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Prenatal Exposure to Bisphenol A Disrupts Fetal Liver Maturation in a Sex-Specific Manner

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Physiology

PRENATAL EXPOSURE TO BISPHENOL A DISRUPTS FETAL LIVER MATURATION IN THE MOUSE IN A SEX-SPECIFIC MANNER

(Thesis format: Monograph)

by

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Abstract

Developmental exposure to BPA is associated with liver dysfunction and diseases in adulthood. However, the effects of BPA on liver development are unknown. To address this question, pregnant mice were exposed to BPA via diet from embryonic day 7.5 (E7.5) to E18.5. At E18.5, fetal livers were collected, and analyzed for changes in the expression of key hepatic maturation markers. We found the following significant protein alterations in BPA-exposed female but not male fetal livers: ***(a)*** mature hepatocyte markers, albumin and glycogen synthase, were decreased; ***(b)*** immature hepatocyte marker, alpha-fetoprotein, was increased; ***(c)*** master transcription factor of hepatocyte differentiation, C/EBP-α, was down-regulated; and ***(d)*** PCNA (cell proliferation marker) was elevated, while caspase-3 (marker of apoptosis) was reduced. These findings demonstrate that prenatal exposure to BPA disrupts molecular maturation of the mouse fetal liver in a sex-specific manner, and suggest that females are more vulnerable to BPA-induced liver dysfunction and diseases.

Keywords

Bisphenol A (BPA), liver development, hepatocyte maturation, sex-specific effect, hepatic dysfunction and disease, albumin, glycogen synthase, alpha-fetoprotein (AFP), CCAAT/ enhancer binding protein alpha (C/EBP- α), PCNA, caspase-3, proliferation, apoptosis

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List of Abbreviations

AFP	alpha-fetoprotein
AR	androgen receptor
BEC	biliary epithelial cell
BPA	bisphenol A
BPA-GA	BPA-glucuronide
C/EBP	CCAAT/enhancer binding protein
CK	cytokeratin
DOHaD	Developmental Origins of Health and Disease
E	embryonic day
EDC	endocrine-disrupting chemical
EGF	epidermal growth factor
EHBD	extrahepatic bile duct
EPA	Environmental Protection Agency
ER	estrogen receptor
ERR	estrogen-related receptor
ES	embryonic stem
G6Pase	glucose-6-phosphatase
GR	glucocorticoid receptor
GS	glycogen synthase

H&E	hematoxylin and eosin
HCC	hepatocellular carcinoma
HGF	hepatocyte growth factor
HNF	hepatocyte nuclear factor
IHBD	intrahepatic bile duct
LOAEL	lowest observed adverse effect level
NOAEL	no observed adverse effect level
NMDRC	non-monotonic dose response curve
OC	Onecut
OSM	oncostatin M
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PEPCK	phosphoenolpyruvate carboxykinase
PFA	paraformaldehyde
PN	postnatal day
SEM	Standard Error of the Mean
STM	septum transversum mesenchyme
SULT	sulfotransferase
TGF	transforming growth factor
TR	thyroid receptor

UGT	UDP-glucuronyltransferase

Chapter 1 – Introduction

1.1 Bisphenol A

Bisphenol A (BPA) is a prominent endocrine-disrupting chemical (EDC) that has fueled major public health concerns due to its association with a wide range of metabolic, reproductive, cardiovascular, and neurological disorders as well as cancer [1–3]. It is one of the highest volume chemicals produced in the world, with over 8 billion pounds being produced annually [4]. As a building block of polycarbonate plastics, as well as a major component of epoxy resins [4–6], BPA is used extensively in the manufacture of a number of consumer goods and products, including reusable food and beverage containers, dental sealants, polyvinyl chloride stretch films, tin can linings, cardboards, and papers used in register receipts [1,4,6,7]. Consequently, BPA has also been detected in water, dust, as well as indoor and outdoor air samples [1,8]. Because BPA is ubiquitously present in the environment, it can exert its harmful effects through multiple routes of exposure including ingestion, inhalation, and dermal absorption [1,8]. Humans are most commonly exposed to BPA via diet [9], as free (active) BPA has a tendency to leach out of food and beverage containers, routinely entering our food and drinks [4,6,10].

1.1.1 Pharmacokinetics

Following ingestion, BPA is absorbed from the intestines and transported to the liver to be metabolized by glucuronidation during first-pass metabolism [11,12].

Unconjugated BPA is rapidly metabolized into inactive BPA glucuronide (BPA-GA) via UDP-glucuronyltransferases (UGTs), most notably UGT2B1, as well as into inactive BPA sulfate metabolites via sulfotransferase (SULT) enzymes [8,13]. BPA metabolism is similar between humans, non-human primates, and rodents in that the major metabolite formed is BPA-GA, accompanied by small amounts of BPA sulfate [11]. However, one species difference exists in terms of BPA elimination. In humans and non-human primates, BPA is cleared from the blood and excreted via the renal system, whereas in rodents, BPA conjugates are eliminated primarily in the feces via bile, with only small amounts excreted in the urine [12]. This species difference in route of clearance has led some to speculate that levels of BPA clearance must also be vastly different between humans and rodents. However, Taylor et al. have confirmed that oral administration results in similar internal exposures to unconjugated BPA in primates and rodents, with a virtually identical rate of clearance [12]. Other studies have also established that the pharmacokinetics of BPA are comparable between humans and rodents [12,14], and regulatory agencies have subsequently deemed rodent models appropriate for assessing the effects of BPA [14].

It has been previously assumed that following oral ingestion, first-pass metabolism of BPA is complete with virtually all ingested BPA being conjugated in the liver [15], attenuating the need for concern in regards to potential health effects underlying BPA exposure. However, this assumption surfaced mainly from the findings of one single study that failed to detect unconjugated BPA in human plasma and urine samples [12]. Importantly, this study was characterized by fundamental design flaws including a limited sample size as well as the use of a relatively insensitive assay [15].

More recent findings from pharmacokinetic studies using both mice [16] and primates [12] have demonstrated that first-pass metabolism is in fact incomplete. Results from several biomonitoring studies in humans also indicate internal BPA exposure, given that measurable concentrations of unconjugated BPA are detected in urine [17], which suggests the following: (a) first-pass metabolism is not complete; (b) a portion of BPA entering the body circumvents first-pass metabolism; and/or (c) BPA metabolites are being deconjugated in the body [14,17]. Indeed, studies using rodents have found that a portion of conjugated BPA undergoes deconjugation via enzymes in the intestine and colon [8]. Glucuronidase enzymes are also apparent in the digestive tracts of humans, especially those of infants, likely indicating their ability to deconjugate and re-activate conjugated BPA [8].

The relative bioavailability of BPA is largely dependent upon route of administration. While orally administered BPA is subject to first-pass metabolism, BPA administered via other routes will bypass this process, leading to higher concentrations of active BPA in circulation [14]. For instance, compared to oral administration, systemic blood concentrations of BPA are markedly higher following subcutaneous or intraperitoneal injection, which indicates higher bioavailability [18]. These findings provide a likely explanation for why the relative potency of BPA is also dependent on exposure route. For example, subcutaneous BPA administration tends to yield effects at much lower doses compared to other routes of exposure [15].

1.1.2 Mechanisms of Action

According to the US Environmental Protection Agency (EPA), an EDC is defined as "an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" [7]. BPA is most commonly referred to an estrogenic EDC because of its ability to disrupt the activity of endogenous estrogens [7]. Estrogens are a group of steroid hormones derived from cholesterol [1], and are critically involved in regulating the growth, differentiation, and function of a number of different target tissues [19]. While estrogens diffuse in and out of all cells, they are retained with high affinity and specificity in target cells via interaction with the estrogen receptor (ER) [1]. The ER is located mainly in the cell nucleus where, once bound by an estrogen, it will undergo a conformational change and bind to response elements in the promoter region of certain estrogen target genes [19,20]. As a result, coregulatory proteins (coactivators or corepressors) are recruited to the promoter region of the target gene, leading to a respective increase or decrease in mRNA levels and associated protein production [21]. ER can also act via non-genomic mechanisms, as outlined by Deroo et al. [21]. In mammals, the two major ER subtypes are ERα and ERβ. These subtypes are encoded by separate genes (ESR1 and ESR2, respectively), and exhibit distinct tissue-specific expression patterns [21]. In tissues where they are coexpressed, ERα and ERβ can sometimes exert differential effects, and can also influence the actions of one another [22].

It is well established that upon binding to nuclear ER, BPA can induce estrogenic signals that modify estrogen-responsive gene expression [7]. BPA can bind to both ERα and ERβ, with a ten times higher affinity for ERβ [2]. Specifically, BPA has been shown to act as an agonist for ERβ, while demonstrating dual effects as an agonist and antagonist in some cell types via ERα [23]. Hence, the activity of BPA is largely dependent on ER subtype and cell type, which can be partially attributed to the differential effect of BPA on the recruitment of coactivator proteins by ERα and ERβ [7,24].

BPA has been classified as a weak estrogen based on its low binding affinity for ER compared to naturally-occurring 17β-estradiol (~10,000-fold lower) [14,25]. Previously, this has led to the notion that BPA might be unable to create an impact in the midst of the already strong effects of endogenous estrogens. However, Rajapakse et al. demonstrate that the combination effect of estradiol and BPA is in fact additive [26]. The spare receptor hypothesis describes how typically, a maximal biological response can be achieved by low concentrations of a hormone, well before receptor occupancy becomes saturated [14]. The presence of these “spare receptors” provides a mechanism for why low doses of an EDC such as BPA might exert a response, regardless of its low affinity for the receptor [25]. In addition, BPA has been shown to interact with ER in a non-classical manner that is distinct from other known groups of ER ligands [25,27], leading to the induction of a unique subset of ER-responsive genes and associated physiological responses [28]. Moreover, while BPA is less potent than estradiol upon binding to nuclear ER, its potency is evidenced to be just as high as that of estradiol when its action is mediated by ERs outside the nucleus [7].

Importantly, the endocrine disrupting actions of BPA extend beyond its ability to modulate the activity of endogenous estrogens. More recently, it has become apparent that BPA can bind to the androgen receptor (AR) and thyroid receptor (TR) as an antagonist [29]. In addition, BPA can bind to the glucocorticoid receptor (GR) as both an agonist and antagonist [30,31], and has also been shown to possess strong binding affinity for the estrogen-related receptor γ (ERRγ) [32]. In addition, BPA can act via variety of intracellular signal transduction pathways directly via mechanisms independent of nuclear hormone transactivation [7,8]. Certain metabolites of BPA have even been suggested to be more potent than the parent compound itself [33].

1.1.3 Dose-Response and Low-Dose Effects

Typically, regulatory testing of a chemical involves the establishment of a lowest observed adverse effect level (LOAEL), which is the lowest dose at which an adverse effect is observable, and/or a no observed adverse effect level (NOAEL), defined as the highest exposure level at which no observable adverse effects are present [14]. After a series of calculations, a reference dose is then produced which becomes the allowable exposure level that is deemed safe for humans [14]. This standard procedure was based on one of the traditional notions in toxicology being that “the dose makes the poison”. This idea implies that the larger the dose of a chemical substance, the greater the physiological response. However, studies of EDCs have challenged this concept, including those of BPA, demonstrating that low-dose effects exist which cannot be predicted by the effects observed at high doses [14]. This is due to the fact that BPA and other EDCs often exhibit non-monotonic versus monotonic dose-response relationships

[34]. Monotonic dose response curves can be linear or non-linear, but the sign of the slope remains constant. In contrast, non-monotonic dose response curves (NMDRCs) present as U-shaped or inverted U-shaped curves, with the sign of the slope changing from positive to negative (or vice-versa) at some point of along the range of examined doses [14]. In other words, BPA's action (or lack thereof) at low doses cannot simply be inferred by the presence or absence of an effect of BPA at a higher dose [34,35]. Thus, the traditional approach of using high-dose testing regimens to assess chemical safety at low doses is particularly problematic for EDCs.

This issue reinforces the importance of environmentally relevant doses in studies examining the effect of an EDC. In animal models, an administered dose of a chemical that creates internal concentrations within the range of what has been measured in the general human population is considered "environmentally relevant" [14]. Environmentally relevant doses are considered "low doses", and the biological effects that arise from environmentally relevant doses are often referred to as "low-dose effects". Importantly, epidemiological studies have repeatedly linked low doses of BPA to adverse health and increased disease prevalence in the general population [14].

1.2 BPA During Pregnancy

EDCs can elicit different effects depending on the life stage during which an animal is exposed [36]. After puberty, exposure to EDCs results mainly in "activational effects", which are transient and generally reversible. Conversely, during critical periods of development, EDCs may exert permanent "organizational effects" that persist even in the absence of subsequent exposure [36]. Exposure to BPA during pregnancy is of

particular concern, as fetal organs are undergoing critical developmental processes. Previous studies indicate that BPA reaches the fetus during pregnancy, and exposure to BPA *in utero* has been shown to alter the development of several fetal organs, including the brain [37,38], heart [39], mammary glands [40–42], ovaries [43,44], uterus [45], testes [46], and lungs [47].

1.2.1 Developmental Origins of Health and Disease

The Developmental Origins of Health and Disease (DOHaD) concept describes how early life environmental perturbations can exert programming effects on the fetus, leading to permanent changes in the body's structure, function, and metabolism, and increasing the risk for disease later in life [48]. This hypothesis was first proposed by David Barker in the 1980's, who revealed that the starvation of Dutch pregnant women during World War I was correlated with an increased risk for adult-onset of cardiovascular and metabolic disease in their underweight offspring [49]. This association was subsequently confirmed in a number of worldwide longitudinal studies [50], and undernutrition during gestation was considered an important early fetal origin of cardiovascular and metabolic disorders [48]. As the DOHaD gradually gained acceptance, the focus of research expanded to incorporate a number of other factors during gestation that might exert programming effects on the fetus [51].

