INTRAUTERINE IMPLANTATION AND BISPHENOL A EXPOSURE

THE IMPACT OF BISPHENOL A EXPOSURE ON IMPLANTATION, STEROID HORMONE EXCRETION, UTERINE MORPHOLOGY AND RECEPTOR EXPRESSION IN INSEMINATED FEMALE CF-1 MICE

By

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Abstract

Bisphenol-A (BPA), used in the production of polycarbonate plastics and epoxy resins, has established estrogenic properties. Early pregnancy in mice is highly sensitive to exogenous estrogens, particularly during the period of blastocyst implantation. Accordingly, I assessed pregnancy outcome, implantation, urinary hormone levels and uterine morphology following BPA exposure. Subcutaneous injections of BPA administered on days 1 through 4 of gestation reduced litter size at a dose of 3 mg/animal/day and decreased the proportion of parturient females at 10 mg/animal/day. Hysterectomies performed on day 6 of pregnancy confirmed a significant disruption of implantation occurring at doses as low as 6 mg/animal/day. Urinary progesterone levels were also reduced by 10 mg/animal/day. Uterine luminal area expanded substantially in response to increasing doses of BPA. Luminal epithelial cell height increased following exposure to 10.125 mg/animal, whereas there were no differences in the number of corpora lutea among conditions. The proportion of cells staining positively for estrogen receptors was affected non-monotonically, showing highest levels at 3.375 mg/animal and lowest levels at 10.125 mg/animal. Similarly progesterone receptor expression measured through western blots related non-monotonically to dose, being highest at 3.375 mg/animal and diminishing with increasing dose. Effects of a single administration of BPA on days 0, 1, or 2 of gestation were also investigated. A single dose of 10 mg reduced the number of implantation sites when given on day 0 or 1, and 6 mg did so on day 1, but there was no such effect of any dose administered on day 2. Exposure to low, environmentally-relevant doses of BPA did not result in any clear reproductive or

hormonal effects. These studies highlight the detrimental effects BPA exposure induces during early pregnancy and provides further evidence of its weak estrogenic properties *in vivo*.

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List of Abbreviation

Endocrine disrupting chemical	EDC
Bisphenol A	BPA
National Health and Nutrition Examination Survey	NHANES
Diethylstilbestrol	DES
Estrogen receptor	ER
Lowest observed adverse effect level	LOAEL
Progesterone receptor	PR
G protein-coupled receptor 30	GPR30
Ano-genital distance index	AGDI
Phosphate buffered saline	PBS
Lactoferrin	Lf
Gestational day	GD

Chapter 1

General Introduction

Some man-made products are known to leach toxic chemicals into the surrounding environment, potentially influencing the development and health of exposed animals (Colborn *et al.*, 1993; Vos *et al.*, 2000). Of particular concern are chemicals that alter the functioning of the endocrine system, commonly referred to as endocrine disrupting chemicals (EDCs). EDCs typically interfere with "the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that is responsible for the maintenance of homeostasis, reproduction, development and/or behavior" (U.S. EPA, 1997).

Bisphenol-A (BPA) is one EDC that has gained attention from researchers for its capacity to mimic steroidal actions both *in vitro* and *in vivo* (see review by vom Saal and Hughes, 2005). BPA is the monomer of polycarbonate plastics and some epoxy resins, and is produced at a rate exceeding 3 billion kilograms per year (Burridge, 2003). It can be found in several household goods such as reusable food containers, polyvinyl chloride stretch films, and water bottles. BPA is used commercially in medical equipment, dental fillings and sealants, and computer and automobile components. BPA-based epoxy resins are used to line metal food cans to reduce rusting and corrosion.

Several studies have shown that BPA will leach into surrounding media from polycarbonate plastics, epoxy resins, and dental composites due to incomplete polymerization, exposure to heat, and contact with acidic or basic compounds (Al-Hiyasat *et al.*, 2004; Brotons *et al.*, 1994; Krishnan *et al.*, 1993; Lazear, 1995; Olea *et al.*, 1996). The abundance of BPA in the environment has resulted in widespread exposure in both humans and wildlife (Colborn *et al.*, 1993; Crain *et al.*, 2007; Kang *et al.*, 2006), with

oral consumption being the primary mode of exposure (Chapin *et al.*, 2008; Kang *et al.*, 2006). In many countries, canned foods account for the majority of the total BPA intake among adults (European Commission, 2002; Miyamoto and Kotake, 2006).

Daily human intake of BPA is dependent on age and diet (Kang *et al.*, 2006). The average intake in pregnant women who provided samples via the Norwegian Mother and Child Birth Cohort study and the National Health and Nutrition Examination Survey in the United States (NHANES) has been estimated to be 0.1 μg/kg/day (Ye *et al.*, 2009). The daily BPA intake of pregnant women in southern Spain was approximately 0.02 μg/kg/day (Mariscal-Arcas *et al.*, 2009). This lower BPA consumption was attributed to greater consumption of fresh foods rather than canned foods and drink (Mariscal-Arcas *et al.*, 2009; Rivas *et al.*, 2007). When ingested, the majority of BPA is conjugated to BPA glucuronide and excreted (Volkel *et al.*, 2005) with an estimated half-life of 6 hours (CERHR, 2007). Over 90% of 2500 human urine samples analyzed from the NHANES had measurable amounts of BPA, suggesting relatively continuous exposure to BPA (Calafat *et al.*, 2008). Within those samples, the highest mean BPA levels were found in children aged 6-11 (Calafat *et al.*, 2008). Recent investigations have shown that median urinary concentrations of BPA in premature infants in neonatal intensive care units are an order of magnitude greater than those of children aged 6-11 (Calafat *et al.*, 2009).

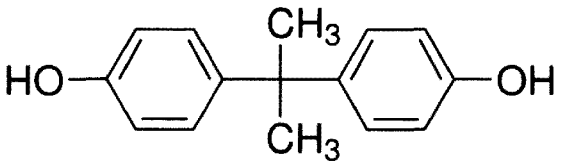
The research reported in this thesis was designed to determine the influences of BPA upon early pregnancy, specifically the process of intrauterine implantation in female mice. Successful blastocyst implantation is critical for the establishment of pregnancy and is known to be very sensitive to estrogens (deCatanzaro *et al.*, 2001, 2006). The

number of pups born, implantation sites, urinary hormone levels, uterine morphology, and uterine receptor and protein expression following peri-implantation BPA exposure was investigated.

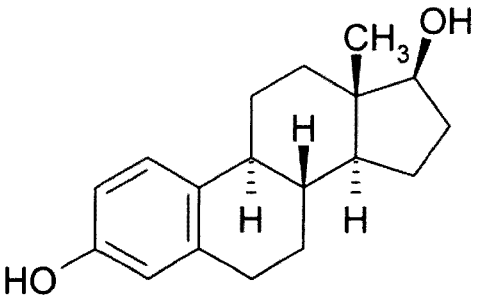
Estrogenic Properties of Bisphenol A

BPA was first reported to have estrogenic properties in the 1930s by Dodds and Lawson (1936) in their search for a synthetic estrogen to be developed for therapeutic use. It was through their research that diethylstilbestrol (DES) was determined to be a potent estrogenic substance with potential pharmaceutical applications (Dodds and Lawson, 1936). More recent investigations have shown that BPA will bind to nuclear estrogen receptors ERα and ERβ (Gould *et al.*, 1998; Kuiper *et al.*, 1998), resulting in estrogen-like signals and eventual modification of gene expression (Matthews *et al.*, 2001; McLachlan, 2001). The relative affinity for binding to these receptors was found to be 1/10,000th that of estradiol, providing further evidence for the weak estrogenic properties of BPA. A comparative study of BPA and 19 other suspected EDCs suggested the minimum structural requirement for estrogenic activity is a 4-OH group on the A-phenyl ring and a hydrophobic moiety at the 2-position of the propane (Kitamura *et al.*, 2005).

Fig. 1. Chemical structure of Bisphenol A and 17β-estradiol



Bisphenol A



17β-Estradiol

In vitro and *in vivo* reports have provided strong support for the estrogenicity of BPA (Schafer *et al.*, 1999; Steinmetz *et al.*, 1997; Takai *et al.*, 2000). For example, it has been observed to increase cell proliferation of MCF-7 breast cancer cells (Howdeshell *et al.*, 2003; Matthews *et al.*, 2001), increase uterine weight following oral ingestion and subcutaneous injections (Matthews *et al.*, 2001), and increase adult male prostate development following *in utero* exposure (Nagel *et al.*, 1997). Initially, the lowest observed adverse effect level (LOAEL) of BPA was reported to be 50 mg/kg/day by the Environment Protection Agency in the United States. This in turn led to the calculation of the acceptable intake or "safe" dose of 50 μg/kg/day by dividing the LOAEL by 1000 to account for uncertainty about factors that include extrapolation of dose levels for animals to humans, the threshold for sensitive humans, and the effects of duration when extrapolating for subchronic to chronic exposure (IRIS, 1988).

Several different endpoints have been used to investigate the estrogenic properties to EDC exposure *in vivo*. The uterotrophic assay measures uterine weight following exposure to a chemical and is a common method for determining the estrogenicity of particular substances. Studies of the uterotrophic response to BPA have produced mixed results. Significant effects have been reported at doses as low as 0.3 mg/kg (Steinmetz *et al.*, 1998). However, other studies did not find an effect at comparable doses, instead finding that much higher doses, in the 100 mg/kg (Markey *et al.*, 2001) to 400 mg/kg range (Ashby *et al.*, 1998), were required to induce a similar response. Various measures of uterine morphology are known to change dramatically in response to estrogen exposure, and thus have also been used to measure of the estrogenic properties of BPA. Subcutaneous administration of either BPA or estradiol to ovariectomized rats significantly increased luminal epithelial height and the thickness of both the stromal and myometrial layers of the uterus (Papaconstantinou *et al.*, 2000; Steinmetz *et al.*, 1998). Uterine stromal cells have also been observed to proliferate following four days of 500 mg BPA/kg in intact rats (Cook *et al.*, 1997).

Estrogen-sensitive steroid receptor and protein expression have also been shown to be altered by BPA exposure. Progesterone receptor (PR) expression increases in a dose dependent fashion in the preoptic and ventromedial areas of the hypothalamus following BPA administration (Funabashi *et al.*, 2003). Changes in steroid receptor expression have also been shown in mouse reproductive tissue following *in utero* BPA exposure, with increases in ERα and PR expression in the uterine luminal epithelium of the endometrium and subepithelial stroma (Markey *et al.*, 2005). BPA can also influence

estrogen-dependent proteins. The expression of lactoferrin, an iron-binding glycoprotein expressed in uterine epithelial cells (Teng *et al.*, 1989), will increase in response to estradiol exposure (Newbold *et al.*, 1992; Pentecost and Teng, 1986). In immature female mice, exposure to 75 mg BPA/kg over a three day period via subcutaneous pump increased lactoferrin expression by over 300%, while 5 μg/kg estradiol increased expression by close to 400% (Markey *et al.*, 2001). However, following three days of administering 100 mg BPA/kg to immature female mice, there was no detectable expression of lactoferrin in the uterus, whereas other estrogenic EDCs such as DES, methoxychlor, and α-zearalanol significantly increased lactoferrin expression (Mehmood *et al.*, 2000). Further investigations are required to clarify these mixed results. Previous to this thesis, it has not been established how BPA influences uterine morphology and steroid receptor and protein expression when administered to inseminated females during the period of blastocyst implantation.

Low Dose Effects of BPA

Some authors of recent *in vivo* investigations have argued that BPA is capable of inducing significant physiological and developmental effects at much lower doses approaching or below the LOAEL of 50 mg/kg/day (Nagel *et al.*, 1997; Richter *et al.*, 2007; vom Saal *et al.*, 1998). Exposure of laboratory animals to BPA doses as low as 2.4 μg/kg has been shown to alter embryonic and pubertal development (Howdeshell *et al.*, 1999; Takai *et al.*, 2001; Tsutsui *et al.*, 1998), sexually-dimorphic development of brain

and behaviour (Fujimoto *et al.*, 2006; Kubo *et al.*, 2001; Nakagami *et al.*, 2009), and maternal behaviour (Palanza *et al.*, 2002).

Further understanding of the actions of estradiol has provided an indication of alternative pathways for BPA and other EDCs to exert their effects at concentrations lower than what would be expected via binding to classical nuclear steroid receptors (Watson *et al.*, 2007). Of particular interest is the membrane steroid receptors, which among others include a variant of the ERα (mER) (Powel *et al.*, 2001; Watson *et al.*, 2007) and G protein-coupled receptor 30 (GPR30) (Thomas and Dong, 2006). Non-genomic receptor actions have been shown to produce high levels of amplification where low concentration levels can induce larger functional changes (see review by Welshons *et al.*, 2006). Binding of a steroid hormone to a classical nuclear receptor typically induces a conformational change in the receptor. This receptor-hormone complex will then interact with specific sites on DNA, leading to altered transcriptional activity and increases in protein synthesis. Measurable effects following nuclear receptor binding are generally observed at least 2-8 hours, and in some cases longer, following the initial binding of the receptor (Gould *et al.*, 1998; Gronemeyer, 1992; Walsh *et al.*, 2005). The effects of hormone binding to non-genomic receptors are typically observed in a shorter amount of time, in the order of seconds to minutes (Aronica *et al.*, 1994; Le Mellay *et al.*, 1997; Pedram *et al.*, 2002).

Non-genomic actions of estradiol have been observed in reproductive tissues including granulosa cells, endometrial cells, and oocytes (Morley *et al.*, 1992; Pietras and Szego, 1975, 1979). Evidence suggests that BPA is able to exert its effects by non-

genomic pathways at low doses in breast cancer cells and pituitary tumour cells (Walsh *et al.*, 2005; Wozniak *et al.*, 2005), and that it is equally potent as estradiol in activating the transcription factor CREB (Quesada *et al.*, 2002). Although BPA has been found to interact with non-genomic receptors *in vitro*, *in vivo* mechanisms and effects have yet to be fully explored (Ryan *et al.*, 2010).

Sensitivity to BPA exposure, particularly at low, environmentally relevant doses, is dependent upon species, strain, and route of administration (Markey *et al.*, 2001; Pottenger *et al.*, 2000). It has also been suggested that exposure to varying levels of androgens *in utero* will influence an animal's sensitivity to BPA (Howdeshell *et al.*, 1999). However, there have been reports that have failed to find supporting evidence for the low dose effects of BPA, using similar genetic strains of animals and routes of administration (Ashby *et al.*, 1999; Cagen *et al.*, 1999; Ema *et al.*, 2001; Tyl *et al.*, 2002). A recent extensive investigation of *in utero* and lactational exposure to BPA did not detect any alteration to sexually dimorphic behaviour, puberty, fertility, or the anatomy of female rats (Ryan *et al.*, 2010). In addition, in research reported elsewhere (Berger *et al.*, in preparation), I found that there was no significant impact of low dose perinatal exposure upon female sexual development and fertility. Due to these inconsistencies, the influence of low dose BPA exposure *in vivo* has been under continued scientific scrutiny (see reviews by Goodman *et al.*, 2009; Gray *et al.*, 2004; Foster *et al.*, 2008).

Implantation

In sexually mature female mammals ovulation generally occurs at the midpoint of the estrous or menstrual cycle (Campbell *et al.*, 1999). If successful copulation has occurred, fertilization will typically take place in the oviduct, where the ovum or ova encounter spermatozoa (see review by Bloch, 1976). Following fertilization, the zygote(s) begins the cleavage process, eventually developing the inner cell mass and the surrounding layer of trophectoderm cells (Campbell *et al.*, 1999; Dey and Lim, 2006; Gardner and Papaioannou, 1975), and migrates down the fallopian tubes arriving at the uterus to begin implantation several days later (Burdick and Pincus, 1935; Greenwald, 1967). There are several hormonal and non-hormonal factors and events that play a role in leading to successful intrauterine implantation. Coordinated actions of the ovarian steroid hormones, progesterone and estrogens, play a critical role in the development of a receptive uterine environment, and embryo development and movement through the oviduct (Harper, 1992; Paria *et al.*, 1993; Roblero and Garavagno, 1979). In mice, implantation of blastocysts typically occurs on gestational day (GD) 3 of pregnancy, with the day of detection of sperm plug being designated as GD 0 (Harper, 1992; Paria *et al..*, 1993). Fluctuations in estradiol levels can influence the retention of zygotes in the oviduct and the rate of transport through the uterus which can ultimately lead to missing the 'window' of implantation and failure to establish pregnancy (Burdick and Whitney, 1937; Greenwald, 1967; Humphry and Martin, 1968; Whitney and Burdick, 1936).

Differentiation of the uterus into a receptive state begins when the blastocyst is capable of two-way interactions that will ultimately create an environment that will

support attachment, initiate implantation, and allow for continued growth (Dey and Lim, 2006; Paria *et al.*, 1993; Yoshinaga, 1988). In the initial days following insemination, pre-ovulatory estrogens and increasing levels of progesterone lead to uterine epithelial and stromal cell proliferation (Dey and Lim, 2006; Huet *et al.*, 1989). A small spike in ovarian estrogen production on the day of implantation further stimulates stromal cell proliferation and inhibits uterine epithelial cell proliferation thereby promoting differentiation (Dey and Lim, 2006; Huet *et al.*, 1989). Altering the estrogen to progesterone ratio in the first few days following insemination will prevent successful intrauterine blastocyst implantation (Gidley-Baird *et al.*, 1986; Safro *et al.*, 1990).

The period surrounding intrauterine implantation of blastocysts can be sensitive to major environmental and social changes, such as physical restraint, temperature extremes, nutritional deprivation, predator exposure, human handling, and other stressors (deCatanzaro and MacNiven, 1992; Euker and Riegle, 1973; Runner, 1959; Weir and De Fries, 1963). Exposure to exogenous androgens and estrogens during this sensitive period will result in a loss of pregnancy (deCatanzaro *et al.*, 1991, 2001; Harper 1967). Implantation is exceptionally sensitive to exogenous estrogens relative to other steroid hormones. Administration of as little as 37 ng of 17β-estradiol to an inseminated female mouse during the window of implantation can terminate pregnancy (deCatanzaro *et al.*, 2001, 2006). Increases in estradiol levels above optimal levels for implantation have been shown to alter the morphology of the uterus and cause it to advance to refractory state through an aberrant expression of genes critical to implantation (Ma *et al.*, 2003). Increased levels of estrogens can also be detrimental to the development of the blastocyst,

slowing its growth, decreasing embryonic adhesion rates, and increasing mortality (Valbuena *et al.*, 2001). Whitney and Burdick (1936) reported that small doses of estradiol delayed ova transport by causing retention of zygotes in the oviduct, however larger doses increased the rate of passage into the uterus (Burdick and Whitney, 1937); in either case implantation may fail as ova arrive at the uterus at an inappropriate time. Administration of as little as 0.4 μg estradiol/day on days 1 to 3 of pregnancy can lead to an increase in the retention of ova in the oviduct and cause significant decreases in the number of ova recovered (Humphrey and Martin, 1968). Clearly, estrogen levels play a critical role from the development of the ova through to successful implantation, and small deviations during this process can have detrimental results through more than one mechanism.

BPA and Pregnancy

Estrogens are well known to have measurable physiological effects on the uterus, including increased uterine wet weight, epithelial cell height, and cell proliferation (Evans *et al.*, 1941; Mukku *et al.*, 1982). BPA exposure has also been shown to have similar impacts on the uterus. Administration of 0.3 mg/kg BPA for four days to ovariectomized rats resulted in two-fold increase in uterine wet weight after three days of exposure (Steinmetz *et al.*, 1998). In immature mice, an increase in uterine wet weight was observed following 3 days of constant exposure to 100 mg/kg by implanted osmotic pumps (Markey *et al.*, 2001). BPA administration has also been shown to increase uterine epithelial cell height in ovariectomized rats and mice (Papaconstantinou *et al.*,

2000; Steinmetz *et al.*, 1998). These alterations to the uterine environment could have significant implications for implantation and pregnancy.

Investigations of exposure during pregnancy confirm that BPA can have deleterious effects. *In utero* investigations have shown that embryonic growth is sensitive to BPA exposure altering rates of development to blastocysts at environmentally relevant doses (Takai *et al.*, 2000). Subcutaneous BPA administration during gestational days (GD) 0 through 7 of pregnancy significantly decreased the number of embryos and uterine weight measured on GDs 10 and 12, and it also decreased the survival rate of neonates within three days after birth (Tachibana *et al.*, 2007). Oral administration of 1000 mg/kg by gavage on GD 1 to 20 produced an increased rate of pregnancy failure, embryonic deaths, and post-implantation loss (Kim *et al.*, 2001). Although these studies have provided an indication of the negative impact BPA can have on gestation and pregnancy outcome, the exposure periods go beyond the peri-implantation period. It is not clear whether BPA is influencing intrauterine blastocyst implantation or causing fetal reabsorption or some other post-implantation effect.

BPA and Hormonal Responses

Changes in endogenous hormone levels are well known to have a wide range of physiological and morphological effects on reproductive tissue, steroid receptors, and protein expression (Leroy *et al.*, 1969; Mendoza-Rodríguez *et al.*, 2003; Mukku *et al.*, 1982; Newbold *et al.*, 1992). BPA also has the capacity to alter similar measures typically sensitive to changes in hormonal levels. As previously mentioned, *in vitro*

investigations have shown the estrogenic properties of BPA, with exposure causing increased proliferation of MCF-7 breast cancer cells (Howdeshell *et al.*, 2003; Matthews *et al.*, 2001). In addition, low level *in vitro* BPA treatment of porcine ovarian granulosa cells resulted in increased basal progesterone levels, FSH-stimulated progesterone secretion and inhibited FSH-induced estradiol levels (Mlynarčíková *et al.*, 2005). Since the impact of BPA on progesterone production reversed at higher concentrations, it was suggested that BPA was not acting strictly by mimicking estradiol but exerting its effects via a host of different hormonal pathways (Mlynarčíková *et al.*, 2005). Furthermore, *in vitro* experimentation has shown that BPA not only has estrogenic properties. BPA also can enhance aromatase activity (Nativell-Serpentini *et al.*, 2003) and act in an anti-androgenic fashion by antagonizing androgen receptors (Lee *et al.*, 2003; Sohoni and Sumpter, 1998; Xu *et al.*, 2005).

BPA can also alter hormone production *in vivo*. Steroid hormone balance in juvenile fish was disturbed following BPA exposure, with an overall increase in estrone levels which lowered the ratio of androgens to estrogens (Labadie and Budzinski, 2006). Pre-pubertal rats administered 2.4 μg BPA/kg/day for fourteen days showed decreased pituitary luteinizing hormone secretion and Leydig cell testosterone production (Akingbemi *et al.*, 2004). BPA administered to adult ovariectomized rats can increase expression of progesterone receptor proteins in the hypothalamus following a single injection of 100 μg (Funabashi *et al.*, 2003) and stimulate the release of prolactin following three days of exposure via an implanted capsule (Steinmetz *et al.*, 1997). Previous investigations of BPA-induced hormonal responses have primarily focused on

exposure *in utero* and during postnatal development (Akingbemi *et al.*, 2004; Ramos *et al.*, 2003; Seta *et al.*, 2006) and have not examined acute impacts during early pregnancy.

