ENVIRONMENTAL CHEMICALS AND STRESS DISRUPT IMPLANTATION

THE IMPACT OF BISPHENOL A IN COMBINATION WITH STRESS AND DIETHYLHEXYL PHTHALATE ON IMPLANTATION, UTERINE MORPHOLOGY, AND ADHESION PROTEIN EXPRESSION IN INSEMINATED FEMALE MICE

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TITLE: The Impact of Bisphenol A in Combination with Stress and Diethylhexyl Phthalate on Implantation, Uterine Morphology, and Adhesion Protein Expression in Inseminated Female Mice

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Abstract

Bisphenol A (BPA), the monomer of polycarbonate plastics and epoxy resins, can disrupt intrauterine implantation of fertilized ova in mice. This effect is also induced by exposure to chronic stress or high doses of diethylhexyl phthalate (DEHP), a plasticizer found in polyvinyl chloride products. I assessed the potential combinatory effects of BPA and stress on blastocyst implantation, uterine morphology, adhesion protein expression, and urinary hormone levels. Subcutaneous injections of BPA administered from gestation days (GDs) 1–4 paired with a stressor (rat exposure across a grid) reduced the number of implantation sites on GD 6 at a dose where neither BPA nor stress had this effect on their own. Uterine luminal area was increased by BPA when paired with stress. BPA reduced epithelial cadherin (e-cadherin), a uterine adhesion protein, independently from the stressor. Urinary estradiol was significantly increased by BPA relative to controls, regardless of stress. In other experiments, effects of concurrent BPA and DEHP administered were assessed. Inseminated female mice were injected with BPA, DEHP, or BPA + DEHP from GDs 1–4. Implantation measured in uteri on GD 6 was disrupted by a combined dose but not by the individual doses. This dose also decreased the amount of e-cadherin and cadherin-11, another adhesion protein expressed by cells, while cadherin-11 was also affected by BPA alone. In further experiments designed to elucidate the interaction of BPA and DEHP, mice were fed ^{14}C-BPA and injected with varied doses of DEHP, then tissues were excised and measured for radioactivity. When given DEHP, males and cycling and peri-implantation females showed increased BPA deposition in reproductive tissues and serum. As people are commonly exposed to both DEHP and

BPA through consumer products, it is important to determine their interactions and also to understand how dose-response is affected by other factors such as stress.

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I am also grateful for my supervisory committee members Dr. Sigal Balshine, Dr. Paul Faure, and Dr. Warren Foster for all their assistance during my PhD. Sigal and Paul have always highlighted the importance of animal behaviour and how it may be influenced by the chemicals in my research. This view on my toxicological work painted a broader picture to the many aspects of toxicology that I did not consider on a daily basis. Warren has always pushed me to be a stronger, independent graduate student and I believe that I am better because of it.

I would also like to thank my colleague, roommate, lab mate, and friend, Tyler Pollock. Throughout our graduate careers we have always discussed our research with each other and we have always provided support. With that said, Tyler has done more to help me than I've been able to reciprocate. He was the first person to teach me basic animal handling and injections when I first arrived to the lab. He has always been very willing to help when needed, and for that I am very grateful. I've also had the pleasure of playing many board games with him to distract us from work, which I have thoroughly enjoyed!

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Finally, I would like to thank Tammy Rosner, who has always supported me and prioritized helping me even when it delayed her own studies. You understand that research comes first, such that I have missed family events and even a wedding, but you always encourage me to relax when I have time.

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List of Abbreviations

Endocrine disrupting chemical	EDC
Estradiol (17β-estradiol)	E_2
Progesterone	P_4
Epithelial-cadherin	e-cadherin
Bisphenol A	BPA
Hypothalamic-pituitary-gonadal	HPG
Gonadotropin-releasing hormone	GnRH
Follicle-stimulating hormone	FSH
Luteinizing hormone	LH
Hypothalamic-pituitary-adrenal	HPA
Adrenocorticotropic hormone	ACTH
Corticotropin-releasing hormone	CRH
Sulfotransferse	SULT
UDP-glucuronosyltransferase	UGT
Estrogen receptor (alpha)	ER(α)
Estrogen receptor (beta)	ER(β)
Membrane estrogen receptor (alpha)	mERα
G-protein couple receptor 30	GPR30
Estrogen-like estrogen receptor (gamma)	ERR-γ
Estrogen-like estrogen receptor (alpha)	ERR-α

Diethylhexyl phthalate	DEHP
Monoethylhexyl phthalate	MEHP
Gestation day	GD
Phosphate buffered saline	PBS
Hydrogen peroxide	H_2O_2
Diaminobenzedine	DAB
Standard error of the mean	S.E.M., S.E.
Analysis of variance	ANOVA
Subcutaneous	sc
Corticosterone	cort
Cadherin-11	Cad-11
Integrin alpha-v beta-3	Integrin αvβ3

Chapter 1

General Introduction

Overview

In current generations, most humans are exposed to various synthetic environmental chemicals on a daily basis. These chemicals are ubiquitous in consumer products such as soap, detergent, body lotion, shampoo, shaving cream, toothpaste, cosmetics, vinyl, shower curtains, consumer plastics, thermal paper, canned foods, and perfumes (Dodson et al., 2012). Wildlife can also be exposed through waste and effluent affecting habitats of many species (Colborn et al., 1993; Vos et al., 2000). Research on many of these substances indicates that they are endocrine disrupting chemicals (EDCs), as they can interfere with hormonal systems or mimic hormones. Known EDCs can affect thyroid function, fertility, development, and behaviour by interfering with conjugating enzymes and interacting with hormone receptors (Berger et al., 2007; Colborn et al., 1993; Pocar et al., 2012; Pollock et al., 2014). Xenoestrogens, estrogen-mimicking chemicals, and anti-androgens have been of particular interest to researchers as they can have deleterious effects on reproduction and are widely used in consumer products and plastics (Kavlock et al., 2002; Toppari et al., 1996).

Exposure routes for EDCs are largely determined by their use in various products. Ingestion is a major route of EDC exposure due to chemicals leaching into foods from packaging and epoxy resins lining canned goods (Muncke, 2009). Some chemicals, such as diethylhexyl phthalate, have even been used directly in the production of fruit juices as clouding agents in order to make them appear more natural (Yang et al., 2013). Inhalation is seen in chemical manufacturing, construction, and building dust (Rudel & Perovich,

2009). Transdermal absorption can also occur through skin contact (Elsisi et al., 1989), dependent on the solubility and molecular size of the chemicals (Beetge et al., 2000).

The aim of the research reported in this thesis was to investigate the interactions of two common EDCs and to determine how stress can influence their potency in mice. I took measures that are clearly perturbed by estrogenic activity, such as intrauterine blastocyst implantation and related events in the uterus. Before discussing the hypotheses that I tested, I will discuss basic reproductive biology and steroid dynamics in female mammals followed by discussion of the two specific EDCs targeted in this thesis.

Mammalian Female Reproductive Biology and Steroid Dynamics

Pregnancy and Implantation

Mammalian pregnancy is complex, with time-specific processes that are sensitive to stress, hormonal imbalance, and estrogenic chemicals. Estrous cycles vary among species, from the six day estrous cycle of a mouse (*Mus musculus*) to the monthly human menstrual cycle, with ovulation occurring around the midpoint of each cycle (Campbell et al., 1999). Proliferation of uterine cells during the early stages of these cycles prepares the uterus for fertilization, and will continue until ovulation (Finn & Martin, 1974). Ovulation is critical for determining pregnancy as it signifies the release of the unfertilized ova or ovum into the oviduct where it can be fertilized by spermatozoa (see review by Bloch, 1976). A fertilized ovum will travel down the oviduct to the uterus and undergo mitotic division, developing into a blastocyst (Wang & Dey, 2006). The blastocyst will then adhere to the epithelial cells of the luminal wall (implantation) through adhesion proteins

both on the wall and the trophoblast, the outer layer of cells of the blastocyst (Paria et al., 1999).

Hormonal dynamics change during the uterine implantation window as 17β-estradiol (E_2) levels decrease and progesterone (P_4) levels increase (McCormack & Greenwald, 1974). This increase in P_4 blocks the epithelial cell proliferation effect of E_2 (Dey et al., 2004), increases stroma cell proliferation (Dey & Lim, 2006), and allows for endometrium attachment of the blastocyst through upregulation of various adhesion proteins, such as epithelial cadherin (e-cadherin; Jha et al., 2006; Potter et al., 1996). P_4 also causes an efflux of fluid from the uterine lumen into the stroma which allows the luminal walls to close around the blastocyst during implantation (Martin et al., 1970; Naftalin et al., 2002). This luminal closure facilitates implantation by restricting movement of the blastocyst to allow adhesion to occur.

Early pregnancy is very sensitive to changes in E_2 and P_4, and perturbations of these hormones can easily lead to pregnancy loss (Ma et al., 2003). Diverse chronic stressors have been shown to disrupt implantation in inseminated female rodents, ungulates, and primates (deCatanzaro, 2011; deCatanzaro & MacNiven, 1992), and evidence implicates a high E_2:P_4 ratio (deCatanzaro et al., 1994; Gidley-Baird et al., 1986; Thorpe et al., 2013). Administration of estrogenic hormones and chemicals, such as E_2 and bisphenol A (BPA), can also terminate pregnancy (Berger et al., 2007; deCatanzaro et al., 2001; Ma et al., 2003).

Hypothalamic-pituitary-gonadal axis

The hypothalamic-pituitary-gonadal (HPG) axis controls reproduction in female

mammals. Hormonal communication among the hypothalamus, pituitary, and ovaries is critical for maintaining pregnancy (Niswender & Nett, 1988) and estrous or menstrual cycling in females (Freeman, 1988; Knobil & Hotchkiss 1988). Generally, the hypothalamus is stimulated by the kisspeptin protein from the arcuate and the anteroventral periventricular nuclei of the hypothalamus which causes secretion of gonadotropin releasing hormone (GnRH; Dungan et al., 2006). Evidence suggests that kisspeptin mRNA expression is increased in the anteroventral periventricular nucleus and decreased in the arcuate nucleus by E_2 (Navarro et al., 2004; Smith et al., 2005). This indicates that E_2 can modulate the release of kisspeptin and, indirectly, other downstream hormones depending on where it binds in the hypothalamus. GnRH then binds to its receptor on the anterior pituitary causing the gonadotrope cells to release the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Schally et al., 1973, Conn & Crowley, 1990). These gonadotropins will travel to the ovaries and cause the follicle cells to release the sex steroids P_4 and E_2 (Jamnongjit & Hammes, 2006).

Continuous production of sex steroids is energetically costly and could be detrimental as high E_2 levels can increase the risk of developing cancer by promoting tumour growth (Cauley et al., 1999). To a degree, excessive E_2 release is prevented by a negative feedback loop, as increased E_2 production inhibits kisspeptin in the arcuate nucleus of the hypothalamus, thereby inhibiting GnRH release (Dungan et al., 2006). This causes more pulsatile release of sex steroids. Female sex steroid release is cyclical. Consistently low levels of E_2 are released to prepare the uterus for pregnancy through

cellular and vascular proliferation, until the proestrus phase of the mouse cycle or the end of the follicular phase in the human cycle. At these points, E_2 acts in a positive feedback loop with the HPG axis to stimulate a surge of LH which stimulates P_4 release and ovulation (Nelson & Kriegsfeld, 2017). This surge may be caused by positive feedback in which E_2 stimulates the anteroventral periventricular nucleus to release kisspeptin (Smith et al., 2011). If fertilization does not occur, E_2 and P_4 levels will decrease to baseline levels and the cycle will begin again. However, if fertilization occurs P_4 will remain elevated to support the pregnancy.

Hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal axis (HPA) is a highly conserved hormonal system that is stimulated by activity and stress. Among many other functions, HPA activity can lead to inhibition of the HPG axis (Viau, 2002). The adrenal cortex will release glucocorticoids, including cortisol and corticosterone, following stimulation from adrenocorticotropic hormone (ACTH) secreted by the pituitary, which in turn was stimulated by corticotrophin-releasing hormone (CRH) from the hypothalamus. Baseline secretion of these hormones follows a circadian rhythm, with levels peaking at the start of an organism's activity cycle (morning for diurnal species and evening for nocturnal species) and levels continually decreasing until after onset of sleep. The release of glucocorticoids directly influences energy levels by increasing blood glucose concentrations. This increase in available glucose is adaptive as it enhances the organism's flight-or-fight stress response by providing additional energy for physical exertion (Dickmeis, 2009).

Glucocorticoids rise substantially above baseline concentrations during stress. While this is generally adaptive, constantly elevated glucocorticoids, as seen in chronic stress, can be detrimental (Sapolsky et al., 2000). To a degree, excess glucocorticoid release is prevented by negative feedback at the pituitary and hypothalamus, as elevated cortisol or corticosterone decreases release of CRH and ACTH. This negative feedback loop can break down if an animal is exposed repeatedly to severe stressors, which can lead to extended periods of increased glucocorticoids.

Stress generally indicates a situation that is non-conducive to reproduction as energy is diverted towards removing or escaping the stressor. This leads to reproductive suppression via the hypothalamus, pituitary, and gonads (Rivier & Rivest, 1991). The release of GnRH by the hypothalamus is decreased in states of stress as CRH binds to its receptor on GnRH neurons to suppress release and suppresses the kisspeptin gene, thus decreasing GnRH release (Matsuwaki et al., 2009). Glucocorticoids can inhibit the HPG axis by stimulating the hypothalamic release of gonadotropin-inhibitory hormone, a peptide involved in suppression of GnRH and gonadotropins (Kirby et al., 2009). Glucocorticoids can also reduce the sensitivity of the gonads to the gonadotropins LH and FSH, thereby decreasing sex steroid production (Charpenet et al., 1982, Sapolsky, 1985). These inhibitory effects on the HPG axis by stress can result in impaired sexual behaviour, anovulation in females, and reduced spermatogenesis in males (McGrady, 1984; Negro-Vilar, 1993; Wingfield & Sapolsky, 2003).

The effects mentioned above result in disruptions of hormone cycling and sexual behaviour from stress-induced reproductive suppression. However, female pregnancy

disruption can occur after successful mating. A severe stressor after insemination can lead to termination of pregnancy in the peri-implantation period (deCatanzaro, 2011; deCatanzaro & MacNiven, 1992). Stressors that can lead to early pregnancy loss can be physiological or psychological, and include physical restraint (Euker & Riegle, 1973), heat (Garcia-Ispierto et al., 2006), overcrowding (Helmreich, 1960), and predator exposure (deCatanzaro, 1988). The reason for pregnancy loss is due to the stressors disrupting E_2 and P_4 levels (Parker & Douglas, 2009; Thorpe et al., 2013).

Endocrine Disruptors Examined in this Research

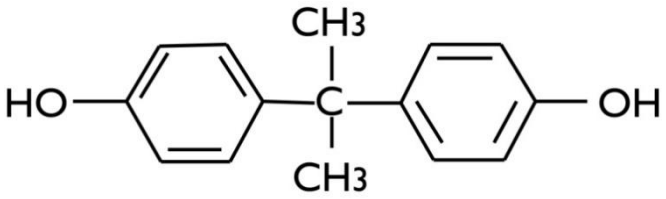
Bisphenol A

BPA, a monomer used in the production of polycarbonate plastics and epoxy resins, can be found in various products such as food storage containers, water bottles, thermal receipt paper, water pipes, and dental sealants (Vandenberg et al., 2007). Due to incomplete polymerization, BPA can leach from these products into the surrounding media. With BPA being used in many consumer products, in developed countries, it is currently very hard to avoid as both people and wildlife are exposed to it daily (Calafat et al., 2005; Crain et al., 2007). The primary route of exposure to BPA is ingestion of contaminated foods and beverages, which accounts for 85–95% of total human exposure (EFSA, 2015). Daily BPA exposure for humans is estimated to be 0.2–0.5 µg/kg/day for individuals aged 2 years or older (Aungst et al., 2014). The tolerable daily intake, an estimate of the daily exposure to humans that is unlikely to produce deleterious effects over a lifetime, is 25 µg/kg in Canada (Health Canada, 2008) and 50 µg/kg in the United

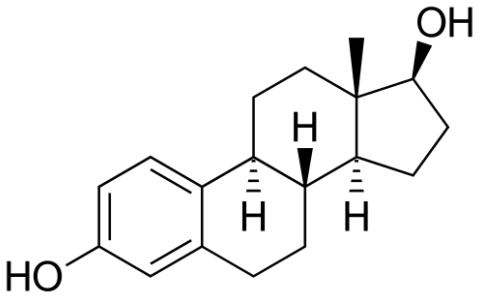
States (U.S. EPA, 1988). Once BPA enters the body it can be inactivated into its conjugated forms, BPA-sulfate and BPA-glucuronide, through the conjugating enzymes sulfotransferase (SULT) and UDP-glucuronosyltransferase (UGT) in the liver and intestines (Hanioka et al., 2008; Pritchett et al., 2002).

Unconjugated BPA competes with E_2 for binding to nuclear estrogen receptors alpha (ERα) and beta (ERβ) at approximately10,000-fold greater concentration (Andersen et al., 1999; Buteau-Lozano et al., 2008; Matthews et al., 2001; Snyder et al., 2000). This indicates that BPA, while much less estrogenic than E_2, can bind and activate ERs at high concentrations. BPA can also bind to membrane-bound ERs, mERα and GPR30 (Dong et al., 2011; Thomas & Dong, 2006; Wozniak et al., 2005). These non-genomic ERs display actions that produce large amplifications that are induced by low concentrations of ligands, including BPA (Wozniak et al., 2005). BPA has recently been found to have a strong binding affinity for another genomic estrogen-related receptor, estrogen-related receptor gamma (ERR-γ), which is closely related to ERα and ERβ (Takayanagi et al., 2006). A study of BPA and other EDCs indicated that the minimum structural requirement for estrogenic activity is a 4-hydroxyl group on the phenyl rings and a hydrophobic moiety at the 2-position of the propane moiety (Fig. 1.1; Kitamura et al., 2005). In addition to these properties, the molecular masses of BPA and E_2 are similar. The estrogenic nature of BPA is corroborated by research, both *in vivo* and *in vitro*, demonstrating that BPA can increase cell proliferation in MCF-7 breast cancer cells (Howdeshell et al., 2003), increase uterine weight following oral ingestion (Matthews et al., 2001), disrupt intrauterine blastocyst implantation (Berger et al., 2007), and increase

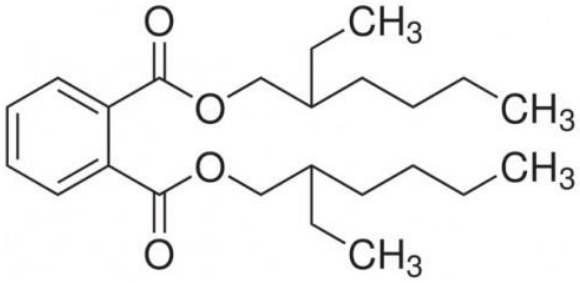
Fig. 1.1. Chemical structures of bisphenol A, 17β-estradiol, and diethylhexyl phthalate.



Bisphenol A



17β-Estradiol



Diethylhexyl Phthalate

uterine luminal epithelial height and luminal area (Berger et al., 2010). Some of the effects of BPA display non-monotonic dose-responses in that either a low and high dose elicits an effect or only a medium dose elicits an effect *in vivo* (Vandenberg et al., 2012). For instance, when given BPA perinatally, female mice had fewer pups relative to controls at the 25 ng and 25 µg doses, but not at the 250 ng dose (Cabaton et al., 2011). Thus, the current regulatory standards for BPA may not be a sufficiently safe precaution. This is further supported by research that has looked at interactions of other EDCs with BPA. Triclosan, an antibacterial found in soaps and toothpaste, co-administered with BPA reduces intrauterine blastocyst implantation in pregnant female mice at doses lower than previously shown (Crawford et al., 2012). Triclosan exposure can also increase the deposition of BPA within various organs (Pollock et al., 2014). This research indicates that BPA, in conjunction with its non-monotonic effects, can interact with other ubiquitous EDCs to elicit deleterious effects which are not incorporated into current regulations.

Diethylhexyl phthalate

Diethylhexyl phthalate (DEHP) is a plasticizer used in the manufacturing of polyvinyl chloride plastics found in medical devices, cosmetics, personal care products, children's toys, flooring, pipes, and clothing (Dodson et al., 2012; Kavlock et al., 2002; Wams, 1987). DEHP can migrate from PVC materials into surrounding media as it is not covalently bound to the polymers in which it is found. Ingestion is the primary route of exposure for DEHP as it can leach from plastics into high fat media such as meat, fish, oils, and dairy (Heinemeyer et al., 2013; Meek & Chan, 1994). Dermal absorption and

inhalation are also routes for exposure (Elsisi et al., 1989; Rakkestad et al., 2007). Human exposure has been estimated to be 1–30 µg/kg/day, with the tolerable daily intake being 50 µg/kg/day in Europe (SCENIHR, 2015) and 20 µg/kg/day in the United States (U.S. EPA, 1987). Treatments utilizing medical bags are an additional exposure route as DEHP can leach into the stored blood and provide an acute dose to the recipient. Blood transfusion patients can receive DEHP concentrations as high as 8,000–10,000 µg/kg/day, with blood in transfusion bags containing up to 83.2 µg/ml of DEHP (Inoue et al., 2005; Koch et al., 2006; SCENIHR, 2015). Once DEHP enters the body, it is rapidly metabolized by esterases and lipases in the gut, liver, and blood into monoethylhexyl phthalate (MEHP), its primary metabolite (Hanioka et al., 2012). MEHP is subsequently conjugated by UGT into its glucuronidated, form, MEHP-glucuronide (Hanioka et al., 2016a, 2016b).