It has now become clear that many diseases have their origins during fetal development [51]. The prenatal period is considered especially critical given the increased sensitivity of tissues undergoing growth, differentiation, and physiological maturation [49]. Accordingly, the fetus is especially vulnerable to the adverse effects of

EDCs, such as BPA. Environmental influences do not usually result in significant defects or malformations that are apparent at birth, but rather manifest as subtle functional changes (increased cell numbers, altered gene expression, etc.) that increase the risk for dysfunction and diseases later in life [49]. Sex-specific effects may also apparent as early as in the developing fetus [49].

1.2.2 Fetal Exposure to BPA

One of the major functions of the placenta is to act as a barrier for the fetus against xenobiotics such as drugs and other compounds [52]. However, the placenta seems to serve as a rather ineffective barrier against BPA. Due to its high lipid-solubility, free BPA can rapidly cross the placenta in both directions, mainly via passive diffusion [53,54]. Although the placenta expresses UGTs and SULTs, studies have shown that only negligible amounts of BPA are conjugated by the placenta [54]. Moreover, BPA has been detected in maternal blood, umbilical cord blood, fetal blood, placental tissue, and amniotic fluid [52,54–56], suggesting that BPA reaches the fetus during pregnancy.

During fetal development, endogenous estrogens are predominantly bound to alpha-fetoprotein (AFP), a high affinity binding protein produced by the fetal liver [14,57]. Accordingly, the developing fetus is protected from excessive estrogen exposure, as relatively low levels of endogenous estrogens circulate as the free, biologically active form [57]. Conversely, BPA possesses a limited binding capacity for AFP [57]. Thus, although BPA possesses a lower affinity for ER compared to estradiol, its estrogenic effects *in utero* are suggested to be greater than would be expected, given that a higher proportion of BPA circulates as the free, biologically active form [57].

Moreover, the Oatp and Mrp family of transporters have been implicated in the maternal-fetal transport of BPA-GA, the concentration of which is reportedly elevated in maternal blood of pregnant women for the entire gestational period [3]. Specifically, studies have shown that BPA-GA is carried from maternal blood vessels to trophoblast cells by the Oatp4a1 (influx) transporter, and then transported to fetal cells from trophoblasts via the Mrp1 (efflux) transporter [52]. Since these metabolites are water-soluble, they are less able to cross the placenta and once excreted in fetal urine, are trapped in amniotic fluid with the potential to be swallowed and re-circulated in the fetus [53]. Although BPA-GA is an inactive metabolite, fetal tissues possess the ability to deconjugate BPA-GA to active BPA via the enzyme β-glucuronidase [52]. The majority of deconjugation occurs in the fetal liver [52], which also represents the major site of BPA accumulation [58]. Deconjugation is also evident in the heart, but to a much smaller degree [52]. This, in combination with the limited drug metabolizing system of the fetus [52,55], further exacerbates fetal exposure to BPA.

1.3 The Liver

The liver is the largest internal organ, accounting for 2-5% of total body mass [59], and is responsible for carrying out a number of exocrine, endocrine, and metabolic functions [60,61] that are essential for the maintenance of overall homeostasis [62,63]. Structurally, the liver is characterized by a complex tissue architecture that is critical for normal hepatic function [59]. The liver also possesses the remarkable capacity to completely regenerate when up to 70% of its volume is removed [60].

1.3.1 Physiological Functions

Hepatocytes are major functional cells of the liver and account for approximately 80% of total liver volume. One of the key functions of hepatocytes is the production of proteins, including albumin (25% of hepatic protein production), lipoproteins, globulins, clotting factors, and certain hormones [64]. Hepatocytes also produce bile for the purpose of aiding in the absorption of fats as well as the excretion of other water-insoluble substances [64]. Subsequent to its production in the liver, bile is transported to the gall bladder for storage until being released into the small intestine during a meal. One of the most critical functions of the liver is the regulation of blood glucose levels. The liver is the major site of gluconeogenesis [64]. Additionally, hepatocytes will store excess glucose as glycogen after a meal, where its synthesis and degradation is hormonally regulated to maintain whole-body blood glucose requirements [64,65]. The liver is also essential for the metabolism of other dietary compounds, as well as detoxification, urea metabolism, and cholesterol synthesis and transport [59,60].

The remaining 20% of the liver is made up of a diverse group of non-parenchymal cells that function in concert with hepatocytes, including biliary epithelial cells (BECs; also known as cholangiocytes), endothelial cells, Kupffer cells (macrophages), stellate cells, and pit cells (natural killer cells) [59]. **Table 1-1** summarizes each cell type and their respective functions.

Cell Type	Population (%)	Position	Function(s)
Hepatocyte	~80%	Parenchyma	- Protein production & secretion - Bile production & secretion - Glucose metabolism - Glycogen metabolism & storage - Detoxification - Cholesterol metabolism - Urea metabolism
Cholangiocyte	~3%	Bile duct epithelium	- Bile transport - Water & bicarbonate secretion
Endothelial cells	~3%	Vasculature	- Form walls of veins, arteries, venules, arterioles - Control blood flow
Kupffer cells	~2%	Sinusoids	- Specialized Macrophages
Stellate cells	~1.5%	Perisinusoidal	- Vitamin A & retinoid storage
Pit cells	rare	Sinusoidal lumen	- Cytotoxic activity

Table 1-1. **Adult Liver Cell Types and Respective Functions.** Hepatocytes are the major functional cells of the liver and account for approximately 80% of total liver volume. The rest of the liver is made up of a diverse group of cell types that function in concert with hepatocytes.

1.3.2 Cellular Architecture

The intrahepatic bile duct (IHBD), portal vein, and hepatic artery run in parallel within the liver, and are collectively referred to as the portal triad [60] (**Figure 1-1**). Each portal triad marks roughly one of six corners of the liver lobule, the basic architectural unit of the liver, which consists of single sheets of hepatocytes (hepatic plates) lined by sinusoidal capillaries [59] (**Figure 1-1**). The lobule is supplied with blood by the portal vein and hepatic artery which, after flowing through the sinusoidal capillaries, exits the lobule via the central vein [59] (**Figure 1-1**). The portal vein sends blood directly to the basal surface of hepatocytes, where the absorption of metabolites and toxins occurs [60]. Hepatocytes are adjoined via tight junctions that form a canaliculus, which receives bile secreted from the apical surface. Bile is then carried through the IHBDs and extrahepatic bile ducts (EHBDs) to the gall bladder for storage [59,60].

The smallest functional unit of the liver is the hepatic acinus, which is positioned between two portal triads, and extends outwards in both directions towards the central veins [66] (**Figure 1-1**). The acinus is divided into three separate zones that each consist of hepatocytes specialized for slightly different functions. Zone 1 is defined according to its proximity to the portal triad. These hepatocytes are exposed to blood with the highest nutrient and oxygen concentrations, and consequently perform the majority of the liver's metabolic functions (glycogenesis, gluconeogenesis, lipid metabolism, and protein synthesis) [66]. Hepatocytes in Zone 3 are positioned closest to the central vein, and are responsible for carrying out glycolysis, urea synthesis, and biotransformation reactions

[66]. Lastly, the functions of hepatocytes in Zone 2 depend mainly on their relative proximity to Zones 1 and 3.

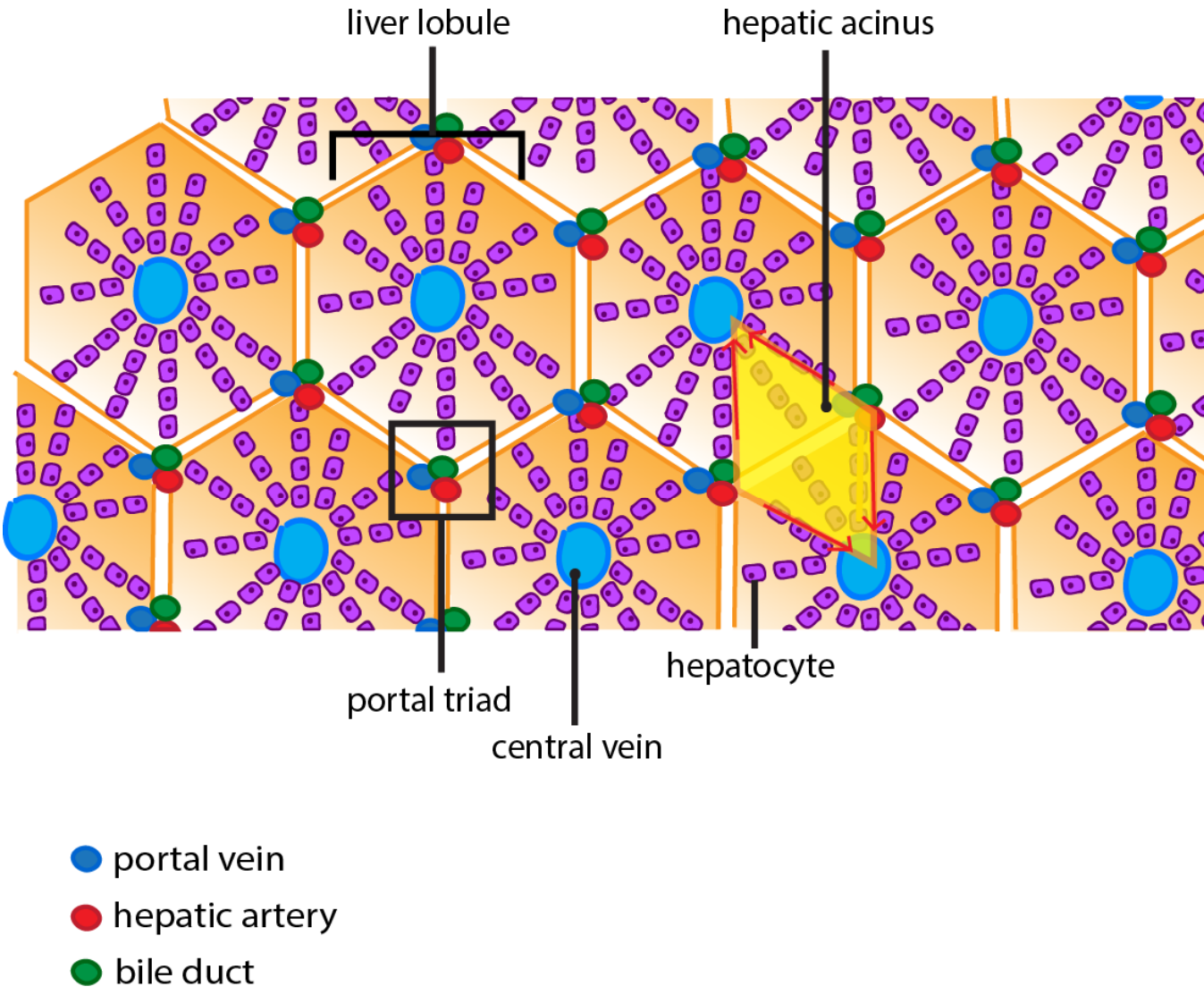

Figure 1-1. Simplified Schematic Diagram of Hepatic Cellular Architecture. The IHBD, portal vein, and hepatic artery make up the portal triad, which marks roughly one of six corners of the liver lobule. Each lobule consists of a central vein from which hepatic plates radiate out. Sinusoidal capillaries are located in between plates of hepatocytes (not shown). The hepatic acinus is positioned between two portal triads, and extends outwards in both directions towards the central veins.

1.3.3 Sexual Dimorphism

The liver is known to exhibit a considerable degree of sexual dimorphism. For example, metabolic function of the liver is known to be markedly sexually dimorphic, which mainly reflects underlying sex differences in the hepatic expression of enzymes such as cytochromes P450, UDP-glucuronosyltransferases, and sulfotransferases [67]. Such enzymes are essential for the metabolism of drugs, fatty acids, environmental toxins, and steroids [67]. Accordingly, the prevalence of a variety of diseases tends to differ between males and females [68]. These patterns are largely mediated by sex-specific secretions in endogenous hormones such as androgens, estrogens, and growth hormone [69].

1.4 Fetal Liver Development

During early gestation, the fetal liver functions largely as a hematopoietic organ [70]. By embryonic day (E) 12.5, the fetal liver is considered the primary site of hematopoiesis [70], with a peak in hematopoietic activity apparent at E13.5 [71]. By E16.5, the major site of hematopoiesis switches to the bone marrow and spleen, at which point the liver begins to acquire its metabolic phenotype [71].

1.4.1 Hepatic Specification and Liver Bud Formation

In the mouse, liver development begins after formation of the definitive gut endoderm at around E8.5 [72,73]. This corresponds to approximately 3 weeks of gestation in the human [61]. The foregut endoderm receives signals from the cardiac mesoderm, septum transversum mesenchyme (STM), and neighbouring endothelial cells that are necessary for hepatic specification [72,74] and subsequent formation of the liver bud [72,75]. Additionally, commitment of the ventral endoderm to a hepatic fate and subsequent liver bud morphogenesis involve the establishment of an essential, complex network of transcription factors [59].