Traditionally, in order to determine hormonal responses in rodents it was necessary to take and analyze blood samples. In smaller rodents such as mice, this typically involves a tail or orbital bleed that requires a large amount of handling and can be very stressful for the animals. Alternatively, terminal blood samples can also be acquired. None of these techniques is ideal for providing multiple samples from a single animal over an extended period of time. In the deCatanzaro laboratory, non-invasive urinary collection techniques were developed and enzyme-linked immunosorbant assays adapted to monitor hormonal variations in a single animal reliably over an extended period of time (deCatanzaro *et al.*, 2003, 2004; Muir *et al.*, 2001). Urinary steroid hormone levels were shown to be related to known systemic trends in female mice (deCatanzaro *et al.*, 2004; Muir *et al.*, 2001). These techniques were employed in this thesis, as they provided an opportunity to monitor hormonal changes following BPA administration during the period of blastocyst implantation.

Objectives and Hypotheses

The majority of studies investigating the estrogenic properties of BPA have focused on the impact of exposure *in utero* or during early development. Authors have previously reported that BPA may have a negative influence on pregnancy outcome; however the period of exposure began prior to mating (Darmani and Al-Hiyasat, 2004, 2006), extended beyond the sensitive period of intrauterine blastocyst implantation (Kim

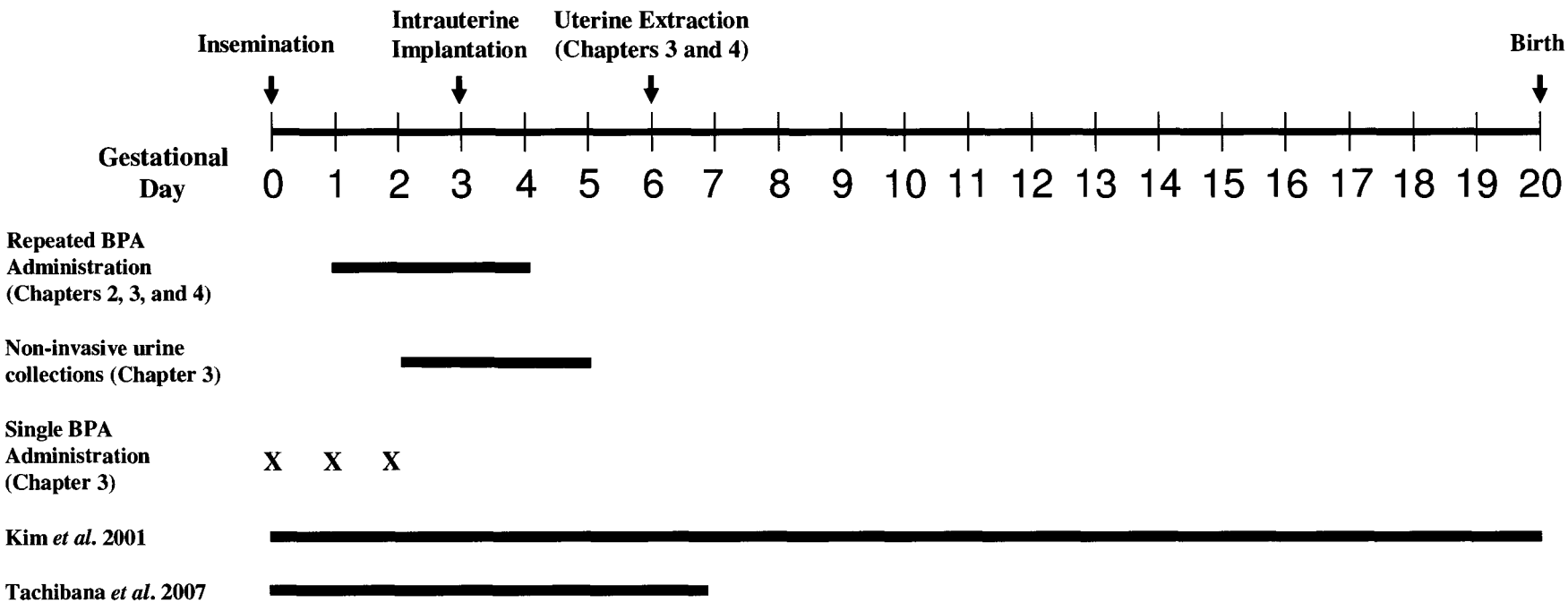
et al., 2001; Tachibana *et al.*, 2007), or began after implantation had occurred (Howdeshell *et al.*, 1999; Zalko *et al.*, 2003). Previous work in the deCatanzaro laboratory has shown that exposure to minute levels of estradiol in the days following insemination will reliably disrupt pregnancy in female mice (deCatanzaro *et al.*, 1991, 2001). This proven estrogen-sensitive paradigm provides a novel way in which to test the estrogenicity of xenoestrogens, one which reflects a critical period of reproduction that would have significant ecological implications. The objectives of experiments provided in this thesis were:

1) to examine the impact of BPA exposure during the first few days following insemination upon implantation and pregnancy;

2) to investigate how BPA exposure could alter uterine morphology, steroid excretion, and uterine steroid receptor and protein expression; and

3) to study a wide range of doses including low, environmentally relevant levels of exposure.

Fig. 2. A timeline highlighting some major gestational events and when BPA administrations and uterine extractions were conducted in the experiments reported in this thesis. The timing of BPA administration of two previous reports that measured pregnancy outcome following exposure is also shown as a basis of comparison.



Pregnancy outcome was initially assessed by counting the number of live pups born. Subsequently, in order to determine the point at which BPA exposure was influencing pregnancy, the number of implantation sites was counted on GD 6. In some experiments, urine was noninvasively collected from females throughout the period of implantation allowing for multiple measurements from a single female. Urinary progesterone and estradiol levels were assessed via enzyme immunoassay procedures. To explore the potential estrogenic effects of BPA exposure further, uterine luminal area and epithelial cell height were measured at gestational day 6. PR and ERα expression in the uterus were determined using immunohistochemical analysis. PR and lactoferrin expression, both sensitive to fluctuating estradiol levels in the uterus, were also determined using western blot techniques.

On the basis of previous reports of the estrogenicity of BPA, it was hypothesized that BPA would interfere with intrauterine implantation of blastocysts. The null-hypothesis was that BPA would not show estrogenic effects when administered during early pregnancy, failing to have impacts on blastocyst implantation, urinary hormonal output, uterine morphology, or uterine receptor or protein expression.

Chapter 2: Berger, R.G., Hancock, T., deCatanzaro, D. (2007) Influence of oral and subcutaneous bisphenol-A on intrauterine implantation of fertilized ova in inseminated female mice. *Reproductive Toxicology*, 23: 138-44.

Abstract: Intrauterine implantation of fertilized ova in inseminated females is sensitive to minute levels of natural estrogens. Bisphenol-A (BPA), a widely used chemical in the

production of polycarbonate plastics and epoxy resins, can be estrogenic. Here we administered BPA during the period of implantation to determine levels of exposure required to terminate pregnancy in mice. Varied doses were given through either injection or ingestion. Subcutaneous injections during days 1-4 of gestation significantly reduced litter size at 3.375 mg/day and substantially reduced the proportion of females that were parturient at 10.125 mg/day. Uterine implantation sites were also significantly reduced in females sacrificed at day 6 after receiving 10.125 mg/day. Exposure to lower doses was without significant effect. When inseminated females' diets were supplemented on days 1-5 with peanut butter contaminated by 0.11-9.0% BPA, litter size and percent parturient were not affected. However, when the animals' diet was exclusively comprised of a mixture of BPA, peanut butter, and powdered chow during days 1-4, an average daily intake of 68.84 mg BPA terminated all pregnancies. No significant effects at lower doses of BPA were seen in number of births or other measures through either mode of administration.

Chapter 3: Berger, R.G., Shaw, J., deCatanzaro, D. (2008) Impact of acute bisphenol-A exposure upon intrauterine implantation of fertilized ova and urinary levels of progesterone and 17β-estradiol. *Reproductive Toxicology*, 26: 94-9.

Abstract: Bisphenol-A (BPA), a monomer used in production of polycarbonate plastics and epoxy resins, has established estrogenic properties. We assessed the impact of acute and repeated subcutaneous BPA administration upon intrauterine implantation of fertilized ova and urinary levels of 17β-estradiol and progesterone in inseminated female

mice. In Experiment 1, females received varied doses of BPA on days 1-4 of gestation. Daily doses of 6.75 and 10.125 mg/animal significantly reduced the number of implantation sites. Urinary progesterone was significantly reduced by the higher dose, but no other dose had an effect on progesterone levels and no dose altered estradiol levels. In Experiment 2, inseminated females received a single dose of BPA on days 0, 1, or 2 of gestation. A single dose of 10.125 mg reduced the number of implantation sites when given on day 0 or day 1, and 6.75 mg on day 1 also produced fewer implantation sites, but there was no such effect of any dose when administered on day 2. These data show a lower threshold for BPA-induced pregnancy disruption than previously reported, also indicating effects of just one exposure. They confirm that this disruption is due to the actions of BPA upon implantation sites, and show that higher doses can influence systemic progesterone levels.

Chapter 4: Berger, R.G., Foster, W.G., deCatanzaro, D. (Submitted). Bisphenol-A exposure during the period of blastocyst implantation alters uterine morphology and perturbs measures of estrogen and progesterone receptor expression in mice.

Abstract: Bisphenol-A (BPA) has estrogenic properties both *in vitro* and *in vivo*. We investigated its impacts upon uterine morphology and estrogen and progesterone receptors after injection on gestational days 1-4 in doses known to disrupt pregnancy. Blastocyst implantation was significantly reduced by doses of 6.75 and 10.125 mg/animal. Uterine luminal area expanded substantially in response to increasing doses of BPA. Luminal epithelial cell height increased following exposure to 10.125

mg/animal, whereas there were no differences in the number of corpora lutea among conditions. The proportion of cells staining positively for estrogen receptors was affected non-monotonically, showing highest levels at 3.375 mg/animal and lowest levels at 10.125 mg/animal. Similarly progesterone receptor expression measured through Western blots related non-monotonically to dose, being highest at 3.375 mg/animal and diminishing with increasing dose. These results suggest that BPA exposure during early gestation acts at the uterus to disrupt intrauterine implantation, consistent with an estrogenic effect.

Chapter 2

Influence of oral and subcutaneous bisphenol-A on intrauterine implantation of fertilized ova in inseminated female mice

Berger, R.G., Hancock, T., deCatanzaro, D. (2007)

Reproductive Toxicology, 23, 138-144.

Authors' Contributions

Robert G. Berger: Formation of experimental design, data collection, literature review, data analysis and manuscript writing.

Trina Hancock: Data collection and development of the oral administration procedure.

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

Additional Support

Undergraduate and Graduate Students: Elliott Beaton, Ayesha Khan, and Katayun Treasurywala provided assistance with experimental procedures.

1. Introduction

Artificial compounds that mimic endogenous steroid hormones may be capable of altering natural processes in both humans and wildlife [1,2]. Bisphenol-A (BPA) is one chemical established to have weak estrogenic properties [3-6]. BPA is primarily used to produce polycarbonate plastics and epoxy resins, and can be found for example in coatings for food cans, in dental sealants, and in reusable drink containers. Heat, contact with either acidic or basic compounds, and incomplete polymerization can cause BPA to leach into the surrounding medium [4,7]. Measurable amounts of BPA may be found in some foods and liquids from lacquer-coated cans [8], sewage plant effluent [9], and marine wildlife and sediment [10]. BPA has been detected in human samples of saliva and urine [11,12] and placental tissue [13].

BPA has been shown to interact with both estrogen receptor subtypes α and β [14,15]. In laboratory animals, exposure has been found to impact embryonic development [16,17], pubertal development [18], weight of prostate glands [19], reproductive tract and organ development [20,21] and maternal behavior [22,23]. Questions remain regarding the level of exposure required to induce some of the aforementioned effects [24]. It has been argued that there may be impacts of BPA at lower, more ecologically-relevant doses than those commonly used in toxicological studies [19,24-27]. Some investigations of low dose exposure with laboratory animals have shown significant effects at levels comparable to those to which humans are regularly exposed [8,19,24,25]. The estimated human daily BPA intake depends on age and varies from about 1.6 μg/kg body weight/day during infancy to 0.4 μg/kg body

weight/day in adulthood [28]. One analysis of adult urine samples showed that 95% of the 394 individuals tested had measurable amounts of BPA with a mean concentration of 1.28 μg/L [11]. BPA has been found in human maternal plasma, fetal plasma, and placental tissue, with medians levels of 3.1 ng/mL, 2.3 ng/mL, and 12.7 ng/mL respectively [29].

Early pregnancy in mammals is exceptionally sensitive to exogenous estrogens. Exposure to minute doses of 17β-estradiol around the period of intrauterine implantation of fertilized ova can terminate pregnancy; other estrogens and to a lesser extent androgens can also have this effect [30-32]. Diverse stressors can also disrupt early pregnancy, an effect that may be attributable to adrenocortical androgens and estrogens [33]. Exposure to novel males or their urine also can disrupt pregnancy in some species [34,35]; the males' urinary androgens and estrogens may be in part responsible [32].

Accordingly, we reasoned that BPA could disrupt early pregnancy, and undertook to determine the lowest effective dose in order to shed light on the potential risk to health in humans and other animals. Although this issue has not previously been examined systematically, there are a few indications from previous studies that BPA exposure during gestation can alter the outcome of pregnancy in laboratory animals. Intraperitoneal administration of BPA to rats during days 1-15 of gestation reduced the number of live fetuses per litter at 85 mg/kg and impaired the establishment of pregnancy at 125 mg/kg [36]. Administration by gavage of 1000 mg/kg/day to rats during gestational days 1-20 produced significant pregnancy failure as well as severe maternal toxicity [37]. Doses of 1250 mg/kg/day by gastric intubation on gestational days 6-15

significantly increased the percentage of fetal reabsorptions in rats [38]. As each of these studies involved BPA administration during the post-implantation period, effects could be due to non-estrogenic properties of BPA impacting upon maternal and/or fetal health.

The present study was designed to investigate the impact upon pregnancy of BPA exposure during the first five days of gestation, timing that coincides with the period of implantation in mice [39]. A wide range of doses was explored in order to address concerns regarding the potential estrogenic properties of BPA at low, ecologically-relevant doses in addition to higher doses. Two forms of administration were examined, subcutaneous injection and ingestion as a food contaminant. Procedures were designed to be as non-invasive as possible, with human handling minimized in order to prevent non-specific impacts of handling on intrauterine implantation [*cf.* 33].

2. Materials and methods

2.1. Mice, insemination procedures, and pregnancy outcome measures

This research was approved by the Animal Research Ethics Board of McMaster University, conforming to the standards of the Canadian Council on Animal Care. Mice were of CF-1 strain from stock obtained from Charles River Breeding Farms of Canada (La Prairie, Quebec). Housing was in standard 28 X 16 X 11 (height) cm polypropylene cages with wire-grid tops. Continuous access to food (Harlan Teklad chow) and water was provided unless noted otherwise. Colony rooms were maintained at 21°C with a reversed 14h light:10h dark cycle.

Sexually naïve female mice aged 3-6 months (28-32 g) were each randomly paired in a cage with a male 5-10 months of age. While paired, female hindquarters were inspected three times daily during the dark phase of their cycle for the presence of sperm plugs. Females were identified as subjects upon detection of intact sperm plugs and randomly assigned to one of the experimental or control conditions. The day of detection was designated as day 0 of pregnancy. Each female was separated from the inseminating male and housed alone on day 1 of pregnancy. Experimental treatment occurred during days 1 to 5 of pregnancy as described below. Age of subjects was counterbalanced across experimental conditions. After treatment, females were left undisturbed for the duration of their pregnancy unless otherwise noted. Commencing on day 17 and continuing until day 25 after sperm plug detection, females were monitored for parturition at least twice daily. Pregnancy outcome was measured by recording the number of pups born on the day of parturition. Mother and pups continued to be observed for 5 days following parturition for any deaths or infanticide. All pups were weaned at 28 days following birth, at which time the number of pups, sex of pups, and weights for each litter were documented. The health of all subjects was closely monitored throughout each experiment.

2.2 Subcutaneous injection

Subcutaneous injections of bisphenol-A (97%, Sigma-Aldrich) dissolved in peanut oil were administered approximately two hours into the dark phase of the lighting cycle on days 1 through 4 of pregnancy. Females were assigned to doses of 0.000,

0.0005, 0.0015, 0.0046, 0.0143, 0.0416, 0.125, 0.375, 1.125, 3.375, and 10.125 mg BPA/animal/day, with sample sizes of 31, 5, 5, 5, 7, 5, 7, 8, 9, 15, and 11, respectively. The volume of peanut oil vehicle varied due to solubility constraints of BPA; doses from 0.0005 to 0.375 mg were dissolved in 0.05 cc, 1.125 and 3.375 mg in 0.15 cc, and 10.125 mg in 0.45 cc peanut oil. Proportional numbers of control subjects were run at these volumes of peanut oil. Different quantities of vehicle did not have an impact on pregnancy outcome in these controls. To minimize the development of skin irritation, each dose at or above 1.125 mg was administered at one of four different sites which included left and right flanks, middle back area, and scruff of the neck. To control any variability in handling stress, controls and BPA exposed groups were given injections in identical locations on the same days of gestation.

2.2 Subcutaneous injection and uterine histology

To confirm that any potential impact of BPA administration on pregnancy outcome was the result of exposure during the period of implantation, uterine histology was conducted on day 6 of pregnancy. Females were randomly assigned to one of two doses, 0.000 mg and 10.125 BPA/animal/day, with sample sizes of 8 and 9 respectively. Similar methods of administration were used as those described above. Subjects were sacrificed in a carbon dioxide chamber and the uterus removed. Any sites of implantation along the uterus were counted.

2.3 Oral administration via diet supplement

Bisphenol-A was mixed with Kraft Extra-Creamy peanut butter until a homogeneous mixture was achieved. One gram of mixture was placed onto each pre-weighed tray (Fisherbrand 2 – 2.5" polystyrene weigh dishes). New trays with fresh peanut butter/BPA mixtures were provided on days 1 through 5 of pregnancy at approximately four hours into the dark portion of the lighting cycle. Trays were hung in the corner of wire cage tops to minimize contamination from bedding, and were checked at least three times daily and removed if all of the mixture had been consumed. Any remnants from the previous dose were weighed and the total amount of BPA consumed was calculated on a daily basis. Concentrations of BPA/peanut butter mixtures were 0.00%, 0.11%, 1%, 3%, and 9% BPA, with sample sizes of 15, 6, 11, 6, and 6, respectively.

2.4 Oral administration via food contamination

This procedure was developed to encourage greater consumption of BPA than was possible with the above method. One part powdered Harlan Teklad 22/5 rodent chow was added to two parts peanut butter. Control subjects received this mixture alone, whereas experimental subjects were provided with a 3% or 6% BPA/peanut butter/chow mixture. There were 6 subjects for each of the 3% group and its controls, and 12 subjects for each of the 6% group and its controls. During days 1 through 4 of gestation, normal pellet chow was removed from all cages and the substitute food provided on pre-weighed hanging trays as described above. A yoked control procedure was implemented whereby

each control subject was given the same amount of peanut butter/powdered chow mixture as had been consumed by a matched BPA-exposed subject on the previous day. This was to ensure that total food consumption was matched in the control and experimental conditions. Fresh mixtures were provided each day at approximately four hours into the dark cycle. Any remnants from the previous dose were weighed and the total amount of BPA consumed was calculated. On day 5 of pregnancy, free access to the normal pellet diet resumed.

3. Results

3.1 Subcutaneous injection

The percent of females that gave birth is presented in Figure 1. A substantial decrease was seen in the 10.125 mg/day condition but not in other conditions. A chi-square test of association comparing condition and whether or not females were parturient was significant, $\chi^2(10) = 34.65$, $p < 0.001$. Figure 2 gives the average number of pups born in each condition. No major changes were seen in the average number born in dosages up to and including 1.125 mg/day. A clear decrease in number born was observed in the 3.375 and 10.125 mg/day groups. Analysis of variance of the number of pups born, including zeros for non-parturient females, was significant, $F(10,97) = 6.99$, $p < 0.0001$. Newman-Keuls multiple comparisons for number of pups born showed that the 10.125 mg/day condition was significantly different from all other conditions ($p < 0.01$), and that the 3.375 mg/day dose was significantly different from the control condition ($p < 0.05$).

Fig. 1. The percent of inseminated females that delivered litters after varied subcutaneous doses of BPA on days 1-4 of gestation. (*) Denotes significant difference from all other conditions ($p < 0.001$).