DEHP is weakly estrogenic as E_2 is 1,000,000-fold more potent at binding to ERs (Buteau-Lozano et al., 2008; Okubo et al., 2003). DEHP can also act as a weak anti-estrogen by inhibiting the activation of ERβ by E_2 at higher concentrations (Takeuchi et al., 2005). These weak binding affinities to ERs may be due to the lack of hydroxyl groups on the phenyl ring and the larger size of the molecule, relative to BPA (Fig. 1.1; Kitamura et al., 2005). DEHP is known to be anti-androgenic in males as it can reduce testes and prostate weight, disrupt spermatogenesis, and cause mild genitalia dysgenesis (Christiansen et al., 2010). Despite its weak estrogenic and anti-estrogenic binding affinities, DEHP has been shown to affect estrogen-sensitive reproductive processes. Examples include reducing intrauterine blastocyst implantation in mice (Li et al., 2012),

disrupting oogenesis and ovulation in zebra fish (Carnevali et al., 2010), decreasing murine fetal oocyte viability (Bonilla & del Mazo, 2010), and inhibiting ovarian steroidogenesis (Svechnikova et al., 2007).

Research Objectives

I reasoned that factors which independently disrupt blastocyst implantation could act additively. I first examined the impacts of a stressor (predator exposure) with BPA. The disruption of E_2 and P_4 levels by stress coupled with the addition of BPA, an estrogenic chemical, could summate and disrupt blastocyst implantation at a lower BPA dose than previously described (Berger et al., 2007). I then examined whether co-administration of DEHP and BPA could produce effects at doses of each chemical that are lower than those previously reported to have effects on their own, similar to actions of triclosan and BPA (Crawford et al., 2012). Finally, I examined impacts of DEHP upon ^{14}C-BPA deposition, reasoning that DEHP and BPA would compete for the same metabolizing enzymes. This would lead to increased ^{14}C-BPA in tissues, similar to the effects of triclosan on ^{14}C-BPA deposition (Pollock et al, 2014).

Although much research has been accomplished on the physiological and behavioural effects of endocrine disruptors, these chemicals must also be studied in combination to better understand any interactions that may occur. The objectives of this thesis are threefold: 1) to assess whether stress and BPA co-administration can impact blastocyst implantation failure in female mice; 2) to assess whether BPA and DEHP interact to reduce blastocyst implantation in mice; and 3) to determine the mechanism for

any BPA and DEHP interaction. The work in this thesis provides a step towards a better understanding of human fertility issues caused by implantation failure through stress and EDCs, both of which are common in developed countries. Furthermore, it addresses the growing concern that regulations based on single chemical studies may not be sufficiently safe as I demonstrate that EDCs can exhibit different potencies depending on the physiological state and the chemical mixture. Thus, I examined the effects of BPA on intrauterine blastocyst implantation in mice in concert with stress and DEHP across three projects emphasizing effects on uterine morphology and uterine luminal adhesion proteins.

In **Chapter 2,** I examine blastocyst implantation in mice exposed to combinations of BPA and stress. In **Chapter 3,** I explore blastocyst implantation in mice exposed to combinations of BPA and DEHP. In **Chapter 4,** I examine the relationship between BPA and DEHP and how they might interact with each other. In **Chapter 5,** I summarize and discuss the results of Chapters 2-4 and suggest areas of future research.

Chapter 2: Borman ED, Foster WG, Greenacre MKE, deCatanzaro D. (2015). Stress lowers the threshold dose at which bisphenol A disrupts blastocyst implantation, in conjunction with decreased uterine closure and e-cadherin. *Chemico-Biological Interactions,* 237, 87-95.

Abstract: Exposure to stress can disrupt blastocyst implantation in inseminated female mice, and evidence implicates elevation of the female's estrogen:progesterone ratio. Exposure to the xenoestrogen, bisphenol A (BPA) can also disrupt implantation. Undisturbed control female CF-1 mice were compared to other females that were exposed

to predators (rats) across a wire-mesh grid during gestation days (GD) 1-4, a procedure that elevates corticosterone but does not on its own disrupt implantation in this genetic strain. They were concurrently exposed to varied doses of BPA that on their own were below the threshold dose sufficient to disrupt implantation. On GD 6, we measured the number of intrauterine implantation sites and extracted their uteri, which subsequently were stained and analyzed for uterine luminal area and epithelial cadherin (e-cadherin), a molecule that causes uterine closure and adhesion of blastocysts to the uterine epithelium. The combination of rat-exposure stress and BPA significantly disrupted implantation and increased uterine luminal area, whereas either manipulation on its own did not. E-cadherin was significantly reduced by exposure to BPA, positively correlated with the number of implantation sites, and inversely correlated with luminal area. BPA exposure was also associated with nonmonotonic perturbation of urinary corticosterone concentrations and increased urinary estradiol concentrations on GD 6. These data are consistent with a potential summation of stress-induced estrogen and xenoestrogen activity.

Chapter 3: Borman ED, Foster WG, deCatanzaro D. (2017). Concurrent administration of diethylhexyl phthalate reduces the threshold dose at which bisphenol A disrupts blastocyst implantation and cadherins in mice. *Environmental Toxicology and Pharmacology*, 49, 105-111.

Abstract: Many people are repeatedly exposed to both bisphenol A (BPA) and diethylhexyl phthalate (DEHP), but there has been little research concerning their effects in combination. Both can disrupt blastocyst implantation in inseminated females, albeit at

high doses. We exposed mice on gestation days (GD) 1–4 to combinations of BPA and DEHP in doses below the threshold necessary to disrupt implantation on their own. On GD 6, there were fewer normally-developed implantation sites and more underdeveloped implantation sites in females given the combined subthreshold doses. Uterine epithelial cadherin (e-cadherin), a protein that assists in blastocyst adhesion to the uterine epithelium, was significantly reduced by these combined doses, but not by the individual doses. A similar trend was seen in integrin αvβ3, another uterine adhesion molecule. Cadherin-11 was disrupted by BPA but not DEHP. These data are consistent with competition of BPA and DEHP for conjugating enzymes.

Chapter 4: Borman ED, Vecchi N, Pollock T, deCatanzaro D. (2017). Diethylhexyl phthalate magnifies deposition of 14C–bisphenol A in reproductive tissues of mice. *Journal of Applied Toxicology,* doi: 10.1002/jat.3484.

Abstract: Endocrine disrupting chemicals are found in diverse common products, including cosmetics, food packaging, thermal receipt paper and plastic containers. This exposes most people in developed countries through ingestion, skin absorption and inhalation. Two ubiquitous endocrine disrupting chemicals, bisphenol A (BPA) and diethylhexyl phthalate (DEHP) can interact in disrupting blastocyst implantation in inseminated females. We hypothesized that DEHP might increase the bioavailability of BPA in tissues by competing for metabolic enzymes. We injected 0, 3, 9 or 18mg DEHP into female and male mice and allowed 30min for the chemical to circulate before giving them a food supplement containing 50μg kg^{-1} ^{14}C–BPA. Animals were dissected 1h following ^{14}C–BPA administration and various tissue samples were acquired. Samples

were solubilized and radioactivity was measured via liquid scintillation counting. In cycling females, DEHP increased BPA deposition in the muscle, uterus, ovaries and blood serum relative to controls. In peri-implantation females, DEHP increased deposition of BPA in the uterus, ovaries and serum relative to controls. In males, DEHP doses increased BPA deposition in serum and epididymis relative to controls. These results are consistent with the hypothesis that DEHP competes with BPA for conjugating enzymes such as UDP- glucuronosyltransferase, thereby magnifying the presence of BPA in estrogen-binding reproductive tissues.

Chapter 2

Stress lowers the threshold dose at which bisphenol A disrupts blastocyst implantation, in conjunction with decreased uterine closure and e-cadherin

Evan D. Borman, Warren G. Foster, Matthew K. E. Greenacre, Cameron C. Muir & Denys deCatanzaro

Chemico-Biological Interactions, 237, 87-95

Author's Contributions

Evan D. Borman: Formation of experimental design, data collection, data analysis, and manuscript writing.

Warren G. Foster: Assistance with data collection and manuscript editing.

Matthew K. E. Greenacre: Assistance with data collection.

Cameron C. Muir: Assistance with data collection.

Denys deCatanzaro: Assistance with concept development and experimental design, data analysis, and manuscript writing.

Abstract

Exposure to stress can disrupt blastocyst implantation in inseminated female mice, and evidence implicates elevation of the female's estrogen:progesterone ratio. Exposure to the xenoestrogen, bisphenol A (BPA) can also disrupt implantation. Undisturbed control female CF-1 mice were compared to other females that were exposed to predators (rats) across a wire-mesh grid during gestation days (GD) 1-4, a procedure that elevates corticosterone but does not on its own disrupt implantation in this genetic strain. They were concurrently exposed to varied doses of BPA that on their own were below the threshold dose sufficient to disrupt implantation. On GD 6, we measured the number of intrauterine implantation sites and extracted their uteri, which subsequently were stained and analyzed for uterine luminal area and epithelial cadherin (e-cadherin), a molecule that causes uterine closure and adhesion of blastocysts to the uterine epithelium. The combination of rat-exposure stress and BPA significantly disrupted implantation and increased uterine luminal area, whereas either manipulation on its own did not. E-cadherin was significantly reduced by exposure to BPA, positively correlated with the number of implantation sites, and inversely correlated with luminal area. BPA exposure was also associated with nonmonotonic perturbation of urinary corticosterone concentrations and increased urinary estradiol concentrations on GD 6. These data are consistent with a potential summation of stress-induced estrogen and xenoestrogen activity.

1. Introduction

A number of external and chemical stimuli can disrupt intrauterine blastocyst implantation in inseminated female mammals. Implantation failure can be caused by diverse stressors, including physical restraint, human handling, excessive heat or cold, predator exposure, and environmental changes [1], and by exposure to novel males (the Bruce effect) [2,3]. While adrenal stress hormones do not appear to be implicated in these effects [4,5], very low doses of exogenous estrogens, especially 17β-estradiol (E_2), mimic both stress-induced early pregnancy disruptions and the Bruce effect [6-8]. To some degree, higher concentrations of circulating progesterone (P_4) may mitigate such pregnancy disruptions [8-10].

Humans and wildlife in current generations are widely exposed to bisphenol A (BPA), the monomer of polycarbonate plastics and epoxies [11-13]. Due to its affinity for estrogen receptors [14,15] and estrogen-related receptors [16], BPA is viewed as a xenoestrogen. Like natural estrogens, oral or injected BPA can disrupt blastocyst implantation, albeit at doses roughly 10,000 times the effective dose of E_2 [17-19]. CF-1 mice given 6.75 mg/animal (approximately 200 mg/kg) daily on gestation days (GD) 1–4 had significantly fewer implantation sites than those given lower doses, while 10.125 mg/animal (300 mg/kg) eliminated pregnancy altogether [19]. However, effects have been observed at 100 mg/kg daily in C57BL6 mice [20], a strain that is much more susceptible to stress and implantation failure [8]. Exposure to triclosan, a common antibacterial substance that exacerbates BPA binding in the uterus [21], also lowers the threshold dose at which BPA disrupts implantation [22]. Although the doses at which

BPA adversely affects implantation in mice are arguably higher than those of common human exposure, women with high urinary concentrations of BPA show greater failure rates in *in vitro* fertilization than do women with lower urinary BPA [23].

Peri-implantation exposure of mice to rats, which naturally predate on mice, is a stress paradigm that effectively induce implantation failure [8,24]. In C57BL6 mice, rat-exposure-induced implantation failure was correlated with elevated E_2 and reduced P_4 [8]. Acute (1 h) exposure of CF-1 mice to rats across a wire-mesh grid elevated adrenocortical corticosterone and P_4 right after exposure, and E_2 levels also rose 4 h after exposure [25]. Other acute stressors that can elevate E_2 include 20 min of swimming stress in rats [26] and lipopolysaccharide exposure in mice [27]. In other studies with pregnant animals, E_2 levels were elevated by chronic restraint stress in rats [28] and sequential exposure to various mild stressors in mice [29].

We reasoned that stress exposure could increase vulnerability to uterine effects of xenoestrogens, given that estrogenicity is a common feature among stimuli that cause blastocyst implantation failure. While estrogen activity is critical in preparing the uterus for implantation [30-32], timing and concentration are critical, as small estrogen elevations interfere with uterine preparation for implantation [33,34], embryo development [35], and ova transport through the oviduct [36]. Accordingly we hypothesized that estrogenic stimuli could summate such that the critical threshold for implantation failure is exceeded. We tested this by examining the rat-exposure stressor in the less sensitive genetic strain of mice (CF-1), titrating the exposure such that it would

not disrupt implantation on its own. We combined this with doses of BPA that are insufficient to disrupt implantation on their own.

We also examined a uterine mechanism that could account for estrogenic impacts on implantation. The uterine lumen, the fluid-filled space within each uterine horn, closes during successful blastocyst implantation, concurrent to blastocyst adhesion to the uterine epithelium [37]. Among other factors, e-cadherin, a molecule secreted by uterine cells, causes blastocyst-epithelial bonding and adhesion of the uterine walls such that they physically enclose the blastocysts [38-42]. Evidence indicates that e-cadherin is promoted by P_4 and inhibited by E_2 [34,41]. We previously observed that uterine luminal area is substantially increased at or just below the threshold dose at which BPA causes implantation failure [19]. E-cadherin is suppressed and uterine luminal area opens during the Bruce effect [37], which is associated with exogenous E_2 from males' excretions [3,7,43] and depressed P_4 [37,44]. In light of evidence cited above, we hypothesized that e-cadherin suppression and uterine luminal opening due to a high E_2:P_4 ratio may be a common mediator of environmentally-induced implantation failures. We therefore measured luminal area and e-cadherin in conjunction with implantation failure in relation to the interaction of stress and BPA exposure.

2. Materials and Methods

2.1. Animals

Female subjects and inseminating males were CF-1 strain mice, and all stimulus rats were males of Long Evans strain, in both cases obtained from Charles River Breeding Farms of Canada (St. Constant, Quebec). CF-1 female subjects were aged 3-5 months. Rats were aged 6-15 months. Unless otherwise stated, animals were housed in standard polypropylene cages with ad libitum access to water and food (8640 Teklad Certified Rodent Chow, Harlan/Teklad, Madison, WI, USA); mouse cages measured 28×16×11 (height) cm whereas rat cages measured 44×23×20 (height) cm. Colony and treatment rooms were maintained on a reversed 14h light: 10h darkness cycle at 21°C. This research was approved by the McMaster University Animal Research Ethics Board, conforming to the standards of the Canadian Council on Animal Care.

2.2. Insemination, rat exposure, BPA injections, and implantation

Female mice were paired with adult males and their hindquarters were observed four times daily. Upon detection of a copulatory plug (GD 0), the female was moved into an exposure cage and randomly assigned to one of eight treatment groups consisting of all combinations of 2 conditions (rat-stressed vs. isolated controls) × 4 BPA doses (0, 3, 4, or 5 mg). Exposure cages were modified 20-cm high polypropylene rat cages with stainless-steel wire mesh (0.5 cm^2 squares) separating a rat compartment (28×23 cm) from a mouse compartment (8.5×23 cm). On GD 1, each control female was placed in an exposure cage with an empty adjacent compartment in a rat-free room, whereas each stressed female was placed in an exposure cage with a rat in the adjacent compartment in another room.

Daily injections of 0, 3, 4 or 5 mg BPA in peanut oil were administered subcutaneously from GD 1-4 for assigned control or rat-stressed mice. The volume of the peanut oil vehicle differed for the doses of BPA due to solubility constraints (3 and 4 mg in 0.2 ml and 5 mg in 0.3 ml). Proportional numbers of 0 mg subjects were run at these vehicle volumes; the results for these mice did not differ and were therefore pooled. On GD 5, the rats were removed from the mice, whereas the female mouse subjects remained in the exposure cages until sacrifice on GD 6. Dissections were performed commencing 4 h into the dark phase of the lighting cycle on GD 6, a time when the success or failure of implantation is clearly established [37]. Females were each sacrificed by cervical dislocation after 2 min of isoflurane anesthetic and weighed. Urine was collected from each subject as the bladder was voided during sacrifice over wax paper using 1 ml syringes and 25 gauge needle. The uterus was then excised via an abdominal incision, and the number of implantation sites was counted. An implantation site was defined as a round protuberance in an otherwise smooth and uninterrupted uterine horn. The two uterine horns were then separated and placed in 1.5 ml Diamed microtubes. Urine samples were stored after collection in such microtubes at -20^{o} C, until hormone analyses were conducted concurrently for all samples as described below.

2.3. Uterine histomorphology

Subsequent analyses were all focused on the 0, 4 and 5 mg BPA dose animals due to a lack of impact upon implantation at the 3 mg dose. Uteri excised from females were immediately fixed in 10% neutral buffered formalin for 48 h at 4°C after which they were stored in 70% ethanol solution at 4°C until the embedding procedure. Samples from both

uterine horns were trimmed of mesentery and cut for embedding. Uterine sections were embedded in paraffin and 5 µm thick sections were cut and mounted on glass slides. Two slides from each subject (one for each uterine horn) were stained with hematoxylin and eosin and mounted for bright-field microscopy. A single trained investigator who was blind with respect to experimental conditions measured the area in a minimum of 3 sections for each subject using 5× field images in ImageJ (National Institutes of Health). Luminal perimeter and area for each individual was calculated based on the average of at least 3 measurements for each uterus.

2.4. Immunohistochemical staining for e-cadherin

Samples embedded in paraffin were used for immunohistochemical staining. Slides were deparaffinized in xylene, rehydrated in descending grades of ethanol (100%, 90%, 70%, and 50%) and rinsed in phosphate buffered saline (PBS). Inhibition of endogenous peroxidase was accomplished by incubating slides in 7 mL of 30% H_2O_2 with 193 mL methanol at room temperature for 30 min followed by a PBS wash. To decrease nonspecific binding, the slides were then incubated with normal goat serum in PBS for 1 h in a covered humidified tray. This was followed by antigen retrieval by citrate buffer (pH 3.0) at 37°C for 30 min and PBS wash. The samples were then incubated at 4°C for 24 h with e-cadherin (H-108 from Santa Cruz, Santa Cruz, CA, USA) polyclonal rabbit antibodies at a dilution of 1:100. On the following day, sections were incubated with biotinylated secondary antibody for 2 h in a covered humidified tray followed by incubation with avidin-biotin peroxidase complex for 2 h separated and followed by a PBS wash. A DAB solution of 50 mg DAB dissolved in 200 mL PBS with

2 drops of H_2O_2 was used to conduct the DAB reaction for 10 min. The reaction was terminated with distilled water and Harris' hematoxylin was used as the counterstain. The sections were then dehydrated through graded ethanol solutions, cleared in xylene and mounted with Permount in preparation for bright-field microscopy. Images were digitally acquired at 100× oil immersion. In order to ensure blind measurement, samples were each labelled with a randomized code. Images were assessed by a trained investigator for presence or absence of staining on the apical border of luminal epithelial cells using ImageJ. The measures were then decoded and matched with conditions. The percent of positively stained cells was used to indicate the proportion of cells expressing the e-cadherin molecule.

2.5 Urinary steroid and creatinine analysis

Enzyme immunoassay methods were previously validated and presented in full [45-47]. Creatinine, E_2, P_4, and corticosterone were obtained from Sigma Chemical Co. Antibodies to these steroids and corresponding HRP conjugates were obtained from the Department of Population Health and Reproduction at the University of California, Davis. Steroid assays were conducted for each sample in duplicate and the average was used for each sample. Creatinine measures were also taken in duplicate, to allow for adjustment for variations in urine hydration. Where steroid measures were adjusted for creatinine, this was achieved by dividing the obtained value by the measurement of creatinine/ml of urine for the particular sample.