The developing embryonic liver first appears as the hepatic diverticulum (E8.5 to E9), a thickened outward extension of the ventral epithelium which is lined by a basement membrane of endothelial cells [60]. Newly specified pre-hepatic cells are called hepatoblasts, bipotential and proliferative hepatic progenitor cells that are capable of giving rise to either hepatocytes or cholangiocytes [61]. Hepatoblasts in the liver bud already begin to express serum protein genes specific to hepatocytes [75]. Following

hepatic specification, the surrounding basement membrane is lost (E9.5) and hepatoblasts migrate as cords to invade the surrounding STM, forming the liver bud [72]. During this time, considerable liver growth is apparent due to rapid proliferation of hepatoblasts as well as increased hematopoietic activity. The STM and hepatic mesenchyme secrete a variety of growth factors that are important for hepatoblast proliferation and migration [60]. In addition, signals that prevent apoptosis and promote cell survival further support liver bud expansion and differentiation [76].

1.4.2 Differentiation of Hepatic Progenitor Cells

Differentiation of bipotential hepatoblasts is initiated at around E13 to E14.5 [60,61,71]. The number of bipotential hepatoblasts gradually reduces as they eventually become unipotent and irreversibly committed to either the hepatocyte or cholangiocyte cell lineage. The majority of parenchymal hepatoblasts will differentiate into hepatocytes, while those residing next to portal veins will become cholangiocytes, which form the luminal epithelium of IHBDs [61]. The correct overall balance between these two cell types is achieved by integrated signaling and transcriptional pathways [75] which act to either induce or repress mechanisms that direct the cell fate of hepatic progenitors [73]. The status of differentiating hepatic progenitor cells can be generally determined with the use of the following respective cell markers: (1) bipotential hepatoblasts express AFP, albumin, and cytokeratin (CK)-19; (2) hepatocytes express AFP and albumin, but not CK-19; and (3) BECs express CK-19, but neither albumin nor AFP [77,78] (**Figure 1-2**). Following differentiation into hepatocytes and cholangiocytes, both cell types will

undergo proliferation to achieve proper liver size [79]. Apoptosis (programmed cell death) is also essential for shaping the liver and controlling overall cell population [80].

Hepatoblasts initially express genes associated with BECs as well as fetal and adult hepatocytes (**Figure 1-2**). Differentiation into cholangiocytes is initiated by signals from the portal mesenchyme (Wnt, TGFβ) to which adjacent hepatoblasts respond, which act to promote BEC-specific transcription factors (OC1, OC2, HNF1β) and suppress hepatogenic transcription factors (HNF4, C/EBP). This results in an upregulation of CK, and a decrease in the expression of genes specific to hepatocytes [60] (**Figure 1-2**). Other factors continue to be secreted by the portal mesenchyme for successful bile duct formation to proceed (Jagged/Notch, EGF, HGF) [60]. During transformation into cholangiocytes, periportal hepatoblasts form a monolayer, then a bilayer of BEC cuboidal precursors, and by E17 and into the perinatal period, the portal mesenchyme begins to surround focal points depicted in the bilayer of biliary precursor cells, while the remaining bilayer cells regress [60].

Conversely, hepatoblasts in the parenchyma do not receive signals from the portal mesenchyme, which causes them to downregulate BEC-associated gene expression, while allowing them to maintain the expression of genes specific to hepatocytes [60] (**Figure 1-2**). By E17, hepatocytes will acquire their characteristic morphology [60], while functional maturation will resume until after birth.

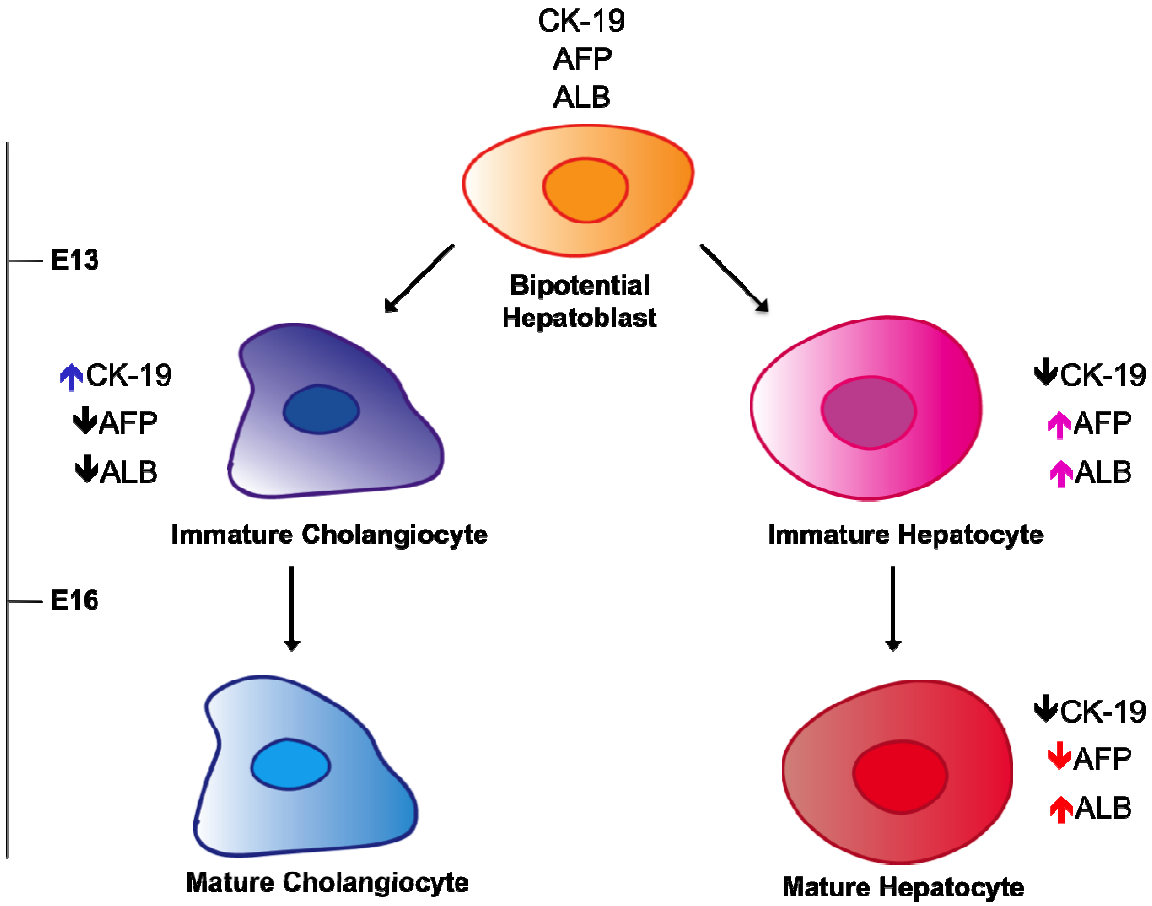

Figure 1-2. Differentiation Markers of Hepatic Progenitor Cells. Bipotential hepatoblasts initially express genes associated with BECs as well as fetal and adult hepatocytes. Hepatic progenitor cells differentiating into cholangiocytes will upregulate genes associated with BECs, while downregulating hepatocyte-specific genes. In contrast, hepatic progenitor cells differentiating into hepatocytes will downregulate BEC-associated gene expression, while upregulating hepatocyte-specific genes.

1.4.3 Functional Maturation of Hepatocytes

Subsequent to lineage segregation and commitment, immature/transitional hepatocytes gradually differentiate into mature hepatocytes, exhibiting progressive functional changes up until several weeks after birth. One major functional transition that takes place is in the production of hepatic plasma proteins. Albumin is the major plasma protein produced by mature hepatocytes, constituting more than half of total plasma protein concentration in the adult [81]. Albumin synthesis takes place exclusively in the liver, and is secreted into circulation as soon as it is manufactured [81] where it becomes involved in the binding and transport of various drugs, hormones, lipids, and anions, and the maintenance of oncotic pressure [82]. Albumin mRNA is first detected in fetal hepatocytes at E10.5 (gestational week 16 in humans), and its expression increases progressively thereafter until it reaches maximal levels in the adult (**Figure 1-3**) [71,83,84].

On the contrary, AFP is the predominant plasma protein produced by the fetal liver, and is largely considered the fetal equivalent of the adult serum albumin [85]. During prenatal development, AFP expression increases steadily from E9 up to E15.5 (~1000-fold), at which time it begins to decline as the liver matures, ultimately becoming undetectable in the adult (**Figure 1-3**) [71,85,86]. This pattern also corresponds to that observed in humans, where AFP production reaches a peak at the end of the first trimester and declines thereafter [66]. Because of the respective temporal expression profiles of albumin and AFP, these two proteins can be effectively used as markers to determine the status of hepatocyte differentiation if delayed or disrupted maturation is

suspected [71]. In particular, albumin is considered a hallmark of mature hepatocytes [60,87,88], while AFP is commonly used as a marker of immature, fetal hepatocytes [89,90].

Another important functional transition is in glycogen storage capability. During most of gestation, hepatic glycogen stores remains low as the fetus obtains an adequate supply of glucose from the mother via the placenta [66,91]. During late gestation (E17.5), hepatocytes start to accumulate significant amounts of glycogen in order to prepare for the extrauterine survival at birth, before the onset of hepatic gluconeogenesis (**Figure 1-3**) [92]. In humans, fetal hepatic glycogen stores measure to 3.4 mg/g of liver tissue at 8 to 9 weeks of gestation, and increase to an average of 50 mg/g immediately prior to term [66,79], amounting to 2-3 times those of adults [79]. This surge in glycogen accumulation at the end of gestation is accompanied by a corresponding increase in glycogen synthase [93], the rate-limiting enzyme of glycogen synthesis [94,95]. Thus, glycogen storage is also used as a marker of mature hepatocytes [88,96,97].

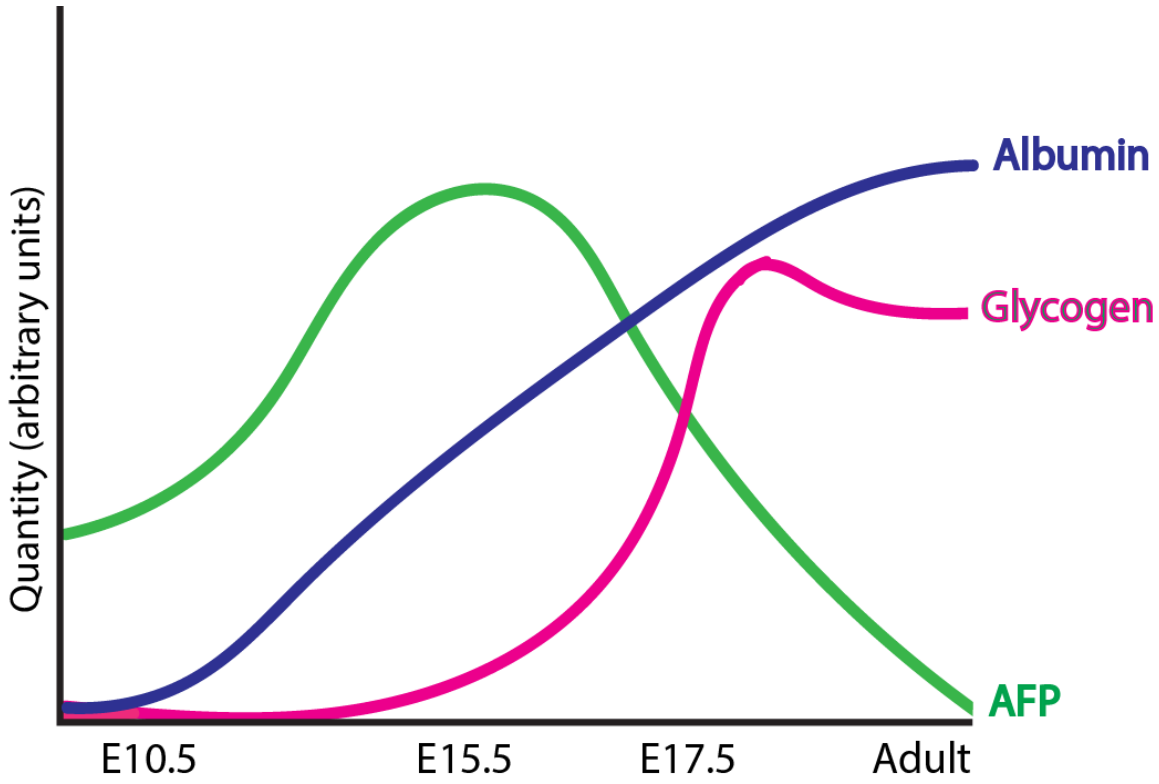

Figure 1-3. Expression Profiles of Hepatocyte Maturation Markers. Albumin expression begins in the fetal liver at E10.5, and steadily increases until reaching maximal levels in the adult. Hepatic glycogen stores remain low until late gestation, at which point hepatocytes accumulate significant amounts of glycogen such that by term, glycogen storage amounts to 2-3 times that observed in the adult. Albumin and glycogen are thus considered hepatocyte maturation markers. In contrast, AFP expression increases steadily from E9 up until E15.5, at which time it begins to progressively decline as the liver undergoes continued maturation. AFP is thus considered a marker of immature, fetal hepatocytes.

1.4.4 Transcriptional Regulation

Hepatocyte differentiation is associated with alterations in the coordinated expression of a number of hepatic genes. These genes are primarily controlled through interactions between several “liver-enriched” transcription factors [98] that are indispensible for the induction and progression of liver development. These include hepatocyte nuclear factors (HNFs) HNF-1, HNF-3, HNF-4, HNF-6, and CCAAT/enhance binding proteins (C/EBPs) [86,98], each of which exhibit their own temporal expression profiles that coincide with distinct stages of hepatic maturation. Quantitative gene expression profiling has revealed that HNF-1, HNF-3, HNF-4, and HNF-6 exhibit particularly similar timelines of expression, with a peak in gene expression apparent at E9.5 and E11.5 [86]. Based on their expression patterns, these transcription factors have been suggested to exert primary roles during the earlier phases of liver development [86]. By contrast, C/EBPs have been implicated during later stages of development, at which time hepatocytes are undergoing functional maturation. Correspondingly, expression levels of C/EBP-α and C/EBP-β are most marked in adult liver tissue [86].

