PFOS-exposed dams, and caution should be exercised in generalizing these results to other PFOS-induced adverse outcomes. Future investigations should explore the potential of C21 or other AT2R agonists in reversing additional PFOS-related complications. Nonetheless, these results suggest that augmenting AT2R activity through pharmacological agonists holds promise as a preventive or therapeutic strategy for managing gestational hypertension and fetal growth restriction associated with PFOS exposure.

4. Materials and Methods

4.1. Animals

All animal procedures were conducted in accordance with the guidelines set by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and were approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee (protocol# V005847). Timed-pregnant Sprague-Dawley rats, aged twelve weeks (with a positive plug indicating gestation day (GD) 1), were obtained from Envigo Laboratories (Indianapolis, IN) on

GD 3. They were housed in a controlled environment with regulated temperature and a 12:12-hour light-dark cycle. The rats were exposed to PFOS at a concentration of 50 μg/mL (CAS #2795-39-3, Sigma Aldrich, St. Louis, MO) through their drinking water from GD 4 to GD 20. As a control group, other rats received drinking water without detectable PFOS. The United States Environmental Protection Agency (US EPA, 2016) established a health advisory limit of 70 parts per trillion (ppt) for PFOS in drinking water [92]. The PFOS exposure at 50 μg/ml used in this study is roughly five times higher than this advisory limit, reflecting exposure levels observed in heavily contaminated regions or occupational settings [86,93,94]. Additionally, this concentration is commonly utilized for testing the effects of PFOS on pregnancy and fetal development [44,84,95]. A subset of Control and PFOS-exposed rats were treated with an AT2R agonist, Compound 21 (C21; VicorePharma, GÖteborg, Sweden), at a dose of 0.3 mg/kg/day subcutaneously from GD 15 to 20. The choice of the C21 dosage was informed by previous studies [96,97], which showed its effectiveness in lowering blood pressure and improving complications associated with hypertension, particularly in hypertensive rats, without affecting control rats. Furthermore, Bosnyak et al. (2011) [98] underscored the striking 4000-fold higher selectivity of C21 for AT2R relative to AT1R, highlighting its potential as a candidate for antihypertensive therapy. On GD 20, blood-pressure measurements and uterine artery ultrasounds were performed on the rats. Subsequently, the rats were euthanized using CO_2 inhalation. Blood samples were taken via cardiac puncture into heparinized vacutainers to obtain plasma. The uterine arteries were collected for vascular reactivity studies and protein isolation. Additionally, the weights of feto-placental units were measured.

4.2. Blood Pressure

At GD 20, blood pressures were noninvasively measured using a tail-cuff method (Kent Scientific, Torrington, CT), as previously described [99,100]. Prior to GD 20, rats were acclimated to the restraint warming chamber for 15 minutes daily for two consecutive days. On the day of blood-pressure measurements, rats were placed in the restraint warming chamber set at 30°C and allowed to rest for 10 minutes to promote dilation of peripheral blood vessels and enhance blood flow to the tail. An occlusion cuff and a volume pressure-recording cuff were applied to the base of the tail. The cuff was programmed to inflate and deflate automatically within a 90-second cycle. Blood pressure was recorded and analyzed using Kent Scientific software. The first five inflation cycles were used for acclimation, and the subsequent five cycles' average was considered as the individual mean blood pressure for each rat.

4.3. Uterine Artery Ultrasound

On GD19, rats were anesthetized with 2% isoflurane in oxygen and placed on a heated platform for ultrasound imaging. The uterine arteries were examined using a 30-MHz transducer and the Vevo 2100 ultrasound system (Visual Sonics, Toronto, ON, Canada), following established procedures [101]. Briefly, the velocities of the main uterine artery were recorded below the bladder and at the point where the main uterine artery branches from the internal iliac artery. From three consecutive cardiac cycles, peak systolic velocity (PSV), end-diastolic velocity (EDV), the area under the peak velocity-time curve, and the R-R interval was measured. The results from these measurements were averaged. To determine the blood flow velocity distribution, the following formula was used: $F = \frac{1}{2} MV\pi (D/2)2$, where MV represents the mean peak velocity over the cardiac cycle (in cm/s), D stands for the diameter (in cm), and F represents the blood flow (in mL/min). To assess the pulsatility of blood velocity waveforms, the

Uterine Artery Resistance Index (RI) was calculated as (PSV-EDV)/PSV, and the Pulsatility Index (PI) was calculated as (PSV-EDV)/MV.

4.4. Ex-vivo Vascular Reactivity Studies

The main uterine artery was carefully excised and freed from any surrounding connective tissues. Arterial ring segments, each measuring 2 mm in length, were then mounted on a wire myograph (Danish Myo Techniques, Aarhus, Denmark) using tungsten wires to enable the recording of isometric tension. Arterial rings were immersed in Krebs physiological solution (KPS) at a temperature of 37°C and gassed with a 95% O2 / 5% CO_2 gas mixture, resulting in a pH of 7.4. The KPS consisted of the following components: NaCl, 118 mM; KCl, 4.7 mM; CaCl2, 2.5 mM; MgSO4, 1.2 mM; KH2PO4, 1.2 mM; NaHCO3, 25 mM; and glucose, 11.1 mM. The rings were allowed to equilibrate in the KPS solution for one hour under resting tension. Subsequently, the arterial rings were normalized using a specialized software package called Myodata from Danish Myotechnology. In the case of endothelium-intact arterial rings, extra precautions were taken to prevent any damage to the endothelial layer. For endothelium-denuded arterial rings, the endothelial layer was gently removed by rubbing the interior of the ring with tungsten wire. The successful removal of the endothelial layer was assessed by the absence of relaxation response to acetylcholine (ACh) in arterial rings that were pre-contracted with a submaximal concentration of phenylephrine (PE).

4.4.1. Assessment of Vascular Contractile Responses

The arterial rings were subjected to an 80mM potassium chloride (KCl) solution until consistent contractions, induced by depolarization, were observed. Following a subsequent round

of washing and equilibration with KPS, the vascular contractile responses were assessed by exposing the rings to increasing cumulative doses of PE ranging from 10^{-9} to $3x10^{-5}$ M, as well as Ang II at concentrations ranging from 10^{-11} to 10^{-7} M.

4.4.2. Assessment of Vascular Relaxation Responses

To evaluate endothelium-dependent relaxation, the response to ACh was measured in arteries pre-contracted with PE, using ACh concentrations ranging from 10^{-9} to 10^{-5} M. For assessing endothelium-independent relaxation, the response to sodium nitroprusside (SNP; 10^{-9} to 10^{-6} M) was measured in arteries without endothelium, which was also pre-contracted with PE. The concentration of PE that induced 80% of the maximal response (pEC80) was utilized for precontraction purposes.

4.5. PLASMA BRADYKININ LEVELS

Plasma bradykinin concentrations were quantified using an enzyme immunoassay kit (Enzo Life Sciences, ADI-900-206, Farmingdale, NY, USA) following the manufacturer's instructions. The assay's detection range spanned from 11.7 to 30,000 pg/ml. For each sample, 100 µl of plasma was used in duplicate for the analysis.

4.6. WESTERN BLOTTING

Arteries were homogenized in ice-cold radioimmunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA) containing a protease inhibitor tablet (Roche, Indianapolis, IN) and phosphatase inhibitor cocktail-2 and -3 (Sigma). Following centrifugation at 14,000 g for 10 minutes at 4°C, the supernatant was aliquoted to measure the concentration of protein (Pierce BCA protein assay kit, Thermo Scientific, Waltham, MA). The supernatant was then re-

suspended in NuPAGE® sample buffer and reducing agent (Invitrogen; Thermo Scientific, Waltham, MA). A total of 30 μg of proteins, along with Precision Plus Standard (Kaleidoscope, Bio-Rad), were loaded into wells on 4% to 12% gradient NuPAGE® Bis-Tris Gels (Invitrogen). Electrophoresis was conducted at 100 V for 2 hours at room temperature, and then the proteins were transferred onto Immobilon-P membranes (Millipore Inc, Billerica, MA) using a mini Blot Module (Invitrogen) at 20 V for 1 hour. The membranes were blocked (5% skim milk) for an hour and then incubated overnight at 4°C with primary antibodies, including AGTR1 (rabbit polyclonal, SAB2100073, 1:1000; Sigma, Burlington, MA), AGTR2 (rabbit monoclonal, ab92445, 1:1000; Abcam, Cambridge, MA), eNOS (rabbit monoclonal, #32027, Cell Signaling Technologies, Danvers, MA), and β-actin (rabbit monoclonal, #4070, 1:5000; Cell Signaling Technologies). After washing, the membranes were treated with secondary antibodies (anti-rabbit conjugated with horseradish peroxidase) for 1 hour and detected using Pierce enhanced chemiluminescence detection kits (Thermo Scientific, Waltham, MA). Densitometric analysis was done with Image J software. The results were normalized and expressed as ratios of the intensity of a specific band to that of β-actin.

4.7. Placental and Fetal weights

Feto-placental units were extracted from the uterus, and the fetuses were sorted into male and female groups based on their anogenital distance. The corresponding placenta was also separated according to the fetal sex. Any excess fluid was carefully blotted from both the fetuses and placentas. Subsequently, the wet weights of the fetuses and placentas were measured using an electronic scale with an accuracy of ± 0.1 mg (Mettler Instrument Corp, Model AE50, Hightstown, NJ).

4.8. Statistical Analysis

Statistical analyses were done using Prism software (GraphPad, San Diego, CA). The data were presented as the mean ± standard error of the mean (SEM). Two-way analysis of variance tests were conducted, followed by Tukey's multiple comparisons tests. Cumulative concentration-response curves were analyzed using a four-parameter sigmoidal curve fitting approach. Contraction responses to PE were expressed as a percentage of its maximal contraction and as percent of 80 mM KCl contraction. Relaxant responses to ACh and SNP were expressed as a percentage of relaxation from the PE-induced contraction. Statistical significance was considered at a P-value of less than 0.05.

Author Contributions:

S.D., B.B., P. Y., and J.M. participated in concept development and study design, performed experimental work, data analysis and drafted the manuscript; R.S. and A. H. performed experimental work; S.K. conceived the conception and study design, obtained funding, provided important intellectual support, and edited the manuscript. All authors have seen and approved the final version.

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Chapter 4

PERFLUOROOCTANE SULFONIC ACID MODULATES EXPRESSION OF PLACENTAL STEROIDOGENESIS-ASSOCIATED GENES AND HORMONE LEVELS IN PREGNANT RATS

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ABSTRACT

Perfluorooctane sulfonate (PFOS) is a widespread and persistent chemical in the environment. Reports show that PFOS is a potential endocrine disruptor; however, the possible effects of PFOS on placental endocrine function are unclear. This study aimed to investigate the endocrine-disrupting effects of PFOS on the placenta in pregnant rats and its potential mechanism. Pregnant rats from gestational days 4 to 20 were exposed to 0, 10, and 50 μg/mL PFOS through drinking water followed by analysis of various biochemical parameters. PFOS dose-dependently decreased fetal and placental weight in both sexes, with a specific decrease in weight of labyrinth but not junctional layer. Plasma progesterone (↑166%), aldosterone (↑201%), corticosterone (↑205%), testosterone (↑45%), luteinizing hormone (↑49%) levels were significantly increased, while estradiol (↓27%), prolactin (↓28%) and hCG (↓62%) levels were reduced in groups exposed to higher doses of PFOS. Real-time quantitative reverse transcriptase-polymerase chain reaction analysis revealed a significant increase in mRNA levels of placental steroid biosynthesis enzymes, including Cyp11A1 and 3β-HSD1 in male placenta and StAR, Cyp11A1, 17β-HSD1 and 17β-HSD3 in female placenta of PFOS dams. Cyp19A1 expression in ovaries was significantly decreased in PFOS dams. mRNA levels for placental steroid metabolism enzyme UGT1A1 increased in male but not in female placenta of PFOS dams. These results suggest that the placenta is a target tissue of PFOS and PFOS-induced dysregulation in steroid hormone production might be related to the altered expression of hormone biosynthesis and metabolism enzyme genes in the placenta. This hormone disruption might affect maternal health and fetal growth.

1. INTRODUCTION

Per- and poly-perfluoroalkyl substances (PFAS) are a group of more than 5000 synthetic chemicals widely used in various consumer and industrial products due to their unique properties, such as water and oil repellence, heat resistance, and stability [1]. Human exposure to PFAS occurs through several routes, including drinking water, intake of PFAS-contaminated fish and foods, and dermal contact with PFAS products [2]. Additionally, occupational exposure and inhalation of PFAS-contaminated indoor air and dust also contribute to human exposure [2]. PFAS are non-degradable, highly persistent in the environment and can bioaccumulate in the human body with estimated half-lives of 3 to 9 years [3]**.** Among the many PFAS, perfluorooctane sulfonic acid (PFOS) is one of the most frequently detected and highly concentrated contaminants in aquatic and terrestrial environments [4]. PFOS has been found in drinking water in many regions of the USA. According to the Environmental Protection Agency's (EPA) study, higher PFOS levels were found in 194 US public water systems that serve about 16.5 million people in 36 states and territories [5]. Another study reported that 6 million people served by 66 public water supplies have PFOS in the range of 1800 ng/L [6]. As per the survey of waterways across the US, PFOS was found in up to 83% of streams and rivers [7]. Studies have shown the prevalent exposure of the general population to PFOS. PFOS has been detected in many human matrices, including blood, urine, breast milk, and amniotic fluid [8-11]. The Center for Disease Control (CDC) Survey indicated that more than 98% of US residents had detectible PFOS in their blood [12]. PFOS was detected in 100% of blood samples in 359 pregnant women collected from California between December 2015 and February 2019 [13]. Given that pregnant women and fetuses are highly sensitive to hazardous environmental substances [14, 15], the potential health effect of PFOS exposure during pregnancy has become a growing concern.

Reproductive hormones are important for pregnancy because they play pivotal roles in the growth and functioning of a broad range of maternal and fetal tissues. Both animal and in vitro studies suggest that PFOS is an endocrine disruptor with impacts on reproductive hormones. High doses of PFOS reduced serum estradiol levels in adult female mice [16] and male monkeys [17] and increased estradiol levels in juvenile zebrafish [18]**.** PFOS exposure in male mice reduced serum testosterone levels [19]. *In vitro* study reported that PFOS decreases the secretion of hCG, progesterone, estradiol, and androstenedione in human granulosa cells and placental syncytiotrophoblasts [20, 21]. *In vitro* bioassays show that PFOS exhibit weak estrogenic and androgenic activity [22, 23]. Conversely, an *in vitro* study reported that PFOS exhibited anti-androgenic activity in breast cells [24]. Molecular docking studies suggest that PFOS binds to human estrogen receptor α (ERα) [25]. Human studies also show that maternal PFOS exposure is associated with disrupted reproductive hormone levels in 6–9 year old young children [26] and 12–17 year old adolescents [27, 28]. However, a limited number of studies investigated the impact of PFOS on hormone levels during pregnancy and the underlying mechanisms.