2.6. Statistical analysis

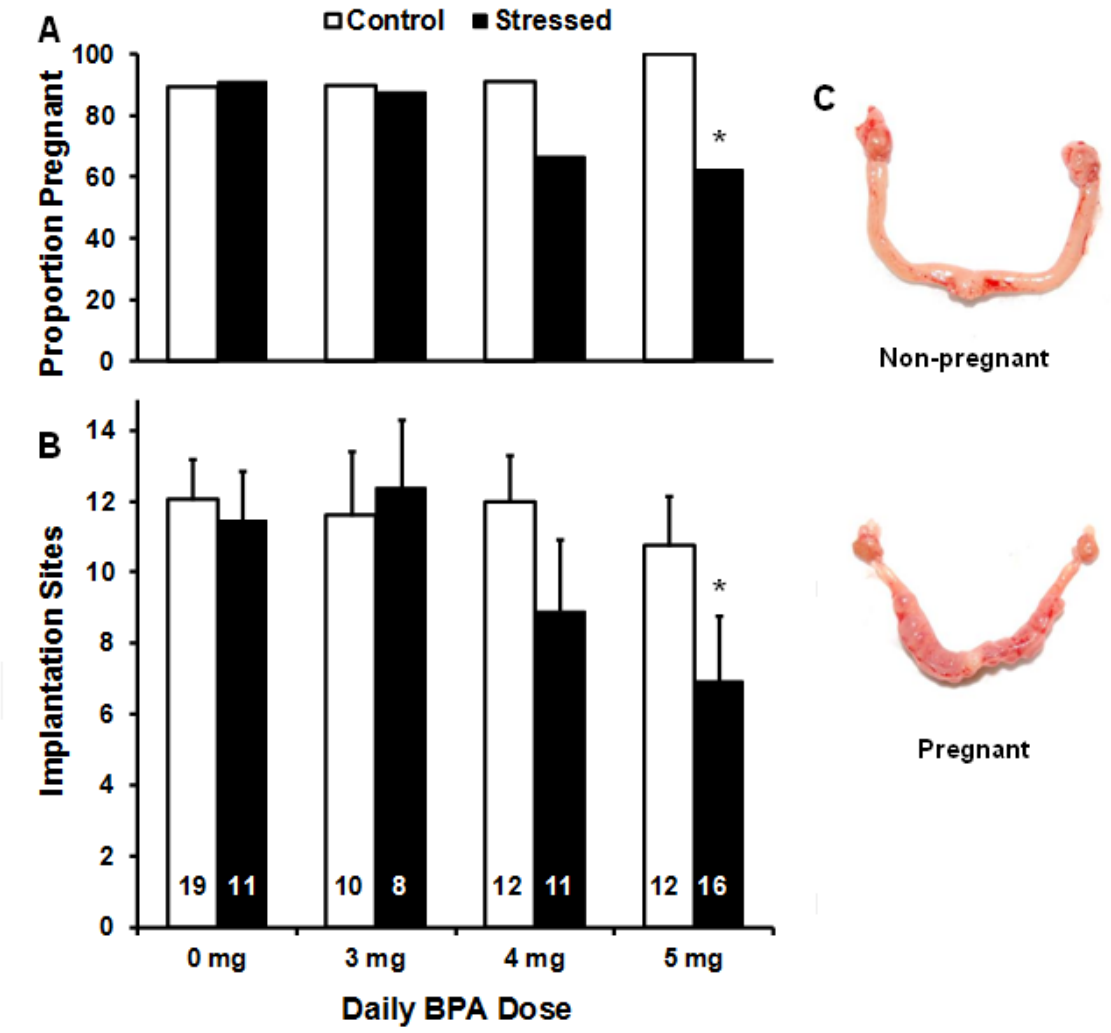
Statistical significance was designated at the conventional α level of $p < 0.05$. The number of implantation sites does not satisfy the assumption of normal distribution necessary for parametric tests, so orthogonal chi-squared tests of association were applied, comparing the presence or absence of any sites in control versus stressed females at each specific BPA dose. For measures of luminal area, luminal perimeter, e-cadherin, and steroid measures, a 2 (control versus stressed) × 3 (BPA dose) factorial analysis of variance (ANOVA) was conducted, followed by multiple pairwise comparisons using Duncan's method. Bivariate correlations were conducted among variables using the Pearson product-moment method, and significance was determined by an associated t-test.

3. Results

3.1. Implantation of blastocysts

The number of implantation sites on GD 6, the percent pregnant, and sample size in each condition are shown (Fig. 2.1). Implantation sites were evident on GD 6 in the majority of females in all conditions, but there were proportionally fewer females that were pregnant in the conditions involving exposure to both stress and either 4 or 5 mg BPA. Exposure to BPA alone without rat exposure at these doses did not reduce the number of implantation sites or the number of females that were pregnant, and the stress alone without BPA exposure also did not have an impact on these variables. Chi-squared

Fig. 2.1. Pregnancy measurements in inseminated female mice that were isolated (Control) or rat-exposed (Stressed) and concurrently given daily injections of 0, 3, 4, or 5 mg BPA on gestation day (GD) 1-4. *Panel A:* The proportion of subjects in each condition that were pregnant on GD 6, as indicated by the presence of any implantation sites. *Panel B:* Mean ± S.E. number of implantation sites on GD 6. Sample size (number of mice) for each condition is given at the base of the bar. *****Denotes a significant difference from the control condition at the same dose, $p < 0.05$. *Panel C:* Representative uteri of pregnant and non-pregnant females.

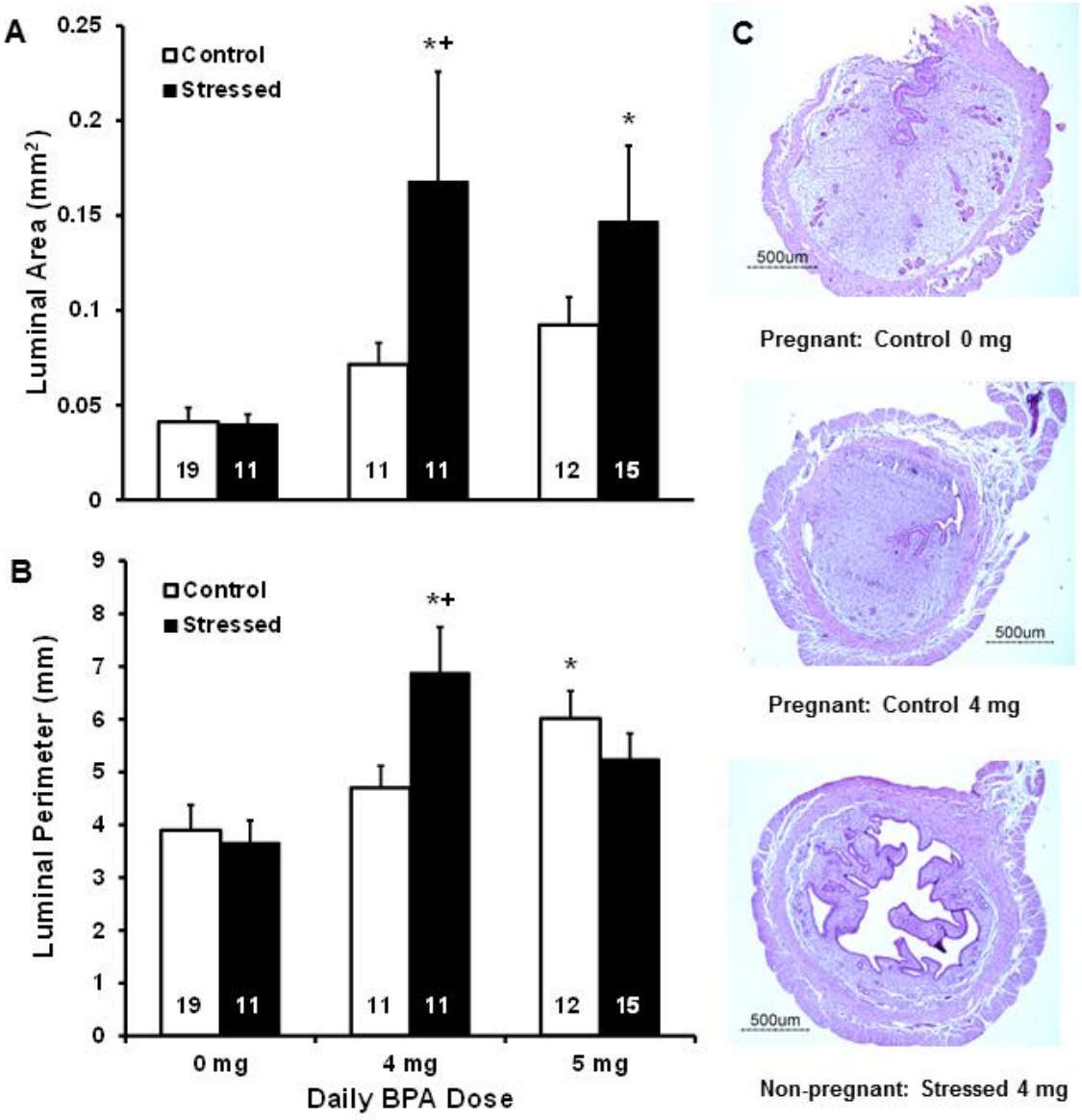


test of association for each dose showed there was no significant difference between control and stressed females in the proportion pregnant at the 0, 3 or 4 mg BPA doses. However, this measure was significant at the 5 mg BPA dose, $\chi(1) = 5.73$, $p < 0.025$. Collectively considering the 4 and 5 mg BPA doses, this measure was also significant, $\chi(1) = 7.34$, $p < 0.01$.

3.2. Uterine luminal area and perimeter length

Quantitative data and typical uterine slices from GD 6 are shown (Fig. 2.2). Generally, luminal area was greater in stressed than in control females, and greater in females given BPA than in those given the vehicle (0 mg BPA) injections. ANOVA on luminal area indicated a significant main effect of stress, $F(1,73) = 4.50$, $p = 0.035$, and of BPA dose, $F(2,73) = 5.28$, $p = 0.007$, but no significant interaction. Multiple comparisons showed that the stressed females given either 4 or 5 mg BPA differed significantly from both the control and stressed females given 0 mg BPA. At 4 mg BPA, the stressed females also differed from the control females. ANOVA on luminal perimeter length showed a significant main effect of BPA dose, $F(2,73) = 8.48$, $p = 0.001$, and a significant interaction of stress and BPA dose, $F(2,73) = 3.77$, $p = 0.027$, but that the main effect of stress was not significant. Multiple comparisons showed that the stressed females exposed to the 4 mg BPA dose differed from both 0 mg BPA groups as well as the control (unstressed) 4 mg BPA group. Also, the control group given 5 mg BPA differed from both 0 mg BPA groups.

Fig. 2.2. Uterine luminal measurements in hemotoxylin and eosin stained uterine sections from inseminated female mice on gestation day (GD) 6 after being isolated (Control) or rat-exposed (Stressed) and concurrently given daily injections of 0, 4, or 5 mg BPA on GD 1-4. *Panel A* shows mean ± S.E. luminal area, whereas *Panel B* shows mean ± S.E. luminal perimeter length. Sample size (number of uteri from distinct mice) for each condition is given at the base of the bar. *****Denotes a significant difference in multiple comparisons from both the control and the stressed groups given the 0 mg BPA dose. +Denotes a significant difference in multiple comparisons from the control group at the same dose. *Panel C:* Representative photomicrographs of the uterine sections from selected conditions. The original magnification was 5×.


A
Control
Stressed
Luminal Area (mm²)
0.25
0.2
0.15
0.1
0.05
0
*+
*
19
11
11
11
12
15
B
Control
Stressed
Luminal Perimeter (mm)
9
8
7
6
5
4
3
2
1
0
*+
*
19
11
11
11
12
15
0 mg
4 mg
5 mg
Daily BPA Dose
C
500um
Pregnant: Control 0 mg
500um
Pregnant: Control 4 mg
500um
Non-pregnant: Stressed 4 mg

3.3. Apical luminal e-cadherin

Quantitative measurements of e-cadherin on GD 6 are given (Fig. 2.3) and stained uterine slices for typical pregnant and non-pregnant females representing each condition are also shown (Fig. 2.4). There was clearly more evidence of e-cadherin staining among pregnant females, generally in association with uterine luminal closure. The proportion of apical luminal epithelial cells with positive staining for e-cadherin was significantly affected by BPA exposure, as indicated by a main effect of dose in ANOVA, $F(2,75) = 24.26$, $p < 0.0001$, but the main effect of stress and the interaction did not reach statistical significance. Multiple comparisons showed that all BPA-exposed groups differed significantly from the two 0 mg BPA groups.

Fig. 2.3. Mean ± S.E. proportion of uterine epithelial cells staining positively for e-cadherin in inseminated female mice on gestation day (GD) 6 after being isolated (Control) or rat-exposed (Stressed) and concurrently given daily injections of 0, 4, or 5 mg BPA on GD 1-4. Sample size (number of uteri from distinct mice) for each condition is given at the base of the bar. *****Denotes a significant difference in multiple comparisons from the both control and stressed 0 mg BPA conditions.

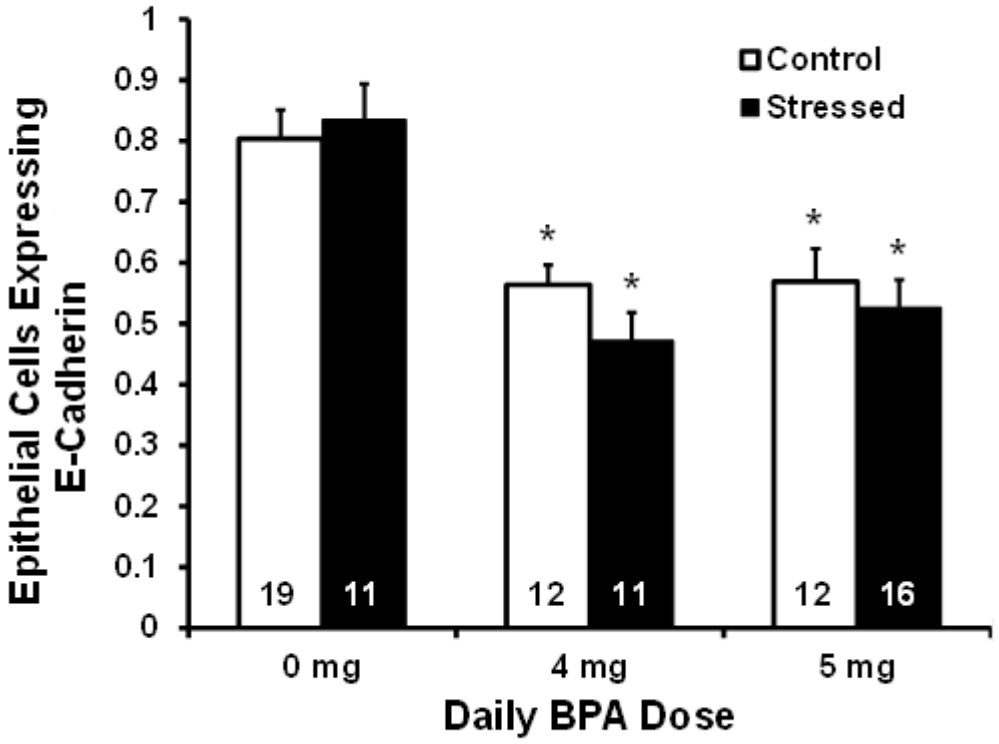
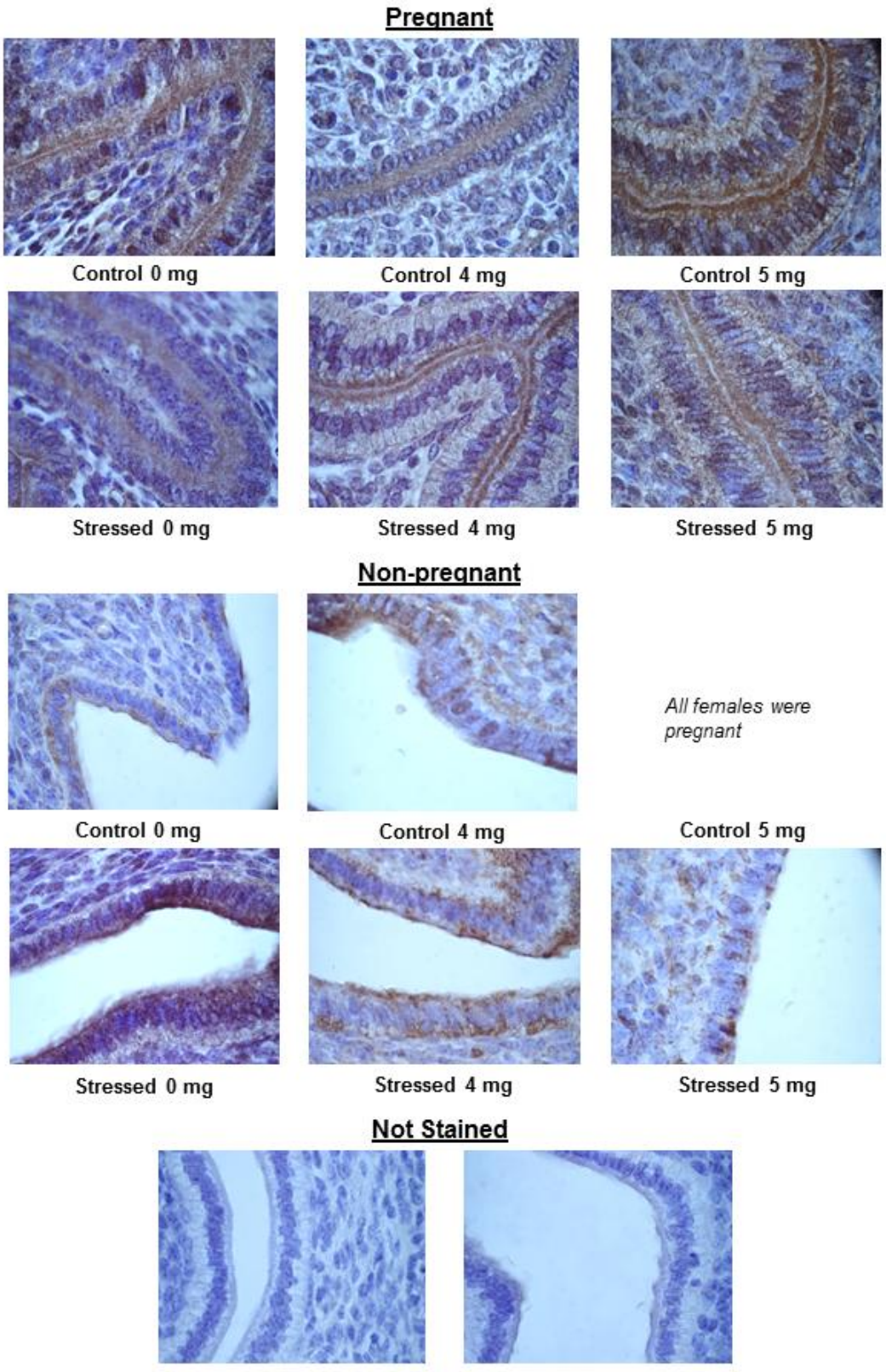


Fig. 2.4. Representative photomicrographs of uterine sections stained immunohistochemically for e-cadherin, showing one pregnant and one non-pregnant female from each condition. Two unstained uterine sections are also shown. The original magnification was 100×.


Pregnant
Control 0 mg
Control 4 mg
Control 5 mg
Stressed 0 mg
Stressed 4 mg
Stressed 5 mg
Non-pregnant
All females were pregnant
Control 0 mg
Control 4 mg
Control 5 mg
Stressed 0 mg
Stressed 4 mg
Stressed 5 mg
Not Stained

3.4. Urinary steroids

Measurements of creatinine-adjusted urinary E_2, P_4, corticosterone, and the E_2:P_4 ratio from terminal samples on GD 6 are given (Fig. 2.5). There was a significant main effect of BPA dose for creatinine-adjusted E_2, $F(1,62) = 4.24$, $p = 0.018$, and for the E_2:P_4 ratio, $F(1,62) = 3.51$, $p = 0.035$, but neither variable showed a main effect of stress nor an interaction. Multiple comparisons indicated that the 5 mg dose animals showed significantly more urinary E_2 and a higher E_2:P_4 ratio than the 0 mg animals. The trends in P_4 were not statistically significant. Creatinine-adjusted corticosterone showed a significant main effect of BPA dose, $F(1,62) = 3.41$, $p = 0.038$, but no main effect of stress or interaction; multiple comparisons showed that the 5 mg BPA dose significantly differed from the 4 mg dose. *3.5. Correlations among variables*

Correlations were calculated among all bivariate combinations of stress vs. control (coded as 1 and 0 respectively), number of implantation sites, presence or absence of pregnancy (coded as 1 and 0 respectively), luminal area, luminal perimeter, percentage of cells staining positive for e-cadherin, and steroid measures (Table 2.1). These correlations included all subjects in conditions involving control and stressed females given 0, 4, or 5 mg BPA. Statistical analysis (t-tests) indicated that pregnancy and the number of implantation sites correlated positively with the percentage of cells staining positively for e-cadherin and the E_2:P_4 ratio, but negatively with luminal area. Exposure to rat-induced stress correlated positively with luminal area and negatively with pregnancy and the number of implantation sites. BPA dose correlated positively with

Fig. 2.5. Mean ± S.E. creatinine-adjusted urinary estradiol (E_2), progesterone (P_4), and corticosterone (Cort), and the ratio of E_2 to P_4 in samples from gestation day (GD) 6 from inseminated females that were either stressed by rat-exposure or undisturbed (control), while concurrently receiving 0, 4, or 5 mg BPA daily on GD 1-4. Sample size (number of mice) for each condition is given at the base of the bar. *****Denotes a significant difference between the 5 mg conditions collectively and the 0 mg conditions. $^{+}$Denotes a significant difference between the 5 mg conditions collectively and the 4 mg conditions.