1.4.5 C/EBP-α

As a member of the bZIP class of leucine zipper transcription factors, C/EBP-α comprises a C-terminal leucine zipper, a basic DNA binding region, and an N-terminal transactivating region [99], and binds DNA in a sequence-specific manner [100]. Expression of C/EBP-α is tissue-specific, with most abundant levels apparent in liver, lung, and fat tissue [101]. While targeted disruption of the C/EBP-β gene fails to produce

substantial consequences on normal liver development [102], a strong mount of evidence supports a critical role for C/EBP-α in hepatocyte maturation, and its involvement in regulating the balance between hepatocyte growth and differentiation. In fact, inadequate maturation of hepatocytes has been attributed specifically to the loss of C/EBP-α [103].

C/EBP-α has been shown to govern the transcription of several hepatocyte-specific genes including albumin, AFP, and glycogen synthase [104,105]. Additionally, C/EBP-α possesses a dominant anti-proliferative effect, and accordingly, its expression is generally restricted to growth-arrested cells [100]. In fetal hepatocytes, C/EBP-α is expressed at around E14.5, which corresponds to the time hepatocytes begin to undergo maturation. Expression of C/EBP-α gradually increases as development proceeds, reaching a maximum in the adult [102]. The developmental increase in C/EBP-α expression corresponds to the expression profile of genes associated with mature hepatocytes, including albumin and glycogen synthase, and is inversely associated with the expression of the immature hepatocyte marker, AFP. Moreover, neonatal livers of C/EBP-α knockout mice exhibit properties characteristic of a dedifferentiated state, including decreased albumin expression, diminished glycogen stores and glycogen synthase expression, significantly elevated AFP levels, as well as increased cell proliferation [106,107].

1.5 Rationale, Objectives, and Hypothesis

1.5.1 Rationale

BPA is one of the most prevalent EDCs in the environment. Of particular concern is exposure to BPA during pregnancy, a critical time during which key organs are undergoing growth and differentiation. Previously, exposure to BPA *in utero* has been shown to alter the development of several fetal organs, including the brain [37,38], heart [39], mammary glands [40–42], ovaries [43,44], uterus [45], testes [46], and lungs [47]. The altered fetal organ development following BPA exposure may provide a fetal origin for various BPA-induced diseases in adult life.

The liver is a key metabolic organ and is essential for the maintenance of overall homeostasis. Proper liver maturation is critical not only for neonatal survival by supplying adequate glucose from hepatic glycogen storage, but also for proper hepatic function later in life [89]. Developmental exposure to BPA is known to be associated with liver dysfunction and diseases, such as hepatic steatosis [108,109], liver tumors [110], metabolic syndrome [111,112], and altered hepatic gene expression [113,114] and DNA methylation profiles [115,116]. However, it is unknown whether these BPA-induced hepatic dysfunctions and diseases have a fetal origin. Therefore, the present study was designed to address this important question by examining the effects of prenatal exposure to BPA on fetal liver maturation using the mouse as an experimental model.

1.5.2 Objectives

(1) To determine the effects of BPA on overall fetal growth morphology (i.e., fetal body weight, litter size and sex ratios)

(2) To examine the effects of BPA on fetal liver structural maturation.

(3) To determine the effects of BPA on fetal liver biochemical maturation.

(4) To determine the effects of BPA on the balance between cell proliferation and apoptosis in the fetal liver.

(5) To ascertain if the effects of BPA on fetal liver maturation are sex-specific.

1.5.3 Hypothesis

We hypothesize that prenatal exposure to BPA disrupts fetal liver maturation in the mouse.

Chapter 2 – Methodology

2.1 Animal Experiments

Breeding pairs of adult C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were housed under standard conditions and provided with food and water *ad libitum*. Polystyrene cages were utilized in order to minimize background exposure to BPA beyond treatment regimen. Mice were maintained at humidity- and temperature-controlled rooms under a normal 12h/12h light-dark cycle. For experiments, 6-8 week old female mice were placed overnight with males, and pregnancy was determined the next morning by the observation of a vaginal plug. Plugged females were separated from males, and gestational days were counted, with presence of a vaginal plug indicating embryonic day 0.5 (E0.5). Pregnant mice were randomly assigned to receive one of the following two diets: (1) control diet (phytoestrogen free food pellets supplemented with 7% corn oil; TD.120176, Harlan Teklad, Madison, WI), or (2) control diet supplemented with 25 mg BPA/kg diet (TD.120466, Harlan Teklad) (**Figure 2-1**). Oral administration was chosen to mimic the most common route of exposure in humans. Feeding was initiated at E7.5, subsequent to successful implantation and just prior to the onset of liver development, and resumed up until E18.5 (**Figure 2-1**). At E18.5, animals were euthanized by CO_2 euthanasia (**Figure 2-1**). Fetuses were recovered by caesarean section, and their weights recorded. In addition, maternal weight, pup number, and the number of reabsorption sites per uterine horn were noted. Fetal livers were collected and either snap frozen in liquid nitrogen and stored at -80°C, or fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. Fetal

limbs were also collected, snap frozen in liquid nitrogen, and stored at -80°C.

2.2 Genotyping PCR

Fetal sex was determined by standard polymerase chain reaction (PCR) for the presence of the male-specific SRY gene. Briefly, DNA was isolated from fetal limb samples. The PCR reactions were carried out using the Platinum Taq DNA Polymerase Kit (cat. no. 10966-026, Invitrogen), with the primers SRY-F (5'-GCA GGT GGA AAA GCC TTA CA-3') and SRY-R (5'-AAG CTT TGC TGG TTT TTG G-3'). PCR amplifications were carried out for 30 cycles (20 seconds at 95°C, 20 seconds at 55°C, and 35 seconds at 72°C) on the Eppendorf Mastercycler® Gradient PCR System (Eppendorf). PCR products were run on a 1% agarose gel, and fetal sex was determined to be male if a DNA product at 271-bp (indicative of the presence of the SRY gene) was observed.

2.3 Histology

Fetal livers were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer solution (PBS) buffer, dehydrated, and embedded in paraffin wax. Using a rocking microtome, the lungs were sectioned; 5-μm sections were transferred to Superfrost Plus microscope slides (Fisher Scientific, Whitby, ON). Sections were stained using a standard hematoxylin and eosin (H&E) protocol [117].

2.4 Protein Extraction and Western Blot Analysis

Western blot analysis was conducted in order to assess protein expression levels, as

described previously [117]. Since limited tissue was available to us, we made the decision to detect protein levels instead of mRNA. Proteins dictate cellular function [118], and a change in mRNA does not necessarily indicate an alteration in protein expression levels [119]. Given that the purpose of this study was not mechanistic, but rather to examine the biochemical maturation of BPA-exposed fetal livers, protein levels were more meaningful and physiologically-relevant to the present study.

First, liver tissues were homogenized in 10 volumes of ice-cold 10 mM sodium phosphate buffer, pH 7.0, containing 0.25 M sucrose. Equal volumes of the homogenates were mixed with SDS gel loading buffer (50 mM Tris·HCl, pH 6.8, 2% wt/vol SDS, 10% vol/vol glycerol, 100 mM DTT, and 0.1% wt/vol bromophenol blue). Equal concentrations of this mixture were then subjected to a 10% SDS-PAGE. After electrophoresis, proteins were transferred to PVDF membranes (Amersham Hybond-P, cat. no. RPN303F, GE Healthcare Lifesciences, Baie D'Urfe, QC) using a Bio-Rad Mini Transfer Apparatus. Nonspecific antibody binding was blocked with 5% wt/vol milk in TBST (0.1% vol/vol Tween-20 in TBS) for 1 hour at room temperature. Membranes were then hybridized with primary antibody (albumin: cat. no. SAB2100098, 1:5,000 dilution, Sigma-Aldrich, Saint Louis, MO; glycogen synthase: cat. no. 3886, 1:1,000 dilution, Cell Signaling Technology, Beverly, MA; AFP: cat. no. sc-8108, 1:1000 dilution, Santa Cruz Biotechnology, Dallas, TX; C/EBP-α: cat no. sc-61, 1:500 dilution, Santa Cruz Biotechnology; PCNA: cat. no. 2586, 1:1,000 dilution, Cell Signaling Technology; caspase-3: cat. no. 9662, 1:1,000 dilution, Cell Signaling Technology; GAPDH: cat. no. IMG-3073, 1:5,000, Imgenex) overnight at 4°C. The membrane underwent 3 × 10 min washes with TBST, and was then incubated with an anti-rabbit secondary antibody (cat.

no. HAF008, 1:500 dilution, R & D Systems) or anti-mouse secondary antibody (cat. no. G-202-C, 1:10,000 dilution, Fisher Scientific) for 1 hour at room temperature. Following another 3 x 10 min washes in TBST, proteins were detected using chemiluminescence (cat. no. WBLUR0500, Luminata Crescendo, Western HRP Substrate; Millipore, Etobicoke, ON). The membrane was viewed using the VersaDoc Imaging System (BioRad, UK). Densitometry was performed on the images and the level of various proteins expressed as percent of controls.

2.5 Statistical Analysis

Results are presented as the mean ± SEM of four different litters. Livers from three pups were pooled per litter. Data were analyzed using Student's t-test. Significance was set at $P < 0.05$.

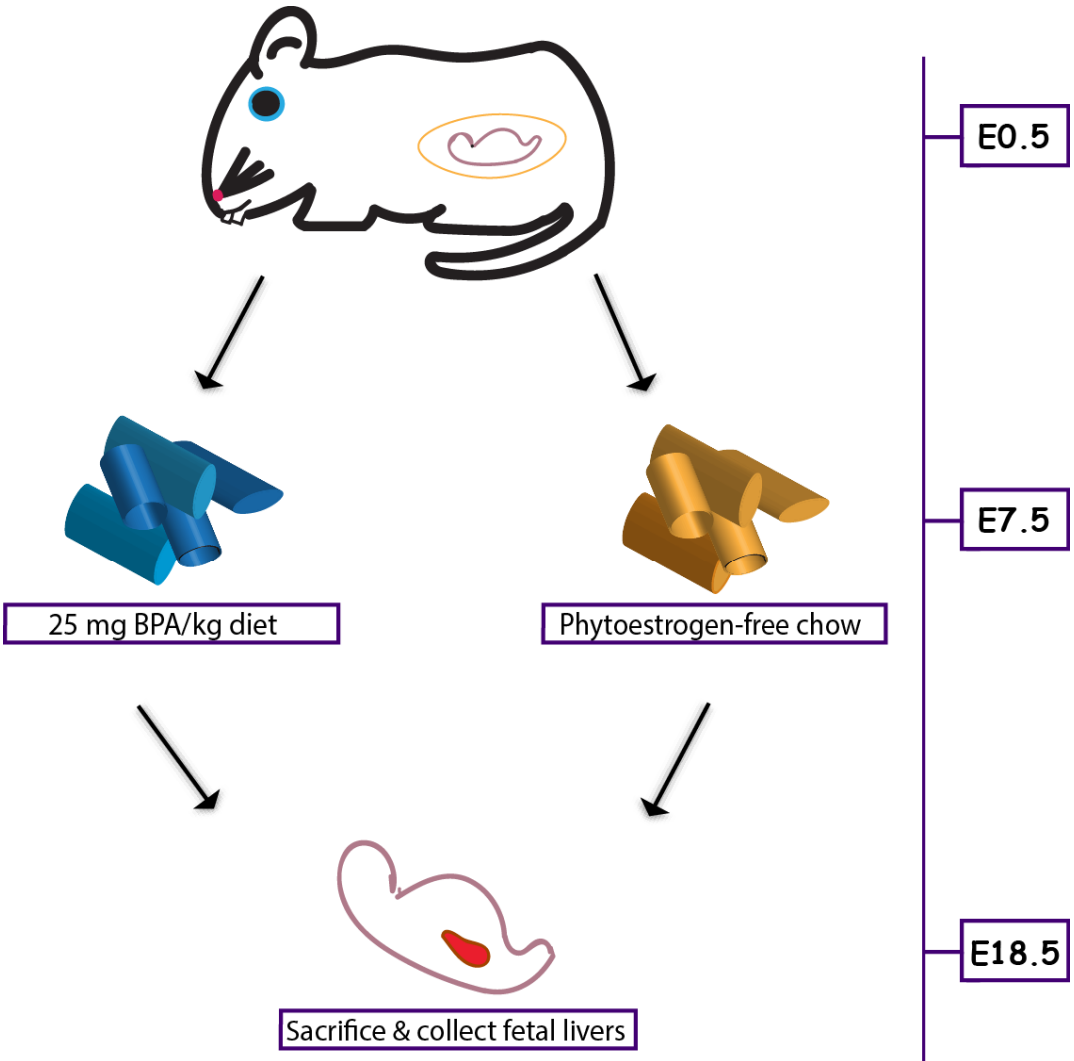

Figure 2-1. Experimental Design. Pregnant mice were randomly assigned to receive one of the following two diets: (1) control diet (phytoestrogen-free food pellets supplemented with 7% corn oil); or (2) treatment diet (control diet supplemented with 25 mg BPA/kg diet). Feeding was initiated at E7.5, subsequent to successful implantation and just prior to the onset of liver development, and resumed up until E18.5. At E18.5, animals were euthanized by CO_2 euthanasia. Fetal livers were collected and either snap frozen in liquid nitrogen and stored at -80°C, or fixed in 4% PFA in 0.1 M PBS.

