THE IMPACT OF PRENATAL EXPOSURE TO LOW DOSES OF BISPHENOLS ON CARDIOVASCULAR OUTCOMES: INVESTIGATION OF POSSIBLE MECHANISMS

by

MARYAM AL MANSI

(Under the Direction of Sheba M.J. MohanKumar)

ABSTRACT

Cardiovascular diseases (CVDs) and hypertension (HTN) are closely linked and influenced by early-life exposures. Prenatal exposure to endocrine-disrupting chemicals (EDCs) like bisphenol A (BPA) can alter heart structure and blood pressure regulation in offspring, potentially increasing CVD risk. HTN incidence is influenced by sex, age, and environmental factors. We examined prenatal exposure of Sprague Dawley rats to low doses of BPA, bisphenol S (BPS), and F (BPF) at levels considered safe for humans. We hypothesized that there will be sex- and chemical-specific alterations to the neurocardiovascular system and female exposure to estradiol (E2) and male exposure to a high-fat diet would exacerbate these effects. Our findings confirm the sex and chemical-specific alterations to the neurocardiovascular system. Both males and females prenatally exposed to EDCs had increased blood pressure accompanied by sympathoactivation of the paraventricular nucleus (PVN) of the hypothalamus. In both sexes, BPS increased Angiotensin II and Endothelin -1 while inducing cardiac muscle fibrosis. BPF increased Aldosterone in both offspring while BPA increased fibrosis and heart weight. There was sex difference with

Corticosterone levels increased in all EDC-treated males but not females and Endothelin-1 increased in males' BPA and BPF offspring but not females. BPF increased left ventricle wall thickness, and heart weight while increasing angiotensin II and inducing fibrosis in male hearts. While in females, all EDCs increased kidney and adrenal weight, and exposure to BPF, and BPS, increased E2 levels. Males show greater sensitivity to BPF exposure, while BPS can cause significant alterations in both sexes. When challenged with a second insult with exposure to a high-fat diet in males, all EDC-treated males had increased blood pressure, BPF offspring had increased sympathoactivation of the PVN and Aldosterone secretion while BPS offspring had increased Angiotensin II, Aldosterone, and Corticosterone levels. As for females challenged with chronic E2, BPS offspring had increased blood pressure, paraventricular sympathoactivation, increased Angiotensin II, and E2 levels accompanied by fibrosis in the heart. These findings raise concerns regarding the low dose EDC exposure on cardiovascular health and show that BPS and BPF are not “safe alternatives” to BPA and can cause more detrimental effects.

INDEX WORDS: Endocrine-disrupting chemicals, prenatal exposure, bisphenols, estradiol, high-fat diet, cardiovascular diseases, hypertension, neurotransmitters, hormones

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Chapter 1

INTRODUCTION

1.1 Endocrine Disrupting Chemicals – Background

Endocrine Disrupting Chemicals (EDCs) started receiving widespread attention in 1996, following the publishing of the book "Our Stolen Future". It is a book that examines the harmful effects of EDCs on human and animal health, including birth defects, developmental disorders, and cancer. The authors support their argument with scientific data from various sources and criticize regulatory agencies for failing to address the issue. The book advocates for a proactive environmental policy that prevents harm rather than just reacting to it. Its impact includes raising public awareness and influencing policy changes such as chemical bans and the creation of the Endocrine Disruptor Screening Program by the US EPA [1]. The endocrine society defined EDC as: "an exogenous chemical, or a mixture of chemicals, that interferes with any aspect of hormone action" [2]. One of the most prominent EDCs is Bisphenol A (BPA), which has extensive applications in various fields, such as manufacturing, food packaging, dental filling, and medical equipment [3]. BPA resins are often used in the lining of canned food and drinks, which can lead to the release of BPA into food or water [3, 4]. Due to its widespread use, BPA is detected in the urine of 93% of Americans [5] and 94% of people in Asian countries, with the highest levels found in Kuwait followed by Korea and India [6]. It is the most widely used chemical, and the BPA market continues to expand at a rate of 6.2%, having already reached a share of more than 7,000 metric tons in 2022. It is projected to grow to over 12,000 metric tons by the year 2031 [7]. BPA was first synthesized in 1891 and identified as estrogenic in 1936 [8].

Urinary levels of BPA were associated with metabolic [9, 10], behavioral [11], cardiovascular [4, 12, 13], reproductive [14, 15], and developmental anomalies [6, 16]. Due to increasing concerns regarding the health effects of BPA, regulations have been put in place to restrict its usage. Consequently, substitutes to BPA, such as Bisphenol S (BPS) and Bisphenol F (BPF) have been developed to help reduce BPA usage [17]. However, these alternatives have been found in various sources including canned foods, human serum, dental sealants, and thermal paper [18-20]. Since these chemicals are structurally analogues to BPA, they have been found to act physiologically like BPA [17]. This indicates that utilizing alternative bisphenols may not necessarily be a safer option and could also present health hazards.

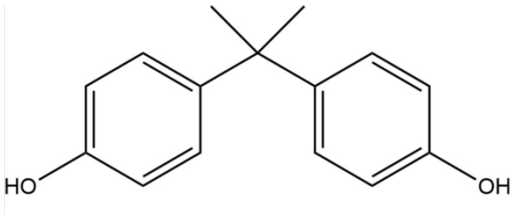
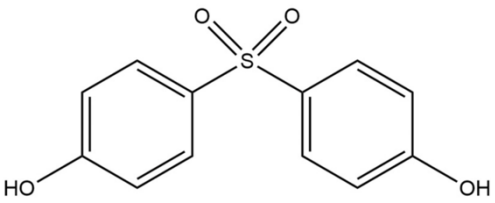
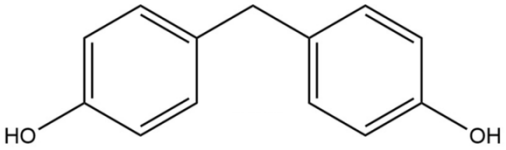
Chemical Properties and Human Exposure of BPA and its Analogues

The similarity of the structure of BPA and its analogues stems from the commonality of having two functional phenol groups on either side connected with isopropyl for BPA, a sulfonyl group in BPS, and methylene for BPF (Table 1.1). BPA and its analogues have a similar route of exposure, most common is through ingestion but it is also possible through inhalation and dermal exposure [21, 22]. The half-life of each chemical as shown in Table 1.1, differs between maternal versus fetal exposure after a single dose of 0.5 mg/kg in sheep model [23]. Fetal exposure has a much higher half-life than maternal, with fetal exposure to BPS having a half-life of 402.2 hour [23]. This is concerning since BPS is the least biodegradable bisphenol followed by BPA and BPF [24]. This would allow BPS to accumulate in the environment and lead to higher exposure during critical development periods and increased risk of disease later in life.

The National Health and Nutrition Examination Survey (NHANES) study conducted in 2013-2014 analyzed the presence of BPA, BPS, and BPF in urine samples of adults and children

in the US. The results showed that these compounds were detected in a high percentage of samples (95.7%, 89.4%, and 66.5%, respectively). Both humans and rats quickly metabolized and eliminated BPA and its substitutes through urine. The study also found that the median urinary levels of BPA, BPF, and BPS in the adult US population were 1.24 μg/L, 0.35 μg/L, and 0.37 μg/L, respectively. Interestingly, individuals with higher income-to-poverty ratios had lower BPA levels, and there was a significant difference in BPF levels between medium and low-income groups with medium groups having higher levels than lower-income groups. Current smokers also had higher urinary BPA levels compared to non-smokers. However, the reasons underlying these associations are not well understood and require further investigation [21]. Due to its large scope of use, BPA and its analogues have been detected not only in the urine [21, 25] but also in the saliva [26], serum [27], breast milk [28], amniotic fluid [29], and fat tissue [25]. These findings suggest that the chemicals can enter and accumulate in the body, potentially leading to adverse health effects. BPA and its analogues have been found to act as estrogenic chemicals and were implicated in many terminal diseases; their physiological effects have a wide range of alterations to the endocrine system (Table 1.1.).

Table 1.1 Properties of BPA and its Analogues

EDC	General Chemical Structure [22]	Sources of Exposure [21, 30]	Half-Life (hr)[23]		Physiological Effects
			Maternal	Fetal	
BPA		Polycarbonate plastics, the lining of food cans, epoxy resin	5.3	52	Estrogenic [31], Reproductive [32-34], Metabolic [35] Behavioral [36] Cardiovascular [37, 38]
BPS		Thermal paper, cleaning products, plastics, shampoos and lotions	3.7	402.2	Estrogenic [31], Reproductive [32-34] Metabolic [35] Behavioral [39] Cardiovascular [37, 40]
BPF		In food, canned food lining, plastic products, epoxy resin	7.7	14.2	Estrogenic [31] Reproductive [34, 41] Metabolic [42] Behavioral [36] Cardiovascular [43]

Note: Bisphenol A (BPA), Bisphenol S (BPS) and Bisphenol F (BPF).

Maternal-Fetal Transfer

The Developmental Origins of Health and Disease (DOHaD) concept, also known as the Barker Hypothesis, links early-life environmental factors to a higher risk of diseases later in life [44, 45]. Poor nutrition during organ development is a significant factor, and recent research has broadened the scope to include immunological, mental health, and reproductive diseases that result from both over- and under-nutrition during fetal development [46]. Stress, diet, and exposure to chemicals can all influence the differentiation process during early embryogenesis

[45]. Environmental factors can influence epigenetic processes like DNA methylation and histone modifications, potentially predisposing cells and tissues to diseases across the lifespan. BPA has been found to cause epigenetic changes through the alteration of the DNA methylation [47]. Hypermethylation reduces the promoter region's affinity for transcription factors while increasing its affinity for certain proteins and enzymes, repressing gene transcription [48]. These environmental influences are particularly harmful during the gestational and early childhood stages [44, 49, 50]. These are the ‘organizational’ states of different systems of the body. When tissues are extremely sensitive to environmental influences on the epigenome, they have distinct 'critical windows' [51]. Compelling evidence suggests a link between developmental chemical exposures such as smoking, lead, and methyl mercury, and later-life illnesses such as obesity, neurodevelopmental disorders, asthma, and immune dysfunctions [49, 51, 52].

EDCs exposure during the critical window of fetal development in utero (‘organizational’ state) is determinantal to the offspring’s health [41, 52-57]. EDCs have been shown to cross the placental barrier and were found in the amniotic fluid, fetal urine, and plasma [23]. Exposure to EDCs on the developing fetus can cause irreversible adverse effects because the fetus's blood-brain barrier, DNA repair mechanisms, or immune system is not yet completely developed [58]. Evaluating the levels of EDCs in utero poses challenges and potential risks for both the mother and fetus. As a result, researchers often rely on animal studies to explore the effects of prenatal or perinatal exposure to EDCs. These animal studies offer valuable insights into the potential consequences of EDC exposure during crucial developmental stages, contributing to our knowledge of the potential risks to human health [59].

Receptor-Mediated Activity and Dose-Response Characteristics

BPA is known for its estrogenic abilities [60, 61], it acts as an agonist for estrogen receptors (ERα and Erβ), displaying a strong affinity for these receptors but lower than estradiol's affinity [62]. The binding affinity of BPA to ERα is 10,000 times lower, and to ERβ is 1,000 times lower, compared to 17β-estradiol (E2) [62]. Relative transactivation of BPA to ERα is 50% and to ERβ is 41% when compared to E2. BPA also exhibits a slightly lower binding affinity to human ERα and ERRγ compared to E2, the biologically active form of estrogen, indicating its partial ability to activate these receptors [63].

BPA also binds to the G protein-coupled receptor 30 (GPR30), also known as G-protein-coupled estrogen receptor [64]. Additionally, BPA interacts with estrogen-related receptor gamma (ERRγ) as an agonist [65]. In fact, there is evidence that even one phenol-hydroxy group is sufficient for bisphenol to have a high affinity to ERRγ, indicating the possibility of structural analogues like BPS or BPF having a similar affinity [65, 66]. High expression of ERRγ in the placenta and its involvement in energy regulation and mitochondrial biogenesis during pregnancy raise concerns about the potential accumulation of bisphenols, such as BPA, in this critical organ [67]. This is particularly worrisome due to the vital role of the placenta in supporting fetal development and maintaining a healthy pregnancy [68].

In addition to BPA's estrogenic effects, it exhibits antiandrogenic activity, interfering with androgen receptor signaling, testosterone levels, and male fertility [69-71]. It has been shown to increase progesterone receptor (PR) expression, further influencing hormonal pathways [72]. BPA can activate the human pregnane X receptor (hPXR), a nuclear receptor involved in xenobiotic metabolism and regulation and elimination processes [73]. It can dock through hydrogen bonding of either hydroxyl groups of the phenol to hPXR, indicating the possibility of

BPA analogues binding as well [74]. Furthermore, BPA acts as an antagonist to thyroid hormone receptors (TRs), preventing triiodothyronine (T3) binding to TR and suppressing gene activation [75].

Research is underway to assess the receptor-mediated activity of BPS and BPF, widely used as replacements for BPA, to understand their relative impacts. BPS was shown to have weaker estrogenic affinity than E2 and is similar to BPA [76]. While BPF shows lower estrogenic ability, once metabolized, it has higher estrogenic activity than BPA [77]. BPS was found to be an estrogen agonist [78] and antagonist [79], and an androgen antagonist [78, 80], and agonist [81]. As for BPF, it acts as an estrogen agonist [78, 82] and an androgen antagonist [78, 82]. Like BPA, BPS, and BPF act antagonistically to TRs. BPA has the highest affinity for binding to TRs, followed by BPF and then BPS. [83, 84].

Traditionally, toxicology studies focused on assessing the toxicity of chemicals at high concentrations before the emergence of endocrine-disrupting chemicals (EDCs). The concept known as "the dose makes the poison" is a fundamental principle in Toxicology, originating from Paracelsus, a Swiss physician, and alchemist, in the 16th century. This principle emphasizes that the toxicity of a substance is determined by its dosage or concentration [85]. However, this was challenged with EDCs, as they can cause more adverse effects at low doses and exhibit non-monotonic dose-response curves (NMDRCs), where the slope changes sign at least once [85]. Low doses are typically defined as those within the typical human exposure range or doses lower than the lowest observed adverse effect level (LOAEL)[85]. Currently, no observed adverse effect level (NOAEL) and LOAEL has been established for BPA;5 mg/kg bw/day,50 mg/kg/day, BPS; 10 mg/kg/day, 60 mg/kg/day and BPF; has not been established;

respectively [86, 87] (EPA 2012, 2014). The complexity of assessing the risk associated with BPA and its analogues is influenced by several factors.

The wide range of mechanisms of action, such as estrogenic and antiandrogenic effects, as well as interactions with specific receptors, underscores the importance of gaining a comprehensive understanding of their impacts on various physiological systems. Furthermore, the concept of low-dose effects challenges the traditional understanding that higher doses always lead to greater toxicity. It highlights the need to carefully consider the dose range and exposure levels when evaluating the risk of BPA and its analogues, as adverse effects can occur even at low concentrations. This necessitates thorough research and risk assessment to ensure the safety of human health and the environment in the presence of these substances.

1.2. Blood Pressure Regulation

Blood pressure regulation is a complex process involving the interplay of multiple organs and intricate cellular interactions. The neurohumoral system, endothelium, sympathetic nerve activation, and immune system all play important roles in maintaining blood pressure within a tightly regulated range. The balance between vasoactive and vasodilative mediators is critical for optimal blood pressure control. Disruptions to this delicate balance can arise from numerous factors, including genetic predisposition, lifestyle choices, environmental influences, and elevated levels of stress. Essential hypertension, affecting a sizable portion of the population, is typically influenced by multiple factors and exhibits variations in prevalence based on age, gender, and race [88-90].

Understanding the physiological mechanisms underlying blood pressure regulation is crucial for comprehending the pathophysiology of hypertension and its associated complications.

Several key mechanisms contribute to blood pressure regulation and can be altered in hypertensive conditions. These include changes in the renin-angiotensin-aldosterone system (RAAS), the hypothalamic-pituitary-adrenal axis (HPA), sympathetic nervous system (SNS) activity, peripheral resistance, calcium handling, endothelial function, and immune response. Disruptions of these mechanisms can lead to chronic hypertension and subsequent organ damage [88, 89, 91].

RAAS plays a critical role in the regulation of blood pressure. It involves the interaction of multiple organs, including the brain, heart, kidney, and adrenals. Within the kidney, renin, and pro-renin are synthesized and stored in the juxtaglomerular cells. Under normal conditions, low sodium levels or low blood pressure stimulate the release of renin, which then cleaves angiotensinogen to produce angiotensin I (Ang I). Ang I is subsequently converted to angiotensin II (Ang II) by the action of angiotensin-converting enzyme (ACE). Ang II can also be further broken down to angiotensin (1-7) (Ang [1-7]). Ang II exerts its effects through activation of the angiotensin type 1 receptor (AT1) or angiotensin type 2 receptor (AT2), while Ang (1-7) acts primarily on AT2 receptors. Activation of AT1 receptors by Ang II leads to vasoconstriction, increased vascular resistance, aldosterone release, and sodium reabsorption. In contrast, AT2 receptors are involved in antiproliferative and vasodilatory effects. Dysregulation of the RAAS, such as increased Ang II production or impaired aldosterone regulation, can contribute to the development of hypertension. Ang II promotes vasoconstriction and aldosterone release, leading to sodium and water retention, increased peripheral resistance, cardiac hypertrophy, and elevated blood pressure [88, 92, 93] (Summarized in Figure 1.1).

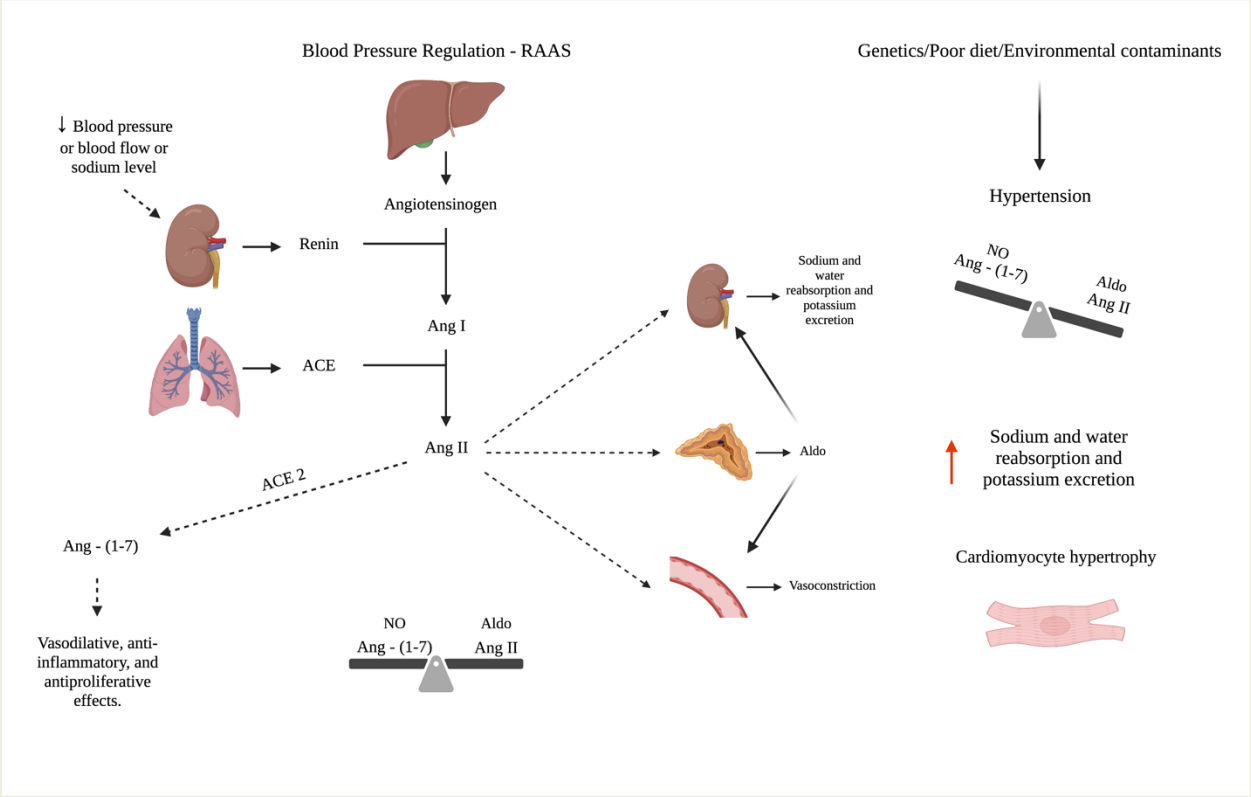


Fig.1.1. Blood pressure regulation through the RAAS vs. hypertensive pathological state. Balance between vasoactive and vasodilative markers. Note: RAAS: renin angiotensin aldosterone system, Ang: angiotensin, NO: nitric oxide, Aldo: aldosterone, ACE: angiotensin converting enzyme. *Created with BioRender.com*

Changes in peripheral resistance, particularly alterations in vascular tone and smooth muscle cell responsiveness, play a critical role in blood pressure regulation. Calcium, a key component in smooth muscle contraction and relaxation, is essential in this process. Decreased plasma calcium levels can potentially lead to increased blood pressure and peripheral resistance, and this can be mediated through various mechanisms involving parathyroid function, the RAAS, and vitamin D. Parathyroid hormone (PTH), released by the chief cells in the parathyroid gland, exerts its effects through the parathyroid hormone receptor type 1 (PTHr-1), which is expressed in endothelial cells, vascular smooth muscle cells, and along the nephron. Decreased calcium levels trigger an increase in PTH and renin, subsequently leading to elevated levels of

Ang II and Aldosterone (Aldo). Aldo, synthesized in the adrenal cortex, acts on the kidneys to increase sodium reabsorption and potassium excretion, resulting in water reabsorption, increased cardiac output, and elevated blood pressure [91, 94].

Calcium levels also have a significant impact on cardiac muscle excitation and relaxation. Sarcoplasmic reticulum calcium ATPase cardiac isoform 2a (SERCA2a) is responsible for actively transporting calcium ions from the cytoplasm back into the sarcoplasmic reticulum in cardiac muscle cells. Its activity is modulated by phospholamban (PLB), an inhibitory protein that controls SERCA2a function. Phosphorylation of PLB relieves its inhibitory effect, allowing for increased calcium reuptake into the sarcoplasmic reticulum and enhancing muscle relaxation. This process is crucial for muscle relaxation, ensuring the availability of calcium ions for subsequent contractions. Disruptions in calcium handling, such as impaired calcium reuptake or altered calcium sensitivity, can lead to increased vasoconstriction and elevated blood pressure. Additionally, reduced expression of SERCA2a has been associated with heart failure, highlighting its importance in maintaining cardiac function and potential therapeutic targets for heart failure treatment [91, 94, 95].

SNS plays a crucial role in the regulation of blood pressure by exerting influence on heart rate, contractility, and vascular tone. Increased sympathetic activity and elevated levels of catecholamines can lead to heightened vasoconstriction and elevated blood pressure. Within the brain, the paraventricular nucleus (PVN) is located bilaterally around the third ventricle. Stimulation of the PVN activates magnocellular and parvocellular neurons, which can release vasopressin or corticotropin-releasing hormone (CRH) through neurohypophysis. Also, the PVN sends projections to the medulla, specifically the rostral ventrolateral medulla (RVLM), and the spinal cord, particularly the intermediolateral nucleus (IML). These connections allow the PVN

to modulate sympathetic outflow. Pre-sympathetic neurons from the PVN project into the thoracolumbar IML nucleus, where they synapse with motor sympathetic preganglionic neurons (SPNs). The axons from these SPNs connect with the autonomic ganglia in the peripheral nervous system. From the ganglia, postganglionic sympathetic neurons extend their axons to innervate various target organs, including the heart, blood vessels, and glands. PVN stimulation of the RVLM has similar effects but to a greater extent. Activation of the SNS by the PVN helps regulate important physiological processes such as blood pressure, heart rate, and responses to stress. Dysregulation of the SNS, commonly observed in hypertension, contributes to sustained increases in peripheral resistance, thereby contributing to elevated blood pressure [88, 89] (Summarized in Figure 1.2).

The endothelium plays a vital role in regulating the tone of the vasculature, exerting both vasodilatory and vasoconstrictive actions. It actively synthesizes and releases various substances that have these effects, including nitric oxide (NO) [96] as a vasodilator and endothelin 1 (ET-1) [97] as a vasoconstrictor. Endothelial dysfunction, characterized by decreased availability of nitric oxide, increased oxidative stress, elevated levels of ET-1, and impaired vasodilation, disrupts the delicate balance between vasoactive factors. This imbalance favors vasoconstriction and contributes to elevated blood pressure [88].

Furthermore, the immune response is involved in the regulation of blood pressure, and there is evidence supporting a connection between inflammation and hypertension. Activation of the immune system can lead to endothelial dysfunction, heightened vascular tone, and structural changes in the blood vessels, all of which contribute to elevated blood pressure. These effects are

mediated, in part, by an increase in reactive oxygen species (ROS), the release of cytokines, and the activity of metalloproteinases [88, 93].

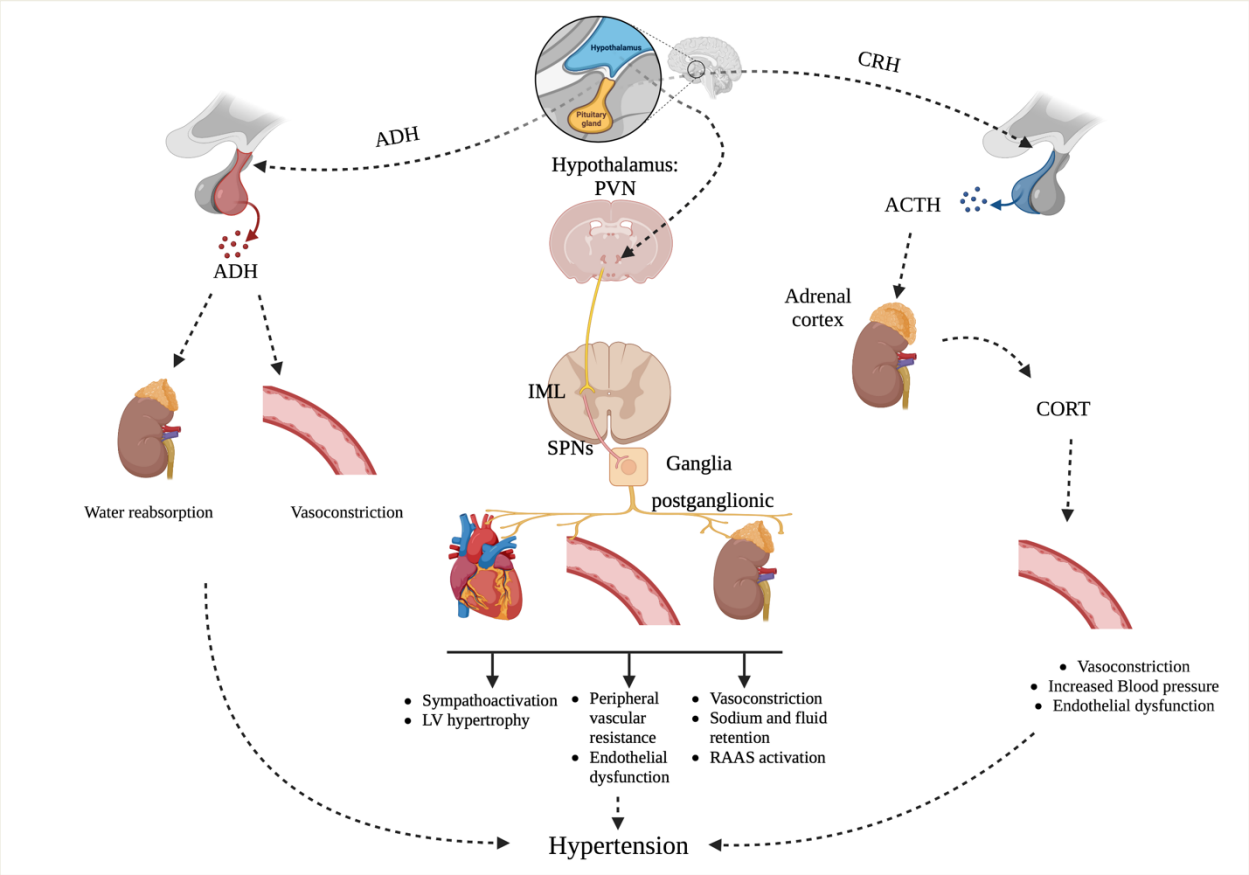


Fig.1.2. Overview of PVN's sympathetic regulation of cardiovascular function. PVN: paraventricular nucleus, IML: thoracolumbar intermediolateral nucleus, SPNs: sympathetic preganglionic neurons, RAAS: renin angiotensin aldosterone system, ADH: antidiuretic hormone or vasopressin, CRH: corticotropin releasing hormone, ACTH: adrenocorticotropic hormone, CORT: cortisol. *Created with BioRender.com*

1.3. EDC Exposure and Hypertension

Hypertension, a chronic medical condition characterized by consistently elevated blood pressure, poses a significant health concern on a global scale. Blood pressure is commonly measured using the ratio between systolic and diastolic blood pressure, reflecting the force exerted on arterial walls during heart contractions and relaxation, respectively. It is widely acknowledged as the most prominent risk factor for CVDs, which remain the leading cause of death worldwide. The implementation of effective preventive measures targeting hypertension is of paramount importance in reducing the annual mortality burden associated with CVDs. Understanding the several factors contributing to elevated blood pressure is crucial for the development of efficient treatment strategies [90]. Several factors can influence blood pressure levels, including age, gender, genetics, lifestyle choices, and environmental factors. Of interest are endocrine disruptors, linked to CVDs and hypertension. These substances have been detected in amniotic fluid, placenta, urine of pregnant women, and even infant urine [49, 59, 98]. Exposure to endocrine disruptors during critical developmental periods can lead to genetic alterations and the subsequent development of diseases later in life [99, 100]. While estrogen is recognized as a cardioprotective hormone, exposure to chemicals that mimic estrogen can disrupt the delicate balance between vasoconstrictors and vasodilators, contributing to abnormal blood pressure regulation and increased hypertension risk [99]. Therefore, exploring the impact of environmental factors and endocrine disruptors on cardiovascular health is crucial for a comprehensive understanding of hypertension and the development of effective preventive and therapeutic interventions.

Emerging evidence from population-based studies has highlighted a connection between Bisphenol exposure and hypertension. Two population-based studies, the Thai National Health

Examination Survey (NHES) 2009 [101] and the Korean Elderly Environmental Study [102], have demonstrated a connection between BPA exposure and hypertension. In the Thai NHES, higher levels of BPA in the blood were associated with an elevated risk of hypertension, particularly among women. This association was independent of estrogen levels and appeared to be more pronounced in postmenopausal women [101]. Similarly, the Korean study found that higher urinary BPA levels were linked to increased blood pressure and decreased heart rate variability. Even in participants with no previous history of hypertension, higher BPA levels were associated with an increased likelihood of developing hypertension [102]. As for the United States population, the National Health and Nutritional Examination Survey (NHANES) conducted in 2003-2004 provided further evidence of the potential health risks associated with Bisphenol A (BPA) exposure. This study revealed a positive association between urinary BPA levels and hypertension in a multiethnic population in the United States. Importantly, this association remained significant even after adjusting for traditional risk factors such as diabetes, obesity, smoking, and cholesterol levels. These findings suggest that BPA may independently contribute to the development of hypertension, emphasizing the need to address BPA exposure as a potential public health concern and further investigate its role in cardiovascular health [103].

The emerging risk of Bisphenol substitutes and their association with cardiovascular health has become a topic of increasing concern in recent years. Limited studies have explored the potential effects of BPS and BPF on CVD using data from different populations. The NHANES conducted between 2013 and 2016 examined for the first time the association between urinary BPS levels and CVD in a diverse age group ranging from 20 to 80 years. The results of this study revealed a positive association between urinary BPS levels and an increased risk of total CVD. Notably, the levels of BPS were found to be higher in the 50-80 years age group

(1.90 ng/ml) compared to the 20-49 years age group (1.26 ng/ml), indicating a potential age-related or dose-accumulation effect [104]. Another study conducted at Wuhan Union Hospital involving 1437 patients further supported the association between urinary BPS and hypertension. The findings demonstrated a linear dose-response relationship, highlighting that higher BPS exposure was associated with an increased risk of hypertension [105]. Furthermore, in the NHANES study, urinary BPF levels were also found to be positively associated with CVD, particularly in female participants and the elderly [106]. These observations suggest that exposure to Bisphenol substitutes may have detrimental effects on cardiovascular health and underscore the need for additional research to better understand the underlying mechanisms and potential preventive measures. Given the widespread use of Bisphenol substitutes in various consumer products, these findings carry significant implications for public health and warrant further investigation (Studies summarized in Table 1.2).

Table 1.2 Association of Bisphenol Exposure with Hypertension and CVD

Study Population	Chemicals Tested	Epidemiological Findings	Reference
BPA			
Thai National Health Examination Survey 2009	Serum BPA and Estradiol	Higher levels of serum BPA are associated with an increased risk of hypertension, particularly in women. The relationship between BPA and hypertension appears to be independent of estrogen levels and may be more pronounced in postmenopausal women.	[101]
The Korean Elderly Environmental study >60yr old male (26.5%) and women (73.5%)	Urinary BPA	Urinary BPA levels were positively associated with blood pressure and negatively associated with heart rate variability. Higher BPA levels increased the odd ratio of hypertension even in participants with no reported hypertension.	[102]
National Health and Nutritional Examination Survey 2003-2004	Urinary BPA	A positive association between urinary BPA levels in multiethnic US population and hypertension. This association was not dependent on traditional risk factors such as diabetes, obesity, smoking or cholesterol levels.	[103]
BPS			
National Health and Nutrition Examination Survey 2013-2016– age 20-80 years	Urinary BPS	Urinary BPS is positively associated with increased risk of total CVD. BPS levels in 20-49 years: 1.26 ng/ml BPS levels in 50-80 years: 1.90 ng/ml	[104]
1437 Patients from Wuhan Union Hospital	Urinary BPS	Urinary BPS is associated with an increased risk of hypertension. BPS and hypertension are associated in a linear dose-response relationship.	[107]
BPF			
National Health and Nutrition Examination Survey 2013-2016– age 20-80 years	Urinary BPF	Urinary BPF levels are positively associated with CVD especially in female participant and elderly.	[106]

Note: Bisphenol A (BPA), Bisphenol S (BPS), Bisphenol F (BPF), and cardiovascular diseases (CVD).

Bisphenol A Cardiovascular Effects

There have been extensive studies on the effects of BPA exposure on fetal cardiovascular health. One study looked at the effects of BPA exposure on cardiovascular parameters in pregnant SD rat females and their fetuses. The results demonstrated significant impacts of BPA on cardiovascular health. Exposure to low and high doses of BPA (0.05 and 0.2 mg/ml in

drinking water) resulted in elevated SBP and DBP during the first and third trimesters of pregnancy. Furthermore, higher BPA exposure led to the upregulation of cardiac microRNAs (miRNAs) associated with cardiac disease in both the dam and fetal hearts. Specifically, miR-17-5p, miR-208a-3p, and miR-210-3p were elevated in the fetuses, while miR-499-5p showed increased expression in the dams. These findings suggest that BPA exposure can induce changes in miRNA expression associated with cardiac dysfunction. Additionally, cardiac fibrosis was observed in both the dam and fetal hearts, indicating structural alterations and potential adverse effects on cardiac tissue. These results emphasize the detrimental effects of BPA exposure on cardiovascular health during pregnancy [14]. Another study focused on evaluating mRNA levels in the fetal hearts of SD rats that were exposed prenatally to BPA. They found that BPA exposure led to a significant increase in the mRNA levels of Myh7, Col5a2, Col1a1, and Col1a2 in the fetal hearts. Myh7 is a gene involved in cardiac muscle contraction, while Col5a2, Col1a1, and Col1a2 are genes associated with collagen synthesis. The observed upregulation of these genes suggests that BPA exposure may have detrimental effects on cardiac muscle function and collagen production in the developing hearts of rat fetuses [108]. Furthermore, another study conducted on rhesus monkeys exposed to BPA during early or late gestation also observed changes in gene expression patterns in the fetal hearts. Specifically, a decrease in Myh6 gene expression in the left ventricle and an increase in Adam12-I gene expression in the right atrium and both ventricles were noted. Myh6 encodes for a protein called alpha-myosin heavy chain, which is a major component of cardiac muscle fibers and Adam12 is a gene that encodes for a protein involved in cell adhesion and signaling processes. These alterations indicate potential impacts on cardiac muscle development and cellular processes, emphasizing the influence of prenatal BPA exposure on gene expression in the developing hearts of fetuses [109]. In a related

study, Patel et al. (2013) investigated the effects of prenatal BPA exposure in C57bl/6n mice. Their findings revealed sex-specific differences in response to BPA. Male mice exposed to BPA exhibited increased body weight, mass index, surface area, and adiposity, along with concentric remodeling of the heart and increased expression of DNA methyltransferase 3a. In contrast, female mice exposed to BPA showed an increase in DBP and alterations in calcium mobility. Notably, exposure to BPA resulted in differential effects on DNA methyltransferase 3a expression and global DNA methylation patterns in males and females. These findings highlight the importance of considering sex-specific responses to BPA exposure and the potential epigenetic modifications induced by prenatal BPA exposure. Collectively, these studies contribute to our understanding of the detrimental epigenetic effects of BPA on cardiovascular health [13].