The initial stage of steroidogenesis is characterized by the conversion of cholesterol into pregnenolone, facilitated by the cholesterol side-chain cleavage enzyme (CYP11A1) [29]. Subsequently, pregnenolone is converted into either dehydroepiandrosterone (DHEA) or progesterone, mediated by steroid 17α-hydroxylase/17,20-lyase (CYP17A1) or 3β-hydroxysteroid dehydrogenase/δ5-4-isomerase type 1 (HSD3B1), respectively. Progesterone serves as a precursor for synthesizing corticosterone and aldosterone, which are facilitated by 18-hydroxylase (CYP11B1)/18-oxidase (CYP11B2) and aldosterone synthase, respectively. Androgens, including testosterone, are synthesized from DHEA and progesterone with the aid of

17β-dehydrogenase 3 (HSD17B3) and HSD3B1 [30]. In the final step, aromatase (CYP19A1) and HSD17B3 catalyze the conversion of androgens into estradiol. The expression of genes encoding the proteins involved in steroidogenesis, as well as their activity, are regulated by peptide hormones such as gonadotropins, including luteinizing hormone (LH) and follicle-stimulating hormone (FSH).

The placenta is an important endocrine organ that synthesizes many hormones, which play important roles in pregnancy maintenance and fetal development. Studies have shown that placenta is a common target of PFOS as it bioaccumulates PFOS [31-33]. *In vitro* studies have shown that PFOS may influence steroidogenic enzyme activities and expressions in different cell types (adrenocortical carcinoma and testicular cells) [34-36]. Nevertheless, until now, no studies have explored the potential effects of PFOS on the placenta *in vivo* to understand the mechanism underlying the change in endocrine hormone levels that results from PFOS exposure. The aims of this study were to investigate the endocrine-disrupting effects of PFOS on the placenta in pregnant rats and to evaluate the mechanism of these effects by exploring the possible involvement of genes associated with hormone biosynthesis and metabolism.

2. Materials and Methods

2.1. Animals

All animal procedures were carried out per the US National Institutes of Health guidelines (NIH Publication No. 85–23, revised 1996) with approval by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison (protocol # V005847). Twelve-week-old timed-pregnant Sprague-Dawley rats (positive plug = gestation day (GD) 1) were obtained on GD 3 from Envigo Laboratories (Indianapolis, IN) and housed individually in a

room with controlled temperature and a 12:12-h light-dark cycle. The rats were randomly divided into 3 groups, exposed either to standard drinking water with no detectable levels of PFOS (control group; n=8) or standard drinking water containing PFOS potassium (CAS #2795-39-3, Sigma Aldrich, St. Louis, MO) at 10 μg/mL (P10 group; n=8) and 50 μg/mL (P50 group; n=8) from GD 4 to GD 20. The selection of PFOS doses (10 μg/ml and 50 μg/ml) was based on prior studies that reported decreased neonatal weight gain [37, 38]. The low dose of 10 μg/ml corresponds to a daily exposure of 1 mg/kg/bodyweight/day PFOS (based on average daily water consumption of 25 ml/day or 10 ml/100 g body weight/day) that was used in the rat developmental toxicity study of Luebker et al. in 2005 [38] to establish the U.S. EPA's drinking water lifetime health advisory level (HAL) of 70 ppt PFOS (US EPA 2016) [39]. The high dose of 50 μg/ml is approximately 5 times higher than the HAL and corresponds to PFOS exposures found in heavily contaminated regions or in occupational settings [37, 40, 41]. In general, toxicity studies in animals have utilized PFOS doses significantly higher than those anticipated in human exposures, with the aim of detecting potential adverse effects and establishing dose-response relationships for evaluating such effects at lower exposures. Rats were fed with a standard breeder chow (D15092401; Research Diets, New Brunswick, NJ) *ad libitum*. On GD 20, rats were euthanized by CO_2 inhalation. Blood was collected by cardiac puncture, and plasma was separated and stored at -80° C until measurement of hormone levels. We collected fetuses placentas from both male and female subjects from the same dams to investigate the potential differential effects of PFOS on placental health. Fetuses and placentas were removed from each uterine horn and excess fluid was blotted from the fetuses before their wet weights were measured with an electronic scale that had an accuracy of ± 0.1 mg (Mettler Instrument Corp, Model AE50, Hightstown, NJ). The placentas were also weighed after the membranes were

trimmed, and were then dissected into junctional and labyrinth zones using a previously described method [42], and the weights of each zone were individually recorded. The two zones can be discerned based on their color, with the labyrinth appearing darker red than the junctional zone. Separation of the two layers involved the use of two forceps, one for grasping below the junctional zone layer and another for sliding along the tines of the first forceps to excise the tissue above it. Three junctional zone placentas from each sex per dam were pooled and processed for mRNA isolation and qRT-PCR analysis. To examine any sex-specific differences in response to PFOS, we conducted comparisons within each sex rather than between sexes (i.e., male vs female). This approach allowed us to thoroughly assess the potential sex-dependent effects of PFOS on placental function and better understand any gender-specific responses. Additionally, ovaries from dams were also collected and processed for mRNA isolation and qRT-PCR analysis.

2.2. STEREOLOGICAL ANALYSIS OF PLACENTA

Placentas were isolated and fixed by overnight immersion in 10% (vol/vol) neutral buffered formalin (ThermoFisher Scientific, Newark, DE). Tissues were dehydrated, embedded in paraffin wax, cut into 4μm serial cross-sections, and mounted on glass slides. After deparaffinization using xylene, sections were rehydrated and stained with hematoxylin and eosin using standard procedure. For each sample, three sections were analyzed, and two placentas per sex per dam were examined. The images were captured using a Keyence BZ-X700 microscope (Keyence Corporation, Itasca, IL). The junctional and labyrinth zone areas relative to the total placental area were quantified using the Image J software (National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/).

2.3 PLASMA HORMONE LEVELS

Concentrations of plasma follicle-stimulating hormone (FSH), luteinizing hormone (LH), hyperglycosylated chorionic gonadotropin (hCG), prolactin, progesterone, corticosterone, corticosterone binding globulin (CBG), aldosterone, testosterone, and estradiol were measured using ELISA kits (**Table 1**) according to the manufacturer's protocols. The hormone levels were quantified using ELISA kits that have been validated and employed in multiple scientific publications. The kits' sensitivity and CVs are as per the manufacturer's specifications.

Table 1. Details of ELISA kits used for hormone analysis.

Hormone	**Catalog no**	**Company**	**Sensitivity**	**Intra-assay; inter-assay variation**
FSH	MBS2502190	MyBioSource	1.88 ng/mL	> 10%; > 10%
LH	ENZ-KIT107	Enzo life sciences	5.2 mIU/mL	> 10%; > 20%
hCG	MBS1600315	MyBioSource	0.22 mIU/mL	< 8%; < 10%
Prolactin	MBS727546	MyBioSource	1.0 ng/mL	> 10%; > 10%
Progesterone	582601	Cayman	10 pg/mL	> 15%; > 20%
Corticosterone	ab108821	Abcam	0.32 ng/mL	> 10%; > 15%
CBG	EKU03464	Biomatik	6.9 ng/mL	> 10%; > 12%
Aldosterone	ADI-900-173	Enzo life sciences	4.7 pg/mL	> 10%; > 20%
Testosterone	ADI-900-065	Enzo life sciences	5.67 pg/mL	> 15%; > 15%
Estradiol	ADI-900-174	Enzo life sciences	14.0 pg/mL	> 10%; > 15%

Samples were analyzed and standard curves were generated simultaneously in all of the plates. Standards, controls, and samples were analyzed in duplicate, and the average was calculated for each sample.

2.4. RNA Isolation and Quantitative Real-time PCR

Total RNA was extracted from junctional zone and ovaries with the RNeasy mini kit (QIAGEN, Valencia, CA). RNA concentration and integrity were determined using a nanodrop spectrophotometer (ThermoFisher Scientific, Newark, DE). One microgram of total RNA was reverse-transcribed to cDNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). cDNA equivalent to 100 ng of RNA was amplified using quantitative real-time reverse transcription-polymerase chain reactions (qRT-PCR), with SYBR green (SsoAdvanced™ Universal SYBR® Green Supermix, Bio-Rad) serving as the fluorophore in a CFX96 real-time thermal cycler (Bio-Rad). PCR primers (**Table 2**) were obtained from a previous study [43] and verified using primer-Blast software. PCR conditions for the SYBR green Gene Expression Assay were 2 min at 50°C and 10 min at 95°C for one cycle, and then 15 s at 95°C and 1 min at 60°C for 50 cycles. Results were calculated based on the $2^{-\Delta\Delta CT}$ method and were expressed as fold change regarding the gene of interest in PFOS dams versus control dams. All reactions were performed in duplicate, and GAPDH was used as the internal control.

Table 2. Sequences of primers used for Q-RT-PCR amplification.

Gene name	**Forward**	**Reverse**

StAR	AGGAAAGCCAGCAGGAGAATG	GTCCATGGGCTGGTCTAGCA
Cyp11A1	TCAAGCAGCAAAACTCTGGA	CGCTCCCCAAATACAACACT
3β-HSD1	AGGGCATCTCTGTTGTCATCCAC	TGCCTTCTCGGCCATCCTTT
Cyp17A1	TGGCTTTCCTGGTGCACAATC	TGAAAGTTGGTGTTCGGCTGAAG
Cyp19A1	CGTGGAGACCTGACAAA	GGATACTCTGCGATGAGA
17β-HSD3	GACCGCCGATGAGTTTG	GGTGCTGCTGTAGAAGAT
SRD5A1	CTCCTGGTCACCTTTGTC	GGTCACCCAGTCTTCAGC
SRD5A3	TCTTGGGAATGATGATGTT	TGCTGGCAGTGGATGAC
UGT1A1	TATTGGTGGGATAAACTGC	TTCCATCGCTTTCTTCT
GAPDH	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA

2.5. Statistical Analysis

Statistical analyses were done using GraphPad Prism (GraphPad Software, San Diego, CA). Data were presented as the mean ± SEM. For each analysis, the 'n' represents the number of litters analyzed. Comparisons between multiple groups were performed with one-way ANOVA followed by Tukey's multiple comparison test, and between two groups were done with an unpaired Student t-test. The normality and homogeneity of the variances were analyzed with the one-sample Kolmogorov–Smirnov test and Anderson-Darling test, respectively. When the data were not normally distributed, a non-parametric Kruskal–Wallis test, with Dunn's multiple comparisons, was used. Differences with a P-value of less than 0.05 were considered statistically significant.

3. Results

3.1. Effect of PFOS on Fetal and Placental Weights

PFOS exposure significantly reduced fetal weights at GD20 to the same degree in both male and female fetuses compared to controls (P10 group: male ↓8% and female ↓7%; P50 group: male ↓15% and female ↓14%; **Fig. 1a**). PFOS exposure also led to a reduction in total placental weight at GD20. The magnitude of reduction in placental weight was similar in male and female placentas in both P10 (male ↓7% and female ↓7%) and P50 groups (male ↓14% and female ↓11%; **Fig. 1b**).

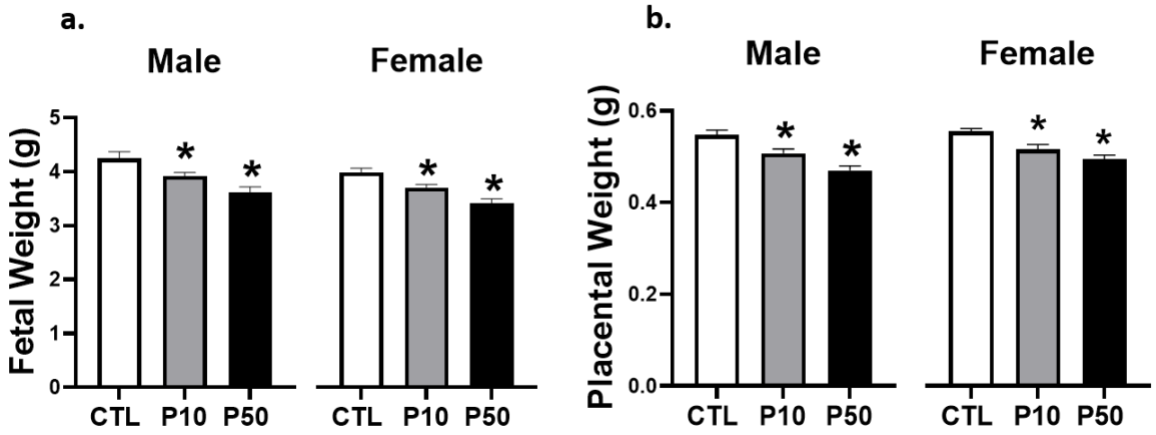

Fig 1. Effect of PFOS exposure on (a) fetal and (b) placental weights. Pregnant rats from gestational days 4 to 20 were exposed to 0 (CTL), 10 (P10), and 50 (P50) µg/mL PFOS through drinking water and fetal and placental weights were measured on gestational day 20. Data are expressed as mean ± SEM of 8 dams in each group. *$p < 0.05$ vs controls in respective sex.

This reduction appeared to be attributable to a significant decrease in labyrinth zone weight in both male and female placentas (P10: male ↓7% and female ↓9%; P50: male ↓12% and female ↓14%; **Fig. 2a**). PFOS exposure had no effect on junctional zone weights (**Fig. 2b**). Accordingly, both male and female labyrinth: junctional zone weight ratios were reduced following PFOS exposure in both P10 and P50 groups. To further quantify placental zones, a stereological

analysis was undertaken. A significant decrease in labyrinth zone area relative to the total placental area was observed in both male and female placentas of the P10 and P50 groups, in comparison to the control group (**Fig. 2c**). However, no significant differences were observed in the junctional zone area between the control group and the groups exposed to PFOS (**Fig. 2c**). The placental efficiency, defined as the ratio of fetal weight to placental weight, was calculated and there was no significant difference observed between the control and PFOS groups, which is in agreement with previous reports [41]. Litter size did not differ significantly among the control (12.2 ± 0.56), P10 (11.8 ± 0.56), and P50 (11.5 ± 0.84) groups. Furthermore, sex ratios (percentage of male offspring per litter - control: 48 ± 4.2%, P10: 50 ± 3.6%, and P50: 49 ± 4.1%) did not exhibit significant differences among the groups.