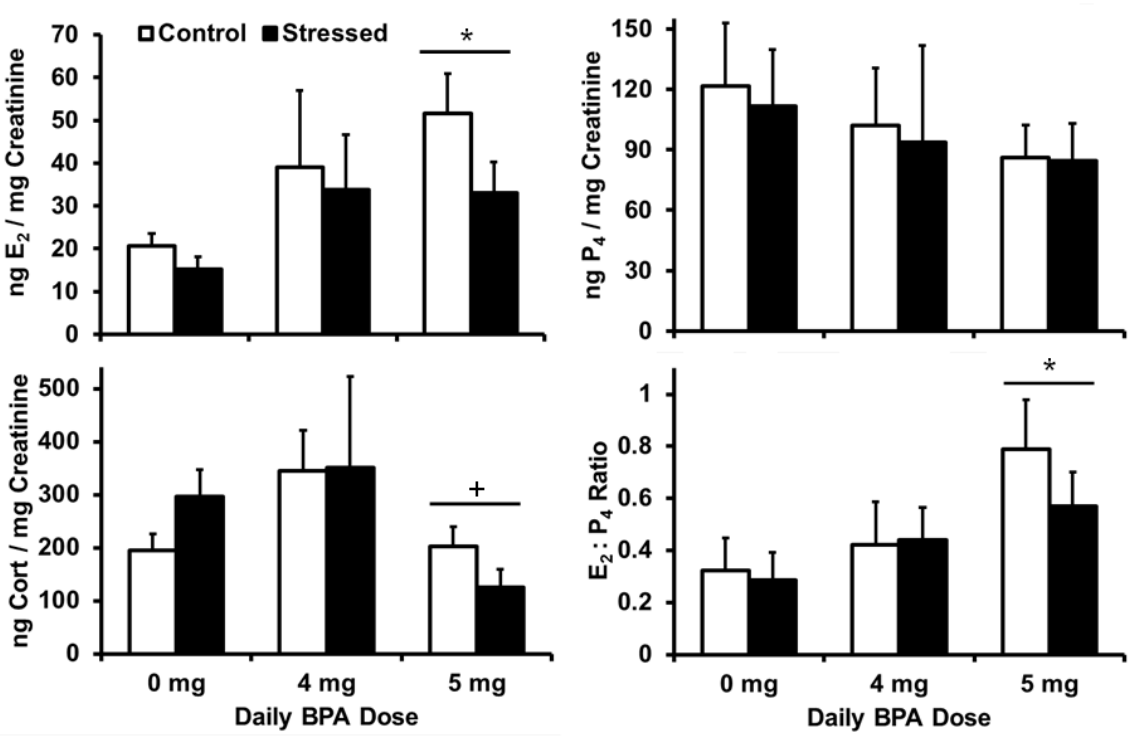


Table 2.1. Correlations among paired variables including BPA dose (dose), stress vs. control (stress), number of implantation sites (sites), uterine luminal area (area), uterine luminal perimeter (perim), uterine e-cadherin (e-cad), creatinine-adjusted urinary estradiol (E_2), creatinine-adjusted urinary progesterone (P_4), the ratio of E_2 to P_4, and creatinine-adjusted urinary corticosterone (cort). Data are from all conditions where inseminated female mice were either stressed by rat-exposure or undisturbed, while also receiving 0, 4, or 5 mg BPA daily on gestation days 1-4. n=77 for all correlations, except those involving steroid measures, where n=68. [a]$p < 0.05$, [b]$p < 0.01$, [c]$p < 0.001$, [d]$p < 0.0001$

	cort	**E_2:P_4**	**P_4**	**E_2**	**e-cad**	**perim**	**area**	**preg**	**sites**
dose	-0.10	0.31[b]	-0.14	0.31[a]	-0.54[d]	0.35[b]	0.32[b]	-0.14	-0.28[a]
stress	0.01	-0.04	-0.06	-0.09	-0.18	0.13	0.26[a]	-0.30[b]	-0.29[a]
sites	-0.11	-0.24[a]	0.13	-0.07	0.44[c]	-0.28[a]	-0.51[d]	0.84[d]	
preg	-0.16	-0.17	0.05	0.07	0.37[b]	-0.22[a]	-0.53[d]		
area	0.07	-0.02	-0.11	-0.14	-0.46[c]	0.71[d]			
perim	0.08	0.12	-0.21	0.10	-0.40[c]				
e-cad	-0.24[a]	-0.01	0.08	0.04					
E_2	0.21	0.38[b]	0.38[b]						
P_4	0.16	-0.31[a]							
E_2:P_4	-0.15								

luminal area, perimeter length, and creatinine-adjusted urinary E_2; and negatively with e-cadherin and number of implantation sites. Being pregnant correlated negatively with urinary corticosterone.

Discussion

We designed this experiment to examine a narrow range of transition in impact of BPA upon intrauterine blastocyst implantation. We chose BPA doses that were below the established threshold at which implantation fails. We used a stress procedure, involving exposure to a rat across a grid, which is established to elevate corticosterone and produce avoidance behavior in CF-1 mice [25]. Whereas this stress procedure on its own can produce a robust disruption of implantation in C57BL6 mice [8], it is insufficient on its own in CF-1 mice. Procedures were designed to be consistent and minimally invasive, given evidence that early pregnancy in mice can be disrupted by physical restraint [48] or human handling [49]. The data show that daily doses of 3, 4, or 5 mg BPA/animal (equivalent to 89, 119, or 148 mg/kg) were insufficient to disrupt implantation when administered on their own. When given in conjunction with the stressor, a trend toward reduced implantation sites emerged at the 4 mg dose and was clearly significant at the 5 mg dose. Uterine luminal area was significantly reduced by the conjunction of stress with either the 4 mg dose or the 5 mg dose. Luminal perimeter was similarly but less consistently affected; luminal area is probably a better measure of luminal opening, as a long irregular perimeter will hold less volume than a shorter, more circular one. E-cadherin was suppressed by either 4 mg or 5 mg BPA, and this occurred regardless of the presence or absence of the stressor.

Implantation failure was clearly associated across conditions with greater uterine luminal area, or a failure of the uterine lumen to close. Also, luminal closure was associated with e-cadherin, apparently binding the uterine walls in our photomicrographs (Fig. 2.4). Luminal closure is an antecedent to implantation that physically promotes proximity of the blastocyst and the uterine wall [50-54]. Luminal closure occurs due to reduced fluid volume in the luminal space following increased fluid absorption by epithelial cells [55] and uterine glands [56]. Luminal closure is associated with increased fluid absorption promoted by P_4, whereas E_2 induces fluid secretion resulting in increased luminal area [52,56-59]. Once the lumen is free of fluid, failure of luminal closure and implantation could also result from E_2-mediated down-regulation of e-cadherin [34]. E-cadherin is up-regulated in the uterus during implantation [39-41], when along with other cadherins it migrates toward the apical (luminal) side of uterine cells [38]. This movement of cadherins promotes the adhesion of epithelial cells between opposing sides of the uterine lumen, facilitating luminal closure around the implanting blastocyst [38]. It also promotes attachment between the blastocyst and uterine wall [60]. In non-receptive human endometrial epithelial cells *in vitro,* forced expression of e-cadherin was found to enhance receptivity for the blastocyst [42].

There are thus at least three mechanisms by which estrogenic activity can impede blastocyst implantation. The first, not studied here, is the disturbance of the timing of blastocyst transport through the oviduct, such that arrival in the uterus is not synchronized with uterine preparation for implantation [30,36]. The second is the influx of fluid into the uterine lumen, as discussed above. The third is the suppression of e-cadherin, also

discussed above. Collectively, these mechanisms can account for the fact that implantation can be disrupted and luminal area opened by novel males, whose excreted E_2 reaches the female's ovaries and uterus [7,61]; by major stressors, which can elevate endogenous E_2 [25,28]; and by BPA, which binds with estrogen receptors [15,62]. Novel males can also suppress e-cadherin [37], and as indicated in the current study so can BPA, but no conclusion can yet be drawn concerning stress on its own from the current data. Accordingly, estrogenicity is a common feature among various stimuli that disrupt implantation, and summation of estrogenic influences could account for the present data showing that two disparate stimuli, each of which could not on its own disrupt implantation and open the uterine lumen, could do so when applied together.

It is unlikely that estrogenic action is the only relevant factor. P_4 suppression, which has been observed in conjunction with the Bruce effect [37], stress-induced implantation failure [8], and BPA-induced implantation failure [18], could also contribute by interfering with P_4-induced fluid efflux from the uterine lumen and decidualization. However, it is unclear whether observed reductions of P_4 in conjunction with implantation failure are cause or consequence of such failure. In the case of BPA, the threshold dose for implantation failure was lower than that for P_4 suppression [18]. Under some circumstances, exogenous P_4 can to some degree counteract the influence of stressors, but those effects are incomplete [10] or conditional on concurrent E_2 levels [8] and may be pharmacological rather than physiological. A contribution of P_4 to the Bruce effect has been posited to occur in response to prolactin suppression during novel male exposure in the Bruce effect [44,63,64]; however the evidence is arguably incomplete

[24]. In the case of stress-induced early pregnancy failure, there are also likely stressor-specific factors, and there is also evidence for a role of immune factors, albeit more during the post-implantation period [65,66].

The urinary steroid measures taken here from samples on GD 6 likely reflect only the aftermath of the impacts of the experimental manipulations, as the females were removed from the rat-exposure cages and given the final BPA injection on GD 4. The trends in urinary E_2 and P_4 are consistent with the hypotheses, but those for P_4 do not reach statistical significance. However, the E_2:P_4 ratio was negatively correlated with the number of implantation sites. Curiously, urinary corticosterone was elevated at the 4 mg BPA dose but apparently suppressed at the 5 mg BPA dose; causes for such a non-monotonic effect are unknown. Previously where it was possible to measure these steroids more proximate to rat-exposure stress, corticosterone was clearly elevated by rat exposure, and P_4 was suppressed and E_2 was elevated in females losing pregnancy due to rat-exposure [8,25].

A practical implication of the current data is the possibility that effects of xenoestrogens such as BPA may be greater in individuals experiencing intense stress and adrenocortical activation. The doses of BPA employed here are unlikely to represent common human exposure, although they may model some forms of industrial exposure. However, this strain of mouse, as indicated above, is relatively less likely to show implantation failure after stressors and BPA than are other strains. Bioactive BPA is observable in the uterus in dose ranges that could represent more common human exposure [62], and very low dose effects of BPA, although controversial, have been

observed with other reproductive parameters, particularly those following perinatal exposure [67,68]. It is possible that the principle of stress-induced reduction in threshold dose might generalize to low-dose effects.

Acknowledgments

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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Chapter 3

Concurrent administration of diethylhexyl phthalate reduces the threshold dose at which bisphenol A disrupts blastocyst implantation and cadherins in mice

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Author's Contributions

Evan D. Borman: Formation of experimental design, data collection, data analysis, and manuscript writing.

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Abstract

Many people are repeatedly exposed to both bisphenol A (BPA) and diethylhexyl phthalate (DEHP), but there has been little research concerning their effects in combination. Both can disrupt blastocyst implantation in inseminated females, albeit at high doses. We exposed mice on gestation days (GD) 1–4 to combinations of BPA and DEHP in doses below the threshold necessary to disrupt implantation on their own. On GD 6, there were fewer normally-developed implantation sites and more underdeveloped implantation sites in females given the combined subthreshold doses. Uterine epithelial cadherin (e-cadherin), a protein that assists in blastocyst adhesion to the uterine epithelium, was significantly reduced by these combined doses, but not by the individual doses. A similar trend was seen in integrin-αvβ3, another uterine adhesion molecule. Cadherin-11, was disrupted by BPA but not DEHP. These data are consistent with competition of BPA and DEHP for conjugating enzymes.

Key words: BPA; DEHP; implantation; e-cadherin; cadherin-11; uterus; blastocyst

1. Introduction

Endocrine disruptors (EDCs) constitute a class of chemicals that can mimic or interfere with hormonal systems. Many of these chemicals are found in common plastics and household products (Dodson et al., 2012; Wams, 1987). Like estradiol (E_2), a potent estrogen, a number of EDCs have been shown to disrupt blastocyst implantation in inseminated female mammals (Berger et al., 2008; Crawford & deCatanzaro, 2012; Li et al., 2012; Xiao et al., 2011). We focused here on the effects of diethylhexyl phthalate (DEHP) and bisphenol A (BPA), undertaking to determine whether these substances could summate or otherwise interact in their impacts on implantation.

DEHP is a plasticizer that is found, for example, in diverse personal care products, medical devices, and various products containing polyvinyl chloride (Dodson et al., 2012; Miles-Richardson et al., 2002; Shelby, 2006). BPA is the monomer of polycarbonate plastics and some epoxy resins, and it is found, for example, in drinking bottles, water pipes, and food storage containers (Vandenberg et al., 2007). These chemicals often occur as contaminants in soil and water in populated regions or near chemical plants at levels varying between 2 µg/L to 30 mg/L for DEHP (Wams, 1987) and 0.14 µg/L to 3.61 mg/L for BPA (Coors et al., 2003; Kolpin et al., 2002). The abundance of these chemicals in modern environments causes daily human exposure through dermal absorption, ingestion, and inhalation; with daily intake estimated as ranging from 1–100 µg/kg for DEHP (Koch et al., 2006) and 0.1–1.6 µg/kg for BPA (European Commission, 2002; US Food and Drug Administration, 2013). While the amount of DEHP and BPA that is typically absorbed is far below that which would be necessary to induce acute toxicity (Pant &

Deshpande, 2012; Wams, 1987), that does not preclude the possibility that exposure to low doses has more subtle detrimental effects in humans and wildlife.

Previous work has shown that blastocyst implantation can be disrupted in mice by five daily injections of DEHP in doses of 1000 mg/kg (Li et al., 2012). Similar administration of BPA over four days disrupts implantation in doses of 100 mg/kg (Berger et al., 2008, 2010; Xiao et al., 2011). These doses clearly exceed levels of common human exposure, although there is evidence that among women undergoing *in vitro* fertilization, urinary concentrations of BPA correlate inversely with success of the procedure (Ehrlich et al., 2012). Moreover, such examinations of threshold doses in mouse models involve particular EDCs in isolation, whereas exposure to EDCs in humans and wildlife typically involves multiple concurrent substances. Recent evidence suggests that some estrogenic chemicals can have additive effects on biological systems. Combined administration of BPA and triclosan, an antibacterial substance found in most soaps and a number of other household products, can cause implantation failure at doses that are insufficient on their own to do so (Crawford & deCatanzaro, 2012). This was corroborated by evidence that concurrent exposure to triclosan can increase uterine deposition of environmentally-relevant oral doses of ^{14}C-BPA in mice (Pollock et al., 2014).

Estrogen activity is critical for uterine receptivity to fertilized ova, but small elevations above optimal concentrations can disrupt blastocyst implantation (Ma et al., 2003; Thorpe et al., 2013). The uterine lumen is a fluid-filled space that closes around blastocysts as they adhere to the epithelium (Rajabi et al., 2014). During implantation the

epithelial cells secrete multiple adhesion proteins, such as e-cadherin, which causes the blastocyst to adhere to the uterine walls and reinforces closure of the uterine lumen (Aplin, 1997; Hyland et al., 1998; Jha et al., 2006; Paria et al., 1999; Rahnama et al., 2009). E-cadherin is dependent on the presence of progesterone (P_4) and E_2, with P_4 increasing and E_2 decreasing expression (Jha et al., 2006; Potter et al., 1996). In addition to e-cadherin, other adhesion proteins are present on the luminal epithelium to assist in implantation (Aplin, 1997). Cadherin-11 (cad-11), is a P_4-mediated protein that is present during decidualization of the uterus and is thought to aid in anchoring the blastocyst to the epithelial cells of the lumen (Chen et al., 1998). Integrin αvβ3 is an endometrial protein that is present in both epithelial cells and stroma, and assists in blastocyst implantation (Ceydeli et al., 2006; Coughlan et al., 2013; Illera et al., 2000; Kang et al., 2014; Lessey et al., 1992; Srinivasan et al., 2009). Regulation of αvβ3 is dependent on an E_2-P_4 balance, with increased E_2 suppressing expression (Lessey et al., 1992; Srinivasan et al., 2009; Widra et al., 1997). The mechanism for estrogenic implantation failure is thought to involve increased estrogen receptor activation, which causes an influx of fluid from the stroma to the lumen while e-cadherin, cad-11, and αvβ3 are downregulated (Borman et al., 2015; Lessey et al., 1992; Martin et al., 1970; Naftalin et al., 2002; Parr, 1983; Salleh et al., 2005; Srinivasan et al., 2009; Widra et al., 1997).

Here we undertook to determine the dose-response of implantation to exogenous doses of DEHP in mice. On that basis we selected doses of DEHP that were below the threshold necessary to disrupt implantation on their own. We examined their impact on implantation when the inseminated mice concurrently received doses of BPA that were

previously established to be insufficient to do so on their own (Berger et al., 2008, 2010). We hypothesized that concurrent administration of DEHP and BPA at such subthreshold doses would summate and disrupt blastocyst implantation, and that this would be associated with estrogenic influences that increase luminal area and decrease e-cadherin, cad-11, and αvβ3.

2. Materials and methods

2.1. Animals and housing

Male and female CF-1 mice (*Mus musculus*) aged 3-6 months were obtained from Charles River Breeding Farms of Canada (La Prairie, Québec). Mice were housed in standard 28 cm x 16 cm x 11 cm (height) polypropylene cages, with *ad libitum* access to food (8640 Teklad Certified Rodent chow; Harlan Teklad, Madison, WI) and water. Colony rooms were maintained at 21^{o} C with a reversed 14:10 h light:dark cycle. This research was approved by the Animal Research Ethics Board of McMaster University in compliance with the guidelines of the Canadian Council on Animal Care.

Sexually naïve female mice were each randomly paired with a CF-1 male. Female hindquarters were inspected three times per day during the dark phase of the light cycle for the presence of vaginal copulatory plugs. The date of a plug was designated as gestation day (GD) 0. Females were pseudo-randomly assigned to one of the experimental conditions with age and weight counterbalanced. On GD 1, each inseminated female subject was housed alone in a clean cage with fresh bedding.

2.2. Chemicals

Both DEHP (≥98% purity, CAS 117-81-7) and BPA (≥99% purity, CAS 80-05-7) were obtained through Sigma-Aldrich, St. Louis, MO.

2.3. Repeated DEHP administration

Subcutaneous injections of DEHP were administered to 103 female mice on GD 1–4. The doses assigned were 0, 1, 3, 9, 18, 27 and 36 mg/animal/day in order to provide a dose-response curve and to determine the lowest dose required to disrupt blastocyst implantation. The mean mass of subjects was 35.9 g. Each dose was dissolved in 0.05 ml peanut oil, with 0 mg controls being administered the same volume. To reduce irritation, injections were given at the scruff of the neck and various locations on the back. Pregnancy outcome was measured on GD 6. Females were sacrificed by cervical dislocation after 2 min of isoflurane anesthetic. Their uteri were excised via abdominal incision and the number of implantation sites counted. An implantation site was defined as a spherical protuberance in an otherwise smooth and uninterrupted uterine horn.

2.4. Combined DEHP and BPA administration

Subcutaneous injections of DEHP and BPA were administered to 70 female mice on GD 1–4. The DEHP dose was 27 mg/animal/day in combination with 2, 3, and 4 mg/animal/day doses of BPA. The mean mass of subjects was 33.2 g. All doses of DEHP were dissolved in 0.05 ml oil while doses of BPA were dissolved in 0.1, 0.2, and 0.3 ml oil respectively. The controls were administered 0.1, 0.2, or 0.3 ml oil and the results were pooled. Injections were administered to minimize irritation as in the first

experiment. Pregnancy outcome was measured on GD 6. Females were sacrificed by cervical dislocation after 2 min of isoflurane anesthetic. Their uteri were excised via abdominal incision and the number of implantation sites was counted. The right uterine horns were then cut medially along the vagina and stored in 10% buffered formalin for 48 h, and subsequently stored in 70% ethanol, both at 4° C.