Some studies also looked at the effects of BPA on blood pressure regulation and its effects on vasoactive and vasodilative markers. In a study conducted by Saura et al. in 2014, the effects of BPA on blood pressure regulation were investigated in CD11 mice. The mice were exposed to BPA at varying concentrations ranging from 4 nmol/L to 400 μmol/L in their drinking water for a duration of 30 days. The results of the study demonstrated a dose-dependent increase in blood pressure with higher concentrations of BPA. This suggests that BPA exposure may contribute to the development of hypertension. Additionally, the study found that BPA exposure had a negative impact on vascular function. Specifically, acetylcholine-induced relaxation, which is an important mechanism for blood vessel dilation, was impaired in mice exposed to BPA. Furthermore, BPA exposure resulted in an increase in the levels of Ang II, as well as endothelial nitric oxide synthase (eNOS) and superoxide accumulation. These changes suggest an alteration in the balance of vasoactive substances and oxidative stress in response to

BPA. Importantly, the study revealed that BPA affected blood pressure regulation by disrupting the coupling of eNOS with AngII/CaMKII-α signaling. This signaling pathway is critical for maintaining vascular tone and blood pressure homeostasis. The uncoupling of eNOS from this pathway due to BPA exposure may lead to a dysregulation of nitric oxide production and subsequent impairment of blood vessel function [110]. Another study investigated the perinatal exposure of BPA at a dose of 5mg/L/day in drinking water. The results of the study revealed several notable findings. Firstly, male offspring exposed to BPA exhibited an increase in SBP at 6 weeks of age, however, no significant changes in SBP were observed in female offspring exposed to BPA. Furthermore, male offspring exposed to BPA demonstrated increased hypertrophic adipocytes, higher adipose tissue mass, and elevated levels of pro-inflammatory cytokines. These findings suggest that perinatal exposure to BPA can have gender-specific effects on cardiovascular health and adipose tissue development, with male offspring being more susceptible to the adverse consequences [111]. In a study by Hu et al. (2016), the effects of BPA exposure on cardiac function and fibrosis were investigated in male SD rats and cardiac fibroblasts. The results showed dose-dependent effects of BPA on cardiac parameters, including impaired cardiac function at the highest dose of 100 mg/kg/day, as indicated by a decrease in ejection fraction and fractional shortening. BPA exposure also led to a dose-dependent increase in cardiac fibrosis, extracellular matrix deposition and increased relative heart weight, indicating the potential contribution of BPA to fibrotic remodeling in the heart and cardiac hypertrophy. In cardiac fibroblasts, BPA exposure resulted in the activation of the ERK1/2 signaling pathway, as evidenced by increased phosphorylation of ERK1/2, and increased expression of transforming growth factor-beta 1 (TGF-β1). The effects of BPA on ERK1/2 phosphorylation and TGF-β1 expression were mediated through ERK signaling and estrogen receptors, as inhibition of ERK

or antiestrogen treatment abolished these effects. [112]. Reventun et al. in 2020, further supported the risk of BPA on cardiac function and cardiac fibrosis. They exposed mice to a dose of 50 mg/kg BW/day for variable durations. The study revealed that BPA exposure resulted in increased HR and prolonged PQ interval and PR segment, indicating alterations in cardiac electrical conduction. Furthermore, BPA exposure led to an increase in SBP and DBP, with the effects becoming more pronounced with longer exposure durations. BPA-induced cardiac hypertrophy and perivascular fibrosis were observed by week 8, accompanied by a decrease in ejection fraction and an increase in interventricular septum thickness, indicative of impaired cardiac function. Additionally, BPA exposure was associated with the development of hemorrhagic cardiac lesions and vascular leakage, characterized by gaps in endothelial cells. BPA exposure also led to a decrease in cardiac vascularization. Mechanistically, it was found that BPA induces endothelial necroptosis through the RIP3/CamKII pathway [113]. As for the effects of BPA on juveniles, Friques et al. investigated the effects in post-weaning male Wistar rats. The rats were exposed to a dose of 0.1 mg/ml (equivalent to 100 μg/kg BW/day) of BPA through gavage administration. The study showed that exposure at a young age led to an increase in SBP, DBP, and MAP. Additionally, BPA exposure resulted in impaired endothelial function, as evidenced by a decrease in acetylcholine-induced relaxation and impaired NO-dependent vasodilation. These effects were likely mediated through increased NADPH oxidase activity, as blocking this enzyme restored normal vasodilatory responses in the BPA-exposed group. Moreover, BPA exposure induced oxidative stress, as indicated by increased levels of reactive oxygen species (ROS) in the aortic cells. This was accompanied by DNA fragmentation, an increase in apoptotic cells, and a decrease in viable cells, suggesting potential cell damage and impaired cellular viability in the aorta [114]. As for chronic adult exposure, Valokola et al. 2019

investigated the effects of BPA exposure on cardiovascular parameters in adult male Wistar rats. The rats were divided into two sets: Set 1 was exposed to BPA at doses of 25 or 50 mg/kg BW/day by gavage, five times a week for four weeks, while Set 2 received BPA at a dose of 50 mg/kg BW/day by gavage, seven times a week for four weeks. The results of the study demonstrated significant effects of BPA on various cardiovascular parameters. In both sets, BPA exposure led to an increase in mean SBP and DBP, as well as prolonged QT, RR, and PQ intervals, indicating alterations in cardiac electrical activity. Furthermore, BPA exposure at both doses resulted in increased body weight, and elevated levels of malondialdehyde and glutathione in heart tissue, indicating oxidative stress, and lymphocytic focal inflammation in the heart muscle fibers. Additionally, the rats exposed to BPA exhibited myocyte cells with enlarged nuclei and hollow spaces in the cytoplasm, suggesting structural changes in the heart tissue. Increased serum levels of CK-MB, a marker of cardiotoxicity, were also observed in the BPA-exposed rats. At the protein level, BPA exposure led to alterations in signaling pathways in the rat heart, as indicated by increased levels of AKT, ERK, and phosphorylated JNK and P38, while decreasing levels of JNK and P38 and phosphorylated AKT and ERK. These findings suggest that BPA exposure can induce cardiovascular dysfunction, oxidative stress, inflammation, and signaling pathway alterations in the heart of adult male rats [115].

Estrogen is usually known for its cardioprotective effects but women on contraceptives are at an increased risk of developing hypertension [116]. Our lab has previously shown that chronic exposure to E2 increases blood pressure and endothelin levels in the paraventricular nucleus [117]. In support of our previous findings, a study conducted by Belcher et al. in 2015 revealed the dose-dependent effects of BPA and ethinyl estradiol (EE) on blood pressure and cardiac parameters. EE exposure showed a trend of increasing blood pressure with an increased

dose, but male and female mice exposed to high doses of BPA showed a decrease in SBP and MAP. Furthermore, higher doses of BPA-induced cardiac hypertrophy in male mice as evident by increased mean heart weight and LV wall thickness. Additionally, male mice exposed to BPA showed increased fibrosis in all doses, suggesting adverse effects on cardiac tissue. On the other hand, female mice exposed to lower doses of BPA exhibited decreased fibrosis in the cardiac tissue. Gene expression analysis of the LV cardiac tissue revealed additional insights. In males, BPA exposure led to a reduction in gene expression associated with the extracellular matrix and collagen, such as MMP8, indicating potential remodeling effects and collagen accumulation. In contrast, female mice exposed to BPA showed an increase in gene expression related to glycosaminoglycan binding and cellular components in the LV cardiac tissue [118]. In a study conducted by Gear et al. (2017), prenatal exposure to SD rats at varying doses of BPA or EE was followed by F1 offspring exposed continuously to either PND 1 to 21, 90, or 6 months of age. The findings revealed that male rats exposed to BPA exhibited myocardial degeneration at PNDs 21 and 90. In contrast, female rats exposed to BPA showed a decrease in collagen accumulation at PNDs 90 and 180, but an increase in the incidence and severity of cardiomyopathy at PND 21, similar to the effects observed in the EE-exposed female rats. Both male and female rats showed myocardial degeneration, suggesting a detrimental impact of BPA and EE on cardiac tissue in both sexes [119]. Another study evaluated the impact of BPA and E2 on cardiac function in hearts and ventricular myocytes from adult SD rats and Erβ knockout mice. They demonstrated that BPA exposure, particularly when combined with E2, had significant and rapid effects on the cardiac health of female subjects. BPA induced arrhythmogenic effects, altered myocyte calcium handling, and increased sarcoplasmic reticulum leak. Crucially, these effects were found to be dependent on the presence of Erβ, as the elimination of this receptor abolished all observed

effects. These results emphasize the involvement of estrogen signaling and suggest that BPA may exert its cardiac effects through interactions with estrogen receptors [120]. BPA's effects on calcium handling are supported by another study that found offspring exposed perinatally to BPA showed alterations in calcium regulation. In male offspring, BPA exposure led to a decrease in the expression of SERCA2a, without changes in the expression of PLB or phosphorylated serine-16 PLB (pS16-PLB). On the other hand, female offspring exhibited an increase in pS16-PLB expression without changes in the PLB expression [121]. The findings suggest that BPA has an inhibitory effect on calcium reuptake in males while having opposite effects in females. This indicates that BPA can disrupt normal calcium regulation and may contribute to the development of cardiac dysfunction. These findings raise concern for females exposed to BPA from daily activities and its interaction with endogenous and exogenous estrogen exposure.

BPA exposure has been shown to disrupt the balance between oxidative stress and antioxidant capacity and impact the cholinergic system. Rats exposed to BPA at doses of 25 mg/kg/day for 6 weeks or 10 mg/kg/day for 6 and 10 weeks exhibited increased oxidative stress markers, such as elevated malondialdehyde levels, while experiencing a reduction in the activity of the antioxidant enzyme catalase and glutathione levels. The study also revealed that BPA exposure disrupted the cholinergic system by reducing the activity of acetylcholinesterase. Additionally, BPA exposure led to reduced levels of NO, indicating impaired endothelial vasodilative function, and an increase in body weight [122]. Similarly, another study by Badawy et al. in 2022 demonstrated that BPA exposure induced oxidative stress, as evidenced by decreased levels of glutathione and increased levels of malondialdehyde. It also resulted in cell vacuolation in the coronary artery tissue, detachment of the endothelium of the intima, and

collagen deposition was observed in the myocardium and coronary artery tissue, suggesting the development of fibrosis vascular system structural damage. Furthermore, immunohistochemical analysis showed increased expression of endothelial eNOS and caspase 3 [123]. Klint et al. (2017) further supported these effects in female Fischer 344 rats and cultured human cardiomyocytes with increased mRNA expression of Vegfr2, Vegf, eNOS, and ACE1 in the rat heart, genes associated with vascular function and regulation. Furthermore, in cultured human cardiomyocytes, they reported an increase in the expression of ACE1, eNOS, IL-8, and NFκβ, genes involved in inflammatory processes and endothelial function. These results suggest that BPA exposure can induce changes in gene expression related to vascular and inflammatory pathways, both in animal and human cardiomyocytes [124].

Diet plays a crucial role in the development and management of HTN. Certain dietary patterns, such as the DASH (Dietary Approaches to Stop Hypertension) diet, have been shown to effectively lower blood pressure and reduce the risk of HTN [125]. The DASH diet emphasizes the consumption of fruits, vegetables, and low-fat dairy products while limiting sodium, saturated fats, and added sugars. And avoiding a diet high in sodium, saturated fats, and processed foods. Excessive sodium intake can lead to fluid retention and increased blood volume, putting additional strain on the blood vessels and heart. Additionally, an unhealthy diet can contribute to obesity and other risk factors for HTN [126]. Some studies have looked at the combined effects of BPA and high fat (HF) or malnourished diet on cardiovascular health. In a study by Liu et al. (2022) mice were exposed to a dose of 500 μg/kg/day of BPA during the perinatal period and the F2 generation was subjected to HF or low-fat diet. In the F1 generation, females exhibited increased body weight, while in F1 males, BPA alone or in combination with the HF diet resulted in increased body weight. In the F2 matrilineal generation, mice exposed to

BPA and HF diet showed myocardial hypertrophy, increased cardiomyocyte size, elevated blood pressure, thickening of the aortic tunica media, and reduced eNOS protein levels in the aorta. These findings demonstrate the long-lasting impact of perinatal BPA exposure on cardiovascular function across generations, with diet playing a modulating role in these effects [127]. Another study showed similar effects but with a malnourished diet. Mice were divided into two groups: normal diet-fed mice and low-protein diet-fed mice and both groups were exposed to a dose of 50 μg/kg BW/day of BPA for 9 days before they were euthanized. In normal diet-fed mice, BPA treatment resulted in an increase in SBP and the percentage of collagen in the heart tissue. The expression of connective tissue growth factor mRNA was also increased, suggesting alterations in tissue remodeling processes. Also, BPA resulted in structural changes in intramyocardial arteries, decreasing lumen diameter, increasing wall thickness, increasing the lumen/wall thickness ratio, and increasing wall cross-sectional area. Interestingly, similar effects were observed in the malnourished mice, suggesting that malnutrition and BPA exposure may have overlapping effects on cardiovascular parameters. Moreover, in the malnourished mice treated with BPA, there was an increase in angiotensinogen and cardiac TGF-β1 mRNA expression, suggesting the potential involvement of these factors in mediating the effects of BPA in the context of malnutrition [128]. Furthermore, our lab conducted a study in 2017 on the effects of prenatal BPA exposure and subsequent overfeeding in adult female Suffolk ewes. The combination of BPA and overfeeding lead to an increase in the systole interventricular septal thickness. Overfeeding alone led to increased body weight and weight gain in the kidneys and lungs. It also resulted in elevated blood pressure and enlargement of the left ventricular area, highlighting the detrimental effects of overfeeding on cardiovascular health [53]. Another study observed that the effects of BPA were not exacerbated by dietary factors. In this study, mice

were exposed to BPA at a concentration of 25 ng/ml in their drinking water during the perinatal period, and the offspring were continuously exposed to 2.5 ng/ml of BPA. The offspring were then given either a standard chow or HF diet. BPA increased pericardial fat accumulation in mice fed a chow diet, however, the effects of BPA on pericardial fat were not exacerbated by the HF diet, suggesting that. Furthermore, the study found that female mice exposed to BPA exhibited an increase in LV mass [121]. The effects of the combination of diet and BPA exposure are controversial and further assessment needs to be done.

Bisphenol S Cardiovascular Effects

Studies evaluating the effects of BPS on hypertension and CVD are extremely limited. In a study conducted by Gao et al. in 2015, the effects of BPS exposure on cardiovascular parameters were investigated in adult female SD rat hearts and isolated ventricular myocytes. The hearts and myocytes were exposed to a concentration of 1 mM BPS. The results of the study demonstrated significant effects of BPS on cardiac function. BPS exposure led to an increase in heart rate, and in the phosphorylation of receptors involved in cardiac contractility, such as PLN and RyR. These receptors' phosphorylation is associated with an increased risk of arrhythmias. It was suggested that these effects could be mediated through the activation of ERβ. These findings suggest that BPS exposure has the potential to affect cardiac function and increase the susceptibility to arrhythmias through receptor phosphorylation, potentially mediated by ERβ activation [129]. In a study conducted by Kasneci et al. in 2017, the effects of BPS exposure on cardiovascular parameters were investigated in adult male mice. The mice were exposed to a dose of 5 μg/kg BW/day of BPS from conception to PND21. Subsequently, the mice underwent surgical induction of myocardial fraction as adults. The results of the study revealed several

significant effects of BPS exposure on cardiac health. Firstly, BPS exposure was associated with an increased incidence of early death, indicating a detrimental impact on overall survival. Additionally, BPS exposure led to a decrease in cardiac function and induced a pro-inflammatory state in the heart. Importantly, BPS exposure promoted cardiac rupture, which is a severe complication associated with cardiac dysfunction. The study also revealed left ventricular dysfunction, further supporting the negative effects of BPS on cardiac health. These findings highlight the adverse consequences of BPS exposure on early mortality, cardiac function, inflammation, and the promotion of cardiac rupture and dysfunction in adult male mice [130]. Furthermore, the effects of BPS exposure on rats exposed to different doses of BPS: 30, 60, and 120 mg/kg BW/day for 30 days were investigated. One significant finding was the dose-dependent decrease in hematological parameters observed in response to BPS exposure (Hemoglobin, red, and white blood cells) indicating potential disruptions in blood cell production or function. Additionally, BPS exposure led to a decrease in coagulation time, this highlights the potential impact of BPS on the body's ability to form clots and respond to injury. Moreover, BPS exposure was associated with an increase in serum calcium levels, which can have implications for calcium regulation and arrhythmia. The study also revealed alterations in lipid profiles, kidney, and liver function in response to BPS exposure [131]. Another study assessed the effects of BPS on embryo development. Zebrafish embryos were exposed to a concentration of 100 µg/L of BPS from 4 to 120 hours post-fertilization. BPS exposure induced cardiac hypertrophy, an increase in atrial size, and a decrease in heart rate, suggesting altered cardiac function [132]. As for the immediate effect of BPS on cardiac function. A study conducted by Ferguson et al. in 2019, perfused the hearts of male and female mice with a concentration of 1 nM of BPA or BPS for 15 minutes, followed by treatment with an Erβ antagonist. The findings of the study revealed

distinct effects of BPA and BPS on cardiac function and calcium handling. In female mice, BPS caused a faster decrease in left ventricular systolic pressure compared to BPA. However, the effects of BPS were abolished when treated with an Erβ antagonist, suggesting the involvement of Erβ in mediating the cardiac effects of BPS. Furthermore, both BPA and BPS exposure resulted in alterations in calcium handling within the heart. In female mice, both BPA and BPS decreased the phosphorylation of PLN at threonine 17, while BPA decreased the phosphorylation of PLN at serine 16 and BPS increased it. In male mice, BPA decreased the phosphorylation of PLN at both threonine 17 and serine 16, while BPS treatment increased the phosphorylation of both sites in the heart. These findings indicate gender-specific effects of BPA and BPS on cardiac parameters and highlight the involvement of Erβ and PLN phosphorylation in mediating these effects [40]. In a study conducted by Connors et al. in 2022, the effects of perinatal exposure to BPS were investigated in the mesenteric arteries of male and female offspring of mice dams. The dams were exposed to a concentration of 250 nM of BPS through their drinking water during the perinatal period. The results of the study revealed gender-specific effects of BPS exposure on mesenteric artery function and estrogen receptor expression. In female mesenteric arteries, BPS exposure led to an increase in acetylcholine-induced vasodilation, indicating enhanced endothelial function and NO-dependent action. Furthermore, females exhibited decreased levels of ERα and ERβ compared to males. Specifically, ERα expression was higher in females, while ERβ expression was higher in males. These findings suggest that perinatal exposure to BPS can induce gender-specific effects on mesenteric artery function and estrogen receptor expression, potentially contributing to differences in vascular responses between male and female offspring [133].

Bisphenol F Cardiovascular Effects

Studies on BPF effects on the heart and cardiovascular function are very limited. In a study conducted by Mu et al. in 2019, the effects of BPF exposure on zebrafish embryos were investigated. The embryos were exposed to different doses of BPF: 0.0005, 0.5, and 5.0 mg/L. The findings of the study revealed significant effects of BPF exposure on cardiac and neuronal development. BPF exposure resulted in a decrease in the number of heartbeats per 20 seconds, indicating altered cardiac function. Additionally, BPF exposure led to impaired motor neuron development. Furthermore, BPF exposure downregulated the expression of cardiac development-related genes, specifically klf2a and igfbp1b. These findings highlight the potential adverse effects of BPF on cardiovascular and neuronal development in zebrafish embryos [134].

In a study conducted by Prudencio et al. in 2021, the effects of BPF exposure on cardiac parameters were investigated using isolated rat heart preparations. The hearts were exposed to a range of concentrations (0.01-100 μM) of BPF for a duration of 15 minutes. The findings of the study revealed a dose-dependent effect of BPF on cardiac conduction. Specifically, BPF exposure led to a slowing of atrioventricular (AV) conduction and an increase in AV node refractoriness. These results suggest that BPF can disrupt the electrical conduction system of the heart, affecting the timing and coordination of the heart's contractions. This information provides valuable insights into the specific effects of BPF on cardiac function and highlights the need for further research to understand the underlying mechanisms and potential implications for cardiovascular health [135]. In a study conducted by Cheng et al. in 2022, the effects of BPF exposure on cardiomyocytes were investigated using H9 ES-derived cardiomyocytes. The cardiomyocytes were exposed to a single dose of 7 ng/ml of BPF through cultured medium. Additionally, the researchers tested the effects of a 1 μM ERβ antagonist, PHTPP, on BPF-

exposed cardiomyocytes. The findings of the study demonstrated that BPF exposure induced hypertrophy, characterized by an increase in cell size and mitochondrial fragmentation. Furthermore, BPF exposure resulted in a decrease in ATP production, suggesting impaired energy metabolism. The expression of hypertrophic-specific markers, such as atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), was increased in response to BPF exposure. Interestingly, calcium levels in the cardiomyocytes exposed to BPF were found to be 10% higher compared to the 8 ng/ml BPA exposure. This increase in calcium levels was attributed to the L-type calcium channel in the cardiomyocytes and the cooperation of ERβ and CnAβ. These findings provide insights into the adverse effects of BPF on cardiomyocytes, including hypertrophy, mitochondrial dysfunction, altered energy metabolism, and disrupted calcium handling, which may have implications for cardiac function [43].

Table 1.3 Gender and dose-specific Cardiac Effects of Exposure to BPA, BPS, and BPF

Exposure	Offspring species	Cardiovascular effects	Reference
BPA			
Exposure of 8wk old mice to 4nmol/L – 400 µmol/L of BPA in water over 30days.	CD11 mice	Dose-dependent inc in BP. Impaired acetylcholine-induced relaxation. Inc in Ang II, eNOS and superoxide accumulation. BPA modulated blood pressure by causing the uncoupling of eNOS from AngII/CaMKII-α signaling	[110]
4×10^{-5} mol/L of BPA in drinking water for 4, 8, and 16 weeks (BPA<50mg/kg BW /day)	CD1 mice	Increased HR, prolonged PQ interval and PR segment. Inc SBP, DBP after 4 weeks and further elevated by 16 weeks. BPA-induced cardiac hypertrophy and perivascular fibrosis by week8. Dec ejection fraction. Diastolic and systolic inc interventricular septum thickness. Inc hemorrhagic cardiac lesion and vascular leakage, gaps in endothelial cells. Dec cardiac vascularization. BPA induces endothelial necroptosis through the RIP3/CamKII pathway.	[113]
BPA- (40 mg/kg/d) BPA plus LC-treated (40 mg/kg/d BPA + 300 mg/kg/d L-carnitine) By gavage for 4 weeks	Adult Wister-albino rats	Vacuolation of cells in the coronary artery tissue and detachment of the endothelium of the intima. Collagen deposition in the myocardium and coronary artery tissue. IHC expression of eNOS and caspase 3, indicating potential vascular disease and apoptosis. Oxidative stress: Dec levels of glutathione (GSH) and increased malondialdehyde (MDA) levels.	[123]
CD-1 mice were exposed to BPA or ethinyl estradiol (EE) through diet at a dose of (0.03-300 ppm) 4-5-5,000 µg/kg or 0.02, 0.2, and 0.15 µg/kg; respectively.	CD-1 mice	Dec SBP and MAP in males exposed to a dose above 5 µg/kg/day and in females exposed to 42300 µg/kg/day. EE had a trend of increasing BP with an increased dose. Males had increased mean heart weight and LV wall thickness in the higher doses. Males also showed increased fibrosis in all doses, while females had decreased fibrosis in low doses. BPA exposure in males reduced gene expression associated with extracellular matrix and collagen in LV cardiac tissue. BPA exposure in females increased gene expression related to glycosaminoglycan binding and cellular components in LV cardiac tissue.	[118]
Prenatal exposure to BPA from GD 11-21 at a dose of 0.5, 5 and 200 µg/kg/day via drinking water.	C57bl/6n mice	BPA 5.0 males and female had increased body weight, body mass index, body surface area, and adiposity. BPA males: Concentric remodeling, inc DNA methyltransferase 3a expression BPA females: Inc Diastolic blood pressure Inc calcium mobility in males and reduced in females. BPA 5.0 females: Inc DNA methyltransferase 3a expression BPA 200 females: Dec DNA methyltransferase 3a expression BPA 0.5 male: Inc global DNA methylation BPA 0.5 females: Dec global DNA methylation	[13]
Exposure of adult sheep to 0.5 mg/kg/day of BPA from GD 30-90	Adult female Suffolk ewes	BPA: Inc natriuretic peptide gene expression in the ventricle. BPA: Dec collagen expressing in right ventricle and modest increase in left ventricle. Overfeeding increased body, kidneys and lung weight and blood pressure and increased left ventricular area.	[53]

Then offspring were challenged with overfeeding in adulthood			
Female mice exposed to perinatal 500 µg/kg/day BPA. F0 females were treated for 10wks before mating. 1. Control low fat diet. 2.Control HF diet 3.BPA 500 µg/kg/day 4. HF diet + BPA Offspring (F1) mated with normal male/female and given low fat diet. F2 generation were given either low fat or high fat diet	Institute of Cancer Research female mice	F0-F1 females: BPA F0 did not gain weight but F1 females did. F1 males: BPA and BPF+HF had increased BW compared to control-LFD. F2 matrilineal: BPA+HFD: myocardial hypertrophy, inc cross-sectional area of cardiomyocytes, inc blood pressure, aortic tunica media thickening, and dec aortic relative eNOS protein.	[127]
Prenatal exposure of rhesus monkeys to 400 µg/kg BW/day to either early or late gestation.	Fetal hearts *of rhesus monkeys (Macaca mulatta)*	Dec in Myh6 gene expression in the left ventricle. Inc in Adam12-I gene expression in the right atrium and both ventricles.	[109]
Dam and offspring exposure to BPA 2.5, 25.0,250, 2500, or 25,000 µg/kg BW/day or 17α-ethinyl estradiol (EE) 0.05 or 0.5 µg/kg BW/day Dams dosing GD6-PND0 F1 dosing PND1-21 or PND90 or 6 months of age.	Male and female SD rats	Male: myocardial degeneration at PND21 and PND90 Female: Dec collagen accumulation at PND90 and 180 and inc cardiomyopathy incidence and severity at PND21 BPA and EE. Male and female: myocardial degeneration.	[119]
1 nmol/L BPA and/or E2	Hearts and ventricular myocytes excised from adult SD rats and Erβ knockout mice	Rapid induction of arrhythmogenic effects in females exposed to BPA, especially when combined with E2. Effects include ventricular arrhythmias and rapid changes in myocyte calcium handling. BPA exposure increases sarcoplasmic reticulum leak. Ablation of Erβ abolished all BPA effects.	[120]
Exposure to BPA 25mg/kg/day for 6weeks or 10mg/kg/day for 6 and 10 weeks	Adult male Wistar albino rats	Inc oxidative stress: Inc malondialdehyde levels and decreased catalase activity. Dec antioxidant capacity: Dec glutathione levels. Impaired cholinergic system: Dec acetylcholinesterase activity. Dec NO levels and inc body weight.	[122]
Pregnant SD rats exposed to 2 and 100 µg/L in drinking water during GD0-19	Pregnant SD rats and fetus	Fetal hearts mRNA levels: Inc in Myh7, Col5a2, Col1a1and Col1a2.	[108]
BPA female exposure to 5, 50, and 500 µg/kg BW/day in drinking water.	Female Fischer 344 rats and cultured human cardiomyocytes	5 µg BPA/kg BW/day increased mRNA expression of Vegfr2, Vegf, eNOS and ACE1 in rat heart. (Exposure in combination with 5% fructose)	[124]

Cultured human cardiomyocytes cells: 10 nM BPA to 1×10^4 nM BPA for 6 hours.		Human cardiomyocytes exposure to 1×10^4 nM BPA: Inc ACE1, eNOS, IL-8 and *NFκβ.*	
C57b1/6n dams perinatal exposure to BPA 25 ng/ml in drinking water from GD11-PND21 Offspring continued exposure to 2.5 ng/ml and divided to either chow or HF diet from PND 21 till 4 month/euthanized	C57b1/6n mice offspring	BPA increased pericardial fat with chow diet, but HF diet did not worsen BPA effects. Increased LV mass in females but not males. Tail-cuff BP showed no significant increase. Altered calcium handing in male and female offspring: Male: Dec SERCA2a expression without changes to PLB or pS16-PLB expression. Female: Inc pS16-PLB expression without changes to PLB.	[121]
Exposure of normal or low-protein diet fed adult male mice to 50 μg/kg BW/day for 9 days before they were euthanized	Male Swiss mice	Normal fed BPA treated male: Inc SBP but not DBP. Inc %collagen in heart tissue. Inc connective tissue growth factor mRNA expression Intramyocardial arteries: dec lumen diameter, inc wall thickness, inc lumen/wall thickness ratio and inc wall cross sectional area. Malnourished mice showed similar effects as BPA. Malnourished +BPA: Inc angiotensinogen and cardiac TGF-β1 mRNA expression.	[128]
Perinatal exposure of female SD rats to 5mg/L /day BPA via drinking water for 2 weeks before pregnancy then during pregnancy and lactation (PND21).	Male and Female SD rat offspring	Inc SBP in BPA males but not females at 6weeks. Male had increased hypertrophic adipocytes, adipose tissue mass and pro-inflammatory cytokines.	[111]
Post weaning (25 days old) male wister rats exposed to 0.1mg/ml (100 μg/kg BW/day by gavage) BPA	Male Wistar rats	Inc in SBP, DBP and MAP. Dec ACH-induced relaxation and impaired NO-dependent vasodilation. This is done probably through NADPH oxidase activity, when blocked, vasodilating effects were reversed. Blocking COX with indomethacin restored normal endothelial function in the BPA group. Inc ROS, DNA fragmentation, apoptotic cells and dec in viable cells in the aorta cells.	[114]
Set 1: Exposed to 25 or 50 mg/kg BW/day by gavage (5times a week for 4 weeks) Set 2: 50 mg/kg BW/day by gavage (7times a week for 4 weeks) then scarified	Adult male Wistar rats	BPA 25 mg/kg: Inc mean SBP and DBP, inc QT, RR and PQ interval. BPA 50 mg/kg: Inc mean SBP and DBP, inc QT, RR and PQ interval. Inc BW, MDA and GSH in heart tissue. lymphocytic focal inflammation in the heart muscle fibers. Myocyte cells with enlarged nuclei and hollow spaces in the cytoplasm. Inc serum CK-MB (cardiotoxicity). Inc AKT, ERK, and phosphorylated JNK and P38 while decreasing JNK and P38 and phosphorylated AKT and ERK protein levels in rat heart.	[115]

Exposure of pregnant SD rat female to 0.05 and 0.2 mg/ml through drinking water from GD2-21then caesarean section performed and hearts were isolated from dam and fetus.	Pregnant SD female rat and their fetus	Inc SBP and DBP in 1st and 3rd trimester in both doses. Upregulation of cardiac miRNA involved in cardiac disease at a dose of 0.2 mg/ml (fetus: miR-17-5p, miR-208a-3p and miR-210-3p, Dam: miR-499-5p) Evidence of cardiac fibrosis in dam and fetal hearts.	[14]
Exposure to BPA at a dose of 5, 20, or 100 mg/kg/day for 30 days	Male SD rats, and cardiac fibroblasts	Dec ejection fraction and fractional shortening at the highest dose. Inc relative heart weight at doses 20 and 100. Dose-dependent inc in cardiac fibrosis and ECM deposition. Cardiac fibroblast exposed to 1 or 10 μM BPA had increased phosphorylated ERK1/2 but no effect on p38 and JNK MAPK signaling. And inc TGF -β1. Treatment with ERK inhibitor or antiestrogen abolished these BPA effects.	[112]
BPS			
30, 60 and 120 mg/kg BW/day BPS for 30days	Adult male SD rats	Dec in Hematological parameters. Dec coagulation time Inc in serum calcium levels. Alteration of lipid profile.	[131]
To excised adult female rat heart and isolated ventricular myocytes to 1mM BPS	Adult female SD rats hearts	Inc heart rate. Phosphorylation of receptors involved in cardiac contractility (PLN and RyR) – Risk of developing arrhythmia. Effects could be mediated by ER_{β} activation	[129]
From conception – PND 21 at a dose of 5 μg/kg BW/day BPS then surgical induce myocardial fraction in adults	Adult male mice	Inc early-death. Dec cardiac function. Inc pro-inflammatory state Promotes cardiac rupture. Left ventricular dysfunction.	[130]
Male and female mice hearts were perfused with 1 nM BPA or BPS for 15min then with Erβ antagonist.	Male and female CD1 mice hearts	BPS decreased left ventricular systolic pressure faster than BPA in females. Erβ antagonist abolished BPS effects. Alteration of calcium handling in BPA and BPS. Female: BPA and BPS decreased phosphorylation of phospholamban at threonine 17. BPA decreases phosphorylation of phospholamban at serine 16 while BPS increases it. Male: BPA had decreased phosphorylation of phospholamban at threonine 17 and serine 16 but BPS treated increased both in the heart.	[40]
Perinatal exposure of mice dams to BPS 250 nM through drinking water.	C57BL/6 mice male and female offspring's mesenteric arteries	Female mesenteric arteries had inc Ach-induced vasodilation, NO-dependent action. Females had decreased levels of ERα and ERβ. ERα was higher in females and Erβ in males.	[133]
Embryos exposure 4-120 hr post fertilization to 100 μg/L BPS	Zebrafish embryo/larvae	Cardiac hypertrophy. Dec heart rate. Inc atrial size.	[132]

BPF			
0.01–100 μM BPA, BPS or BPF for 15min	hiPSC-CM (iCell cardiomyocytes, female donor #01434) Female SD rats cannulated hearts	In isolated rat heart preparations, exposure to BPF demonstrated a dose-dependent effect of slowing atrioventricular (AV) conduction and increasing AV node refractoriness. BPA was the most potent inhibitor of the sodium and calcium channel	[135]
Exposure to cultured medium a single dose of 7ng/ml BPF Also tested 1 μM ERβ antagonist PHTPP on BPF cardiomyocytes	H9 ES-derived cardiomyocytes	Induced hypertrophy and mitochondrial fragmentation. Dec ATP production. Inc in the hypertrophic -specific markers ANP and BNP. Calcium levels were 10% higher than 8ng/ml BPA, through L-type calcium channel in cardiomyocytes and the cooperation of ERβ and CnAβ.	[43]
Zebrafish embryos exposed to 0.0005, 0.5 and 5.0 mg/L BPF	Zebrafish	BPF decreased heartbeats/20sec and motor neuron development. Downregulated expression of cardiac development related genes (klf2a and igfbp1b).	[134]
Note: Bisphenol A (BPA), Bisphenol S (BPS), and Bisphenol F (BPF).			

1.4. Study Aims

The effects of BPA and its analogues on cardiovascular function have raised significant concerns due to their detrimental impact on endothelial function, cardiac development, and blood pressure regulation as discussed above. These effects have contributed to the growing incidence of CVDs and increased mortality rates worldwide. Importantly, the toxic effects of these EDCs are particularly pronounced during critical periods of development, potentially resulting in long-lasting consequences for cardiovascular health across generations. However, our understanding of the specific effects of BPS and BPF on cardiovascular health is still limited, with only a handful of studies investigating their impact in this context. Furthermore, females are exposed to both endogenous and exogenous estrogen throughout their lifetime, including using birth control pills or post-menopausal hormonal therapy, which has been associated with adverse effects on blood pressure regulation and overall cardiovascular well-being. To further investigate these concerns, a series of experiments were conducted to examine the effects of BPA and its

analogues on the cardiovascular system, with the aim of testing specific hypotheses related to their impact on cardiovascular function.

1. Prenatal EDC exposure is anticipated to elicit distinct modifications in blood pressure control, monoaminergic neurotransmitter activity within the pivotal cardiovascular regulatory brain region, and hormonal and cardiac fibrosis responses, varying between male and female offspring.
2. Exogenous estradiol exposure during adulthood will amplify the neuro-cardiovascular effects induced by prenatal exposure to EDCs in female rat offspring.
3. High-fat diet in adulthood will exacerbate the neuro-cardiovascular effects induced by prenatal EDC exposures specifically in male rat offspring.