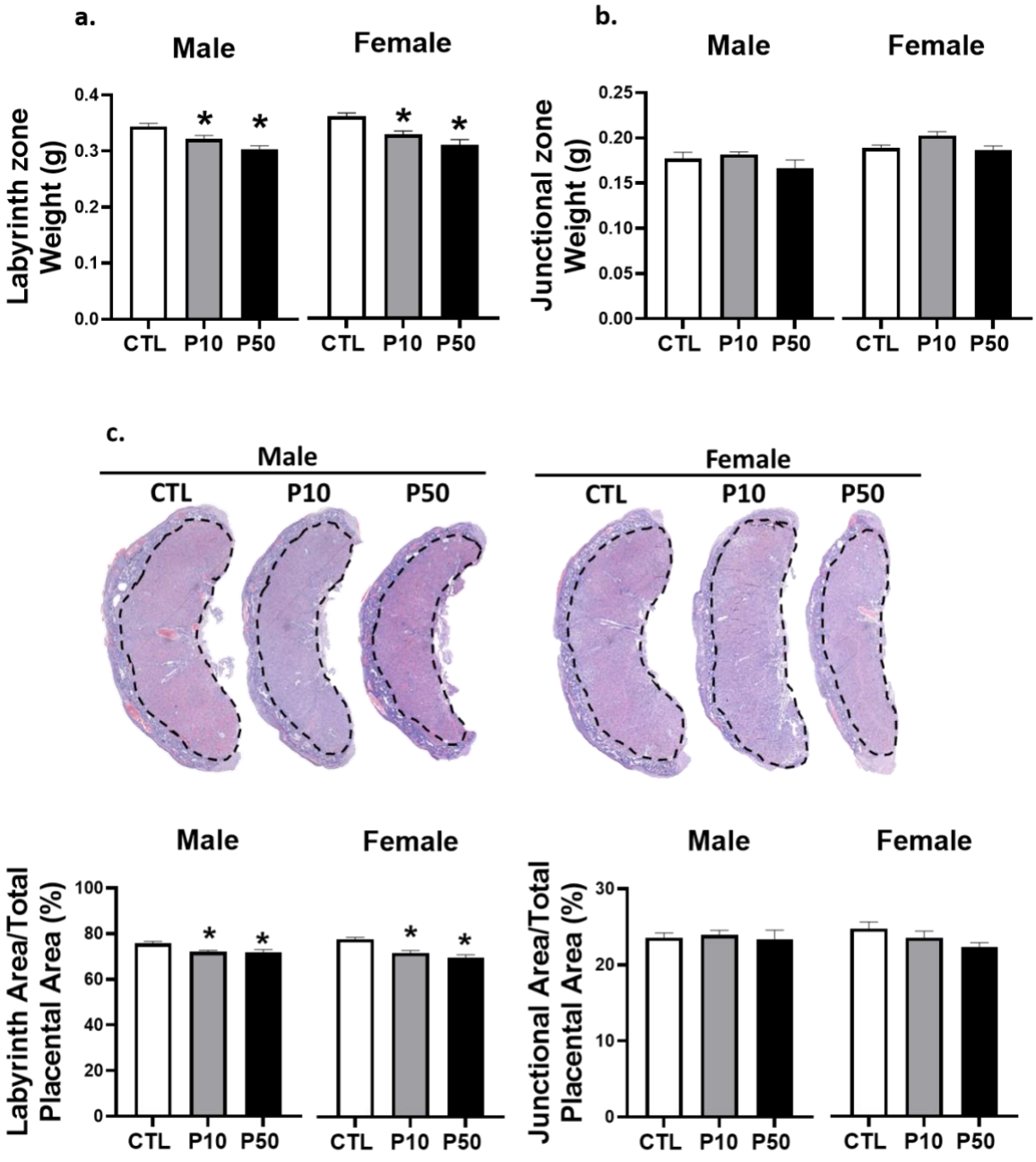

Fig 2. Effect of PFOS exposure on (a) labyrinth and (b) junctional zone weights and (c) stereological measurement of the sizes of the placental zones. Representative photomicrographic images of male and female placenta from each group with labyrinth area outlined by a dashed line are shown at the top panel. The quantitative labyrinth and junctional zone area relative to the total placental area is shown below. Pregnant rats from gestational days 4 to 20 were exposed to

0 (CTL), 10 (P10), and 50 (P50) μg/mL PFOS through drinking water and labyrinth and junctional zone weights and area were measured on gestational day 20. Data are expressed as mean ± SEM of 8 dams in each group. *$p < 0.05$ vs controls in respective sex.

3.2. Effect of PFOS on sex hormones levels

As shown in **Fig. 3**, PFOS exposure altered maternal sex steroid hormone levels. Compared to controls, PFOS exposure in the P50 group resulted in significantly higher levels of progesterone (↑166%), aldosterone (↑201%), and testosterone (↑45%) ($p < 0.05$), but these hormone levels were unaffected in the P10 group (**Fig. 3**). Corticosterone levels were higher in both P10 (↑180%) and P50 (↑205%) groups than in controls ($p < 0.05$, **Fig. 3**). In contrast, CBG levels were reduced in both P10 (↓7%) and P50 (↓11%) groups than in controls ($p < 0.05$, **Fig. 3**). Estradiol levels were unaffected in P10 but reduced in the P50 group (↓27%) than in controls ($p < 0.05$) (**Fig. 3**).

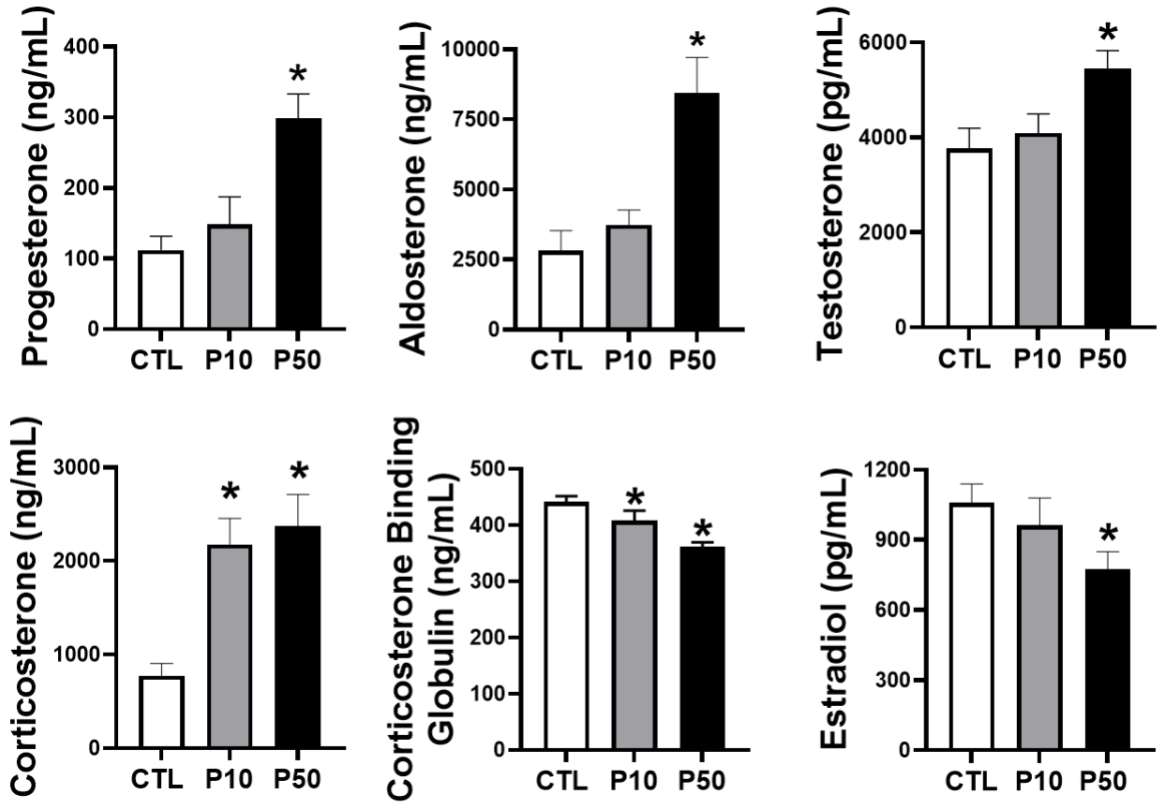

Fig 3. Effect of PFOS exposure on maternal steroid hormone levels. Pregnant rats from gestational days 4 to 20 were exposed to 0 (CTL), 10 (P10), and 50 (P50) μg/mL PFOS through drinking water. Blood was collected through cardiac puncture, plasma was separated and hormone levels were measured using commercial ELISA kits. Data are expressed as mean ± SEM of 8 dams in each group. *p< 0.05 vs. controls.

The effect of PFOS on peptide hormonal levels is shown in **Fig. 4**. PFOS exposure did not affect maternal FSH levels but resulted in higher LH levels in P50 (↑49%) group than in controls ($p > 0.05$). The hCG levels were significantly lower in the P10 (↓47%) and P50 (↓62%) groups than in the controls ($p < 0.05$). The prolactin levels tended to be lower in the P10 group but were significantly decreased in the P50 group (↓28%) than in the controls ($p < 0.05$).

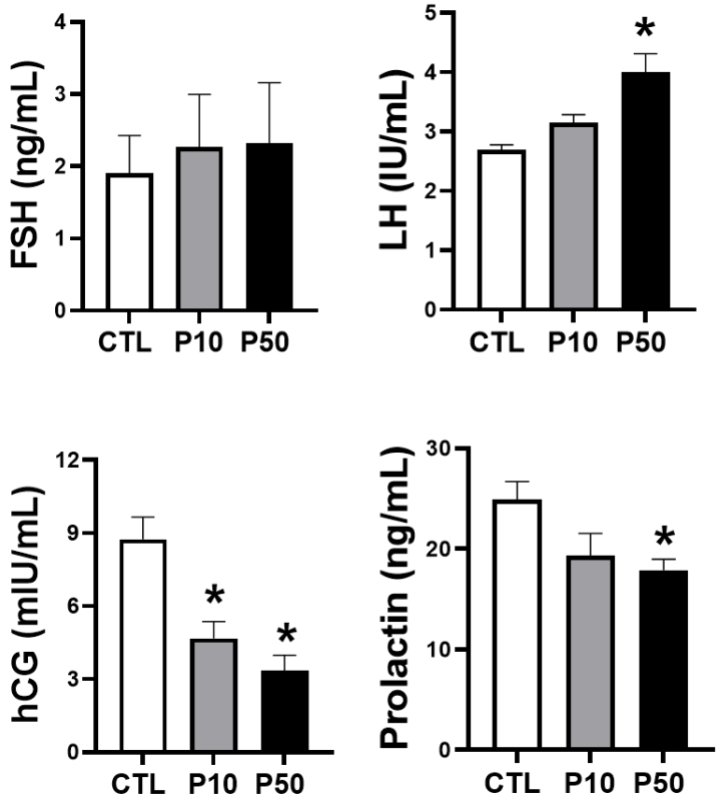

Fig 4. Effect of PFOS exposure on maternal peptide hormonal levels. Blood was collected through cardiac puncture, plasma separated, and hormone levels were measured using commercial ELISA kits. Data are expressed as mean ± SEM of 8 dams in each group. *p< 0.05 vs. controls.

3.3. Effect of PFOS on steroidogenic enzymes

As shown in **Fig. 5**, mRNA expression of genes responsible for hormone biosynthesis, including steroidogenic acute regulatory protein (StAR), cytochrome P450 11A1 (Cyp11A1), 3-beta hydroxysteroid dehydrogenase (3β-HSD1), cytochrome P450 17A1 (Cyp17A1), cytochrome P450 19A1 (Cyp19A1) and 17-beta hydroxysteroid dehydrogenase (17β-HSD3), were expressed in the junctional zone of rat placenta. PFOS exposure significantly increased the expression

Cyp11A1 and 3β-HSD1 in the male placenta while it increased StAR, Cyp11A1, 17β-HSD1 and 17β-HSD3 in the female placenta.

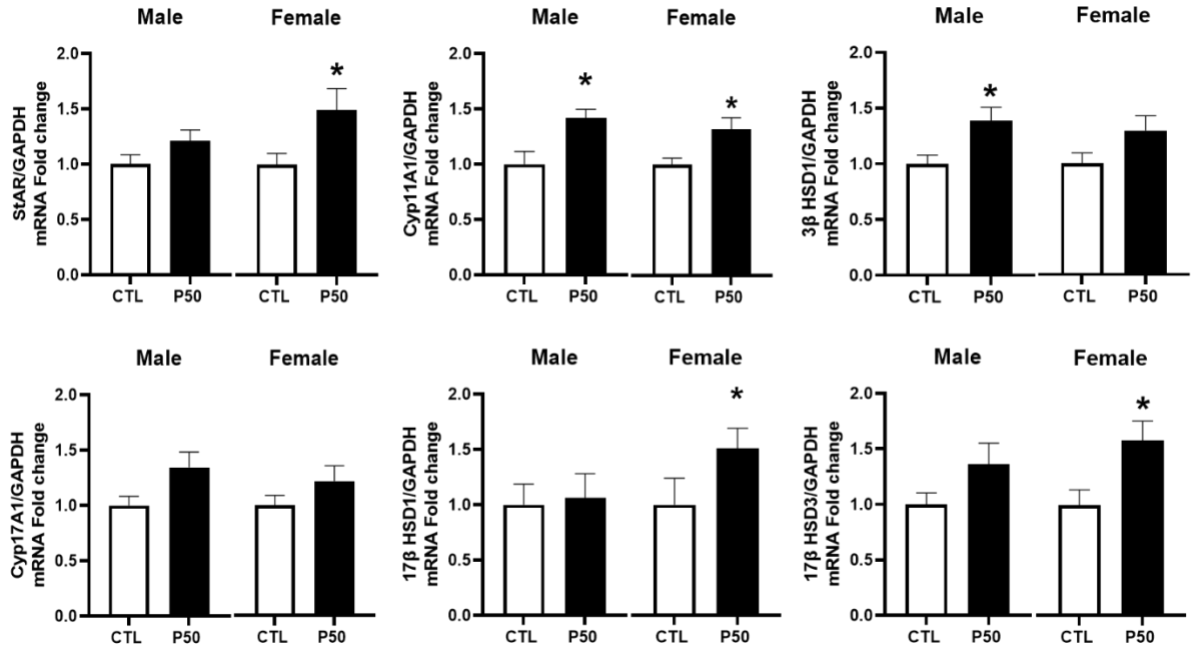

Fig 5. Effect of PFOS exposure on mRNA expression of genes related to hormone biosynthesis in the junctional zone of placenta. Real-time reverse transcriptase PCR was used to assess mRNA expression. Quantitation of candidate genes was normalized relative to GAPDH. Data are expressed as mean ± SEM of 8 dams in each group. *p< 0.05 vs. controls in respective sex.