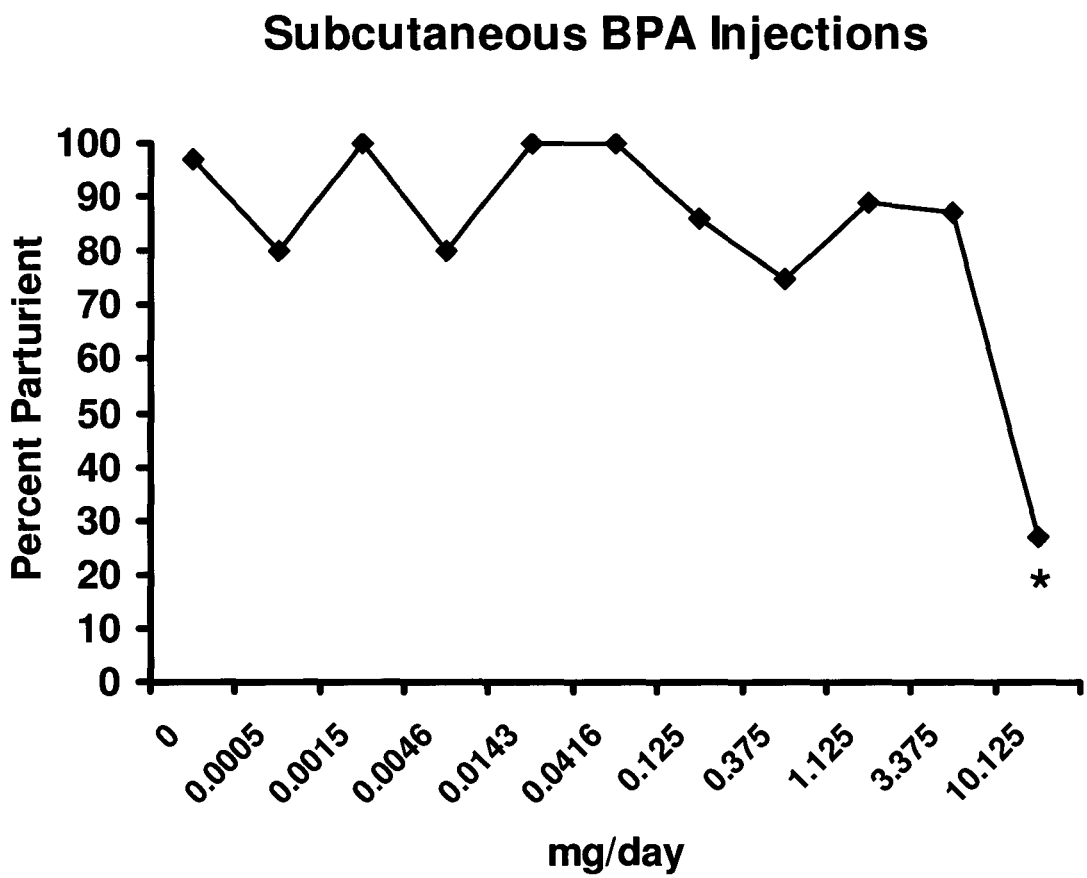
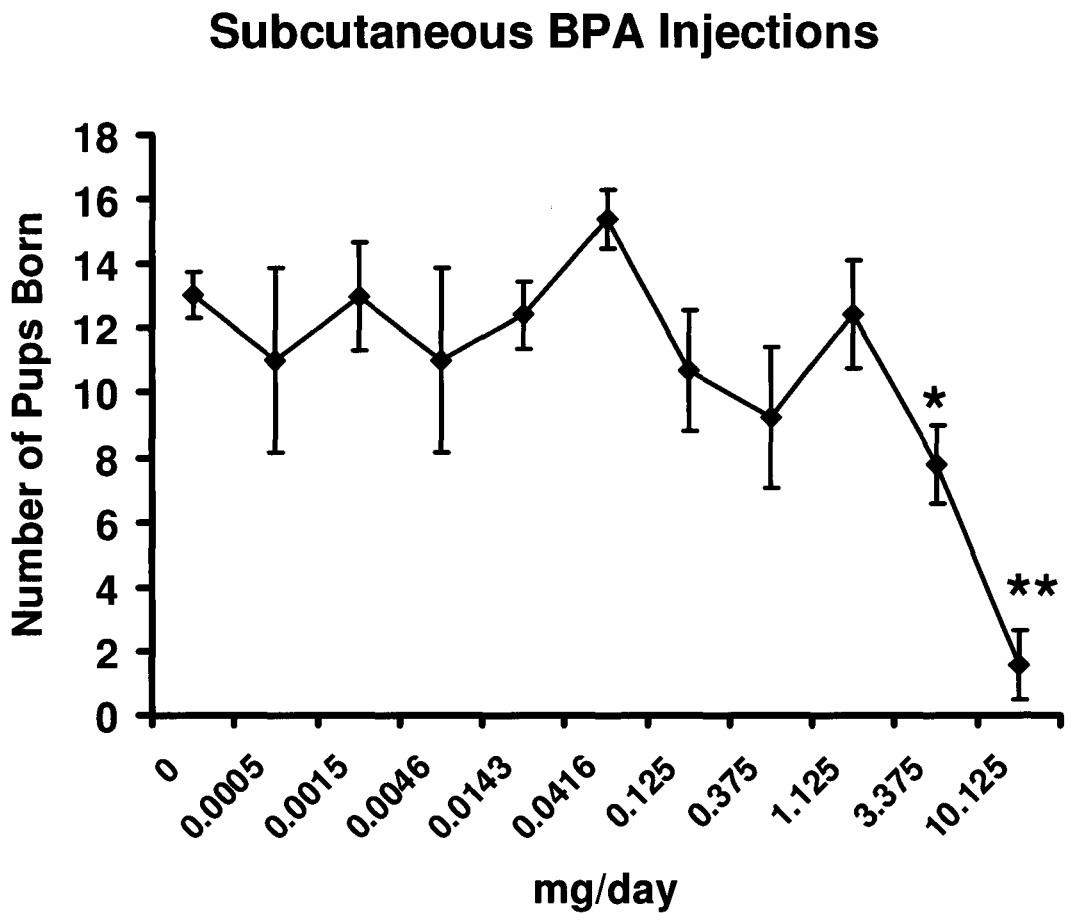


Fig. 2. The mean (±S.E.) number of pups born to inseminated females after varied subcutaneous doses of BPA on days 1-4 of gestation. (*) Denotes significant difference from control condition ($p < 0.05$). (**) Denotes significant difference from all other conditions ($p < 0.01$).



Animals exposed to BPA levels of 1.125, 3.375, and 10.125 mg/day showed some irritation around the site of injection approximately 4-7 days after the final administration. This appeared to be due to excessive grooming of the area surrounding the injection site, particularly at the scruff of the neck. Animals otherwise appeared healthy. In a few cases of more severe irritation, a 50:50 solution of hydrogen peroxide and saline was applied. One female receiving the 10.125 mg dose was sacrificed due to such irritation on day 12 of gestation; uterine histology revealed no sites of implantation or fetuses. A control female sacrificed at the corresponding point of gestation had 13 identifiable fetuses. Table 1 shows remaining measures from this experiment. A number of individual pups or full litters did not survive until weaning, however this was not systematically related to conditions. There were two partially cannibalized litters in the 0.0015 dose. There was one full litter cannibalized and two litters partially cannibalized among control females. Variation in sex ratio among surviving pups at 28 days of age did not reach significance in a test of association relating conditions to sex of pups, $\chi^2(10) = 12.89, p < 0.25$. Average weight of pups was influenced by litter size and competition for resources from the dam as there were very small litters in the highest two doses.

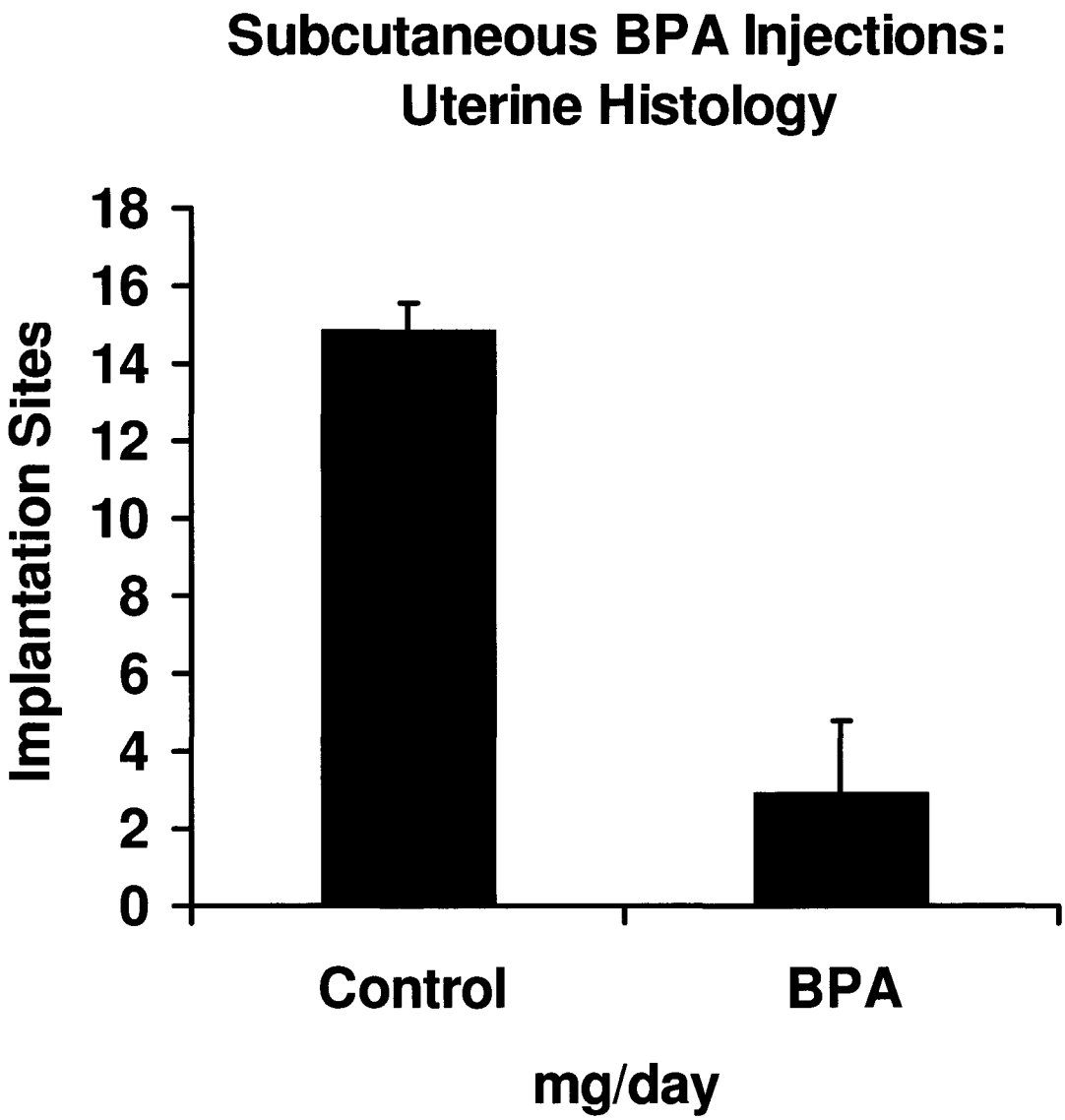
Table 1. Pregnancy outcome from inseminated female mice given subcutaneous injections of peanut oil and varying concentrations of bisphenol-A on days 1-4 of pregnancy.

Condition (mg/day)	No. of Subjects	No. Parturient	Weight/Pup at Weaning (g)	Sex Ratio (M:F)
0.0000	31	30	19.8±0.2	1.10
0.0005	5	4	18.9±0.5	0.73
0.0015	5	5	20.7±0.8	1.33
0.0046	5	4	18.9±0.6	1.71
0.0143	7	7	20.1±0.7	1.39
0.0416	5	5	19.4±0.4	1.42
0.125	7	6	20.2±0.4	0.95
0.375	8	6	19.9±0.3	1.00
1.125	9	8	19.4±0.3	0.67
3.375	15	13	22.0±0.5	0.89
10.125	11	3	23.6±0.7	0.71

3.2 Uterine histology following subcutaneous injection

The number of implantation sites of control and BPA-exposed subjects is shown in Figure 3. All eight control females showed multiple implantation sites, with a range of 12 to 17 per female. Six of 9 BPA-treated females showed no implantation sites, with 1, 9, and 16 evident in remaining females in this group. A chi-squared test of association showed that the proportion of females with any implantation sites differed between the two groups, $\chi^2(1) = 8.24$, $p < 0.005$. A two-sample t-test on the number of implantation sites per female showed significance, $t(15) = 5.61$, $p = 0.0001$.

Fig. 3. The mean (±S.E.) number of implantation sites per inseminated female following subcutaneous administration of 10.125 mg/animal BPA on days 1-4 of gestation as compared to vehicle-injected controls.



3.2 Oral administration via diet supplement

Figure 4 shows the percent parturient in each condition. There were no apparent trends among conditions. Table 2 shows remaining measures for this experiment. Consumption of BPA was calculated for each of the exposed conditions by using the weight of BPA/peanut butter mixture which remained unconsumed on a daily basis. There was a substantial decrease in consumption of the mixture in the higher dose conditions, particularly the 9% BPA condition. Analysis of variance comparing the total average daily BPA consumption in each group showed that there was a significant difference among the conditions, $F(3,16) = 3.24$, $p < 0.001$. The percent of pups surviving after birth to weaning from the zero control to the highest dose was respectively 98.2, 92.7, 84.2, 90.5, and 76.1%; two whole litters were lost in the 9% condition, apparently due to cannibalization. The sex ratio of pups at weaning did not reach statistical significance in a test of association relating conditions and total number of male and female pups, $\chi^2(4) = 4.15$, $p < 0.50$.

Fig. 4. The percent of inseminated females that delivered litters after oral administration of varied doses of BPA in a peanut butter diet supplement on days 1-5 of gestation.

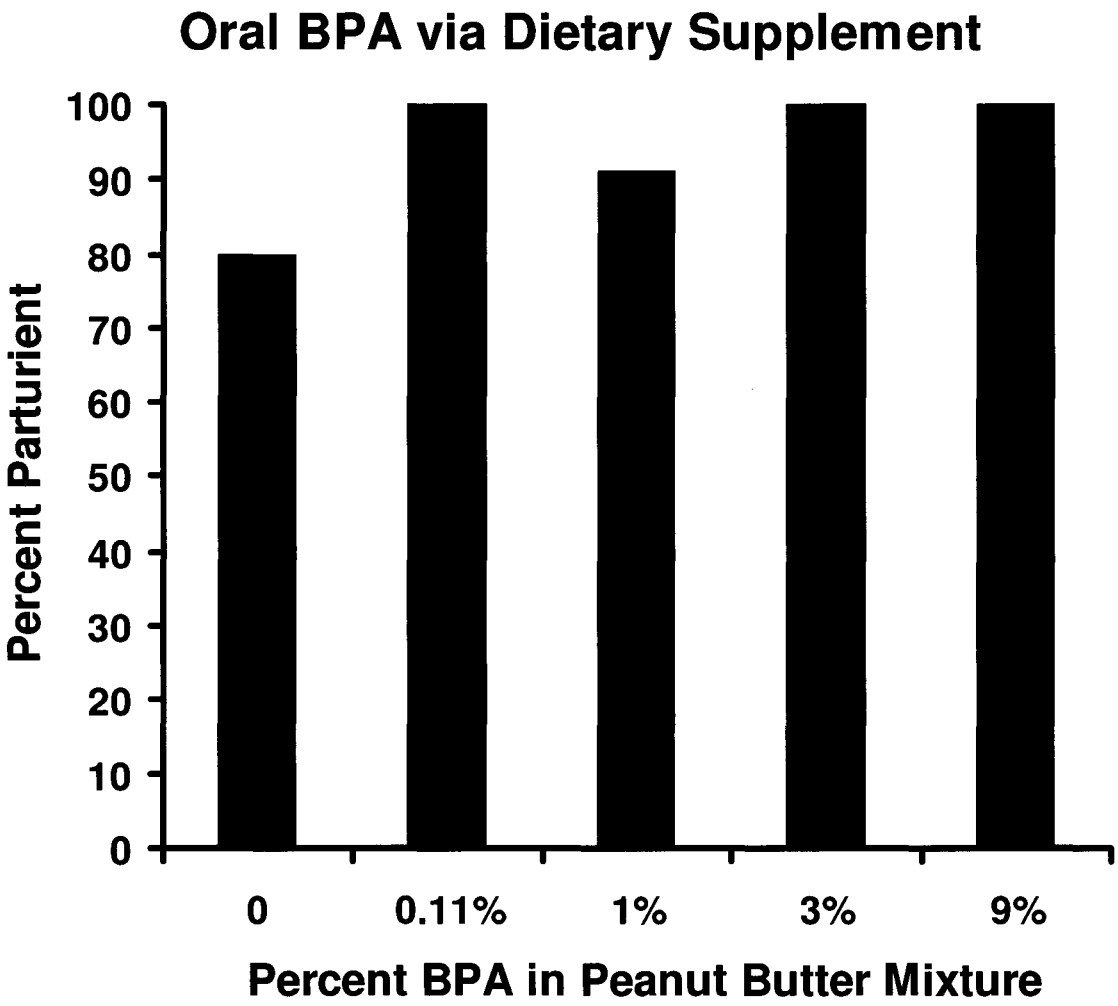


Table 2. Summary of data from inseminated female mice given various concentrations of BPA in a supplemental 1g peanut butter mixture on days 1-5 of pregnancy.

Condition (% BPA)	No. of Subjects	No. Parturient	Supplement Intake (g/day)	Daily BPA Intake (mg)	No. of Pups at Birth	Weight/Pup at Weaning (g)	Sex Ratio (M:F)
0%	15	12	0.89±0.04	0.00±0.00	11.9±1.6	20.1±0.4	1.16
0.11%	6	6	0.98±0.02	1.08±0.02	13.7±1.1	21.9±0.3	1.24
1%	11	10	0.84±0.04	8.33±0.26	12.1±1.3	21.2±0.3	0.75
3%	6	6	0.57±0.07	16.50±1.04	15.8±0.5	20.9±0.2	1.02
9%	6	6	0.16±0.03	13.59±0.85	11.8±2.1	21.4±0.4	1.16

3.3 Oral administration via food contamination

Figure 5 gives the percent parturient for all conditions. Table 3 gives all remaining measures for this experiment. There was no impact of the 3% mixture upon the percent of females that delivered litters or upon litter size. However, none of the 6% BPA-treated animals was parturient. For the 6% mixture, there was a reduction in the total amount of food consumed as compared to animals in the 3% mixture condition. Although yoked control animals for the 6% group also received less food, 11 of 12 were parturient. A chi-square test of association, comparing the proportion parturient in the 6% exposed group and yoked controls was significant, $\chi^2(1) = 20.31$, $p < 0.001$. The percent of pups surviving to weaning was 97.2% for the 3% BPA group, 95.1% for the controls yoked to the 3% group. In the control females yoked to the 6% group, one litter was euthanized post-natally due to poor health and another was lost due to a combination of neonatal death and cannibalization. Among pups surviving to weaning, there were no clear effects of condition on sex ratio or weight of pups.

Fig. 5. The percent of inseminated females that delivered litters after ingesting BPA via a peanut butter/powdered chow mixture as a modified diet on days 1-4 of gestation. One day following consumption, yoked controls were given the same amount of mixture consumed.

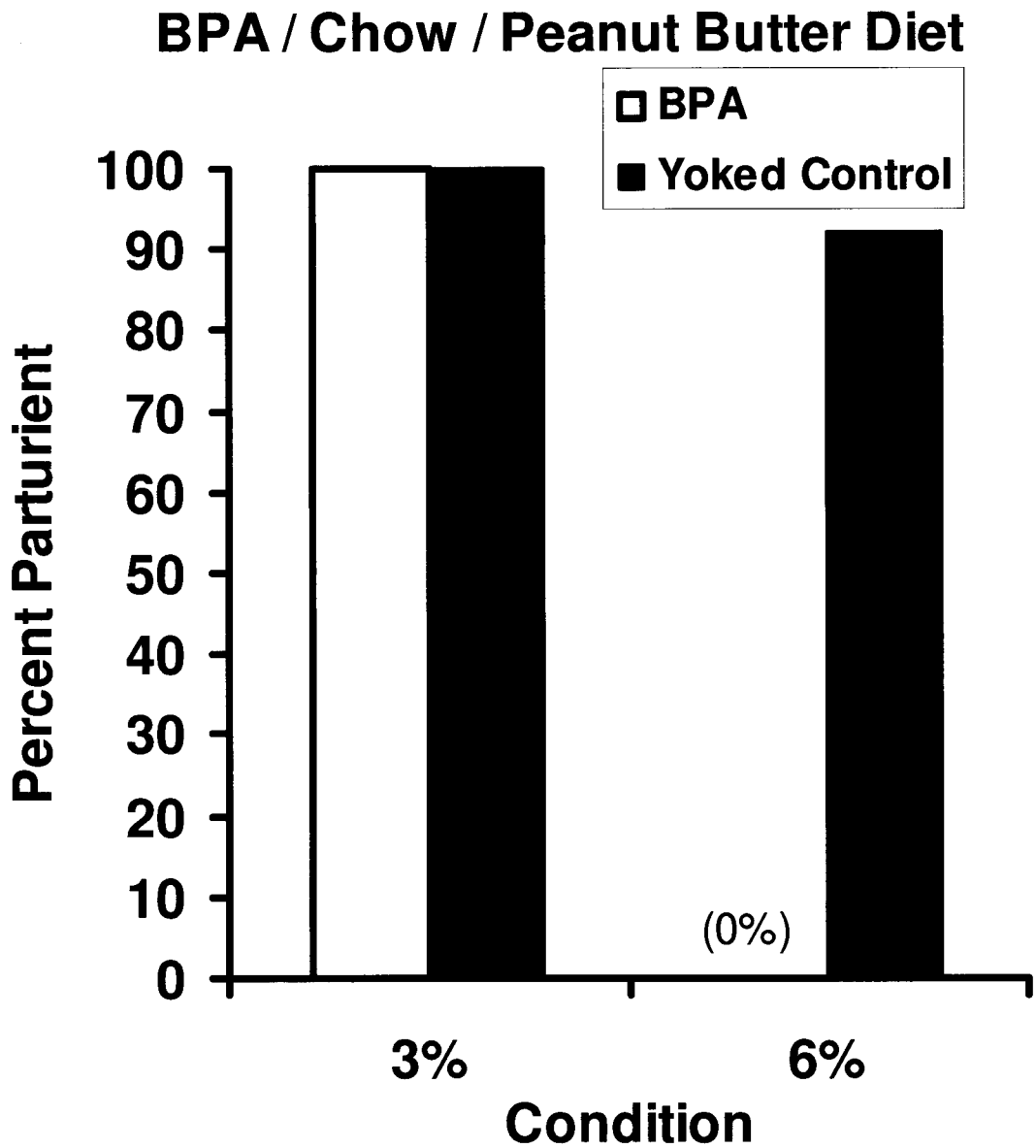


Table 3. Summary of data from the BPA / peanut butter / powdered chow alternative diet mixture. BPA was either 3% or 6% of the mixture. Each "yoked control" female was paired with a BPA subject, receiving the same amount of peanut butter / chow mixture as the BPA subject ate on the same day of gestation.

Condition	No. of Subjects	No. Parturient	Daily Food Intake (g)	Daily BPA Intake (mg)	No. of Pups at Birth	Weight/Pup at Weaning (g)	Sex Ratio (M:F)
3%	6	6	2.28±0.19	66.7±13.5	11.8±1.5	19.7±0.5	1.23
Control	6	6	2.28±0.19	0.00±0.00	13.3±0.9	19.8±0.4	1.22
6%	12	0	1.22±0.14	68.8±17.1	0.0±0.0	-	-
Control	12	11	1.22±0.14	0.00±0.00	11.6±1.4	18.4±0.3	0.98

4. Discussion

High doses of BPA by ingestion or injection during the period of implantation will terminate pregnancies in inseminated female mice. Subcutaneous injections resulted in a significant decrease in the average number of pups at 3.375 mg/day. At 10.125 mg/day, there was a significant reduction in the number of pregnancies, both when females were allowed to give birth and when sites of implantation were inspected via uterine histology on day 6 after insemination. Oral administration of BPA mixed in a 1 g peanut butter dietary supplement while normal chow was available did not have any significant effect on pregnancy. Mice reduced their intake of this supplement at 3% and even more so at 9% BPA. When normal chow was taken away and an altered diet provided during the period of administration, a complete block of pregnancies was observed with BPA concentrations at 6% of the diet, which was associated with an average consumption of 68.8 mg/day. There was some interaction of BPA ingestion and decreased food intake; females in the 3% BPA group remained pregnant after ingesting almost as much BPA as those in the 6% group. However, dietary restriction alone was not sufficient to disrupt pregnancy, as most control females gave birth despite identical food intake to that consumed by females in the 6% BPA group.

In comparison, administration of less than 0.15 μg/day of 17β-estradiol during the period of implantation significantly reduces the number of births in inseminated female mice [30-32]. Estrone and estriol have such effects commencing at about 9 μg/day, while androgens have weaker effects with impacts of doses in the order of 25 to 100 μg/day

[30,31]. When administered through subcutaneous injection, estradiol is approximately 20 to 60 thousand times more potent by mass than is BPA.

In mice, the time period during which the uterus is receptive to implantation of a blastocyst is very narrow, and the rate of transport of fertilized ova down the fallopian tube is critical [39]. Elevated plasma estradiol levels alter the speed at which fertilized ova travel through the fallopian tubes [40-44]. Exposure to high levels of BPA could have had a similar effect. BPA exposure may have also been influential at the level of the embryo. Elsewhere [25], exposure to low concentrations of BPA significantly increased developmental rates of embryos to blastocysts while high levels of exposure had the opposite effect. Areas in the hypothalamus also play a critical role in mediating hormonal levels during the period of implantation and may be influenced by BPA exposure. Small quantities of estradiol can influence the onset of estrus through receptors in the ventromedial hypothalamus [45], a process that may be mimicked by high levels of BPA exposure. BPA can increase the expression of progesterone receptor proteins in the ventromedial and preoptic areas of the hypothalamus [46]. An increase in progesterone receptor cells could significantly alter hypothalamic mechanisms and subsequently affect the onset of estrus and receptivity of the uterus [46].