2.5. Uterine histomorphology

Subsequent analyses were all focused on the 0, 27 mg DEHP, 3 mg BPA, and 27 mg DEHP + 3 mg BPA conditions due to a significant effect in developed implantation sites at the 27 mg DEHP + 3 mg BPA dose. Stored uteri were trimmed of mesentery and horizontal cross-sections were cut for embedding. Uterine sections were embedded in paraffin wax and 5 µm sections were cut and mounted on glass slides. Uteri were stained with hematoxylin and DAB solution in preparation for e-cadherin measurement. A single, trained investigator who was blind with respect to the conditions from which specific samples derived measured luminal area using 5 x bright-field microscopy images obtained from an Olympus BH-2 microscope with a Lumenera Infinity 1 camera in ImageJ software (National Institutes of Health). Luminal area was measured by tracing the luminal perimeter in ImageJ and utilizing the program for area calculation from the perimeter. The area for each subject was the average from a minimum of 2 horizontal cross-sections (Borman et al., 2015; Rajabi et al., 2014).

2.6. Immunohistochemistry staining for e-cadherin, cad-11, and integrin αvβ3

Paraffin embedded samples were mounted on slides and deparaffinized in xylene followed by rehydration in 100%, 90%, 70%, and 50% ethanol and washed in phosphate

buffered saline (PBS). Endogenous peroxidase was blocked through 7 mL 30% H_2O_2 and 193 mL of methanol for 30 min. Slides were then washed in PBS and, to decrease non-specific binding, were incubated with normal goat serum for 2 h at room temperature. This was followed by antigen retrieval at 37 °C in citrate buffer (pH 3.0) for 30 min. Slides were then washed in PBS and incubated at 4 °C with e-cadherin antibody (H-108 from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), cad-11 antibody (Catalog #717600 from ThermoFisher Scientific, Waltham, MA USA), or integrin αvβ3 antibody (Catalog #sc-7312 from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 24 h. Following this, slides were washed in PBS and incubated with biotinylated antibody for 2 h at room temperature followed by a wash. Slides were incubated with an avid-biotin peroxidase complex for 2 h at room temperature and were washed in PBS prior to immersion in a DAB solution of 50 mg DAB dissolved in 200 mL PBS with 2 drops of 30% H_2O_2 for 10 min. The DAB staining was terminated with distilled water and Harris' hematoxylin was used to counter stain. The sections were then dehydrated in graded ethanol solutions, cleared in xylene, and then mounted using Permount for bright-field microscopy. Pictures of the cells were obtained using an Olympus BH-2 microscope with a Lumenera Infinity 1 camera at 40x magnification. A rater who was blind to the condition of the tissues performed visual counts of e-cadherin and cad-11 positive cells (those adjacent to a dark brown color on the apical wall of the luminal epithelium) and negative cells (without such color). For each subject, the proportion of cells that were positive was calculated. As integrin αvβ3 was primarily found within the stroma, stains were assessed on a continuum based on staining strength in place of cell counting and

were labeled as no stain (0), weak (1), medium (2), or strong (3) to elucidate any differences.

2.7. Statistical analysis

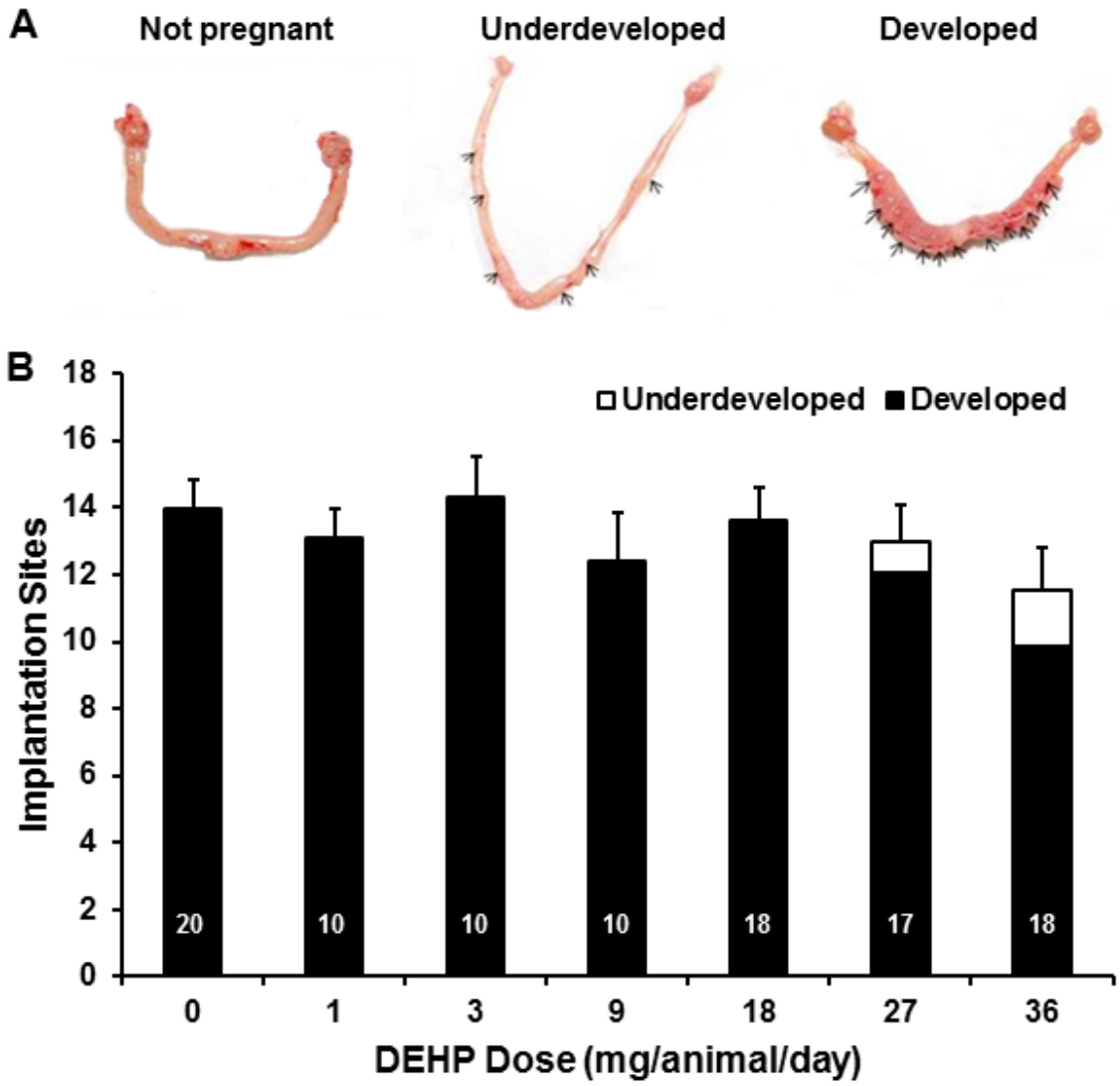
Differences between treatments were analyzed by Kruskal-Wallis rank sum test as Bartlett's test showed unequal variances across treatments for implantation sites, underdeveloped implantation sites, and developed implantation sites. Significant Kruskal-Wallis tests were followed by Dunn's multiple comparisons between all pairs of treatment combinations with Holm's alpha correction. Measures of luminal area, e-cadherin, cad-11, and integrin αvβ3 were analyzed by analysis of variance (ANOVA) to assess differences among treatments. Significant ANOVA results were followed by Newman-Keuls multiple pairwise comparisons among treatments. For all tests, the threshold level of statistical significance was set at the $p<0.05$ convention.

3. Results

3.1. Repeated DEHP administration

The number of implantation sites on GD 6 in the uterine horns of inseminated females was counted (Fig. 3.1) and a Kruskal-Wallis test was conducted. No significant difference was found in the number of implantation sites between dose treatment groups, $\chi^2(6) = 5.27$, $p=0.510$. However, it was visually noted that some of the implantation sites were underdeveloped in mice that were administered higher doses of DEHP. There was a significant difference among conditions in the number of underdeveloped implantation

Fig. 3.1. A) Pictures of typical uteri of inseminated female mice displaying variation in pregnancy on GD 6: Mouse uteri with no implantation sites (Not pregnant), underdeveloped implantation sites, and normally developing implantation sites (Developed). B) Mean ± S.E. number of developed (black bar) and underdeveloped (open bar) implantation sites for the total number of implantation sites (sum of developed and underdeveloped sites) for all DEHP doses. Sample sizes are given within each bar.

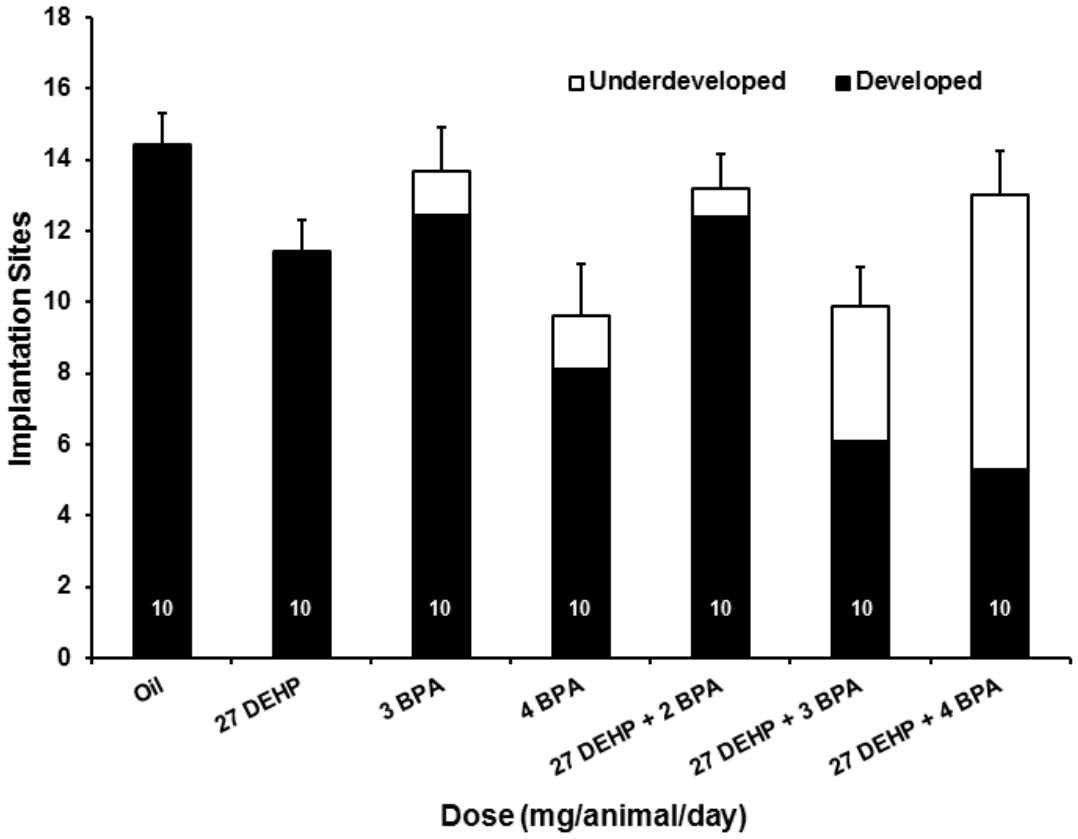


sites, $\chi^2(6) = 26.36$, $p<0.001$. Multiple comparisons indicated a greater number of underdeveloped sites in the 36 mg dose compared to controls ($p<0.001$) and the 3 and 9 mg doses ($p<0.01$). While the number of underdeveloped sites differed, the total number of developed sites only approached significance, $\chi^2(6) = 12.03$, $p = 0.061$. Pearson product-moment correlations were conducted comparing developed, underdeveloped, total implantation sites, and dose. Dose was negatively correlated with developed implantation sites, $r = -0.28$, $p<0.01$, while underdeveloped sites were positively correlated with dose, $r = 0.422$, $p<0.001$. Dose was not significantly correlated with the total number of implantation sites, $r = -0.157$, $p = 0.11$.

3.2. Combined DEHP and BPA administration

The number of implantation sites on GD 6 and the sample size of each condition are shown (Fig. 3.2). Results indicated a significant difference between conditions, $\chi^2(6) = 16.56$, $p<0.05$. Multiple comparisons showed that the 27 mg DEHP + 3 mg BPA and the 4 mg BPA doses had fewer implantation sites than did the controls ($p<0.05$). Subjects in the study exhibited similar underdeveloped implantation sites to those seen in Experiment 1. Analysis of underdeveloped implantation sites revealed a significant difference, $\chi^2(6) = 42.89$, $p<0.0001$, with multiple comparisons indicating the 27 mg DEHP + 3 mg BPA and 27 mg DEHP + 4 mg BPA doses had more underdeveloped sites than controls. There were fewer developed sites in the higher combined doses, $\chi^2(6) = 26.05$, $p<0.0001$; both the 27 mg DEHP + 3 mg BPA and the 27 mg DEHP + 4 mg BPA groups differed from the controls ($p<0.01$ and $p<0.001$, respectively).

Fig. 3.2. Mean ± S.E. number of developed (black bar) and underdeveloped (open bar) implantation sites for the total number of implantation sites (sum of developed and underdeveloped sites) for individual and combined doses. Sample sizes are given within each bar.



3.3. E-cadherin

Measurements of e-cadherin are illustrated (Fig. 3.3) and representative stained uterine slices are given for each condition (Fig. 3.4). ANOVA indicated differences among conditions, $F(3,22) = 3.60$, $p=0.029$, and multiple comparisons indicated that only

Fig. 3.3. A) Mean ± S.E. percentage of uterine epithelial cells staining positively for e-cadherin in inseminated female mice on gestation day (GD) 6 after daily injections of oil, 27 mg DEHP, 3 mg BPA, or 27 mg DEHP and 3 mg BPA on GD 1-4. Sample size (number of distinct uteri) is given within the bars. *Denotes a significant difference in multiple comparisons from the control (oil vehicle) condition. B) Mean ± S.E. percentage of uterine epithelial cells staining positively for cad-11 in inseminated female mice on gestation day (GD) 6 after daily injections of oil, 27 mg DEHP, 3 mg BPA, or 27 mg DEHP and 3 mg BPA on GD 1-4. Sample size (number of distinct uteri) is given within the bars. $^{+}$Denotes a significant difference in multiple comparisons from the 27 mg DEHP condition.

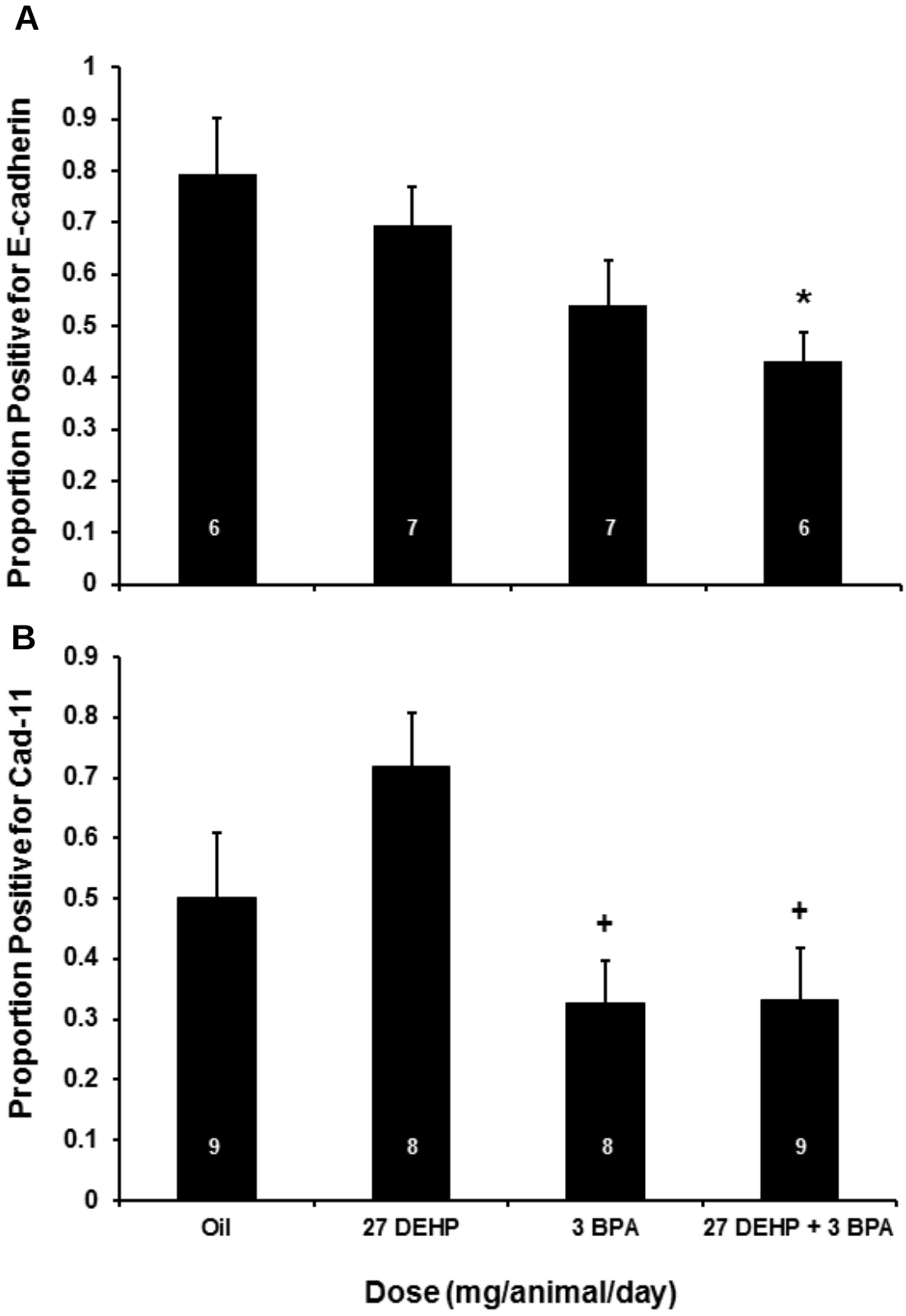
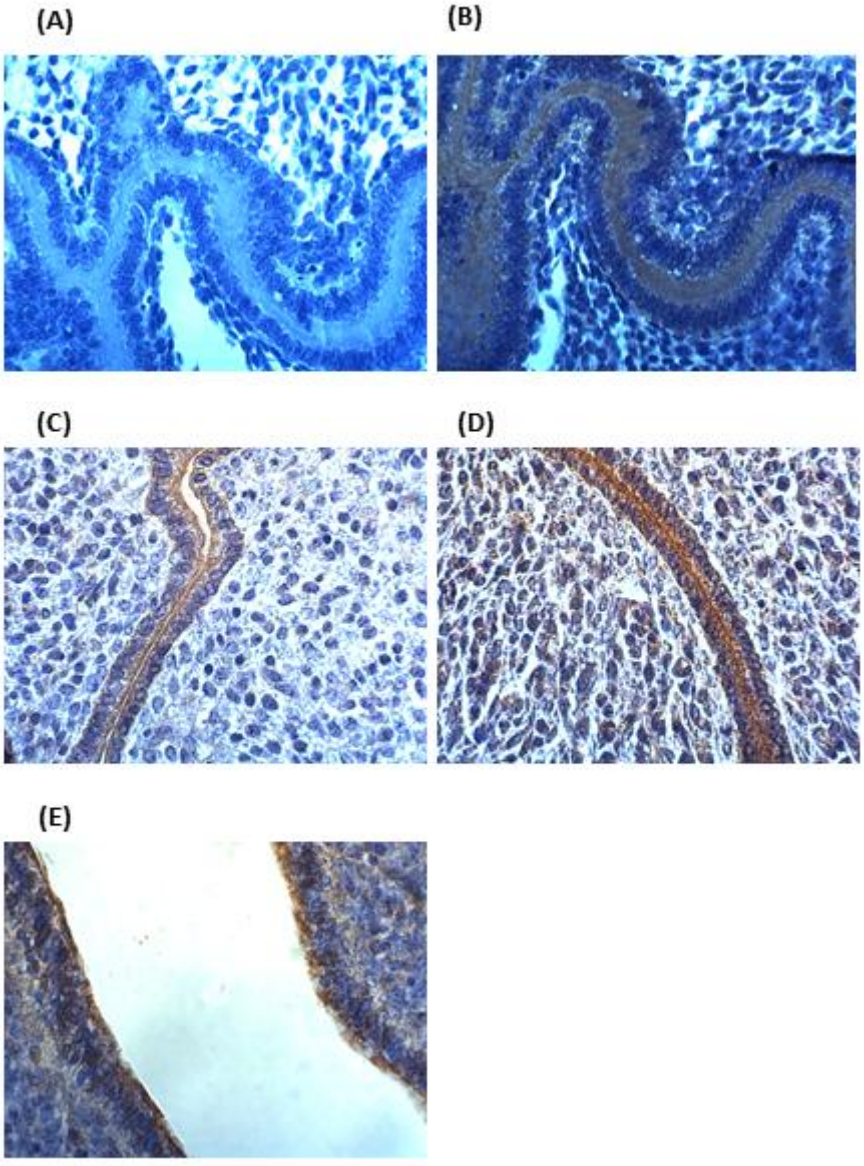

A
Proportion Positive for E-cadherin
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
6
7
7
6
*
B
Proportion Positive for Cad-11
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
9
8
8
9
+
+
Oil
27 DEHP
3 BPA
27 DEHP + 3 BPA
Dose (mg/animal/day)

Fig. 3.4. Representative photomicrographs of uterine sections stained immunohistochemically for e-cadherin, showing (A) an unstained section, (B) a pregnant female in the 3 mg BPA condition, (C) a pregnant female in the 27 mg DEHP condition, (D) a pregnant female in the control condition, and (E) a non-pregnant female in the 27 mg DEHP + 3 mg BPA condition.



the 27 mg DEHP + 3 mg BPA condition had significantly less e-cadherin on the apical luminal epithelium than did the controls.