Cyp19A1 is absent in the rat placenta; therefore, the conversion of testosterone to estrogen predominantly takes place in the ovaries in rodents [44-46]. Analysis of ovaries showed that PFOS significantly decreased the expression of Cyp19A1 compared to controls (**Fig. 6**).

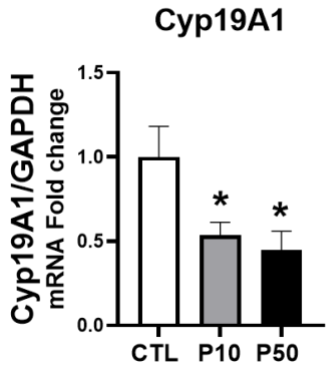

Fig 6. Effect of PFOS exposure on Cyp19A1 expression in the maternal ovaries. Real-time reverse transcriptase PCR was used to assess mRNA expression and was normalized relative to GAPDH. Data are expressed as mean ± SEM of 8 dams in each group. *p< 0.05 vs. controls.

Analysis of expression of genes involved in steroid hormone metabolism showed that PFOS increased the expression of UDP-glucuronosyltransferase 1A1 (UGT1A1) in the male but not female placenta (**Fig. 7**). PFOS did not affect the expression of steroid 5α-reductase (SRD5A1 and SRD5A3) in both the male and female placentas (**Fig. 7**).

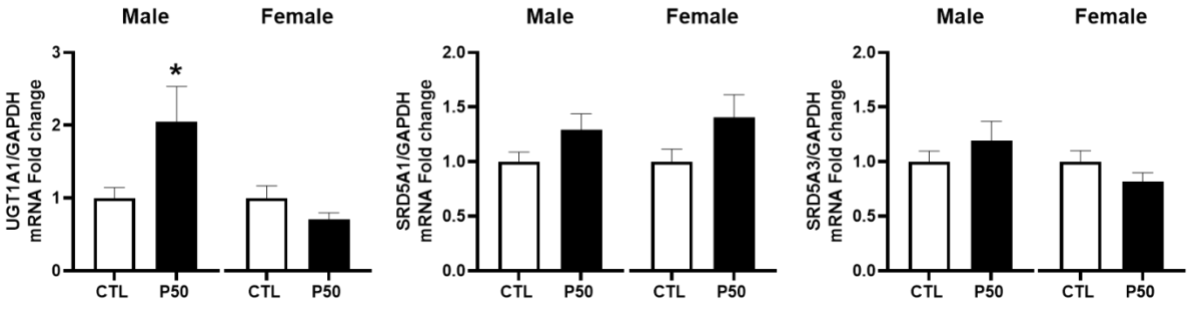

Fig 7. Effect of PFOS exposure on mRNA expression of genes related to steroid hormone metabolism in the junctional zone of placenta. Real-time reverse transcriptase PCR was used to assess mRNA expression. Quantitation of candidate genes was normalized relative to GAPDH.

Data are expressed as mean ± SEM of 8 dams in each group. *p< 0.05 vs. controls in respective sex.

4. Discussion

To our knowledge, the present study is the first to examine the effects of PFOS on placental steroidogenesis during rat pregnancy. In this study, maternal PFOS exposure resulted in a dose-dependent reduction in fetal weights, with the magnitude of fetal growth restriction being similar in both male and female fetuses. This fetal growth-suppressive effect of PFOS is consistent with the meta-analysis that shows that the birth weight decreases by 3.22 g per 1 ng/mL increase in serum PFOS concentrations in pregnant women [47]. However, analysis of sex-specific associations between prenatal PFOS exposure and fetal growth restriction in humans has shown mixed results. Some show a greater effect in male fetuses [48], while others report a higher impact in female fetuses [49]. Some studies also show no sex difference in the relationship between PFOS and fetal weight [47, 50]. The potential reasons behind these observed differences in human studies are unclear. However, many factors such as study design, population size, ethnicity, the level and timing of exposure, presence of other PFAS and environmental contaminants and other factors such as maternal age, health status, and lifestyle can influence the effects of PFOS on fetal growth. Further focused studies are needed to fully understand the potential sex-specific impacts of PFOS on fetal growth.

Decreased placental weight has been observed in pregnant rats with elevated levels of PFOS [41, 51, 52]. This study provides new evidence that the placenta of both male and female fetuses are affected to a similar magnitude. Further, this study identifies that the placenta from the pregnant rats exposed to PFOS specifically decreases the labyrinth layer but not the

junctional zone. This suggests that the population of cells in the labyrinth zone is more susceptible to PFOS. The labyrinth zone comprises syncytiotrophoblasts and a dense network of blood vessels lined by endothelial cells [44, 45]. Studies show that PFOS interferes with the proliferation and differentiation of syncytiotrophoblasts [21, 53] and impairs endothelial function [37, 41, 54]. Moreover, *in vitro* treatment of cells with PFOS reduced angiogenesis [55], suggesting that PFOS may impair blood vessel formation. Since the labyrinth zone plays an important role in facilitating nutrient transport from the mother to the fetus, this PFOS-induced reduction in size and vascularity of the labyrinth zone could lead to impaired nutrient transport and may contribute to fetal growth restriction. However, further studies are needed to confirm this notion.

The junctional zone, in contrast, does not contain fetal blood vessels but constitutes the main endocrine compartment of the placenta. It produces many hormones like progesterone and estrogens for maintaining pregnancy and ensuring proper fetal development. This study showed that PFOS exposure disrupted the normal hormonal balance and led to increased circulating levels of progesterone, corticosterone, aldosterone, and testosterone, as well as reduced estradiol levels. To determine whether PFOS interferes with hormone balance by altering the mRNA expression of enzymes involved in steroid hormone synthesis, we measured the mRNA levels of many steroidogenic enzymes in the junctional zone of the rat placenta. Our results showed that PFOS exposure increased mRNA expression of steroidogenic enzymes in a sex-dependent manner. In the male placenta, Cyp11A1 and 3β-HSD1 were increased, while in the female placenta StAR, Cyp11A1, 17β-HSD1, and 17β-HSD3 were increased. The sex-specific impact of PFOS on the expression of steroidogenic genes is consistent with previous studies in zebrafish, where PFOS were shown to have transcriptional-level responses that differed by gender [21].

Nevertheless, the PFOS-induced increase in expression of steroidogenic genes in both sexes in this study was in the direction favoring increased synthesis of progesterone, corticosterone, aldosterone, and testosterone. Additionally, exposure to PFOS leads to a reduction in the levels of CBG. This CBG reduction could lead to an increase in the concentration of corticosterone that is available to bind to receptors and exert biological effects. Therefore, PFOS exposure may result in an elevation in bioavailable corticosterone levels. As such, these findings suggest that the PFOS-induced upregulation of these enzymes may contribute to increased steroid hormone levels in pregnant rats.

Unlike the human placenta, the rat placenta does not produce estrogen due to the absence of the enzyme aromatase. The aromatase in the corpus luteum uses the testosterone produced by the placenta as the substrate for the biosynthesis of estrogen [44-46]. Thus, although the rat placenta does not produce estrogen, it helps sustain estrogen synthesis [44-46]. This study found that exposure to PFOS decreased aromatase expression in the ovaries in both P10 and P50 groups; however, the decrease in estradiol levels was significant only in the P50 group, indicating that mRNA expression may have to decrease below a threshold level to reflect in changes in estradiol level. The current study findings are consistent with the studies that show that PFOS concentration-dependently inhibited aromatase expression and activity in primary human placental syncytiotrophoblasts [21] and JEG-3 trophoblast cells [56]. Reduced estradiol levels were also detected following PFOS exposure in adult mice and monkeys [16, 17]. Consistently, human studies also show that PFOS exposure is negatively associated with estradiol levels and positively associated with testosterone levels [57].

In addition to biosynthesis enzymes, metabolism enzymes also modulate hormone homeostasis. Expression of steroid 5-alpha-reductases (SRD5A1 and SRD5A3), which metabolizes progesterone and testosterone, were unaffected by PFOS exposure. UDP-glucuronosyltransferase 1A1 (UGT1A1) is an enzyme that catalyzes the formation of estrogen glucuronide. This glucuronidation reaction is an important step in estrogen metabolism and elimination, especially during pregnancy in mice and humans [58]. The increased mRNA expression of UGT1A1 in the male placenta indicates its potential contribution to the reduced maternal estradiol levels in PFOS-exposed pregnant rats. The mechanism by which PFOS exposure contributes to the upregulation of UGT1A1 expression remains unclear. Nevertheless, the upregulation of UGT1A1 expression may promote the metabolism of estradiol, leading to a further reduction in estradiol levels, in addition to the reduction mediated by the decreased activity of Cyp19A1. Thus, decreased estradiol production due to reduced aromatase expression and increased estradiol breakdown due to enhanced UDP-glucuronosyltransferase 1A1 expression may contribute to lower estradiol levels in rats exposed to PFOS during pregnancy. The mechanism by which PFOS affects the transcription of aromatase and other steroidogenic genes is a relatively unexplored area of toxicology; however, one hypothesis suggests that PFOS might regulate steroidogenic genes by modulating nuclear receptors [59, 60]**.**

During pregnancy, hCG is the first hormonal signal from the placenta to the mother and is mainly secreted by the placental syncytiotrophoblasts [61]. hCG plays an essential role in pregnancy maintenance by stimulating steroidogenesis in the placenta [62, 63]. Studies show that hCG influences estrogen synthesis by altering aromatase expression and activity in the placenta [64]. Hyperglycosylated human chorionic gonadotropin (hCG-H) is a larger variant of the hormone hCG characterized by the presence of extensive carbohydrate side chains. It is

exclusively produced by cytotrophoblast cells and constitutes a significant portion, up to 90%, of the total measurable hCG levels in serum and urine [65, 66]. The primary function of hCG-H is to promote invasion of trophoblast cells during early pregnancy, and it is also believed to have potential roles in modulating immune cells and endothelial function in the uterus during pregnancy [67]. It has been hypothesized that a deficiency in hCG-H may contribute to the high incidence of pregnancy failures in humans [66]. hCG is also shown to play an important role in promoting steroidogenesis in pregnant rats [68, 69]**.** In the present study, plasma hCG-H levels in the PFOS-exposed rats were significantly decreased and might have contributed to reduced aromatase expression and estradiol levels. Further studies are needed to determine how PFOS decreases hCG-H levels and if this mediates the PFOS-induced decrease in estradiol levels. Placental prolactin stimulates the growth of the placenta and the mammary glands and plays a role in fetal growth and pregnancy maintenance [70]. PFOS exposure decreased prolactin hormone production in pregnant rats. In the placenta, trophoblast cells are the main source of prolactin production and secretion, and estrogen levels are related to the proliferation and differentiation of these cells [71]. This study demonstrated that the plasma levels of estradiol were significantly decreased in the PFOS-exposed pregnant rats, which may affect the development and differentiation of placental trophoblast cells in pregnant rats and contribute to the reduced prolactin levels. This is consistent with studies that show that PFOS decreases prolactin levels with reduced mammary gland differentiation and delayed epithelial involution in mice [72]. In contrast, a recent study showed a null association between PFOS and prolactin levels in pregnant women [73]**.** This difference could be due to the sampling timing (prolactin levels were measured between 12 and 29 weeks of gestation, while prolactin levels at term pregnancy or during lactation are considered a better indicator). PFOS exposure in the current

study increased LH levels in maternal plasma with no change in FSH levels. Studies show that increases in testosterone levels increase LH levels [74, 75]. However, further studies are needed to investigate if PFOS directly impacts LH production or is secondary to increased testosterone levels.

The present study has several limitations that warrant consideration. First, our focus on mRNA levels may not reflect protein or activity levels, and future studies that evaluate target enzyme protein levels, activity status, and specific placental cell populations could augment our understanding of how PFOS modulates placental steroidogenesis. Second, we solely examined the effects of PFOS, and since typical human exposures involve various combinations of PFAS, it is important for future research to assess the combined toxic potential of commonly encountered PFAS mixtures. Third, interspecies differences may exist that limit the relevance of our findings to humans. Despite both rats and humans having a discoid hemochorial placenta, there are other differences in the maternal-placental-embryo unit, such as the labyrinthine vs. villous structure, the number of offspring per pregnancy (∼12 vs. ∼1), and gestation length (∼21d vs. ∼280d). Although we chose the genetically diverse outbred Sprague Dawley rat for our study, strain variations in PFOS sensitivity should be investigated in future work. Nonetheless, an important strength of our research is that mRNA expression of steroidogenic genes is bolstered by the hormone levels and feto-placental weight data which was obtained from the same animals. Furthermore, by collecting male and female placentas from the same dams, we were able to assess inherent sex differences in steroidogenic gene expression, offering valuable context for interpreting prenatal PFOS-induced sex disparities in the developmental programming of adult diseases, as reported in recent studies [37]

In conclusion, the placenta might be a target tissue of PFOS in pregnant rats. PFOS exposure decreased the circulating hCG, prolactin and estradiol levels while increasing plasma progesterone, aldosterone, corticosterone and testosterone levels. Based on the present results, we speculated that hormone biosynthesis and metabolism enzymes might contribute to the altered maternal hormone levels after PFOS exposure during pregnancy.

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Declaration of Competing Interest:

The authors declare no conflict of interest.

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Chapter 5

PERFLUOROOCTANE SULFONIC ACID (PFOS) EXPOSURE DURING PREGNANCY INCREASES BLOOD PRESSURE AND IMPAIRS VASCULAR RELAXATION MECHANISMS IN THE ADULT OFFSPRING.

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Running Title: Reduced endothelium-dependent relaxation in PFOS offspring

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ABSTRACT

Perfluorooctanesulfonate (PFOS) is a persistent environmental agent. We examined whether PFOS exposure during pregnancy alters blood pressure in male and female offspring, and if this is related to sex-specific changes in vascular mechanisms. PFOS was administered through drinking water (50 µg/ml) to pregnant Sprague-Dawley rats from gestational day 4 until delivery. PFOS-exposure decreased maternal weight gain but did not significantly alter feed and water intake in dams. The male and female pups born to PFOS mothers were smaller in weight by 29% and 27%, respectively. The male PFOS offspring remained smaller through adulthood, but the female PFOS offspring exhibited catch-up growth. The blood pressure at 12 and 16 weeks of age was elevated at similar magnitude in PFOS males and females than controls. Mesenteric arterial relaxation to acetylcholine was reduced in both PFOS males and females, but the extent of decrease was greater in females. Relaxation to sodium-nitroprusside was reduced in PFOS females but unaffected in PFOS males. Vascular eNOS expression was not changed, but phospho (Ser^{1177})-eNOS was decreased in PFOS males. In PFOS females, both total eNOS and phospho (Ser^{1177})-eNOS expression were reduced. In conclusion, PFOS exposure during prenatal life (1) caused low birth weight followed by catch-up growth only in females (2) lead to hypertension of similar magnitude in both males and females; (2) decreased endothelium-dependent vascular relaxation in males but suppressed both endothelium-dependent and -independent relaxation in females. The endothelial dysfunction is associated with reduced activity of eNOS in males and decreased expression and activity of eNOS in females.