Chapter 3 – Results

3.1 Effects of BPA on Fetal Body Weight, Litter Size, and Sex Ratio

As a first step in determining the effect of prenatal exposure on fetal organ maturation, we examined fetal body weight, litter size, and litter sex ratio at E18.5. We found that there was no difference in fetal body weight, litter size, or sex ratio (expressed as percentage of females per litter) between control and BPA-exposed fetuses (**Figure 3-1 A-C**).

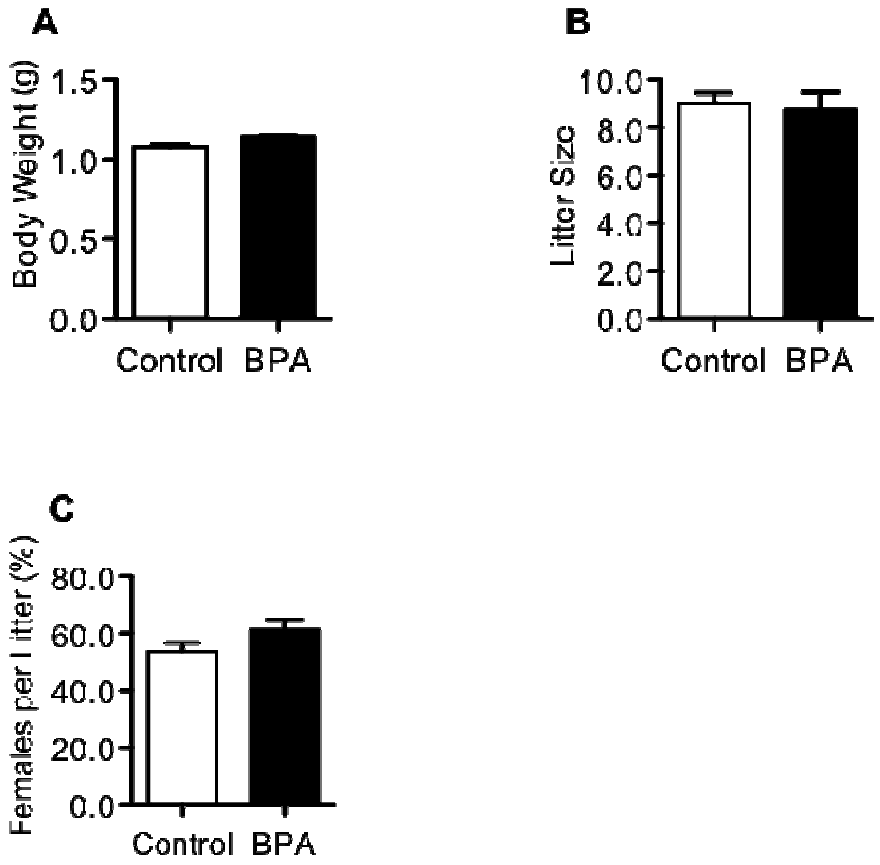

Figure 3-1. Effects of BPA on Fetal Body Weight, Litter Size, and Litter Sex Ratio. Pregnant mice were fed a control diet (phytoestrogen free food pellets) or the control diet supplemented with 25 mg of BPA/kg food pellets from embryonic day (E) 7.5 to 18.5. On E18.5, mice were sacrificed and fetal body weight (*A*) and litter size (*B*) were recorded. Sex ratio of litters were expressed as the percentage of females per total number of pups in the litter (*C*). Data are presented as the mean ± SEM (n = 20).

3.2 Effects of BPA on Fetal Liver Histology

Standard histological analysis was performed in order to determine whether prenatal exposure to BPA affects structural maturation of fetal livers at E18.5. Upon analysis, we found that there were no observable structural differences in BPA exposed fetal livers (**Figure 3-2 B and D**) compared to controls (**Figure 3-2 A and C**).

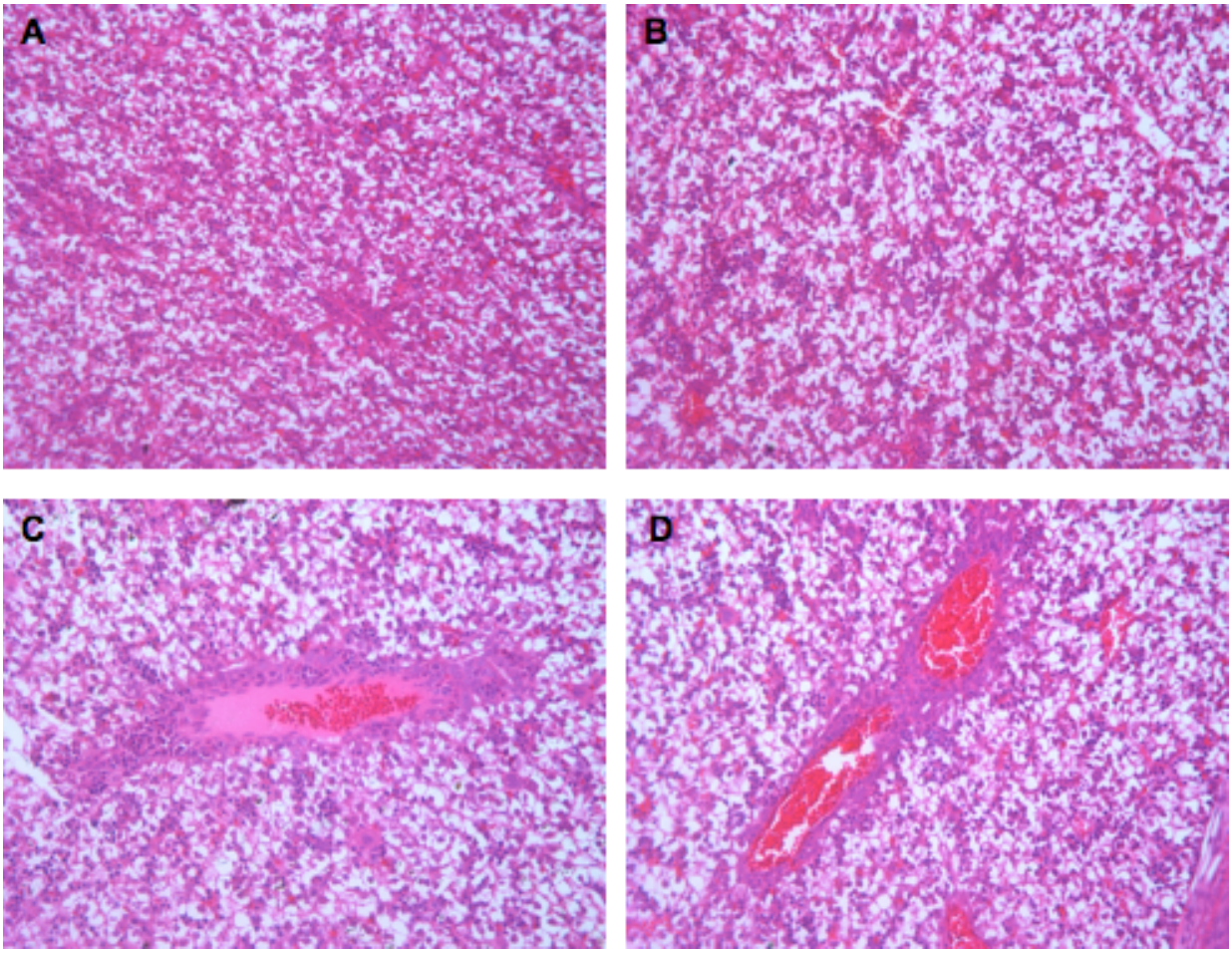

Figure 3-2. Effects of BPA on Fetal Liver Histology. Pregnant mice were fed a control diet (phytoestrogen free food pellets) or the control diet supplemented with 25 mg of BPA/kg food pellets from embryonic day (E) 7.5 to 18.5. On E18.5, mice were sacrificed and fetal livers were collected, fixed and subjected to histological analysis. Representative fetal liver sections from mice fed the control diet (*A* and *C*) and the control diet supplemented with 25 mg BPA/kg diet (*B* and *D*) are shown (n = 3).

3.3 Effects of BPA on Albumin Protein Expression

To determine whether prenatal exposure to BPA affected fetal liver maturation, we first examined albumin expression, a well-known marker of, and the most abundant protein synthesized by, mature hepatocytes [60,87,88]. We found that levels of albumin protein were significantly decreased in BPA-exposed female fetal livers when compared to controls (35% of control; **Figure 3-3 A and C**). In marked contrast, the abundance of albumin protein was not altered in the fetal livers of BPA-exposed males (**Figure 3-3 B and D**).

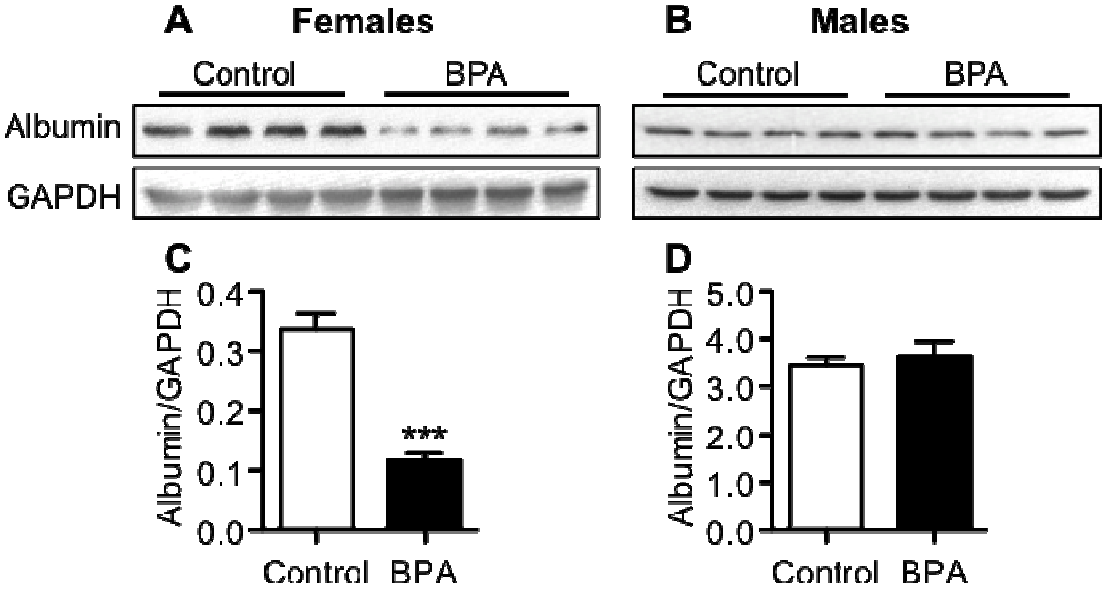

Figure 3-3. Effects of BPA on albumin protein expression. Pregnant mice were fed a control diet (phytoestrogen free food pellets) or the control diet supplemented with 25 mg of BPA/kg food pellets from embryonic day (E) 7.5 to 18.5. On E18.5, mice were sacrificed and fetal livers were collected, flash frozen in liquid nitrogen, and stored at -80°C. Levels of albumin protein in female (*A* and *C*) and male (*B* and *D*) fetal livers were determined by western blot analysis. Data are presented as the mean ± SEM (****P* < 0.001; n = 4 litters, livers from three pups were pooled per litter).

3.4 Effects of BPA on Glycogen Synthase Protein Expression

Glycogen storage is an important marker of mature hepatocytes [88,96,97]. Prior to birth, a rapid accumulation of glycogen in the fetal liver is accompanied by a corresponding increase in glycogen synthase [93,120]. We next determined changes in the expression of glycogen synthase. We found that the level of glycogen synthase protein was significantly decreased in the fetal livers of female BPA-exposed mice when compared to controls (60% of control; **Figure 3-4 A and C**). However, there was no change in the level of glycogen synthase protein in BPA-exposed male fetal livers (**Figure 3-4 B and D**).

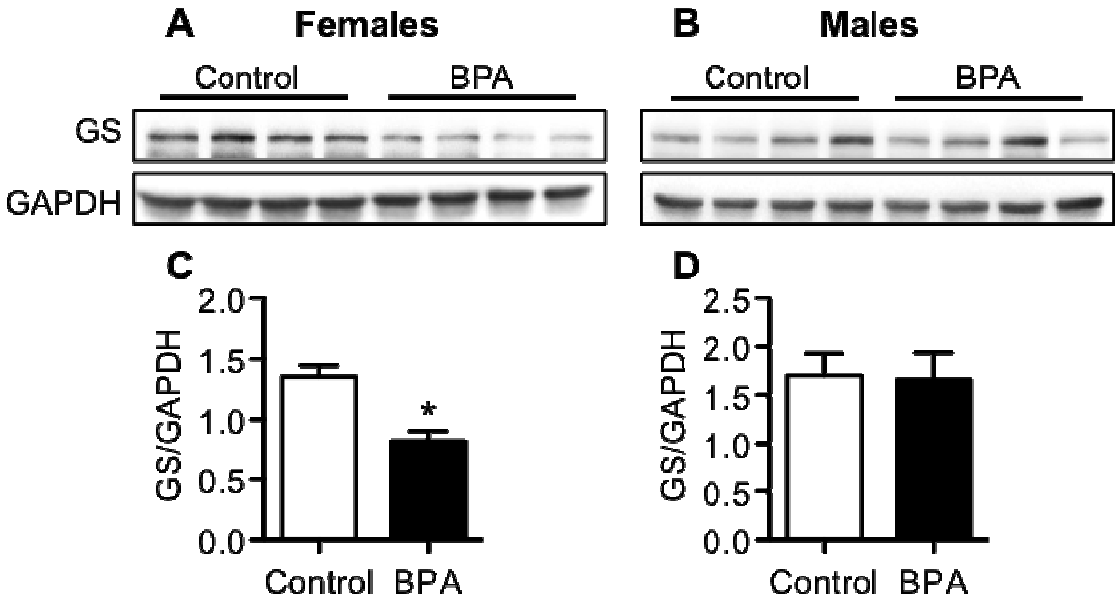

Figure 3-4. Effects of BPA on glycogen synthase protein expression. Pregnant mice were fed a control diet (phytoestrogen free food pellets) or the control diet supplemented with 25 mg of BPA/kg food pellets from embryonic day (E) 7.5 to 18.5. On E18.5, mice were sacrificed and fetal livers were collected, flash frozen in liquid nitrogen, and stored at -80°C. Levels of glycogen synthase (GS) protein in female (*A* and *C*) and male (*B* and *D*) fetal livers were determined by western blot analysis. Data are presented as the mean ± SEM (**$P < 0.01$; n = 4 litters, livers from three pups were pooled per litter).