Although BPA was given during the period of implantation and seemed to mimic the effects of exposure to estrogens, other mechanisms could have influenced the results. Some evidence indicates that BPA is anti-androgenic [47]; the potential impact of this upon implantation is unclear, as exogenous androgens' effects on implantation are generally assumed to be due to their metabolism to estrogens [33,48]. Adverse maternal

health effects of BPA administration that could not be readily observed may have had an impact. As a potential stressor, irritation observed around injection sites with the highest doses of subcutaneous BPA could have contributed, however many females were parturient despite signs of this irritation in the second and third highest doses. Although some post-implantation impact of BPA lingering in the dam's system cannot be entirely excluded at higher doses, results from the uterine histology experiment indicate that pregnancy disruption occurred during the period of implantation.

Standard chow, in both powder or pellet form, was used in all conditions and has high levels of soy as an ingredient. Phytoestrogens from the chow may have acted in either an additive or inhibitory manner with administered BPA. This interaction could potentially impact the dose response curve reported here. In addition, phytoestrogens or unknown contaminants in peanut butter could have impacted the lowest effective dose levels of BPA when ingested with the powdered chow mixture. Importantly, no adverse effects on litter size or number of parturient females were detected in any of the control subjects exposed to peanut butter and powdered chow. Subcutaneous administration disrupted pregnancy at substantially lower doses than did oral administration. This is to be expected as bioavailability of BPA is dependent on the route of administration [28,49].

Low oral and subcutaneous doses were investigated to address concerns about potential risks of BPA at this level [19,24,25]. Such low doses did not produce any significant effects. The lowest subcutaneous doses of BPA examined here are similar in concentration to the minimal doses of exogenous estradiol necessary to disrupt implantation [30-32]. It would seem unlikely that BPA would have effects at doses

below those required for the most powerful estrogen. Estrogens and androgens impact implantation with a monotonic dose-response; doses above the threshold dose tend to disrupt implantation completely [30-32]. The amount of BPA required to disrupt pregnancies consistently in mice through both oral and subcutaneous administration was much higher than that typically found in the environment [26,28]. It remains to be determined whether lower doses of BPA could have additive or synergistic effects with other environmental estrogens.

Acknowledgments

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Chapter 3

Impact of acute bisphenol-A exposure upon intrauterine implantation of fertilized ova and urinary levels of progesterone and 17β-estradiol

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Robert G. Berger: Concept, formation of experimental design, data collection, literature review, data analysis and manuscript writing.

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Denys deCatanzaro: Assistance with concept development, data analysis and manuscript editing.

Additional Support

Ayesha Khan: Assisted with ano-genital distance measurements and enzyme-linked immunoassay procedures.

Undergraduate Students: Adam Guzzo and Elaine Lewis provided assistance with experimental procedures.

1. Introduction

Bisphenol-A (BPA) is established to have estrogenic properties both *in vitro* and *in vivo* [1-3]. BPA, used primarily in polycarbonate plastics and epoxy resins, has been shown to leach into surrounding media due to incomplete polymerization, exposure to heat, and contact with acidic or basic compounds [2,4]. Produced at a rate exceeding 3 billion kilograms per year [5], BPA has been detected, for example, in human urine [6], saliva [7], and placental tissue [8]. Exposure to BPA is considerable in both humans and wildlife, carrying potential impacts on reproductive and developmental health [9-11]. Embryonic and pubertal development [12-14], weight of prostate glands [15], and reproductive tract and organ development [16,17] have all been altered in laboratory animals exposed to BPA. Many such effects have been found at low, ecologically-relevant doses [*e.g.* 15,18,19], although some researchers have failed to replicate these findings [20,21], resulting in calls for further investigation of low dose effects [22,23].

Intrauterine implantation of fertilized ova is extremely sensitive to exogenous estrogens [24]. Administration of as little as 140 ng 17β-estradiol to an inseminated female mouse during the window of implantation can terminate pregnancy [25,26]. Subcutaneous and oral administration of BPA during early gestation can similarly terminate gestation or reduce litter size [27], however in concentrations approximately 20 to 60 thousand times greater than those of 17β-estradiol [25,27]. Although this suggests that BPA disrupts implantation by binding to estrogen receptors, endocrine responses to BPA exposure can be diverse and complex [28]. For example, BPA can affect expression of progesterone receptor proteins in the hypothalamus [29], act as an androgen antagonist

at the receptor [30], increase testosterone levels [31], and stimulate release of prolactin [32].

The current study was designed to delineate the minimal dose necessary to disrupt early pregnancy, determine the impact of a single exposure, clarify whether effects are on implantation *per se*, and determine whether steroid levels are altered by BPA exposure. Intrauterine implantation sites were measured directly, unlike previous measures of litter size [27]. This was designed to preclude post-implantation effects, given evidence that BPA can affect embryo survival and formation of the placenta [33] as well as post-implantation reabsorption and fetal death [34]. We also measured unconjugated estradiol and progesterone in exposed and control animals, using enzyme immunoassays on non-invasively collected urine samples which have been shown largely to reflect systemic trends in female mice [35-37]. Previous investigations [31,38,39] of BPA-induced hormonal responses primarily focused on exposure *in utero* and during development, but typically utilized invasive measures and have not examined acute impacts during early pregnancy. Finally, we related the impact of BPA to variation among inseminated females in ano-genital distance, an established reflection of differential exposure to sex hormones *in utero* [40-42] and a factor that can influence sensitivity to BPA [13,43] and alter urinary hormone levels [44].

2. Material and Methods

2.1 Animals and Housing

CF-1 mice were of stock from Charles River Breeding Farms of Canada (La Prairie, Quebec). During the exposure period, subjects were individually housed in a urine collection apparatus measuring 15x21x13 (height) cm. These were constructed of clear Plexiglas with a stainless steel wire-grid floor with open squares measuring 0.5 cm^2, raised approximately 1 cm above a clean flat Teflon coated stainless-steel surface. Wire tops allowed continuous access to food (8640 Teklad Certified Rodent chow; Harlan Teklad, Madison, Wisconsin) and water. Colony rooms were maintained at 21°C with a reversed 14h light:10h dark cycle. Subjects were sexually naïve female mice aged 3-6 months with an average weight of 35 g at the commencement of experimental procedures. The health of all animals was closely monitored throughout the experiments. This research was approved by the McMaster University Animal Research Ethics Board, conforming to standards of the Canadian Council on Animal Care.

2.2 AGDI Measurements

Prior to commencement of the experiment, an ano-genital distance index (AGDI) was generated for each female. Individual ano-genital distance (mm) was determined using a Mastercraft digital calliper to measure the distance between the base of the genital papilla and proximal end of the anal opening. AGDI was calculated by dividing the ano-genital distance (mm) by body mass (g) and multiplying the resultant value by 100 [40]. Care was taken to ensure that the ano-genital region was neither stretched nor compressed

during measurements. All indices were calculated by experimenters well trained in animal handling. Females were then immediately isolated in the collection apparatus for a one week adaptation period and left undisturbed until mating.

2.3 Mating and Pregnancy Outcome

Following the adaptation period, females were removed from the collection apparatus and randomly paired with a CF-1 male aged 5-12 months in standard polypropylene 28x16x11 (height) cm cages with continuous access to food and water. Female hindquarters were subsequently inspected three times daily during the dark phase of the light cycle for the presence of a vaginal sperm plug. The day of sperm plug detection was designated as day 0 of pregnancy. Females with vaginal plugs were pseudo-randomly assigned to one of the experimental or control conditions. Age and weight were counterbalanced across all conditions. On day 1 of pregnancy, females were returned to the same collection cage they inhabited prior to mating, with exception of the day 0 single dose groups which were isolated following sperm plug detection. Pregnancy outcome was measured on day 6 of gestation at 3-6 h after commencement of the dark phase of the light cycle. Females were sacrificed by CO_2 asphyxiation, uteri were excised via an abdominal incision, and the number of implantation sites was counted. An implantation site was operationally defined as a clear, discrete protuberance in either horn of the uterus, which is otherwise smooth and uninterrupted.

2.4 BPA Administration

In Experiment 1, subcutaneous injections of BPA (97%, Sigma-Aldrich) dissolved in peanut oil were administered on days 1 through 4 of pregnancy. Females were assigned to doses of 0, 0.0005, 0.0045, 0.05, 0.125, 1.125, 3.375, 6.75, and 10.125 mg BPA/animal/day, approximately 0, 0.01, 0.1, 1.5, 3.5, 30, 100, 200 and 300 mg BPA/kg body weight, with sample sizes of 46, 11, 3, 10, 3, 4, 12, 6, and 9 respectively. Doses were selected from those used previously and chosen to provide a wide range of BPA exposure, including doses known to disrupt pregnancy partially or completely [27]. The volume of peanut oil vehicle varied due to solubility constraints of BPA; doses from 0.0005 to 0.0.125 mg were dissolved in 0.05 ml, 1.125 mg in 0.15 ml, 3.375 in 0.3 ml, and 6.75 to 10.125 mg in 0.45 ml peanut oil. Proportional numbers of control subjects were run at these volumes of peanut oil. To minimize the development of skin irritation, each injection was administered at a different site, including the left and right flanks, middle back area, and scruff of the neck. Both control and experimental subjects were given injections in identical locations on the same day of gestation. All injections were administered 3-5 h into the dark cycle.

In Experiment 2, a single subcutaneous injection of BPA (97%, Sigma-Aldrich) dissolved in peanut oil was administered on day 0, day 1, or day 2 of pregnancy 3-5 h after commencement of the dark phase of the lighting cycle. Females were assigned to doses of 0, 6.75, and 10.125 mg BPA/animal/day. Sample sizes were 9, 9 and 8 for day 0; 8, 7 and 8 for day 1; and 8, 9 and 10 for day 2. These doses were selected through analysis of hormonal results from Experiment 1 which indicated a significant impact of

BPA exposure following a single administration. Day 0 administrations were given immediately following sperm plug detection, counterbalancing for time across groups. All injections were administered at the scruff of the neck.

2.6 Urine Collection

Samples were collected following the commencement of the dark phase of the light cycle, when mice generally deposit pools of urine [35-37]. Collections occurred on days 2 through 5 of pregnancy, with the exception of the day 0 single administration group where collections occurred on days 1 through 5. To collect urine samples, the apparatus was gently placed on a clean surface covered with wax paper at the beginning of the collection period. Urine was aspirated from deposited pools with a 1 ml syringe and 25-gauge needle. Caution was used to ensure that urine was not contaminated with feces, food, or water. The 4 h collection period commenced 30 min prior to the start of the dark phase of the light cycle. Samples were stored in coded 1.5 ml micro-tubes and frozen immediately at -20°C until chemical analyses were performed simultaneously for all samples at a later date.

2.7 Urinary Hormonal Analysis – Assay Procedures

Enzyme immunoassay procedures generally followed those described for other mammals [45]. Validations for this procedure in adult female mice were previously reported from this laboratory [35,36]. Creatinine, 17β-estradiol, and progesterone were obtained from Sigma Chemical Co. (St Louis, MO). Antibodies to 17β-estradiol and

progesterone and corresponding horseradish peroxidase conjugates were obtained from the Department of Population Health and Reproduction at the University of California (Davis). Nunc Maxisorb plates were first coated with 50 µl of antibody stock diluted at 1:10 000 in a coating buffer (50 mmol bicarbonate buffer l^{-1}, pH 9.6) and stored for 12–14 h at 4°C. Wash solution (0.15 mol NaCl l^{-1} containing 0.5 ml of Tween 20 l^{-1}) was added five times to each well using an automated strip washer (Bio-Tek Instruments Inc. model Elx50) to rinse away any unbound antibody. Immediately, 50 µl phosphate buffer per well was added to each well. The plates were incubated at room temperature (21°C) for 2 h for estradiol determination and 1 h for progesterone determination before adding standards, samples, or controls.

Urine samples were diluted 1:8 in phosphate buffer before they were added to the plate. Standard curves were derived by serial dilution from a known stock solution. Estradiol stock (2000 pg ml^{-1}) was first diluted 1:3 and then serially diluted, yielding values of 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9, 1.95, 0.98, 0.46 and 0.23 pg ml^{-1}. Progesterone standard (5000 pg ml^{-1}) stock was serially diluted to 2500, 1250, 650, 325, 162.5, 81.25, 40.63, 20.31, 10.16, 5.08, 2.54, 1.27, and 0.63. For all assays, 50 µl estradiol or progesterone horseradish peroxidase was added to each well, with 20 µl of standard, sample, or control for estradiol or 50 µl of standard, sample, or control for progesterone. All samples were run in duplicate. The plates were incubated for 2 h at room temperature. Subsequently, plates were washed and 100 µl of a substrate solution of citrate buffer, H_2O_2 and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) were added to each well and the plates were covered and incubated while shaking at room

temperature for approximately 30 to 60 min. The plates were then read with a single filter at 405 nm on the microplate reader (Bio-Tek Instruments Inc., model Elx 808). In all assays, absorbances were obtained, standard curves were generated, a regression line was fit to the most sensitive range of the standard curve (typically 40–60% binding) and samples were interpolated into the equation to obtain a value in pg per well.

Following convention [*e.g.* 35,36], the concentration of urine in samples was adjusted for creatinine to compensate for variations in fluid intake and output. Standard creatinine values of 100, 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 μg ml^{-1} were used. Urine sample were diluted to 1:36.34 urine: phosphate buffer (0.1 mol l^{-1} sodium phosphate buffer, pH 7.0 containing 8.7 g of NaCl and 1 g of BSA per litre). Dynatech Immulon flat bottom plates were used and 50 μl per well of standard was added together with a solution of 50 μl distilled water, 50 μl 0.75 mol l^{-1} NaOH and 50 μl 0.4 mol l^{-1} picric acid. The plates were shaken and incubated at room temperature for 30 min, then measured for absorbance on a plate reader with a single filter at 490 nm. Standard curves were generated, regression lines were fit, and the regression equation was applied to the absorbency for each sample. Steroid measurements were divided by creatinine values per ml of urine.

2.8 Statistical Analysis

In Experiment 1, one-way analysis of variance was conducted comparing the number of implantation sites to condition. This was followed by Newman-Keuls multiple comparisons. AGDI was related to number of implantation sites and urinary steroid

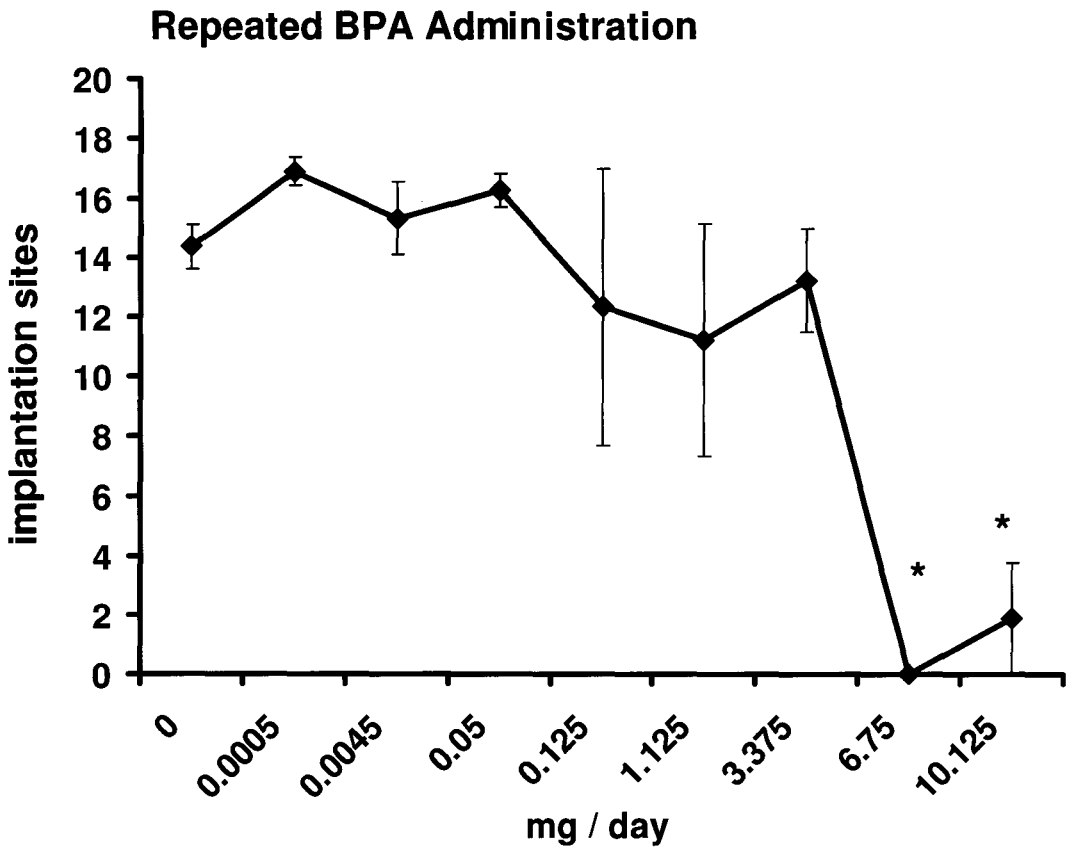
levels via Pearson product-moment correlations with associated t-tests to determine significance. For urinary steroid and creatinine measures, a two-way factorial analysis of variance was conducted, treating condition as between-subjects and repeated daily measure as within-subjects. The unweighted-means method was used due to unequal sample sizes among conditions. In Experiment 2, planned orthogonal t-tests were conducted comparing each dose with the control (0 mg BPA) dose for the respective day. A two-way factorial analysis of variance was conducted for urinary steroid and creatinine measures, treating condition as between-subjects and repeated daily measure as within-subjects.

3. Results

3.1 Experiment 1

There were no significant differences among groups of control subjects receiving different quantities of vehicle; therefore these were collapsed in subsequent statistical analysis. The average number of implantation sites found in each of the conditions on day 6 of pregnancy is shown in Figure 1. A clear decrease was observed in both the 6.75 and 10.125 mg/day groups; in the former dose no animals showed any implantation sites, while only one animal did at the 10.125 mg dose. Analysis of variance on the number of implantation sites was significant, $F(8, 94) = 13.49$, $p < 0.0001$. Multiple comparisons showed that the 6.75 and 10.125 mg/day conditions were significantly different from all others ($p < 0.01$). No other significant differences were observed.

Fig. 1. The mean (±S.E.) number of implantation sites in inseminated females after subcutaneous injections of bisphenol-A (BPA) on days 1-4 of gestation. * Denotes significant differences from control condition ($p < 0.01$).



When AGDI was related to number of implantation sites, with all groups collapsed excluding the 6.75 and 10.125 doses that significantly impacted implantation, there was a significant negative correlation, r (101) = -0.33, $p < 0.001$. A test of this relationship between AGDI and the number of implantation sites among controls alone approached the conventional significance level in two-tailed probability, r (43) = -0.24, $p = 0.0523$.

Animals exposed to BPA levels 3.375, 6.75 and 10.125 mg/day showed some irritation around the site of injection, typically on days 4-6 of pregnancy. This appeared to be due to excessive grooming of the area surrounding the injection site. No such irritation was observed at lower doses or in controls and animals appeared healthy otherwise.

Urinary creatinine levels of experimental and control females are presented in Table 1. Analysis of variance showed a significant main effect of condition, $F(8,94) = 2.19$, $p = 0.0344$, and day, $F(3,282) = 3.35$, $p = 0.0192$, with no significant interaction. The highest dose group, 10.125 mg BPA, had the lowest creatinine measurements on days 2 and 4 of pregnancy. Mean creatinine-adjusted progesterone levels across days 2-5 of pregnancy are shown in Table 2. Figure 2 shows that progesterone levels of the 10.125 mg BPA/day group are clearly lower when compared to controls. Analysis of variance indicated a significant difference between groups, $F(8,94) = 4.80$, $p = 0.0002$, and an effect of day, $F(3,282) = 41.96$, $p < 0.0001$. Similar trends were observed when non-adjusted progesterone was examined. The daily mean values for creatinine-adjusted urinary 17β-estradiol for all groups are shown in Table 3. No significant differences in

mean estradiol levels were found between groups. An effect of day was present, $F(3,282) = 7.17$, $p = 0.0003$.

Fig. 2. Mean (±S.E.) urinary progesterone levels (ng/mg creatinine) for days 2-5 of gestation in inseminated females administered 0 or 10.125 mg bisphenol-A (BPA) on days 1-4. * Denotes significant differences between groups ($p < 0.0005$).

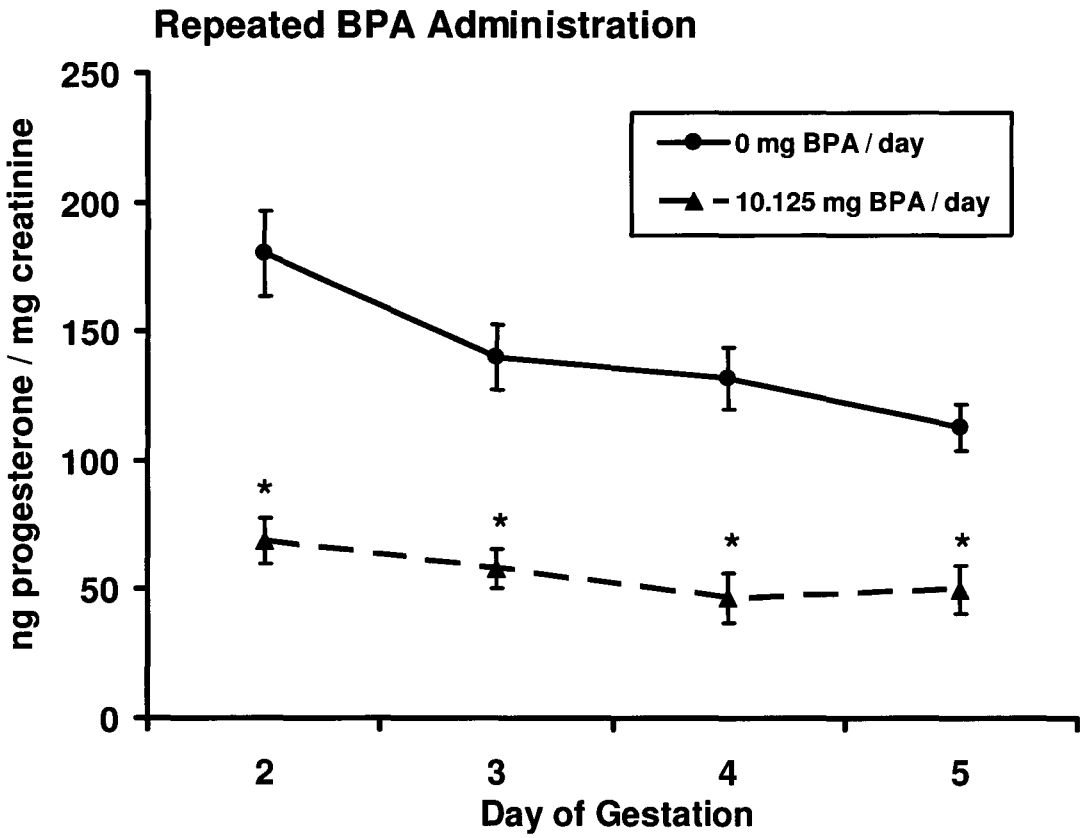


Table 1. Mean (±S.E.) urinary creatinine values (mg/ml) for days 2-5 of gestation in inseminated females administered repeated doses of BPA (mg/day) on days 1-4.