1. INTRODUCTION

Poly- and perfluoroalkyl substances (PFAS) are a family of fluorinated compounds with unique physical and chemical properties such as high stability and strong lipophobic and hydrophobic properties [1-3]. They are extensively used in the manufacture of consumer products such as disposable food packaging, cookware, outdoor gear, furniture, and carpet. Additionally, applications of PFASs in the automotive, semiconductor, electronics, and aviation industries are well documented [4, 5]. As a result, these chemicals have garnered considerable international attention due to their environmental persistence and global occurrence in humans. The chemicals of this class are detected in groundwater [6, 7] and drinking water [8]. The EPA Office of Water established a health advisory exposure level of 70 ng/L for PFOS (US EPA 2016) [9]. But the majority of the communities in the US tend to be exposed to PFOS levels above EPA's recommendations. For example, studies report that more than twenty-five US communities and 126 military sites contain PFOS levels above EPA's helath advisory levels [10-13]. Another study report that about 6 million people served by 66 public water supplies have PFOS in the range of 1800 ng/L [8]. The US Center for Disease Control and Prevention reported that PFOS is detected in 98% of human blood in the United States [14], including in reproductive-age women. PFAS has biological half-lives of up to several years [15]. PFAS crosses the placental barrier [16-18], and therefore it is critical to determine any adverse health effects that may result in their offspring from PFAS exposure in pregnant women.

Among the PFAS family, perfluorooctanesulfonate (PFOS) is one of the most concerning members [19, 20]. Prenatal exposure to PFAS may affect fetal growth and have lasting effects on offspring health. Several systematic reviews and meta-analyses have reported inverse associations between maternal concentrations of PFOS during pregnancy, and low birth weight,

and their association was regarded as likely to reflect a causal relationship [21-24]. Some studies report that the maternal serum concentrations of PFOS strongly correlate with low birth weight in female infants only [25-27]. Assessing postnatal weight gain revealed no difference in growth trajectories [28] while some other report accelerated growth specifically in male [29] or female offspring [30] following prenatal PFOS exposure. No animal studies have verified the sex-specific impacts of maternal PFOS exposure on birthweight and growth trajectories.

In addition to their effect on fetal growth, maternal PFOS levels during pregnancy have been related to reproductive and endocrine disturbances [31-33], neurodevelopmental impairments [34, 35], and glucose intolerance [36, 37] in the offspring. The neurobehavioral and glucose metabolic outcomes were more dysregulated in male than in female prenatal PFOS exposed offspring [36, 38]. Given that the cardiovascular system begins to develop in utero**,** there is the potential for hypertension risk to be affected by environmental exposures during this period [39, 40]. However, only one study has evaluated hypertension risk in offspring following their exposure to PFOS during early-life windows of susceptibility [40]. Additionally, the vascular mechanisms linking prenatal PFOS exposure and adult hypertension are unknown. The endothelium is known to release endothelium-derived relaxing factors such as nitric oxide (NO) [41]. NO diffuses to the adjacent vascular smooth muscle cell, where it stimulates the enzyme guanylate cyclase leading to increased cGMP production and smooth muscle relaxation [41, 42].

The purpose of this study was to test the hypothesis that maternal PFOS exposure leads to low birth weight with sex-specific changes in growth trajectories and blood pressure and vascular mechanisms in the male and female offspring. To test this hypothesis, we used the offspring of normal pregnant rats and offspring of pregnant rats exposed to PFOS through drinking water. Experiments were designed to investigate (1) whether birth weight, growth rate,

and blood pressure are altered in PFOS compared with control offspring; (2) whether the vascular constriction to the α-adrenergic agonist phenylephrine is enhanced in PFOS compared with control offspring; (3) whether the endothelium-dependent and vascular relaxation to acetylcholine are reduced in PFOS compared with control offspring; and (4) whether the changes in growth trajectories and vascular constriction/relaxation in blood vessels of PFOS offspring vary in a sex-dependent manner.

2. Material and Methods

2.1. Animals

All procedures were approved by the Animal Care and Use Committee at the University of Wisconsin-Madison and were in accordance with those published by the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996). Twelve-week-old timed-pregnant Sprague-Dawley rats (gestation day 3, weighing between 175 and 225 g (positive plug = gestation day 1) were obtained from Envigo laboratories, (Indianapolis, IN) and housed in a room with controlled temperature and a 12:12-h light-dark cycle. The rats were randomly divided into two groups, housed individually, and exposed to standard drinking water (control group) or drinking water containing PFOS potassium (CAS #2795-39-3, Sigma Aldrich, St. Louis, MO) at 50 µg/ml from gestation day 4 to until delivery. This concentration of PFOS is proportional to 5 mg/kg/bodyweight based on average daily water consumption of 25 ml/day (10 ml/100 g body weight/day) and was selected based on previous studies that showed fetal growth restriction [43]. The dose of 50 µg/ml through drinking water used in this sudy is comparable to the rat developmental toxicity study of Luebker at al. 2005 [44] that provided a lowest effect dose that was used to set the reference dose within the

U.S. EPA's drinking water health advisory level of 70 ppt PFOS (US EPA 2016) [9]. Rats were fed with a standard breeder chow (D15092401; Research Diets, New Brunswick, NJ) ad libitum. Daily food and water intake and body weight gain were monitored. All pregnant rats were allowed to deliver at term. At delivery, all dams were placed on standard drinking water, and the pups were weaned at 21 days of age. The litter sizes and birth weights of control and PFOS pups were recorded within 2 hours after delivery. Pups were considered as significant IUGR if their birth weight was more than 2 standard deviations below the mean body weight of the control litter. Litter sizes were adjusted to 8 pups per dam (pups of extreme weights were sacrificed) and weighed every two weeks after that. Blood pressures were measured at 8, 12, and 16 weeks of age and were sacrificed at 16 months of age to isolate mesenteric arteries for vascular function and Western blotting. Female offspring were studied at the diestrus stage of the estrous cycle based on vaginal cytology [45].

2.2. Measurement of Blood pressure

The mean blood pressure was measured in conscious rats using a CODA computerized noninvasive blood pressure system (Kent Scientific, Litchfield, CT) as described previously [46]. The rats were first preconditioned to a restraint warming chamber for 2 days. Conditioning was performed daily for 15 minutes between 8:00, and 10:00 am, and then blood pressure was measured the following day. Rats were allowed to rest quietly for 10 minutes in a plexiglass restrainer placed in a warming chamber preset at 30°C to dilate peripheral blood vessels and stimulate blood flow to the tail. An occlusion cuff and a volume pressure-recording cuff were applied to the base of the tail. The cuff was programmed to inflated and deflated automatically within 90 seconds. Blood pressure was recorded and analyzed using Kent Scientific software.

Results from the first 5 inflation cycles were discarded, and the average obtained from the next 5 cycles was taken as the individual mean blood pressure of that rat.

2.3. PREPARATION OF MESENTERIC ARTERIES

The rats were euthanized by CO_2 inhalation, and the mesenteric arcade was excised and immersed in oxygenated Krebs physiological salt solution (KPS) (in mM): NaCl, 119; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.17; $NaHCO_3$, 25; KH_2PO_4, 1.18; EDTA, 0.026; and d-glucose, 5.5. Mesenteric arteries (2-mm segments of the third-order branch of the superior mesenteric artery, 150- to 200-μm diameter) were dissected free of fat and connective tissue and mounted using tungsten wires on a wire myograph (Danish Myo Techniques, Aarhus, Denmark) for the recording of isometric tension. The tissues were incubated for 15 min in KPS at 37°C, which was gassed with 95% O_2 and 5% CO_2 to maintain pH 7.4, and allowed to equilibrate for 30 minutes before normalization to an internal diameter of 0.9 of L13.3kPa by using a normalization software package (Myodata; Danish Myotechnologies). For endothelium-intact mesenteric rings, extreme care was taken to avoid injury to the endothelium. For endothelium-denuded mesenteric rings, the endothelium was removed by gently rubbing the ring interior with tungsten wire. Removal of the endothelium was verified by the absence of relaxation to acetylcholine (ACh) in arterial rings precontracted by a submaximal concentration of phenylephrine (PE).

2.3.1. Assessment of vascular contractile responses

The arterial rings were exposed to 0.08 mol/L potassium chloride (80 mM KCl) until reproducible depolarization-induced contractions were achieved. After the second round of

washing and equilibration with KPS, vascular contractile responses to cumulative additions of PE (10^{-9} – $3x10^{-5}$ mol/L), which causes α_1-adrenoceptor–induced contractions, were determined.

2.3.2. Assessment of vascular relaxation responses

Endothelium-dependent relaxation was assessed by ACh (10^{-9} –10^{-5} mol/L) -induced relaxation in PE-precontracted arteries. The PE concentration used for precontraction was that required to produce 80% of the maximal response (pEC_{80}). Endothelium-independent relaxation was determined by sodium nitroprusside (SNP) (10^{-9}–10^{-6} mol/L) -induced relaxation in PE-precontracted endothelium-denuded arteries.

2.4. WESTERN BLOTTING FOR eNOS PROTEIN QUANTIFICATION

The mesenteric arteries were homogenized in ice-cold RIPA buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin; Cell signaling Technology, Danvers, MA) containing a protease inhibitor tablet (Roche, Indianapolis, IN) and phosphatase inhibitor cocktail-2 and -3 (Sigma, St Louis, MO). Tissue lysates were centrifuged (14,000 g for 10 min at 4°C), and the protein content was measured using the Pierce BCA protein assay kit (Thermo Scientific, Waltham, MA). The supernatant was resuspended in the NuPAGE® LDS sample buffer and reducing agent (Invitrogen; Thermo Scientific, Waltham, MA). Proteins (30 μg) alongside Precision Plus Standard (Kaleidoscope, Bio-Rad Laboratories, Hercules, CA) were resolved on 4 to 12% gradient NuPAGE® Bis-Tris Gels (Invitrogen) at 100 V for 2.0 hours at room temperature and then transferred onto Immobilon-P membranes (Millipore Inc., Billerica, MA) at 100 V for 1.5 hours. The membranes

were blocked with 5% bovine serum albumin for 1 hour and then incubated overnight at 4°C with primary antibodies. The primary antibodies were rabbit monoclonal eNOS (1:1000; Catalog No. 32027, Cell Signaling, Danvers, MA) and rabbit polyclonal phospho-eNOS (Ser^{1177}) (1:500; Catalog No. PA5-17917, Thermo Fisher Scientific, Waltham, MA) and rabbit polyclonal β-actin (1:2000; Catalog No. 4967, Cell Signaling, Danvers, MA). After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for one hour and then developed using the Pierce ECL detection kits (Thermo Scientific). The densitometric analysis was done using Image J software. Results were expressed as ratios of band intensity to that of β-actin.

2.5. STATISTICAL ANALYSIS

Data are presented as mean ± SEM. Data analysis was done using GraphPad Prism for Windows (GraphPad Software, San Diego, CA). Cumulative concentration-response curves were analyzed by computer fitting to a 4-parameter sigmoid curve. Contraction responses to PE were calculated as a percent of its maximal contraction. Relaxant responses to ACh were calculated as percent inhibition of the PE-induced contraction. E_{max} (maximal responses) and pD_2 values (concentration that produces 50% effect) were then obtained. pD_2 values were determined by regression analysis and expressed as negative log molar concentration. The normality and homogeneity of the variances were analyzed using the one-sample Kolmogorov–Smirnov test and Anderson–Darling test, respectively. Statistical analysis was performed using repeated measures of ANOVA followed by Dunnet's post hoc test and unpaired Student's t-test for comparison of single observation between control and PFOS groups. Where data were not normally distributed, a non-parametric Kruskal-Wallis test, with Dunn's multiple comparisons

were used. Data from several vascular rings of the same rat were averaged and presented as the datum for 1 rat, with the n value representing the number of dams. Differences are considered statistically significant at $p<0.05$.

3. RESULTS

3.1. MATERNAL OUTCOMES

Maternal total weight gain during the dosing period was significantly in lower in PFOS dams (97 ± 5.1 g) compared with control dams (119 ± 3.6 g). When expressed as daily weight gain through the dosing period, PFOS dams gained significantly less weight on gestational day 20 and 21 than controls (**Figure 1A**). Daily feed and water intake were not significantly different between the control and PFOS dams (**Figure 1B** and **1C**). The length of gestation (control: 22 ± 0.1; PFOS: 21 ± 0.9 days), number of pups per litter (control: 12.3 ± 2.6; PFOS: 11.9 ± 1.8) and sex ratios (percent males per litter – control: 47 ± 3.9%; PFOS: 50 ± 4.5%) were not different between control and PFOS groups.

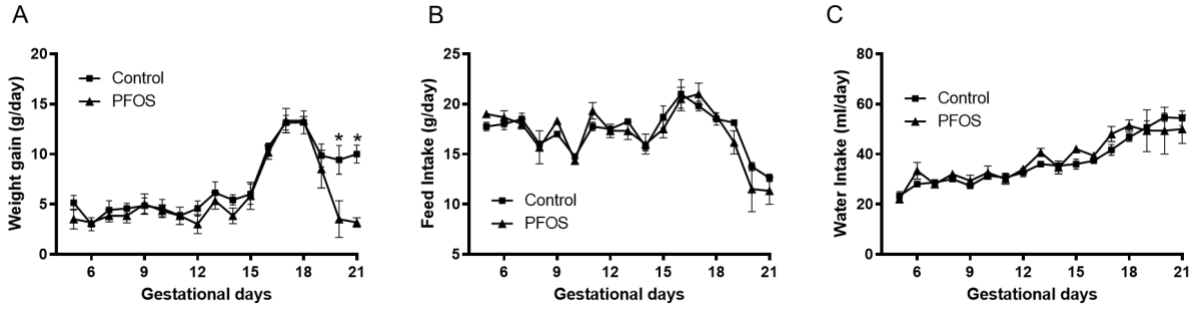

Figure 1. Daily weight gain and feed and water intake during pregnancy. (A) Daily weight gain of dams was measured and expressed as g/day (B) Daily feed intake in pregnant rats was quantified in grams. (C) Daily water intake in pregnant rats was quantified in milliliters. Animals were exposed to standard drinking water or water containing PFOS (50 µg/ml) from gestation

day 4 until delivery. Values are expressed as mean ± SEM of 6 animals in each group. *p<0.05 vs. control group at respective time point.