3.5 Effects of BPA on AFP Protein Expression

To gain further insight into the effects of BPA on fetal liver maturation, we examined changes in the expression of AFP, a well-known marker of immature hepatocytes [89,90]. We found that levels of AFP protein were significantly increased in BPA-exposed female fetal livers when compared to controls (143% of control; **Figure 3-5 A and C**). In contrast, AFP protein abundance was not changed in BPA-exposed male fetal livers (**Figure 3-5 B and D**).

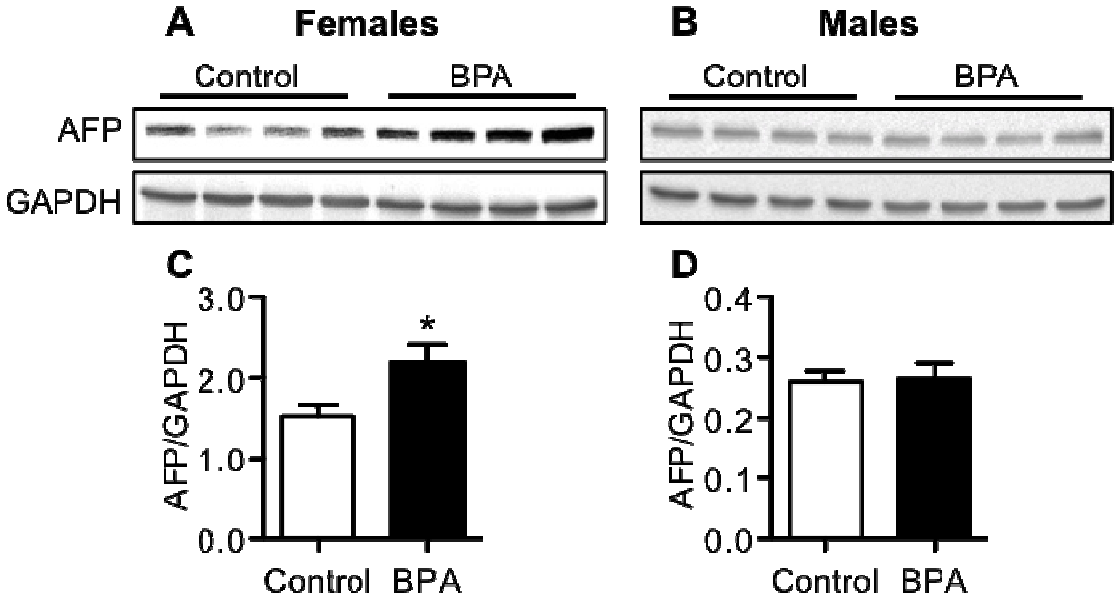

Figure 3-5. Effects of BPA on AFP protein expression. Pregnant mice were fed a control diet (phytoestrogen free food pellets) or the control diet supplemented with 25 mg of BPA/kg food pellets from embryonic day (E) 7.5 to 18.5. On E18.5, mice were sacrificed and fetal livers were collected, flash frozen in liquid nitrogen, and stored at -80°C. Levels of AFP protein in female (*A* and *C*) and male (*B* and *D*) fetal livers were determined by western blot analysis. Data are presented as the mean ± SEM (*$P < 0.05$; n = 4 litters, livers from three pups were pooled per litter).

3.6 Effects of BPA on C/EBP-α Protein Expression

Given that C/EBP-α is a master transcription factor essential for hepatocyte differentiation [86,121], we sought changes in the expression of this transcription factor following exposure to BPA. We showed that levels of C/EBP-α protein were decreased by 50% in BPA-exposed female fetal livers when compared to controls (**Figure 3-6 A and C**). By contrast, the level of C/EBP-α protein in BPA-exposed male fetal livers was not changed (**Figure 3-6 B and D**).

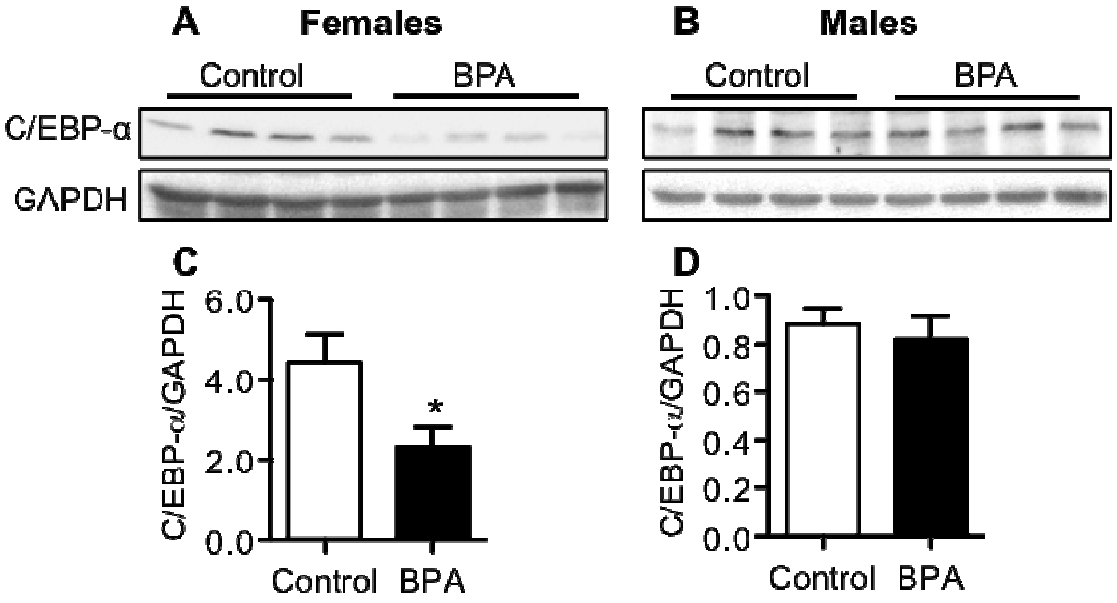

Figure 3-6. Effects of BPA on C/EBP-α protein expression. Pregnant mice were fed a control diet (phytoestrogen free food pellets) or the control diet supplemented with 25 mg of BPA/kg food pellets from embryonic day (E) 7.5 to 18.5. On E18.5, mice were sacrificed and fetal livers were collected, flash frozen in liquid nitrogen, and stored at -80°C. Levels of C/EBP-α protein in female (*A* and *C*) and male (*B* and *D*) fetal livers were determined by western blot analysis. Data are presented as the mean ± SEM (*$P < 0.05$; n = 4 litters, livers from three pups were pooled per litter).

3.7 Effects of BPA on PCNA Protein Expression

Normal organ growth and maturation depends critically on the right balance between cell proliferation and apoptosis [80]. Thus, we sought to determine if this balance might be perturbed in BPA-exposed fetal livers. To do so, we first examined the proliferative status of the BPA-exposed fetal liver by analyzing the expression of PCNA, a universal marker of cell proliferation. We showed that levels of PCNA protein were up-regulated significantly in BPA-exposed female fetal livers when compared to controls (160% of control; **Figure 3-7 A and C**). In contrast, the level of PCNA protein was unchanged in BPA-exposed male fetal livers (**Figure 3-7 B and D**).

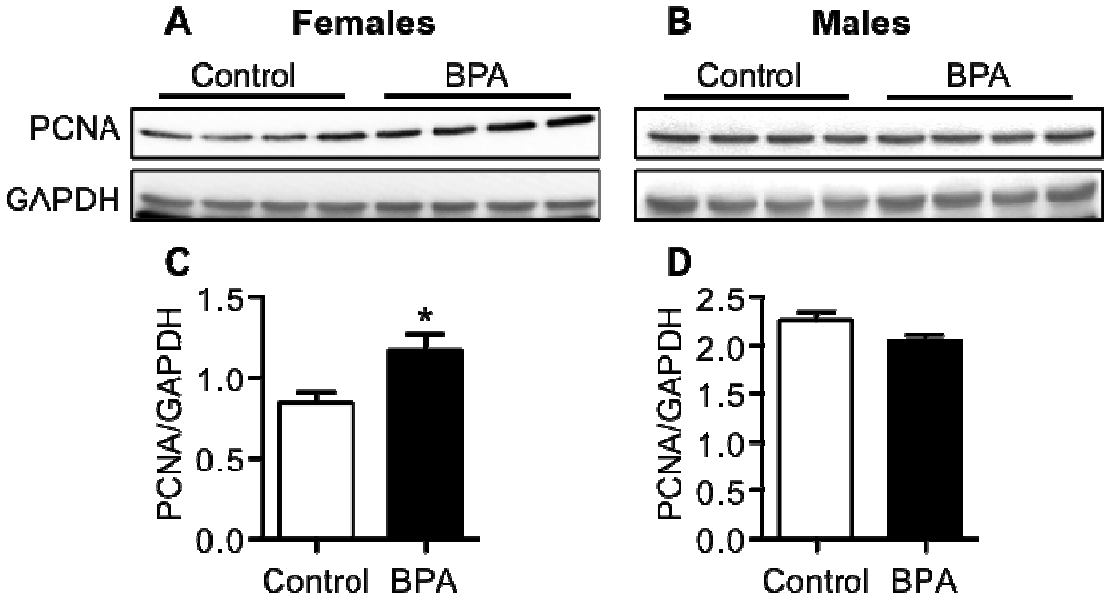

Figure 3-7. Effects of BPA on PCNA protein expression. Pregnant mice were fed a control diet (phytoestrogen free food pellets) or the control diet supplemented with 25 mg of BPA/kg food pellets from embryonic day (E) 7.5 to 18.5. On E18.5, mice were sacrificed and fetal livers were collected, flash frozen in liquid nitrogen, and stored at -80°C. Levels of PCNA protein in female (*A* and *C*) and male (*B* and *D*) fetal livers were determined by western blot analysis. Data are presented as the mean ± SEM (*$P < 0.05$; n = 4 litters, livers from three pups were pooled per litter).

3.8 Effects of BPA on Caspase-3 Protein Expression

We then examined the effect of BPA on the expression of caspase-3, a universal marker of apoptosis. We found that protein levels of both procaspase-3 (60% of control; **Figure 3-8 A and C**) and cleaved caspase-3 (45% of control; **Figure 3-8 A and 6E**) were significantly down-regulated in BPA-exposed female fetal livers when compared to controls. By contrast, no changes in the protein level of either procaspase-3 (**Figure 3-8 B and D**) or cleaved caspase-3 (**Figure 3-8 B and F**) were observed in livers of BPA-exposed male fetuses.