Day\Dose	0	0.0005	0.0045	0.05	0.125	1.125	3.375	6.75	10.125
2	0.49±0.02	0.54±0.03	0.43±0.08	0.47±0.03	0.37±0.05	0.42±0.04	0.49±0.03	0.38±0.04	0.35±0.04
3	0.49±0.02	0.55±0.03	0.38±0.06	0.49±0.04	0.39±0.03	0.46±0.04	0.46±0.04	0.36±0.04	0.41±0.04
4	0.42±0.02	0.56±0.02	0.37±0.04	0.44±0.03	0.35±0.02	0.43±0.02	0.40±0.03	0.42±0.02	0.28±0.02
5	0.49±0.02	0.58±0.03	0.44±0.06	0.51±0.03	0.29±0.03	0.49±0.03	0.46±0.03	0.43±0.03	0.44±0.03

Table 2. Mean (±S.E.) urinary progesterone values (ng/mg creatinine) for days 2-5 of gestation in inseminated females administered repeated doses of BPA (mg/day) on days 1-4.

Day\Dose	0	0.0005	0.0045	0.05	0.125	1.125	3.375	6.75
2	180±16	132±16	308±143	165±43	452±197	297±58	189±22	353±33
3	140±13	111±11	184±46	157±29	233±106	189±19	171±27	233±50
4	132±12	112±13	231±59	126±22	222±71	191±40	177±37	180±33
5	113±9	109±10	204±37	109±30	157±67	152±33	119±14	162±28

Table 3. Mean (±S.E.) urinary 17β-estradiol values (ng/mg creatinine) for days 2-5 of gestation in inseminated females administered repeated doses of BPA (mg/day) on days 1-4.

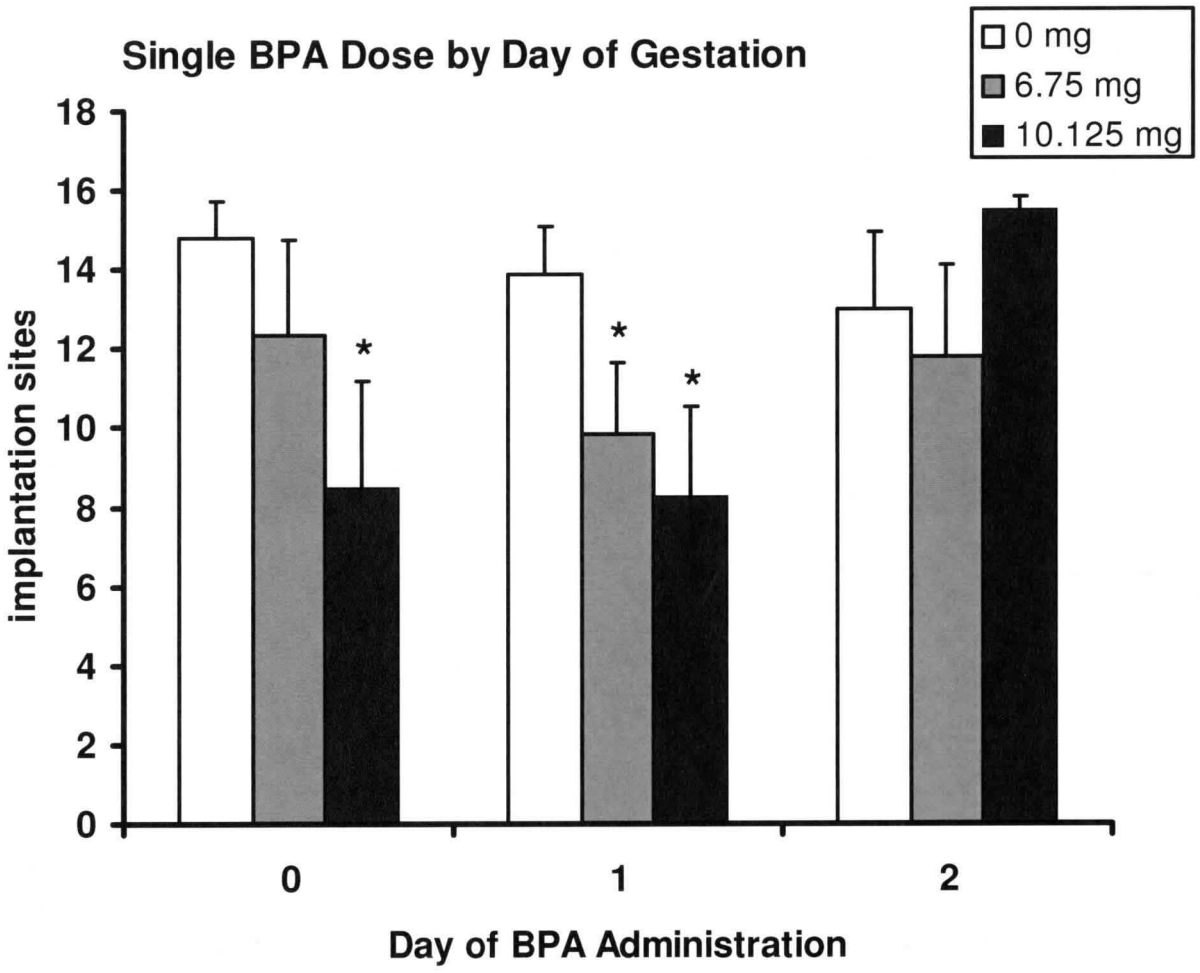
Day\Dose	0	0.0005	0.0045	0.05	0.125	1.125	3.375	6.75	10.125
2	14.0±0.7	11.7±0.6	13.0±0.3	13.0±1.1	11.2±1.9	13.7±0.7	15.8±1.4	14.2±2.8	11.8±0.7
3	13.5±0.8	12.0±0.6	13.0±1.6	14.6±1.6	10.8±1.0	11.2±0.9	16.4±1.5	16.4±2.8	11.6±0.6
4	14.5±1.0	12.4±0.9	12.9±0.9	14.2±1.3	11.2±2.3	12.1±1.0	15.2±0.6	14.5±2.3	13.1±0.6
5	11.5±0.8	11.2±0.5	10.7±0.9	11.1±0.9	11.7±0.2	11.0±1.7	13.4±0.9	12.1±2.8	8.9±0.7

There was a significant negative correlation between the creatinine-adjusted progesterone level across groups and AGDI on day 2, r(101) = -0.234, $p = 0.017$, day 3, $r(101) = -0.249$, $p = 0.011$, and day 4, $r(101) = -0.322$, $p = 0.013$ of pregnancy. This negative correlation was also observed when progesterone and AGDI values of control subjects only were analyzed: day 2, $r(43) = -0.389$, $p = 0.008$; day 3, $r(43) = -0.426$, $p = 0.004$; day 4, $r(43) = -0.456$, $p = 0.002$; and day 5, $r(43) = -0.339$, $p = 0.022$. No significant correlations were found between estradiol levels and AGDI.

3.2 Experiment 2

Implantation sites for all conditions are reported in Figure 3. In those treated on day 0 of gestation, there was a significant decrease in the number of sites following a single administration of 10.125 mg BPA, $t(14) = 1.98$, $p = 0.034$. Among those treated on day 1, both the 6.75 mg, $t(13) = 2.05$, $p = 0.030$, and the 10.125 mg, $t(14) = 2.20$, $p = 0.022$, groups showed a significant decrease in number of sites. There was no significant effect of single BPA doses given on day 2 of pregnancy.

Fig. 3. The mean (±S.E.) number of implantation sites in inseminated females after a single subcutaneous injection of varied doses of bisphenol-A (BPA) on day 0, 1, or 2 of gestation. * Denotes significant differences from control condition ($p < 0.05$).



Among animals treated with a single dose on day 0 of gestation (see Table 4), creatinine levels showed a significant effect of day, $F(4,92) = 2.86$, $p = 0.027$, but no other significant effects. Analysis of variance comparing creatinine-adjusted progesterone measures among all three doses showed only a significant effect of day of measurement, $F(4,92) = 7.28$, $p = 0.0001$. However, when just the high dose (10.125 mg) was compared to controls, significant effects of condition, $F(1,15) = 5.84$, $p = 0.027$, and day, $F(4,60) = 8.77$, $p < 0.0001$, were observed. There were no significant differences in creatinine-adjusted urinary estradiol levels in animals treated on day 0.

Among animals treated on day 1 (see Table 4), urinary creatinine levels showed a significant effect of day, $F(3,63) = 10.01$, $p < 0.0001$, and there was a significant interaction between day and group, $F(6,63) = 3.95$, $p = 0.0023$. In the creatinine-adjusted progesterone values, there were no significant differences observed among groups; however there was a slight trend for decreasing values on days 3 and 4 in animals administered 10.125 mg BPA/day. In addition, there was a significant effect of day, $F(3,63) = 13.28$, $p < 0.0001$, but no interaction. A significant effect of day was also seen in the creatinine-adjusted estradiol levels, $F(3,63) = 8.46$, $p = 0.0002$, but no effect of group nor any interaction.

Among animals treated on day 2 (see Table 4), urinary creatinine levels did not show a significant effect of group but did show a significant effect of day, $F(3,72) = 4.03$, $p = 0.0106$. Analysis of variance showed no significant impact of BPA administration on mean adjusted progesterone or estradiol levels. A significant effect of day was observed in creatinine-adjusted progesterone values, $F(3,72) = 6.75$, $p = 0.0007$.

Table 4. Mean (±S.E.) urinary creatinine (mg/ml), 17β-estradiol (ng/mg creatinine) and progesterone values (ng/mg creatinine) for days 2-5 of gestation in inseminated females exposed to a single dose of BPA (mg/day) on day 0, 1 or 2.

		Creatinine			Progesterone			17β-Estradiol		
Day\Dose		0	6.75	10.125	0	6.75	10.125	0	6.75	10.125
	1	0.50±0.05	0.51±0.07	0.56±0.09	131±27	135±40	229±52	17.4±3.1	21.1±2.6	23.9±4.2
Day 0	2	0.40±0.04	0.47±0.07	0.47±0.05	68±11	108±23	140±24	17.8±2.0	19.0±2.2	18.0±2.0
Exposed	3	0.40±0.06	0.50±0.08	0.44±0.07	81±17	89±23	125±33	21.8±3.6	15.7±1.4	18.8±2.7
	4	0.45±0.07	0.42±0.07	0.43±0.05	80±13	114±28	133±25	17.1±1.4	17.8±2.4	18.2±2.2
	5	0.47±0.05	0.45±0.06	0.42±0.05	78±17	116±26	104±16	17.6±2.1	18.0±2.2	18.1±1.4
Day 1	2	0.36±0.03	0.45±0.03	0.48±0.04	221±41	237±73	210±43	18.4±0.9	26.6±3.3	19.5±1.1
Exposed	3	0.41±0.02	0.38±0.03	0.36±0.03	168±23	115±16	118±22	17.3±1.2	20.5±2.7	20.7±2.1
	4	0.38±0.03	0.38±0.03	0.33±0.02	183±27	121±30	93±14	18.1±0.6	21.6±2.1	19.5±1.5
	5	0.42±0.03	0.45±0.03	0.44±0.02	142±22	81±8	89±11	14.7±0.8	17.5±2.2	16.8±1.3
Day 2	2	0.36±0.02	0.44±0.04	0.37±0.03	132±23	93±12	272±99	12.5±1.0	13.1±0.9	16.2±2.9
Exposed	3	0.31±0.03	0.42±0.04	0.36±0.02	142±32	105±15	203±58	12.9±1.3	13.5±0.6	19.1±2.8
	4	0.32±0.03	0.37± 0.02	0.35±0.02	75±7	69±7	168±41	13.0±1.0	13.5±0.9	17.2±2.0
	5	0.40±0.05	0.42±0.03	0.43±0.04	72±12	56±7	101±21	9.9±0.7	10.8±0.5	11.4±1.9

4. Discussion

These data demonstrate a lower threshold for BPA-induced pregnancy disruption than previously reported [27]. In Experiment 1, no females given 6.75 mg BPA/day on days 1 through 4 of gestation showed any signs of blastocyst implantation. Moreover in Experiment 2, inseminated female mice exposed to a single injection of 10.125 mg BPA on the day of, or 6.75 mg BPA one day following insemination, showed a significant decrease in the number of implantation sites. In the present study, intrauterine implantation sites were counted directly, indicating that previously observed reductions in litter size in response to BPA [27] are mediated by a disruption of intrauterine blastocyst implantation rather than post-implantation effects.

Our results also indicate that BPA exposure around the time of intrauterine implantation can significantly influence maternal urinary excretion of progesterone. A decrease in urinary progesterone levels seen from day 2 through 5 of pregnancy coincided with a disruption in implantation in the highest repeated dose group. Previous studies [36,37] have shown that urinary progesterone levels typically rise in female mice following insemination and are sustained at high levels through pregnancy while gradually declining in the latter half of gestation. Increased progesterone is well established to be associated with the maintenance of pregnancy [46]. However, in the 6.75 mg repeated-administration subjects, progesterone levels were maintained despite the absence of implantation sites, indicating that BPA can disrupt implantation independently of its impact on progesterone levels. Reductions in the number of implantation sites without a corresponding drop in progesterone levels were also observed

in response to single doses of BPA given on days 0 and 1 of gestation. Indeed, there was an enhancement of progesterone levels in the high single-dose animals treated with BPA on day 0 of pregnancy. This increase in progesterone could be due to influences of BPA on the negative feedback regulation of gonadotropins [47] or effects upon progesterone production at the granulosa cells [48].

Urinary estradiol levels were not significantly impacted by any single or multiple dose of BPA. In normal mouse pregnancy, urinary 17β-estradiol levels are less dynamic than progesterone in early gestation but show a spike at day 7 [37], well after implantation. BPA is known to pass through the blood-brain barrier and to influence areas of the hypothalamus [27,49,50] and hormone production [31,32,38]. Although the present analysis of BPA-exposed inseminated mice showed a significant impact on urinary progesterone levels, it did not indicate a clear, dose-dependent response of urinary progesterone and estradiol.

Results from single dose administrations indicate the impact of subcutaneous BPA on implantation is not immediate. Implantation of blastocysts typically occurs on day 3 of pregnancy [51,52]. Single doses of BPA administered on days 0 and 1 had a significant influence; however exposure on day 2 was too late to have an impact. Day 1 administration proved to be the most effective, with a dose of 6.75 mg significantly reducing the number of implantation sites, the lowest dose at which we observed an effect in any experiment. Estrogens help prepare the uterus for implantation [51], but implantation itself is vulnerable to small rises in estrogens [25,26]. Timing of estrogenic activity is critical. In mice, the period of uterine receptivity to blastocyst implantation is

very narrow [52] and minute increases of estradiol can influence the receptiveness of the uterus [53]. Estrogens are also among the critical factors affecting the rate of passage of fertilized ova through the fallopian tubes [51,54-56]. Exposure to high levels of BPA could, through estrogenic action, interfere with the normal rate of passage. BPA exposure may have also been influential at the level of the embryo. Low concentrations of BPA can increase the developmental rates of embryos to blastocysts, while high levels of exposure can have the opposite effect [57].

AGDI did not appear to play a major role in the susceptibility of females to BPA exposure [*cf.* 13,43]. When data from all groups were collapsed, excluding those groups where BPA influenced implantation, AGDI was negatively correlated with the overall number of implantation sites. Previous investigations have shown that female mice exposed to lower levels of testosterone *in utero*, which correlates to smaller AGDI [40], produce more young in their first litter [58]. This trend was not disrupted by exposure to BPA. In addition, exposure to BPA did not influence the significant negative correlation found between AGDI and progesterone levels. This relationship was observed across all females on days 2 through 4 of pregnancy and in controls alone on all four days of collections.

It is clear that intrauterine implantation is far less sensitive to BPA exposure than are many other parameters, especially those impacted by BPA exposure during prenatal and early postnatal development [*e.g.* 13,59-62]. The requirement for relatively high doses of BPA may be explained by recent evidence indicating a metabolic barrier against BPA in adult rat uterine endometrium [63]. When the rat uterus was perfused with BPA,

most of the BPA was glucuronidated in the epithelium, resulting in transport of glucuronides to the serosal side of the uterus, while a small fraction of BPA was unmodified and transported to the mucosal side of the uterus [63].

The results from the current investigation demonstrate that BPA, a chemical found in numerous consumer goods, can disrupt intrauterine implantation following single or multiple administrations. BPA-induced pregnancy disruption requires exposure to levels that are approximately 12 thousand times those for 17β-estradiol, 225 times those of estrone or estriol, or 75 times those of testosterone [*cf.* 25]. This is consistent with published estimates of the relative affinity of BPA for estrogen receptors [64]. We studied a wide range of BPA doses in order to address concerns regarding the possibility of low, ecologically relevant dose effects. Although the dose levels necessary for adverse effects may require quantities above the estimated range of environmental exposure [15,18,19], a single high-dose exposure can clearly alter natural processes involved in implantation.

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Chapter 4

Bisphenol-A exposure during the period of blastocyst implantation alters uterine morphology and perturbs measures of estrogen and progesterone receptor expression in mice

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Undergraduate Student: Nazanin Rajabi provided assistance with experimental procedures.

1. Introduction

Bisphenol-A (BPA) is the monomer used in the production of polycarbonate plastics and some epoxy resins. Produced at a rate exceeding 3 billion kilograms per year [1], BPA can be found in several household goods such as reusable food containers, polyvinyl chloride stretch films, and water bottles. It can leach into surrounding media, including food products, due to incomplete polymerization and exposure to heat, acidic, or alkaline conditions [2-4]. BPA has been detected in human urine [5,6], umbilical cord blood [7], colostrum [8], and breast milk [9,10]. Both *in vitro* and *in vivo* investigations have shown that BPA has estrogenic properties [11-13].

In laboratory animals, exposure to 25 ng BPA/kg /day can influence fertility [14,15] while doses ranging from 5 to 300 mg/kg/day can alter hormonal output [16-18]. BPA exposure at doses as low as 2.4 μg/kg has also been shown to alter embryonic and pubertal development [19-21] and sexually-dimorphic development of brain and behavior [22,23]. Several studies suggest that environmentally-relevant doses of BPA at or below 5-50 μg/kg can have estrogenic effects [15,20,24], whereas other investigations failed to find such evidence [13,25-28,29]. Due to these inconsistencies, the influence of BPA exposure *in vivo* has been under continued scientific scrutiny [30-32].

Previous investigations in our laboratory have shown that BPA exposure during the sensitive period of blastocyst implantation will disrupt pregnancy [13]. Dietary or subcutaneous BPA on days 1-4 of gestation, at dose levels of 68 and 10.125 mg/animal/day respectively, significantly decreased litter size and the percentage of females that gave birth [13]. Daily subcutaneous doses of 6.75 mg/animal (approximately

200 mg/kg) reduced the number of uterine implantation sites, whereas a single injection of 6.75 mg/animal on day 0 or 10.125 mg BPA on day 1 of gestation was sufficient to disrupt implantation [18]. Exposure to 10.125 mg (approximately 300 mg/kg/day) on gestational days 1-4 was also found to reduce urinary progesterone levels [18]. Lower, environmentally relevant doses did not affect pregnancy, implantation, or hormonal output [13,18]. These results resemble the disruption of early pregnancy by low doses of exogenous estrogens. Subcutaneous injections of as little as 37 ng/animal/day 17β-estradiol, and somewhat higher doses of other estrogens and androgens, can terminate gestation [33-35]. Administration of BPA beyond the implantation period until day 7 of pregnancy can also disrupt placental functions and reduce the number of embryos, uterine weight, and offspring survival [36,37].

Previous investigations have indicated that BPA can alter uterine morphology. Proliferation in uterine epithelial cells and increased luminal epithelial height have been observed in ovariectomized rats following injections of BPA [38,39]. In intact rats, proliferation of uterine stromal cells was seen following four days of 500 mg/kg [40]. BPA can also influence estrogen-sensitive steroid receptor and protein expression. In female mice exposed to BPA *in utero*, increases have been observed in expression of estrogen receptor alpha (ERα) and progesterone receptor (PR) in the uterine luminal epithelium of the endometrium and subepithelial stroma [41]. Furthermore, BPA has been shown to influence uterine expression of lactoferrin (Lf) [42]. Lf is an estrogen-sensitive glycoprotein that is present in the mouse uterus, the expression of which fluctuates over the estrous cycle and the period of blastocyst implantation [43-45]. In

response to 75 mg BPA/kg/day over a three day period, Lf expression increased by over 300% in immature female mice [42]. However, elsewhere, the expression of Lf was undetectable in the uterus after three days of 100 mg BPA/kg/day in immature female mice, whereas other xenoestrogens including DES, methoxychlor, and α-zearalanol, significantly increased Lf expression [46]. The response of Lf to BPA exposure therefore remains unclear and calls for further investigation.

The influences of BPA upon uterine morphology and steroid receptor expression have not previously been investigated during early pregnancy around the time of blastocyst implantation. Although estrogenic action of BPA could readily explain its impacts on implantation, it remains conceivable that other specific or nonspecific actions of BPA could be responsible, especially given that diverse stressors can disrupt implantation [*reviewed in* 47]. The current study was designed to examine cellular and receptor mechanisms through which BPA might interfere with intrauterine implantation, including indicators of estrogenic activity. We examined uterine morphology and estrogen-sensitive steroid receptor and protein expression following BPA exposure around blastocyst implantation. Uterine luminal area and cell height, number of ovarian corpora lutea, and the expression of ERα, Lf, and PR were analyzed. Subcutaneous injections on days 1-4 of gestation were used in doses known to influence litter size, blastocyst implantation, and urinary progesterone excretion [13,18].

2. Methods

2.1 Animals and mating procedures

CF-1 mice were of stock originating from Charles River Breeding Farms of Canada (La Prairie, Quebec). Animals were housed in standard 28 cm x 16 cm x 11 cm (height) polypropylene cages with wire-grid tops. Continuous access was provided to water and food (8640 Teklad Certified Rodent chow; Harlan Teklad, Madison, Wisconsin). Glass water bottles were used to decrease inadvertent BPA exposure. Rooms were maintained at 21°C with a reversed 14-h light:10-h dark cycle. Sexually naïve female mice aged 3-5 months with an average weight of 35 g were each randomly paired with a CF-1 male aged 5-10 months. Following pairing, female hindquarters were inspected three times daily during the dark phase of the light cycle for the presence of a vaginal sperm plug. The day of sperm plug detection was designated day 0 of pregnancy. On day 1 of pregnancy, 1-3 hours after commencement of the dark phase of the light cycle, each female was isolated and randomly assigned to one of the experimental conditions, with age and weight counterbalanced across conditions.