The experimental protocol involved the oral administration of BPA, BPS, or BPF to pregnant Sprague-Dawley dams during gestational days 6-21. The female and male offspring were subsequently assessed for various parameters in adulthood, including blood pressure, heart rate, hormonal levels, brain neurotransmitter levels, heart fibrosis, left ventricle wall thickness, and expression changes of vasoconstrictor molecules in small blood vessels. Another group of animals underwent the same assessments but were subjected to additional challenges in adulthood. Male offspring were exposed to a HF diet challenge, while female offspring had estradiol pellets implanted. These experiments aimed to investigate the sex-specific long-term effects of prenatal exposure to BPA, BPS, or BPF and their interaction with diet and hormonal challenges in adulthood.

Chapter 2

Prenatal Exposure of Sprague Dawley Rats to Bisphenol A Analogues Alters Blood Pressure Regulation and Induces Cardiovascular Dysfunction in Sex-Specific and Dose-Dependent Manner

Al Mansi et al. to be submitted to *Chemosphere.*

2.1. Abstract:

Cardiovascular diseases (CVD) are the primary cause of death globally, with hypertension being a major risk factor. Environmental contaminants, which are often overlooked, have been linked to the development of hypertension. Bisphenol A (BPA) analogues, such as bisphenol S (BPS) and bisphenol F (BPF), are commonly found in BPA-free products and their cardiovascular effects on prenatal exposure are largely unknown. This study was conducted on pregnant Sprague-Dawley rats to investigate the cardiovascular effects of prenatal exposure to 5 µg/kg BW of BPA or BPS, or 1 µg/kg BW of BPF. Adult male and female offspring were implanted with radiotelemetry for continuous monitoring of blood pressure and heart rate. The offspring were sacrificed after data collection and plasma levels of aldosterone (Aldo), corticosterone (CORT), and angiotensin II (Ang II) levels were measured using a radioimmunoassay (RIA). Neurotransmitter levels in the paraventricular nucleus (PVN) were analyzed using HPLC-EC. Masson trichrome staining was employed to measure the level of fibrosis in the heart. The thickness of the left ventricle (LV) wall was measured using eSlide Viewer. Endothelin 1 (Endo-1) levels were measured in small vessels in lung tissues using immunohistochemistry (IHC). Offspring prenatally exposed to BPA and its analogues had significantly increased SBP and MAP. Male offspring prenatally treated with BPA had elevated levels of CORT, DA, 5HT, Endo-1, and a reduced ratio of 5HIAA/5HT and DOPAC/DA in the PVN. Whereas females exposed to BPA only showed elevated levels of NE. Both had fibrotic hearts and increased relative heart weight but only females showed thickening of the LV wall and the weight of the kidney and adrenal gland. BPS-treated offspring had more adverse findings, with both genders showing an increase in Ang II, NE, 5HT, heart fibrosis, and Endo-1. Males exposed to BPS had also an increase in CORT, DA, LV wall thickness, and heart weight. However, BPS also had female-specific effects such as an increase in kidney and adrenal gland weight. As for BPF, male offspring had an increase in Ang II, Aldo, CORT, NE, 5HT, Endo-1, heart fibrosis, LV wall thickness, and heart, kidney, and lung weight. While in females BPF treated offspring did not show as adverse effects as in males but had an increase in CORT, 5HT, and kidney and adrenal weight. Prenatal exposure to BPS or BPF seems to have more adverse effects than BPA and those effects are sex and dose specific.

Keywords: Bisphenol A (BPA), Bisphenol S (BPS), Bisphenol F (BPF), Endocrine Disrupting Chemical (EDC), Endothelin 1 (Endo-1), sex differences, hypertension.

2.2. Introduction

Bisphenol A (BPA, 2,2-bis[4-hydroxyphenyl]propane) is a plasticizing chemical produced in high volume and is a ubiquitous environmental contaminant [136]. Over the last two decades, research has indicated that BPA acts as an endocrine disruptor and produces a variety of adverse effects [137, 138]. As a result, it is slowly being replaced in the market by its analogues, Bisphenol S (BPS) and Bisphenol F (BPF). Unfortunately, the biological effects of these compounds have not been studied extensively. In this study, our objective is to explore the effects of prenatal exposure to BPA, BPS, and BPF on cardiovascular function in adult offspring.

Exposure during the prenatal period is critical since the fetus cannot metabolize the chemicals, resulting in prolonged exposures that could favor alterations at the cellular or tissue level, leading to conditions that manifest in adulthood [139]. BPA and its analogues were associated with metabolic, behavioral, reproductive, and cardiovascular diseases [9, 34, 40, 107, 140, 141]. BPA levels in urine have been positively associated with hypertension regardless of age, sex, smoking status, ethnicity, body mass index, or the presence of diabetes [103]. Since 92% of women have BPA in their urine [142], given that BPA alternatives are structural analogues of BPA, it is highly likely that pregnant women are also exposed to them. In fact, there are reports that BPA, BPS, and BPF are present in the urine of pregnant women [143, 144]. A study conducted on pregnant and nonpregnant Sprague Dawley Rats exposed the females to 7mg/kg or 100 mg/kg BPF found that in pregnant females, the BPF was found in the placenta, uterus, fetuses, and amniotic fluid [145]. These exposures are also known to adversely affect hematobiochemical parameters in BPA-exposed pregnant women [143] and certain parameters of fetal growth, such as the length of the femur and abdominal and head circumference [57]. Recently, urinary levels of BPA and its analogues have been linked to obesity in U.S. children and adolescents [146];

moreover, BPA analogues have been associated with metabolic disorders and lipid dysfunction depending upon the gender and the type of analogue, BPS levels were positively associated with obesity in U.S. men, but BPF levels were associated with obesity in women [10]. The effects of these chemicals seem to be sex-specific, with certain analogues affecting males more than females and vice versa, depending on the chemical type, dose, and time of exposure.

Although multiple studies have found associations between urinary BPA and hypertension [4, 102, 103], studies that describe the association between BPS and BPF and hypertension are limited. An epidemiological study in men of age 56 years and above found modest associations between urinary BPA levels and systolic (SBP), diastolic (DBP), and mean arterial pressure (MAP) [105]. Stronger associations were found between urinary BPS levels and SBP, while no such association was found between blood pressure and BPF levels [105].

The current study aimed to investigate whether exposure to chemicals such as BPA, BPS, or BPF during the critical period of fetal development would cause hypertension when the offspring develops into an adult. To test this, pregnant Sprague-Dawley (SD) rats were orally dosed with Bisphenol A or its analogues during gestational days (GD) 6-21. Telemeters were implanted in adult offspring, and changes in blood pressure were monitored. We hypothesized that exposure to BPA analogues would increase blood pressure in a sex-specific manner, and their effects are more determinantal than BPA.

To further evaluate the mechanisms by which BPA and its analogues might produce their effects, we also investigated the effects on the neuroendocrine system that governs cardiovascular function. The paraventricular nucleus (PVN) of the hypothalamus plays a critical role in blood pressure regulation and influences blood pressure through various pathways. Increases in the levels of neurotransmitters such as Norepinephrine (NE) or Serotonin (5HT) could have a direct impact

on blood pressure. Also, hormones, such as Angiotensin II (Ang II) and Aldosterone (Aldo), are known to directly influence blood pressure. The thickness of the left ventricle (LV) wall and heart fibrosis could also be implicated through an increase in endothelin 1 (Endo-1) levels. Therefore, we investigated the changes in these parameters in offspring that were prenatally exposed to BPA and its analogues.

2.3 Materials and Methods

2.1. Experimental Animals and Treatment

Adult female and male Sprague Dawley rats (3 mo old) were purchased from Envigo (Indianapolis, IN) and housed in light-(12:12 light-dark cycle) and temperature-(23 ± 2 °C, 50 ± 20% relative humidity) controlled animal rooms. Food (PicoLab-LabDiet 5053) and water were provided *ad libitum*. All animal procedures were approved by the University of Georgia Institutional Animal Care and Use Committee (IACUC) and were carried out according to the NIH guideline for the care and use of laboratory animals. Females were set for breeding and pregnant dams were randomly assigned to one of four treatment groups: Control (10µl Phosphate Buffered Saline (PBS), *n*=9), Bisphenol A (5µg/kg BW/day, *n*=6), Bisphenol S (5µg/kg BW/day, *n*=13), and Bisphenol F (1µg/kg BW/day, *n*=10) as previously described [34]. The dams were dosed orally every day based on their body weight from GD 6-21. The offspring were weaned three weeks after birth and were group housed in polypropylene cages until further experimentation. The dose of EDC was lower than the current Environmental Protection Agency (EPA) recommended no-observed-adverse-effect-level (NOAEL)(BPA;5 mg/kg bw/day, BPS; 10 mg/kg/day, BPF; not established)(EPA 2012, 2014).

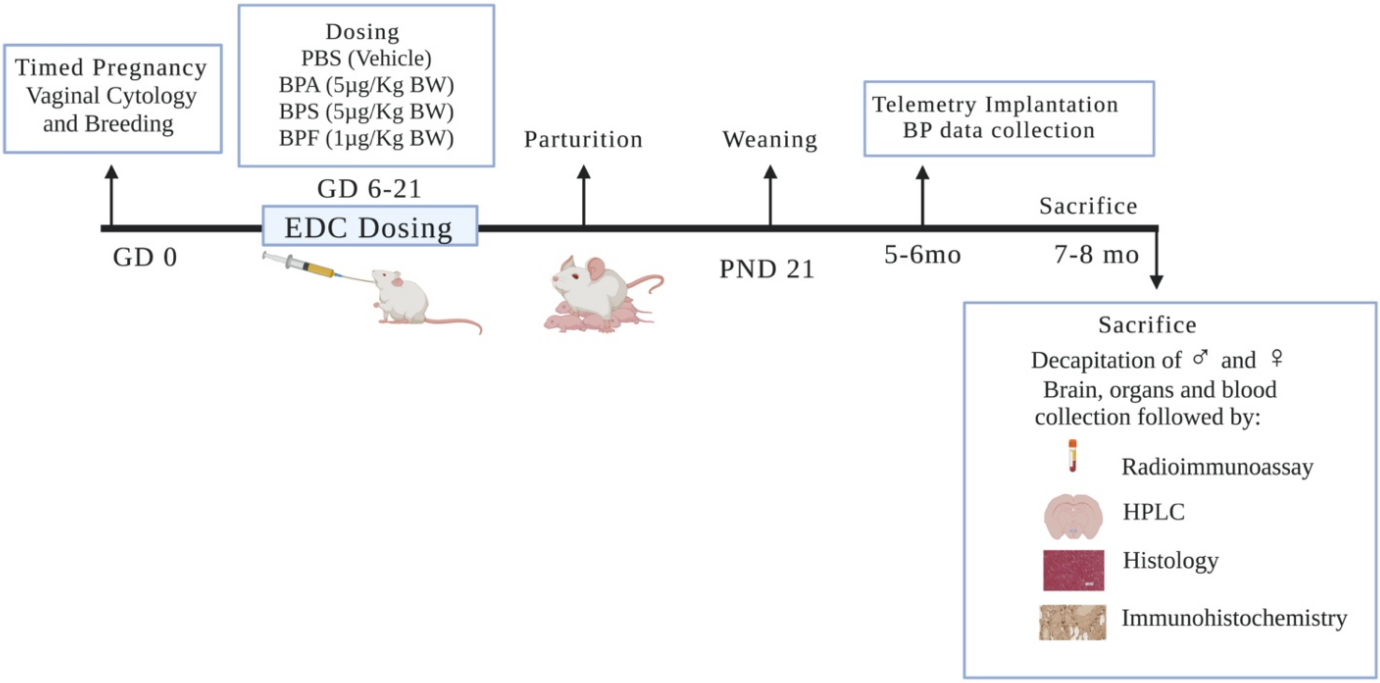


Fig. 2.1. Overall study scheme. Pregnant SD rats were administered orally with Control (10µl Phosphate Buffered Saline (PBS), *n*=9), Bisphenol A (5µg/kg BW/day, *n*=6), Bisphenol S (5µg/kg BW/day, *n*=13), and Bisphenol F (1µg/kg BW/day, *n*=10) from GD 6-21. The offspring were weaned at PND 21 and around 5-6 months of age they were implanted with a radiotelemetry device. Blood pressure and heart rate were monitored continuously for 24 hours, after which the animals were sacrificed. The brain, trunk blood, hearts and lungs were collected for measurement of neurotransmitters, hormones, histology, and immunohistochemistry, respectively. Created with BioRender.com

2.2. Radiotelemetry Implantation Surgery and Hemodynamic Measurements

Male and female offspring were allowed to reach adulthood and implanted with a radiotelemeter (Data Sciences International; HD-S10) in the femoral artery as previously described [147, 148]. Briefly, the animals were anesthetized with isoflurane, the ventral abdomen, and the inside of the left thigh was shaved and cleaned using aseptic techniques. Meloxicam 1 mg/kg BW and Enrofloxacin 5 mg/kg BW were administered subcutaneously as prophylactics. A 2-centimeter incision was made in the left groin to expose the femoral artery that emerges from the body wall. Superficial fascia and fat were bluntly dissected to expose the femoral artery, vein, and nerve. A ligature was placed at the distal end of the femoral artery close to the knee and a retention suture was placed close to the body wall to restrict blood flow through the femoral artery. A subcutaneous pocket was created in the ventral abdomen to hold the transmitter, and the transmitter was placed in this pocket. A drop of lidocaine was instilled into the femoral artery to prevent contraction of the arterial wall and a hole was made in the arterial wall under a dissection microscope using fine scissors. The hole was distended using Dumont vessel cannulation forceps and the transmitter catheter was inserted into the femoral artery for up to 2 cm. Ligatures were placed around the arterial wall containing the catheter to hold the catheter in place. The fascia on the catheter was sutured with simple interrupted sutures, and the skin was sutured with horizontal mattress sutures. The transmitter was turned on by a magnet and its functioning was monitored using a wireless radio. The animals were allowed to recover in independent cages and closely monitored every day for seven days. Meloxicam was administered every day for 3 days post-surgery. After recovery, the transmitter was turned on and data was collected at 10 min intervals over a 24-hr. Systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), and heart rate (HR) were collected. Animals were sacrificed by rapid decapitation. The females were in an

estrus state at the time of sacrifice. The brains were harvested, and the trunk blood was centrifuged to extract the plasma. All tissues were stored at -80 ˚C until further analysis.

2.3. Hormone Assays

Plasma levels of corticosterone (CORT), angiotensin II (Ang II), and aldosterone (Aldo) were measured using a double antibody radioimmunoassay according to the manufacturer's protocol. We used the following assays: CORT: Catalog #07120102 (MP Biomedicals, Irvine, CA) Ang II: Catalog # 07120102 (Phoenix Pharmaceuticals, Inc., Burlingame, CA) Aldosterone: Catalog # MG13051 (IBL International GmbH, Hamburg, Germany). The samples were added in duplicate and analyzed according to the assay protocol. Values were expressed as ng/ml for CORT and pg/ml for Ang II and Aldo (%CV <10).

2.4. Brain Sectioning and Microdissection

A cryostat (Slee, London, UK) maintained at -10°C was used to section the brains at 300 µm thickness. Subsequently, the paraventricular nucleus (PVN) was microdissected on a cold stage using the Palkovits procedure with the help of the Rat Brain Atlas - *the Rat Brain in Stereotaxic Coordinates – 7th Edition* as a reference [149]. The punches included all subdivisions of the PVN. Microdissected tissue was stored at -80°C until further analysis.

2.5. Neurotransmitter Analysis by HPLC-EC

HPLC-EC was used to analyze the PVN for norepinephrine (NE), dopamine (DA), and serotonin (5HT) as previously described [150]. Brain punches were briefly homogenized in 0.05 M perchloric acid and placed on ice, and an aliquot was used for protein estimation (Pierce,

Rockford, IL). The remaining homogenate was centrifuged for 8 minutes at 18,000 × g at 4°C. The supernatant was injected with an internal standard (dihydroxybenzylamine, 0.05 M) into the autoinjector for HPLC analysis. The HPLC-EC system is comprised of a 5-µm ODS reverse phase C-18 column (Phenomenex, Torrance, CA), a SIL-20AC autoinjector, a CTO-20AC column oven (Shimadzu, Columbia, MD) maintained at 37°C, and an LC-4C detector (Bioanalytical Systems, West Lafayette, IN). The mobile phase flow rate was maintained at 1.8 ml/min using an LC-20AD pump (Shimadzu, Columbia, MD). The chromatograms were analyzed for neurotransmitter concentrations using the Class VP software v 7.2 (Shimadzu, Columbia, MD). Neurotransmitter concentrations in tissue samples were expressed as pg/µg of protein. Protein levels in tissue punches were measured using the micro bicinchoninic acid assay (MicroBCA assay, Pierce, Rockford, IL). The absorbance was measured using the ELX 800 microplate reader.

2.6. Histology of heart tissues.

The heart was collected and stored in formalin. They were cut at the same location, from the apex, subjected to routine paraffin embedding and sectioning. Sections were stained with Masson's trichrome and scanned using an Aperio scanner (Leica Biosystems, Wetzlar, Germany) and visualized in the eSlide Viewer. The thickness of the left ventricle wall was measured using the eSlide Viewer at five distinct locations. The extent of interstitial and perivascular fibrosis was analyzed using ImageJ software according to Kennedy et al., 2006 [151].

2.7. Immunohistochemistry

Paraffin-embedded lung sections were stained with Endo-1 monoclonal antibody, HRP conjugated: Catalog # bsm-0954M-HRP (Bioss Antibodies, Woburn, MA) to detect Endo-1 levels

in small blood vessels of the lung. It was done by first deparaffinizing the slides with Xylene, following which the slides were hydrated with graduated ethanol then cooked using a pressure cooker for 28 minutes while immersed in citrate buffer. The slides were allowed to cool then rinsed with 1X PBS. After rinsing, the slides were immersed in permeabilization solution for 15 minutes, followed by another rinse with PBS. The tissues were then blocked with Casein Blocker in PBS for 1 hour. The blocking solution was then drained, and the slides were washed with BPS. Endo-1 antibody was diluted 1:200 in PBS and the tissues were covered with the antibody and left overnight at room temperature. The next day, the antibody was removed, and the slides were thoroughly washed with PBS. Following the wash, for the final step, DAB substrate kit: Catalog # SK – 4100 (Vector Laboratories, Newark, CA) was used to stain Endo-1. Procedure was done based on instructions given by the company. The slides were then scanned using an Aperio scanner (Leica Biosystems, Wetzlar, Germany) and visualized for analysis using the eSlide manager.

2.8 Statistical Analysis

Prism 9.0 software (GraphPad, Inc., San Diego, CA) software was used to perform statistical analysis. Two-way ANOVA was used followed by Tukey's multiple comparison test or Holm-Šídák test for treatment or sex differences, respectively. It was used to detect sex-specific changes in blood pressure, hormones, fibrosis, immunohistochemistry, organ weights and neurotransmitter measurements. One-way ANOVA was used followed by Tukey's multiple comparison test to assess EDC treatment effect in neurotransmitters in the PVN and circulating hormonal levels. A p-value < 0.05 was considered statistically significant. Data were expressed as mean ± standard error of the mean (SEM).

2.4 Results

3.1. BPA Analogues Increase Blood Pressure in a Chemical and Sex-Specific Manner

Figure 2.2 displays the hourly and mean telemetry data (mean ± SEM) for SBP, DBP, MAP, and HR.

Panels A-C specifically focuses on SBP. We observed a significant influence of EDC exposure ($p<0.0001$) and sex ($p=0.0083$) on SBP. Specifically, Panel A shows the hourly SBP for male offspring, revealing a significant surge in SBP for animals exposed to BPA, BPS, and BPF (145.5±1.82, 152.4±4.52, and 146.30±1.28, respectively; $p<0.0001$) compared to the control group (119.3±3.19). Contrastingly, Panel B shows that females also exhibited a rise in SBP after exposure to BPA and BPS, but only a slight increase with BPF (140.0±2.28, 140.5±2.17; $p<0.05$, vs. 133.2±2.18; respectively) compared to the control group (129.2±1.20).

As for sex differences, BPS and BPF-exposed females had significantly lower SBP than males ($p<0.05$). However, this difference wasn't significant amongst BPA-exposed offspring. Interestingly, all treatment groups for both sexes initially showed an increase in SBP as shown in panels A and B, except for females exposed to BPF, which had a similar pattern as control offspring.

While no clear shifts in DBP were seen as shown in panels D-F, treatment and sex-specific effects were significant ($p=0.0288$, $p<0.0001$; respectively). Specifically, female offspring prenatally exposed to BPA showed a slight increase compared to the control group (93.67±2.45 vs. 85.76±1.22; $p=0.078$). Control females exhibited lower DBP than males ($p<0.01$), yet no sex differences were evident in EDC-treated animals.

For DBP, the pattern of an hourly increase was unclear in male offspring, while female offspring displayed a pattern similar to SBP, with BPA and BPS starting at a higher DBP than the control and BPF groups as illustrated in panel E.

Regarding MAP, depicted in Panels G-I, significant EDC exposure and sex effects were observed ($p<0.0001$, $p=0.0017$; respectively). Similar to SBP, females had significantly lower MAP compared to males in the BPS and BPF-treated groups ($p<0.05$).

A pattern analogous to SBP was observed in MAP for EDC exposure, with males showing a significantly higher MAP for all EDCs (BPA, 116.55±1.57; $p<0.05$, BPS, 122.73±4.00; $p<0.001$, and BPF 116.15±1.14; $p<0.05$) compared to the control group (107.33±2.10). Notably, only female offspring exposed to BPA showed a significant increase in MAP (114.71±2.53; $p<0.05$), while BPS offspring showed an increase but was not statistically significant (113.92±1.90; $p=0.0548$) compared to the control (105.19 ±1.24). As for MAP hourly graphs, it shows a pattern like SBP, with all treatment groups showing increased MAP from the start of data collection.

As for HR (Panels J-L), both EDC treatment and sex had significant effects ($p=0.0101$, $p=0.0003$; respectively). BPS-treated females exhibited a higher HR than the control group (388.37±10.31 vs. 339.33±22.32; $p<0.05$). Furthermore, females treated with BPS had significantly higher HR than their male counterparts (388.37±10.31 vs. 343.48±5.56; $p<0.05$). The hourly HR pattern was similar to SBP and MAP at the onset, with an increase in HR across all treatments. However, HR across all treatments appeared to stabilize by the end of tracking, except for the BPS-treated female group.

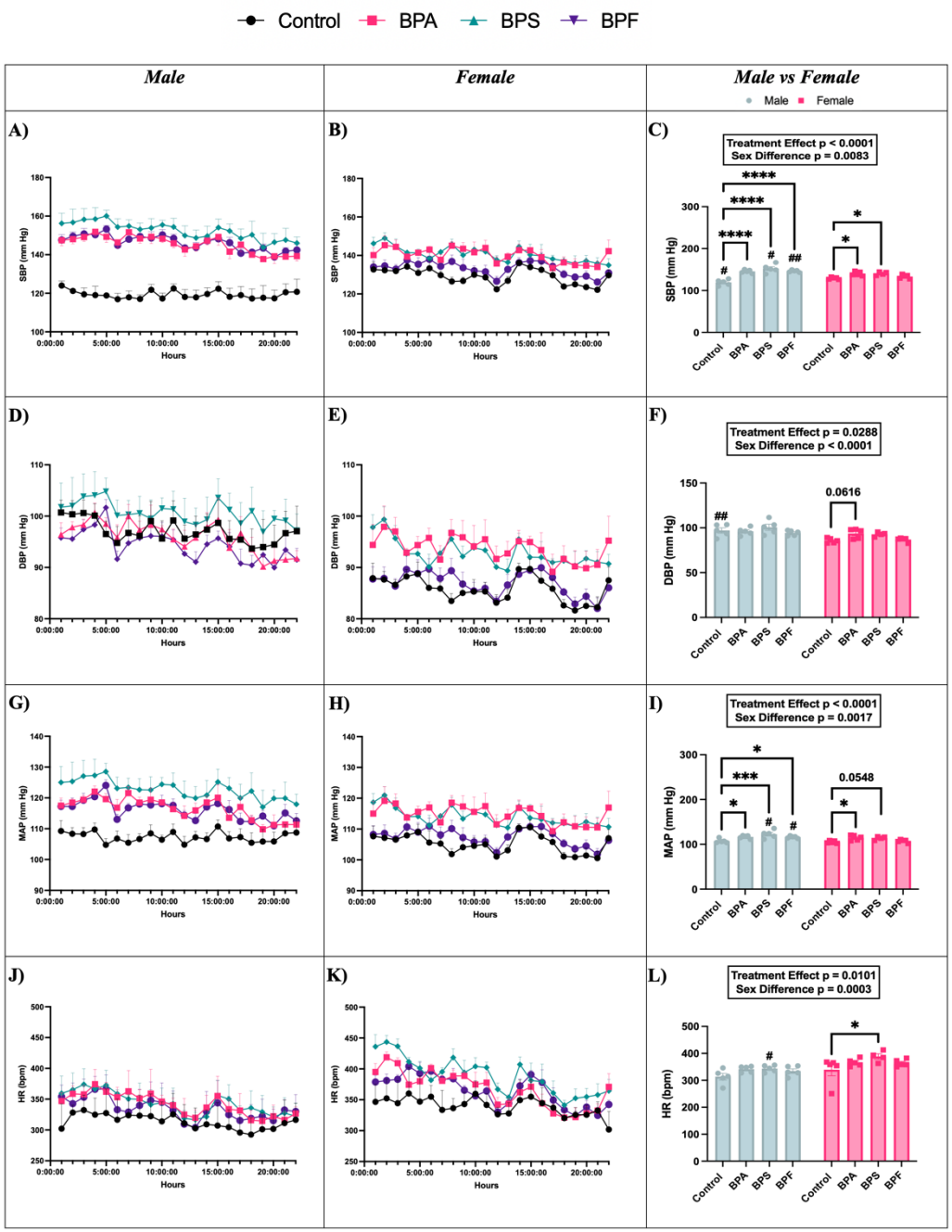


Fig. 2.2. Sex-specific effects of prenatal exposure to Bisphenol-A and its analogues on cardiovascular parameters. The offspring were prenatally exposed to PBS, 5µg/kg BW/day of BPA, 5µg/kg BW/day of BPS or 1µg/kg BW/day of BPF. SBP (A-C), DBP (D-F), MAP (G-I), and HR (J-K) are depicted. Males are represented with blue bars and females with pink bars. "*" indicates $p<0.05$, "**" $p<0.01$, "***" $p<0.001$, and "****" $p<0.0001$ compared to the

corresponding controls of the same sex. "#" Indicates $p<0.05$, "##" $p<0.01$, "###" $p<0.001$, "####" $p<0.0001$ compared to the corresponding female rats. Data were analyzed by two-way ANOVA, followed by Tukey's multiple comparison test or Holm-Šídák test for treatment or sex differences, respectively. The error bars represent the standard error of the mean (SEM).

3.2. Hormone Levels

Levels of Ang II, Aldo, and CORT are depicted in Figure 2.3. Ang II levels (pg/ml; Mean ± SEM) are depicted in 3A (Male) and 3B (Female) and 3C (Male vs Female). Male offspring showed a pattern of increased levels of Ang II with EDC treatment. Significantly higher levels were observed in BPS (75.31±11.86; p=0.0336) followed by the highest in BPF (86.86±8.77; p=00052) compared to their control counterparts (32.85±9.70). Females show a different pattern compared to males with BPA and BPF having similar levels whereas BPS offspring are significantly higher than control (86.45±12.20 vs. 39.50±6.45; p=0.0013). Two-way ANOVA showed no significant sex differences (p=0.5187) but showed treatment effect ($p<0.0001$) consistent with the findings from one-way ANOVA.

Aldo levels (pg/ml; Mean ± SEM) are shown in the second row in 3D (Male) and 3E (Female) and 3F (Male vs Female). One-way ANOVA showed significant EDC exposure in males (p=0.0017) and females (p=0.0310). Male offspring exposed to BPF prenatally showed significantly higher Aldo levels than controls (193.78±27.44 vs. 74.69±10.58; p=0.0075) while BPS offspring had increased levels (119.21±36.32), but they were not statistically significant. Females exposed to BPA or BPS had increased levels of Aldo compared to controls (167.43±13.51, and 140.60±20.53 vs. 137.60±9.20) but they were not statistically significant.

Two-way ANOVA showed no significant sex differences ($p=0.1074$) or treatment effect ($p=0.2534$).

We also tested the levels of circulating CORT (ng/ml; Mean ± SEM). Male CORT is shown in 3G, female in 3H and male vs female in 3I. One-way ANOVA showed a significant treatment effect in male offspring ($p<0.0001$) and female ($p=0.0119$). Male offspring prenatally exposed to BPA, BPS, or BPF showed an increasing pattern of CORT. BPF-treated offspring showed the highest levels (170.63±22.38; $p<0.0001$) followed by BPS (160.04±12.33; $p=0.0001$) and BPA (130.32±14.68; $p=0.0043$) and they were all significantly higher than control (46.67±9.60). Whereas female offspring showed a similar pattern of increase with BPF CORT levels significantly higher (304.19±13.52; $p=0.0083$) than controls (214.91±19.38) and the highest among the EDC group but BPA (238.24±20.59) and BPS (248.81±16.90) were not statistically significant. Two-way ANOVA showed significant sex differences ($p<0.0001$) and treatment effect ($p<0.0001$) consistent with the findings from one-way ANOVA.

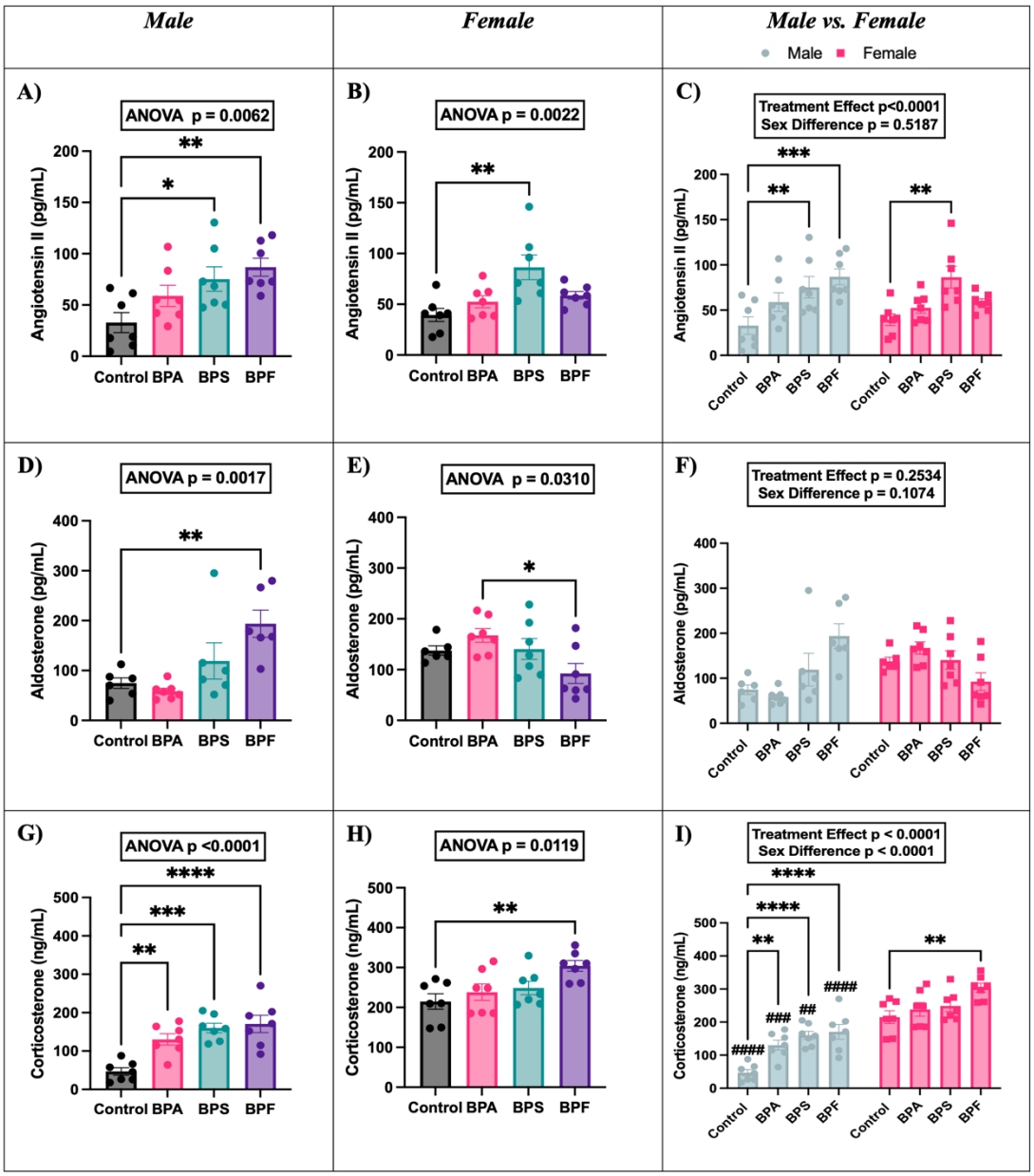


Fig. 2.3. Effects of prenatal exposure to Bisphenol-A and its analogues on circulating hormone levels. The offspring were prenatally exposed to PBS, 5µg/kg BW/day of BPA, 5µg/kg BW/day of BPS, or 1µg/kg BW/day of BPF. Ang II (A, B&C), Aldo (D, E&F), and CORT (G, H&I) are depicted. Males are represented with blue bars and females with pink bars. "*" indicates $p<0.05$, "**" $p<0.01$, "***" $p<0.001$, and "****" $p<0.0001$ compared to control. "##" indicates $p<0.01$, "###" $p<0.001$, "####" $p<0.0001$ compared to the corresponding female rats. Data were

analyzed by one-way ANOVA (Columns 1&2) and two-way ANOVA (Column 3) followed by Tukey's multiple comparison test or Holm-Šídák test for treatment or sex differences, respectively. The error bars represent the standard error of the mean (SEM).

3.3. Neurotransmitter Levels

Neurotransmitters concentration (pg/µl protein; mean ± SEM) in the PVN is depicted in Figure 2.4, n=6-7 in each group. Male levels are represented in Fig 4A, D, and G while females are in Fig 4 B, E, and H. Sex differences are shown in Fig 4 C, F, and I.

Male NE levels showed significant ANOVA effects with $p=0.0056$. Offspring prenatally treated with BPS or BPF had significantly higher NE levels (27.24±3.32 or 27.90±2.19; $p<0.05$, $p<0.01$; respectively) than control (17.13±0.98) while BPA treated offspring had increased levels, they were not statistically significant (24.22±1.09; $p=0.1033$). Female NE levels showed ANOVA EDC treatment effect with $p=0.0016$. Similar to males, BPS-treated offspring had a significant increase in NE (33.70±3.09; $p<0.01$) as well as BPA-treated offspring (30.38±2.35; $p<0.05$) compared to controls (19.13±1.94). Unlike male offspring, BPF-treated female offspring were not statistically significant in one-way ANOVA but showed increased levels compared to controls (28.33±1.92). Two-way ANOVA of NE levels showed significant treatment ($p<0.0001$) and sex differences ($p=0.0228$), results were consistent with one-way ANOVA except for female BPF, where it showed statistical significance ($p<0.05$) compared to control females.

As for DA levels, we observed significance in male offspring ($p=0.0020$) but not female ($p=0.0916$). Post-hoc analysis of male offspring showed significantly higher levels of DA in BPA and BPS-treated offspring (2.78±0.44 and 2.40±0.41, with $p<0.01$ and $p<0.05$; respectively) compared to controls (0.90±0.17). BPF-treated males had increased levels but were not statistically

significant (1.60±0.12, p=0.4197). Two-way ANOVA of DA levels showed significant treatment (p=0.0221) and sex-specific effects (p<0.0001). Male offspring showed significantly lower DA levels in control, BPA, BPS, and BPF (0.90±0.17, 2.78±0.44, 2.40±0.41and 1.60±0.12; respectively) than females (8.43±1.26, 10.56±1.14, 6.76±0.95, 7.26±0.91; respectively).

Prominent changes were also observed in 5HT levels in PVN of male (p=0.0001) and female (p<0.0001) offspring. Male offspring showed significantly higher levels of 5HT in BPA, BPS, and BPF-treated offspring (9.84±0.89, 7.83±0.42, and 7.38±0.85, p<0.0001, p<0.01 and p<0.01; respectively) compared to controls (3.75±0.72). Whereas female offspring showed a significant increase in BPS and BPF-treated offspring (9.97±0.93 and 11.44±0.51, p<0.0001 respectively) compared to their control counterparts (2.77±0.55). BPA-treated female offspring showed increased levels of 5HT but were not statistically significant (4.92±0.61, p=0.1415). Two-way ANOVA showed significant treatment effects (p<0.0001) consistent with the one-way ANOVA findings but showed no sex-specific effects (p=0.8827).