3.4. Cadherin-11

Measurements of cad-11 are illustrated (Fig. 3.3B) and representative stained uterine slices are given for each condition (Fig. 3.5). ANOVA showed a significant effect of condition, $F(3,30) = 3.92$, $p = 0.018$. Multiple comparisons showed that the two conditions involving BPA showed reduced cad-11 on the apical luminal epithelium compared to the condition involving exposure to DEHP alone.

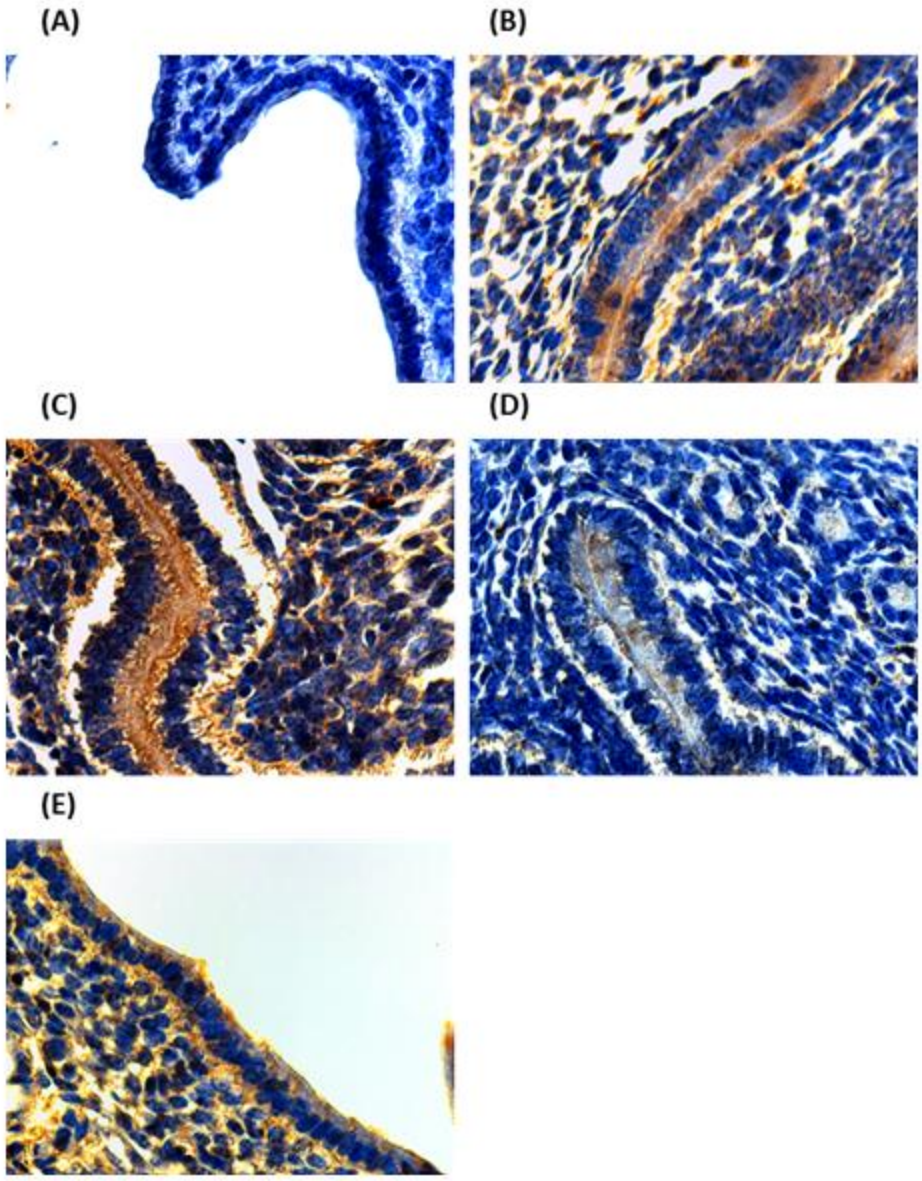
3.5. Integrin αvβ3

Analysis of integrin αvβ3 staining using ANOVA indicated a marginally non-significant effect of condition, $F(3,31) = 2.668$, $p = 0.065$. There was an overall trend of mean staining strength being lower, with the control condition having the strongest rating (2.00 ± 0.33) and the 27 mg DEHP and 3 mg BPA combined dose having the weakest rating (0.89 ± 0.20), with the 27 mg DEHP (1.75 ± 0.36) and 3 mg BPA (1.50 ± 0.27) doses in between.

3.6. Luminal area

The mean luminal areas for the oil, 27 DEHP, 3 BPA, and 27 DEHP + 3 BPA conditions were 5.08, 8.69, 5.18, and 7.96 μm^2, respectively. Despite there being fewer observed implantation sites and positive e-cadherin staining in the combination conditions, luminal area did not differ significantly among conditions, $F(3,20) = 0.367$, $p = 0.778$.

Fig. 3.5. Representative photomicrographs of uterine sections stained immunohistochemically for cad-11, showing (A) an unstained section, (B) a pregnant female in the control condition, (C) a pregnant female in the 27 mg DEHP condition, (D) a pregnant female in the 3 mg BPA condition, and (E) a non-pregnant female in the 27 mg DEHP + 3 mg BPA condition.



4. Discussion

Our data show that the dose response curves for DEHP and BPA are quite different. DEHP's effect is much weaker, and increasing doses gradually produce only a partial disruption of implantation. In contrast, there is an abrupt transition in the impact of BPA from no effect to complete disruption of implantation over a narrow dose range (Berger et al., 2008, 2010). We found here that implantation was significantly disrupted by a combination of 27 mg (approximately 750 mg/kg) DEHP and 3 mg (approximately 100 mg/kg) BPA, doses that were insufficient on their own to have such an effect. Much of this effect was due to some implantation sites being underdeveloped. This combined dose produced significantly lower levels of e-cadherin relative to controls. It is unclear whether underdeveloped sites are a result of delayed implantation, delayed development of the blastocyst, or blastocyst resorption, but some evidence indicates that implantation was delayed in studies with other EDCs where there were similar observations (Crawford & deCatanzaro, 2012; Xiao et al., 2011).

Luminal closure is crucial to implantation as it promotes blastocyst proximity with the uterine walls (Finn & Martin, 1969; Hedlund et al., 1972; Mayer et al., 1967; Reinius, 1967). Luminal volume is mediated by the balance of E_2 and P_4. E_2 promotes luminal opening as fluid is secreted by the epithelial cells and uterine glands, increasing luminal space, whereas P_4 promotes luminal closure by causing an efflux of fluid (Martin et al., 1970; Naftalin et al., 2002; Salleh et al., 2006). Blastocyst implantation is negatively correlated with luminal opening and positively correlated with e-cadherin on the apical lumen (Berger et al., 2010; Borman et al., 2015; Rajabi et al., 2014). However, our results

did not show increased luminal area at the doses that decreased e-cadherin. As e-cadherin can bind the luminal walls together, e-cadherin downregulation may be required prior to luminal opening, and it is possible that administration of the combined dose from GD 1–4 provided insufficient time to increase the luminal space, but sufficient time to reduce e-cadherin expression.

The reduced levels of cad-11 observed in both BPA conditions indicate reduced P_4 production, as P_4 upregulates cad-11 expression and BPA decreases P_4 production (Chen et al., 1998; Peretz & Flaws, 2013). Higher cad-11 in the 27 mg DEHP condition could be due to the fact that DEHP can increase levels of progesterone (Mlynarĉíková et al., 2009), although the literature contains conflicting results on this matter (Hannon & Flaws, 2015). In contrast to the effects on cad-11, the trend in integrin αvβ3 resembles that in e-cadherin. Integrin αvβ3 is typically detected on the embryo surface where it can interact with the uterine epithelium (Sutherland et al., 1993). As were unable to isolate the blastocyst during tissue sectioning, we observed more integrin αvβ3 in the stroma than in the luminal epithelium.

The mechanism underlying the joint effect of DEHP and BPA upon blastocyst implantation remains to be determined. One hypothesis is that both chemicals bind to estrogen receptors and their individual effects summate to affect blastocyst implantation. Notably, BPA has a higher binding affinity than DEHP and will outcompete for estrogen receptor binding (Buteau-Lozano et al., 2008). DEHP can decrease serum concentrations of E_2 and increase serum concentrations of testosterone through decreased aromatase expression, thus making E_2 and BPA summation unlikely to occur (Davis et al., 1994;

Pocar et al., 2012). Another hypothesis about the interaction of DEHP and BPA considers their influence on enzymes that conjugate the chemicals into their inactive forms. The main enzymes for BPA conjugation are UDP-glucuronosyltransferase (UGT) and estrogen sulfotransferase (SULT1E1) (Hanioka et al., 2008; Nishiyama et al., 2002). Evidence indicates that substances that inhibit SULT1E1 can exacerbate the effects of estradiol and BPA (Crawford & deCatanzaro, 2012; Pollock et al., 2014, 2016). DEHP is not reliant on SULT1E1 to be conjugated (Koch et al., 2006), but its metabolite monoethylhexyl phthalate (MEHP) is conjugated by UGT (Hanioka et al., 2016). As both BPA and MEHP are conjugated by UGT, MEHP may recruit UGT activity and thereby allow additional BPA to remain in its free form. This would be similar to the effects of triclosan, which can competitively inhibit BPA glucuronidation through UGT (Wang et al., 2004) and magnify the binding of BPA at the uterus (Pollock et al., 2014). Triclosan also lowers the threshold dose at which BPA suppresses implantation (Crawford & deCatanzaro, 2012). In addition to enzyme competition, high doses of DEHP can increase endogenous β-glucuronidase (Seth et al., 1976), an enzyme that hydrolyzes BPA-glucuronide into free BPA (Nakagawa & Tayama, 2000). Thus, BPA would be the chemical driving the effect of implantation failure.

People and wildlife are concurrently exposed to multiple endocrine disruptors that influence estrogen receptors and mechanisms of metabolism. When these substances are each considered in isolation, the doses required to influence pregnancy negatively are typically much greater than doses that could be considered equivalent to common human exposure. However, risk assessment needs to consider the common context of concurrent

exposure to other endocrine disruptors. Chemicals such as BPA, parabens, and phthalates are present in items used every day, such as personal care products, clothing, furniture, and various plastics, and amounts up to 1000 mg/kg can be found in some pillow protectors and shower curtain vinyl (Dodson et al., 2012). Occupational exposure to high concentrations also needs to be considered. Future investigation is necessary to determine the estrogenic effects of different chemicals in combination in order to best protect humans and wildlife.

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Chapter 4

Diethylhexyl phthalate magnifies deposition of ^{14}C-bisphenol A in reproductive tissues of mice

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Author's Contributions

Evan D. Borman: Formation of experimental design, data collection, data analysis, and manuscript writing.

Nicholas Vecchi: Assistance with data collection.

Tyler Pollock: Assistance with experimental design, data collection, and manuscript editing.

Denys deCatanzaro: Assistance with concept development and experimental design, data analysis, and manuscript writing.

Abstract

Endocrine disrupting chemicals (EDCs) are found in diverse common products, including cosmetics, food packaging, thermal receipt paper, and plastic containers. This exposes most people in developed countries through ingestion, skin absorption, and inhalation. Two ubiquitous EDCs, bisphenol A (BPA) and diethylhexyl phthalate (DEHP), can interact in disrupting blastocyst implantation in inseminated females. We hypothesized that DEHP might increase the bioavailability of BPA in tissues by competing for metabolic enzymes. We injected 0, 3, 9, or 18 mg DEHP into female and male mice and allowed 30 min for the chemical to circulate before giving them a food supplement containing 50 μg kg^{-1} ^{14}C-BPA. Animals were dissected 1 h following ^{14}C-BPA administration and various tissue samples were acquired. Samples were solubilized and radioactivity was measured via liquid scintillation counting. In cycling females, DEHP increased BPA deposition in the muscle, uterus, ovaries, and blood serum relative to controls. In peri-implantation females, DEHP increased deposition of BPA in the uterus, ovaries, and serum relative to controls. In males, DEHP doses increased BPA deposition in serum and epididymis relative to controls. These results are consistent with the hypothesis that DEHP competes with BPA for conjugating enzymes such as UDP-glucuronosyltransferase, thereby magnifying the presence of BPA in estrogen-binding reproductive tissues.

Introduction

Diethylhexyl phthalate (DEHP) is one of the most commonly used plasticizers for the manufacture of polyvinyl chloride (PVC) plastics, and can be found in materials including flooring, pipes, clothing, medical devices, personal care products, and children's toys (Dodson *et al*., 2012; Kavlock *et al*., 2002; Wams, 1987). It enters the environment through effluent and direct release into the atmosphere following incineration of waste (Wams, 1987). Exposure in the general population is primarily through diet, as DEHP can leach from plastics into high fat media such as dairy products, fish, meat, and oils (Heinemeyer *et al*., 2013; Meek and Chan, 1994). Inhalation and dermal contact can also be routes of absorption (Elsisi *et al*., 1989; Rakkestad *et al*., 2007). Human exposure has been estimated to be 1–30 μg kg^{-1} day^{-1} on average; however, concentrations of 8,000–10,000 μg/kg/day have been estimated in patients undergoing blood transfusions due to DEHP leaching from PVC medical bags into stored blood up to a concentration of 83.2 μg ml^{-1} (Inoue K *et al*., 2005; Koch *et al*., 2006; SCENIHR, 2015).

At high doses DEHP can negatively affect various aspects of reproduction. For example, DEHP can reduce blastocyst implantation on the uterine wall of inseminated female mice (Li *et al*., 2012), reduce sperm viability in male mice (Pocar *et al*., 2012), disrupt oogenesis and ovulation in zebra fish (Carnevali *et al*., 2010), and increase ovarian and testicular diseases through transgenerational epigenetic mechanisms in rats (Manikkam *et al*., 2013). Some of these effects can be mimicked by much lower concentrations of exogenous 17β-estradiol (E_2). However, DEHP has very weak affinity

for estrogen receptors alpha (ERα) and beta (ERβ), requiring a concentration approximately 1,000,000-fold greater than E_2 (Buteau-Lozano *et al*., 2008; Okubo *et al*., 2003; Takeuchi *et al*., 2005). Thus, direct estrogen receptor activation is unlikely to be the mechanism underlying DEHP's toxic effects. There is clear evidence that DEHP can act as an anti-androgen in males (Chang *et al*., 2015; Christiansen *et al*., 2010), and some evidence that it can perturb hypothalamic GnRH and uterine function in prepubertal females (Liu *et al*., 2016).

Bisphenol A (BPA) is another ubiquitous substance, used primarily in the production of polycarbonate plastics and epoxy resins. It is found in many consumer and medical products such as drinking bottles, food storage containers, thermal paper, water pipes, and dental sealants (Vandenberg *et al*., 2007). Like DEHP, the main route of exposure for BPA is ingestion of contaminated food and beverages, accounting for 85–95% of total human exposure (EFSA, 2015). The US Food and Drug Administration estimated an average BPA exposure of 0.2–0.5 μg kg^{-1} day^{-1} for individuals aged 2 years and older (Aungst *et al*., 2014). The maximum daily intake set by the U.S. Environmental Protection Agency is 50 μg kg^{-1} day^{-1} (EPA, 1988). Unconjugated BPA competes with 17β-estradiol, at 10,000-fold greater concentration, for binding to nuclear ERα and ERβ (Andersen *et al*., 1999; Buteau-Lozano *et al*., 2008; Matthews *et al*., 2001; Snyder *et al*., 2000). BPA can also bind to membrane-bound estrogen receptors, GPR30 and mERα, in some instances much more potently than it does to conventional nuclear receptors (Dong *et al*., 2011; Thomas and Dong, 2006; Wozniak *et al*., 2005).

Recent research (Borman *et al*., 2017) demonstrated that combined administration of DEHP and BPA reduced blastocyst implantation in inseminated female mice at doses below the threshold necessary for DEHP or BPA to have such action on their own (Berger *et al*., 2010; Li *et al*., 2012). As excessive E_2 is established to disrupt blastocyst implantation (deCatanzaro, 2015; deCatanzaro *et al*., 2001; Gidley-Baird *et al*., 1986; Ma *et al*., 2003), and BPA has much greater affinity for estrogen receptors than does DEHP, we suggest that BPA drives blastocyst implantation failure when co-administered with DEHP. This could occur similarly to actions of triclosan, another endocrine disruptor with low affinity for estrogen receptors that nevertheless can impede blastocyst implantation in mice (Crawford and deCatanzaro, 2012). Triclosan administration increases endogenous urinary E_2 and concentration of exogenous ^{3}H-E_2 in the uterus of female mice (Pollock *et al*., 2016). Triclosan also increases ^{14}C-BPA concentration in reproductive tissues of mice, most likely via interference with conjugating enzymes (Pollock *et al*., 2014). Consistent with this hypothesis, BPA and monoethylhexyl phthalate (MEHP), a major metabolite of DEHP, share conjugative activity through UDP-glucuronosyltransferase (UGT) (Hanioka *et al*., 2008, 2012, 2016; Ito *et al*., 2005).

The purpose of the present study was to determine whether administration of DEHP could alter the deposition of ^{14}C-BPA in various mouse tissues. We were specifically interested in reproductive tissues as they contain substantial concentrations of estrogen receptors (Couse *et al*., 1997; Kuiper *et al*., 1997). We hypothesized that DEHP administration would increase BPA deposition in reproductive tissues.

Materials and Methods

Animals and housing

CF-1 mice were acquired from Charles River Breeding Farms of Canada (St. Constant, Quebec). Female subjects were aged 2–4 months and male subjects were aged 2–6 months. Animals were housed in standard polypropylene cages with *ad libitum* access to water and food (8640 Teklad Certified Rodent Chow, Harlan/Teklad, Madison, WI, USA). The colony was maintained at 21°C with a reversed 14 h light:10 h darkness cycle. The procedures for this research were approved by the Animal Research Ethics Board of McMaster University and conformed to the standards of the Canadian Council on Animal Care.

Chemicals and materials

DEHP (bis(2-ethylhexyl) phthalate, CAS 117-81-7) was obtained from Sigma-Aldrich, St. Louis, MO. ^{14}C-BPA ([ring-[14C](U)]-BPA, in ethanol, 3.7 MBq ml^{-1}, 4.07 GBq mmol^{-1}) was obtained from Moravek Biochemicals, Brea, CA. SOLVABLE solubilization cocktail, Ultima Gold scintillation cocktail, and 8-ml Midi-Vial scintillation vials were obtained from Perkin Elmer, Waltham, MA.

Bisphenol A deposition in selected tissues of cycling females

At 8 h after start of the dark phase of the cycle on the first day of the experiment, 32 female subjects were individually housed, weighed, and subsequently given 1 g of peanut butter in a petri dish to prevent dietary neophobia. At the start of the dark phase of the cycle on the second day, animals were pseudorandomly assigned to conditions involving a subcutaneous (sc) injection of 0, 3, 9, or 18 mg DEHP dissolved in 0.05 ml

peanut oil vehicle, with equal numbers and age counterbalanced (n = 8 per condition). After 30 min, each animal was placed in an empty polypropylene cage without rodent chow and water, then given 0.2 g of peanut butter mixed with 50 µg kg^{-1} ^{14}C-BPA. They were allowed 1 h to consume the peanut butter, after which they were anesthetized with isoflurane and serum samples were obtained via cardiac puncture. Animals were sacrificed by cardiac perfusion with 20 ml saline. Following procedures previously described (deCatanzaro and Pollock, 2016; Pollock *et al*., 2014, 2016), samples of the heart, lungs, muscle from the hind leg, abdominal adipose, uterus, ovaries, liver, and a cross-section of the kidney encompassing the medulla and cortex were obtained and placed in pre-weighed scintillation vials. Following tissue collection, vials were re-weighed to measure the wet mass of each tissue.

Bisphenol A deposition in selected tissues of peri-implantation females

Female mice were each housed with a stimulus breeder male. The hindquarters of females were inspected on three occasions daily during the dark phase of the lighting cycle for the presence of a vaginal copulatory plug. When a copulatory plug was detected, the female was isolated in a clean cage and the day of insemination was labelled as gestation day 0. On gestation day 2, females were weighed and pre-exposed to peanut butter as described above for cycling females. At the start of the dark phase of the cycle on gestation day 3, animals were pseudorandomly assigned in equal numbers to conditions involving sc injection of 0, 3, 9, or 18 mg DEHP (n = 8 per condition). After 30 min, each animal was housed without rodent chow and water, given ^{14}C-BPA in

peanut butter to consume over 1 h, then anesthetized, perfused, and dissected for tissues as described above for cycling females.