2.2 BPA administration

Subcutaneous injections of BPA (97%, Sigma-Aldrich) dissolved in peanut oil were administered on days 1-4 of pregnancy. Females were assigned to doses of 0, 3.375, 6.75, and 10.125 mg/animal/day, equivalent to approximately 100, 200, and 300 mg/kg respectively. The volume of peanut oil vehicle varied due to solubility constraints of BPA, with the 3.375 mg dose dissolved in 0.3 ml and the 6.75 and 10.125 mg doses in

0.45 ml peanut oil. Proportional numbers of control subjects were run at these vehicle volumes. In order to minimize the development of skin irritation, each injection was administered at a different site, including the left and right flanks, middle back area, and scruff of the neck. Control and BPA-treated subjects were given injections in identical locations on the same day of gestation. All injections were administered 2-4 h following the onset of the dark phase of the light cycle by a single experienced investigator.

2.3 Blastocyst Implantation

On gestational day 6 (GD 6) females were each weighed and then sacrificed by CO_2 asphyxiation, and uteri were excised via an abdominal incision. A subset of females from each experimental group was randomly chosen for assessment of blastocyst implantation (0 mg, n = 6; 3.375 mg, n = 5; 6.75 mg, n = 4; 10.125 mg, n = 4). Uteri and ovaries were excised and placed in 2% NaOH in PBS for 1 h, and the number of implantation sites was counted [48].

2.4 Uterine and Ovarian Histomorphology

The uteri and ovaries of the remaining females sacrificed on GD 6 (0 mg, n = 6; 3.75 mg, n = 8; 6.75 mg, n = 9; 10.125 mg, n = 8) were trimmed of mesenteries and fixed in 10% neutral buffered formalin for 48 h at 4°C. Three 1-cm pieces of the uterus were taken from randomly chosen areas along the uterine horn. The ovaries and uterine sections were then embedded in paraffin. Sections of 5 μm were cut and mounted on glass slides. Slides were deparaffinized in xylene and rehydrated in descending grades of

ethanol and rinsed in phosphate buffered saline (PBS) 2 times (5 min each). One slide from each subject was then stained with hematoxylin and eosin for morphological evaluation. An average uterine luminal epithelial cell height was generated for each subject by measuring 20 randomly selected cells on each of the three uterine sections in X40 field. Average luminal area was generated by measuring the area in all 3 sections for each subject. Both cell height and area were calculated using Image Pro Plus (v. 4.5, Silver Spring, MD). The number of corpora lutea was calculated by counting the number in both ovaries and generating an average number per individual.

2.5 Immunohistochemical staining

Slides were deparaffinized in xylene and rehydrated in descending grades of ethanol and rinsed in phosphate buffered saline (PBS) 2 times (5 min each) and immersed in citrate buffer for antigen retrieval (pH 6.0) for 30 min and then washed in PBS. Normal goat serum in PBS (1 h in covered humidified tray) was used to decrease nonspecific binding. This was followed by incubation with PR (Ab-13 from Thermo Scientific, Nepean, ON, Canada) or ERα (H-184) polyclonal rabbit antibodies at optimal dilutions of 1:100 for PR and 1:200 for ERα for 24 h at 4°C. Non-immune serum was used as a negative control on each slide. Sections were incubated with the biotinylated secondary antibody for 2 h in a covered humidified tray and then washed in PBS and incubated with avidin-biotin peroxidase complex at 37°C for 1 h. Sections were then again washed in PBS before being placed in diaminobenzidine (DAB) (50 mg DAB dissolved in 200 ml PBS with 2 drops H202). For PR, this DAB reaction was conducted

for 2 min, whereas for ERα the DAB reaction was monitored for each slide under microscope and terminated after 30-60 sec as deemed appropriate. Carazzi's hematoxylin was used to counterstain the slides which were then dehydrated through graded ethanol solutions, cleared in xylene, and mounted with Permount for bright-field microscopy. Images were acquired digitally using a microscope coupled to an Image Analysis System (Image Pro Plus 4.5, Silver Spring, MD) and the presence or absence of staining in luminal epithelial cells and stroma was assessed by a single experienced investigator blind to condition.

2.6 Western Blotting

An additional 8 females per group were sacrificed on GD 6 and their uterine tissue removed and trimmed of mesenteries and frozen immediately frozen in a bath of 2-methylbutane (>99%, Sigma-Aldrich) on dry ice and then kept at -80°C until use. Proteins were extracted from tissues by homogenization in RIPA buffer containing 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 0.5% deoxycholic acid sodium salt (Sigma-Aldrich) and Complete Mini protease inhibitor (1 tablet per 10 mL; Roche Applied Science, Laval, Que., Canada). Homogenates were centrifuged at 10,000 X g for 10 min at 4°C and supernatants collected. The Bradford method was used to determine the total protein [49]. Equal amounts of protein (40 μg) were denatured by heating at 100°C for 5 min and then electrophoresed on 12% acrylamide gels (Thermo Scientific, Rockford, IL) at 100 V for 1 h. Proteins were then transferred to a polyvinylidene difluoride membrane (Bio-Rad Labs, Hercules, CA) at 40 V for 1.5 h. Membranes were blocked overnight

with 5% skim milk in 0.01 M PBS and 0.1% Triton-X at 4°C on a rocking platform. The following day, membranes were incubated for 2 h at room temperature with rabbit polyclonal PR Ab-13 (1:200; Thermo-Fisher Scientific, Fremont, CA) antibody or Lf H-65 antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) in 5% skim milk solution on a rocking platform. β-Actin (1:15000; AbCam, Cambridge, MA) was used as a loading control for each membrane. Following primary antibody incubation, membranes were washed and incubated with horseradish peroxidase conjugated secondary antibody in 5% skim milk solution (1:5000) for 1 h at room temperature on a rocking platform. Membranes were washed thoroughly and reactive protein detected using ECL-plus chemiluminescence substrate and high performance chemiluminescence film (Amersham Pharmacia Biotech, Oakville, ON). Band intensity was quantified using ImageJ software (v 1.42). Test bands were normalized to the control band in each blot.

2.7 Statistical Analysis

For all analyses, statistical significance was designated at the conventional level of $p < 0.05$. For each quantitative measure, dose was first treated as a categorical condition within a one-way analysis of variance; where significance was identified all multiple pairwise comparisons were examined using the Newman-Keuls method. Given that BPA dose was a continuous ratio-scale variable, it was also related to each quantitative measure through linear regression, with the associated r^2 value tested via an F-test. A quadratic regression was also conducted for each measure and the increment in R^2 over

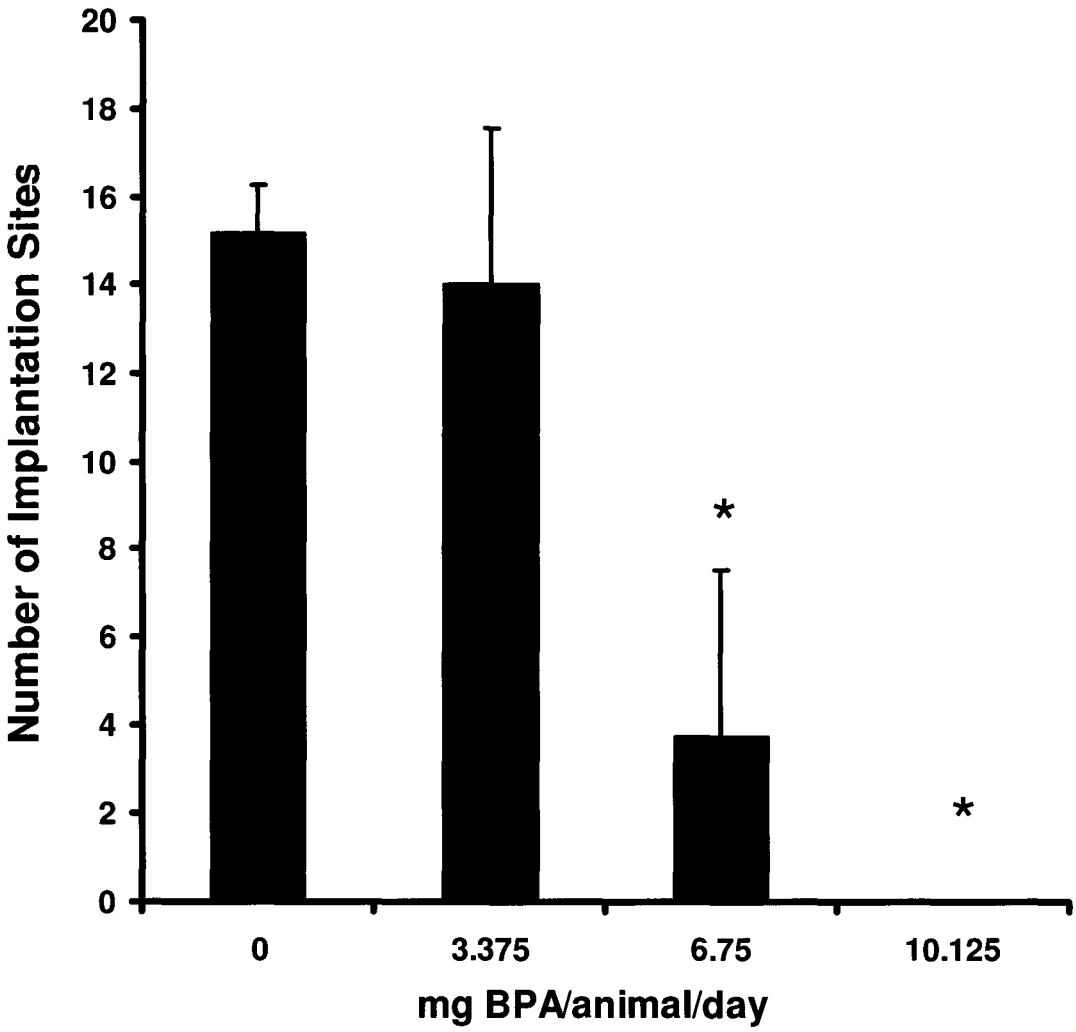
the linear regression was tested for significance via an *F*-test [50]. If that increment was significant the quadratic model was adopted and reported.

3. Results

3.1 Weight Gain and Implantation of Blastocysts

There were no significant differences between the two groups of controls administered different quantities of vehicle; these groups were therefore combined for subsequent statistical analysis. The mean weight (±S.E.) of all females just prior to the first injection was 26.2±4.27 g and was very similar among conditions. The weight gain to GD 6 was 1.75±0.50, 1.77±0.48, 0.21±0.69, and 0.00±0.70 g with n = 12, 13, 13, and 12 females respectively in the 0, 3.375, 6.75, and 10.125 mg BPA conditions. Analysis of variance on this weight gain showed an effect that approached statistical significance, $F(3,46) = 2.58$, $p = 0.064$. Linear regression on this gain did show significance, $F(1,48) = 6.55$, $p = 0.013$. As shown in Fig. 1, there were clearly fewer implantation sites in animals given doses of 6.75 and 10.125 mg BPA compared to controls. Analysis of variance showed a significant effect of condition, $F(3,15) = 8.59$, $p = 0.002$; multiple comparisons indicated that both of the higher two doses differed from both of the other two conditions. Linear regression comparing dose and the number of implantation sites was statistically significant, $r^2 = 0.577$, $F(1,17) = 23.24$, $p < 0.001$.

Fig. 1. The mean (±S.E.) number of implantation sites on gestational day 6 following subcutaneous injections of bisphenol-A (BPA) on days 1-4 of gestation. * denotes significant differences from the control condition.



3.2 Uterine and Ovarian Histomorphology

As seen in Figs. 2 and 3, the average luminal area increased substantially with increasing BPA dose. The average luminal area significantly differed across groups, $F(3,26) = 8.56$, $p < 0.001$, with dose-dependent increases. Multiple comparisons indicated that both of the higher two doses differed from both of the other two conditions. Linear regression comparing dose and luminal area was also statistically significant, $r^2 = 0.577$, $F(1,28) = 26.29$, $p < 0.001$. As shown in Fig. 4, uterine epithelial cell height also differed across conditions. Analysis of variance indicated a significant effect, $F(3,27) = 4.07$, $p = 0.016$; multiple comparisons showed that the high dose condition differed significantly from the control and low-dose conditions. Linear regression similarly showed significance, $r^2 = 0.296$, $F(1,29) = 12.20$, $p = 0.002$. The number of corpora lutea was 12.3±3.1, 9.9±2.1, 15.2±1.7, and 10.7±3.2 respectively in the 0, 3.375, 6.75, and 10.125 mg BPA conditions; none of the statistics conducted on this measure achieved or approached statistical significance.

Fig. 2. The mean (±S.E.) uterine luminal area of inseminated females on gestational day 6 following subcutaneous injections of bisphenol-A (BPA) on days 1-4 of gestation. * denotes significant differences from the control condition.

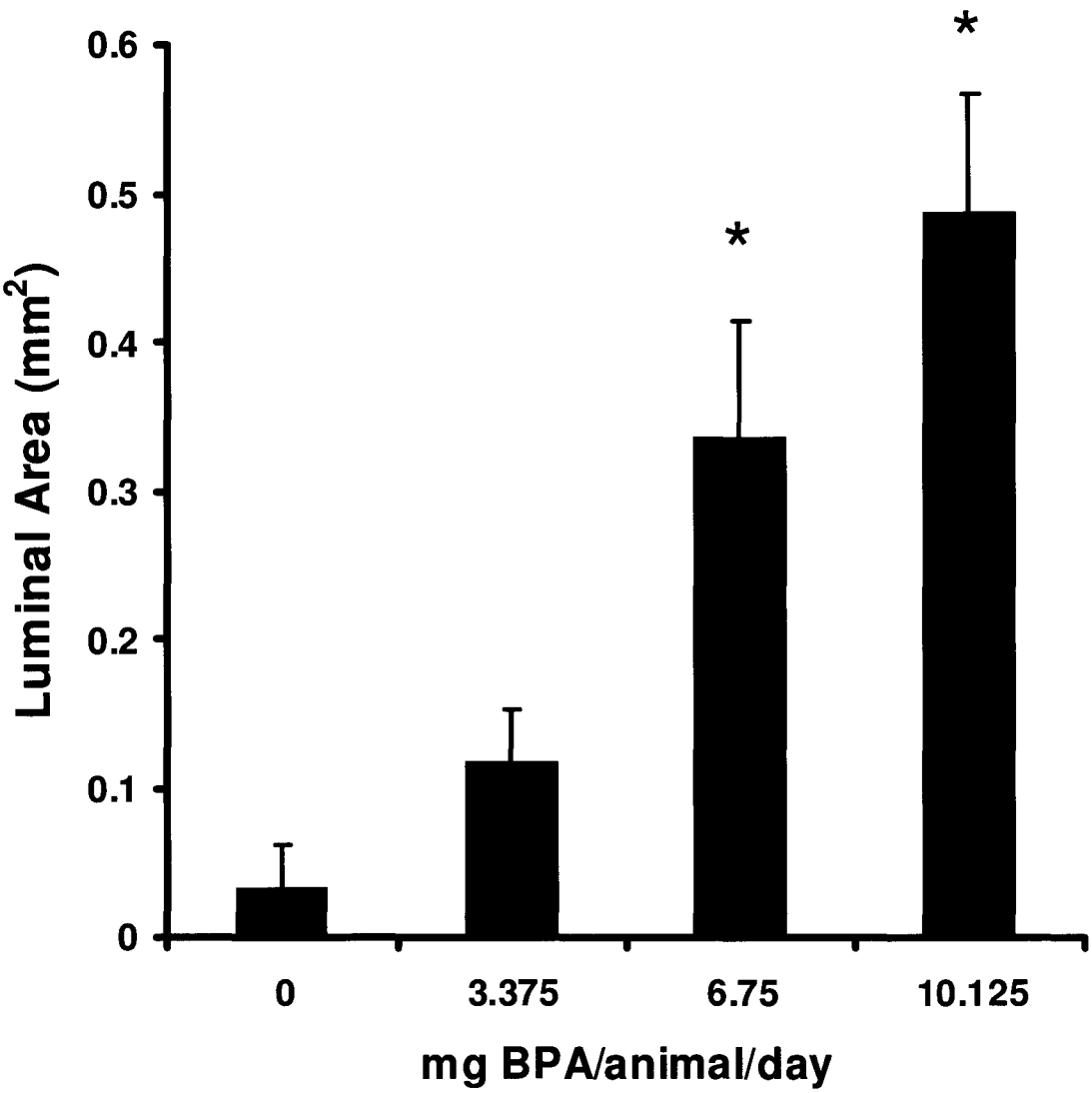


Fig. 3. Representative uterine sections on gestational day 6 following subcutaneous injections of bisphenol-A (BPA) on days 1-4 of gestation. Panels A = 0, B = 3.375, C = 6.75, and D = 10.125 mg BPA/animal/day. LU = lumen; SC = Stroma.

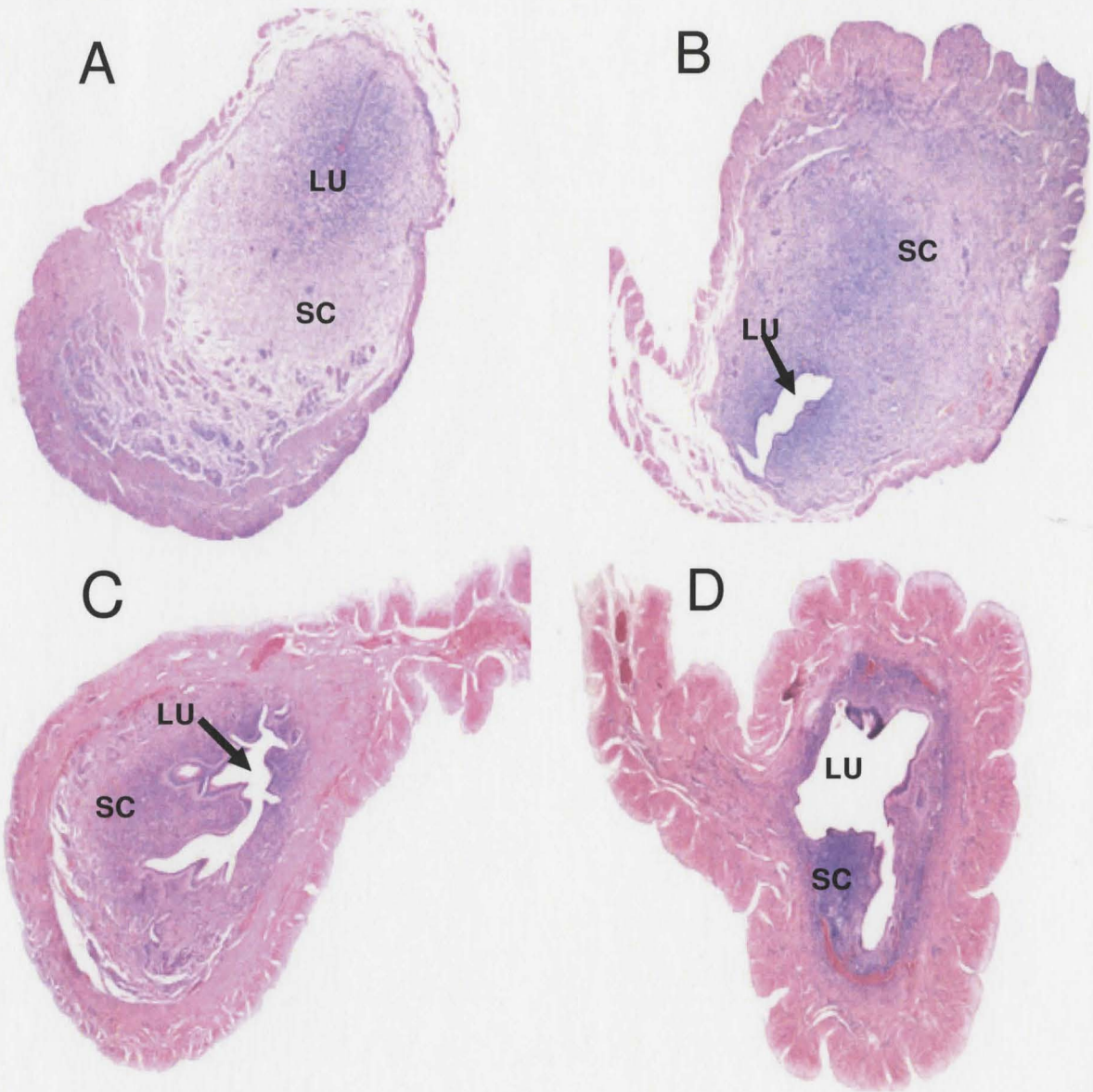
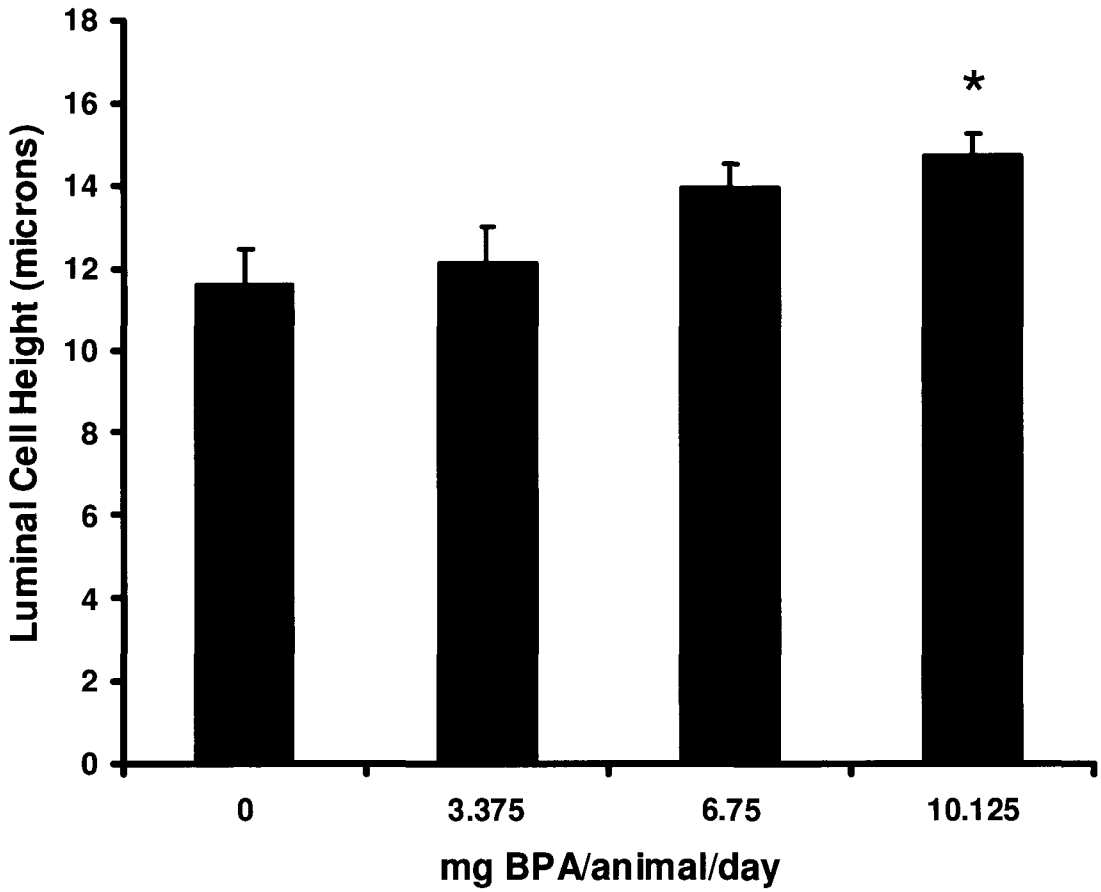


Fig. 4. The mean (±S.E.) uterine luminal epithelial cell height on gestational day 6 following subcutaneous injections of bisphenol-A (BPA) on days 1-4 of gestation. * denotes a significant difference from the control condition.