3.2 Offspring birth weight and growth rate

The birth weight of PFOS pups was 4.7 ± 0.06 g in males and 4.3 ± 0.05 g in females, and this was significantly lower by 29% and 27% compared with the respective males and females in the control group (males: 6.6 ± 0.13 g; females: 5.9 ± 0.12 g) (**Figure 2**). Both control and PFOS offspring showed significant increases in body weight with age.

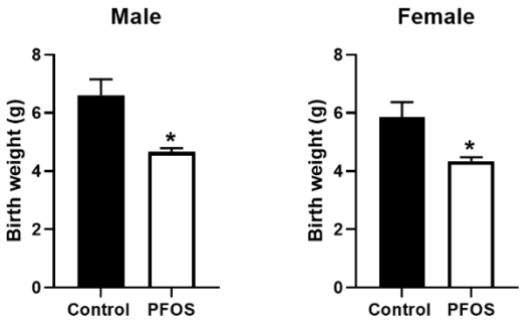

Figure 2. Birth weight of male and female pups born to control and PFOS exposed dams. Data expressed as mean ± SEM of 6 litters in each group. *p<0.05 vs. controls in respective sex.

However, the males' growth curves were parallel through adulthood, with the body weights of PFOS males consistently lower compared with control males (**Figure 3A**). On the other hand, the body weights of PFOS females were lower compared with control females up to 9 weeks of age, but the difference disappeared at 12 and 16 weeks (**Figure 3B**). The feed and water intake in male and female PFOS offspring were comparable to their respective sexes in controls (data not shown).

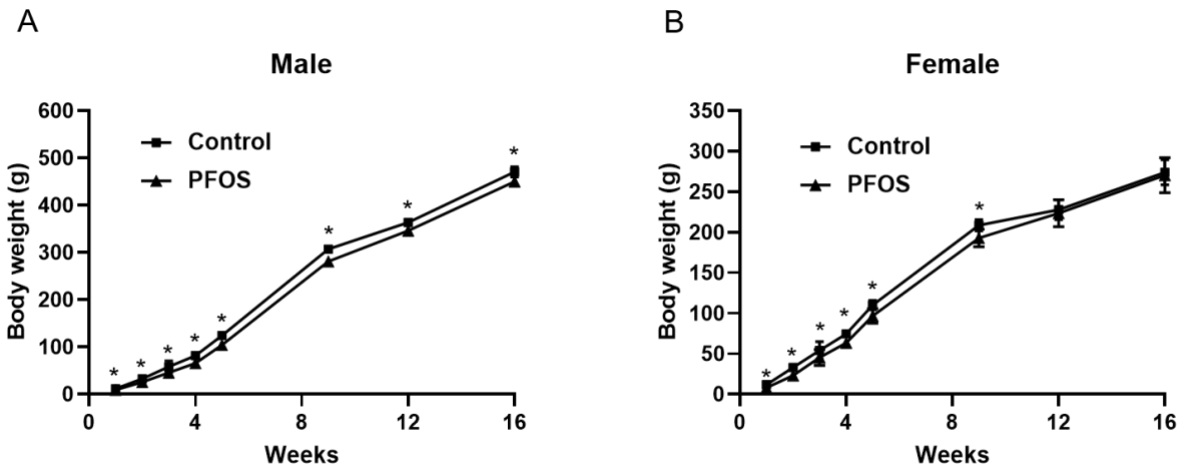

Figure 3. The growth rate of control and PFOS offspring. Bodyweight of male and female offspring from birth to 16 weeks of age. Weights of pups from a litter were averaged with the n value representing the number of litters. Data expressed as mean ± SEM of litters born to 6 dams in each group. *p<0.05 vs. control group at respective time point.

3.3. Mean Arterial Pressure

Mean arterial pressure was comparable between control and PFOS males at 8 weeks of age, but PFOS males had higher arterial pressure than controls at 12 weeks (11 mmHg higher; $P < 0.05$) and 16 weeks of age (20 mmHg higher; **Figure 4A**). In the female offspring, arterial pressure was similar between control and PFOS groups at 8 weeks of age, but PFOS females had significantly higher arterial pressure at 12 weeks (17 mmHg higher; $P < 0.05$) and 16 weeks of age (20 mmHg higher; **Figure 4B**).

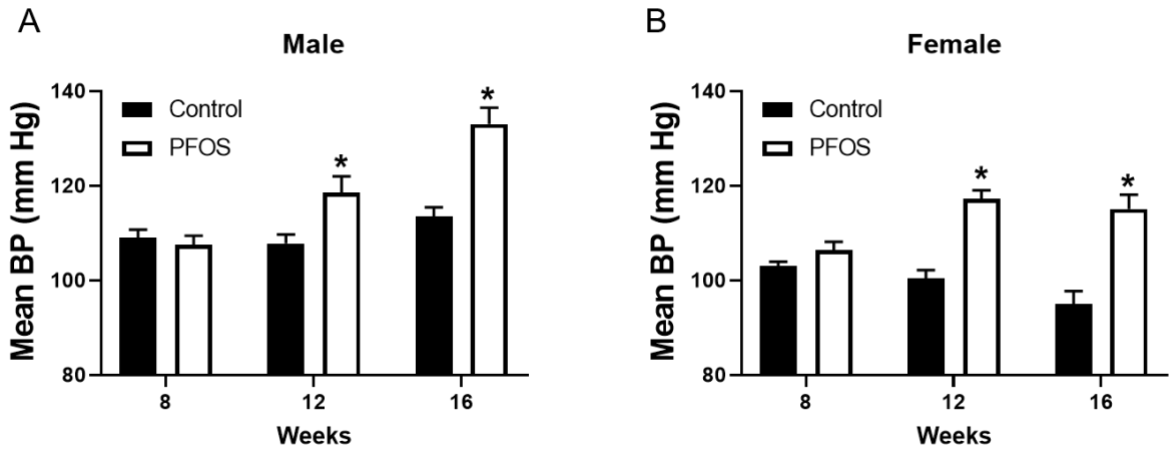

Figure 4. Temporal changes in mean blood pressure in male and female offspring of control and PFOS exposed dams. Mean blood pressure was measured in conscious rats using a CODA computerized noninvasive blood pressure system at 8, 12, and 16 weeks of age (n=6 in each group). *p<0.05 vs. respective control.

3.4. CONTRACTILE RESPONSES

In endothelium-denuded vascular rings contractile responses to KCl, a determination of depolarization-induced vessel contraction, and cumulative doses of PE, a measurement of α_1-adrenoceptor–induced contraction, were similar in arterial segments from PFOS males and females compared to their respective controls (**Figure 5A** and **B** and **Table 1**).

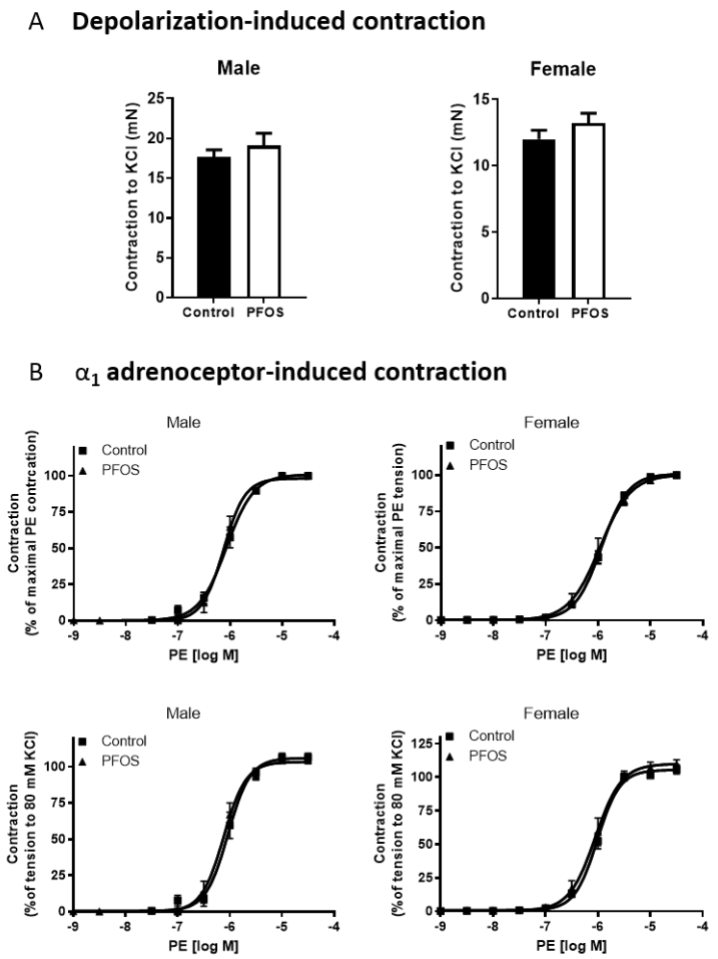

Figure 5. Endothelium-independent vascular reactivity in the mesenteric arteries of adult male and female offspring of control and PFOS exposed dams. Mesenteric arterial rings were isolated from adult males and females from control and prenatal PFOS exposed groups. Vascular contractile responses were taken to (A) KCl (80 mM), and (B) cumulative additions of PE. PE contraction was measured and presented as a percentage of maximum PE contraction and percentage of KCl (80 mM) contraction. Values are given as means ± SEM of 6 rats in each group.

3.5. ENDOTHELIUM-DEPENDENT RELAXATION RESPONSES

ACh induced relaxation in PE-precontracted (10^{-6} M) endothelium-intact arterial rings were significantly reduced in PFOS males and females compared with their respective sex in controls (**Figure 6A, left panel**). Sensitivity for ACh was significantly decreased in PFOS males compared to control males (**Figure 6A, left panel,** and **Table 1**). ACh sensitivity was also significantly reduced in PFOS females compared to control females (**Figure 6A, right panel,** and **Table 1**). The magnitude of the rightward shift, compared to respective sex in controls, was higher in the PFOS females (pD_2 value increased by 0.6 log units; **Figure 6B**) than PFOS males (pD_2 value increased by 0.3 log units; **Figure 6A,** and **Table 1**). Similarly, the ACh-induced maximal responses (E_{max}) was reduced by 20% in PFOS females and 8% in PFOS males compared to respective sex in controls (**Figure 6A,** and **Table 1**).

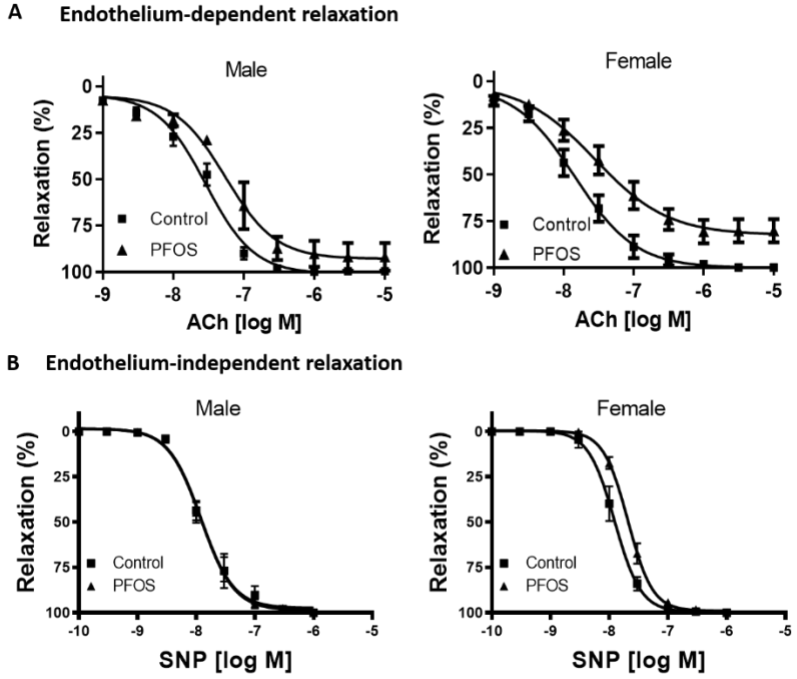

Figure 6. Endothelium-dependent and -independent vascular relaxation in the mesenteric arteries of adult male and female offspring of control and PFOS exposed dams. (A) Endothelium-dependent relaxation. Mesenteric arterial rings were precontracted with PE and examined for relaxation to cumulative additions of acetylcholine (ACh). (B) Endothelium-independent relaxation. Mesenteric arterial rings were precontracted with PE and examined for relaxation to sodium nitroprusside (SNP). Values are given as means ± SEM of 6 rats in each group**.**

3.6. ENDOTHELIUM-INDEPENDENT RELAXATION RESPONSES

Endothelium-independent vascular relaxation to SNP was not different in arterial rings from PFOS males than control males (**Figure 6B, left panel**). SNP-induced relaxation was significantly decreased in PFOS females compared to control females (**Figure 6B, right panel**).

Table 1. Contraction and relaxation responses in mesenteric arterial rings of Control and PFOS offspring.

Variable	Control	PFOS
KCl, 80 mM, Active stress		
Male	17.7 ±0.86	19.1 ±1.57
Female	12.0 ±0.67	13.2 ±0.72
PE pD_2 [-log M]		
Male	6.12 ±0.03	6.17±0.03
Female	6.00 ±0.02	6.04 ±0.04
PE, E_{max},($3x10^{-5}$ mol/L, % of KCl Contraction)		
Male	106 ± 2.84	104.8 ±2.48
Female	105.8 ±3.33	109.7 ±3.41
ACh, pD_2 [-log M]		
Male	7.2 ± 0.08	7.5 ± 0.03*
Female	7.2 ± 0.11	7.8 ± 0.06*

ACh, E_{max},(10^{-5} mol/L, % Relaxation)		
Male	99.9 ± 0.06	91.6 ± 7.31
Female	100 ± 0.00	80.2 ± 6.03*
SNP, pD_2 [-log M]		
Male	7.9 ± 0.03	7.9 ± 0.03
Female	7.6 ± 0.03	7.9 ± 0.02*

All other abbreviations are as defined in the text. Data represent mean±SEM of measurements in arterial rings from 6 rats of each group. *Measurements in PFOS are significantly different (P<0.05) from respective sex in control.