Bisphenol A deposition in selected tissues of males

Males were pre-exposed to peanut butter, assigned in equal numbers to conditions involving injection of 0, 3, 9, or 18 mg DEHP (n = 8, 6, 8, 8 respectively), and administered ^{14}C-BPA following procedures that were identical to those described above for cycling females. Equal numbers were prepared, but two males in the 3 mg DEHP dose were excluded as they did not finish eating the peanut butter. After 1 h, they were then anesthetized, perfused, and dissected for tissues as described for females, except that the reproductive tissues collected were one testis, vesicular-coagulating (VC) gland, preputial gland, and epididymis.

Tissue processing for liquid scintillation counting

Following previously published protocols (Guzzo *et al*., 2012, 2013), tissue samples were solubilized by adding 1 ml of SOLVABLE to each vial after tissue weighing. The vials were then placed in a 50°C water bath for 2 h and then agitated for 1 min before being placed back in the water bath for an additional 2 h to complete solubilization. The vials were removed from the water bath and allowed to cool for 10 min before adding 5 ml of Ultima Gold scintillation cocktail to each vial. The vials were then agitated for 10 min to promote mixing of the scintillation cocktail and tissue. After agitation, the vials were transferred and stored in the darkness chamber of a TriCarb 2910 TR Liquid Scintillation Analyzer (PerkinElmer) overnight to eliminate background noise. Radioactivity was measured the next morning for 5 min per vial, and final adjusted

estimates for the amount of radioactivity per sample in disintegrations/min (dpm) were automatically calculated by the accompanying Quanta-Smart software package. The dpm measure was standardized to the weight of the sample wet mass as dpm mg^{-1} tissue and then converted to ng BPA g^{-1}.

Serum processing for liquid scintillation counting

Following collection, blood samples were stored in microtubes and allowed to coagulate for at least 30 min. The samples were then centrifuged at 1500 g for 10 min, then for each sample 10 µl serum was transferred to a scintillation vial filled with 5 ml of Ultima Gold. Vials containing the serum and scintillation cocktail were agitated for 10 min to promote mixing. Measurement of radioactivity was the same as described above but is reported as ng BPA ml^{-1}.

Statistical analyses

Differences among treatments were examined by analysis of variance (ANOVA) for each tissue. Significant ANOVA was followed by Newman-Keuls multiple comparisons of all pairs of treatments in each tissue. Statistics were focused on treatments within tissues and not among tissues because of the unavoidable possibility of differential impacts of perfusion upon tissues. Bartlett's test showed equal variances across treatments. The threshold for ascribing statistical significance for all tests was a comparison-wise error rate of $\alpha < 0.05$.

Results

Distribution of radioactivity in cycling females

Radioactivity was measured in peripheral tissues of cycling females that received an injection of DEHP followed by oral administration of 50 µg kg^{-1} ^{14}C-BPA (Fig. 4.1). ANOVA was conducted on each of the tissues and serum, and showed significant effects of treatment in muscle, $F(3, 28) = 6.83$, $p = 0.001$; uterus, $F(3, 28) = 5.36$, $p = 0.005$; ovaries, $F(3, 28) = 3.15$, $p = 0.040$; and serum, $F(3, 28) = 7.69$, $p = 0.001$. Multiple comparisons indicated that females given the 18 mg DEHP dose showed greater levels of radioactivity than those given the 0 mg dose (controls) in the muscle, uterus, ovaries, and serum. There were similar but non-significant trends in radioactivity in most other tissues at higher DEHP doses.

Distribution of radioactivity in peri-implantation females

Radioactivity was measured in peripheral tissues of peri-implantation females that received an injection of DEHP followed by oral administration of 50 µg kg^{-1} ^{14}C-BPA (Fig. 4.2). ANOVA was conducted on each of the tissues and serum, and showed significant effects of treatment in the uterus, $F(3, 28) = 5.37$, $p = 0.005$; ovaries, $F(3, 28) = 4.20$, $p = 0.014$; and serum, $F(3, 28) = 3.85$, $p = 0.020$. Multiple comparisons indicated that females given the 3, 9, and 18 mg DEHP doses showed greater levels of radioactivity than controls in the uterus, while only those given the 9 mg dose differed from controls in the ovaries and serum. Radioactivity showed similar but non-significant non-monotonic trends in relation to DEHP dose, peaking at 9 mg, in all other tissues except the kidney.

Fig 4.1. Mean (+SE) concentration of radioactivity, expressed as ng BPA equivalent g^{-1} or ml^{-1}, in the (A) heart, lung, muscle, adipose, uterus, ovary, (B) liver, kidney, and serum of cycling females following sc injection of 0, 3, 9, or 18 mg DEHP and subsequent oral administration of 50 µg kg^{-1} ^{14}C-BPA (n = 8 per condition). Difference from 0 mg DEHP treatment in same tissue: *p<0.05, +p<0.01.

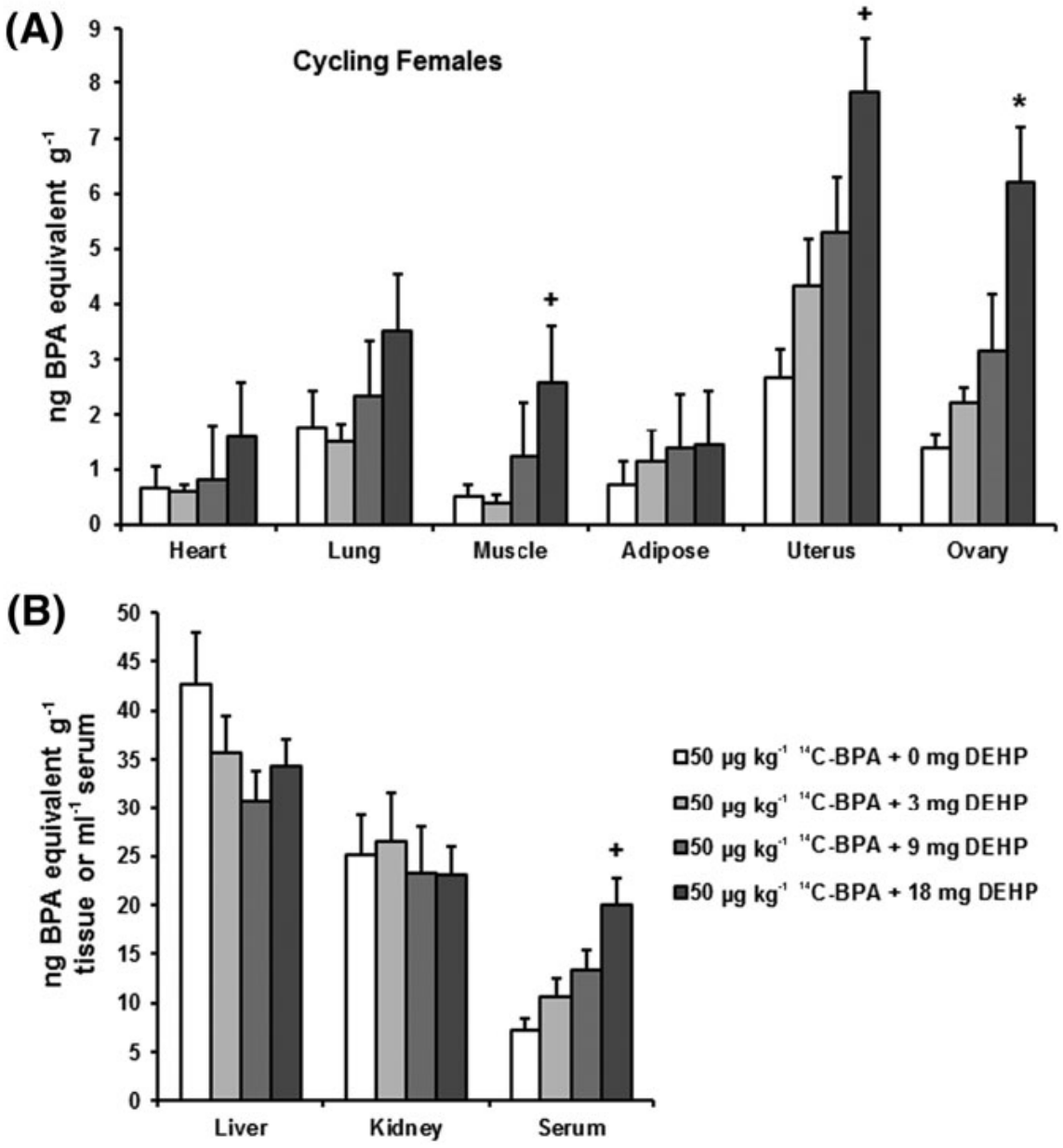
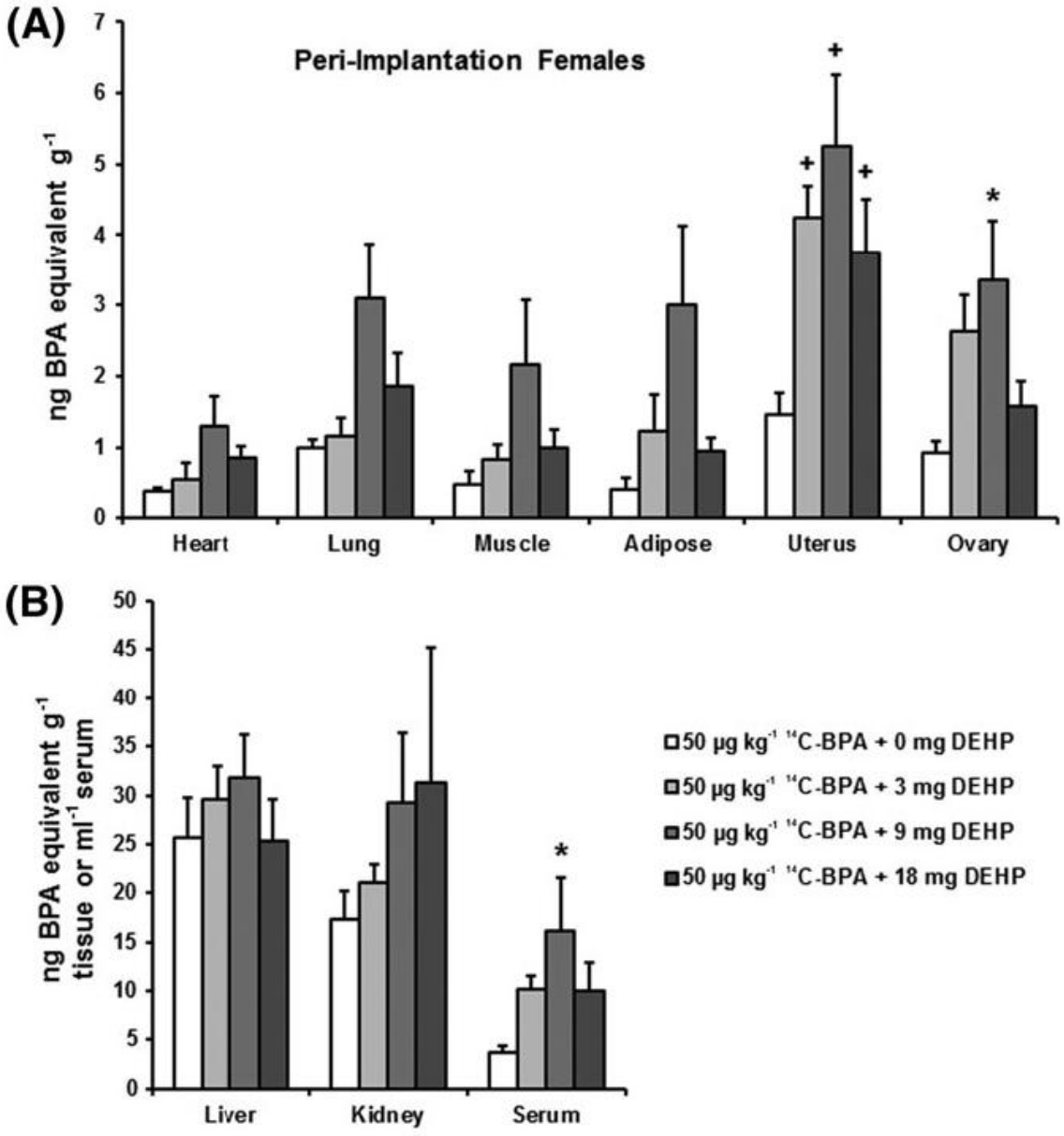


Fig 4.2. Mean (+SE) concentration of radioactivity, expressed as ng BPA equivalent g^{-1} or ml^{-1}, in the (A) heart, lung, muscle, adipose, uterus, ovary; and (B) liver, kidney, and serum of peri-implantation (gestation day 3) females following sc injection of 0, 3, 9, or 18 mg DEHP and subsequent oral administration of 50 µg kg^{-1} ^{14}C-BPA (n = 8 per condition). Difference from 0 mg DEHP treatment in same tissue: *p<0.05, +p<0.01.



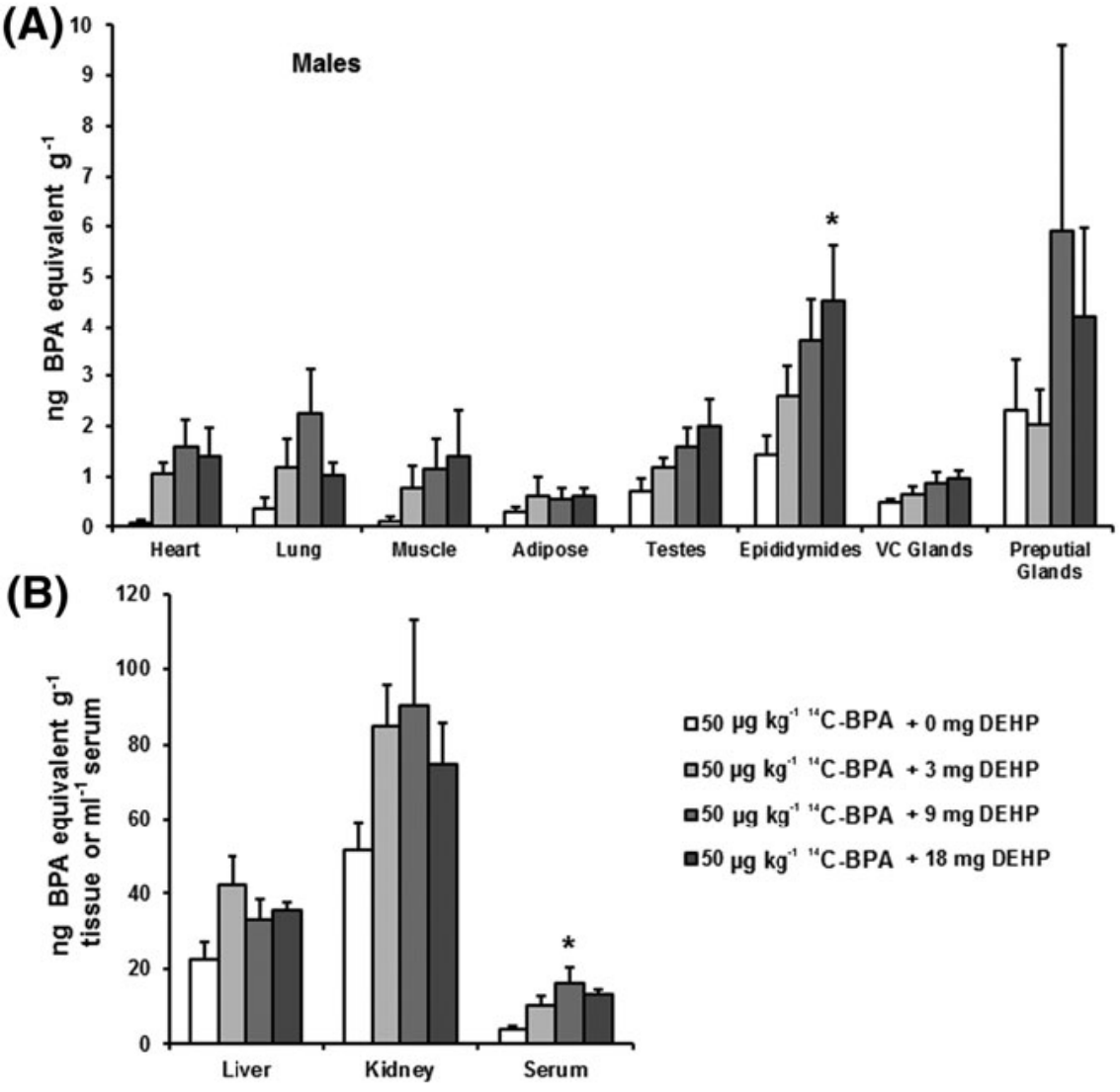
Distribution of radioactivity in males

Radioactivity was measured in peripheral tissues of males that received an injection of DEHP followed by oral administration of 50 µg kg^{-1} ^{14}C-BPA (Fig. 4.3). ANOVA was conducted on each of the tissues and serum, and showed significant effects of treatment in the epididymides, $F(3, 27) = 3.29$, $p = 0.036$, and serum, $F(3, 27) = 3.84$, $p = 0.021$. Multiple comparisons revealed that males given the 18 mg DEHP dose showed greater radioactivity than controls in the epididymides, while males given the 9 mg DEHP dose showed greater radioactivity than controls in serum. Although no other tissue showed significantly increased levels of ^{14}C-BPA, radioactivity trended toward significance at higher doses of DEHP in most other tissues.

Discussion

These data demonstrate that exposure to DEHP increases the concentration of BPA and its metabolites in specific adult male and female tissues. In animals given ^{14}C-BPA, pre-treatment with DEHP significantly increased radioactivity in the muscle, uterus, ovaries, and serum in cycling females; the uterus, ovaries, and serum in peri-implantation females; and the epididymis and serum of males. The profile of radioactivity across tissues of females was consistent with previous experiments that looked solely at ^{14}C-BPA deposition (Pollock and deCatanzaro, 2014), where the majority of orally-administered 50 µg kg^{-1} ^{14}C-BPA in the uterus was unconjugated and bioavailable. The greatest impact of DEHP administration in the present study was in the uterus and ovaries of females, and the epididymis of males. All of these tissues are characterized by expression of nuclear ERα and ERβ, with the uterus and ovaries demonstrating high

Fig 4.3. Mean (+SE) concentration of radioactivity, expressed as ng BPA equivalent g^{-1} or ml^{-1}, in the (A) heart, lung, muscle, adipose, testes, epididymis, vesicular-coagulating (VC) glands, preputial glands, (B) liver, kidney, and serum of males following sc injection of 0, 3, 9, or 18 mg DEHP and subsequent oral administration of 50 µg kg^{-1} ^{14}C-BPA (n = 8, 6, 8, 8 respectively). Difference from 0 mg DEHP treatment in the same tissue: *p < 0.05.



expression and the epididymis demonstrating moderate expression (Couse *et al*., 1997; Kuiper *et al*., 1997). Additionally, GPR30 expression is found in various tissues, including the mammary glands, ovaries, uterus, thyroid, lungs, liver, intestine, brain, testes, epididymis, vas deferens, prostate, and seminal vesicles (Brailoiu *et al*., 2007; Hazell *et al*., 2009; O'Dowd *et al*., 1998; Owman *et al*., 1996; Prossnitz *et al*., 2008; Takada *et al*., 1997). These tissues are also affected by BPA through disruption of oogenesis in the ovaries (Eichenlaub-Ritter and Pacchierotti, 2015), reduced sperm motility in the epididymides (Chitra *et al*., 2003), and reduced uterine receptivity (Berger *et al*., 2010; Xiao *et al*., 2011). Where exposure to DEHP increases the presence of BPA in these tissues, effects such as these could occur at lower doses of BPA than originally thought.

Our data are consistent with the hypothesis that BPA and DEHP compete for conjugating enzymes. Multiple metabolic factors may be contributing to the observed dose curves. MEHP, the primary metabolite of DEHP, is conjugated by UGT isoforms 1A3, 1A7, 1A8, 1A9, 1A10, 2B4, and 2B7 (Hanioka *et al*., 2012, 2016). BPA is also conjugated by similar isoforms, specifically 1A1, 1A9, 2B4, and 2B7 (Hanioka *et al*., 2008); however BPA has greater binding affinity for UGT than does MEHP (Ito *et al*., 2005). This difference in binding affinities is a determining factor for enzymatic competition by MEHP, as more of the chemical is required to compete for the enzyme. Conjugation of BPA is not solely accomplished by UGT, as sulfotransferase (SULT) conjugates BPA into its sulfonated form (Pritchett *et al*., 2002; Wen *et al*., 2013), whereas DEHP may not have effects on sulfotransferase expression (Witzmann *et al*., 1996).

Nevertheless, the main metabolite of BPA is BPA-glucuronide (Pritchett *et al*., 2002; Yokota *et al*., 1999).