3.3 Immunohistochemical Staining

For PR staining, uterine epithelial cells were generally negative, and where positive staining was encountered it was focal, weak, and confined primarily to the nucleus. As the staining pattern was inconsistent and generally absent it was determined to be inconclusive and Western blot analysis of uterine homogenates would be more reliable, as reported below. Staining for ERα is shown in Figs. 5 and 6. There was a non-monotonic relationship between BPA dose and positive staining for ERα, with the highest values being evidence in the 3.375 mg dose and the lowest values in the 10.125 mg dose. Analysis of variance on these ERα data indicated a significant effect of condition, $F(3,27) = 3.82$, $p = 0.021$; multiple comparisons indicated a significant difference between the 10.125 and the 3.375 mg dose conditions. There was a significant quadratic trend indicated by multiple regression, $R^2 = 0.228$, $F(2,28) = 4.13$, $p = 0.026$.

Fig. 5. The mean (±S.E.) percent of uterine luminal epithelial cells staining for estrogen receptor alpha (ERα) on gestational day 6 following subcutaneous injections of bisphenol-A (BPA) on days 1-4 of gestation. ** denotes a significant difference from the 3.375 mg condition.

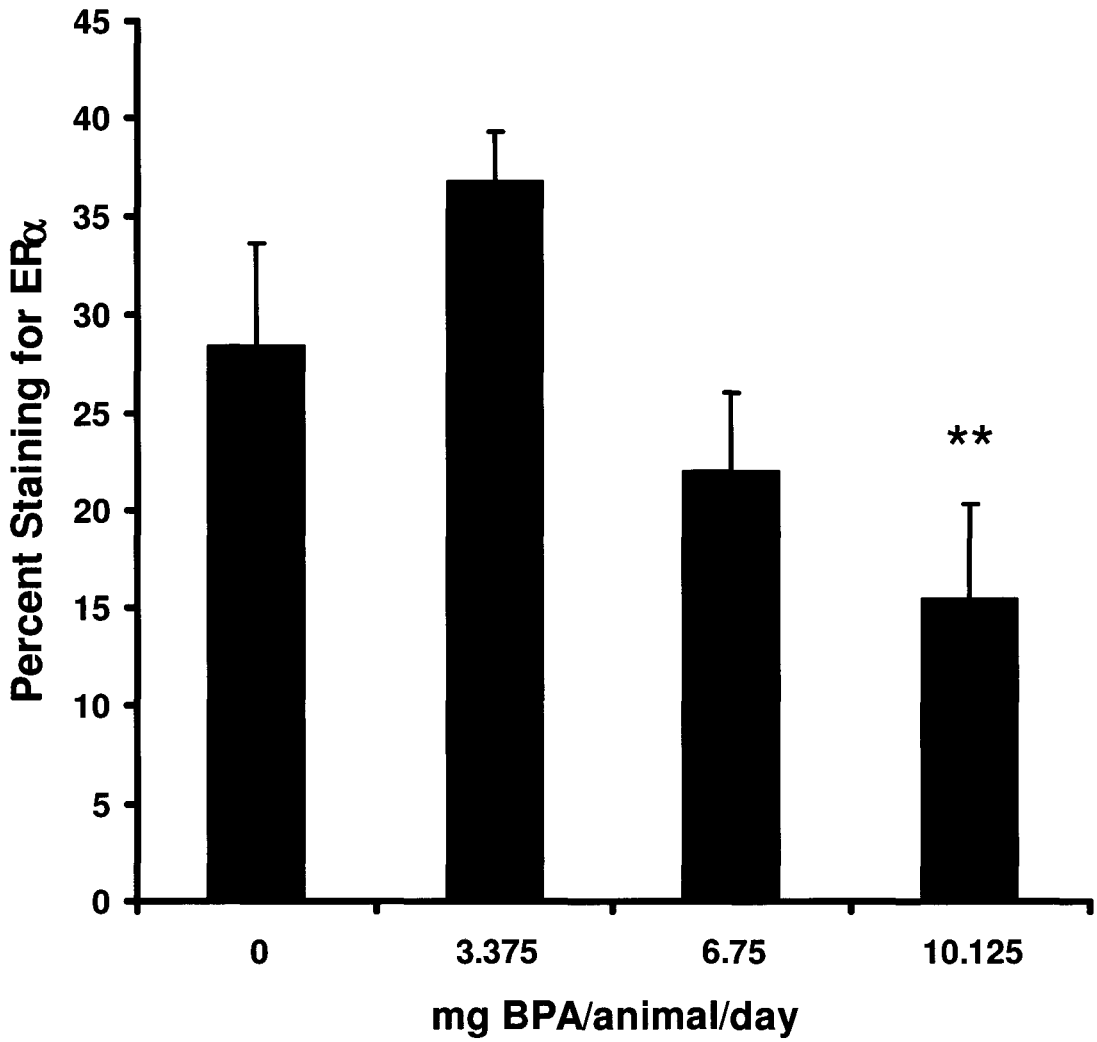
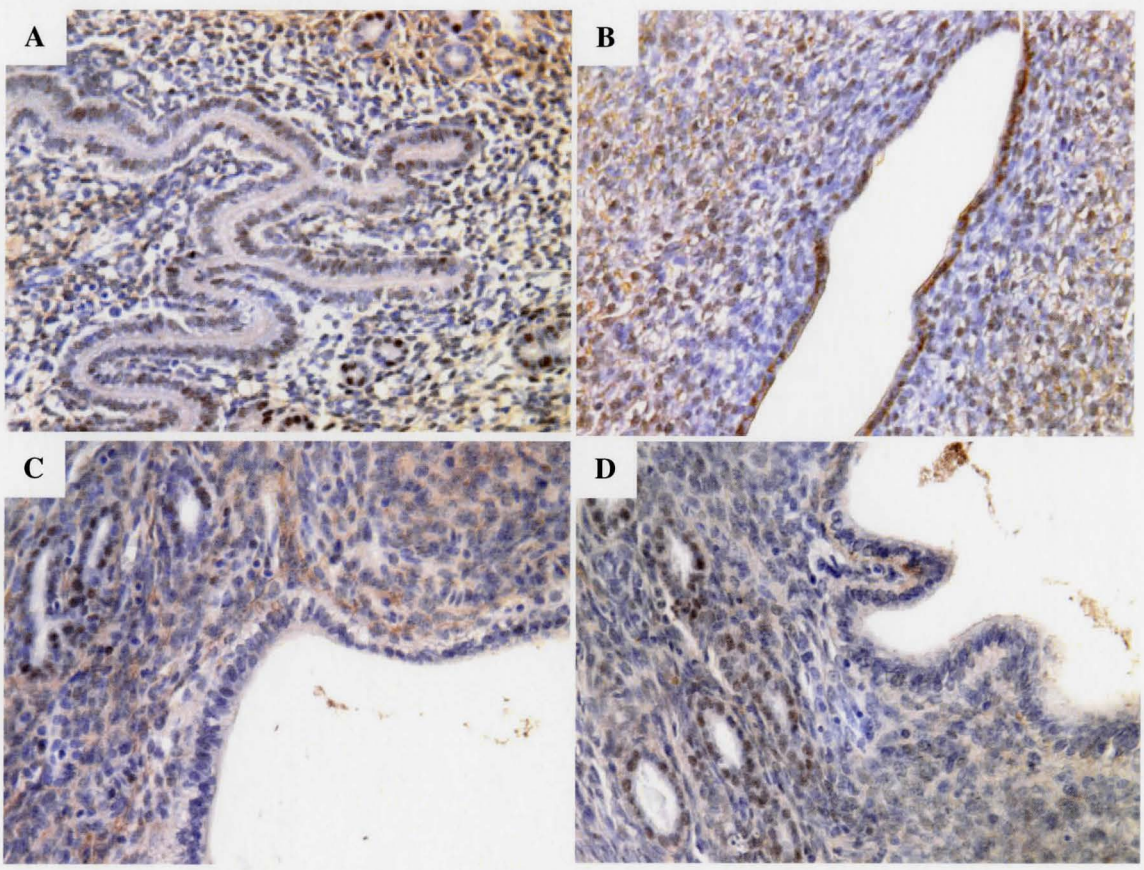


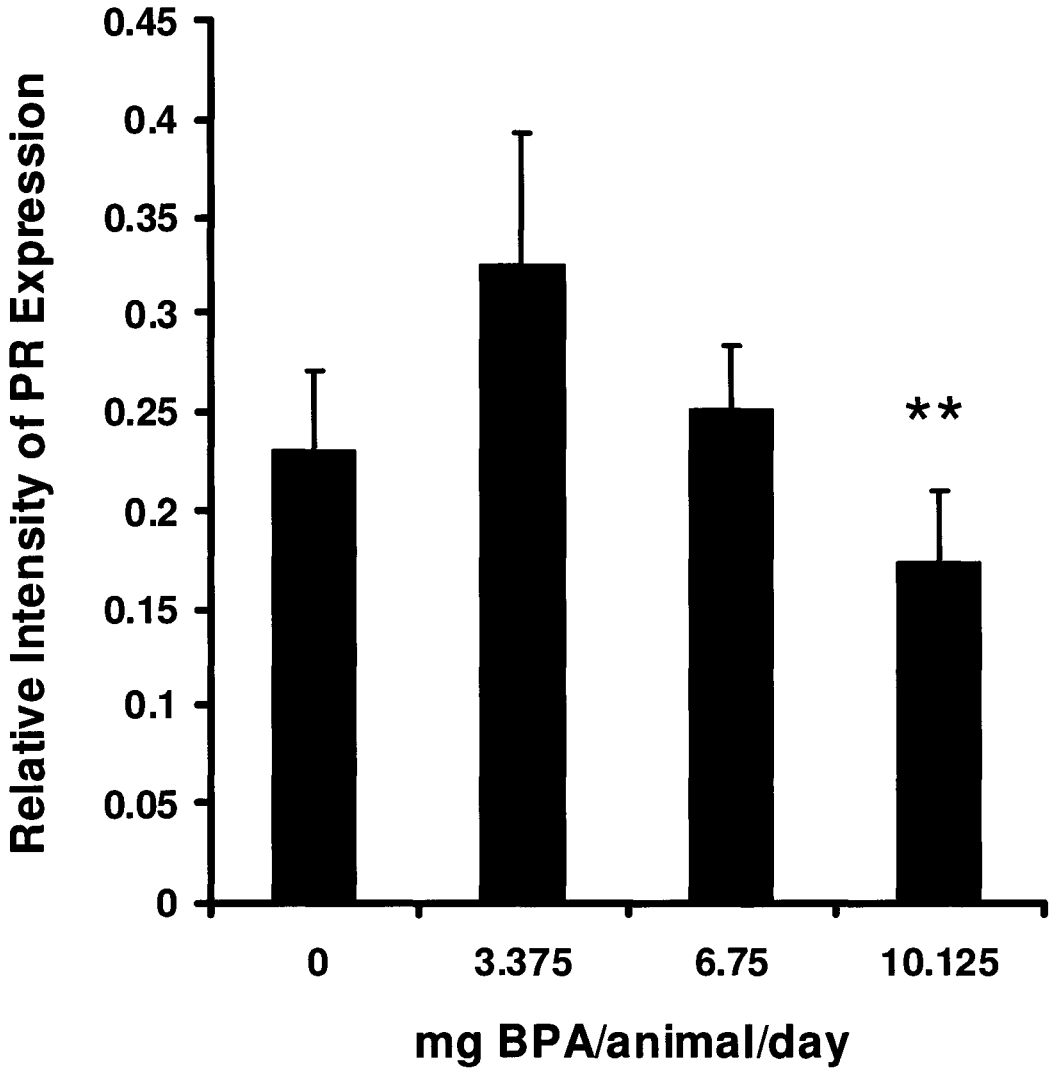
Fig. 6. Representative uterine sections on gestational day 6 following subcutaneous injections of bisphenol-A (BPA) on days 1-4 of gestation, stained for estrogen receptor alpha (ERα). Panels A = 0, B = 3.375, C = 6.75, and D = 10.125 mg BPA/animal/day. Original magnification 400 X.



3.4 Western Blotting

PR Western blotting techniques showed a detectable band between 100 and 150 kDa that was determined to mark the expression of PR-B. There was no detectable band between the 75 and 100 kDa markers that would represent PR-A. As shown in Fig. 7, there was a non-monotonic trend across increasing doses. Analysis of variance comparing the expression of PR between groups approached but did not reach the conventional level of statistical significance, $F(3, 27) = 2.61$, $p = 0.072$; multiple comparisons indicated a difference between the 3.375 and 10.125 mg doses. A quadratic regression indicated a significant trend, $R^2 = 0.220$, $F(2,27) = 3.80$, $p = 0.043$. Lactoferrin expression showed a high degree of variability within groups and a small trend toward decreased expression with increased BPA dose, with values of 7.12±1.62, 5.31±1.90, 4.17±1.24, and 4.38±1.69 respectively in 0, 3.375, 6.75, and 10.125 mg BPA conditions; none of the statistics conducted on this measure achieved or approached statistical significance.

Fig. 7. The mean (±S.E.) relative intensity of uterine progesterone receptor (PR) expression on gestational day 6 following subcutaneous injections of bisphenol-A (BPA) on days 1-4 of gestation, as measured via western blot. ** denotes a significant difference from the 3.375 mg condition.



4. Discussion

This is the first study to investigate uterine morphology and steroid receptor expression in response to BPA exposure around the sensitive period of intrauterine implantation of blastocysts. The data demonstrate that high-dose BPA exposure during this period disrupts intrauterine implantation while dramatically altering uterine morphology. Following daily injections of 6.75 or 10.125 mg BPA per animal on days 1-4 of gestation, females showed a substantial expansion in uterine luminal area and an increase in luminal epithelial cell height. Immunohistochemical analysis showed the presence of ERα expression in luminal and glandular epithelial cells and in the stroma. ERα in the luminal epithelium showed a non-monotonic relationship to dose, peaking at the low (3.375 mg/animal) dose, then diminishing with higher doses. The estrogen-receptor activated protein, Lf, did not vary significantly among conditions. Similarly to ERα, PR expression was modulated as a non-monotonic function of BPA dose, with some evidence of a rise with the lowest dose and declines with increasing dose.

The epithelial cells of the uterine lumen are known to be sensitive to exogenous estradiol, undergoing proliferation within 20-24 hours following a single injection [51]. BPA administration has also been shown to increase uterine epithelial cell height in ovariectomized mice [38] and rats [39]. In the present study, a significant increase in uterine luminal epithelial cell height was observed at both the higher BPA doses in samples collected on day 6 of gestation, suggesting a weak estrogenic effect. Samples in the present investigation were collected approximately 48 hours following the final

administration of BPA; the observed increase in cell height could vary dependent on the timing of administration and sampling.

A dose-dependent increase in uterine luminal area was observed, showing strong significance at the higher two BPA doses. An appropriate uterine environment and embryo development are both critical for successful implantation [52]. Coordinated actions of progesterone and estrogens play a critical role in development of a receptive uterine environment, embryo development, and embryo migration through the oviduct [53,54]. Changes in uterine morphology would have implications for the success of blastocyst implantation. Estrogen and progesterone actions are critical in the regulation of uterine cell proliferation, establishing a window of receptivity to blastocyst implantation [52,55,56]. This window is very narrow in mice and sensitive to changes in steroid levels [53,57]. Small increases in estradiol levels can cause alterations in uterine PR and gene expression, leading the uterus to enter a refractory state and thereby decreasing the probability of successful implantation [57]. The activation of dormant blastocysts, in which estradiol may play a role [58], is also critical for successful implantation [53]. Blastocyst transport from the oviduct to the uterus is also sensitive to estrogens and may result in accelerated embryo transport, leading to potential implantation failure [59-61]. BPA has been shown to decrease the rate of development of blastocysts [62]. The gross alterations in uterine morphology observed in the present study suggest that BPA can also exert its influence at the level of the uterus, affecting its receptivity for blastocyst implantation.

Expression of ERα and PR in uterine luminal cells fluctuates over the estrous cycle and varies dependent on the phase of early pregnancy [63,64]. PR expression decreases substantially following implantation while high levels of ERα expression are maintained for an additional day or two before also decreasing [63]. The expression of PR in the uterus is sensitive to estradiol exposure, showing overall increases in expression [65], however estradiol also acts to repress PR expression in the uterine epithelium [66,67]. Increases in PR expression at the low BPA dose in the current study could be due to weak estrogenic effects of BPA. However, this possible estrogenic response was not observed in any other measures and it is currently not possible to dissociate these trends from the indirect influence of intrauterine implantation failure on PR expression.

Following confirmation of the presence of ERα in the uterus, the expression of the estrogen sensitive glycoprotein Lf in uterine homogenates was analyzed using Western blotting techniques. There were no detectable increases in Lf expression in response to BPA administration. In the early phases of implantation, Lf expression has been found amongst underlying decidualizing cells of implanting blastocysts in mouse uteri [68] and in preimplantation mouse embryos [69]. Leading up to the period of implantation, a decrease in Lf expression occurs in both the maternal endometrium and in the embryo to low, but detectable levels [68,69]. Previous investigations of Lf expression in immature mouse uteri that used similar doses of BPA as in the current study were contradictory [42,46]. In the present investigation there were no detectable increases in Lf expression in response to BPA administration. This would be consistent with the possibility that

disruption of blastocyst implantation following BPA administration is not induced via binding to estrogen receptors, however further research is necessary to clarify this.

The observations reported here are likely dependent on the timing of measurement. Uterine extraction occurred 48 hours following the final administration of BPA, which followed previously developed methods [18]. Previous studies investigating PR and Lf expression in response to estradiol, DES, or BPA have taken samples immediately following exposure [42] or 12-24 hrs following the final administration [46,67]. BPA is thought to reach its metabolic half life within hours of administration [70,71]. Following subcutaneous administration, over 90% of BPA will be metabolized and excreted within 48 hours [72], therefore at the time of sampling circulating levels of BPA may have been too low to continue exerting effects. BPA may have also acted via non-genomic actions, including membrane steroid receptor and G protein-coupled receptor 30 [73,74]. However, the effects of non-genomic pathways are typically observed in the order of seconds to minutes [75-77]. Previous investigations using a single dose of BPA have shown the most sensitive period prior to implantation is gestational day 1, 48 hours prior to implantation, while a higher dose on day 2 had no effect [18]. This time frame suggests that BPA's actions are not induced by non-genomic pathways.

The observed disruption of pregnancy and blastocyst implantation reported here and previously [13,18] may also be due to general toxic or stress-induced alterations at the level of the uterus. Pre- and post-implantation pregnancy loss and maternal toxicity have been reported in response to doses of 1000 mg BPA/kg orally administered on GD

0-20 in rats [37]. Although there was a decrease in maternal weight following BPA exposure in the present study, it was assumed this was due to the disruption of pregnancy. Single administrations of 10.125 mg BPA on the day of insemination (GD 0) or 6.75 mg on GD 1 were sufficient to reduce the number of implantation sites counted on GD 6 [18]. This single administration may have produced more of an estrogenic response compared to the repeated doses of BPA on GD 1-4 used here.

In summary, pregnancy disruption was accompanied by a profound increase in luminal volume together with decreased expression of ERα and PR in BPA exposed rats. Taken together we suggest that BPA acts through the estrogen receptor in our model to decrease PR expression and disrupt uterine physiology and pregnancy. Future directions include examination of the time course for the observed changes in order to define the BPA mechanism underlying these changes.

Acknowledgments

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Chapter 5

General Discussion

Rationale

The experiments in this thesis were designed to investigate the estrogenic properties of BPA when administered to inseminated female CF-1 mice during the period surrounding blastocyst implantation. The peri-implantation period is known to be highly sensitive to exogenous 17β-estradiol (deCatanzaro *et al.* 2001, 2006). Therefore given BPA's reputation as an estrogenic compound, pregnancy outcome, number of implantation sites, urinary hormonal output, uterine morphology, and steroid receptor and protein expression were investigated following exposure. The measures selected have been shown to be sensitive to natural endocrine fluctuations and administration of exogenous steroid hormones or EDCs (deCatanzaro *et al.*, 2001, 2006; Markey *et al.*, 2001; McMaster *et al.*, 1993; Tan *et al.*, 1999).

Previous investigations have shown that BPA exposure can have significant effects on development, sexual differentiation, and maternal behaviour (Howdeshell *et al.*, 1999; Palanza *et al.*, 2002; Rubin *et al.*, 2006). However, many of these studies have involved exposing animals in late gestation and/or during early postnatal development, with measures taken during subsequent maturation. Others have examined how BPA can affect pregnancy *per se*; however exposure periods either started well before the implantation period or continued through fetal development (Kim *et al.*, 2001; Tachibana *et al.*, 2007; Takai *et al.*, 2000). Many of these studies utilized gavage techniques for oral administration of BPA, which are likely to stress the animals due to the amount of human handling involved. The experiments reported in this thesis were among the first to focus directly on the period of implantation, use non-invasive oral administration and urinary

steroid measurement techniques, and investigate hormonal, receptor, and protein responses to BPA exposure.

Overview of Results

In the experiments reported in Chapter 2, the impact on pregnancy outcome of oral and subcutaneous BPA exposure around the period of blastocyst implantation was examined. These were the first experiments to focus on the impact of short-term peri-implantation exposure, demonstrating a clear disruption of pregnancy. In addition, a natural feeding procedure was used wherein animals were free to ingest daily doses without additional handling and induced stress. This is desirable given that diverse stressors can influence hormonal dynamics and lead to pregnancy loss (deCatanzaro and MacNiven, 1992). Subcutaneous injections of 3.375 mg/day on gestational days (GD) 1-4 resulted in a decrease in the average number of pups born, whereas doses of 10.125 mg/day reduced the percentage of females that were parturient when compared to animals administered peanut oil vehicle control. Oral doses of 68.8 mg/day on GD 1-4 were also found to decrease the percentage of females that were parturient, completely eliminating all pregnancies. BPA did not influence the average weight of individual pups at weaning (postnatal day 28), nor did it influence the ratio of males to females. A wide range of doses was investigated, encompassing those comparable to common exposure levels in humans and wildlife up to the maximal doses that the animals could reasonably accommodate. BPA had no measurable influence on pregnancy outcome at low doses from 0.5 μg/day to 4.5 μg/day or approximately 12.5 μg/kg/day to 112.5 μg/kg/day,

which are surrounding the calculated acceptable or "safe" dose of 50 μg/kg/day (IRIS, 1988). The decreases observed in the proportion of females that were parturient and the number of pups they delivered suggests that BPA, at doses greater or equal to 3.375 mg/day, may have been acting as a weak estrogen, thereby disrupting implantation.