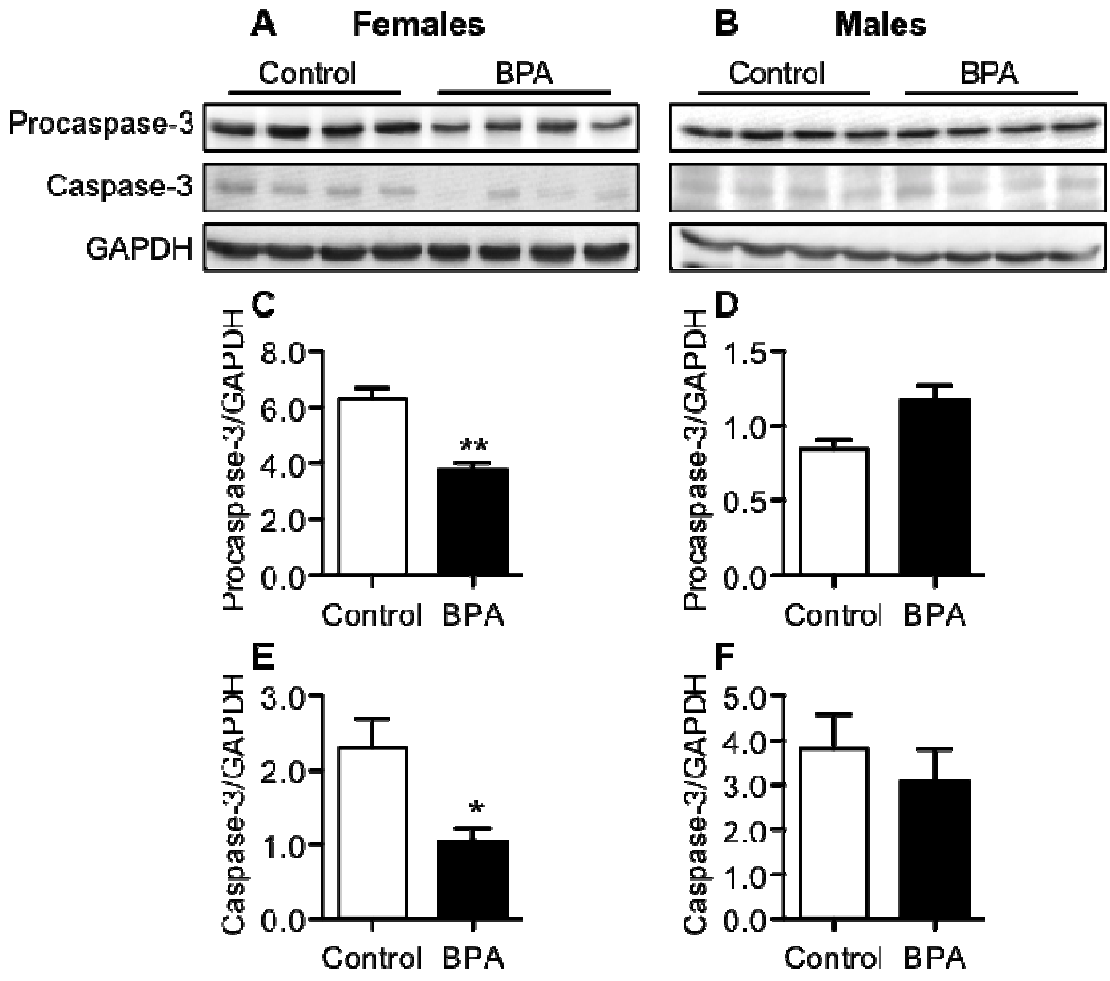

Figure 3-8. Effects of BPA on caspase-3 protein expression. Pregnant mice were fed a control diet (phytoestrogen free food pellets) or the control diet supplemented with 25 mg of BPA/kg food pellets from embryonic day (E) 7.5 to 18.5. On E18.5, mice were sacrificed and fetal livers were collected, flash frozen in liquid nitrogen, and stored at -80°C. Levels of procaspase-3 protein and cleaved caspase-3 protein in female (*A* and *C*) and male (*B* and *D*) fetal livers were determined by western blot analysis. Data are presented as the mean ± SEM (*$P < 0.05$, **$P < 0.01$; n = 4 litters, livers from three pups were pooled per litter).

Chapter 4 – Discussion

There is robust evidence that adverse events in early life can permanently alter organ growth and function, leading to a wide range of diseases later in life, including cardiovascular, metabolic, neurological, reproductive, and behavioral disorders as well as cancers [49,51]. Although developmental exposure to BPA has been shown to cause liver dysfunction and diseases, the effects of BPA on liver development had never been explored. In the present study, we addressed this important question, and demonstrate that *in utero* exposure to environmentally relevant doses of BPA via maternal diet disrupts female, but not male, fetal liver maturation in the mouse. Thus, our findings suggest a fetal origin for BPA-induced liver dysfunction and metabolic diseases.

4.1 Dosage

The dosage of BPA used, 25 mg BPA/kg diet (equivalent to 5 mg/kg/day), in the present study was chosen to mimic BPA exposure at environmentally relevant levels, and had been shown by us previously not to alter fetal body weight when examined at E18.5 [47]. Further, no changes were apparent in litter size or litter sex ratio. This dosage is one tenth of the NOAEL of 50 mg/kg/day for rodents, as defined by the US EPA [122]. BPA exposure was initiated at E7.5, subsequent to successful implantation and just prior to the onset of liver development, and continued until E18.5, one day before term. Although maternal blood BPA levels were not measured in the present study, they were estimated to be 20 ng/ml at the maximum. This estimation was based on a previous study, in which pregnant mice were fed 5 mg BPA/kg diet for a total of 28.5 days (prior to mating and throughout pregnancy), and maternal plasma levels of BPA were found to be 4 ng/ml

[38]. Thus, the estimated maternal circulating levels of BPA in our study are within the range of 0.5–22.3 ng/ml, which has been reported in pregnant women of the US [56].

4.2 BPA Decreases Albumin Protein Expression in Fetal Female Livers

In the mouse, liver development begins at E9. At approximately E14, bipotential hepatoblasts begin to differentiate into either hepatocytes or bile duct epithelial cells [71]. By E16, these two distinct cell types become irreversibly differentiated but continue to undergo maturation for several weeks after birth [75], at which time they are considered mature hepatocytes and cholangiocytes, respectively. In adults, hepatocytes are the main functional units of the liver accounting for nearly 80% of the total liver volume [59,60]. The adult liver in mammals produces a myriad of proteins and enzymes that are crucial for maintaining homeostasis, the most abundant of which is albumin, constituting more than half of total plasma proteins [81]. Albumin is first expressed in fetal hepatocytes at E12, and its expression increases progressively thereafter until it reaches maximal levels in the adult [71]. Consequently, albumin is considered a hallmark of hepatocyte maturation [60,87,88]. As a first step in examining the effects of BPA on fetal liver maturation, we analyzed albumin protein expression. We found that levels of albumin protein were significantly reduced in BPA-exposed female fetal livers when compared to controls. In marked contrast, BPA had no effect on albumin protein expression in male fetal livers, demonstrating that BPA disrupts fetal liver maturation in a sex-specific manner.

4.3 BPA Decreases Glycogen Synthase Protein Expression in Fetal Female Livers

Glycogen accumulation is another key feature of mature hepatocytes, and an important marker of hepatic maturation [88,96]. During most of gestation, hepatic glycogen store remains low as the fetus obtains an adequate supply of glucose from the mother via the placenta [91]. During late gestation, hepatocytes start to accumulate significant amounts of glycogen in order to prepare for the extrauterine survival at birth [92]. This surge in glycogen accumulation is accompanied by a corresponding increase in glycogen synthase [93,120], the rate-limiting enzyme of glycogen synthesis [94,95]. In the present study, therefore, we examined glycogen synthase protein expression and used it as a surrogate of glycogen accumulation. We showed that similar to its effects on albumin, prenatal BPA exposure significantly downregulated fetal liver glycogen synthase expression in females but not males. This finding further supports our conclusion that BPA disrupts fetal liver maturation in a sex-specific manner. Although glycogen content was not measured in the present study, previous studies have found that changes in fetal glycogen accumulation are directly correlated with alterations in glycogen synthase expression [96,123]. In addition, one previous study has shown that the developmental increase in fetal liver glycogen synthase expression is critically determined by the status of hepatocyte differentiation rather than substrate availability [120].

4.4 BPA Increases AFP Protein Expression in Fetal Female Livers

To provide further insight into the effects of BPA on fetal liver maturation, we then examined the expression of AFP, a marker of immature fetal hepatocytes [89,90]. AFP is the predominant fetal plasma protein produced by the fetal liver [85], and is considered the fetal equivalent of the adult serum albumin. During fetal development, the liver produces increasing amounts of AFP from E9 up to E15.5, at which time, AFP production begins to decline as the liver matures, ultimately becoming undetectable in the adult [86]. Here, we showed that in female livers, prenatal BPA exposure resulted in a significant increase in AFP protein expression at E18.5. In contrast, AFP protein levels in BPA-exposed male fetal livers were comparable to those of non-exposed controls. Taken together, the distinct changes in the expression of all three markers corroborate each other, and provide powerful evidence that prenatal exposure to BPA severely impairs fetal hepatic maturation only in females and not males.

4.5 BPA Decreases C/EBP-α Protein Expression in Fetal Female Livers

C/EBP-α is a master transcription factor essential for hepatocyte maturation, and is necessary for the activation of several genes associated with differentiated hepatocytes [121]. As the liver undergoes maturation, C/EBP-α expression increases progressively until reaching a maximum in the adult [86]. Because of its temporal expression profile during liver development, C/EBP-α is also considered a marker of mature hepatocytes.

Consequently, we determined whether prenatal BPA exposure affected the expression of this key transcription factor in fetal livers. We found that C/EBP-α protein levels were significantly decreased in BPA-exposed female fetal livers. Similar to the other markers of hepatic maturation, the level of C/EBP-α protein remained unaltered in BPA-exposed male fetal livers. The reduced hepatic C/EBP-α expression in BPA-exposed female fetal livers not only supports our conclusion that BPA disrupts fetal hepatic maturation in a sex-specific manner, but also suggests that the altered expression of albumin, glycogen synthase and AFP is likely a result of the decreased C/EBP-α expression.

Indeed, neonatal livers of C/EBP-α knockout mice exhibit properties characteristic of a dedifferentiated state. In an *in vivo* study by Wang et al., C/EBP-α knockout mice displayed normal body weight and gross organ morphology, however they failed to survive more than one day after birth due to impaired energy homeostasis [107]. In particular, C/EBP-α knockout mice displayed significantly decreased glycogen stores at both E18 and 1 hour postpartum. In fact, while control mice produced abundant levels of hepatic glycogen stores, those void of C/EBP-α exhibited virtually none [107]. These diminished glycogen stores were accompanied by a 50-70% decrease in glycogen synthase mRNA levels, which suggests that the lack of hepatic glycogen was a direct consequence of insufficient glycogen synthase [107]. Thus, although glycogen was not directly measured in the present study, it is highly likely that hepatic glycogen stores are reduced as a consequence of significantly decreased glycogen synthase. Conversely, C/EBP-α knock-in mice demonstrate earlier fetal hepatic glycogen deposition (E15.5) in comparison to their wild type littermates, with a corresponding increase in glycogen synthase expression [123].

Wang et al. also demonstrated C/EBP-α knockout mice display significantly reduced albumin mRNA at 2 hours after birth, which remained low at both 7 and 32 hours postpartum, suggesting that C/EBP-α regulation is required for the transcriptional induction of the albumin gene [107]. Because albumin expression was not completely absent, it is likely that the albumin gene might be fully transactivated by the activity of C/EBP-α together with some other liver-enriched transcription factor(s) known to bind to the albumin promoter [107]. This reduced albumin was also recapitulated in another *in vivo* C/EBP-α knockout model by Flodby et al. [106], and consistent with results from an *in vitro* model of C/EBP-α knock-in hepatocytes where albumin mRNA and protein levels were increased [104]. In addition, neonatal C/EBP-α knockout mice exhibit significantly elevated levels of AFP, indicative of a less differentiated state [106].

4.6 BPA Increases Cell Proliferation Marker PCNA in Fetal Female Livers

A proper balance between cell proliferation and apoptosis is essential for organ development, including that of the liver [80]. Thus, we also determined whether prenatal BPA exposure disrupted this balance in the fetal liver. First, we examined the proliferative status of the fetal liver by analyzing the expression of PCNA, a universal marker of cell proliferation. One of the main characteristics of a differentiated cell is a marked decrease in proliferation. As hepatocytes mature during fetal development, their proliferative activity progressively decreases as they approach term [124]. Hence, increased expression of proliferative markers is likely indicative of impaired hepatic differentiation and maturation [96]. In the present study, we found that levels of PCNA

protein were significantly elevated in BPA-exposed female but not male fetal livers. It is interesting to note that prenatal BPA exposure has been shown to increase proliferation in the pituitary gland of female but not male mice at birth [125].

Given that we revealed a significant decrease in C/EBP-α expression in female fetal livers, it is especially noteworthy that this transcription factor is known to exert powerful inhibitory effects on cell proliferation. For instance, hepatocyte proliferation is induced in C/EBP-α knockout mice [106,126], whereas C/EBP-α overexpression results in significantly reduced proliferative activity [127]. C/EBP-α is also highly expressed in terminally differentiated tissues and in non-dividing hepatocytes [128]. Furthermore, during liver regeneration [128–130] and tumorigenesis [104], instances during which proliferative activity is positively driven, C/EBP-α is significantly downregulated, which is accompanied by a corresponding re-activation of AFP [123]. Thus, it is tempting to speculate that the increased PCNA expression, and by inference hepatic proliferation, as observed in the present study may be a consequence of decreased C/EBP-α.

4.7 BPA Decreases Apoptotic Marker Caspase-3 in Fetal Female Livers

During normal fetal development, an appropriate level of apoptosis is necessary for controlling cell population and deleting abnormal or genetically damaged cells [80,131]. Moreover, organ homeostasis is critically regulated by the balance between cell gain and cell loss [132]. Given that female fetal livers exhibited aberrant cell proliferation in response to BPA exposure, we then sought to examine if BPA exerted an effect on apoptosis. Previous studies have shown that prenatal exposure to BPA decreases

apoptosis in the fetal mammary gland, leading to delayed lumen formation at E18 in the mouse [41,42]. With regards to the liver, Xia et al. found that BPA exposure during pregnancy and lactation resulted in enhanced apoptosis in male offspring at 15 and 21 weeks of age [109], but females were not examined.

Apoptotic cell death is carried out via two major pathways: the extrinsic (death receptor) pathway, and the intrinsic (mitochondrial) pathway. The two main initiator caspases associated with each of those pathways are caspase-8 and caspase-9, respectively [133]. These two pathways converge, and apoptosis is carried out by the cleavage of the executioner protein caspase-3 [134]. Thus, in the present study, we analyzed the expression of caspase-3 (a universal apoptotic marker) to determine the effect of prenatal BPA exposure on apoptosis in fetal livers. Our results showed a significant decrease in levels of both pro- and cleaved (active) caspase-3 in BPA-exposed female fetal livers, suggesting that BPA reduced both the expression and activity of caspase-3. By contrast, neither pro- nor cleaved caspase-3 protein levels were altered in BPA-exposed male fetal livers.