3.7. eNOS EXPRESSION AND PHOSPHORYLATION

Western blot analysis using tissue homogenates of endothelium-intact mesenteric arteries and anti-eNOS antibody showed a prominent band at ~140 kDa with optical density was not significantly different between control males and PFOS males (**Figure 7A**). The enzyme eNOS possesses multiple phosphorylation sites, and Ser^{1177} is the most widely investigated phosphorylation site in the systemic vasculature. Phosphorylation at Ser^{1177} activates eNOS and increases in NO production [47-49]. Examination of the phosphorylation status of eNOS at Ser^{1177}, as an indicator of eNOS activity state, demonstrated that the optical density of phospho-eNOS was significantly lower in PFOS males than in control males (**Figure 7A**). The optical density of eNOS was significantly lower in mesenteric arteries from PFOS females than in control females (**Figure 7B**). In addition, phosphorylation at Ser^{1177} was significantly lower in the mesenteric arteries of PFOS females compared with control females (**Figure 7B**).

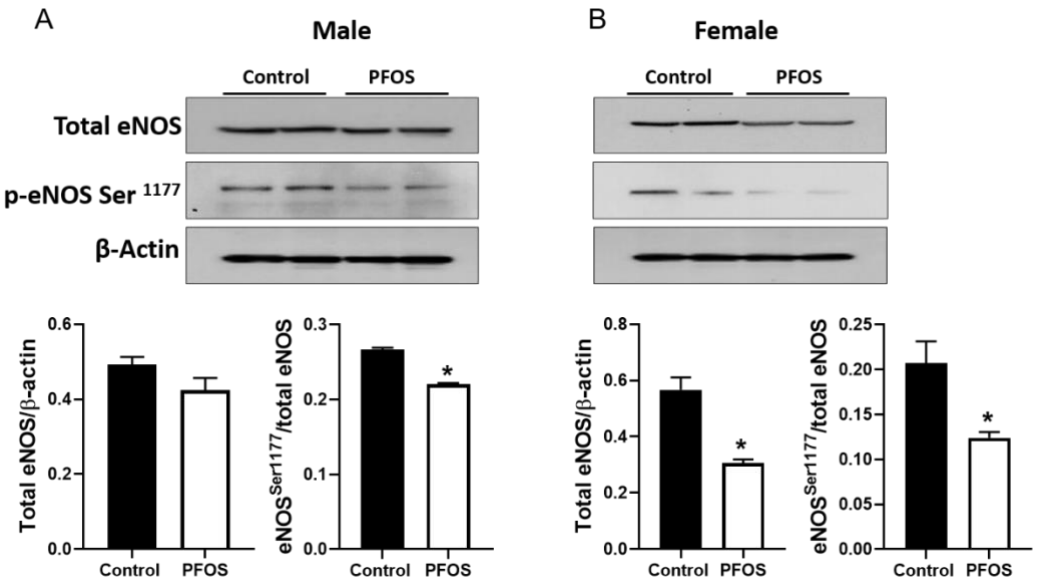

Figure 7. Endothelial nitric oxide synthase (eNOS) protein expression in the mesenteric arteries of adult male and female offspring of control and PFOS exposed dams. Protein was isolated from mesenteric arteries and probed for total eNOS and eNOS phosphorylated at Ser^{1177}. Representative Western blots for eNOS, phospho-eNOS, and β-actin are shown at the top; blot density obtained from densitometric scanning of eNOS normalized to actin, and phospho-eNOS normalized to total eNOS is shown at the bottom. Values are given as means ± SEM of 6 rats in each group. *p<0.05 vs. control group.

4. DISCUSSION

The present study's goal was to determine whether PFOS exposure through drinking water during pregnancy resulted in hypertensive male and female offspring that exhibit impaired endothelium-dependent vascular relaxation and enhanced vascular contraction. The main findings are as follows: (1) Birth weight and later growth are significantly lower in both male

and female PFOS offspring compared with respective sex in controls; (2) PFOS males remained smaller through adulthood while PFOS females exhibited catch-up growth (3) Blood pressure is elevated to similar magnitude in both PFOS males and females compared with respective sex in controls; (4) Vascular contractile responses in mesenteric arteries are not altered in both sexes; (5) the endothelium-dependent vascular relaxation is reduced in PFOS males, and endothelium-dependent and –independent relaxation is reduced in PFOS females; and (6) the reduced relaxation in PFOS males relates with decreased eNOS activity and in PFOS females relates with decreased eNOS expression and activity.

Cardiovascular diseases (CVD) are the leading cause of mortality and morbidity in the US. Among CVD, hypertension ranks first, affecting more than 73 million people—nearly 1 in 3 adults—in the United States. Hypertension directly increases a patient's risk of coronary heart disease, leading to stroke or heart attack. In spite of increased efforts to prevent, treat, and control hypertension, the prevalence of hypertension has not decreased. In fact, an increase in the incidence of hypertension is expected [50]. Although several genes have been identified to be associated with hypertension, the genetic component of this condition cannot account for the dramatic increase in the prevalence of CVD in recent years. It is now well established that a variety of insults, when experienced in the prenatal period, can have long-term influences on the individual's health. Given the prolonged period that PFASs were used, a potential exists for PFASs to constitute a significant ***in utero*** exposure for offspring now reaching the age of hypertension diagnosis. In the present study, we focused on ***in utero*** exposure to PFOS and subsequent risk for hypertension and vascular dysfunction in the male and female offspring.

The exposure of rats to PFOS through drinking water during pregnancy resulted in offspring with a significant reduction in birth weight compared to controls. Previous studies of

maternal PFOS exposure through diet or oral gavage have produced similar birth weight results in rats and mice (reviewed in [22]). Also, strong associations between prenatal exposure to PFOS and low birth weight have been reported in seven human epidemiological studies (reviewed in [23]). This study provides new data that the magnitude of decrease in birth weight following maternal PFOS exposure is similar for male and female offspring. This is in line with clinical observations in humans [51, 52], although some studies reported a greater impact, specifically in males [53] or females only [54]. The latter sex-specific effects reported in human studies need to be carefully interpreted as those reports involved high-risk pregnancies and were criticized for poor study design, which could have skewed the results towards specific sex. In general, our findings indicate that maternal PFOS exposure has fetal growth inhibitory properties. The decreased maternal weight gain noted in PFOS dams at term could reflect reduced fetal weight as exponential fetal growth (approximately 1 g day^{-1}) occurs at this stage of gestation [55]. The feed and water intake were unaltered in PFOS dams suggesting that the observed effects of PFOS on fetal growth in this model may not be secondary to nutritional intake in the dams.

PFOS is reported to cross the placenta [16-18] and has been detected in human amniotic fluid [56] and umbilical cord blood [57]. Thus, increases in maternal PFOS levels could increase in fetal PFOS levels and directly affect fetal growth and/or energy homeostasis. It will be interesting to directly inject/expose the fetus/newborn to PFOS and assess its impact on growth. Alternatively, increased maternal PFOS levels could reduce fetal growth through impaired placental function. In mammals, the primary determinant of intrauterine growth is the placental supply of nutrients to the fetus [58]. PFOS is shown to decrease trophoblast migration [59] and increase reactive oxygen species production [60]. Whether PFOS exposure affects placental

development, and nutrient transport capacity is yet to be investigated. Thus, further studies are needed to examine the exact mechanism of how maternal PFOS causes fetal growth restriction.

Although both the male and female PFOS offspring were equally growth restricted, the male PFOS offspring remained smaller through adulthood, while the females exhibited catch-up growth between 10–16 weeks of age. This is consistent with the clinical observations where girls with prenatal exposure to PFOS were smaller at birth, but these differences were eliminated by 9 months of age and weighed heavier at 20 months of age than controls [25, 30]; however, such catch-up growth was not evident in the boys with prenatal PFOS exposure [61]. It is possible that prenatal exposure to PFOS is associated with more enduring suppression of growth in males. Whether the catch-up growth observed PFOS females is due to increased adiposity/muscle mass or improved feed intake/efficiency is unknown, and why this occurs only in the female offspring needs to be investigated further. Prenatal exposure to PFOS is shown to delay puberty and alter sex steroid hormone levels, especially leading to decreases in plasma estrogen and androgen levels [62, 63]. Future studies should consider examining if the underlying change in hormones contributes to alterations in growth trajectories over time.

It is now well established that a variety of insults, when experienced in the prenatal period, can have long-term influences on the health of the individual. In the present study, prenatal PFOS exposure leads to blood pressure increase in the offspring, with males and females having similar onset (12 weeks) and magnitude of blood pressure increase (mean increase of 20 mmHg at 16 weeks in PFOS males and females vs. respective sex in controls). This suggests that prenatal PFOS exposure equally affects males and females. This is consistent with the previous reports in the offspring of pregnant rats given PFOS through oral gavage

during pregnancy [40]. These observations suggest that exposure to PFOS during pregnancy negatively influences their offspring's cardiovascular function in both sexes.

In order to investigate alterations of vascular function in the offspring of PFOS dams, vascular contractile responses to PE was investigated. It is well known that PE mediates vascular smooth muscle contraction by activating the α_1 adrenergic receptor. Both the male and female PFOS offspring were similarly responsive to PE compared to their respective sexes in control, suggesting that the vascular smooth muscle contractile responses have been preserved in the PFOS offspring. The KCl contraction data are consistent with the PE data and indicate that the unaltered vascular contraction PFOS offspring is not limited to a particular agonist/receptor coupling mechanism in the vascular smooth muscle. Additionally, PFOS offspring does not appear to have hypertrophy or hyperplasia of vascular smooth muscle cells, as the contractile response to membrane depolarization by KCl, a receptor-independent response [64], was unaltered compared to controls. The finding that relaxation to ACh was reduced in male and female PFOS offspring compared to respective controls suggests that prenatal PFOS exposure inhibits the endothelium-dependent relaxation pathway. The magnitude of ACh-induced appears to be more pronounced in the PFOS females than males (mean pD_2 increase of 0.6 log units in females vs. 0.3 log units in males and mean decrease of maximal relaxation by 20% in females vs. 8% in males).

One important vasodilator released from endothelial cells is NO [65], and a large number of published studies demonstrated that endothelial dysfunction in arteries is caused by the loss of NO biological activity [66]. To further investigate the possible role of NO synthesis/release in the impaired endothelium-dependent relaxation, we found that eNOS mRNA and protein expression and the amount of activated phospho-eNOS, which indicates its activity was reduced

in PFOS female offspring. In contrast, eNOS expression was not altered, but its activity was decreased in the PFOS male offspring. These observations suggest that NO synthesis by endothelial cells is impaired due to prenatal PFOS exposure. The precise mechanism by which prenatal PFOS exposure could decrease the amount and/or activity of eNOS is not clear and remain to be studied.

The NO produced by endothelial cells promotes vascular relaxation by activating guanylate cyclase and increasing cGMP production in vascular smooth muscle [42]. Although a decrease in eNOS expression/activity could explain the decreased relaxation in arteries from PFOS offspring, this response is also possible due to a change in vascular smooth muscle sensitivity to relaxation by NO. The observation that relaxation of endothelium-denuded arteries by SNP, an exogenous NO donor, was not significantly different between PFOS male offspring and control males provided evidence that prenatal PFOS does not affect the sensitivity of vascular smooth muscle to relaxation and thereby suggests that the reduced ACh-induced relaxation in PFOS male offspring is more likely due to decreased synthesis of NO from endothelial cells. On the other hand, SNP-induced relaxation in the endothelium-denuded arteries was less than that in control females. These data suggest that prenatal PFOS exposure in the females may attenuate vascular relaxation by an additional endothelium-independent mechanism involving direct effects on the cellular mechanisms of vascular smooth muscle relaxation. The molecular mechanisms responsible for reduced endothelium-independent relaxation in the PFOS female offspring is unknown. If there are structural changes related to differential vascular cell growth and proliferation in PFOS female offspring, should be examined in future studies.

Although the present study suggests that the decrease in endothelial cell and/or vascular smooth muscle function contributes to the observed elevation in arterial pressure, vascular

dysfunction may also be secondary to arterial pressure alterations. Further analysis of longitudinal changes in vascular function and blood pressure is essential to establish a cause-effect relationship. Prenatal PFOS exposure is also shown to impair neurodevelopment [34] and reduce nephron counts [40]; thus, the present data do not rule out the contribution of neuronal and renal mechanisms for vascular dysfunction and blood pressure increase. Previous studies have shown that plasma testosterone and estradiol levels are lower in PFOS offspring than controls [62, 63]. Testosterone and estrogen have been shown to modulate eNOS expression/activity and NO production via both genomic and nongenomic mechanisms [67, 68], which may contribute to regulation eNOS expression/activity. Future studies should also explore the arteries isolated from gonadectomized PFOS offspring with or without hormone replacement to determine whether changes in steroid hormones contribute to the vascular function.

5. Conclusions:

In conclusion, PFOS exposure during prenatal life leads to fetal growth restriction with postnatal catch-up in females, but not males and the development of hypertension with similar magnitude in both sexes. These hypertensive progenies have impairments in mesenteric vascular function in a sex-dependent manner with suppression of endothelium-dependent vasodilator pathway in males and deficits in both endothelium-dependent and endothelium-independent relaxation in females.

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Declaration of Competing Interest:

The authors declare no conflict of interest.

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Chapter 6

SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

Perfluorooctane sulfonate (PFOS), a ubiquitous environmental contaminant, poses a significant threat to maternal and fetal health. Emerging research indicates a potential link between PFOS exposure and the development of hypertensive disorders of pregnancy (HDP). Maternal health complications due to HDP can have lifelong consequences for both mother and child. To elucidate the mechanisms underlying this association, we investigated the effects of PFOS on maternal vascular adaptations, placental endocrine function, and long-term offspring cardiovascular health. Using a clinically relevant exposure model, we aimed to identify potential therapeutic targets for mitigating the detrimental effects of PFOS during pregnancy.

MATERNAL VASCULAR ADAPTATIONS:

To investigate the effects of PFOS on vascular adaptations during pregnancy, we employed a pregnant Sprague-Dawley rat model. PFOS was administered continuously through drinking water, providing a more clinically relevant simulation of human environmental exposure compared to the daily single-dose oral gavage used in many toxicological studies. Our findings demonstrate that elevated PFOS levels during pregnancy induce hypertension and cardiac hypertrophy in dams (**Figure 1**). Mechanistically, PFOS exposure altered angiotensin receptor expression in the uterine artery (↑AT1R, ↓AT2R), promoting heightened vasoconstriction. Additionally, PFOS impaired endothelium-dependent vasodilation, likely due to reduced eNOS expression. These combined effects suggest PFOS-induced disruption of uterine artery hemodynamics, potentially contributing to the observed fetal and placental growth

restriction.