The ratio of 5HIAA/5HT and DOPAC/DA is presented in Fig.2.5. Male offspring 5HIAA/5HT and DOPAC/DA are shown in Fig.5A and D respectively while females in B and E. Sex differences are shown in panels C and F.

Male and female offspring showed a significant treatment effect (p=0.0010 and p<0.0001, respectively) in 5HIAA/5HT ratio. Post-hoc analysis showed that BPA, BPS, and BPF-treated male offspring had a significantly lower ratio (0.95±0.11, 1.08±0.07 and 1.28±0.14; p<0.01; respectively) than controls (3.34±0.77). Whereas females exposed to BPS or BPF (1.21±0.13 or 1.39±0.08, p<0.01) but not BPA (5.12±1.04, p=0.7573) showed significantly lower levels than their control (4.35±0.28). Two-way ANOVA of the 5HIAA/5HT ratio had significant treatment (p<0.0001) and sex differences (p=0.0003). Treatment effects were consistent with one-way

ANOVA. BPA-treated male offspring had a significantly lower ratio (0.95±0.11, $p<0.0001$) than females (5.12±1.04).

As for DOPAC/DA ratio, male offspring showed significant treatment effects ($p=0.0013$) but not females ($p=0.7507$). Male post-hoc analysis showed significantly lower ratio in BPA, BPS and BPF treated offspring (0.31±0.03, 0.41±0.07 and 0.53±0.08, $p<0.01$, $p<0.01$ and $p<0.05$; respectively) than their control counterparts (1.49±0.38). Two-way ANOVA showed significant treatment ($p=0.0006$) and sex differences ($p<0.0001$). Treatment findings were consistent with one-way ANOVA. Holm-Šídák post-hoc test showed significantly lower control female ratio (0.12±0.03; $p<0.0001$) than control males (1.49±0.38).

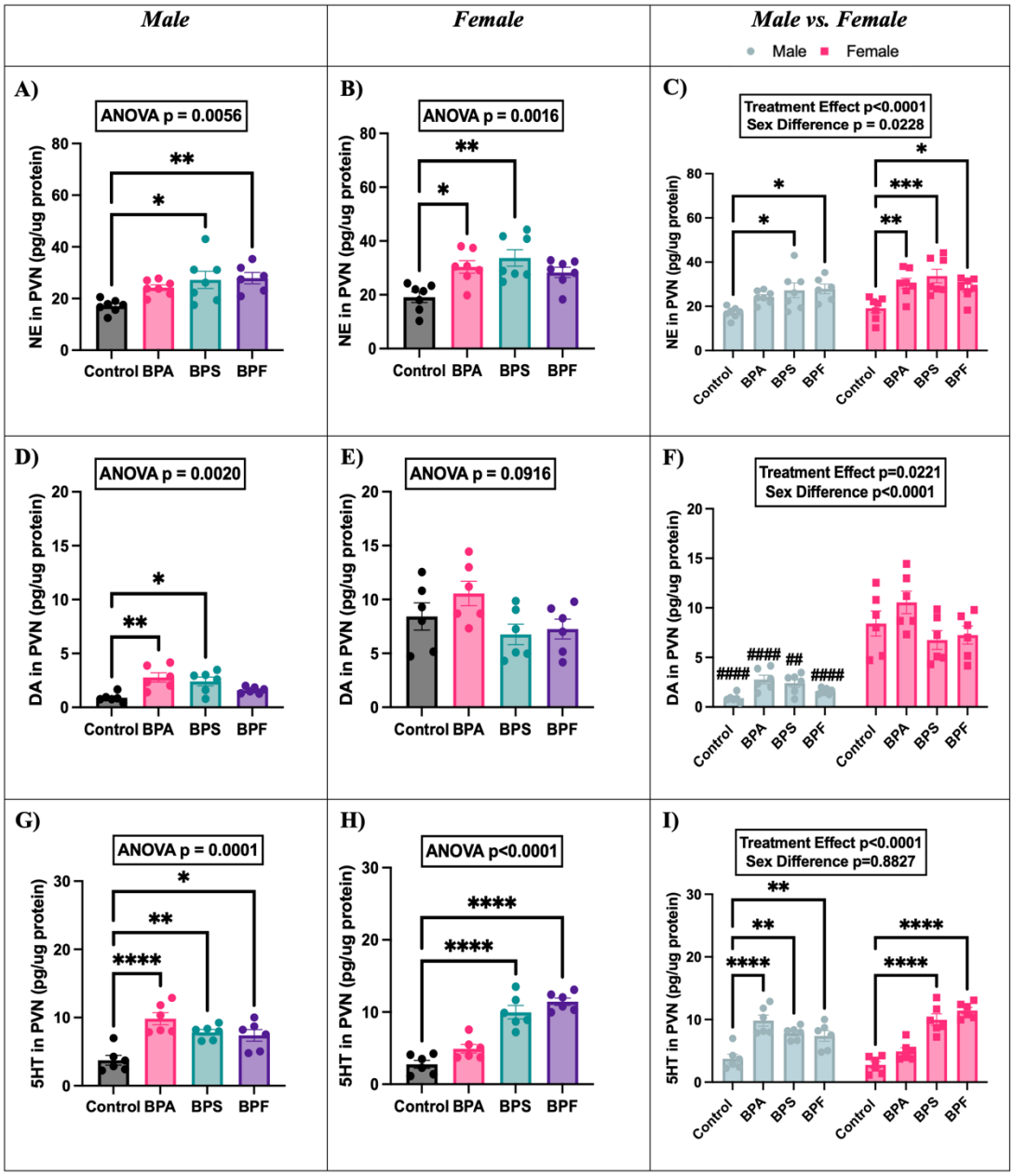


Fig. 2.4. Sex-specific effects of prenatal exposure to Bisphenol-A and its analogues on the neurotransmitter level in the PVN. The offspring were prenatally exposed to PBS, 5µg/kg BW/day of BPA, 5µg/kg BW/day of BPS, or 1µg/kg BW/day of BPF. NE (panels A-C), DA (panels D-F), and 5HT (panels G-I) are depicted. Males are represented with blue bars and females with pink bars. "*" indicates $p<0.05$, "**" $p<0.01$, "***" $p<0.001$ and "****" $p<0.0001$ compared to control.

"#" indicates p<0.05, "##" p<0.01, "###" p<0.001, "####" p<0.0001 compared to the corresponding female rats. Data were analyzed by one-way ANOVA (Columns 1&2) and two-way ANOVA (Column 3) followed by Tukey's multiple comparison test or Holm-Šídák test for treatment or sex differences, respectively. The error bars represent the standard error of the mean (SEM).

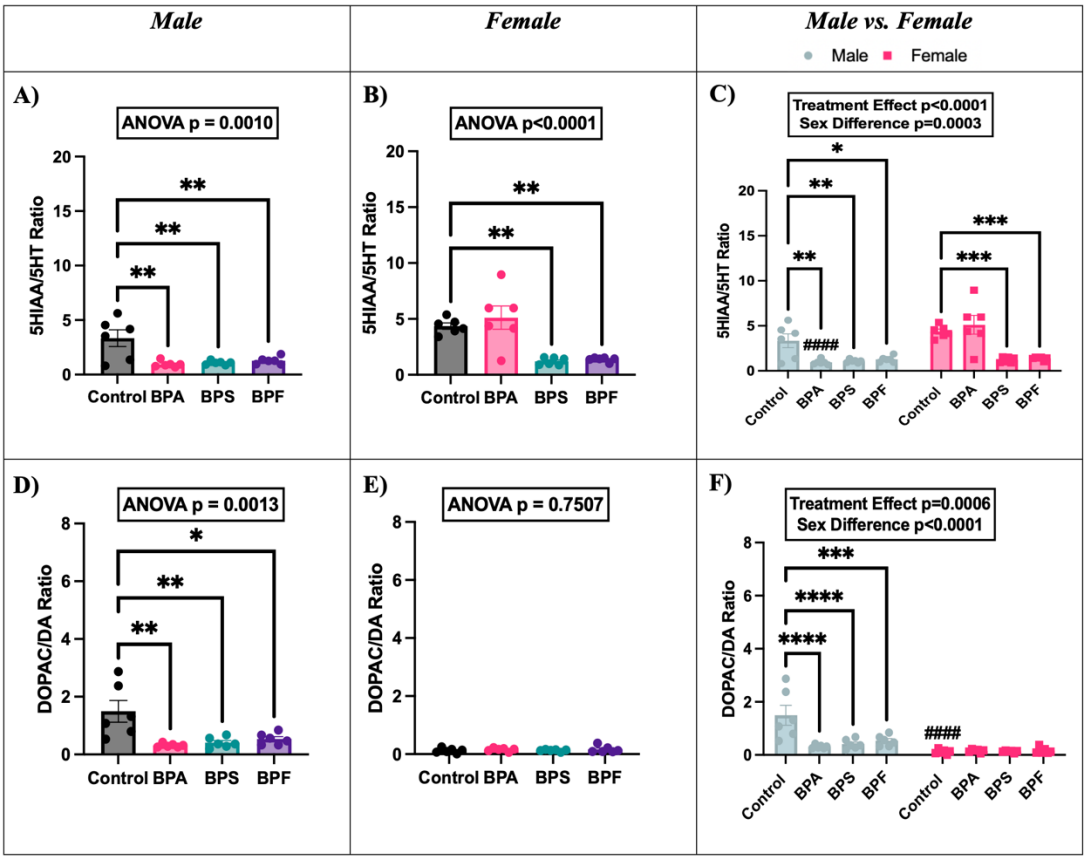


Fig. 2.5. Sex-specific effects of prenatal exposure to Bisphenol-A and its analogues on the neurotransmitter ratio in the PVN. The offspring were prenatally exposed to PBS, 5µg/kg BW/day of BPA, 5µg/kg BW/day of BPS, or 1µg/kg BW/day of BPF. 5HIAA/5HT (panels A-C) and DOPAC/DA (panels D-F) are depicted. Males are represented with blue bars and females with pink bars. "*" indicates p<0.05, "**" p<0.01, "***" p<0.001, and "****" p<0.0001 compared to their controls. "####" indicates p<0.0001 in terms of sex effects. Data were analyzed by one-way

ANOVA (Columns 1&2) and two-way ANOVA (Column 3) followed by Tukey's multiple comparison test or Holm-Šídák test for treatment or sex differences, respectively. The error bars represent the standard error of the mean (SEM).

3.4. Prenatal Bisphenol A and Analogues Exposure Induces Cardiac Fibrosis and Hypertrophy in Offspring

The extent of fibrosis is shown in Fig. 2.6 for females A-D and males E-H and the quantitative analysis of 5 areas of every offspring heart was measured using ImageJ software shown in Fig.6I (%fibrotic area; mean ± SEM, n=5-8/group). LV wall thickness was measured in at least 5 areas of each heart using eSlide manager (mm; mean ± SEM, n=5-8/group) shown in Fig.6J. Relative heart weight (g/Kg BW; mean ± SEM, n=7/group) is shown in Fig.6K.

There was a significant treatment effect in the extent of fibrosis ($p<0.0001$) but no sex-specific significance ($p=0.5552$). The extent of fibrosis in female heart tissue is shown in panel A-D. Panel A shows very little blue stain compared to BPA(B), BPS (C), and comparable color to BPF(D). When quantified BPA and BPS had significantly more %fibrosis (1.66±0.11 and 1.53±0.15, $p<0.0001$ and $p<0.001$; respectively) than the control (0.61±0.09). Whereas BPF had increased levels but was not statistically significant (0.92±0.11). Male fibrosis staining is shown in pane E-H, control offspring (E) shows less blue staining than BPA (F), BPS (G), and BPF (H). When quantified, BPA, BPS, and BPF-treated offspring had a significantly higher extent of fibrosis (1.18±0.14, 1.47±0.40, 1.81±0.18, $p<0.05$, $p<0.01$ and $p<0.0001$; respectively) than control (0.54±0.09).

We measured LV wall thickness and found significant treatment ($p=0.002$) and sex ($p=0.0052$) differences (Fig.6J). Males prenatally exposed to BPS or BPF had significantly thicker

LV wall (3.69±0.01 or 3.60±0.17, $p<0.01$ or $p<0.05$; respectively) than control offspring (3.17±0.07) while BPA-treated male offspring had increased thickness but not statistically significant (3.36±0.22). However, in females, only BPA-treated offspring showed increased LV wall thickness (3.45±0.08, $p<0.05$) compared to the control (3.03±0.08). BPS and BPF female offspring showed increased but not statistically significant changes (3.24±0.04 and 3.23±0.11). Sex-specific effects were evident in BPS-treated offspring, males exposed to BPS had significantly thicker walls (3.69±0.01) than females (3.24±0.04).

Relative heart weight is shown in panel K. There were significant treatment ($p<0.0001$) and sex ($p<0.0001$) effects. The increase in relative heart weight was consistent with fibrosis and LV wall thickness. Male offspring exposed to BPA, BPS or BPF had significantly increased heart weight (3.52±0.07, 3.48±0.09 or 3.54±0.07, $p<0.01$, $p<0.05$ and $p<0.01$; respectively) than control (3.22±0.07). Whereas females exposed to BPA had significantly higher heart weights (3.96±0.03) than control (3.60±0.05). BPS and BPF-treated females had increased relative heart weight but were not statistically significant (3.74±0.03 and 3.81±0.08). Control, BPA, BPS, and BPF-treated males had significantly lower relative heart weights (3.22±0.07, 3.52±0.07, 3.48±0.09 and 3.54±0.07, $p<0.001$, $p<0.0001$, $p<0.05$ and $p<0.05$; respectively) than females (3.60±0.05, 3.96±0.03, 3.74±0.03 and 3.81±0.08; respectively).

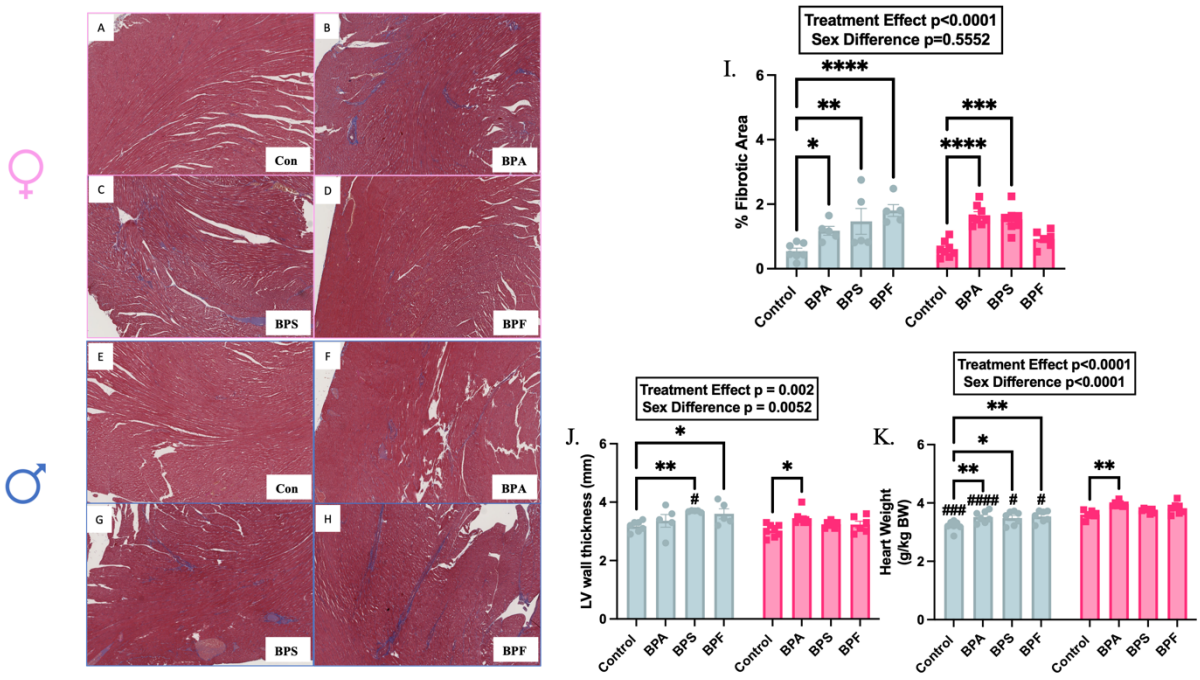


Fig. 2.6. Sex-specific effects of prenatal exposure to Bisphenol-A and its analogues on the heart. The offspring were prenatally exposed to PBS, 5µg/kg BW/day of BPA, 5µg/kg BW/day of BPS, or 1µg/kg BW/day of BPF. Panels A-H represents the extent of fibrosis in the heart tissue of each treatment group with females shown in panels A-D and males E-H. Fibrosis is stained blue with Masson trichrome stain. The percent fibrotic area was quantified using ImageJ software and is shown in panel I. Panel J represents the left ventricle wall thickness measured using eSlide manager software and panel K is the relative heart weight of these offspring. Males are represented with blue bars and females with pink bars. "*" indicates $p<0.05$, "**" $p<0.01$, "***" $p<0.001$ and "****" $p<0.0001$ compared to control. "#" indicates $p<0.05$, "##" $p<0.01$, "###" $p<0.001$, "####" $p<0.0001$ compared to the corresponding female rats. Data were analyzed by two-way ANOVA, followed by Tukey's multiple comparison test or Holm-Šídák test for treatment or sex differences, respectively. The error bars represent the standard error of the mean (SEM).

3.5. Prenatal Bisphenol A and its Analogues Effects on Organ Weights

Table 2.1. shows the relative organ weight (g/Kg BW; mean ± SEM, n=6-7/group). Two-way ANOVA p-values are provided in the table for each organ. There was a significant treatment effect in Kidney, Adrenal, and Lung tissue ($p<0.0001$, $p<0.0001$, and $p=0.0249$; respectively). There were significant sex differences across all organs presented.

BPF-treated males had significantly increased relative kidney and lung weights. Whereas female offspring prenatally exposed to BPA, BPS or BPF had significantly higher relative kidney and adrenal weights than their corresponding controls.

Table 2.1. Sex-specific effects of prenatal exposure to Bisphenol-A and its analogues on organ weights.

Relative Organ Weight (g/Kg BW)	**Control**	**BPA**	**BPS**	**BPF**	**Sex Differences *p*-value**	**Treatment Effect *p*-value**
Kidney					0.0271	<0.0001
Male	6.29±0.21	6.74±0.10$^{\#\#\#}$	6.79±0.12	6.96±0.05**		
Female	6.06±0.16	7.54±0.08****	6.87±0.18***	7.21±0.13****		
Adrenal					<0.0001	<0.0001
Male	0.13±0.01$^{\#\#\#\#}$	0.15±0.01$^{\#\#\#\#}$	0.14±0.02$^{\#\#\#\#}$	0.16±0.01$^{\#\#\#\#}$		
Female	0.23±0.01	0.32±0.01****	0.29±0.02**	0.33±0.02****		
Lung					<0.0001	0.0249
Male	4.06±0.11$^{\#\#}$	4.57±0.32	4.38±0.12$^{\#\#\#}$	5.18±0.48*		
Female	5.38±0.11	5.46±0.20	6.12±0.38	5.90±0.22		
Spleen					<0.0001	0.1681
Male	1.59±0.03$^{\#\#\#}$	1.65±0.03$^{\#\#\#\#}$	1.61±0.03$^{\#\#\#\#}$	1.66±0.03$^{\#\#\#\#}$		
Female	1.91±0.04	2.01±0.08	1.99±0.06	2.06±0.07		

Note: BPA, bisphenol A; BPS, bisphenol S; BPF, bisphenol F. Measures were obtained from offspring with prenatal exposure to PBS (control), BPA (5 µg/kg BW), BPS (5 µg/kg BW), or BPF (1 µg/kg BW). Data (N=6-7/group) are presented as mean ± SEM. "*" indicates $p<0.05$, "**" $p<0.01$, "***" $p<0.001$ and "****" $p<0.0001$ compared to control. "#" indicates $p<0.05$, "##"

p<0.01, “###” p<0.001, “####” p<0.0001 compared to the corresponding female rats. Data were analyzed by two-way ANOVA, followed by Tukey’s multiple comparison test or Holm-Šídák test for treatment or sex differences, respectively.

3.6. Prenatal Exposure to BPA Analogues increases Endothelin -1 Expression in small blood vessels.

Endothelin-1 (Endo-1) levels in lung tissue are depicted in Fig.2.7I (count; mean ± SEM, n=5-6/group). Fig.2.7-A-H depicts lung tissue images using eSlide Manager at 9X; Endo-1 staining in the lung vessels is shown as dark brown circles. Fig.7-A-D illustrates the extent of Endo-1 staining in male offspring exposed to saline, BPA, BPS or BPF, respectively. As for Fig.7-E-H illustrates the same treatments but for female offspring. Based on the images EDC-treated offspring show more brown staining of Endo-1 than their control counterparts. Quantitative analysis of Endo-1 showed that there was a significant effect of EDC exposure (p=0.0001) and sex effect (p=0.0060) on Endo-1 levels. Male offspring prenatally treated with BPA, BPS, or BPF (29.71 ± 5.64, 29.61 ± 2.65, and 28.10 ± 6.11 respectively; p<0.01) had significantly higher Endo-1 levels than control (6.30 ± 1.26). Whereas only females prenatally exposed to BPS (25.06± 5.22; p<0.05) showed significantly higher Endo-1 levels than their control counterparts (4.19 ± 1.06). BPA and BPF-treated female offspring showed increased levels of Endo-1 staining but were not statistically significant (15.26±6.37 and 12.23± 4.00; respectively).

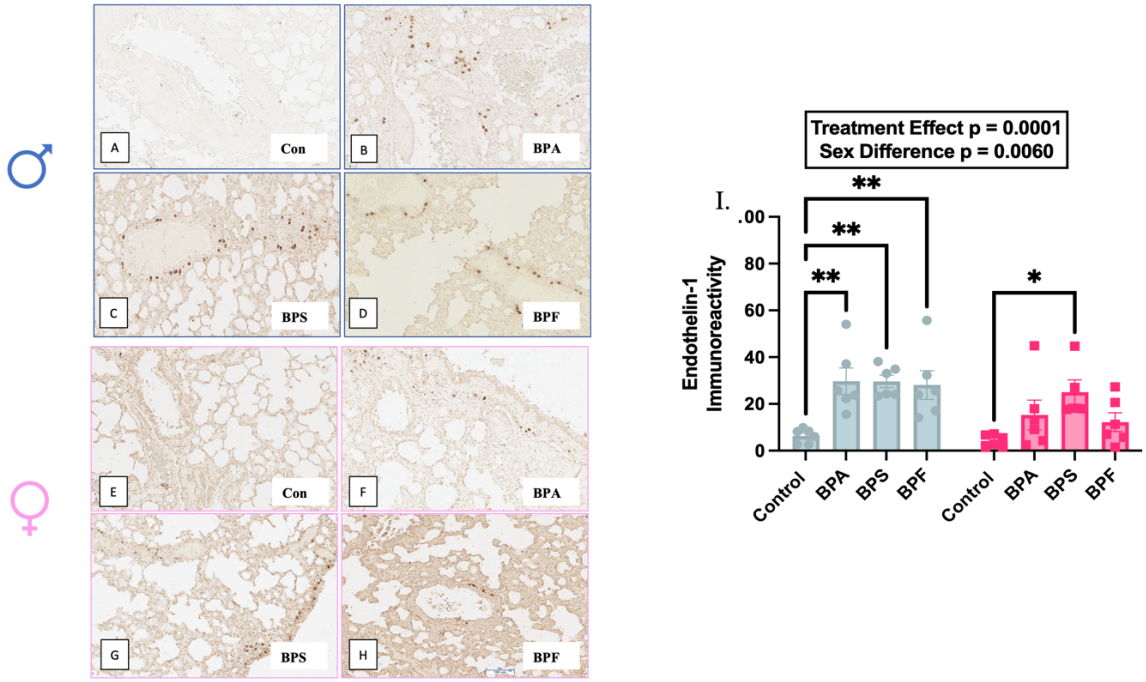


Fig.2.7. Endothelin-1 levels in large and small blood vessels of the lung following prenatal exposure to BPA and its analogues. The offspring were prenatally exposed to PBS, 5µg/kg BW/day of BPA, 5µg/kg BW/day of BPS or 1µg/kg BW/day of BPF. Panels A-D and panels E-H represent male and female IHC images of Endo-1 expression in blood vessels, respectively. Panel 'I' represents image quantitative analysis of Endo-1 positive cells in males (blue) and female (Pink). "*" indicates $p<0.05$, "**" $p<0.01$, "***" $p<0.001$ and "****" $p<0.0001$ in terms of treatment effects. Data were analyzed by two-way ANOVA, followed by Tukey's multiple comparison test or Holm-Šídák test for treatment or sex differences, respectively. The error bars represent the standard error of the mean (SEM).

2.5. Discussion

The findings from this study demonstrate for the first time that prenatal exposure to low doses of bisphenol analogues impacts SBP, MAP, and HR in a sex- and chemical-specific manner. While the BPA or BPS-exposed dams were treated with these chemicals at 5 µg/kg/day, dams exposed to BPF at the same dose had low offspring survival and high abortion rates, so the dose was reduced to 1 µg/kg BW/day as previously reported [34]. Interestingly, BPF at a dose of 1 µg/kg BW/day significantly increased SBP and MAP in males but not females. Suggesting that it has a very narrow safety range and has sex-specific effects. A slightly higher dose of BPF could have increased the risk of hypertension for females, but this needs further investigation. Unlike female offspring, male rats prenatally exposed to BPA, BPS, or BPF had 22 – 28 % higher SBP and 8 – 14 % higher MAP levels than controls, with the biggest increase observed in BPS-treated male offspring. While in females, we observed an 8 – 9% increase in MAP and SBP in BPA and BPS-treated offspring with a moderate increase in BPF offspring with a 2 – 3% increase compared to control offspring. BPS-treated females were the only ones that showed significantly elevated levels of HR, with a 14% elevation from their control counterparts. While males exposed to BPS had a 10% increase in HR but was not statistically significant. Notably, these increases were apparent in adult offspring, although the exposure was limited to when they were *in utero*. These results show that prenatal exposure to BPA and BPS at a dose of 5 µg/kg BW/day and BPF at a dose of 1 µg/kg BW/day was still able to induce adverse effects in offspring, contrary to the belief that very low doses would not cause harmful effects. To our knowledge, this is the first report to demonstrate that prenatal exposure to a minimal dose of BPS or BPF leads to increased blood pressure in offspring.

The results of the present study are consistent with the findings of a cross-sectional study in China assessing the association between urinary bisphenol levels and hypertension in males aged 55 and above, which found associations between increased blood pressure and exposure to low doses of BPA and BPS [105]. Jiang et al. did not find an association between BPF and hypertension, but that may be because of the limited enrollment of individuals with urinary BPF levels [105]. One study conducted in mice to evaluate the effects of prenatal exposure to BPA at low doses of 0.5 or 5 ug/kg/day BPA from gestation through sacrifice or 200 ug/kg/day from GD11 – PND21 showed that female, but not male mice, continuously exposed to BPA had increased SBP. Their data also showed increased left ventricular relative wall thickness and concentric remodeling in males' cardiac structure along with global DNA methylation [13]. This is consistent with our results which show that prenatal exposure to low doses of BPA and its analogues leads to increased blood pressure and increased LV wall thickness (Fig. 2,6). When we further investigated the heart histology to assess for fibrosis, we found that BPA analogues increased fibrosis and relative heart weight (Fig. 6). Our findings are alarming since BPS and BPF have been detected in urine, fetal cord, and serum, and have been found to have a longer metabolic half-life and are absorbed more rapidly than BPA [18, 145, 152, 153].

Bisphenols are characterized as EDCs, and these chemicals can alter hormone levels and physiological pathways in the body, leading to many impairments that lead to reproductive, metabolic, cardiovascular, and behavioral disorders, especially if exposed during critical developmental or gestational periods [99, 154-156]. The Renin-Angiotensin-Aldosterone System (RAAS) is a crucial mechanism that controls blood pressure and involves several organs such as the lungs, kidneys, heart, vasculature, and brain. Prorenin in the kidneys is converted to renin, which cleaves angiotensinogen into Angiotensin I (Ang I) and then activated into Angiotensin II

(Ang II) by Angiotensin Converting Enzyme (ACE). Ang II acts on the angiotensin II type 1 receptor (AT1R) to increase blood pressure, stimulate Aldosterone release, and increase arteriole pressure through vasoconstriction. The effects of Ang II are not limited to peripheral action, as Ang II can also act centrally by exerting effects on the hypothalamus and posterior pituitary [92]. Endogenous Ang II in PVN increases vasopressin response and blood pressure [157]. It acts on the AT1R of PVN and increases NE release [158]; it can excite PVN neurons through AT1R by reducing GABAergic synaptic input rather than excitation of glutamatergic neurons [159].

Consequently, we investigated the effects of prenatal exposure to BPA and its analogues on the levels of circulating hormones and monoamines in the PVN, while also exploring gender differences in CORT, Ang II, and Aldo levels. Males prenatally exposed to BPA, BPS or BPF had increased CORT levels while only females exposed to BPF showed a significant increase in circulating levels. This is consistent with a study that exposed female breeders to a comparable dose as our study, 2 ug/kg/day of BPA perinatally found that male offspring CORT levels were significantly higher than control males, but not BPA females [141]. Findings suggest that male offspring display a heightened sensitivity to BPA exposure. On the other hand, there exists a possibility that a higher dosage of BPA may have led to elevated levels of CORT in females. One study that exposed Wistar rat dams to a higher dose of BPA at 40 ug/kg/day for 42 days perinatally found that female offspring had significantly higher CORT levels than control females and their male counterparts, and exposure to stressful behavioral tests increased corticosterone levels in both male and female offspring [160]. As for BPS and BPF, one study exposed offspring perinatally to BPS at 10 and 50 μg/kg/day and found no changes to CORT levels compared to control animals [161]. To our knowledge, there is very limited studies investigating the effects of bisphenols on

CORT levels. Our study is the first to establish the effects of prenatal exposure to low doses of BPF or BPS on CORT levels.

In 2018, a study investigated the impact of BPA exposure on male mice. The mice were subjected to 10mg/kg of BPA for 12 weeks, and a significant increase in Ang II levels was observed 24 hours after the final dose [15]. Another study found that exposure to 0.4 μM BPA led to a significant rise in blood pressure and Ang II levels in aortic rings [110]. However, research on the effects of BPA on Ang II levels is limited. Although two previous studies showed an upregulation of Ang II, a study exposing male SD rats to 4 mg/kg BW found no significant differences between controls and BPA-exposed animals [162]. While our study did not find any significant changes to Ang II levels in BPA-treated offspring, there was a notable increase in Ang II levels in offspring prenatally exposed to BPS or BPF. Furthermore, our study is the first to establish that BPF-exposed males had elevated levels of Ang II and Aldo. This is the first study to investigate the effects of prenatal exposure to BPA and its analogues on Aldo levels in rats. It should be noted that the impact of BPA and its analogues on Ang II levels is influenced by several factors such as dose, time, and duration of exposure. Although we have found alterations to the circulating levels of Ang II, we are unaware of the central levels in the PVN. Ang II can act as a neurotransmitter in the PVN to regulate sympathetic activation of the cardiovascular system [163].

The PVN is a key cardiovascular regulatory region in the hypothalamus. To understand the effects of these EDCs on the monoaminergic activity in the PVN, we measured monoamine levels and the turnover rate of their metabolites in the PVN. In male offspring, there was a significant increase in NE in BPS and BPF offspring and a modest increase in BPA. In comparison, females had significantly higher levels of NE in BPA and BPS but only a modest increase in BPF. Chronic psychosocial stress and repeated stress can cause increased activity and vasopressin mRNA

expression in the PVN, which can lead to heart hyperactivity and increased blood pressure [89, 164]. The parvocellular neurons of the PVN modulate the HPA axis and stress responses, and play a central role in the autonomic adjustment of blood pressure to environmental challenges, by controlling sympathetic outflow towards the heart, blood vessels, and kidneys [89].

In addition, male and female offspring exposed to BPA and its analogues showed an increase in the levels of 5HT in the PVN, except for BPA-treated females, for whom the increase was not statistically significant. Serotonergic nerve fibers in the PVN can stimulate the release of the neurohypophysial hormone vasopressin; vasopressin can act on blood vessels to cause vasoconstriction and cause fluid retention in the kidneys [165, 166]. This is also consistent with our findings of increased relative kidney weight in BPF males and in BPA, BPS, and BPF-treated females. Further investigation of the mechanism of action involving levels of vasopressin and serotonin in Bisphenol-treated offspring needs to be explored.

Furthermore, DA levels were elevated in male offspring exposed prenatally to BPA or BPS. The presence of dopaminergic neurons in the PVN may be involved in regulating sympathetic activity by directly affecting the D1 receptor. This can lead to the hyperpolarization of many neurons, including sympathetic preganglionic neurons, in spinal cord slices. Although DA may not directly cause PVN excitatory responses, it could lead to some inhibitory effects. The PVN's increase in dopamine levels in male offspring exposed to BPA or BPS may serve as a defensive mechanism against elevated blood pressure levels [167]. One study investigated the effects of perinatal exposure to 250 ng/kg BPA on dopaminergic levels in the medulla oblongata of male and female mice offspring and found that BPA increased DA levels in males but not in females. Additionally, the study found that BPA reduced the DOPAC/DA ratio in males but not in females

[168]. The results of this study align with our own findings, but our study is the first to investigate the effects of prenatal exposure to BPA and its analogues on PVN.

Prenatal exposure to BPA and its analogues decreased the 5HIAA/5HT ratio as well as DOPAC/DA ratio in males PVN. Whereas female offspring had a decrease in BPS and BPF offspring 5HIAA/5HT ratio but not BPA and no significance in DOPAC/DA ratio. Our findings suggest that the PVN neurotransmitters are not readily metabolized by monoamine oxidase. Additionally, BPA exposure resulted in a significant decrease in MAO-B activity in male offspring medulla oblongata [168]. This will possibly lead to extended vasoactive effects of 5HT in the PVN.

In our findings, prenatal exposure to BPA and its analogues lead to cardiac fibrosis and left ventricular hypertrophy (Fig.6). Females exposed prenatally to BPA or BPS had a significant increase in fibrosis with a pattern in which BPA and BPS had the highest increase and BPF the least. Whereas males showed opposite patterns of increasing fibrosis in BPA, BPS, and BPF, with BPS and BPF showing the most profound increase (Fig.6). These findings are associated with hypertensive heart disease. It is characterized by increased collagen I and III synthesis and reduced or unchanged levels of degradation [169]. Growth factors such as Transforming Growth factor beta (TGF-β) can upregulate Gli2 by activating Smad3 [170]. Upregulation of TGF-β can increase collagen I and III and act as a profibrotic factor [169]. TGF-β stimulation of the fibrotic ALK5 / Smad3 pathway is also characterized by elevated Endo-1 levels [171].

Endo-1 is an endothelial-derived vasoconstrictor peptide that can be found in various cells, including lungs, cardiomyocytes, neurons, and kidneys [172]. Elevated levels of Endo-1 have been shown to induce vasoconstriction in endothelial cells, leading to increased blood pressure, as well as contributing to the development of lung and cardiac fibrosis. [172-175]. To assess whether Endo-1 is involved in Bisphenol-related increased blood pressure and fibrosis, Endo-1 levels were

assessed in small blood vessels in the lung. Our findings indicate that Endo-1 levels were found to be significantly higher in male offspring treated with BPA, BPS, and BPF and in female offspring treated with BPS (Fig.2.7). These findings are correlated with the BP data, and we found a similar pattern in cardiac fibrosis (Fig.2.6). Our study is the first to establish a relationship between Endo-1 and Bisphenol exposure. To our knowledge, there are no in vitro studies available yet that test this possible mechanism of action of Bisphenol analogues; however, a recent study was carried out on human adenocarcinoma alveolar basal epithelial cells, which were exposed to 10 nM BPS for 48 hours, and found that BPS elevated the expression of TGF-β and increased phosphorylation of Smad2/3, leading to in vitro migration of human non-small cell lung cancer [176]. The findings from this study highlight the need for continued research on the potential health effects of Bisphenol exposure, particularly during pregnancy.

2.6. Conclusions

The study revealed that prenatal exposure to low doses of BPA, BPS, and BPF at 5 μg/kg BW/day and 1 μg/kg BW/day, respectively, has a sex-specific impact on blood pressure regulation. Specifically, male offspring had significantly increased SBP and MAP after treatment with BPA, BPS, and BPF, while female offspring exhibited significant increases in SBP and MAP only after treatment with BPA and BPS, with a modest increase noted in BPF-treated offspring. In addition, females treated with BPS had a higher heart rate than their control counterparts. BPS and BPF exposure led to increased NE levels in male offspring, while BPA and BPS exposure resulted in increased NE levels in female offspring, indicating that BPA and its analogues may affect sympathetic nerve activation through the PVN. Furthermore, exposure to BPA, BPS, and BPF resulted in increased 5HT levels in male offspring, and BPS and BPF exposure led to increased

5HT levels in female offspring, and BPA and its analogues affected monoamine turnover ratio of DA and 5HT. Organ-level changes were also noted, including increased fibrosis in the heart, increased LV wall thickness, and heart weight, as well as increased Endo-1 levels in the lungs and blood vessels and increased organ weights in females such as kidney and adrenal glands. Overall, this study is the first to establish the cardiovascular effects of prenatal exposure to low doses of BPS and BPF. The results are concerning and require further investigation.