While decreased albumin, decreased glycogen synthase, increased AFP, and increased proliferation in BPA-exposed females can be largely attributed to the lack of C/EBP-α, there is no evidence that this transcription factor directly affects apoptosis in hepatocytes. In one study, C/EBP-α was found to ameliorate liver fibrosis in mice through the induction of apoptosis in hepatic stellate cells [134]. However, apoptosis of hepatocytes generally remained unaltered [134]. In the present study, suppressed apoptosis in BPA-exposed females could potentially be a direct consequence of the upregulated cell replication [132], especially given that both of these processes were

altered strictly in females, and not males. Indeed, results from previous studies have indicated that cell proliferation and apoptosis are coupled processes, and hence, suppression of apoptosis may occur as part of a proliferative response [135]. In any case, decreased apoptotic activity is particularly unfavourable during organogenesis. Especially in combination with the observed increase in cell proliferation, suppression of apoptosis could cause hyperplasia, which creates an expanded population of cells from which those with oncogenic mutations could arise.

4.8 Sex-Specific Effects

The most striking findings of the present study are the sex-specific effects of BPA on fetal liver maturation. We are not the first to demonstrate that male and female fetal livers display differential vulnerability in response to an adverse *in utero* environment. For example, maternal protein and nutrient restriction [136,137], maternal smoking [138,139], prenatal caffeine exposure [140], and prenatal cadmium exposure [141] have been shown to alter fetal hepatic gene expression and DNA methylation in a sex-dependent manner. Although the precise molecular mechanisms underlying the sex-specific effects in the present study and those reported previously remain largely unknown, it is possible that fetal sex steroid hormones may play a role [142]. A likely contributing factor may also be sex chromosome complement, because chromosomal differences have been shown to dictate the responses of male and female cells to environmental stressors even before the production of fetal sex hormones [143,144]. It is also possible that these sex-specific effects could be mediated indirectly via differential

effects of BPA on male and female placentas [145]. Obviously, future studies will be required to determine the precise mechanisms underlying this phenomenon.

Our present findings also suggest females are more vulnerable to BPA-induced hepatic damage. This contention is consistent with some but not all of the previously reported sexual dimorphism associated with the BPA-induced adult liver phenotypes, which appear to be largely dependent on the dose as well as the exposure time and duration. For instance, although maternal exposure to BPA throughout pregnancy and lactation has been shown to induce metabolic disorders in both male and female adult offspring, the effects on females were more profound [112]. In contrast, Alonso-Magdalena et al. found that prenatal exposure to BPA (E9 to E16) disrupted glucose homeostasis in adult male but not female offspring [111]. In another study, Weinhouse et al. found that exposure to BPA throughout gestation and lactation led to a dose-dependent incidence of liver tumors in both male and female adult offspring [110]. Although these effects were not different between sexes, it is interesting to note that while males are normally two to four times more likely to develop hepatocellular carcinoma (HCC), this sexual dimorphism disappeared as a result of perinatal BPA exposure [110]. This BPA-induced increased vulnerability to HCC in adult females is especially interesting in the context of the present study's findings for C/EBP-α, given that C/EBP-α knock-in mice have been shown to exhibit reduced susceptibility to HCC compared to wild-types [123], and also given its suspected role as a tumor suppressor in other tissues [146].

Developmental exposure to BPA has also been shown to promote aberrant DNA methylation profiles [115,116] as well as altered gene expression [113] in adult offspring. However, sex-specific effects are unknown as differences between sexes were never

compared in these studies. For example, Ma et al. revealed glucokinase promoter hypermethylation in adult male offspring as a result of early life BPA exposure, but female offspring were never examined [116]. In another study, Somm et al. showed that perinatal BPA exposure resulted in the overexpression of several hepatic metabolic genes in adult female offspring, but gene expression in male livers was not analyzed [113]. Early life exposure to BPA also contributes to the development of hepatic steatosis in adult male offspring [108,147] but this phenomenon has never been examined in BPA-exposed females.

4.9 Conclusions

In the present study, we demonstrate for the first time that prenatal exposure to environmentally relevant doses of BPA via maternal diet impairs female, but not male, fetal liver maturation in the mouse. Although the long-term consequences of the present findings remain to be determined, it is tempting to speculate that the disrupted hepatic maturation observed in the present study may result in permanent alterations in hepatic function, ultimately leading to hepatic dysfunction and diseases later in life. In addition, these findings suggest that females are more vulnerable to BPA-induced hepatic damage.

4.10 Limitations & Future Directions

Although we provide strong evidence that BPA impairs the biochemical maturation of female fetal livers, the liver also undergoes structural maturation to acquire a specialized tissue architecture important for supporting its associated functions [148]. In the present study, no definitive conclusion can be made regarding the effects of prenatal

BPA exposure on structural maturation of fetal livers. While there were no observable structural abnormalities in the livers of BPA-exposed fetuses upon standard H&E staining, this histological analysis was carried out on a small sample size with males and females pooled together, prior to the realization of a sex-specific effect. Thus, an impairment in the structural maturation of female fetal livers could potentially be masked by the strong lack of effect of BPA on the livers of male fetuses. Unfortunately, further analysis was not possible in the present study since the limited store of fixed tissues was never genotyped. Future studies should explore this by analyzing structural markers of maturation [96]. Moreover, one of the most utilized indices for assessing effects of xenobiotics is organ weight [149]. In the present study, fetal liver weights failed to be recorded at the time of dissection. However, neonatal livers of C/EBP-α knock-out mice exhibit a normal liver size [106,150], which leads us to believe that liver weights of BPA-exposed fetal females likely remain normal in the present study. Nevertheless, this question should be addressed in future studies.

Our study employed the use of an *in vivo* mouse model to demonstrate the effects of prenatal BPA exposure on fetal liver maturation in the mouse at E18.5, just prior to term (E19). This time point was chosen since our goal was to determine whether a fetal origin might exist for BPA-induced hepatic disease and dysfunction. Nevertheless, hepatocytes continue to undergo functional maturation for several weeks after birth, and C/EBP-α is also essential for continued postnatal differentiation of hepatocytes. For example, the rapid depletion of glycogen stores at birth is paralleled by the immediate onset of hepatic gluconeogenesis [151]. The acquired gluconeogenic capacity of the liver is supported by the onset of gluconeogenic enzymes, including glucose-6-phosphatase

(G6Pase) and the rate-limiting enzyme, phosphoenolpyruvate carboxykinase (PEPCK), at postnatal day (PN) 1 [152–154]. C/EBP-α binds to the promoter regions of these genes and activates their expression [155,156]. In fact, neonatal C/EBP-α knockout mice exhibit delayed and reduced PEPCK and G6Pase expression, which is largely responsible for their severe dysregulation in glucose homeostasis and resultant hypoglycemia [107,155]. In the present study, given that levels of C/EBP-α are significantly decreased in BPA-exposed female livers at E18.5, it is likely that these important gluconeogenic enzymes are also downregulated in the neonatal liver, and that the consequences of prenatal BPA exposure on hepatocyte maturation extend into the postnatal period. Future studies are necessary in order to address these questions.

Furthermore, it is unknown as to whether the effects observed in the present study persist into adulthood. Liver-specific knockout models have revealed that C/EBP-α continues to play an important role in the adult. Specifically, these studies have demonstrated that C/EBP-α is necessary for transcription of critical gluconeogenic genes (glycogen synthase, G6Pase, PEPCK), as well as bilirubin UGT, which is required for the detoxification of serum bilirubin [157,158]. In addition, adult C/EBP-α knockout mice have been shown to exhibit glucose intolerance, increased hepatic steatosis, and increased serum cholesterol levels [158]. Thus, programmed under-expression of hepatic C/EBP-α could have negative consequences in the adult. Interestingly, a subset of these profiles have already been established in adult mice subjected to early life BPA exposure. It would be interesting to understand the extent to which downregulated C/EBP-α in the fetus contributes to these phenotypes, if at all.

Lastly, we have yet to elucidate the molecular mechanism underlying the effect of BPA on female fetal liver maturation. We propose that upon binding to an intracellular receptor, BPA acts to downregulate C/EBP-α protein expression, which leads to a downregulation of albumin and glycogen synthase, an upregulation of AFP, as well as increased PCNA expression (cell proliferation marker) and decreased caspase-3 expression (marker of apoptosis) (**Figure 4-1**). However, the precise mechanism remains unclear, and future studies will be necessary in order to address this question.

It is conceivable that prenatal exposure to BPA impairs female fetal liver maturation through aberrant estrogen signaling. As previously mentioned, BPA acts mainly via interaction with ER. The predominant isoform in hepatocytes is ERα [159,160], and studies have shown that hepatic ER concentrations increase in the fetus during later stages of gestation in parallel with maternal estrogens [161]. In other cell types that coexpress C/EBP-α and ER, estradiol has been shown to induce C/EBP-α activation [162]. Additionally, BPA has been shown to act via an ER-mediated pathway to increase C/EBP-α expression in human adipose stem cells [163].

BPA has also been shown to bind to the GR as both an agonist and an antagonist [30,31]. Glucocorticoids are important for regulating the expression of many hepatic genes. Specifically, the GR regulates transcription via DNA binding, as well as through cross-talk with other transcription factors [164,165]. Studies have shown that during late gestation, glucocorticoids promote hepatic maturation. For example, glucocorticoids accelerate the decline of AFP while enhancing albumin expression [166]. In addition, glucocorticoid have been shown to induce glycogen synthase expression and glycogen

accumulation [167]. Glucocorticoids have also been shown to induce G_1 cell cycle arrest in a rat hepatoma cell line, which required the induction of C/EBP-α as a mediator [165].

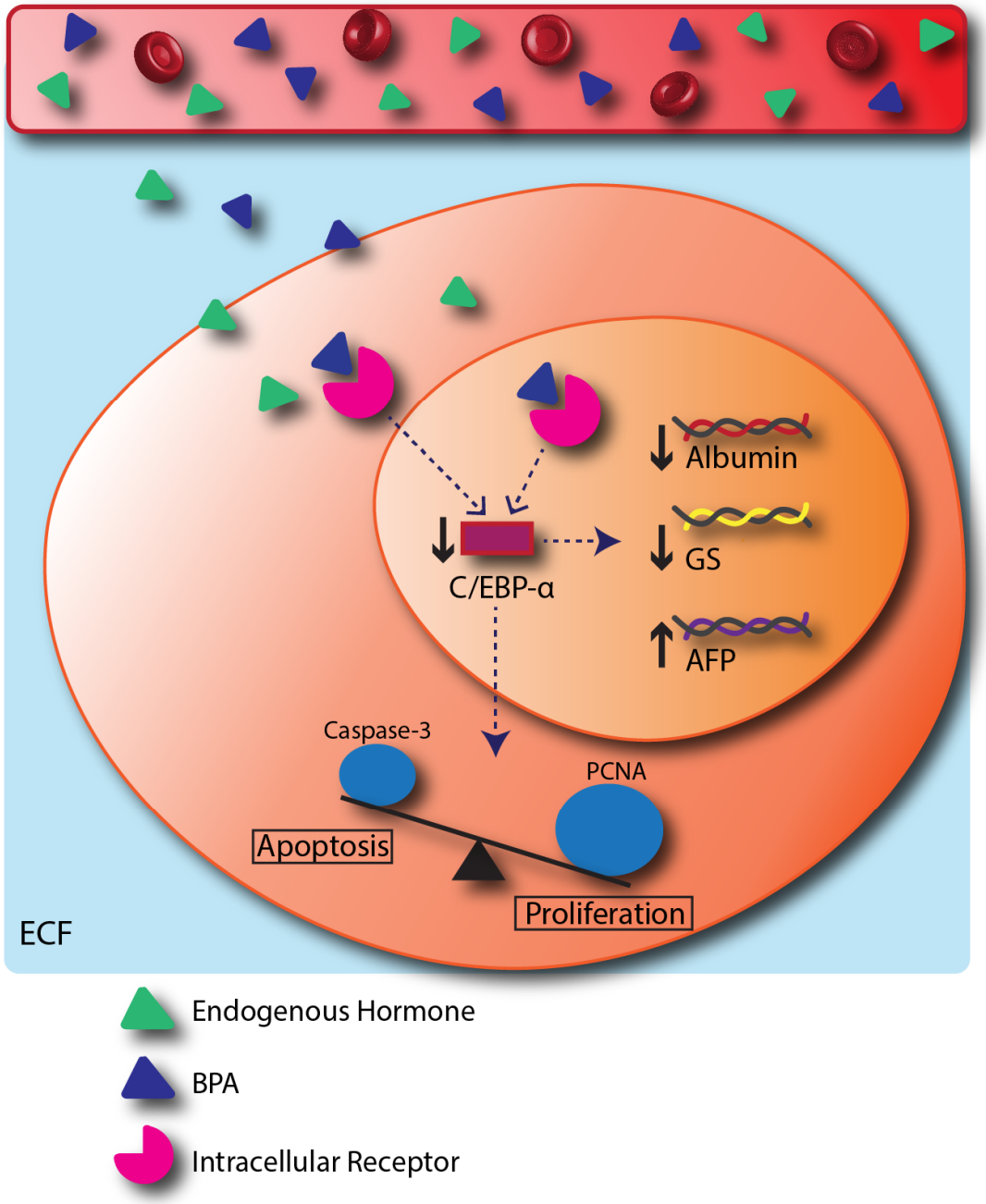

Figure 4-1. Proposed Mechanism. Upon binding to an intracellular receptor, BPA acts to downregulate C/EBP-α, which leads to decreased expression of albumin and glycogen synthase, increased expression of AFP, as well as a perturbed balance between cell proliferation and apoptosis.

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2013-2015

Publications:

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