Other potential interactions of DEHP and BPA would not produce the observed results. Competition between DEHP and ^{14}C-BPA in binding to estrogen receptors would, at large DEHP doses, progressively reduce radioactivity in tissues with high concentrations of estrogen receptors such as the uterus, ovaries, and epididymis. Similarly, competition between DEHP and BPA for molecules that carry these lipophilic substances in circulation would likely reduce transport of ^{14}C-BPA to such tissues, lowering measures of radioactivity as DEHP doses increased. As our data clearly demonstrate that increasing DEHP doses progressively increase radioactivity in serum and specific tissues containing high concentrations of estrogen receptors, we suggest that competition for limited resources that conjugate and metabolize BPA is the most plausible explanation.

Peri-implantation females showed statistically significant effects at lower DEHP doses than did the cycling females. This may be attributed to a decrease in UGT during pregnancy (Inoue H *et al*., 2005; Wen *et al*., 2013). With reduced presence of UGT, less MEHP may be required to occupy the enzyme sufficiently to allow BPA to persist in tissues. This suggests that pregnant females may be more sensitive to combinations of chemicals that interfere with UGT enzymatic activity, which has been observed in both rodents and humans (Isoherranen & Thummel, 2012; Matsumoto et al., 2002). Also, a non-monotonic trend of DEHP doses was seen in a number of measures on the peri-implantation females, unlike the monotonic influence of increasing dose seen in cycling

females and males. Peri-implantation females are highly sensitive to minute variations in estrogen concentrations (deCatanzaro *et al*., 2001; Ma *et al*., 2003; Thorpe *et al*., 2013) and vulnerable to xenoestrogen exposure (*e.g.* Berger *et al*., 2010; Borman *et al*., 2017 ; Crawford and deCatanzaro, 2012). It is possible that these sensitivities alter pharmacokinetics, however reasons for the observed non-monotonic trends in peri-implantation females remain to be determined.

People and wildlife are concurrently exposed to multiple endocrine disruptors that influence estrogen receptor activity. These chemicals have typically been studied alone, with little research on effects of concurrent exposure to more than one substance. Our data demonstrate that BPA and DEHP interact *in vivo* to increase orally administered ^{14}C-BPA in various tissues, and we believe this is caused by competition for shared conjugating enzymes. While the DEHP doses used in this study exceed those of typical human exposure, they may be relevant for individuals who work in the manufacturing of products and materials that contain the chemical. This work furthers our understanding how combinations of various endocrine disruptors may interact. Indeed, research on the potential interactions of endocrine disrupting chemicals should be further expanded as there is ubiquitous exposure to these chemicals via personal care products, clothing, and various plastics (Dodson *et al*., 2012). One such interaction comes from a study demonstrating that DEHP and butyl paraben could disrupt ovarian steroidogenesis, leading to attenuated E_2 output, only when administered concurrently (Guerra et al., 2016). Further research on such interactions would seem warranted in order to better protect humans and wildlife.

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Conflicts

The authors have no conflicts of interest.

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Chapter 5

General Discussion

Overview

The research in this thesis was undertaken to determine whether the threshold dose at which BPA influences ovo-implantation and associated uterine dynamics is reduced by concurrent maternal stress and exposure to another common EDC, DEHP. Implantation was disrupted by a combination of BPA and stress, which were insufficient to cause the effect alone. Implantation was similarly disrupted by combined doses of BPA and DEHP that were each below the threshold dose required for implantation disruption on their own. Implantation disruption in each instance was associated with a reduction in adhesion proteins that are necessary for uterine luminal closure. Administration of DEHP increased the deposition of a low dose of ^{14}C-BPA in the uterus and other reproductive tissues, supporting the hypothesis that DEHP competes for BPA-metabolizing enzymes.

This research is meaningful in that it demonstrates that BPA interacts with both stress and DEHP to produce deleterious reproductive effects at a BPA dose lower than previously reported. Concurrent exposure to these EDCs and stress is common and can be a concern for human fertility. Many women that have concerns about infertility have difficulty becoming pregnant, which may be influenced by the factors described in this research. The estrogenic activity involved in EDC implantation disruption may also play a role in ovarian and uterine carcinogenesis, as estrogenic chemicals reach these tissues in unconjugated and bioactive form. Regulatory agencies, such as Health Canada and the U.S. Environmental Protection Agency, have not sufficiently considered the interactions of other chemicals and physiological states on chemicals of interest, such as BPA, when developing current safety regulations.

Summary of Results

In the research reported in **Chapter 2**, I examined the impacts of combinations of stress and BPA on blastocyst implantation in inseminated mice. Exposure to both stress and 5 mg BPA over 4 days reduced the number of blastocyst implantation sites measured on gestation day (GD) 6. As blastocyst implantation was unaffected by either that dose of BPA alone or by the stressor alone, the results from the combined treatment indicate some sort of summation of BPA and stress. Uterine luminal area was increased at the 4 and 5 mg BPA + stress conditions, but e-cadherin was reduced by BPA regardless of the stress condition. Urinary steroid measures collected on GD 6 indicated that the 5 mg BPA dose increased urinary E_2, but decreased measured corticosterone. The lack of an effect of stress on these measures may be attributable to the removal of the predatory stressor a full day before urine collection. Overall, these results indicate that blastocyst implantation was disrupted by BPA and stress through a combination of reduced e-cadherin expression on uterine luminal epithelial cells and increased uterine luminal area. The study also showed blastocyst implantation disruption using a BPA dose below the previously established threshold, when combined with stress.

In the research reported in **Chapter 3**, I investigated potential combinatory effects of BPA and DEHP on intrauterine blastocyst implantation in inseminated mice. In the first experiment, I established the dose-response of implantation to DEHP, finding that a dose of 36 mg DEHP over 4 days was sufficient to produce a partial disruption of blastocyst implantation in mice. The second experiment utilized DEHP and BPA doses that did not show any significant effects on their own on blastocyst implantation and

administered them in a combined dose over GDs 1-4. Investigation on GD 6 showed a significant decrease of total implantation sites relative to controls. There was also an increase in underdeveloped implantation sites, characterized by their white colour and spherical shape while being flush with the outer layer of the uterus. The combined dose condition also produced a significant decrease in both e-cadherin and cadherin-11 in on the uterine luminal epithelium, with the 3 mg BPA dose also decreasing cadherin-11 levels. This work demonstrates that BPA and DEHP can interact to disrupt blastocyst implantation by increasing luminal area and reducing levels of these adhesion proteins. Triclosan is another EDC that has been found to interact similarly with BPA to disrupt blastocyst implantation. Triclosan competes for a conjugating enzyme that inactivates BPA, thus allowing free BPA to activate ERs (Crawford et al., 2012; Pollock et al., 2014). Accordingly, I reasoned that as DEHP and BPA share the UGT conjugating enzyme, the high DEHP dose may be saturating the enzyme and allowing unconjugated BPA to persist in tissues.

In the research reported in **Chapter 4**, I evaluated the interaction of DEHP and BPA via biomonitoring radiolabeled BPA in various tissues after subcutaneous administration of DEHP in mice. Results indicated that concurrent exposure to DEHP can increase measured BPA radioactivity within the uterus, ovaries, and serum of peri-implantation and cycling females as well as the epididymides and serum of males. Significant increases in radioactivity were seen in doses as low as 3 mg DEHP in peri-implantation females, although the majority of significance was found at the 9 mg and 18 mg DEHP doses. This increase in BPA deposition is consistent with competition between

the main metabolite of DEHP, MEHP, and BPA for the same UGT enzyme isoforms (Hanioka et al., 2008; 2012; 2016). As greater quantities of DEHP were administered to animals, MEHP would be preferentially conjugated by UGT over BPA due to its higher concentration. This would allow more BPA to remain free in tissues with high ER expression. Peri-implantation females demonstrated significant increases in measured radioactivity at lower doses than did cycling females. This difference might be attributable to lower endogenous UGT expression in females during pregnancy, which would mean that less MEHP would be required to saturate the enzyme (Wen et al., 2013). This research helps to explain the interaction of BPA and DEHP that was demonstrated in **Chapter 3**, as it appears that the introduction of DEHP in higher quantities increased the deposition of BPA in the uterus. This would allow BPA to bind and activate ERs over an extended period of time, driving effects such as disruption of blastocyst implantation.

Exacerbating the Effects of BPA on Pregnancy

Estrogens, estrogenic chemicals, and stress can each disrupt early pregnancy on their own (Berger et al., 2008; deCatanzaro et al., 2011; Thorpe et al., 2013; **Chapters 2 & 3**). This disruption can be mediated by a variety of factors, including reduced adhesion protein expression for uterine closure and implantation (Jha et al., 2006; Rahnama et al., 2009; **Chapters 2 & 3**), the uterus prematurely entering a refractory stage (Ma et al., 2003), and delayed or accelerated blastocyst transport through the reproductive tract (Greenwald, 1967). Much research has been conducted on the effects of BPA on various aspects of reproduction, but this body of work has generally ignored other factors such as

stress and concurrent exposure to other EDCs. My work has investigated the effects that these factors exert in conjunction with BPA.

Stress & BPA

The phenomenon of early pregnancy loss due to stress is well documented in mammals (deCatanzaro & MacNiven, 1992; deCatanzaro, 2011). Relevant studies of the effects of stress on humans are sparse and correlational, but show that major stressors correlate with pregnancy complications such as spontaneous abortion, preterm labour, and growth retardation of the offspring (see review by Mulder et al., 2002). The majority of human pregnancy loss occurs within the first trimester (Allison et al., 2011). While many other causative factors can be involved, including genetic, endocrine, anatomical or disease processes, stress is among the causes of miscarriages (Arck, 2001). As such, stress can be concerning to individuals seeking to bear children. While modern day humans are not typically susceptible to predatory stress, human stressors can be caused by a variety of other factors that can be difficult to avoid such as personal illness, divorce, family deaths, parental care, financial woes, relationships issues, and work problems. The common occurrence of stress is an important reason to investigate its effects in conjunction with a ubiquitous EDC with known effects on the uterus and early pregnancy.

High amounts of stress can increase endogenous E_2 in pregnant mammals (MacNiven & deCatanzaro, 1992). Evidence suggests that the origin of this E_2 is from the adrenal cortex (Thorpe et al., 2014). The origin is suspected to be from the adrenals rather than the gonads for two reasons. First, activation of the HPA axis inhibits the HPG axis, thus limiting the release of E_2 from the gonads of the females (Charpenet et al., 1982;

Kirby et al., 2009; Sapolsky, 1985). Second, the adrenals release androgens which can be aromatized into E_2 (Golovine et al., 2003; Payne & Hale, 2004). This rise of E_2 during stress is not from ovarian production as the effect has been found in ovariectomized female mice exposed to predatory stress (Thorpe et al., 2014). This stress-induced increase in E_2 may be a contributing factor to the implantation disruption that was observed in the data reported in **Chapter 2**. E_2 decreases expression of e-cadherin (Jha et al., 2006; Potter et al., 1996), thereby contributing to implantation disruption. However, the reduction of e-cadherin observed in the data reported in **Chapter 2** was attributable to BPA alone, regardless of the stressor. This may be a result of BPA saturating ERα, which downregulates e-cadherin mRNA expression, such that the E_2 increase is not required. Administration of E_2 causes an influx of fluid from the uterine endometrium into the uterine lumen, causing it to expand (Salleh et al., 2005). This would explain the apparent increase in uterine luminal area observed in the data of **Chapter 2**. The implantation disruption observed with the combination of BPA and stress may be a result of BPA reducing uterine e-cadherin and E_2 and BPA estrogenically summating to increase luminal area. Alternatively, E_2 activity at ERs may be increased by BPA as they share conjugating enzymes, sulfotransferase and UDP-glucuronosyltransferase (Hanioka et al., 2008; Nishiyama et al., 2002; Raftogianis et al., 2000). This enzyme competition would allow unconjugated E_2 to remain active and bind to ERs, leading to reduced uterine e-cadherin and increased luminal area. Another possibility includes BPA binding to ERR-γ to increase the expression of the ERR-α, another estrogen-related receptor, gene (Zhang & Teng, 2007). ERR-α activation can induce the transcription of several enzymes that can

lead to increased estrogen levels and ERα activity (Stein & McDonnell, 2006). This increase in estrogen levels and ERα activity may lead to reduced uterine e-cadherin and increased luminal area.

Overall, the results from **Chapter 2** demonstrate a concern for women who want to have children as a currently ubiquitous endocrine disruptor, BPA, can interact with stress, which many women experience regularly, to disrupt uterine blastocyst implantation.

DEHP & BPA

The results reported in **Chapter 3** demonstrate that two ubiquitous EDCs interact to disrupt early pregnancy. A combined dose of BPA + DEHP decreased the total number of implantation sites, but also increased the proportion of underdeveloped implantation sites. These underdeveloped implantation sites indicate that the introduction of a high DEHP dose combined with BPA may cause either an implantation delay or resorption of blastocysts. This effect is most likely due to administration of DEHP, as underdeveloped sites were noted during single dose injections in experiment 1 of **Chapter 3**. Similar to the data of **Chapter 2**, the combined dose of BPA and DEHP reduced the amount of adhesion protein expressed by the epithelial cells, a process thought to be driven by BPA. No significant effect was found in luminal area data reported in **Chapter 3,** which differed from the results in **Chapter 2**. This difference may have occurred for two reasons. First, stress may have a greater impact than DEHP on luminal closure, as stress induces a rise of E_2 that can lead to an influx of fluid into the uterine lumen, whereas DEHP cannot induce such an effect as it is estrogenically weak (Buteau-Lozano et al.,

2008; Okubo et al., 2003; Takeuchi et al., 2005). Second, as luminal area was increased at the 4 mg BPA dose in **Chapter 2** but not at the 3 mg BPA dose in **Chapter 3**, the 3 mg BPA dose may not have been sufficient to disrupt luminal closure.

The interaction between BPA and DEHP was investigated in the experiments of **Chapter 4** to elucidate their combined effect on blastocyst implantation. The results are consistent with the hypothesis that MEHP, the main metabolite of DEHP, can compete for the UGT enzyme at high doses, allowing BPA to persist in tissues. This would let BPA bind to ERs and elicit the effects described in **Chapter 3**. Although mine was the first study to investigate this interaction *in utero,* other EDCs have been shown to compete more potently for enzymes that conjugate BPA (Pollock et al., 2014). Triclosan, an anti-bacterial additive in soaps, is a notable example of this as it can compete for UGT more potently than does DEHP, while also competing for sulfotransferase, another conjugating enzyme (Hanioka et al., 2016; Wang et al., 2004). Using the paradigm described in **Chapter 4,** triclosan administration increased BPA deposition in reproductive tissues and serum at doses lower than the DEHP doses used in the experiments of **Chapter 4** (Pollock et al., 2014).

It is important to note that DEHP is not the only other chemical that can interact with BPA to affect early pregnancy. Triclosan can also interact with BPA to disrupt blastocyst implantation (Crawford & deCatanzaro, 2012), similarly to DEHP as reported in **Chapter 3**. This is concerning and begs the question: what happens when more EDCs are combined? It is possible that a combination of chemicals can exert a negative reproductive effect at more environmentally relevant doses. Studies of women

undergoing *in vitro* fertilization suggest that increased human EDC exposure increases risk of implantation failure (Ehrlich et al., 2012; Hauser et al., 2016). This suggests that current regulations on daily exposure levels may be insufficient for public safety.

Current regulations for BPA and DEHP (U.S. EPA, 1988, 2007; SCENIHR, 2015) are based on single chemical dose-response studies which do not incorporate effects due to interactions. EDCs are initially evaluated for harmful effects and, if harmful, a dose-response evaluation is developed. Following this evaluation, a risk characterization is conducted which incorporates the exposure levels of the chemical and its dose-response. Based on the conclusions of the evaluation, toxic chemicals can be released into the environment as long as adverse effects upon both human and wildlife are averted (Zoeller et al., 2012). My work has shown that stress and DEHP can affect the threshold dose of BPA that exerts a negative reproductive effect. The doses of DEHP employed in these experiments likely exceed those that would be environmentally relevant to humans, except possibly in certain occupational settings. However, additional investigation is warranted to ascertain whether environmentally-relevant doses of various EDCs administered in mixture can replicate this adverse effect. The work presented in this thesis has potential implications for regulations regarding EDCs.

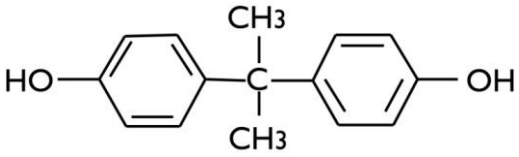
Future Directions

This thesis demonstrates that BPA administered alongside stress or DEHP during blastocyst implantation has deleterious effects on early pregnancy in inseminated female mice. The interactions that BPA had with stress and DEHP caused adverse reproductive

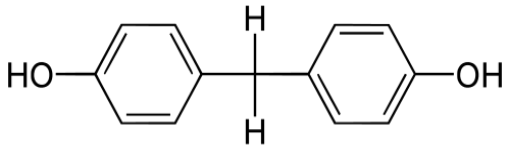
effects at lower individual doses than previously described. The impact of this work is diminished by the fact that the doses of BPA and DEHP remain above environmental relevance; however, the research in this thesis is an important step towards determining whether environmentally relevant doses are a concern. **Chapter 4** describes a paradigm that indicates potential interactions of DEHP with BPA through enzyme competition, using an oral dose of ^{14}C-BPA that is the EPA reference dose, which is the maximum acceptable daily oral dose (U.S. EPA, 1988). In the future, this paradigm could be adapted to assess the impacts of concurrent exposure to multiple chemicals. Our laboratory has utilized this paradigm to assess the influence of various EDCs, including triclosan, tetrabromobisphenol A, parabens, and DEHP in mixture on the deposition of ^{14}C-BPA. Results of this work show an increase in ^{14}C-BPA deposition using the listed EDCs at more environmentally relevant doses (unpublished). However, the application of more than two EDCs on blastocyst implantation using a similar paradigm as described in **Chapter 3** has yet to be accomplished.

The research reported in this thesis has primarily focused on the effects of BPA; however, new analogues are currently replacing BPA in various products. In 2010 the Canadian Government banned the sale of BPA in polycarbonate baby bottles, which was followed by a ban in the European Union in 2011 (Government of Canada, 2010; European Commission, 2011). Due to increased public concern and government regulations, analogues were quickly developed and produced to replace BPA. These chemicals share a common structure with BPA which includes two hydroxyphenyl groups (Fig. 5.1).

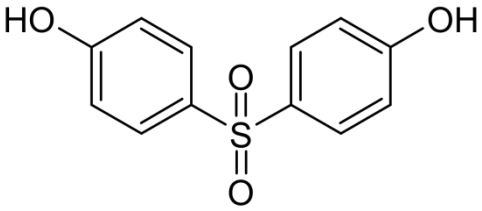
Fig. 5.1. Chemical structures of Bisphenol A, Bisphenol F, Bisphenol S, and Bisphenol AF.



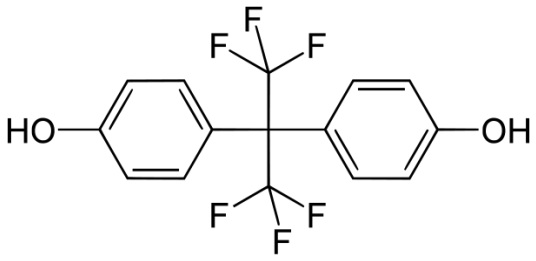
Bisphenol A



Bisphenol F



Bisphenol S



Bisphenol AF

There are currently 16 different BPA analogues; with BPF, BPS, and BPAF being the primary substitutes in polycarbonate plastics and epoxy resins (Chen et al., 2016). BPF can be found in varnishes, adhesives, water pipes, plastics, coatings of food packaging, and dental sealants (Cabaton et al., 2009). BPS can be found in epoxy glues, can coatings, and thermal receipt paper (Naderi et al., 2014). BPAF can be found in electronics, optical fibers, and specialty polymers (Baradie et al., 2005; Konno et al., 2004; Matsushima et al., 2010). Preliminary ER binding assays on these analogues indicated that, compared to BPA, BPAF has higher ER potency while BPF and BPS have slightly lower ER potency (Chen et al., 2016). Due to their ability to bind to ERs, I would suspect that these

chemicals can also disrupt blastocyst implantation in inseminated female mice at doses similar to those of BPA. These analogues also share the BPA-conjugating enzyme UGT, which transforms the analogues into their glucuronidated forms (Skledar et al., 2015; Yabusaki et al., 2015). Therefore, deposition of these analogues may increase *in vivo*, as other chemicals that compete for this conjugating enzyme are introduced in mixture in a similar manner as illustrated in **Chapter 4**.

With the growing use of BPA analogues and the sparse amount of research currently available, the potential for adverse effects prior to informed regulation may be inevitable. The work presented in this thesis raises concern for the overlap of exposure with BPA and its analogues, as the structures, estrogenic activity, and conjugation of these chemicals are very similar. Thus, they may interact more strongly with each other than they do with dissimilar chemicals, such as DEHP.

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