Acknowledgments

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CHAPTER 3

Prenatal Exposure of Sprague Dawley Rats to Bisphenol A, S, or F and Effects of Exposure to Exogenous Estrogen in Adulthood on the Cardiovascular System

Al Mansi et al. to be submitted to *Chemosphere.*

3.1. Abstract:

Recent research suggests that prenatal exposure to Endocrine Disrupting Chemicals (EDCs) such as Bisphenol A (BPA) and its analogs, Bisphenol S (BPS) and Bisphenol F (BPF), can pose significant health risks. These EDCs, which may act as exogenous estrogens, can affect developmental processes by crossing the placenta to the fetus. Additionally, hypertension (HTN) prevalence is known to be sex-dependent and increases with age. There is a shift in blood pressure regulation that occurs during menopause, likely tied to changes in the sex hormones. Our study investigated the cardiovascular effects of prenatal exposure to BPA and its analogs, coupled with postnatal chronic estradiol (E2) exposure in female rats. Pregnant Sprague Dawley rats were exposed to saline, 5 μg/kg BW of BPA or BPS, or 1 μg/kg BW of BPF, and adult female offspring were implanted with E2 or sham pellets to assess the combined cardiovascular effects. All animals were also implanted with radiotelemetry transducers for continuous monitoring of the blood pressure-related parameters. We conducted a comprehensive examination of cardiovascular function, particularly investigating the exposures' effects on the hypothalamus's paraventricular nucleus (PVN) in regulating blood pressure and involvement of peripheral circulating hormones such as angiotensin II (Ang II), aldosterone (Aldo), and corticosterone (CORT). Finally, we assessed cardiac alterations by evaluating left ventricle wall thickness and the presence of heart fibrosis. Prenatal BPA and BPS offspring with sham-implant in adulthood had a significant increase in SBP, DBP, and MAP. BPS also had increased HR levels. As for BPF-offspring, they had increased DBP. When hit with E2 exposure in adulthood, BPS offspring had significantly higher SBP, DBP, and MAP. BPS offspring, whether sham or E2 implanted, their increased blood pressure was accompanied by PVN sympathoactivation and renin-angiotensin system (RAS) activation. As for BPF offspring with a sham implant, their increase in DBP was accompanied by an increase in serotonin (5HT) levels in PVN and increased circulating CORT. Exposure to BPF prenatally and E2 in adulthood led to significantly higher levels of the Aldo hormone. BPA exposure increase in blood pressure was accompanied by PVN's sympathoactivation and increase in left ventricle wall thickness. All E2-treated offspring had decreased Dopamine (DA) levels in PVN and increased DOPAC/DA ratio. All EDC-exposed offspring whether exposed to E2 in adulthood or sham had increased cardiac fibrosis. Exposure to E2 seems to have adverse activational effects while prenatal exposure to BPA and its analogues causes detrimental organizational effects on the neuro-cardiovascular system.

3.2. Introduction:

Several recent studies investigating the possible detrimental health effects of EDCs have increased awareness and concern about exposure to these chemicals during the prenatal period [177]. While pregnant women are susceptible to many diseases during pregnancy due to their reduced immunity, their offspring is at risk of developing many pathologies postnatally and diseases that can follow them through adulthood [178]. Exposure to EDCs during the critical prenatal period can cause chemicals to cross the placenta to the fetus and bioaccumulate in the fetus and affect key developmental processes [178]. Women are constantly exposed to estrogen, whether from endogenous or exogenous sources. Exogenous estrogen could come from EDCs like Bisphenol A (BPA) or its analogs, Bisphenol S (BPS) or F (BPF) [17], and later in life, women are exposed to extra estrogen from hormonal therapy (HRT) or oral contraceptives (OC) [179]. While estrogen is known for its vasodilative and cardioprotective abilities, exposure to BPA was found to block its protective effects by binding and activating non-classical estrogen pathways [180]. BPA, BPS, and BPF were found to have estrogenic [31, 181] and anti-androgenic [181, 182] effects and were demonstrated to bind estrogen receptors and alter endogenous estrogen [31, 181, 183, 184].

The prevalence of hypertension (HTN) is sex-dependent and increases with age. While premenopausal women generally have a lower prevalence of HTN compared to men, the situation changes during menopause, leading to a significant increase in HTN rates [185]. Postmenopausal women exhibit a four-fold higher incidence of HTN compared to premenopausal women [186]. These observations suggest the involvement of sex hormones, particularly estrogen, in the regulation of blood pressure. In premenopausal women, estrogen, primarily in the form of 17β-estradiol (E2), is released from the ovaries. However, as women enter postmenopausal stages, the

adrenal gland and adipose tissue become the main sources of estrogen, with estrone being the primary hormone [187]. This hormonal shift may contribute to the increased blood pressure observed in ovariectomized mice [188]. The use of oral estrogen HRT has been associated with increased blood pressure [116, 189, 190]. However, it is worth noting that the cessation of oral contraceptive use tends to reduce the increased risk, suggesting that estrogen may exert activational (reversible or irreversible) effects on the cardiovascular system [116, 191]. Prenatal exposure to androgens has been found to increase blood pressure, but this effect can be reversed with gonadectomy [192]. Similarly, in a study conducted on CD-1 mice, exposure to ethinyl estradiol (EE) through diet resulted in dose-dependent effects on cardiovascular parameters, with a trend of increasing blood pressure observed at higher EE doses [118]. These findings suggest that both prenatal exposure to androgens and exogenous estrogen exposure can influence blood pressure regulation.

Exposure to BPA, BPS, or BPF during critical stages of fetal development has been associated with increased blood pressure and notable physiological and morphological changes in the heart [12, 193, 194]. However, studies investigating the effects of prenatal exposure to BPS and BPF on blood pressure are limited. Nonetheless, BPA has been shown to exhibit dose-dependent cardiovascular effects. A recent study explored the effects of exposure of Sprague Dawley rats to BPA through 5 or 20 ppm drinking water from day 2 to 21 of pregnancy. The results revealed that dams exposed to BPA had significantly higher SBP compared to the control dams. Additionally, morphological changes were observed in the heart muscle of the exposed dams, and fetal hearts displayed an increased presence of heart fibrosis in a dose-dependent manner [14]. This study demonstrated a significant dose-dependent increase in SBP, with a minimum dose of 0.4 μM being sufficient to cause a significant alteration in blood pressure. Furthermore, an

accumulation of Angiotensin II (AngII) was detected in the carotid arteries, suggesting a potential mechanism contributing to the blood pressure changes induced by BPA [194].

The objective of this study was to investigate the cardiovascular effects resulting from prenatal exposure to BPA and its analogues, followed by postnatal challenge with chronic E2 exposure in female rats. This study aimed to assess the distinct contributions of organizational effects (prenatal exposure) and activational effects (adulthood exposure) on cardiovascular outcomes and explore the potential interplay between these two factors. By employing a double-hit paradigm, we sought to gain insights into the combined impact of prenatal exposure to BPA and its analogues and the subsequent activation of hormonal pathways during adulthood, providing a comprehensive understanding of their effects on the cardiovascular system.

Briefly, pregnant Sprague-Dawley (SD) rats were orally administered BPA or its analogues, BPS and BPF, from gestational days 6 to 21. In adult female offspring, telemetry systems were implanted for continuous blood pressure measurements along with either sham or E2 pellet implantation. The study aimed to comprehensively examine various aspects of cardiovascular function, focusing on blood pressure parameters and both central and peripheral effects. A key area of investigation was the paraventricular nucleus (PVN) of the hypothalamus, a vital regulator of blood pressure, and its involvement in neurotransmitter pathways, particularly norepinephrine (NE) and serotonin (5HT). Furthermore, the study aimed to elucidate the impact of hormones such as estradiol (E2), corticosterone (CORT), angiotensin II (Ang II), and aldosterone (Aldo) on blood pressure regulation. Cardiac alterations were assessed by evaluating left ventricle (LV) wall thickness and the presence of heart fibrosis. The overarching goal was to identify and characterize changes in these parameters in rat offspring exposed to BPA and its analogues during prenatal development, as well as chronic E2 exposure during adulthood.

3.3. Materials and Methods

2.1. Experimental Animals and Treatment

Adult female and male Sprague Dawley rats (3 mo old) were purchased from Envigo (Indianapolis, IN). Animals were housed at the University of Georgia animal facility in light-(12:12 light-dark cycle) and temperature-(23 ± 2 °C, 50 ± 20% relative humidity) controlled rooms. Food (PicoLab-LabDiet 5053) and water were provided *ad libitum*. All animal procedures were approved by the Institute Committee for Animal Care and Use at the University of Georgia and carried out in accordance with the NIH Laboratory Animal Care and Use Guidelines. Females were set for breeding, and pregnant dams were randomly assigned to one of four treatment groups: Control (10µl Phosphate Buffered Saline (PBS), *n*=9), Bisphenol A (5µg/kg BW/day, *n*=6, Catalog No. 239658; Lot MKBH2096V; ≥99% purity), Bisphenol S (5µg/kg BW/day, *n*=13, Catalog No. 43034; Lot BCBV2462; ≥98% purity), and Bisphenol F (1µg/kg BW/day, *n*=10, Catalog No. 51453; Lot BCBQ5566V; ≥98% purity) as previously described [34]. The dams were dosed orally with gavage every day based on their body weight from GD 6-21. The offspring were weaned three weeks after birth and were group housed until further experimentation. The dose of EDC was lower than the current Environmental Protection Agency (EPA) recommended no-observed-adverse-effect-level (NOAEL) (BPA;5 mg/kg bw/day, BPS; 10 mg/kg bw/day, BPF; not established) (EPA 2012, 2014). In all the studies and the results described below, the 'dams' are the experimental unit.

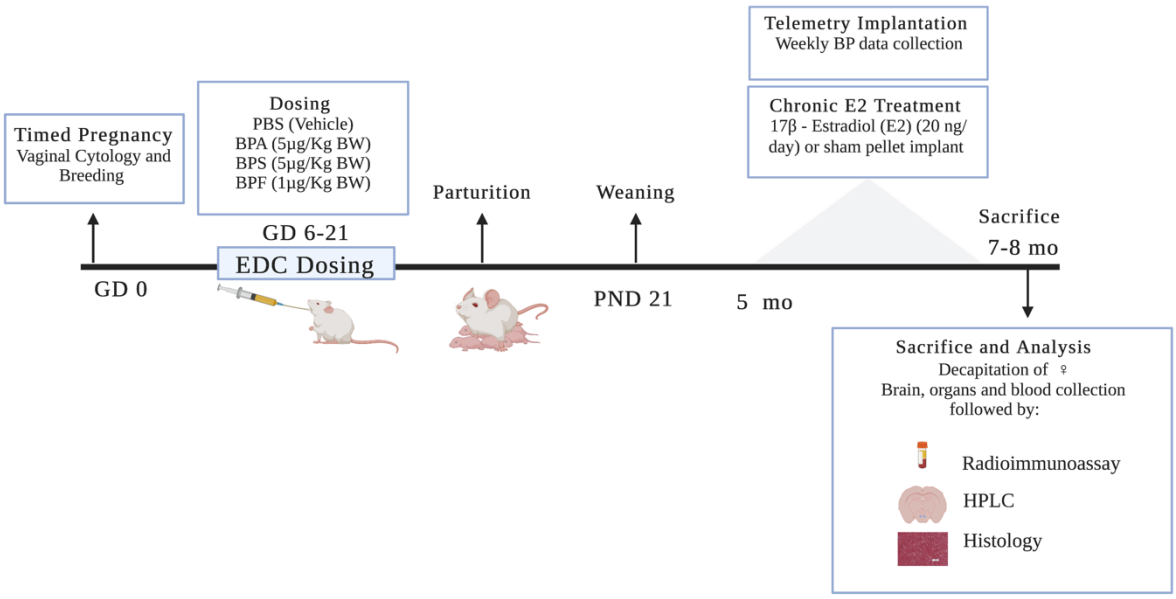

Fig. 3.1. Overall study scheme. Pregnant SD rats were administered orally with Control (10µl Phosphate Buffered Saline (PBS), *n*=9), Bisphenol A (5µg/kg BW/day, *n*=6), Bisphenol S (5µg/kg BW/day, *n*=13), and Bisphenol F (1µg/kg BW/day, *n*=10) from GD 6-21. The offspring were weaned at PND 21, and around 5 months of age they were implanted with a radiotelemetry device followed by implantation of Estradiol 17β or sham pellet. Blood pressure and heart rate were monitored continuously for 24 hours every week for 10 weeks, after which the animals were sacrificed. The brain, trunk blood, hearts, and lungs were collected to measure neurotransmitters, hormones, and histology. *Created with BioRender.com*

2.2. Radiotelemetry Implantation Surgery and Hemodynamic Measurements

Female offspring were permitted to mature to adulthood, and then a radiotelemeter (Data Sciences International; HD-S10) was implanted in the femoral artery following established procedures [147, 148]. Briefly, the animals were anesthetized with isoflurane, and sterile

techniques were employed to shave and clean the ventral abdomen and the inside of the left thigh. Prophylactic administration of Meloxicam (1 mg/kg BW) and Enrofloxacin (5 mg/kg BW) was performed subcutaneously. A 2-centimeter incision was made in the left groin to expose the femoral artery. The superficial fascia and fat were gently dissected to visualize the femoral artery, vein, and nerve. A ligature was placed at the distal end of the femoral artery near the knee, and a retention suture was positioned close to the body wall to restrict blood flow through the femoral artery. A subcutaneous pocket was created in the ventral abdomen to hold the transmitter, which was then inserted into the pocket. Lidocaine was applied to the femoral artery to prevent arterial wall contraction, and a hole was carefully created in the arterial wall using fine scissors under a dissection microscope. The hole was expanded using Dumont vessel cannulation forceps, and the transmitter catheter was inserted into the femoral artery to a depth of up to 2 centimeters. Ligatures were placed around the arterial wall containing the catheter to secure it in place. The fascia around the catheter was sutured with simple interrupted sutures, and the skin was closed with horizontal mattress sutures. The transmitter was activated using a magnet, and its functionality was monitored wirelessly. The animals were placed in separate cages to recover and closely monitored daily for a period of seven days. Meloxicam was administered for three consecutive days post-surgery. Once fully recovered, the transmitter was activated, and data collection was conducted at 10-minute intervals over a 24-hour period every week for eleven weeks. Systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), and heart rate (HR) were recorded.

2.3. Chronic Exogenous Estradiol Exposure

After stabilizing the animals following telemetry implantation, two female rat offspring from each dam were selected for the study. One group received a slow-release 17β-Estradiol (E2)

pellet implant (n=6-8/group) (1.8 µg, Innovative Research America, Sarasota, FL). These E2 pellets released 20 ng per day over a 90-day period. The other group underwent a sham implantation and served as the control (n=6-8/group). Animals were sacrificed by rapid decapitation following BP collection. The females were monitored using vaginal cytology and sacrificed in an estrus state. The brains were harvested, and the trunk blood was centrifuged to extract the plasma. All tissues were stored at -80 ˚C until further analysis.

2.4. Hormone Assays

Plasma levels of estradiol (E2), corticosterone (CORT), angiotensin II (Ang II), and aldosterone (Aldo) were measured using a double antibody radioimmunoassay according to the manufacturer's protocol.

We employed the following assays: E2: Catalog #0713810-CF and CORT: Catalog #07120102 (MP Biomedicals, Irvine, CA) Ang II: Catalog # 07120102 (Phoenix Pharmaceuticals, Inc., Burlingame, CA) Aldosterone: Catalog # MG13051 (IBL International GmbH, Hamburg, Germany). The samples were added in duplicate and analyzed according to the assay protocol. Values were expressed as ng/ml for CORT and pg/ml for E2, Ang II, and Aldo (%CV <10).

2.5. Brain Sectioning and Microdissection

Brain sections were cut at a thickness of 300 µm using a cryostat (Slee, London, UK) maintained at -10°C. Following that, the paraventricular nucleus (PVN) was microdissected from the sections using the Palkovits procedure on a cold stage. The Rat Brain Atlas, specifically the 7th Edition of The Rat Brain in Stereotaxic Coordinates, was used as a reference to guide the

microdissection and ensure the inclusion of all PVN subdivisions [149]. The microdissected tissue was then stored at -80°C until it was ready for further analysis.

2.6. Neurotransmitter Analysis by HPLC-EC

The PVN samples were subjected to HPLC-EC for measurement and analysis of neurotransmitters to determine the levels of norepinephrine (NE), dopamine (DA), and serotonin (5HT), following the established methodology [150]. In summary, brain punches were homogenized in 0.05 M perchloric acid and kept on ice. A portion of the homogenate was used for protein estimation (Pierce, Rockford, IL), while the remaining homogenate underwent centrifugation at 18,000 × g for 8 minutes at 4°C. The resulting supernatant was mixed with an internal standard (dihydroxybenzylamine, 0.05 M) and injected into the HPLC autoinjector for analysis. The HPLC-EC system consisted of a 5-µm ODS reverse phase C-18 column (Phenomenex, Torrance, CA), a SIL-20AC autoinjector, a CTO-20AC column oven (Shimadzu, Columbia, MD) maintained at 37°C, and an LC-4C detector (Bioanalytical Systems, West Lafayette, IN). The mobile phase flow rate was set at 1.8 ml/min using an LC-20AD pump. The chromatograms were processed using Class VP software v 7.2 for neurotransmitter concentration analysis. Neurotransmitter levels in the tissue samples were reported as pg/µg of protein. Protein levels in the tissue punches were measured using the micro bicinchoninic acid assay (MicroBCA assay, Pierce, Rockford, IL), and the absorbance was recorded using an ELX 800 microplate reader.

2.7. Histology of heart tissues.

The hearts were collected from sham and E2 implanted females and fixed in formalin for preservation. Subsequently, they were cut at a consistent position, starting from the apex, and underwent standard paraffin embedding and sectioning procedures. The resulting sections were stained with Masson's trichrome and then scanned digitally using a Leica Biosystems Aperio scanner (Wetzlar, Germany). The scanned images were accessed and examined using the eSlide Viewer software. The thickness of the left ventricle wall was measured at five specific locations within the images using the eSlide Viewer. To assess the extent of heart fibrosis, ImageJ software was utilized, following the methodology outlined by Kennedy et al. in 2006 [151].

2.8 Statistical Analysis

Prism 9.0 software (GraphPad, Inc., San Diego, CA) software was used to perform statistical analysis. Two-way ANOVA was used, followed by Tukey's multiple comparison test to detect changes in blood pressure, hormones, fibrosis, immunohistochemistry, and neurotransmitter measurements. Sex differences were assessed with post hoc Šídák test. Control sham versus E2 effects were assessed using an unpaired two-tailed t-test. A p-value < 0.05 was considered statistically significant. Data were expressed as mean ± standard error of the mean (SEM).

3.4. Results

3.1 Exposure to BPA analogs and exogenous estradiol increases blood pressure in a chemical-specific manner.

Figure 3.2 displays the weekly and mean telemetry data (mean ± SEM) for SBP, DBP, MAP, and HR for sham or E2 implanted offspring. Panels A-C represents SBP, there was a significant EDC exposure effect ($p<0.001$) but not E2 treatment ($p=0.093$). Sham females exposed to BPA or BPS showed increased SBP (133.8 ± 1.8, $p<0.01$; 132.8 ± 2.1, $p<0.05$, respectively) compared to their control counterparts (125.2 ± 1.1). As for E2 implanted female offspring, BPS-treated females showed significantly higher SBP (135.0 ± 1.5, $p<0.05$) than their control counterparts (128.9 ± 1.2). Showing that BPS had a persistent increase in blood pressure independent of E2 treatment. The overall pattern of SBP in sham (column A) and E2 (column B) shows that sham-implanted females prenatally treated with BPA, BPS, or BPF had increased SBP from week 1 and gradually increased with age, with BPF being the closest to control offspring. This pattern is reversed with E2 treatment as BPA becomes the closest to control and BPF the furthest along with BPS, but in both treatments, the pattern shows increased SBP with age. Based on observed changes, prenatal exposure to BPA analogs increased SBP and deteriorated with age.

DBP is represented in panels D-F. There was a significant EDC exposure effect ($p<0.0001$) but not an E2 effect ($p=0.895$). BPA, BPS, and BPF- sham-treated offspring showed significantly higher DBP (89.2 ± 1.9, $p<0.05$; 90.5 ± 1.3, $p<0.01$; 88.6 ± 0.8 $p<0.05$, respectively) than the control (83.7 ± 0.7). Like SBP, DBP showed a similar pattern with only E2-treated- BPS offspring showing significantly increased DBP (93.4 ± 2.8, $p<0.001$) than their control counterparts (85.4 ± 1.3). DBP pattern in sham female offspring showed a similar pattern to SBP, with BPA and BPS females having increased SBP from week 1. In contrast, BPF starts at a similar level to control but,

with age, significantly increases (Fig. 2D). E2 treatment seems to return DBP levels to normal for BPA and BPF-treated offspring but not in BPS (Fig. 2E).

MAP levels are illustrated in panels G-I. MAP levels follow a similar pattern and significance as SBP. There was a significant EDC effect ($p<0.0001$) but not an E2 effect ($p=0.85$). Sham-implanted BPA and BPS-treated offspring showed significantly higher MAP levels (109.0 ± 2.1, 108.8 ± 1.3; $p<0.01$, respectively) than their control counterparts (102.2 ± 1.0). As for E2-treated offspring, similar to SBP and DBP, only BPS-treated offspring significantly increased MAP (108.7 ± 2.2; $p<0.05$) compared to their control (103.4 ± 0.7). MAP pattern in sham-implanted females showed a similar pattern as SBP and DBP, BPA and BPS treated offspring showed increased levels from the first measurement while BPF and control started at about the same level and then BPF continued to increase with age while the control stayed relatively the same throughout the measurements period. E2-treated offspring showed a pattern in MAP like DBP, with BPS having the highest levels. Compared to Fig. 2G MAP sham-implanted, Fig. 2H E2-implanted females show higher levels of MAP. Still, EDC treatment is the most detrimental effect, and E2 does not seem to protect the offspring from the prenatal effects of BPA and its analogs.

HR levels are displayed in panels J-L. There is a significant EDC exposure effect ($p=0.0157$) but not an E2 treatment effect ($p=0.7415$). BPS-sham-implanted females showed significantly higher HR (342.2 ± 5.7, $p<0.05$) than their control counterparts (328.6 ± 2.7). E2 treatment relatively reduced HR levels of BPS-treated offspring. Based on the weekly pattern depicted in Fig 2 J and K, in sham-treated females, BPS HR was elevated from the first week and continued to be elevated throughout the observation period. HR levels were reduced when BPS-treated offspring were implanted with an E2 pellet.

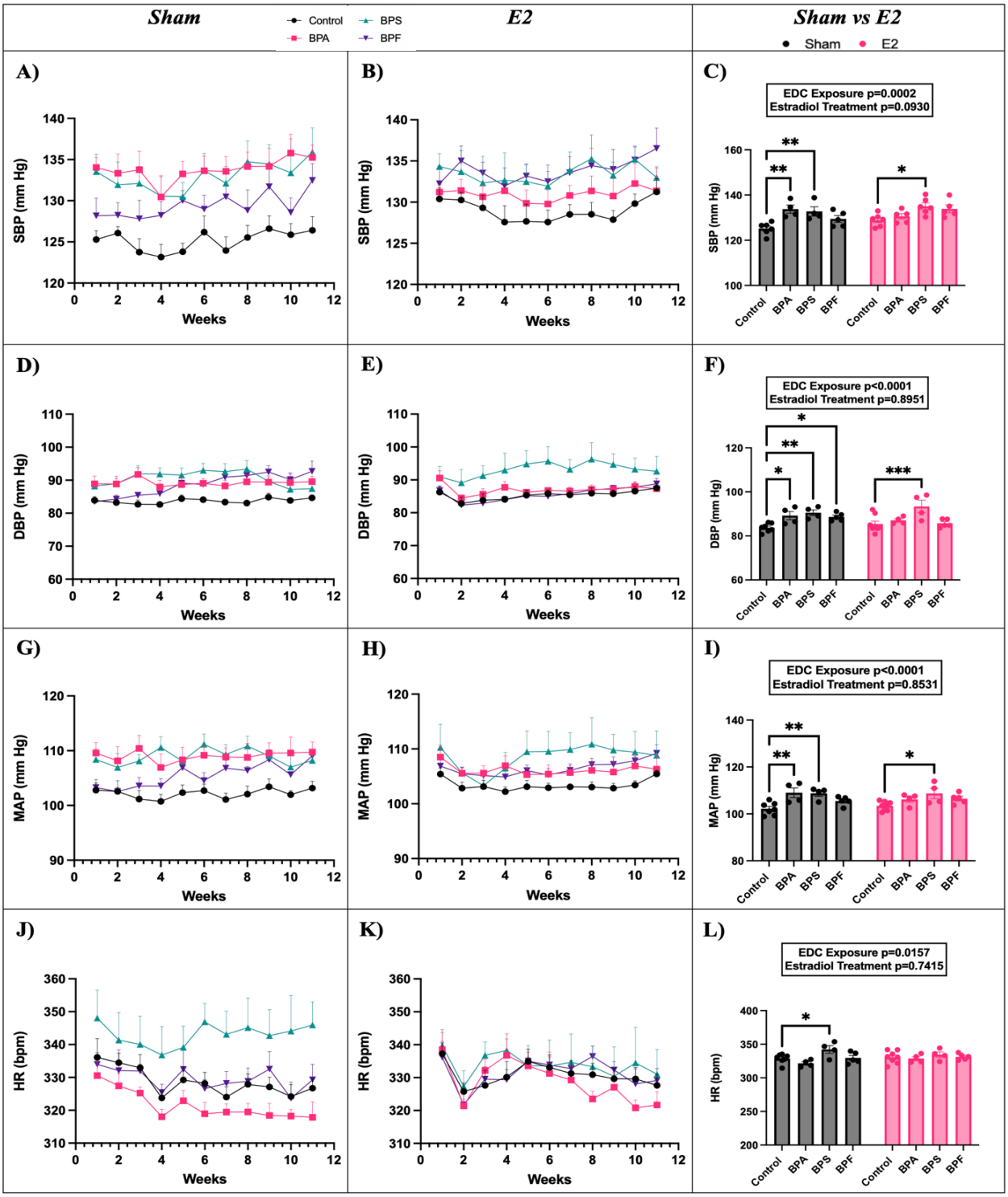

Fig.3. 2. Effects of prenatal exposure to Bisphenol-A and its analogs followed by exposure to exogenous estradiol in adulthood on females' cardiovascular parameters. The offspring were prenatally exposed to PBS, 5µg/kg BW/day of BPA, 5µg/kg BW/day of BPS, or 1µg/kg BW/day

of BPF. SBP (A-C), DBP (D-F), MAP (G-I), and HR (J-L) are depicted. "*" Indicates $p<0.05$, "**" $p<0.01$, "***" $p<0.001$ compared to the corresponding controls of the same implant. Data were analyzed by two-way ANOVA and Tukey's multiple comparison test. The error bars represent the standard error of the mean (SEM).

3.2. Neurotransmitter Levels

Neurotransmitters concentration (pg/µl protein; mean ± SEM) in the PVN is depicted in Figure 3.3, n=6-7 in each group.

NE levels are illustrated in Fig 3A-C, panel A depicts one-way ANOVA of females implanted with sham pellet and B with E2 pellet whereas C is a comparison between the two, analyzed with two-way ANOVA. There was a significant increase in NE levels (ANOVA $p=0.0016$) in BPA and BPS sham-treated offspring (30.4 ± 2.4, $p<0.05$ and 33.7 ± 3.1, $p<0.01$; respectively) compared to their control (19.1 ± 1.9). Whereas panel B, shows no significant effect in E2-treated females $p=0.2484$, but there is a pattern of increase in BPA (27.5 ± 3.1) and BPS (31.3 ± 4.1) compared to their controls (22.8 ± 1.9). Two-way ANOVA shows a significant EDC treatment effect($p=0.0009$) but not an E2 effect ($p=0.5512$) on the NE in PVN.

DA levels in the PVN are shown in panels D-F. female offspring showed no EDC treatment significance ANOVA $p=0.0906$. While there was a slight increase in sham-BPA offspring (10.6 ± 1.1) and a decrease in sham-BPS (6.8 ± 0.9) and sham-BPF (7.3 ± 0.9) offspring compared to their sham-control (8.4 ± 1.3). When treated with chronic E2 DA levels dropped significantly ($p<0.0001$).

5HT levels are depicted in Fig 3G-I, panel G represents the significant effects of sham-implanted bisphenol offspring on 5HT levels in the PVN ($p<0.0001$) whereas panel H illustrates

the combined effect of bisphenols and chronic E2 ($p=0.0190$). In panel G there is a pattern of increased 5HT levels in BPA (4.9 ± 0.6), BPS (10.0 ± 0.9), and BPF (11.4 ± 0.5) sham-implanted offspring compared to controls (2.8 ± 0.6) with a significant increase in BPS and BPF females ($p<0.0001$). As for panel H, there is also an increased pattern of 5HT but to a lower extent than sham implanted except for BPS offspring that were exposed to chronic E2 (11.5 ± 2.2; $p<0.05$) compared to E2-controls (5.4 ± 1.1). BPF E2 treated offspring had increased 5HT levels (9.2 ± 1.1) but they were not statistically significant. Two-way ANOVA showed no significant E2 effect ($p=0.2475$). Once again, BPS offspring showed persistent increase regardless of E2 treatment.

Monoamine turnover ratios are shown in Figure 3.4. Fig 3.4 A-C depicts the ratio of DOPAC to DA whereas D-F is the ratio of 5HIAA to 5HT. Chronic E2 exposure increased the ratio of DOPAC to DA for all treatments ($p<0.0001$) but there was not any bisphenol exposure effect, consistent with the findings in DA levels (Fig. 3F).

As for the ratio of 5HIAA/5HT, sham, and E2-implanted offspring show significant effects ($p<0.0001$ and $p<0.05$; respectively). In sham offspring, BPS and BPF females had significantly reduced levels (1.2 ± 0.1, and 1.4 ± 0.1; respectively, $p<0.01$) while BPA had comparable levels (5.1 ± 1.0) to their control counterparts (4.4 ± 0.3). BPF-treated offspring in combination with chronic E2 exposure had significantly reduced 5HIAA/5HT ratio (1.1 ± 0.1, $p<0.05$) compared to control (1.7 ± 0.3) while BPA and BPS had reduced levels ($1.4 \pm 0.1$and 1.2 ± 0.1) but they were not statistically significant. Panel F shows a two-way ANOVA significant treatment ($p<0.0001$) and E2 effect ($p<0.0001$). Chronic E2 treatment significantly reduced control (1.7 ± 0.3, $p<0.001$) and BPA (1.4 ± 0.1, $p<0.0001$) levels compared to sham-implanted control (4.4 ± 0.3) and BPA (5.1 ± 1.0).

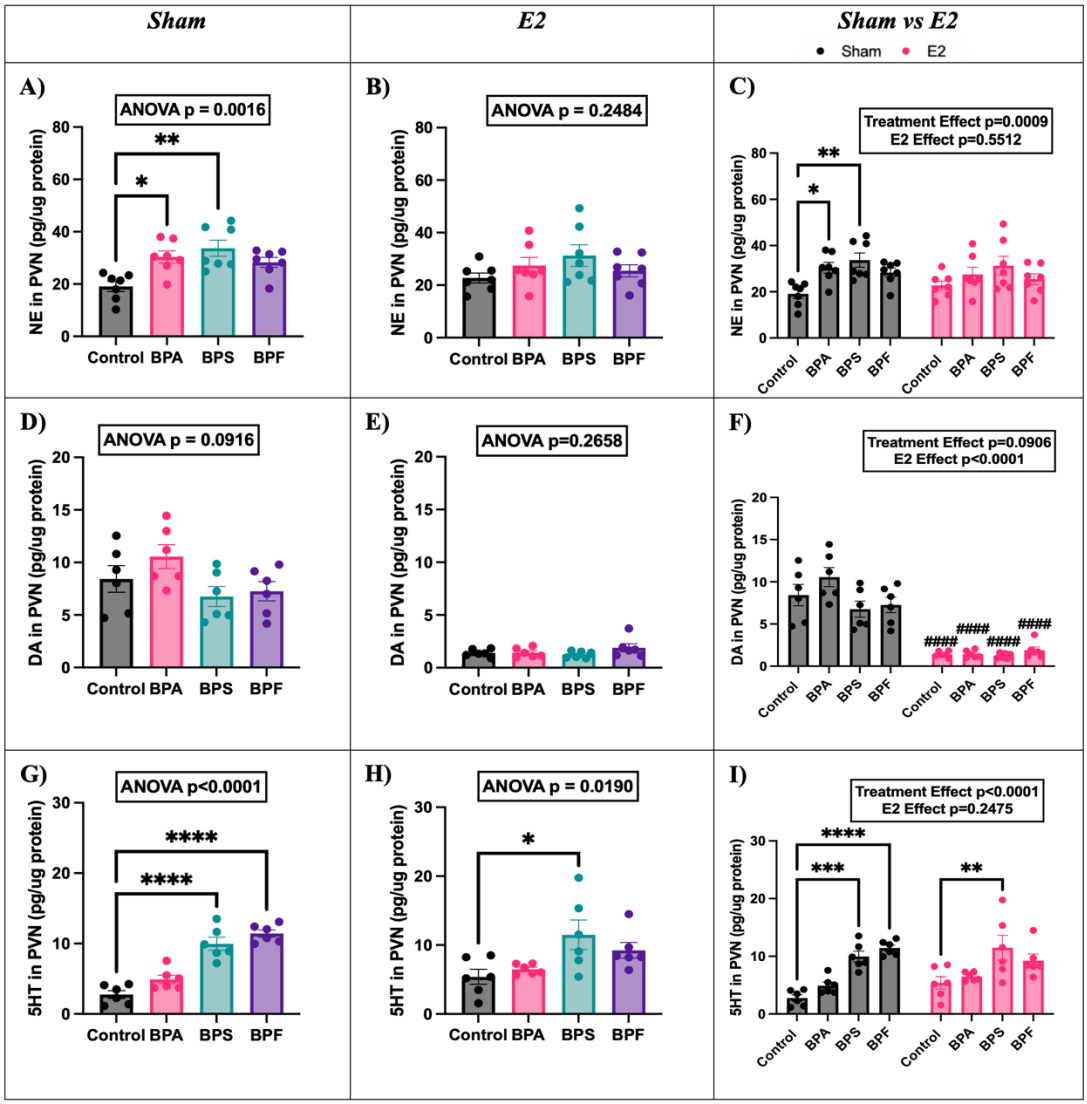

Fig. 3.3. Effects of prenatal exposure to Bisphenol-A and its analogs followed by exposure to exogenous estradiol in adulthood on females' monoamines levels in the paraventricular nucleus. NE (A-C), DA (D-F), and 5HT (G-I) are depicted. "*" Indicates $p<0.05$, "**" $p<0.01$, "***" $p<0.001$, "****" $p<0.0001$ compared to the corresponding controls of the same implant. "####" indicates $p<0.0001$ estradiol treatment effect compared to the corresponding treatment with the sham implant. Data were analyzed by two-way ANOVA and Tukey's or Šídák multiple comparison test. The error bars represent the standard error of the mean (SEM).