THERAPEUTIC INTERVENTION WITH AT2R AGONIST:

A novel aspect of this study was the exploration of a therapeutic intervention using the AT2R agonist Compound 21 (C21) to mitigate PFOS-induced hypertension during pregnancy. C21 treatment successfully normalized blood pressure in PFOS-exposed dams, potentially by improving uterine artery blood flow and reducing Ang II-mediated vasoconstriction. Furthermore, C21 restored endothelial-dependent vasodilation in PFOS-exposed dams, an effect associated with increased uterine artery eNOS expression and elevated plasma bradykinin levels. Importantly, C21 treatment improved fetal and placental growth outcomes in PFOS-exposed dams, likely through the restoration of vascular function and enhanced uterine blood flow.

PLACENTAL ENDOCRINE DISRUPTION BY PFOS:

Our study revealed sex-specific alterations in placental endocrine function following PFOS exposure. While fetal and placental weights decreased in both sexes, the reduction in placental weight was primarily localized to the labyrinth zone. Significant changes in maternal plasma steroid hormone levels were observed, including increases in progesterone, aldosterone, corticosterone, and testosterone, along with decreased estradiol, hCG, and prolactin. These hormonal disruptions were associated with sex-specific alterations in the expression of key steroidogenic enzymes (Cyp11A1, 3β-HSD1, 17β-HSD1, 17β-HSD3) and the steroid metabolism enzyme UGT1A1. Importantly, the rat placenta lacks aromatase (Cyp19A1), and we observed decreased ovarian Cyp19A1 expression in PFOS-exposed dams. These findings strongly suggest that the placenta is a direct target of PFOS-induced endocrine disruption.

LONG-TERM OFFSPRING CONSEQUENCES OF PRENATAL PFOS EXPOSURE:

We investigated the long-term consequences of prenatal PFOS exposure on adult offspring. Offspring from PFOS-exposed dams exhibited lower birth weights. While male PFOS offspring remained smaller throughout adulthood, females exhibited catch-up growth. Both male and female PFOS offspring developed hypertension after 12 weeks of age. Endothelium-dependent vascular relaxation was impaired in both sexes, with a more pronounced effect in females. Interestingly, endothelium-independent relaxation was only reduced in female PFOS offspring. While vascular eNOS expression was unchanged in males, PFOS exposure decreased eNOS activity (phospho-Ser1177-eNOS). In females, both total eNOS and phospho-Ser1177-eNOS expression were reduced. These findings highlight the potential for prenatal PFOS exposure to program the development of long-term cardiovascular risks.

LIMITATIONS AND FUTURE DIRECTIONS:

While this work has advanced our understanding of the mechanisms by which PFOS disrupts maternal health and contributes to the pathogenesis of HDP, several important questions remain.

1. THE COMPLEXITY OF PFAS EXPOSURE:

- **Individual PFAS Variation:** Our study focused on PFOS as a representative PFAS. However, environmental exposure involves a diverse array of PFAS compounds with potentially distinct pharmacokinetics and mechanisms of action. Future studies should

systematically investigate how variations in PFAS structure and properties influence pregnancy outcomes.

- **Mixture Effects:** Humans are exposed to combinations of PFAS and other environmental pollutants. Research is needed to explore potential additive or synergistic effects of these mixtures on maternal and fetal health.

2. Elucidating Mechanisms of PFOS-Induced Cardiovascular Dysfunction:

- **Cardiac Impact:** Having established PFOS-induced uterine artery dysfunction and left ventricular hypertrophy, it is crucial to determine if similar hyperactivation of Ang II signaling drives PFOS-related cardiac pathology. Further investigation should also consider the role of PFOS-induced oxidative stress and inflammation in cardiovascular impairment.
- **Endothelial Dysfunction:** Investigating the precise mechanisms by which PFOS inhibits eNOS is vital. Examining whether PFOS disrupts VEGF and PlGF signaling, which play key roles in pregnancy-related vascular adaptations, could provide valuable insights. Additionally, exploring whether PFOS alters gene splicing patterns related to endothelial dysfunction warrants exploration.

3. Placental Impacts of PFOS Exposure:

- **Labyrinth Zone Effects:** While we have investigated the endocrine function of the placental junctional zone, thorough studies of the labyrinth zone's response to PFOS are needed. These should focus on PFOS-induced changes in placental vascularization and the potential consequences for nutrient transport.

- **Nutrient Transport Mechanisms:** Our preliminary studies indicate PFOS disrupts placental glucose (**Figure 2**), amino acid (**Figure 3**), and DHA transport (**Figure 4**). Determining whether this occurs primarily through reduced placental vascularization or by directly targeting nutrient transporters is essential. Immunostaining could reveal if PFOS selectively impacts specific zones and transporter localization within the placenta.

4. TRANSLATIONAL RELEVANCE:

- **Human Studies:** Replicating key findings using human placental and omental arteries would strengthen the translational relevance of our rat model results. This would have significant implications for understanding PFAS risks in humans, guiding future public health interventions and clinical practice.

CONCLUSIONS:

This thesis investigated the impact of PFOS exposure on maternal and offspring cardiovascular health. Utilizing a rat model that mimics human exposure routes, we elucidated molecular mechanisms linking maternal PFOS exposure to the development of hypertensive disorders of pregnancy (HDP). Key findings include:

- **Maternal Cardiovascular Disruption:** PFOS exposure induces maternal hypertension, cardiac hypertrophy, and impairs uterine artery function through alterations in angiotensin receptor signaling and endothelial dysfunction.
- **Placental Endocrine Disruption:** PFOS significantly alters the placental steroidogenic profile and disrupts steroid hormone synthesis in a sex-specific manner, highlighting the placenta as a direct target of its toxicity.

- **Offspring Outcomes:** Prenatal PFOS exposure leads to persistent hypertension and endothelial dysfunction in adult offspring, with potential sex-specific differences in severity.
- **Therapeutic Potential:** Targeting the angiotensin system with AT2R agonists demonstrates promise in mitigating PFOS-induced hypertension during pregnancy, providing a potential avenue for intervention.

This work underscores the urgent need for research investigating the mechanisms of PFOS toxicity to protect maternal and fetal health. It also highlights the importance of identifying therapeutic strategies to mitigate the adverse effects of environmental pollutants on pregnancy and long-term offspring health.

FIGURES:

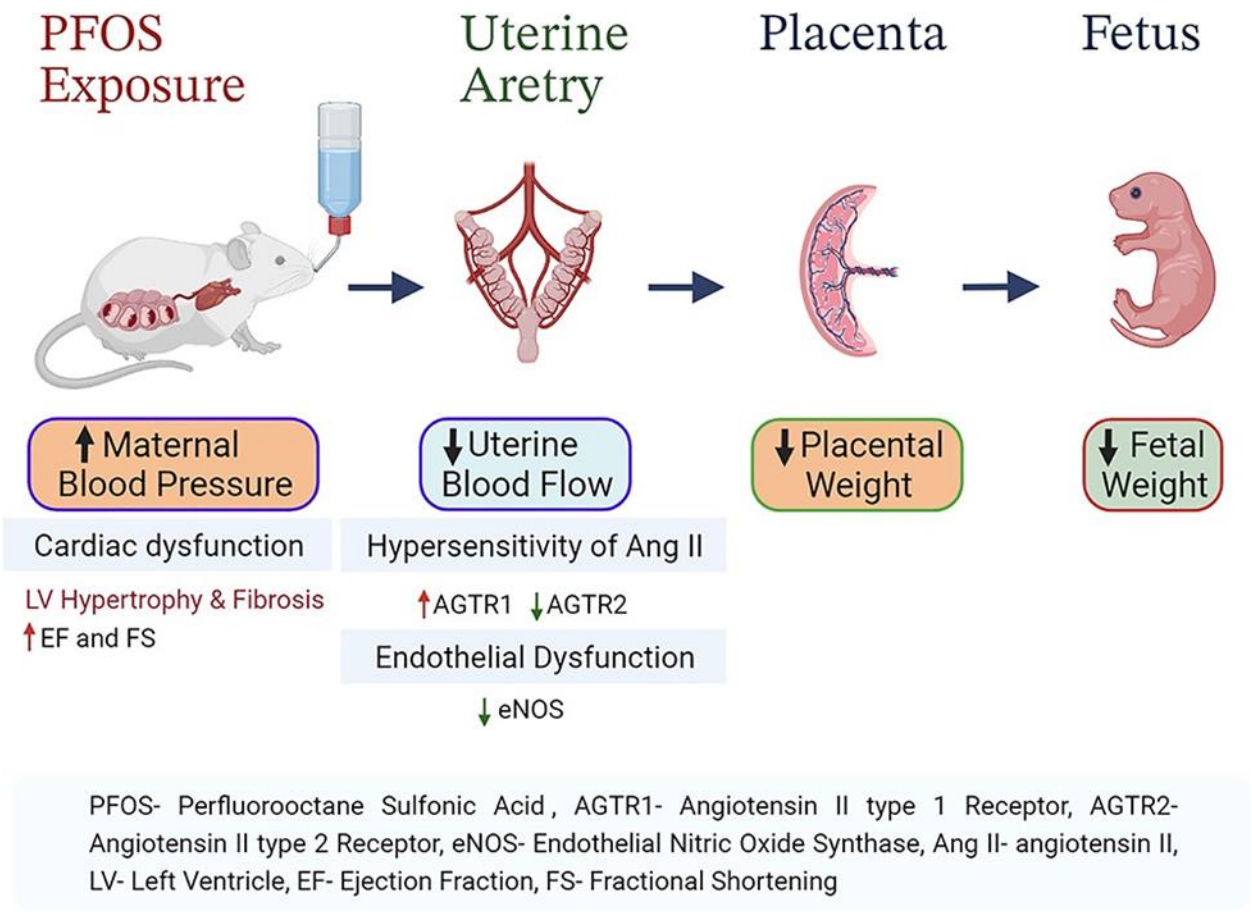

Figure 1. Molecular Mechanisms Linking Maternal PFOS Exposure to Gestational Hypertension and Fetal Growth Restriction.

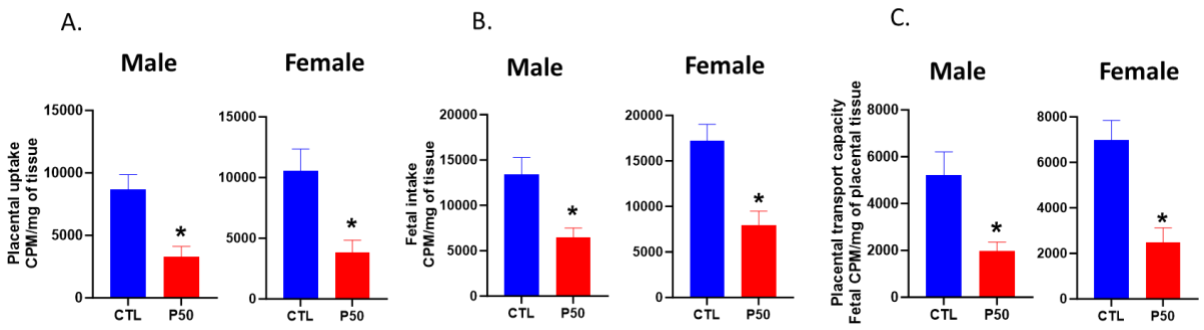

Figure 2. Placental Glucose Transport in Control and PFOS Dams. Placental glucose transport was measured using 3-O-methyl-D-[3H] glucose. (A) Placental uptake (placental counts per minute (CPM) per mg of the placenta). (B) Transport to the fetus (fetal CPM per mg of fetal tissue). (C) Placental transport capacity (fetal CPM per mg of the placenta). Values are given as means ± S.E.M. *P < 0.05 vs controls.

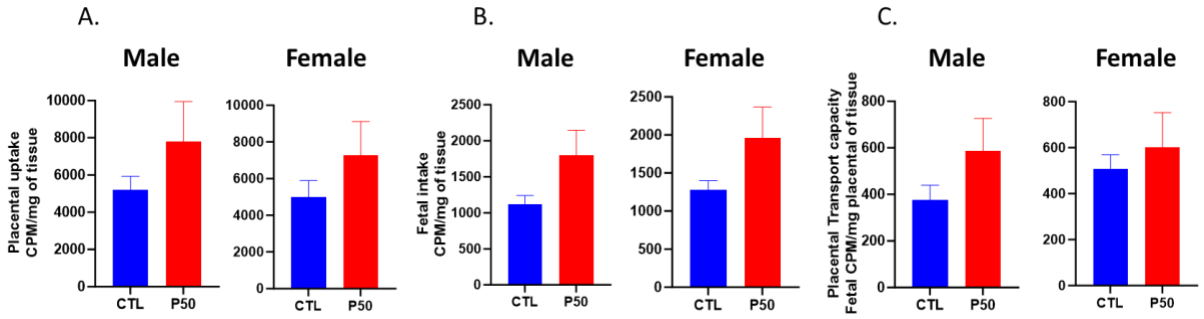

Figure 3. Placental Amino Acid Transport in Control and PFOS Dams. Placental amino acid transport capacity was measured using [14C]-methylamino isobutyric acid. (A) Placental uptake (placental CPM per mg of the placenta). (B) Transport to the fetus (fetal CPM per mg of fetal tissue). (C) Placental transport capacity (fetal CPM per mg of the placenta). Values are given as means ± S.E.M. *P < 0.05 vs controls.

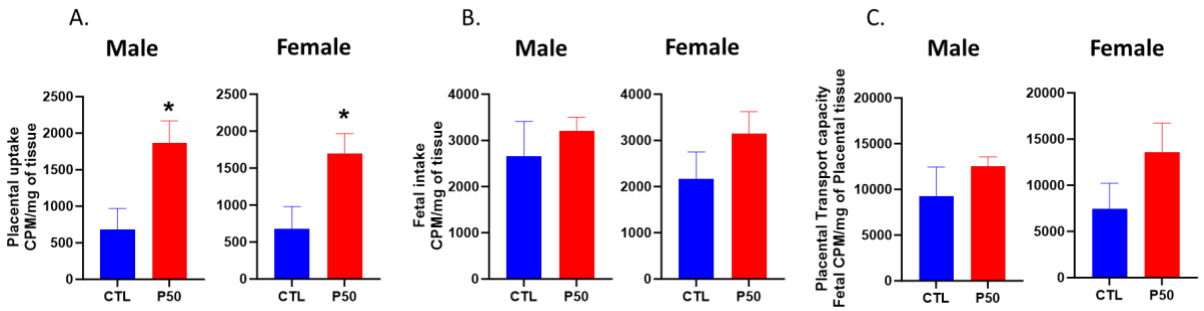

Figure 4. Placental DHA Transport in Control and PFOS Dams. Placental DHA transport capacity was measured using [14C]-Docosahexaenoic acid. (A) Placental uptake (placental CPM per mg of the placenta). (B) Transport to the fetus (fetal CPM per mg of fetal tissue). (C) Placental transport capacity (fetal CPM per mg of the placenta). Values are given as means ± S.E.M. *P < 0.05 vs control.