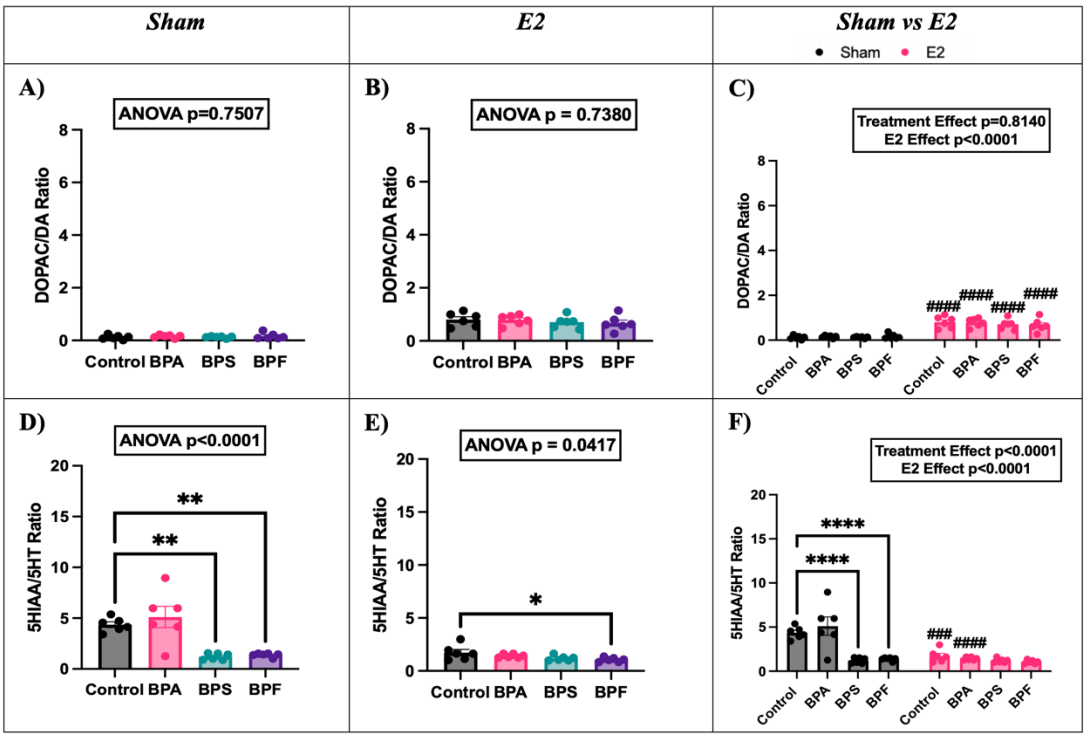

Fig.3.4. Effects of prenatal exposure to Bisphenol-A and its analogs followed by exposure to exogenous estradiol in adulthood on females' monoamines turnover ratio levels in the paraventricular nucleus. DOPAC/DA (A-C), and 5HIAA/5HT (D-F) are depicted. "*" Indicates $p<0.05$, "**" $p<0.01$, "****" $p<0.0001$ compared to the corresponding controls of the same implant. "###" $p<0.001$, and "####" indicates $p<0.0001$ estradiol treatment effect compared to the corresponding treatment with the sham implant. Data were analyzed by two-way ANOVA and Tukey's or Šídák multiple comparison test. The error bars represent the standard error of the mean (SEM).

3.3. Hormonal Levels

Circulating levels of Ang II, Aldo, CORT, and E2 are depicted in Figure 3.5, n=5-7 in each group.

Ang II levels (pg/ml; Mean ± SEM) are shown in Fig 3.5A-C. Sham-implanted female offspring had a significant ANOVA effect in circulating Ang II levels ($p=0.0022$) and in chronic adulthood E2 exposure ($p=0.0036$). While BPA, BPS, and BPF had increased Ang II levels (52.5 ± 5.9, 86.5 ± 12.2, and 58.8 ± 3.8; respectively) compared to control (39.5 ± 6.4), only BPS sham implanted females showed significance ($p<0.01$) as shown in Fig 5A. E2-treated offspring showed a similar pattern in Fig 5B, with BPS showing significantly higher levels of Ang II (72.0 ± 9.4, $p<0.01$) compared to their control counterparts (36.1 ± 3.2). Two-way ANOVA showed a significant EDC exposure effect ($p<0.0001$) consistent with one-way ANOVA but no E2 treatment effect (0.1717). Chronic E2 treatment slightly reduced Ang II levels but was not statistically significant and BPS-treated offspring had elevated levels of Ang II independent of E2 treatment.

Aldo levels (pg/ml; Mean ± SEM) are depicted in Fig 3.5D-F, chronic E2 treatment had a significant effect on circulating Aldo levels ($p=0.0002$) but no EDC treatment effect ($p=0.3460$) as shown in Fig 5F. There was an upward pattern of increase in BPA (152.8 ± 18.8), BPS (152.8), and BPF (306.3 ± 63.6) compared to control-E2 offspring (174.3 ± 25.1), but they were not statistically significant. While in sham-implanted offspring, BPF females had the lowest Aldo levels (92.4 ± 19.7) followed by BPS (140.6 ± 20.5) and BPA (167.4 ± 13.5) compared to their control counterparts (129.4 ± 5.1), they were not statistically significant either. E2-implanted BPF females had significantly higher Aldo levels than sham ($p<0.001$).

As for CORT (ng/ml; Mean ± SEM) which is shown in Fig 3.5G-I, there was significant EDC treatment ($p=0.0100$) and chronic E2 exposure effect ($p=0.0006$) as shown in Fig 5I. There

is a trend of increased levels in sham-implanted offspring with the lowest in BPA (238.2 ± 19.4) followed by BPS (248.8 ± 16.9) and highest in BPF (304.2 ± 13.5) compared to their control counterparts (214.9 ± 19.4), but only BPF was a statistically significant increase ($p<0.01$). An opposite trend is observed in these bisphenols when exposed to chronic E2 in adulthood, BPF offspring had the lowest levels (211.4 ± 17.4) significantly lower than sham implanted BPF ($p<0.01$) followed by BPS (213.7 ± 22.6) and BPA (218.1 ± 13.8) compared to their control (176.0 ± 12.2), these increases were not statistically significant. These findings show that E2 treatment is capable of attenuating the elevated CORT levels induced by prenatal exposure to BPF.

Circulating E2 levels (pg/ml; Mean ± SEM) were measured and are shown in Fig 3.5J-L. Females were implanted with an E2 pellet capable of releasing 20 ng of E2 per day, it is expected to observe a slight increase in E2 levels. E2 levels showed a significance in sham implanted offspring ($p=0.0099$, Fig 5J) and in E2-implanted ($p=0.0043$, Fig5K), while two-way ANOVA showed significant EDC effect ($p<0.0001$, Fig5L), there was no significance between sham and E2 treatment ($p=0.4002$, Fig5L). Sham-implanted offspring had significantly higher E2 levels in BPS (168.2 ± 12.8; $p<0.01$) and BPF (153.9 ± 19.9; $p<0.05$) treated offspring compared to their control (103.0 ± 5.4), while BPA offspring had increased levels (141.4 ± 8.5) but was not statistically significant. A similar pattern is observed post-exposure to chronic E2 but only BPS was statistically significant (179.9 ± 10.7; $p<0.05$) compared to their control (132.7 ± 5.4) while BPA E2 levels went down (129.4 +11.4) and BPF maintained its relative levels (151.8 ± 10.1).

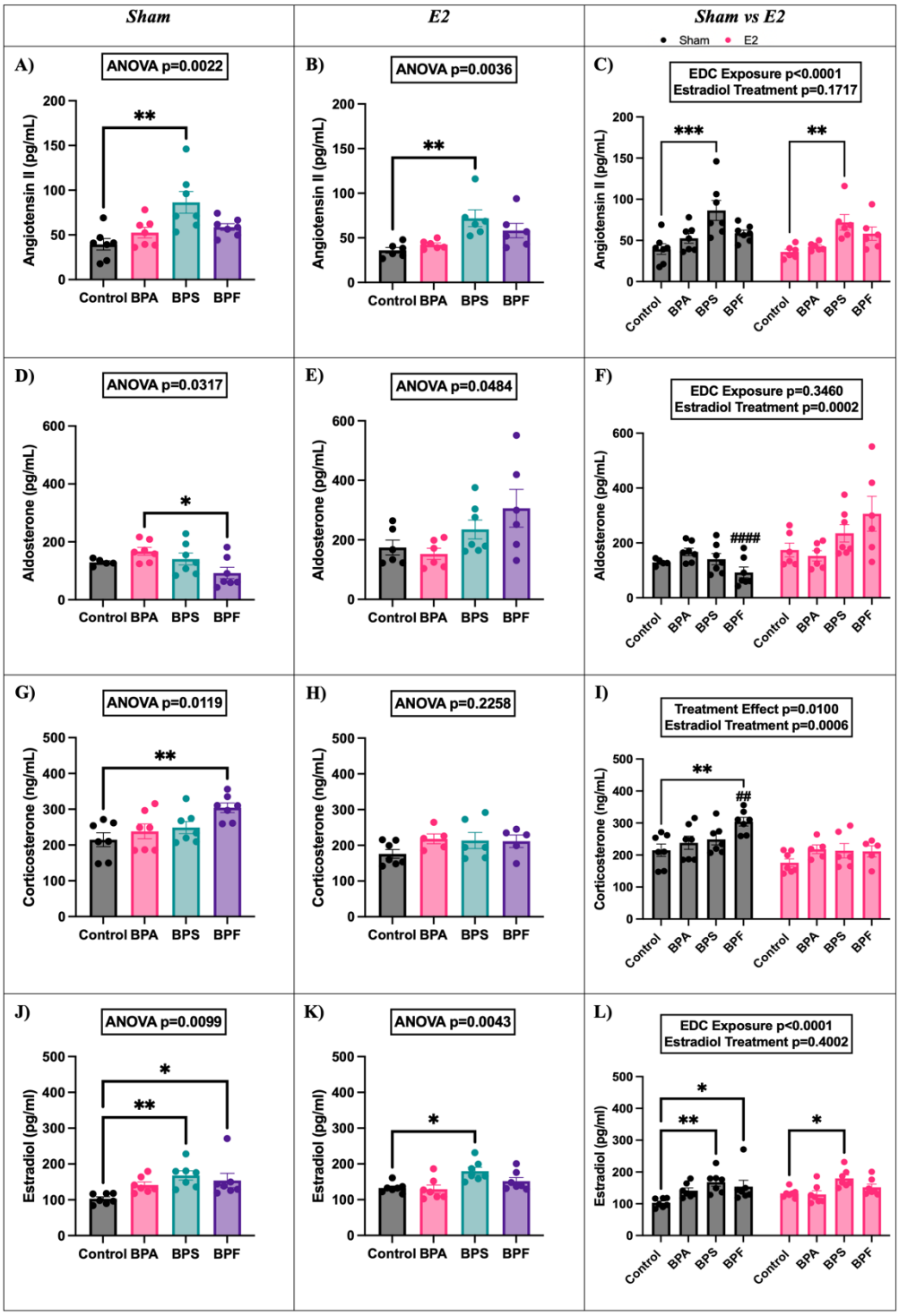

Fig. 3.5. Effects of prenatal exposure to Bisphenol-A and its analogs followed by exposure to exogenous estradiol in adulthood on females' circulating hormone levels. The offspring were prenatally exposed to PBS, 5µg/kg BW/day of BPA, 5µg/kg BW/day of BPS, or 1µg/kg BW/day

of BPF followed by exposure to E2 pellet in adulthood. Ang II (A-C), Aldo (D-F), CORT (G-I), and E2 (J-L) are depicted. "*" Indicates $p<0.05$, "**" $p<0.01$, "***" $p<0.001$ compared to the corresponding controls of the same implant. "##" indicates $p<0.01$, and "###" $p<0.001$ compared to their E2 exposed counterparts. Data were analyzed by two-way ANOVA and Tukey's or Šídák's multiple comparison test. The error bars represent the standard error of the mean (SEM).

3.4. Effects of BPA and its Analogues on Cardiac Muscle

The effects of prenatal exposure to Bisphenol analogues followed by chronic exposure to E2 in adulthood on LV wall and cardiac muscle fibrosis is shown in Fig. 3.6 A and B. LV wall thickness was measured in at least 5 areas of each heart using eSlide manager (mm; mean ± SEM, n=5-8/group) there was a significant ANOVA effect on offspring with E2 treatment ($p=0.0293$) but not EDC exposure ($p=0.0928$). Control offspring exposed to E2 in adulthood had significantly thicker LV wall (3.4 ± 0.04) than control-sham offspring (3.025 ± 0.1). Sham-implanted BPA offspring had a thicker LV wall (3.45 ± 0.1) and to a lower extent in BPS (3.24 ± 0.04) and BPF (3.23 ± 0.1) compared to control but wasn't significant in two-way ANOVA.

%fibrotic area was evaluated to assess collagen deposit in the cardiac muscle (mean ± SEM, n=5-8/group). There was a significant ANOVA effect found in prenatal EDC exposure ($p<0.0001$) and E2 postnatal exposure ($p=0.0318$). Overall, sham and E2 implanted offspring showed similar patterns of increase in fibrosis. In sham-implanted offspring, BPA (1.7 ± 0.1; $p<0.0001$) and BPS (1.5 ± 0.1; $p<0.0001$) had significant increases in % fibrotic area while BPF was the lowest increase (0.9 ± 0.1) compared to their control counterparts (0.6 ± 0.1). As for E2-treated offspring, BPS had the highest increase (2.0 ± 0.3; $p<0.0001$) followed by BPA (1.7 ± 0.2; $p<0.0001$) and BPF (1.2 ± 0.1; $p<0.05$) compared to control-E2 (0.7 ± 0.1).

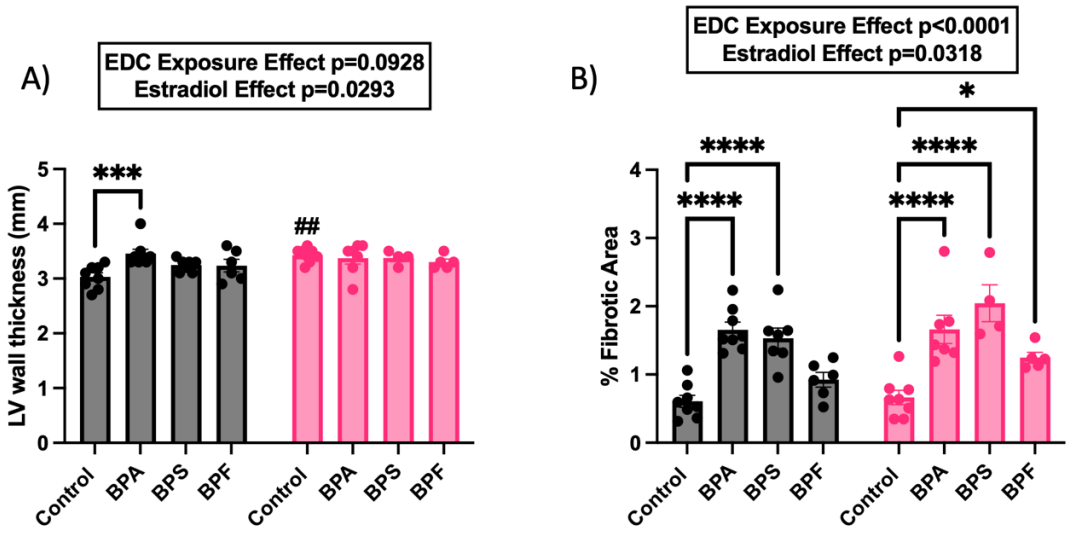

Fig. 3.6. Effects of prenatal exposure to Bisphenol-A and its analogs followed by exposure to exogenous estradiol in adulthood on cardiac tissue. The offspring were prenatally exposed to PBS, 5µg/kg BW/day of BPA, 5µg/kg BW/day of BPS, or 1µg/kg BW/day of BPF followed by exposure to E2 pellet in adulthood. Left ventricle wall thickness (A), and %fibrotic area (B) are depicted. "*" Indicates $p<0.05$, "**" $p<0.01$, "***" $p<0.001$, and "****" $p<0.0001$ compared to the corresponding controls of the same implant. "##" indicates $p<0.01$ compared to their E2 exposed counterparts. Data were analyzed by two-way ANOVA followed by Tukey's or Šídák's multiple comparison test. The error bars represent the standard error of the mean (SEM).

3.5 Effects of Chronic Exposure to Exogenous Estradiol in Adulthood on Females

The summarized exposure effects of estradiol to control females are shown in Fig 3.7 and Table 3.1. All data is analyzed by unpaired two-tailed t-test and data is represented as mean ± SEM. E2 levels were measured to confirm the increase of E2 with E2 implantation ($p<0.01$, Fig 7C). Chronic exposure to E2 increased SBP ($p<0.05$) and LV wall thickness ($P<0.001$). Indicating the

possible determinantal effects of chronic exogenous estrogen on healthy females' cardiovascular health. When the neurotransmitter levels were assessed in key regulatory region in the brain (PVN), DA levels were found to be significantly reduced ($p<0.001$) while the ratio of DOPAC/DA was significantly increased ($p<0.001$) indicating that DA is being converted to DOPAC at a higher extent than control (Fig 7D-E). The opposite effect is observed in the 5HT and 5HIAA/5HT ratio, where there is an increased level of 5HT and significantly reduced level of 5HIAA/5HT ratio ($p<0.0001$) indicating possible increased production of 5HT and reduced reuptake (Table 1). No significant changes were detected in DBP, MAP, HR, or in hormones such as Ang II, Aldo, or CORT or %fibrotic area or NE or 5HT in the PVN (means and p values are summarized in Table 1).

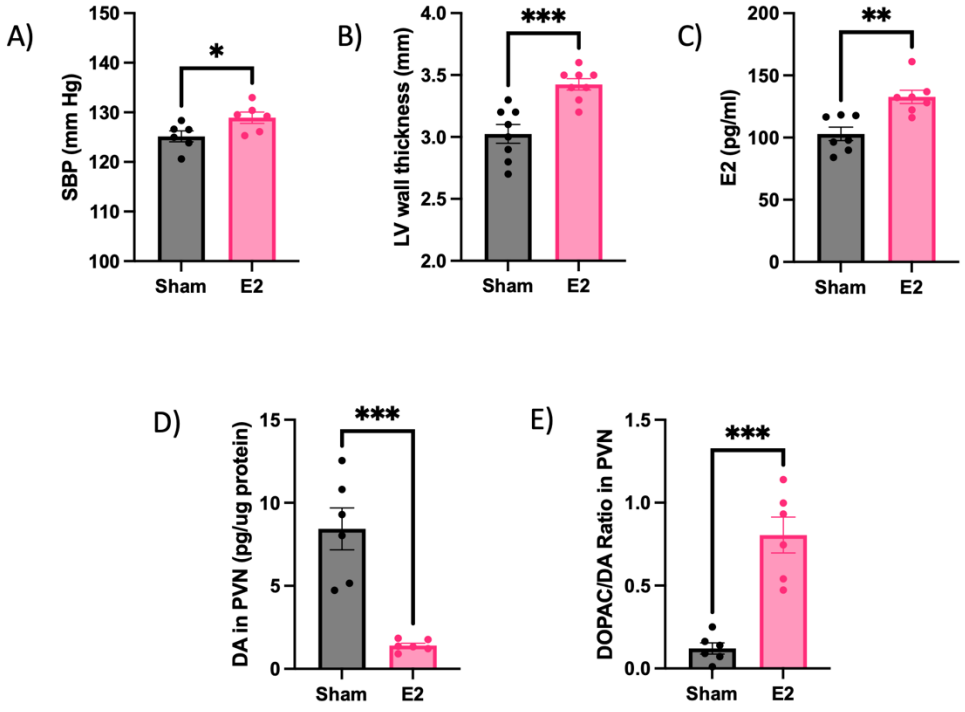

Fig. 3.7. Effects of exposure to exogenous estradiol in adulthood on females. SBP (A), left ventricle wall thickness (B), Estradiol levels (C), and DA (D) and DOPAC/DA ratio in PVN (E)

are depicted. "*" Indicates p<0.05, "**" p<0.01, and "***" p<0.001 between sham and E2. Data were analyzed by unpaired t-test. The error bars represent the standard error of the mean (SEM).

Table 3.1. Effects of exposure to exogenous estradiol in adulthood on neurohumoral factors.

Parameter	Sham	E2	Unpaired t-test p value	p value summary
Blood Pressure				
DBP (mm Hg)	83.7 ± 0.7	85.4 ± 1.3	0.3095	ns
MAP (mm Hg)	102.2 ± 1.0	103.4 ± 0.7	0.325	ns
HR (bpm)	328.6 ± 2.7	330.7 ± 3.2	0.6244	ns
Hormones				
AngII (pg/mL)	39.5 ± 6.4	36.1 ± 3.2	0.6593	ns
CORT (pg/mL)	214.9 ± 19.4	176.0 ± 12.2	0.1156	ns
Aldo (ng/mL)	129.4 ± 5.1	174.3 ± 25.1	0.1451	ns
Cardiac Muscle				
Heart Fibrosis (%fibrotic area)	0.6 ± 0.1	0.7 ± 0.1	0.6805	ns
PVN Neurotransmitters (pg/µl protein)				
NE	19.13 ± 1.9	22.75 ± 1.9	0.2065	ns
5HT	2.8 ± 0.6	5.4 ± 1.1	0.0582	ns
5HIAA/5HT	4.4 ± 0.3	1.7 ± 0.3	<0.0001	****

Note: DBP, diastolic blood pressure, MAP, mean atrial pressure, HR, heart rate, Ang II, angiotensin II, CORT, corticosterone, Aldo, aldosterone, PVN, paraventricular nucleus, NE, norepinephrine, 5HT, serotonin, 5HIAA/5HT, the ratio of 5HIAA metabolite of 5HT to 5HT, E2, estradiol implant capable of releasing 20 ng/day for 90days. Data (N=5-8/group) are presented as mean ± SEM.

3.5. Discussion

The aim of this study was to investigate the potential organizational effects of prenatal exposure to BPA and its analogues, and the activational effects of the subsequent challenge with chronic E2 exposure during adulthood, on the neuro-cardiovascular system. The objective was to determine whether the double hit paradigm would lead to a more detrimental or protective role on blood pressure and neurohumoral factors.

To assess the baseline of the effects of chronic E2 exposure in adulthood. We investigated the effects on SD rats that were prenatally exposed to saline. Our results demonstrated that exposure to E2 alone resulted in notable increases in SBP and LV wall thickness. Also, we observed a decrease in DA levels and an elevation in the DOPAC/DA ratio within the hypothalamus' PVN. These findings align with previous studies conducted in our laboratory, which also reported increased SBP with chronic E2 exposure [117], as well as increased superoxide levels in the rostral ventrolateral medulla (RVLM) [195]. The predominant projection of PVN neurons is towards the RVLM, rather than a direct projection of PVN neurons into the spinal cord [196] which suggests that E2 may exert its influence on SBP by activating the PVN-RVLM neural pathway, which is involved in sympathetic regulation. This potential mechanism suggests that E2-induced sympathoactivation of PVN-RVLM neurons may contribute to the observed increase in SBP. Further investigations are warranted to elucidate the underlying mechanisms and the precise role of the PVN-RVLM pathway in mediating the effects of E2 on blood pressure.

We further assessed the impact of prenatal exposure to BPA and its analogues, followed by chronic E2 exposure at a dose of 20ng/day in adulthood, on the cardiovascular system. This

comprehensive investigation aimed to elucidate the potential interactions and combined effects of these exposures on cardiovascular parameters.

Sham-implanted offspring exposed prenatally to BPA exhibited elevated levels of SBP, DBP, and MAP. This was accompanied by increased NE levels in the PVN, LV wall thickness, and cardiac fibrosis. Conversely, offspring exposed to both prenatal BPA and adulthood E2 showed similar blood pressure levels and LV wall thickness as sham-implanted offspring and did not significantly differ from control offspring exposed to E2 alone. However, like sham-implanted BPA offspring, these individuals displayed increased heart fibrosis. Notably, no significant differences in fibrosis were observed between sham and E2-treated offspring. In terms of neurotransmitters, the PVN analysis revealed decreased DA levels and an increased DOPAC/DA ratio in E2 offspring, along with a decreased 5HIAA/5HT ratio. Numerous studies have contributed to our understanding of the effects of BPA on blood pressure regulation and cardiac health. BPA has been shown to have dose-dependent effects on SBP and DBP, with some studies reporting an increase in blood pressure with BPA exposure [13, 14, 110, 113-115, 128]. Interestingly, there are also findings indicating that BPA can lower SBP at higher doses, while exposure to ethinyl estradiol leads to dose-dependent blood pressure elevation [118]. These results highlight the complexity of BPA's effects on cardiovascular parameters and the importance of considering dose and other factors. In addition to blood pressure, BPA has been associated with structural changes in the heart. Consistent with our findings, previous research has demonstrated that BPA can increase LV wall thickness and fibrosis in female animals [118]. The impact of BPA on LV mass, wall thickness, and collagen content suggests a potential role in altering the cardiac structure and function [121, 128]. Furthermore, prenatal exposure to BPA has been demonstrated to affect calcium handling and reuptake in cardiac tissue, providing a potential mechanistic

explanation for its impact on cardiovascular health. Calcium is vital for the proper contraction and relaxation of cardiac muscles, and any deviations in calcium handling can disrupt contractility and vascular tone. Multiple studies have documented the link between BPA exposure and alterations in calcium handling and reuptake, supporting the hypothesis that BPA-induced disturbances in calcium homeostasis contribute to its effects on the cardiovascular system [13, 120, 121]. Our study adds to the existing body of literature by providing evidence that BPA's effects on blood pressure may be mediated through the activation of PVN neurons, as indicated by the observed increase in NE levels. The PVN is an important brain region involved in the regulation of sympathetic outflow and cardiovascular function. The activation of PVN neurons can lead to increased sympathetic activity, vasoconstriction, and subsequent changes in blood pressure. The link between BPA, PVN activation, and NE release suggests a potential mechanism through which BPA influences blood pressure regulation.

BPS, an analog of BPA, has gained attention due to its widespread use as a BPA substitute in various consumer products. While the effects of BPA on cardiovascular health have been extensively studied, the specific impacts of BPS on the cardiovascular system are still relatively unknown. We observed more adverse effects in BPS offspring compared to BPA exposure. Sham-implanted offspring showed significant increases in SBP, DBP, MAP, and HR. Prenatal exposure to BPS resulted in elevated circulating levels of Ang II and E2, and increased neurotransmitter levels in the PVN, including NE and 5HT. Analysis of the serotonin to 5HIAA ratio indicated a decrease in the conversion rate, suggesting a slower breakdown of 5HT compared to controls. Furthermore, BPS exposure led to increased levels of fibrosis in the heart muscle. Surprisingly, when BPS exposure was combined with E2 exposure in adulthood, the effects were similar to those observed in sham offspring. This suggests that BPS effects are not affected or amplified by

E2, or that the additional negative impact of E2 is not as detrimental as the effects of BPS on the cardiovascular system. Offspring exposed to chronic E2 also exhibited increased SBP, DBP, and MAP, along with elevated levels of 5HT, while NE levels remained similar to sham, as did 5HT turnover. Additionally, chronic E2 exposure resulted in increased Ang II and E2 levels and demonstrated increased fibrosis in the heart. The only notable effect of the combination treatment was a decrease in DA levels and an increased DOPAC/DA ratio compared to controls.

The role of DA in blood pressure regulation remains uncertain, but it is possible that reduced DA levels may contribute to the maintenance and enhancement of the blood pressure increase induced by Ang II through their impact on renal function [197]. To the best of our knowledge, our research group is the first to demonstrate the effects of prenatal exposure to BPS on offspring's blood pressure parameters. Previous studies have provided some insight into the cardiac effects of BPS exposure. Gao et al. (2015) reported that exposure to excised adult female hearts and ventricular myocytes resulted in an increased heart rate [129]. While Ferguson et al. (2019) demonstrated that perfusion of BPS in CD1 mice hearts led to a decrease in left ventricular systolic pressure [40]. Additionally, a study conducted on zebrafish embryos showed that BPS exposure caused a reduction in heart rate and induced cardiac hypertrophy [132] Furthermore, studies have suggested the potential of BPS to disrupt calcium handling, which may contribute to cardiac arrhythmias [40, 129]. Importantly, our study represents the first investigation to shed light on the effects of prenatal BPS exposure on the sympathoactivation and hormonal effects of the PVN, contributing to a deeper understanding of the cardiovascular consequences associated with BPS exposure.

Compared to BPS, offspring exposed to BPF exhibited fewer adverse effects on the Neurocardiovascular system. However, these effects were observed at a dose five times lower than that of BPS. Like BPS-exposed offspring, sham-implanted females demonstrated increased DBP,

elevated levels of 5HT, and a decreased 5HIAA/5HT ratio. BPF offspring also displayed increased circulating levels of CORT and E2, along with elevated fibrosis in the heart. In the case of combined exposure, BPF offspring exposed to E2 in adulthood exhibited blood pressure levels similar to sham-implanted offspring but not significantly higher than the control group exposed to E2 alone. These offspring showed a slight increase in 5HT, a significant decrease in the 5HIAA/5HT ratio, and an increase in heart fibrosis. Compared to sham-implanted offspring, E2-implanted BPF offspring demonstrated a significant reduction in DA levels and an increased DOPAC/DA ratio, along with elevated Aldo levels.

To the best of our knowledge, our study is the first to report the effects of prenatal exposure to BPF on offspring's blood pressure and neurohumoral factors. Previous studies conducted on zebrafish embryos found that BPF exposure resulted in a decreased heart rate and downregulation of cardiac development genes [134]. Additionally, a study on cultured cardiomyocytes demonstrated the effects of BPF on cardiac muscle, including induced hypertrophy and increased calcium levels [43]. However, there is limited research available to fully understand the effects of BPF exposure. Hence, our study contributes significant insights by suggesting that prenatal exposure to BPF may alter cardiovascular function and stress response in offspring.

3.6. Conclusion

The available evidence, including our own study, highlights the intricate and multifaceted effects of BPA and its analogues on blood pressure and cardiac health. Among the studied compounds, BPS has the most detrimental impact on the neuro-cardiovascular system, followed by BPA and BPF. BPS exposure increased blood pressure, potentially through involving the renin-angiotensin system and sympathoactivation of the PVN. BPA-induced hypertension may be

mediated by sympathoactivation of the PVN. In the case of BPF, the observed increase in DBP could be influenced by 5HT levels within the PVN and circulating CORT levels. Furthermore, BPA and its analogues have been shown to induce cardiac fibrosis, indicating the need for further exploration into the underlying mechanisms involved in fibrotic processes. Additionally, investigating the effects of these bisphenols on kidney function is crucial, as disturbances in renal function may contribute to their impact on blood pressure regulation. Based on these findings, further research is warranted to fully elucidate the intricate mechanisms through which BPA and its analogues exert their effects on the cardiovascular system. Understanding these mechanisms will provide valuable insights for the development of targeted interventions and the promotion of cardiovascular health.

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CHAPTER 4

Prenatal Exposure to BPA, BPS, and BPF and postnatal exposure to High-Fat Diet: Impacts on Neurohumoral Parameters in Adult Male Sprague Dawley Rats

Al Mansi et al. to be submitted to *Chemosphere.*

4.1. Abstract:

High-fat diet is linked to increased blood pressure levels and a higher risk of cardiovascular conditions like stroke and coronary artery disease. Cardiovascular diseases (CVDs) are the leading cause of mortality globally, with hypertension as a significant risk factor. Environmental contaminants have been associated with hypertension, yet they're often overlooked. The cardiovascular effects of BPA analogues Bisphenol S (BPS), and Bisphenol (BPF) are not well understood, especially regarding prenatal exposure and interaction with a high-fat diet. These analogues are commonly found in BPA-free products, raising concerns about their impact on cardiovascular health. The primary objective of this study was to assess the organizational and activational effects of BPA and its analogues in combination with a high-fat diet, on the neuro-cardiovascular system of male offspring. To achieve this, pregnant Sprague-Dawley (SD) rats were exposed to 5 µg/kg BW of BPA or BPS, or 1 µg/kg BW of BPF during the prenatal period. The adult male offspring were surgically implanted with radiotelemetry devices to enable continuous monitoring of blood pressure and heart rate. Subsequently, the offspring were subjected to a high-fat diet for a duration of two weeks. After the data collection period, the animals were sacrificed, and plasma levels of aldosterone (Aldo), corticosterone (CORT), and angiotensin II (Ang II) were measured using a radioimmunoassay (RIA). Furthermore, neurotransmitter levels in the paraventricular nucleus (PVN) were analyzed using high-performance liquid chromatography with electrochemical detection (HPLC-EC). Additionally, the extent of fibrosis in the heart and the thickness of the left ventricle (LV) wall were measured. Prenatal exposure to BPA, BPS, or BPF as well as exposure to a high-fat diet in adulthood, resulted in elevated blood pressure levels in all offspring. In BPA-exposed offspring, the increase in blood pressure was accompanied by elevated serotonin (5HT) levels in the PVN and increased CORT levels. Similarly, BPS-exposed offspring exhibited increased blood pressure along with PVN sympathoactivation and elevated CORT and Ang II levels. In the case of BPF, blood pressure elevation was accompanied by PVN sympathoactivation and increased levels of Ang II, Aldo, and CORT. When the EDC-exposed offspring were subjected to a high-fat diet, the blood pressure elevation in BPS offspring was primarily attributed to increased levels of Ang II, Aldo, and CORT. On the other hand, blood pressure elevation in BPF offspring was mainly due to PVN sympathoactivation and increased Aldo levels. All EDC-exposed offspring exhibited increased cardiac muscle fibrosis, while BPS and BPF offspring also displayed increased thickness of the LV wall.

Keywords: Bisphenols, Endocrine Disrupting Chemical (EDC), high-fat diet, hypertension.

4.2 Introduction:

The prevalence of obesity and metabolic complications has been steadily rising in recent years, driven by numerous factors including poor dietary habits, sedentary behavior, and exposure to environmental pollutants [111, 198]. Hypertension (HTN) is strongly associated with these conditions and is influenced by age, gender, genetics, lifestyle choices, and environmental factors [90]. Endocrine-disrupting chemicals (EDCs) have been implicated in cardiovascular diseases (CVDs) and HTN, highlighting their impact on human health [37, 103, 106]. Dietary interventions, such as the Dietary Approaches to Stop Hypertension (DASH) diet, have demonstrated effectiveness in lowering blood pressure and reducing the risk of HTN [126].

Recent studies have shed light on the strong correlation between obesity, metabolic dysfunction, and the development of severe comorbidities and premature mortality [177, 199]. Of the emerging contributing factors is exposure to environmental pollutants, particularly EDCs [146, 177]. Bisphenol A (BPA), a prominent EDC found in many food sources, can be absorbed through various routes and has been linked to elevated blood pressure and the development of CVDs[102, 200]. Concerningly, BPA substitutes like bisphenol F (BPF) or bisphenol S (BPS), which are being used as alternatives due to regulatory restrictions on BPA, have also shown adverse health effects, indicating that these alternatives may not be safer [31, 40, 43, 106, 131, 201].

Notably, exposure to EDCs during the fetal developmental stage, known as the "organizational" period, has been found to have detrimental effects on offspring health. EDCs can cross the placental barrier and are detected in amniotic fluid, fetal urine, and plasma [18, 98, 202]. Such exposure during this critical period can lead to irreversible adverse outcomes due to the limited protective mechanisms present in the developing fetus [54, 58].

Key studies investigating the effects of BPA exposure and/or poor diet have revealed significant associations between BPA exposure and increased body weight, blood pressure, and cardiovascular disease in offspring across multiple generations [53, 127, 128, 203]. One study by Liu et al. 2022, found that perinatal exposure to 500 μg/kg/day BPA and exposure to a high-fat diet to the F1 generation could induce detrimental cardiovascular effects such as increased blood pressure, myocardial hypertrophy, and increased cross-sectional area of cardiomyocytes [127]. Other studies conducted in malnourished mice [128] and adult female Suffolk ewes [53] have also reported concerning findings regarding blood pressure, heart disease, and alterations in gene expression, highlighting the serious health consequences associated with BPA and poor diet. In a separate study conducted by Hsu et al. in 2019, the effects of perinatal BPA exposure and a high-fat diet revealed that BPA exposure led to an increase in blood pressure and when combined with a high-fat diet, the detrimental impact on adult male blood pressure was further intensified. However, prenatal treatment with resveratrol was found to mitigate these effects, suggesting a potential protective role of resveratrol against the adverse cardiovascular consequences of BPA exposure and a high-fat diet [203]. To our knowledge, there are currently no studies that have investigated prenatal exposure to BPS or BPF and a high-fat diet on cardiovascular function. However, there are recent studies that have investigated the effects of combined exposure to BPA analogues and diet on lipid and glucose metabolism [201, 204, 205].

This study's objective was to assess the organizational effects of prenatal exposure to BPA and its analogues and the activational effects of challenge with the second insult of a high-fat diet in adulthood on male offspring's neurohumoral parameters. We hypothesized that prenatal exposure to BPS or BPF would cause more detrimental effects on the cardiovascular system than BPA and the activational effects of a high-fat diet exposure in adulthood would exacerbate those effects.

To evaluate this, pregnant Sprague-Dawley (SD) rats were administered BPA, BPS, or BPF orally during gestational days (GD) 6-21. Telemeters were implanted in the adult male offspring for blood pressure and heart rate monitoring. Following a baseline period, the offspring were subjected to a high-fat diet for two weeks. In addition to evaluating blood pressure levels, the study sought to elucidate the impact of BPA and its analogues on neurotransmitter levels in key hypothalamic cardiovascular control area, the paraventricular nucleus (PVN), such as Norepinephrine (NE), Serotonin (5HT), Dopamine (DA) and their metabolites. Furthermore, circulating hormones, including Angiotensin II (Ang II), Aldosterone (Aldo), and Corticosterone (CORT), which are known to play crucial roles in the regulation of blood pressure through various physiological pathways, including the renin-angiotensin-aldosterone system (RAAS) were assessed. Additionally, the investigation encompassed the assessment of left ventricle (LV) wall thickness and the presence of heart fibrosis, providing insights into the potential effects of BPA and its analogues on cardiac muscle structure and function.

4.3. Materials and Methods

2.1. Experimental Animals and Treatment

Sprague Dawley rats, both adult females and males at 3 months of age, were sourced from Envigo (Indianapolis, IN) and housed in controlled animal facilities. The housing conditions included a 12:12 light-dark cycle, a temperature of 23 ± 2 °C, and a relative humidity of 50 ± 20%. The rats were provided with unrestricted access to PicoLab-LabDiet 5053 food and water. All animal-related procedures were performed in accordance with the guidelines set by the University of Georgia Institutional Animal Care and Use Committee (IACUC) and complied with the NIH guidelines for the ethical treatment and use of laboratory animals. Females were set for breeding

for the experiment, pregnant dams were randomly assigned to one of four treatment groups: Control (administered 10 µl Phosphate Buffered Saline (PBS), n=9), Bisphenol A (5 µg/kg BW/day, n=6), Bisphenol S (5 µg/kg BW/day, n=13), and Bisphenol F (1 µg/kg BW/day, n=10), as previously described [34]. BPF dose was selected to be lower than BPA and BPS due to high abortion rates at 5 µg/kg BW/day [34]. The dams were dosed by oral gavage daily based on their body weight from gestation day (GD) 6-21. Following this, offspring were weaned at three weeks of age and group-housed until further experimentation as shown in Figure 1. The selected dose of EDC was lower than the current Environmental Protection Agency (EPA) recommended no-observed-adverse-effect-level (NOAEL)(BPA;5 mg/kg bw/day, BPS; 10 mg/kg/day, BPF; not established)(EPA 2012, 2014).

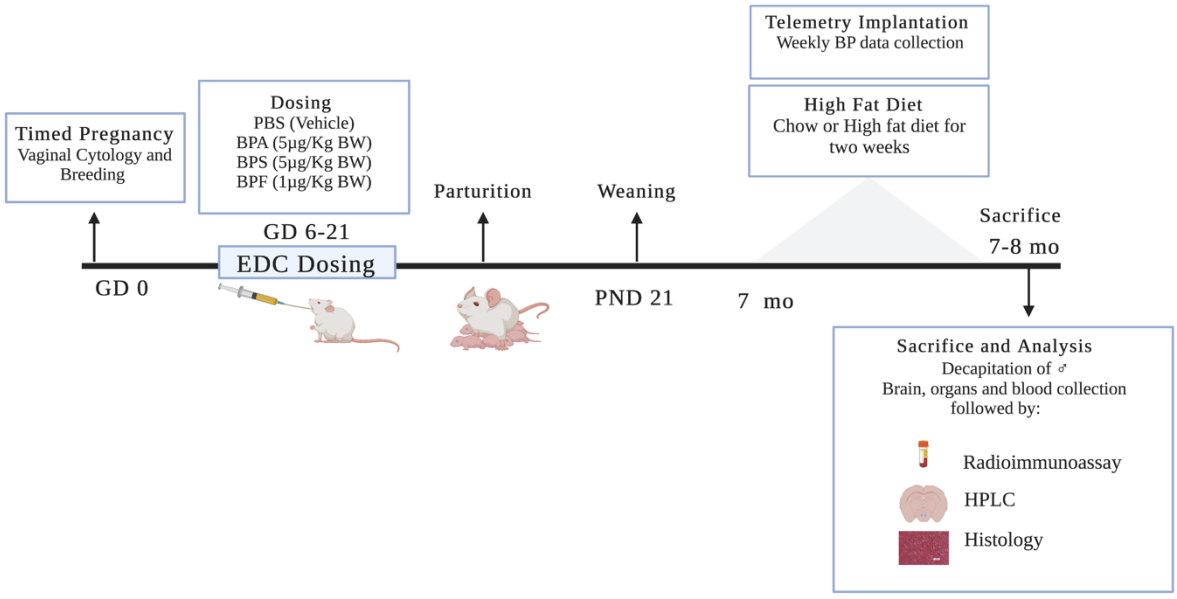

Fig. 4.1. Overall study scheme. Pregnant SD rats were exposed to Control (10µl Phosphate Buffered Saline (PBS), *n*=9), Bisphenol A (5µg/kg BW/day, *n*=6), Bisphenol S (5µg/kg BW/day, *n*=13), and Bisphenol F (1µg/kg BW/day, *n*=10) from GD 6-21 by oral gavage. The offspring were weaned at PND 21 and at 7 months of age they were implanted with a radiotelemetry device, after

one-week animals were given a high-fat diet for two weeks. Blood pressure and heart rate were monitored continuously for 24hrs every week for three weeks, after which the animals were sacrificed. The brain, trunk blood, and hearts were collected for the measurement of neurotransmitters, hormones, and histology, respectively. Created with BioRender.com

2.2. Radiotelemetry Implantation Surgery and Hemodynamic Measurements

Male offspring were allowed to reach adulthood and then underwent surgical implantation of a radiotelemetry device (Data Sciences International; HD-S10) in the femoral artery as previously described [147, 148]. Briefly, the animals were anesthetized with isoflurane, and aseptic techniques were used to prepare the ventral abdomen and inside of the left thigh. Prophylactic doses of Meloxicam (1 mg/kg BW) and Enrofloxacin (5 mg/kg BW) were administered subcutaneously. A 2 cm incision was made in the left groin to expose the femoral artery, and the surrounding tissues were bluntly dissected to visualize the artery, vein, and nerve. Ligatures were placed to restrict blood flow, and a subcutaneous pocket was created in the abdominal region to hold the transmitter. To prevent arterial wall contraction, lidocaine was applied to the femoral artery, followed by the creation of a hole in the arterial wall using fine scissors under a dissection microscope. The hole was enlarged using Dumont vessel cannulation forceps, and the transmitter catheter was then inserted into the femoral artery to a depth of up to 2 cm. The transmitter catheter was inserted into the femoral artery and secured with ligatures. The incision was closed using sutures, and the functioning of the transmitter was monitored using a wireless radio. The animals were monitored closely for seven days and received daily doses of Meloxicam for 3 days post-surgery.

Post recovery, the transmitter was turned on and off using a magnet, and data were collected at 10 min intervals over a 24-hr every week for three weeks. Systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), and heart rate (HR) were measured. Animals were sacrificed by rapid decapitation. The brains were harvested, and the trunk blood was centrifuged to extract the plasma. All tissues were stored at -80 ° C until further analysis.

2.3. Experimental Dietary Paradigm

After the implantation of telemetry devices, the animals underwent a recovery period and their initial blood pressure measurements were recorded while they were on a standard chow diet. The chow diet consisted of 13% fat, 24% proteins, and 62% carbohydrates (Pico Lab Rodent Diet 20; LabDiet). Subsequently, the animals were subjected to the second insult of the experimental paradigm, which involved a high-fat diet. This high-fat diet has an energy density of 4.7 Kcal/g, containing 45% kcal from fat, 20% kcal from proteins, and 35% kcal from carbohydrates. (Formulated by E. A. Ulman, Ph.D., Research Diets, Inc; Product #D12451; Fisher Scientific) ad libitum. Blood pressure monitoring was conducted for a two-week duration under the high-fat diet until the animals were sacrificed as illustrated in Figure 1.

2.4. Hormone Assays

Plasma concentrations of corticosterone (CORT), angiotensin II (Ang II), and aldosterone (Aldo) were quantified using a double antibody radioimmunoassay, following the guidelines provided by the manufacturer. The following assays were used: CORT: Catalog #07120102 (MP Biomedicals, Irvine, CA) Ang II: Catalog # 07120102 (Phoenix Pharmaceuticals, Inc., Burlingame, CA) Aldosterone: Catalog # MG13051 (IBL International GmbH, Hamburg,

Germany). The samples were added in duplicate and analyzed according to the assay protocol. Values were expressed as ng/ml for CORT and pg/ml for Ang II and Aldo (%CV <10).

2.5. Brain Sectioning and Microdissection

Brain sections with a thickness of 300 μm were obtained using a cryostat (Slee, London, UK) maintained at -10°C. The paraventricular nucleus (PVN) was then microdissected from the brain sections using the Palkovits procedure on a cold stage, with guidance from the Rat Brain Atlas - the Rat Brain in Stereotaxic Coordinates – 7th Edition as a reference [149]. The microdissected tissue, which encompassed all subdivisions of the PVN, was subsequently stored at -80°C for further analysis.

2.6. Neurotransmitter Analysis by HPLC-EC

The PVN samples were analyzed for norepinephrine (NE), dopamine (DA), and serotonin (5HT) levels using HPLC-EC (high-performance liquid chromatography with electrochemical detection) following a previously described method [150]. Briefly, brain punches were homogenized in 0.05 M perchloric acid, and a portion of the homogenate was used for protein estimation using the Pierce MicroBCA assay (Rockford, IL). The remaining homogenate was centrifuged, and the supernatant was injected with an internal standard (dihydroxybenzylamine, 0.05 M) into the HPLC-EC system. The HPLC-EC system is comprised of a 5-μm ODS reverse phase C-18 column (Phenomenex, Torrance, CA), a SIL-20AC autoinjector, a CTO-20AC column oven (Shimadzu, Columbia, MD) maintained at 37°C, and an LC-4C detector (Bioanalytical Systems, West Lafayette, IN). The mobile phase flow rate was maintained at 1.8 ml/min using Shimadzu's LC-20AD pump (Columbia, MD). The chromatograms were analyzed using

Shimadzu's Class VP software v 7.2. The neurotransmitter concentrations in tissue samples were expressed as pg/µg of protein. Protein levels in tissue punches were measured using the MicroBCA assay, and the absorbance was measured using the ELX 800 microplate reader.

2.7. Histology of heart tissues.

The hearts were collected and fixed in formalin, and sections were obtained by cutting from the apex and subjected to standard paraffin embedding and sectioning procedures. These sections were stained with Masson's trichrome and digitally scanned using an Aperio scanner (Leica Biosystems, Wetzlar, Germany). The scanned images were viewed and analyzed using the eSlide Viewer software. The thickness of the left ventricle wall was measured at five different locations using the eSlide Viewer. The extent of heart fibrosis was quantified using ImageJ software following the methodology described by Kennedy et al., 2006 [151].

2.8 Statistical Analysis

Prism 9.5.1 software (GraphPad, Inc., San Diego, CA) software was used to perform statistical analysis. Two-way ANOVA was used followed by Tukey's multiple comparison test or Holm-Šídák test for EDC or diet effect, respectively. It was used to detect EDC and high fat diet alterations to blood pressure, hormones, fibrosis, and neurotransmitter measurements. One-way ANOVA was used followed by Tukey's multiple comparison test to assess EDC treatment effect on neurotransmitter levels in the PVN. An unpaired student two-tailed t-test was employed to assess the effects of a chow versus high-fat diet on control animals. A p-value <0.05 was considered statistically significant. Data were expressed as mean ± standard error of the mean (SEM).

4.4. Results

3.1. BPA and its analogues increase blood pressure in male offspring in a chemical and diet specific manner.

Figure 4.2 displays the weekly and mean telemetry data (mean ± SEM) for SBP, DBP, MAP, and HR for chow and high-fat diet-exposed males.

Panel A represents weekly SBP, and panel B represents mean SBP diet differences between chow and high-fat exposed offspring. There was a significant EDC treatment and diet effect ($p<0.0001$ and $p=0.0004$; respectively). In chow offspring, BPA, BPS, and BPF prenatally exposed offspring had significantly higher SBP (145.5±1.8, 152.4±4.5, and 146.3±1.3; respectively, $p<0.0001$) than their control counterparts (119.3±3.2). A similar pattern is observed in high-fat-exposed offspring, with significantly higher levels in BPA, BPS, and BPF (149.9±2.4, 157.2±5.1, and 150.3±1.7; respectively, $p<0.05$) compared to their controls (138.5±2.3) and BPS having the highest levels ($p<0.001$). As for diet-specific differences, controls exposed to a high-fat diet showed significantly higher SBP (138.5±2.3; $p<0.001$) compared to chow offspring (119.3±3.2). However, when prenatally exposed to BPA and its analogues, there is no significance between chow and high-fat diet, possibly indicating that the organizational effects caused by prenatal exposure to BPA and its analogues have caused chronic modifications and saturation in SBP activation. Moreover, there was a 3% elevation in SBP post exposure to high-fat diet in all EDC treatments in comparison to the 16% increase between controls, indicating the possibility that while an increase is not significant, alteration exists, and chronic effects may develop later in life. Overall, Panel A illustrates the pattern of SBP over the three-week monitoring period. Initially, BPA and its analogues displayed elevated levels compared to the control group before the high-fat diet. Following exposure to the high-fat diet, the control group exhibited a significant increase

in SBP by the first week of high-fat diet exposure, whereas the increase in SBP for the BPA and analogue-exposed offspring was minimal. However, by the third week, there was a slight rise in SBP observed in the offspring exposed to EDCs.

As for DBP, represented in panel C&D, there was not a significant EDC or diet effect ($p=0.1667$, and $p=0.5846$; respectively). Panel C represents the weekly changes of DBP, before high-fat diet exposure (week 1) and after exposure (weeks 2 and 3). During the first week, BPS offspring had 4% higher levels of DBP than controls whereas BPA and BPF had similar values to controls. After exposure to the high-fat diet, Control, BPA, and BPS stayed consistent whereas BPF had a 4% increase in DBP compared to control chow. However, in the final week of measurements, BPA, BPS, and BPF had increased levels in DBP (2%, 7%, and 3%; respectively) than high-fat controls of the same week. Indicating the possibility of alterations to DBP following long-term high-fat diet exposure.

MAP is displayed in panels E and F, there was a significant EDC treatment and diet effect ($p<0.0001$, and $p=0.0236$; respectively). In chow-exposed offspring, there was a significant increase in MAP in BPA and BPS-treated offspring (116.5 ± 1.6; $p<0.05$, and 122.7 ± 4.0; $p<0.001$; respectively) compared to controls (107.3 ± 2.1). BPF offspring had an 8% increase in MAP with respect to their control but was not statistically significant. As for high-fat exposed offspring, only offspring exposed prenatally to BPS had a significant increase in MAP (124.9 ± 4.6; $p<0.05$) compared to their control counterparts (114.2 ± 1.5). However, BPA and BPF offspring had a 4-6% increase in MAP but it was not statistically significant. Overall, panel E illustrates the pattern of changes in MAP before and after high-fat diet exposure. In the first week, BPA and BPF offspring had 8-9% higher MAP, while BPS had 14% higher MAP than control offspring. Following a high-fat diet, control offspring MAP levels increase to over 6% higher levels than chow feeding. While

for EDC-exposed offspring we see a more modest increase of 2-4%. With the highest increase was observed in BPF-treated offspring over the two weeks of exposure to high fat. BPS offspring, again show a pattern like SBP and DBP with increased levels from chow treatment and a steady minimal increase with a high-fat diet.

As for HR levels, they're displayed in panels G&H, there was a significant treatment ($p=0.0008$). In chow-exposed offspring, prenatal treatment with BPA and BPS showed significantly higher levels of HR (341.3±5.2, and 343.5±5.6; $p<0.05$) compared to their controls (313.4±12.3). Contrastingly, high-fat diet-treated offspring showed a significant increase in HR only in BPF-treated offspring (353.0±3.2; $p<0.05$) compared to their control (322.4±5.4). There was not a significant diet effect ($p=0.1443$), however, high-fat exposed BPF offspring had slightly higher HR levels (353.0±3.2) than chow diet-fed offspring (334.4±8.3). Panel G provides an overview of the changes in heart rate (HR) patterns. Initially, offspring exposed to BPA, BPS, and BPF exhibited HR levels that were 7-10% higher than the control group. However, after one week of being on a high-fat diet, both the control and BPF-exposed offspring showed an increase in HR. By week 3, the HR levels in the control group returned to normal, while in the BPF-exposed group, HR continued to increase, reaching up to 6% higher than the pre-diet levels. Additionally, at week 3, the HR levels in the BPA, BPS, and BPF groups were 6-9% higher than those of the corresponding control group.

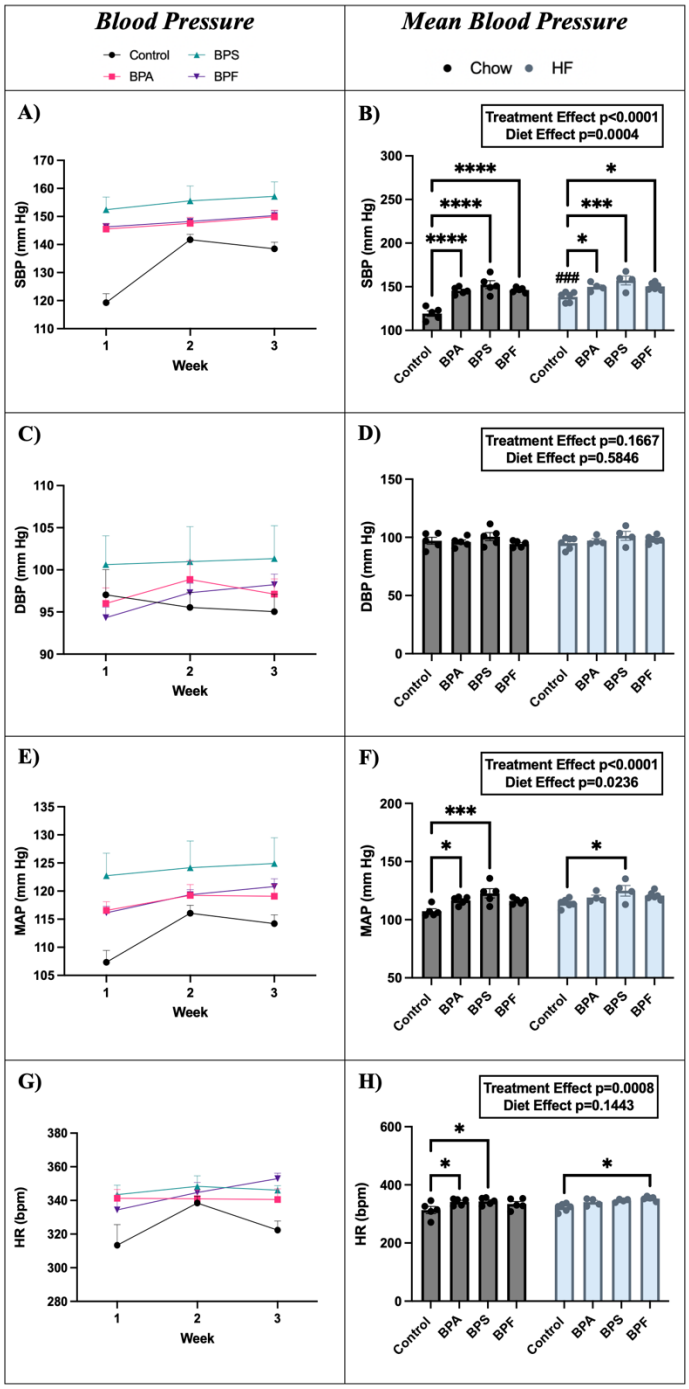

Fig. 4.2. Effects of prenatal exposure to Bisphenol-A, S, or F on male's cardiovascular parameters. The offspring were prenatally exposed to PBS, 5µg/kg BW/day of BPA, 5µg/kg BW/day of BPS or 1µg/kg BW/day of BPF. SBP (A-B), DBP (C-D), MAP (E-F), and HR (G-H) are depicted. Panels A, C, E, and G represent weekly blood pressure or heart rate measurements based on treatment. Week 1 is pre-diet and weeks 2 and 3 with high fat diet exposure. Males exposed to the chow diet are represented with black bars and high-fat diet with blue bars. "*" indicates $p<0.05$, "**" $p<0.01$, "***" $p<0.001$, and "****" $p<0.0001$ compared to the corresponding controls with same diet. "###" $p<0.001$ comparing high fat to chow diet males. Data were analyzed by two-way ANOVA, followed by Tukey's multiple comparison test or Holm-Šídák test for treatment or diet differences, respectively. The error bars represent the standard error of the mean (SEM).

3.2. Neurotransmitter levels in the PVN

Neurotransmitters concentration and monoamine turnover ratio (pg/µl protein; mean ± SEM) in the PVN are depicted in Figures 4.3 and 4, n=6-7 in each group.

NE levels are shown in panels A-C, panel A illustrates significant one-way ANOVA ($p=0.0056$) effects of prenatal exposure to BPA and its analogues followed by exposure to regular diet. BPS and BPF treated offspring had significantly higher levels of NE in PVN (27.2±3.3; $p<0.05$, and 27.9±2.2; $p<0.001$; respectively) than their control counterparts (17.1±1.0). As for BPA offspring, their NE levels were elevated by 41% with respect to the controls but was not statistically significant. Panel B illustrates the significant effects of high fat diet and EDCs exposure on NE levels ($p=0.0016$). Offspring prenatally exposed to BPF followed by adulthood exposure to a high-fat diet had significantly higher NE levels (40.5±4.8; $p<0.01$) than their control counterparts (23.8±2.6). Levels of NE with a high-fat diet were decreased in BPA and BPS offspring but increased by 39-45% in control and BPF compared to chow offspring. Again, similar to blood pressure alterations we see that BPF offspring are not only affected by organizational alterations due to prenatal exposure but also by activational effects due to exposure to a high-fat diet in adulthood.

As for DA levels, which are represented in panels D-F, panel D showed significant one-way ANOVA effects on chow offspring ($p=0.0020$) and panel E, on high-fat exposed offspring ($p=0.0243$). In chow offspring, prenatal exposure to BPA and BPS had significantly higher levels of DA (2.8±0.4; $p<0.01$, and 2.4±0.4; $p<0.05$; respectively) than controls (0.9±0.2). As for BPF, while it had 78% higher levels of DA than control offspring, it was not statistically significant. The opposite pattern is seen in high-fat fed offspring with BPF-treated offspring having significantly higher levels of DA (7.0±1.3; $p<0.05$) than their control counterparts (3.6±0.2). While

BPA and BPS had only a 9-11% increase from high-fat control offspring, which was not statistically significant. Panel F shows a significant diet effect ($p=0.0001$), post hoc analysis shows that BPF offspring had an increase of over 300% post-high-fat diet ($p<0.0001$) compared to controls.

5HT levels are depicted in panels G-I. Two-way ANOVA showed significant EDC treatment and diet effect ($p<0.0001$, and $p=0.0077$; respectively). In chow-treated offspring, prenatal exposure to BPA, BPS, and BPF significantly increased 5HT levels in the PVN (9.8±0.9; $p<0.0001$, 7.8±0.4; $p<0.01$, and 7.4±0.8; $p<0.01$; respectively) compared to their control counterparts (3.7±0.7). The opposite pattern is observed in high-fat offspring, BPF offspring had the highest and only significant increase in 5HT levels (8.3±1.0) compared to their controls (4.2±0.6). As for BPA and BPS high-fed offspring, they had an increase of 16-28% in 5HT levels compared to high-fat controls, but it was not statistically significant. However, a high-fat diet decreased 5HT levels in BPA and BPS offspring, with a significant decrease in BPA (5.4±0.3; $p<0.001$) compared to chow-fed BPA offspring (9.8±0.9).

Monoamine turnover ratios are shown in Figure 4.4. Panels A-C illustrate the effects of chow and a high-fat diet on the 5HIAA/5HT ratio in the PVN. As shown in panel C, two-way ANOVA showed significant EDC treatment ($p=0.0002$) but not diet effect ($p=0.0547$). In chow offspring, BPA, BPS, and BPF offspring had significantly lower levels of 5HIAA/5HT ratio (0.9±0.1; $p<0.0001$, 1.1±0.1; $p<0.0001$, and 1.3±0.1; $p<0.001$; respectively) than their controls (3.3±0.8). Within high-fat offspring, there is no significant EDC effect ($p=0.3486$) as shown in panel B. However, there is a trend of decreasing levels, with BPS lowest and BPA highest compared to high-fat controls (34-7%). There seems to be decreased turnover ratio in EDC-treated offspring and high-fat treatment, with data consistent with 5HT levels in Fig.3 G-I.

Similar findings are observed for the turnover ratio of DOPAC/DA as shown in panels D-F. There is a significant EDC treatment and diet effect ($p=0.0007$ and $p<0.0001$; respectively) as shown in panel F. Chow offspring had a significantly lower ratio in BPA, BPS, and BPF treated offspring (0.3 ± 0.03; $p<0.0001$, 0.4 ± 0.07; $p<0.0001$, and 0.5 ± 0.08; $p<0.001$; respectively) compared to their control counterparts (1.5 ± 0.4). High fat-treated controls had significantly lower levels (0.1 ± 0.02; $p<0.0001$) than chow controls (1.5 ± 0.4), as for BPA, BPS, and BPF they were reduced by 64-72% lower levels than chow EDC treated offspring; respectively; however, that reduction was not statistically significant. While within high-fat exposed offspring, there wasn't any significance ($p=0.5262$) as shown in panel E. Based on these findings, there seems to be an alteration in DA and 5HT reuptake and conversion by monoamine oxidase caused by exposure to BPA and its analogues as well as high-fat diet.

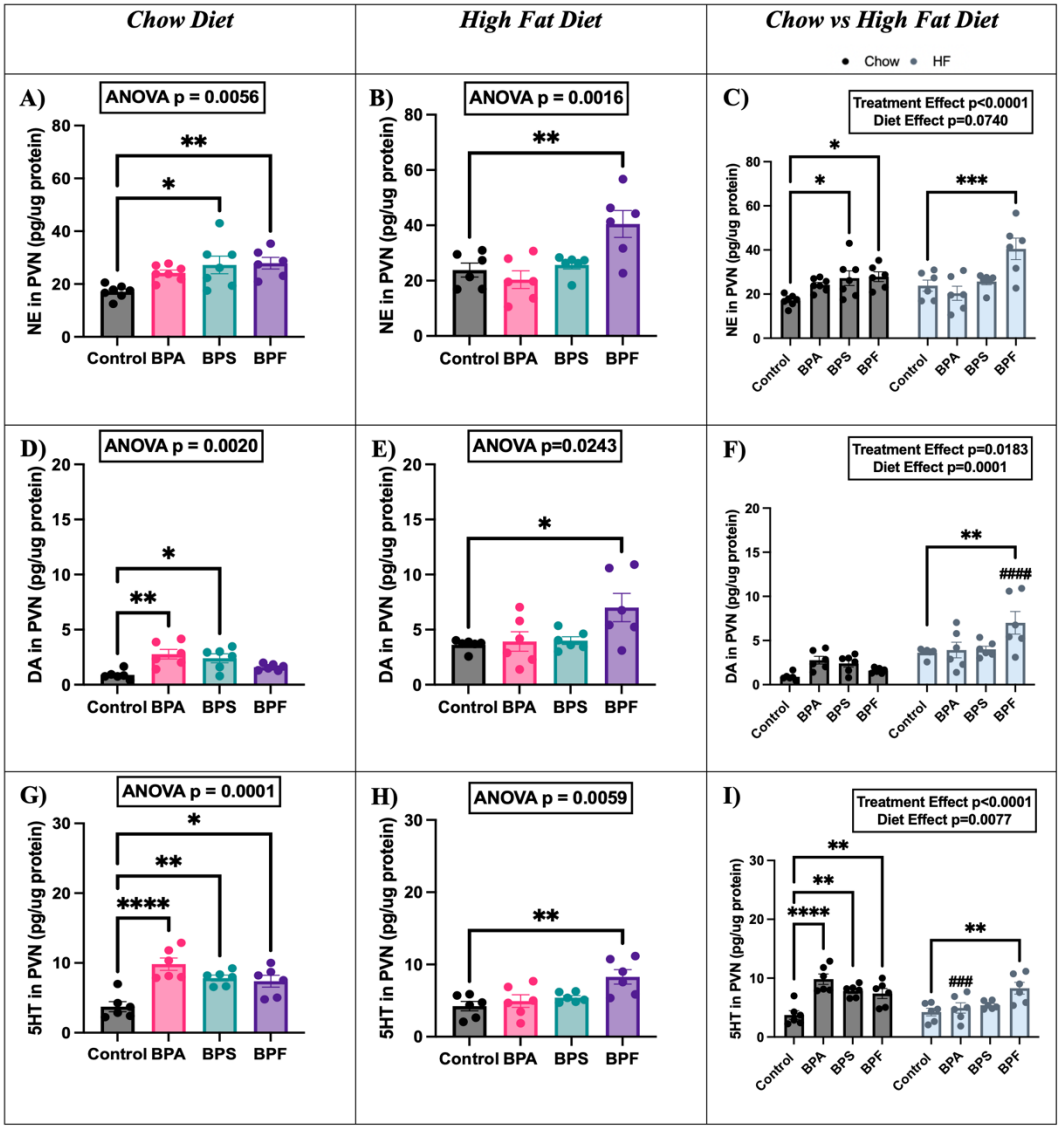

Fig. 4.3. Prenatal exposure to Bisphenol-A and its analogues followed by a high-fat challenge in adulthood on male's neurotransmitter level in the PVN. NE (panels A-C), DA (panels D-F), and 5HT (panels G-I) are depicted. Males exposed to the chow diet are represented with black bars and high-fat diet with blue bars. Columns 1 and 2 illustrate the separate one-way-ANOVA EDCs effects of a chow or high-fat diet; respectively. "*" indicates $p<0.05$, "**" $p<0.01$, "***" $p<0.001$ and "****" $p<0.0001$ compared to the corresponding controls with same diet. "###" $p<0.001$, "####" $p<0.0001$ comparing high fat to chow diet males. Data were analyzed by

one-way ANOVA (Columns 1&2) and two-way ANOVA (Column 3) followed by Tukey's multiple comparison test or Holm-Šídák test for treatment or diet differences, respectively. The error bars represent the standard error of the mean (SEM).

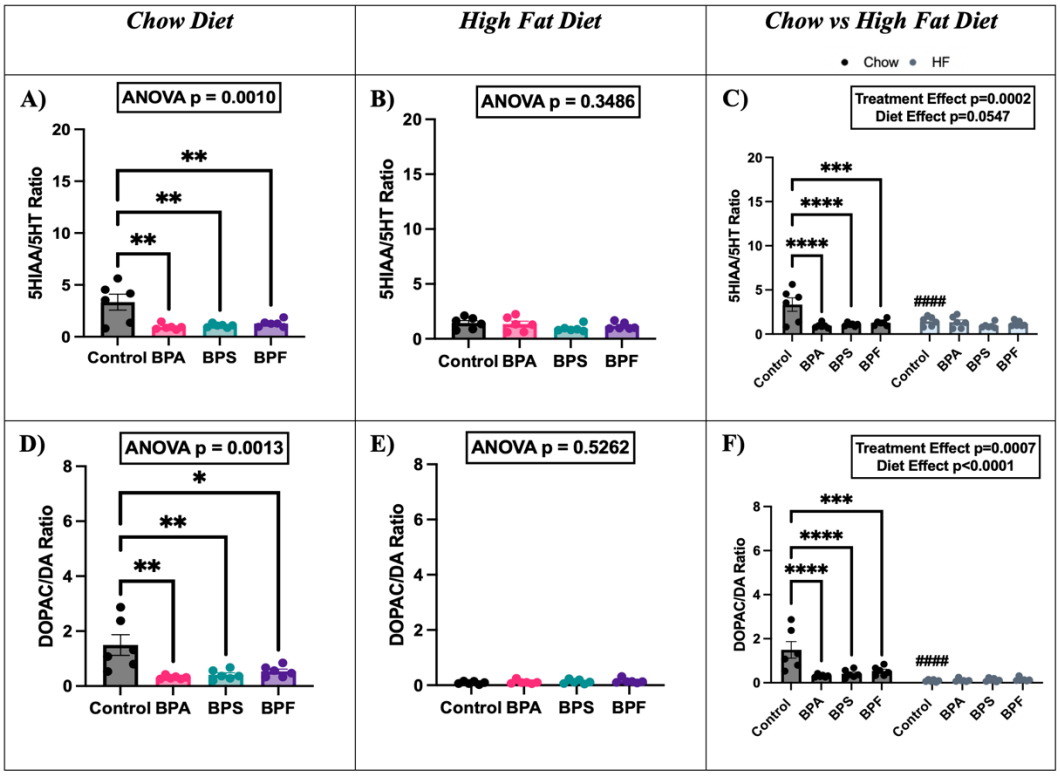

Fig. 4.4. Prenatal exposure to Bisphenol-A and its analogues followed by a high-fat challenge in adulthood on male's neurotransmitter ratio level in the PVN. 5HIAA/5HT (panels A-C) and DOPAC/DA (panels D-F) are depicted. Males exposed to the chow diet are represented with black bars and high-fat diet with blue bars. Columns 1 and 2 illustrate the separate one-way-ANOVA EDCs effects of a chow or high-fat diet; respectively. "*" indicates $p<0.05$, "**" $p<0.01$, "***" $p<0.001$ and "****" $p<0.0001$ compared to the corresponding controls with same diet. "####" $p<0.0001$ comparing high fat to chow diet males. Data were analyzed by one-way ANOVA

(Columns 1&2) and two-way ANOVA (Column 3) followed by Tukey's multiple comparison test or Holm-Šídák test for treatment or diet differences, respectively. The error bars represent the standard error of the mean (SEM).

3.3. Circulating hormonal levels

Ang II, Aldo, and CORT Circulating levels are depicted in Figure 4.5, n=5-7 in each group.

Ang II levels (pg/ml; Mean ± SEM) are shown in Fig 4.5A, there is a significant EDC treatment and diet effect ($p<0.0001$, and $p=0.0141$; respectively). In chow-fed offspring, there is an increasing trend with BPS and BPF having significantly higher levels of Ang II (75.3±11.9; $p<0.01$, and 86.9±8.8; $p<0.001$; respectively) compared to their controls (32.8±9.7). As for BPA-treated offspring, they had a 79% increase in Ang II levels, but they were not statistically significant. As for high-fat-fed offspring, only BPS-treated offspring had significantly higher levels of Ang II (74.3±5.1; $p<0.05$) than their controls (32.2±2.9). As for BPA and BPF high-fat-fed males had 18-46% increased levels of Ang II with respect to their controls, but it was not statistically significant. Surprisingly, a high-fat diet reduced Ang II levels in all offspring, with a significant decrease in BPF compared to chow-fed BPF offspring (47.1±4.7 vs. 86.9±8.8).

As for Aldo's levels (pg/ml; Mean ± SEM) are depicted in Fig 4.5B, there was a significant EDC treatment and diet effect ($p<0.0001$, and $p=0.0002$; respectively). In chow-treated EDC offspring, a similar pattern to Ang II is observed, but only BPF-treated offspring showed a significant increase in Aldo levels (193.8±27.4; $p<0.01$) compared to their controls (74.7±10.6). as for BPS treated offspring, they had a 60% increase in Aldo compared to controls but was not statistically significant. High-fat fed offspring showed a similar pattern to chow-offspring but had significantly higher levels in BPS and BPF-treated offspring (236.2±35.2, and 238.5±30.0;

respectively, $p<0.05$) than their control counterparts (134.2±16.8). High-fat exposure generally increased levels of Aldo in all offspring. Specifically, BPS-treated offspring had a significant increase in Aldo levels compared to chow-fed offspring (236.2±35.2 vs. 119.2±36.3; respectively, $p<0.05$).

CORT levels (ng/ml; Mean ± SEM) are shown in Figure 4.5C. There is a significant EDC treatment and diet effect ($p<0.000$, and $p=0.0007$; respectively). In chow-fed offspring, there was a trend of increasing CORT levels in BPA, BPS, and BPF (130.3±14.7; $p<0.01$, 160.0±12.3; $p<0.0001$, and 170.6±22.4; $p<0.0001$; respectively) compared to controls (46.7±9.6). As for high-fat diet-fed offspring, only BPS-treated animals had significantly higher levels of CORT (204.6±22.1; $p<0.05$) compared to their control counterparts (135.8±19.1). BPA and BPF had higher levels of CORT (27% and 17%; respectively) than their controls but levels were not statistically significant. High-fat diet increased levels of CORT compared to a chow-fed diet ($p<0.01$), however, no significant increase is observed between EDC-treated animals.

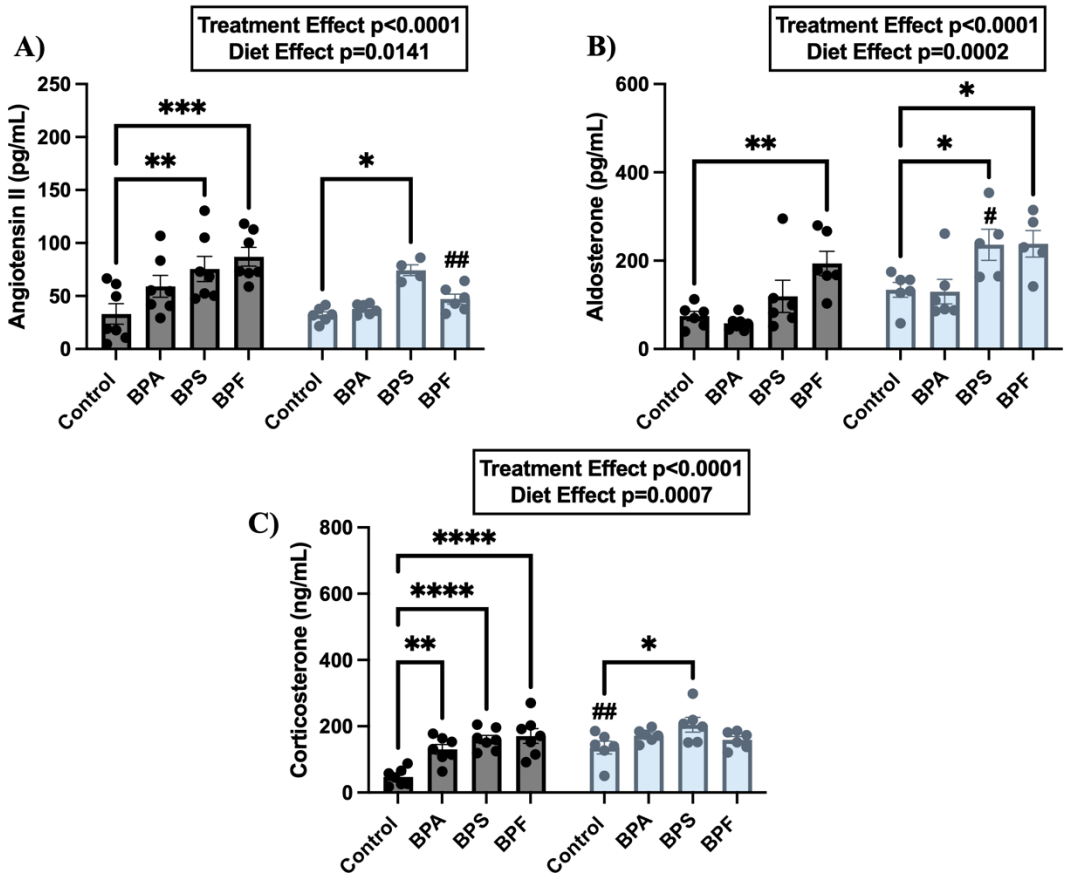

Fig. 4.5. Prenatal exposure to Bisphenol-A and its analogues followed by a high-fat challenge in adulthood on male's circulating hormones. Ang II (A), Aldo (B), and CORT (C) are depicted. Males exposed to the chow diet are represented with black bars and high-fat diet with blue bars. "*" indicates $p<0.05$, "**" $p<0.01$, "***" $p<0.001$ and "****" $p<0.0001$ compared to the corresponding controls with same diet. "#" $p<0.05$ and "##" $p<0.01$ comparing high fat to chow diet males. Data were analyzed by two-way ANOVA followed by Tukey's multiple comparison test or Holm-Šídák test for treatment or diet differences, respectively. The error bars represent the standard error of the mean (SEM).

3.4. Effects of prenatal exposure to BPA and its analogues and diet on cardiac muscle

The effects of prenatal exposure to BPA and its analogues followed by exposure to chow or high-fat diet in adulthood on cardiac muscle was assessed by evaluating %fibrotic area and LV wall thickness as presented in Figure 4.6.

% Fibrotic area in the cardiac muscle (mean ± SEM, n=4-8/group) is shown in Figure 4.6A, at least 5 images were taken at 8X per heart and analyzed using NIH ImageJ software. There was a significant EDC treatment and diet effect ($p<0.0001$ and $p=0.0007$; respectively). In chow-fed offspring, the extent of fibrosis was significantly increased in BPA, BPS, and BPF (1.2±0.1; $p<0.05$,1.5±0.4; $p<0.001$, and 1.8±0.2; $p<0.0001$; respectively) compared to their controls (0.5±0.1). High-fat diet-fed animals showed a similar pattern of increasing levels in prenatally treated offspring with BPA, BPS, and BPF (0.8±0.1; $p<0.05$,1.05±0.1; $p<0.01$, and 1.2±0.1; $p<0.001$; respectively) compared to controls (0.3±0.03). Compared to the chow diet, high-fat exposure reduced fibrosis extent in all offspring.

LV wall thickness (mm; mean ± SEM, n=5-8/group) is shown in Figure 4.6 B. LV wall thickness was measured in at least 5 areas of each heart using the eSlide manager software. There was a significant EDC treatment but no diet effect ($p=0.0225$ and $p=0.1340$; respectively). In chow offspring, the LV wall was significantly thicker in BPS and BPF-treated offspring (3.7±0.02, and 3.6±0.2; respectively, $p<0.05$) than their controls (3.2±0.1). BPA-treated offspring only had a 6% increase and was not considered statistically significant. As for high-fat-fed offspring, there were no significant changes. However, BPF-treated offspring had a 6% increase from controls with the same diet.

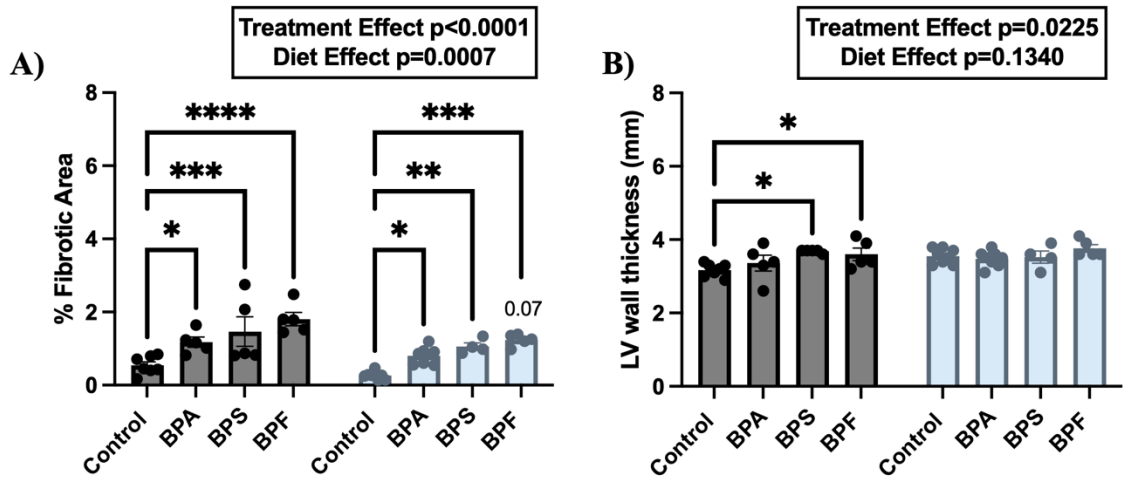

Fig. 4.6. Prenatal exposure to Bisphenol-A and its analogues followed by a high-fat challenge in adulthood on male's cardiac structure. % Fibrotic area (A), and LV wall thickness (B) are depicted. Males exposed to the chow diet are represented with black bars and high-fat diet with blue bars. "*" indicates $p<0.05$, "**" $p<0.01$, "***" $p<0.001$ and "****" $p<0.0001$ compared to the corresponding controls with the same diet. "p=0.07" compares high fat BPF to chow diet males. Data were analyzed by two-way ANOVA followed by Tukey's multiple comparison test or Holm-Šídák test for treatment or diet differences, respectively. The error bars represent the standard error of the mean (SEM).

3.5 Effects of exposure to high-fat diet in adulthood on males

The summarized effects of a high-fat diet on control males are depicted in Figure 4.7 and Table 4.1. All data is analyzed by unpaired two-tailed t-test and data is represented as mean ± SEM. High-fat diet significantly increased SBP and MAP ($p<0.001$ and $p<0.05$; respectively) (Fig. 7A-B). This alteration in blood pressure was accompanied by increased Aldo ($p<0.05$) and CORT levels ($p<0.01$) (Fig. 7C-D) and increased NE and DA levels in the PVN ($p<0.05$ and $p<0.0001$; respectively) and decreased ratio of DOPAC/DA and 5HIAA/5HT ($p<0.01$ and $p<0.05$;

respectively) (Fig. 4.7 E-F and Table 1). Indicating increased accumulation of DA and 5HT in the PVN. As for effects on cardiac muscle which are depicted in Figure 4.7 panels G-H, there was a significant decrease in the extent of fibrosis ($p<0.05$) and increased LV wall thickness ($p<0.01$). The neurohumoral parameters of high-fat diet exposure are summarized in Table 1.

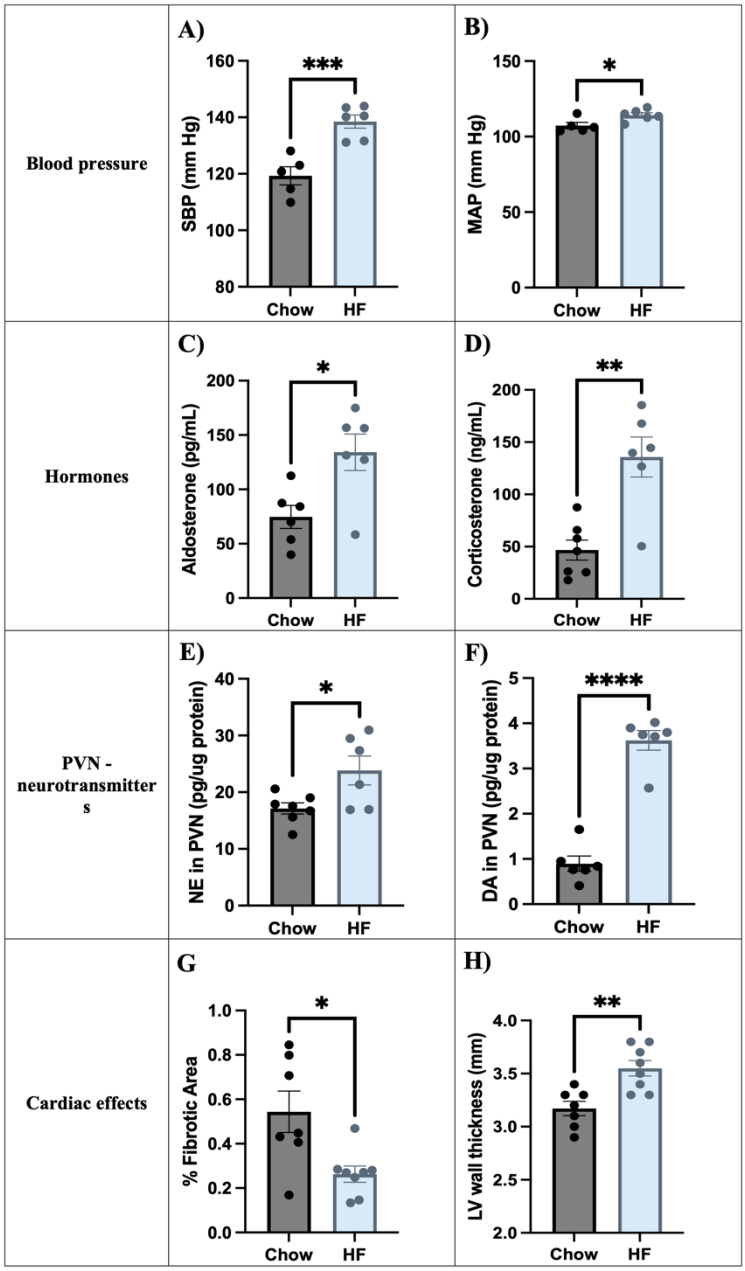

Fig. 4.7. Prenatal exposure to Bisphenol-A and its analogues followed by a high-fat challenge in adulthood on control males neurohumoral factors. SBP (A), MAP (B), Aldo (C), CORT (D), NE in PVN (E), DA in PVN (F), % Fibrotic area (G), and LV wall thickness (H) are depicted. Males exposed to the chow diet are represented with black bars and high-fat diet with blue bars. "*" indicates $p<0.05$, "**" $p<0.01$, "***" $p<0.001$, and "****" $p<0.0001$ compared to chow fed animals. Data were analyzed by students' unpaired two-tailed t-tests. The error bars represent the standard error of the mean (SEM).

Table 4.1. Effects of exposure to a high-fat diet in adulthood on neurohumoral factors.

Parameter	Chow Diet	High Fat Diet	Upaired t-test p value	p value summary
Blood Pressure				
DBP (mm Hg)	97.0 ± 3.0	95.1 ± 2.1	0.5873	ns
HR (bpm)	313.4 ± 12.3	322.4 ± 5.4	0.493	ns
Hormones				
Ang II (pg/mL)	32.9 ± 9.7	32.2 ± 2.9	0.9565	ns
PVN Neurotransmitters (pg/ul protein)				
5HT	3.7 ± 0.7	4.2 ± 0.6	0.6263	ns
DOPAC/DA	1.5 ± 0.4	0.09 ± 0.02	0.0041	**
5HIAA/5HT	3.3 ± 0.8	1.4 ± 0.2	0.0385	*

Note: DBP, diastolic blood pressure, HR, heart rate, Ang II, angiotensin II, PVN, paraventricular nucleus, 5HT, serotonin, 5HIAA/5HT, the ratio of 5HT metabolite to 5HT, and DOPAC/DA ratio of DA metabolite to DA. Data (N=5-8/group) are presented as mean ± SEM

4.5. Discussion

The aim of this study was to evaluate the organizational effects caused by prenatal exposure to BPA and its analogues and the activational effects caused by exposure to a high-fat diet in adulthood on males' neuro-cardiovascular system. The objective was to find whether exposure to the double hit paradigm will cause more detrimental effects on blood pressure and cardiovascular outcomes.

We initially examined the impact of a high-fat diet on the neurohumoral parameters of control animals. Our findings revealed that exposure to a high-fat diet during adulthood resulted in activation effects on the neuro-cardiovascular system of male offspring. These effects were characterized by increased SBP and MAP, along with elevated levels of circulating Aldo and CORT. Additionally, there was evidence of sympathoactivation in the hypothalamic PVN. Notably, the high-fat diet-induced changes in neurotransmitter levels within the PVN, including increased levels of NE, and DA while reducing the ratios of DOPAC/DA and 5HIAA/5HT, suggesting an accumulation of DA and 5HT. Furthermore, the high-fat diet resulted in cardiac muscle alterations, including increased LV wall thickness and a reduction in the percentage of fibrotic area. Although a reduction in fibrosis was observed, it was accompanied by the presence of vacuolation and degeneration in the cardiac muscle. Further investigations will be conducted in the future to explore these phenomena in more detail. Our findings are consistent with available research about the effects of a high-fat diet on neurohumoral parameters. Activation of the PVN and increase of CORT and Aldo indicates activation of the hypothalamic-pituitary-adrenal (HPA) axis which is an important neuroendocrine stress system regulation axis, and the mineralocorticoid Aldo regulates blood pressure through regulation of fluid and electrolytes balance through the RAAS. Aldo is strongly associated with obesity and blood pressure control, in fact, weight loss

leads to decreased Aldo and blood pressure levels [206, 207]. The activation of these two pathways increases blood pressure and causes alterations in the neuro-cardiovascular system. In fact, a cross-sectional study found that the HPA axis dominates RAAS in positive association with the hypertension [208]. One study found that exposure to a high-fat diet increased CORT levels and the second hit with chronic mild stress augmented that increase accompanied by increased vasopressin mRNA expression in the hypothalamus [209]. High-fat diet alone has determinantal effects on the neuro-cardiovascular system, so we wanted to investigate how exposure prenatally to BPA and its analogue followed by exposure to a high-fat diet in adulthood would affect males' cardiovascular health. Men are at increased risk of cardiovascular diseases and their risk increases with age, exposure to environmental contaminants and a high-fat diet could lead to more determinantal effects [210].

Based on the findings in the study, prenatal exposure to BPS or BPF has been found to cause more severe effects on the neuro-cardiovascular system than BPA. In BPA offspring fed a chow diet, SBP increased by 22%, and MAP and HR by 9% and this was probably mediated by the increased 5HT levels in the PVN and decreased 5HIAA/5HT ratio as well as increased circulating CORT levels. These alterations led to increased fibrosis in the heart muscle. When challenged with a high-fat diet in adulthood, BPA offspring had 8% increased SBP compared to high-fat controls but compared to chow BPA the increase was only 3%, this was accompanied by decreased 5HT levels in the PVN. Our findings are consistent with recent studies that investigated the multigenerational effect of exposure to BPA and a high-fat diet. They found that the combination in the F1 generation leads to increased blood pressure [127, 203]. However, Hsu et al 2019 reported exacerbated effects with a high-fat diet but our results showed contrasting findings [203]. The findings from this study suggest that BPA's offspring increased blood pressure

may be mediated through the sympathoactivation of PVN and indirectly through the HPA axis. 5HT in the PVN is known to stimulate the release of corticotropin-releasing-hormone (CRH) into the median eminence which then stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary gland and then the release of CORT from the adrenal gland [211].

While similar findings are observed in BPS-treated offspring, there seem to be more detrimental effects than BPA in chow and high-fat diet-fed offspring. Chow-fed offspring had 28% higher SBP, and 10-14% higher MAP and HR than controls and a 5% increase in SBP and MAP with respect to BPA. In support of this, another study found increased HR levels in excised rat hearts [129] but when done on mice hearts, BPS decreased left ventricular systolic pressure [40]. However, there is not enough studies investigating the effects of BPS exposure on blood pressure, but it was found to be positively associated with CVD and HTN [21, 105]. Furthermore, they had increased levels of NE, DA, and 5HT accompanied by a decreased ratio of 5HIAA/5HT and DOPAC/DA. BPS offspring had increased levels of circulating hormones such as Ang II and CORT. These findings were also accompanied by alteration to the cardiac muscle by increased LV wall thickness and fibrosis. Combined exposure with a high-fat diet, increased SBP and MAP by 9-14% higher than high-fat controls but only a 3% increase from chow-fed BPS. PVN's neurotransmitter levels were comparable to chow offspring and were not significantly increased from high-fat controls, however, Aldo levels were significantly increased and higher than chow offspring accompanied by increased Ang II and CORT. While to our knowledge there are no data reporting effects of BPS on Ang II levels, BPA is capable of increasing Ang II [110, 162]. BPS and a high-fat diet also had increased fibrosis in the cardiac muscle. Findings shed light on BPS's possible mechanism of action through the activation of the RAAS system and possibly the HPA axis. While a high-fat diet alone altered levels of NE and 5HT in the PVN, combined exposure did

not worsen the levels. Instead, the levels of NE and 5HT were comparable to chow-fed offspring and high-fat BPS offspring had 31-33% higher NE and 5HT than control chow.

Males' prenatal exposure to BPF had a plethora of effects on neurohumoral parameters regardless of diet. Chow-fed offspring had 23% increased SBP which was accompanied by hypothalamic PVN sympathoactivation and activation of RAAS and HPA axis. There was an increased level of NE, 5HT, and decreased ratio of 5HIAA/5HT and DOPAC/DA in the PVN. Circulating hormones such as Ang II, Aldo, and CORT were significantly elevated. And alterations of cardiac muscle such as LV hypertrophy and cardiac fibrosis were detected. When combined with a high-fat diet, BPF had 9% increased levels of SBP and HR and this was accompanied by an increase in NE, DA, and 5HT in PVN and increased Aldo levels by 78% and induced cardiac muscle fibrosis. While there are studies on zebrafish [134], and cardiomyocytes [43] exposure to BPF, data is still limited effects of BPF on cardiovascular function. However, these studies found BPF to induce cardiac hypertrophy and increase calcium levels [43], decrease heart rate, and reduce cardiac development genes expression [134]. Our study is the first to investigate the effects of exposure to BPF and challenge those offspring with a high-fat diet to study the effects on the neuro-cardiovascular system. Based on our findings, BPF can alter RAAS and HPA axis and cause cardiac muscle remodeling. These findings warrant the need for more investigations to assess the risk of BPF on the cardiovascular system.

5. Conclusion

The study has demonstrated that both prenatal exposure to BPA and its analogues BPS and BPF, and a high-fat diet in adulthood have a detrimental impact on the male neuro-cardiovascular system. BPS and BPF appeared to cause more severe effects than BPA, leading to significant increases in SBP, MAP, and HR. These effects were mediated through the activation of both the HPA axis and the RAAS, leading to increases in circulating CORT, Aldo, and neurotransmitter levels in the hypothalamic PVN. Alongside these alterations, cardiac muscle remodeling, including increased LV wall thickness and fibrosis, was also observed. While the high-fat diet alone induced similar changes, combined exposure to BPA, BPS, or BPF prenatally and a high-fat diet in adulthood resulted in only marginal increases in blood pressure compared to a high-fat diet alone. However, it led to augmented Aldo levels in BPS offspring. The findings highlight the potential risks posed by BPA and its analogues on cardiovascular health, especially when combined with a high-fat diet. It provides crucial insights into their mechanisms of action, which seem to involve the activation of stress response systems and neurotransmitter changes in the brain, leading to alterations in cardiovascular function and structure. Our findings suggest a need for further investigations, particularly into BPS and BPF, whose impacts on the cardiovascular system remain largely unexplored.

Acknowledgments

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CHAPTER 5

DISCUSSION

5.1. Summary of Findings

Exposure to EDCs is widespread and almost inevitable, raising concern for the wide range of diseases and health consequences associated with them. The study aimed to investigate the effects of prenatal exposure to low doses of BPA and its analogues BPS and BPF on the neurocardiovascular system. The research focused on assessing the sex-specific impact of prenatal EDC exposure on blood pressure and exploring potential mechanisms through central and humoral parameters. Additionally, a double-hit paradigm was employed, involving exposure to chronic estradiol in females and a high-fat diet in males in adulthood. Offspring were exposed to these chemicals *in utero* and were maintained till adulthood then changes in cardiovascular function were tracked in both males and females. Trunk blood, brain tissue, and organs were collected at sacrifice for analysis of hormonal and neurotransmitter levels as well as target organ effects. The major findings of the study are summarized in Figure 5.1. These findings shed light on the effects of prenatal EDC exposure on the neurocardiovascular system.

Prenatal exposure to BPA, BPS, or BPF and its effects on cardiovascular function – sex differences.

To establish whether prenatal exposure to BPA and its analogues affects blood pressure parameters, the first major aim of the study focused on the effects of these chemicals on male and female offspring. We found striking effects, with increased blood pressure in males exposed to BPA, BPS, and BPF and in females to BPA and BPS. Male offspring exposed to BPS and BPF had significantly higher levels of SBP and MAP compared to females. Whereas females exposed to BPS had significantly higher HR elevation. Surprisingly those increases in blood pressure in males were associated with increased Ang II and CORT levels while in females BPS increased blood pressure was accompanied by increased Ang II levels. To assess whether sympathoactivation through the PVN is affecting blood pressure and hormonal parameters, we measured neurotransmitter levels and found that there were increased NE and 5HT levels in all EDC-treated offspring. Levels of the 5HIAA/5HT ratio were significantly lower in all EDC-treated animals except for BPA-treated females, which had higher levels than male offspring. Endo-1 immunostaining in small blood vessels of the lung was found to be high in all males but only in BPS-treated females. As for cardiac muscle, male offspring exposed to EDCs had increased relative heart weight but in females only BPA offspring. Heart weight was significantly higher in all females compared to males. Males exposed to BPS and BPF had increased LV wall thickness whereas only BPA-treated females showed that increase consistent with relative heart weight. There were increased fibrosis levels in the cardiac muscle in all EDCs male and female except for BPF-treated females. Based on these findings, BPS seems to be detrimental to both males and females while BPF is more detrimental to male offspring and BPA to a lower extent than BPS and BPF. BPS exposure seems to increase blood pressure through activation of PVN and subsequent

RAAS system activation in both males and females but also HPA axis activation in males. As for BPF, in males' activation of PVN and RAAS, and HPA, while in females, through PVN and HPA axis. BPA in females through sympathoactivation of PVN and in males through 5HT in PVN and HPA axis. These findings shed light on the importance of assessing the health consequences of exposure to BPS and BPF and the criticality of evaluating sex differences as these EDCs could cause organizational sex-specific alterations in the HPA axis and RAAS system.

Prenatal exposure to BPA, BPS, or BPF followed by exogenous chronic estradiol exposure and its effects on cardiovascular function.

Our second aim was focused on the assessment of organizational vs. activational effects of a second insult on female offspring by exposure to chronic 17β-estradiol (E2) in adulthood. Offspring received a similar protocol except when implanted with telemetry, females were also implanted in a subcutaneous pocket with sham or E2 pellet, and measurements of blood pressure continued for 11 weeks following which they were sacrificed, and trunk blood and organs were collected.

Our experiment yielded intriguing findings that support and validate previous results from our laboratory regarding the effects of chronic E2 exposure on blood pressure. It appears that these effects are likely mediated through alterations in neurotransmitters within the PVN. The control offspring exposed to E2 exhibited increased SBP, reduced DA levels, increased DOPAC/DA ratio, and decreased 5HIAA/5HT ratio. These findings provide further insights into the mechanisms underlying the impact of E2 exposure on blood pressure regulation and neurotransmitter dynamics in the PVN. As for EDC-treated offspring, BPS had increased levels of SBP, DBP, and MAP with sham and chronic E2. BPA offspring had increased SBP, DBP, and MAP in sham but not in E2,

similarly for BPF the increase in DBP in sham is not observed in E2. These findings suggest that there are differences in the mechanism of action of BPS compared to BPA and BPF related to E2.

In our study, we further investigated the effects of EDCs on neurohumoral parameters and examined the potential role of E2 in mediating these effects. Sham-treated offspring exposed to BPS displayed increased levels of NE and 5HT in the PVN, while E2-implanted offspring showed elevated 5HT levels only. E2 treatment resulted in decreased DA levels across all offspring, accompanied by an increased ratio of DOPAC to DA. In BPF offspring, there was an increase in 5HT levels in sham-treated but not E2-treated offspring, similarly, BPA offspring exhibited increased NE levels in sham-treated but not E2-treated offspring. These findings suggest that estradiol-induced changes in blood pressure may be mediated through alterations in neurotransmission in the PVN. Interestingly, circulating E2 levels were elevated in sham-treated and E2-implanted BPS offspring, but only in sham-treated BPF offspring and not in BPA offspring. These differences could be attributed to factors such as the metabolism and clearance rates of the EDCs, their specific interactions with hormone receptors, and variations in their absorption and distribution within the body. In terms of hormonal levels, BPS offspring showed increased levels of Ang II in both sham-treated and E2-treated groups, while BPF offspring exhibited elevated CORT levels, which returned to normal with E2 exposure. Notably, all EDC-treated animals displayed increased cardiac fibrosis, except for sham-implanted BPF offspring.

The findings regarding the effects of BPS on blood pressure are consistent regardless of chronic E2 exposure. BPS increases blood pressure through the activation of the PVN and the RAAS. On the other hand, the effects of BPA and BPF are more complex. In the presence of E2, BPA, and BPF exhibit a decrease in blood pressure compared to the control group with E2. However, this decrease is not observed when compared to the sham group. In sham offspring, BPA

and BPF increase in blood pressure is likely mitigated through sympathoactivation of the PVN and activation of the HPA axis, respectively. Further research is necessary to fully understand the effects of these EDCs and their interactions with estrogen.

Prenatal exposure to BPA and its analogues followed by high-fat diet exposure in adulthood on males' neurohumoral parameters.

Our third aim focused on male offspring's organizational and activational effects of exposure to a high-fat diet in adulthood. A similar protocol was utilized except that after the acquisition of pre-diet parameters, offspring were given a high-fat diet for two weeks and were continuously monitored till sacrifice.

The effects of a high-fat diet alone on the neuro-cardiovascular system were intriguing. It led to increased blood pressure through the activation of the hypothalamic PVN and the HPA axis, along with an increase in mineralocorticoid aldosterone. In offspring exposed to EDCs, males exposed to a high-fat diet exhibited increased blood pressure regardless of the specific bisphenol exposures. However, the increase in blood pressure with a high-fat diet was only modest (3%) compared to the chow diet in EDC-treated offspring. This suggests that either the activational changes take longer than two weeks to develop in the presence of prenatal BPA and its analogues' organizational effects, or the receptors are altered by prenatal exposure, preventing further exacerbation of these effects by the high-fat diet.

Among the EDC-treated offspring, BPS and BPF had more detrimental effects on the neuro-cardiovascular system. BPS offspring exhibited increased activation of the RAAS and HPA axis regardless of diet exposure, but the high-fat diet further elevated circulating Aldo levels. In the case of BPF-treated offspring, they displayed increased sympathoactivation of the PVN and

activation of the RAAS and HPA axis in both chow and high-fat diet groups. BPA chow offspring showed increased sympathoactivation of the PVN and subsequent activation of the HPA axis, but this effect was not observed in the high-fat diet-fed offspring.

This experiment provided great insight into the effect of prenatal exposure to EDCs on males' cardiovascular health and bring attention to the severe effects of high fat regardless of exposure.

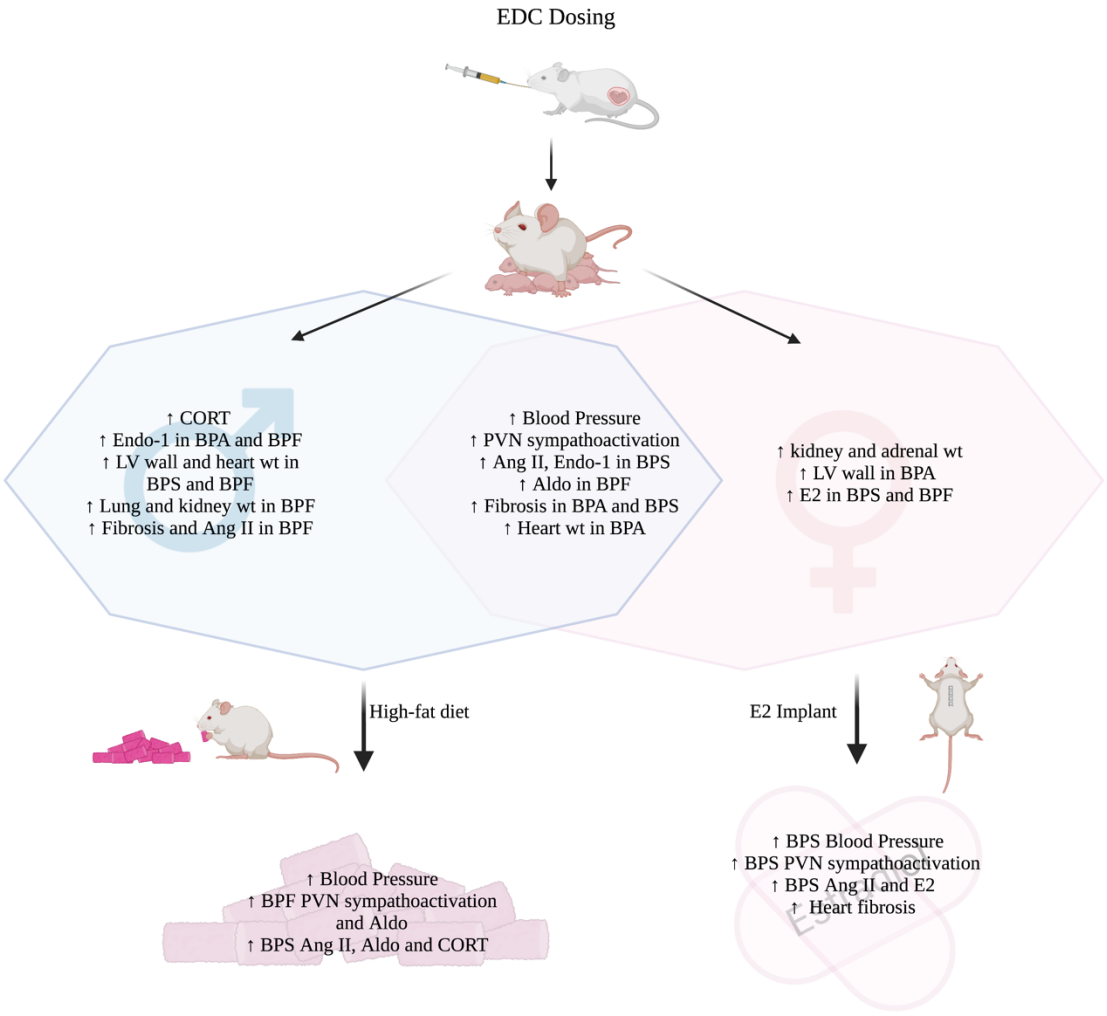

Fig 5.1. Overall study findings. Sprague Dawley (SD) rats, adult females, and males were acquired from Envigo and allowed to mate. Pregnant SD rats were exposed to Control (10µl

Phosphate Buffered Saline (PBS), *n*=9), Bisphenol A (5µg/kg BW/day, *n*=6), Bisphenol S (5µg/kg BW/day, *n*=13), and Bisphenol F (1µg/kg BW/day, *n*=10) from GD 6-21 by oral gavage. The offspring were weaned at PND 21 and when they reached adulthood, they were implanted with a radiotelemetry device, and sex differences were acquired. In another set of animals, males were given chow or a high-fat diet, and females were implanted with sham or E2 pellets. Note: EDCs: endocrine disrupting chemicals, BPA: bisphenol A, BPS: bisphenol S, BPF: bisphenol F, PVN: paraventricular nucleus, CORT: corticosterone, Aldo: aldosterone, Ang II: angiotensin II, E2: estradiol, LV: left ventricle, and wt: weight. *Illustration created with biorender.com.*

5.2. Future Directions

The findings presented in these studies have sparked further curiosity and highlight the need for continued investigations to determine the mechanism of action of EDCs and possible ways to ameliorate these effects.

Ongoing experiments have been initiated to investigate the effects of EDCs on perivascular adipose tissue, as well as the expression of vasoconstrictor factors such as Visfatin and Endo-1. Additionally, the impact of a high-fat diet on cardiac muscle structure is being assessed. These investigations are currently in progress and will provide valuable insights once the data analysis is completed. Understanding the underlying mechanisms by which these EDCs induce fibrosis, such as investigating TGF β and GLi2 expression, would provide valuable insights. Exploring the presence of fibrosis in the kidneys and its impact on filtration function is also a promising area for future investigation. Therefore, there is a need for further exploration of the effects of prenatal bisphenols on kidney health and filtration processes. These areas of study will

contribute to a more comprehensive understanding of the effects of EDCs on various organs and physiological processes.

Exploring the effects of hormonal changes and EDCs on various receptors involved in cardiovascular regulation, such as AT1R, mineralocorticoids, vasopressin, and ER subtypes (ERα and ERβ), is crucial for a comprehensive understanding of their impact on the neuro-cardiovascular system. Additionally, investigating other important cardiovascular regulatory areas in the brain, including the subfornical organ (SFO) and rostral ventrolateral medulla (RVLM), would provide valuable insights. Brain punches from these regions have been collected and are awaiting further experimentation to elucidate their role in EDC-induced cardiovascular dysfunction. Moreover, evaluating oxidative stress in the SFO and investigating the effects of circulating Ang II on the SFO-PVN-RVLM pathway would contribute to our understanding of the underlying mechanisms involved. These future directions hold great potential for uncovering novel aspects of EDC-mediated cardiovascular alterations and should be pursued to enhance our knowledge in this field.

Lastly, further exploration of the epigenetic modifications in cardiovascular development-specific genes in the hypothalamus and heart muscle would provide valuable insights into the underlying mechanisms of action. Investigating these epigenetic alterations could shed light on the long-term effects of BPA, BPS, and BPF exposure on cardiovascular health. Additionally, given the limited information available on BPS and BPF, there are numerous other directions that can be pursued to deepen our understanding of their effects on the neuro-cardiovascular system. These potential avenues of research hold great promise for uncovering novel aspects of EDC-induced cardiovascular dysfunction and should be considered in future investigations.

5.3. Conclusions

This study investigated the effects of *in utero* exposure to low doses of BPA, BPS, and BPF on blood pressure regulation and the neuro-cardiovascular system. It revealed sex-specific impacts, with males showing significant increases in blood pressure with all EDC treatments, while females exhibited increases with BPA and BPS treatments. EDC exposures also affected neurotransmitter levels and led to cardiac muscle remodeling. Additionally, the study found that both prenatal EDC exposure and a high-fat diet in adulthood had detrimental effects on the male neuro-cardiovascular system, with BPS and BPF causing more severe effects. As for females, BPS caused more severe effects regardless of exposure to chronic E2. These effects were mediated through the activation of renin-angiotensin and stress response system, and alterations in neurotransmitter levels in the PVN. The study highlights the need for further research to fully understand the mechanisms and risks associated with EDC exposure to cardiovascular health. We hope that the findings from this study will contribute to raising awareness about the potential health risks associated with EDC exposure. It is our intention that these data prompt a reevaluation of current EDC regulations, with the aim of improving public health and minimizing the adverse effects on cardiovascular well-being.

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