In the experiments reported in Chapter 3, subcutaneous doses identical to those used in Chapter 2 were used, with the addition of an intermediate dose of 6.75 mg/day. Blastocyst implantation was assessed via uterine extraction on GD 6, and urinary steroid levels were measured throughout the exposure period. These experiments were the first to use non-invasive urinary collection techniques to profile the effect of BPA exposure on urinary steroid output. The data indicated a lower threshold for BPA-induced pregnancy disruption than what was reported in Chapter 2, with 6.75 mg/day on GD 1-4 reducing the number of implantation sites observed on GD 6. There was not a decrease in the number of implantation sites in females administered 3.375 mg/day on GD 1-4; this suggests that pregnancy loss following implantation may have contributed to the reduction in the number of pups born at this dose in the experiments of Chapter 2. Urinary progesterone was significantly reduced by doses of 10.125 mg/day on GD 1-4 compared to controls, with the reduction becoming evident on GD 2, or 24 hours following the first injection of BPA. No other dose influenced progesterone levels and no dose altered estradiol levels. The impact of a single exposure on GD 0, 1, or 2 was also examined, providing the first demonstration that one dose of BPA could be sufficient to disrupt intrauterine implantation. A dose of 10.125 mg on GD 0 or 6.75 mg on GD 1 resulted in a significant decrease in the number of implantation sites counted on GD 6. Urinary progesterone and

17β-estradiol levels were not altered by exposure to a single dose of BPA on GD 0, 1, or 2. In summary, these results suggest that BPA is acting to disrupt implantation at repeated doses of 6.75 mg/day and 10.125 mg/day, and that a single exposure to BPA in the first two days following insemination is sufficient to disrupt intrauterine implantation. The urinary hormone analyses suggest that the observed disruption in implantation could occur independently of BPA's influence on progesterone levels.

The experiments reported in Chapter 4 were designed to determine whether BPA was acting via estrogenic pathways to exert its effects, employing doses that influenced pregnancy and blastocyst implantation in Chapters 2 and 3. Analysis of uterine morphology on GD 6 showed a profound increase in luminal area in response to doses of 6.75 and 10.125 mg on GD 1-4. There was also a small but significant increase in uterine epithelial cell height in females exposed to the highest dose of 10.125 mg/day. The number of corpora lutea did not differ among groups. Immunohistochemical staining for estrogen receptor alpha (ERα) indicated a non-monotonic relationship to dosage, with a trend toward an increase at the low (3.375 mg/kg) dose with a decreasing trend with higher doses. The staining of progesterone receptor (PR) was inconsistent and highly variable. To explore PR expression further, Western blotting techniques were used. In addition, the expression of lactoferrin, an estrogen-dependent protein, was analyzed via Western blot. PR expression showed a non-monotonic relationship to BPA dose, similar to that seen for ERα staining, whereas the expression of lactoferrin did not differ significantly among the groups.

Influence of BPA on Blastocyst Implantation and Pregnancy

The establishment of early pregnancy has been shown to be sensitive to environmental factors such as temperature extremes, nutritional deprivation, predator exposure, human handling, and various other forms of stress (deCatanzaro and MacNiven, 1992; Euker and Riegle, 1973; Runner, 1959; Weir and De Fries, 1963). Blastocyst implantation, which typically occurs on GD 3 in mice (Harper, 1992; Paria *et al.*, 1993), is particularly sensitive to exogenous administration of androgens and estrogens (deCatanzaro *et al.*, 1991). Data obtained in the experiments of Chapter 2 demonstrate that both ingestion and subcutaneous injections of BPA are capable of disrupting pregnancy in a manner that resembles the influence of exogenous 17β-estradiol (deCatanzaro *et al.*, 1991, 2001), albeit at much higher doses. The minimum dose required to have a significant impact on pregnancy outcome was approximately 20 thousand times greater than the lowest effective dose of 17β-estradiol (deCatanzaro *et al.*, 1991). This value is consistent with initial estimates of BPA's affinity for estrogen receptors (Kuiper *et al.*, 1998). Low, ecologically valid doses of BPA administered in the experiments of Chapter 2 had no measurable impact on pregnancy outcome. As the number of pups born was employed as a measure of BPA's effects on pregnancy in the experiments of Chapter 2, it remained to be confirmed whether the observed results were due to a disruption of implantation or complications arising later in gestation. The results of the experiments of Chapter 3 further supported BPA's ability to disrupt pregnancy and provided evidence that the pregnancy disruption shown in Chapter 2 was due specifically to a failure of blastocyst implantation.

The inclusion of oral administration is important for BPA investigations, as this is the primary mode of exposure in humans (Chapin *et al.*, 2008; Kang *et al.*, 2006). In the oral ingestion experiment reported in Chapter 2, the dose of BPA required to disrupt pregnancy was approximately 10 times greater than that of the subcutaneous injection experiments. The bioavailability of BPA , defined as "the amount of parent compound reaching the systemic circulation" (Pottenger *et al.*, 2000,) changes significantly dependent upon the route of administration (Pottenger *et al.*, 2000; Upmeier *et al.*, 2000). Subcutaneous or intraperitoneal injections have substantially higher rates of bioavailability compared to oral ingestion, due to first pass metabolism of ingested substances (Knaak and Sullivan, 1966). It has been suggested that metabolism of BPA is mediated by intestinal and hepatic enzymes (Pottenger *et* al., 2000), known to be associated with the metabolic clearance of phenols in rodents (Cassidy and Houston, 1984). Previously, others have shown that BPA ingestion from GD 1 through 20 will have adverse effects on pregnancy outcome (Kim *et al.*, 2001). However, the data presented in Chapter 2 are the first to show that oral ingestion of BPA specifically during the period of implantation can disrupt pregnancy.

The coordinated actions of estradiol and progesterone help to prepare a uterine environment that is receptive for implantation (Harper, 1992; Paria *et al.*, 1993). A number of mechanisms have been established to mediate the detrimental impact of minute increases in estradiol during the peri-implantation period upon the establishment of pregnancy (deCatanzaro *et al.*, 2001, 2006). Elevated levels of estradiol prior to blastocyst implantation can lead to aberrant expression of genes causing the uterus to

enter a refractory state, thus creating an inhospitable environment for implantation (Ma *et al.*, 2003). Variations in circulating estradiol levels can further lead to implantation failure by influencing blastocyst growth (Valbuena *et al.*, 2001) and embryo transport from the oviduct to the uterus (Burdick and Whitney, 1937; Humphry and Martin, 1968; Whitney and Burdick, 1936). The results from Chapters 2 and 3 suggested that BPA may have been acting as a weak estrogen, and thus the observed disruption of pregnancy may have been due to any one of these factors or some combination.

The single exposure experiment reported in Chapter 3 provided insight into the time course of BPA's actions leading to the loss of pregnancy. A dose of 6.75 mg on GD 1 caused a significant reduction in the number of implantation sites, suggesting maximal sensitivity to a single BPA exposure approximately 48 hours prior to blastocyst implantation on GD 3 (Harper, 1992; Paria *et al.*, 1993). The measurable effects of bound nuclear receptors are generally not observed until a number of hours following activation (Gould *et al.*, 1998; Gronemeyer, 1992; Walsh *et al.*, 2005), whereas non-genomic receptor actions are typically observed in the order of seconds to minutes (Aronica *et al.*, 1994; Le Mellay *et al.*, 1997; Pedram *et al.*, 2002). Therefore the increase in sensitivity to BPA on GD 1, combined with no measurable effect of exposure on GD 2, suggest that if BPA induced its effects via estrogenic pathways, it likely did so via nuclear receptors.

The results of this single administration study may have important implications for one time, high-level industrial exposure. To date there is very little published research concerning impacts of industrial exposure to BPA; however recent investigations have

shown that occupational exposure levels of BPA can have significant detrimental effects (He *et al.*, 2009; Li *et al.*, 2010). Male Chinese workers involved in the manufacturing of BPA and epoxy resin were shown to have increased risk of sexual dysfunction, including reduced sexual desire and erectile and ejaculation difficulty (Li *et al.*, 2010). As this is the first finding of occupational BPA exposure having significant health effects, there have been calls for continued epidemiological investigations not only to determine possible mechanisms but also to provide replication of these results (Sharpe, 2010). The data of this thesis suggest that one specific focus of such epidemiological research should be the fertility of young women exposed to high levels of BPA acutely or chronically.

The results of the experiments reported in Chapters 2 and 3 do not support previous reports of significant impacts of low doses of BPA approaching or below recommended daily allowances (see review by Palanza *et al.*, 2008). Many of the studies showing low dose effects have involved post-implantation *in utero* exposure and/or administration during early postnatal development (Howdeshell *et al.*, 1999; Markey *et al.*, 2005). The perinatal period is well known to be sensitive to small alterations in steroid hormone levels, which can significantly alter development (Clark and Galef, 1988; vom Saal *et al.*, 1981). BPA has been shown to pass through the placental barrier and transfer from the maternal rat to the fetus following oral exposure (Miyakoda *et al.*, 1999). BPA has also been detected in fetal reproductive organs and brain in as little 30 minutes following subcutaneous injections (Uchida *et al.*, 2002). Although the results from the research paradigm reported in this thesis do not show low dose effects, this particular endpoint may not be as sensitive as different measures in other paradigms.

How BPA Exposure Affects Steroid Hormone Excretion and Receptor Expression

Appropriate hormonal levels are critical for the overall health and wellbeing of an animal. Deviations from typical levels can have significant physiological and morphological effects. Appropriate hormonal variations are particularly important for the establishment of pregnancy, which requires coordinated release of estradiol and progesterone (Dey and Lim, 2006). These steroid hormones are vital for creating an environment that will support implantation coincident to the arrival of blastocysts to the uterus (Dey and Lim, 2006; Huet *et al.*, 1989; Paria *et al.*, 1993). Changes in steroid receptor and protein expression are also known to be associated with the preparation of blastocyst invasion (Das *et al.*, 1997; McMaster *et al.*, 1993; Tan *et al.*, 1999).

The experiments conducted in Chapters 3 and 4 were the first to investigate the influence of BPA exposure on estrogen and progesterone excretions and receptor expression during the peri-implantation period in inseminated mice. The significant decrease in urinary progesterone levels following administration of 10.125 mg BPA/day coincided with the observed disruption of implantation. Urinary progesterone levels typically rise in female mice in the days following insemination and begin to decrease during the latter half of gestation (deCatanzaro *et al.*, 2003, 2004), dynamics known to be associated with the maintenance of pregnancy (McCormick and Greenwald, 1974). Interestingly, in the 6.75 mg/day repeated dose group there was not a similar decrease in progesterone levels despite a complete absence of blastocyst implantation. A previous report showed a drop in serum progesterone levels in pseudopregnant mice following repeated injections of 200 mg BPA/kg on pseudopregnancy days 4-7 (Spencer *et al.*,

2002); however, in the same report, BPA administration on pseudopregnancy days 0-3 did not influence progesterone levels (Spencer *et al.*, 2002).

The results in Chapter 3 are the first to show a significant decrease in progesterone on GD 1. BPA may have exerted its effects by influencing granulosa progesterone production (Mlynarčíková *et al.*, 2005) and/or the negative feedback regulation of gonadotropins (Tohei *et al.*, 2001). Progesterone levels did not decrease in animals administered a single dose of BPA on GD 0 and 1, despite a reduced number of implantation sites. These results suggest that BPA is able to disrupt implantation independently of its impact on progesterone excretion. There were no measurable dose-dependent changes in urinary estradiol levels following either the repeated or the single BPA exposure. This would suggest that if BPA mediated its effects by acting as an estrogen, it did so without significantly influencing endogenous estradiol levels by altering aromatization or the hypothalamic-pituitary-gonadal axis as measured by urinary excretions.

Variations in exposure to testosterone *in utero* have been shown to influence the onset of sexual maturation in females (Clark and Galef, 1988) and length of estrous cycles (vom Saal *et al.*, 1981). A female fetus positioned between two males will be exposed to more androgens than is a female surrounded by two other females. The anogenital index (AGDI) is an indirect measurement that is positively correlated with *in utero* androgen exposure (Vandenbergh *et al.*, 1995). The sensitivity of animals exposed to endocrine-disrupting chemicals has been reported to be altered by differing levels of *in utero* steroid hormone exposure (Howdeshell *et al.*, 1999). Following *in utero* BPA

administration, females positioned between two other females *in utero* were more susceptible to BPA's developmental effects, reaching sexual maturation earlier than those that were surrounded by two males and controls that were not exposed to BPA (Howdeshell *et al.*, 1999).

Results from the experiments conducted in Chapter 3 showed that AGDI was negatively correlated with progesterone excretion and the number of young in the first litter of inseminated females. These results are in agreement with previous findings that females positioned between two males tend to have smaller litters *in utero* (Kinsley *et al.*, 1986). Females exposed *in utero* to higher levels of testosterone have also been shown to produce fewer young in their first litter (Vandenbergh *et al.*, 1995). The results from Chapter 3 show that BPA exposure during the peri-implantation period did not significantly alter these correlations. In addition, AGDI did not correlate with any observable effect on the sensitivity of females to BPA.

In order to explore further the observed changes in hormone excretions following BPA exposure, experiments in Chapter 4 examined steroid receptor expression in the uterus following the repeated dose paradigm. Expression of both ERα and PR is known to fluctuate in relationship to estrous cycling and to be dynamic during the peri-implantation period (Mote *et al.*, 2006; Tan *et al.*, 1999). On the day of implantation, ERα expression is known to be high in glandular epithelium and lower in luminal epithelial cells and stroma, whereas PR expression is predominantly found in the luminal epithelium and stroma (Das *et al.*, 1997; Tan *et al.*, 1999). Following implantation, PR expression decreases to undetectable levels in uterine luminal cells, whereas ERα

expression remains present in luminal cells before eventually decreasing and moving to stromal cells adjacent to the luminal epithelium around GD 7 (Das *et al.*, 1997; Tan *et al.*, 1999). *In utero* BPA exposure can influence steroid receptor expression in adulthood, with increases in uterine ERα and PR observed later in life (Markey *et al.*, 2005). BPA can also reduce ER-mRNA expression in pseudopregnant rats following subcutaneous injections of 200 mg/kg during the equivalent of the peri-implantation period (Spencer *et al.*, 2002).

The experiments from Chapter 4 were the first to investigate receptor expression during the implantation period in inseminated female mice following BPA exposure. Immunohistochemical analysis for ERα and Western blots for PR both showed that expression trended somewhat upward following repeated doses of 3.375 mg BPA/day and then decreased with the higher doses of 6.75 and 10.125 mg/day. PR expression in the uterus is sensitive to fluctuations in estradiol levels, showing overall increases in expression with increased estradiol (Milgrom *et al.*, 1973). However, the uterine epithelium is an exception, as estradiol acts there to repress PR expression (Parczyk *et al.*, 1997; Tibbetts *et al.*, 1998). Increases in PR expression at the 3.375 mg/day dose, albeit statistically non-significant, may have been due to a weak estrogenic response. In Chapter 2 this dose decreased the number of pups born, however it did not alter the number of implantation sites counted in the experiments of Chapters 3 and 4 or alter estradiol and progesterone excretions in the data of Chapter 3. The dose-dependent decrease observed in PR expression may have also been due to non-steroidal effects of BPA, such as non-specific stress-related or toxic responses. In addition, the observed

changes in steroid receptor expression and the indirect influence intrauterine implantation failure cannot be disassociated.

BPA and Uterine Morphology

The preparation of the uterine environment is critical for successful blastocyst implantation and further development of embryos. In mice, preparation for blastocyst implantation through cell proliferation and differentiation is initiated and controlled by estrogens and progesterone. Progesterone secretion in the days following insemination is critical for stromal cell proliferation, which is enhanced by a small increase in estrogen secretion on GD 3. The epithelial cells of the uterine lumen are known to be sensitive to exogenous estradiol, undergoing proliferation within 20-24 hours following a single injection (Mukku *et al.*, 1982). Exposure to BPA can also increase uterine epithelial cell height in ovariectomized rats and mice (Papaconstantinou *et al.*, 2000; Steinmetz *et al.*, 1998).

The data presented in Chapter 4 showed significant morphological changes in the uteri on GD 6 following BPA exposure on GD 1-4. There was a strong dose-dependent increase in luminal area as well as a small but significant increase in uterine epithelial cell height. Changes in uterine morphology prior to blastocyst implantation could have profound implications for the establishment of pregnancy. Through the combined actions of estradiol and progesterone and the activity of the blastocyst, a very narrow window of uterine receptivity is established (Huet *et al.*, 1989; Paria *et al.*, 1993). This window is sensitive to changes in steroid levels, with small increases in estradiol leading to the

uterus entering a refractory state (Ma *et al.*, 2003). The increase in uterine epithelial cell height suggests a weak estrogenic action by BPA. Epithelial cells are sensitive to estrogen exposure and will change shape, becoming longer and more cylindrical with increased levels of estrogens (Mukku *et al.*, 1982).

The changes in uterine morphology observed in Chapter 4 suggest that BPA exerted its influence at the level of the uterus, which led to the observed disruption of pregnancy and blastocyst implantation reported in all three data chapters of this thesis. BPA's specific actions at the uterus may vary dependent on the period of pregnancy at which animals are exposed (Spencer *et al.*, 2002). During pseudopregnancy days 0-3 when pre-ovulatory estrogens are critical for epithelial and stromal cell proliferation (Dey and Lim, 2006; Huet *et al.*, 1989), rats exposed to BPA show an increase in uterine wet weight and protein expression (Spencer *et al.*, 2002). However on pseudopregnancy days 4-7, when increased levels of progesterone are crucial for the maintenance of pregnancy, administration of BPA significantly reduced uterine wet weight, protein expression, serum progesterone levels, and ERα mRNA levels (Spencer *et al.*, 2002). The repeated BPA dose exposure period reported in Chapters 3 and 4 overlapped the pre- and post-decidual period. The observed implantation failure and changes in uterine morphology may have been the result of BPA exerting a variety of effects at different time points during the exposure period.

BPA and Lactoferrin

The expression of lactoferrin in the mouse uterus is known to be induced by estrogens (Pentecost and Teng, 1986), to fluctuate in conjunction with estrous cycling (Newbold *et al.*, 1992), and to be present in uterine decidualizing cells at the site of blastocyst implantation (McMaster *et al.*, 1993). Markey *et al.* (2001) reported that there were changes in lactoferrin expression in immature female mice following exposure to BPA, however other researchers (Mehmood *et al.*, 2000) have not shown any changes in its expression following exposure to similar BPA levels. The experiments in Chapter 4 were the first to investigate the impact of BPA exposure during the peri-implantation period on lactoferrin expression in the uterus. These data showed no significant change in lactoferrin expression following BPA exposure. A decreasing trend was observed with higher doses of BPA, however there was a large amount of variability within each group and this trend did not reach statistical significance with the sample size investigated.

If BPA were acting through nucleic estrogen receptors, an increase in lactoferrin expression would be expected. Although the results of Chapter 4 did not show this increase, they do not eliminate the possibility that BPA's influence on blastocyst implantation was via estrogenic pathways. In Chapter 4, uteri were excised on GD 6, 48 hours following the last of four daily subcutaneous BPA injections. BPA's influence on lactoferrin expression may have been missed by sampling at this time rather than earlier. In addition, previous reports have found differences in the actions of BPA dependent on the stage of pregnancy during which exposure occurs (Spencer *et al.*, 2002). The single BPA exposure reported in Chapter 3 showed that implantation is most sensitive to BPA

administration on GD 1. BPA may have had a greater estrogenic effect prior to the day of implantation.

Methodological Limitations

It is well established that exogenous estradiol administration during the first few days following insemination disrupts pregnancy in female mice (deCatanzaro *et al.*, 1991, 2001, 2006). The methodologies used in this thesis were based upon these previous investigations, which allowed a comparison of the relative estrogenicity of BPA administered during the peri-implantation period. However the measures reported in Chapters 3 and 4, which were designed to provide further information regarding the mechanisms leading to BPA's disruption of pregnancy, had not been thoroughly investigated using exogenous estradiol treatment. The experimental design would have therefore been improved by inclusion of a positive control group that received estradiol. As BPA is a synthetic compound with a wide range of endocrine-disrupting effects, direct comparison to another known synthetic estrogenic compound such as diethylstilbestrol (DES) may also have been beneficial. For instance, in Chapter 4 it was not possible to dissociate the direct impact of BPA upon uterine morphology and protein and receptor expression from the more indirect consequences of failure of blastocyst implantation. Determining how the uterus would respond to natural estrogen or DES exposure would provide further insight into the mechanisms and pathways used by BPA. However, as it was important to investigate a range of doses and maintain a substantial number of

animals in each group, at the time of experimentation it was not feasible to include these positive control groups.

Animals subcutaneously administered BPA doses above 1.125 mg/day developed irritation at the site of injection. In order to minimize this irritation, four different injection sites were used, however irritation continued to develop, particularly at the scruff of the neck. The sores that developed were clearly causing some discomfort to the animals as they would continually scratch the area. Signs of irritation around the injection site were not indicative of pregnancy or implantation failure, as some animals in both the 1.125 and 3.375 mg groups developed irritation but maintained pregnancy. Notably, other investigations conducted in our laboratory (Shaw and deCatanzaro, 2009), wherein the influence of butyl- and propyl-parabens on pregnancy was examined, also showed similar irritation around the injection site; nevertheless animals were able to maintain their pregnancy, even at doses three times higher than the greatest dose of BPA used in the experiments of this thesis.

Future Directions

Evidence presented in this thesis shows that BPA exposure during the period of blastocyst implantation has deleterious effects on the establishment of pregnancy in inseminated female mice. Chapter 4 presents evidence suggesting that BPA may be acting at the level of the uterus, altering the sensitive environment necessary for successful implantation. The measurements reported in Chapters 3 and 4 were taken on GD 6, 48 hours following the final subcutaneous administration of BPA. BPA is thought

to reach its metabolic half life within hours of administration (Volkel *et al.*, 2002, 2005). Maximal plasma levels are reached within the first hour over following intravenous BPA exposure (Upmeier *et al.*, 2000) and 90% of BPA will be metabolized and excreted within 48 hours following subcutaneous administration (Kurebayashi *et al.* 2003). Sampling on GD 6 may be too late to observe many of the effects of BPA exposure. It would be beneficial to take samples throughout the exposure period on GD 2, 3, 4, and 5. This would provide insight into the progression of results that were observed in Chapters 3 and 4. In addition, investigation on GD 2 would provide further information regarding the impact of a single exposure to BPA on what was shown to be the most sensitive day as reported in Chapter 2.

As previously mentioned, the route of administration influences the bioavailability of BPA in adult animals (Pottenger *et al.*, 2000; Upmeier *et al.*, 2000), as first pass metabolism associated with oral intake will not occur if BPA is subcutaneously injected. Chapter 2 included two routes of BPA exposure, subcutaneous injection and oral ingestion. As oral ingestion is the primary route of exposure in humans, it would be beneficial to conduct similar investigations to those reported in Chapters 3 and 4 to determine whether oral exposure would have similar effects on implantation, uterine morphology, and receptor and protein expression. A wide range of doses would provide an indication as to whether oral ingestion of low environmentally-relevant doses of BPA can influence uterine morphology and protein and steroid receptor expression.

In Chapter 4, changes in uterine morphology and the expression of lactoferrin and PR were used as indicators of the estrogenic properties of BPA. The PR analysis suggests

that BPA may have acted through estrogenic pathways to influence blastocyst implantation; however the results from the analysis of lactoferrin expression did not provide further support for this claim. As described above, further research analyzing these measures throughout the exposure period would likely produce more conclusive data. An additional method to determine whether BPA exerts its influence via estrogenic mechanisms would be to administer an estrogen inhibitor concurrently. Previous reports have shown that tamoxifen administration can counteract BPA's effects on pre-implantation mouse embryos (Takai *et al.*, 2000), whereas ICI 182,780 attenuated BPA's ability to induce estrogen-dependent uterine gene expression (Papaconstantinou *et al.*, 2001). Using these or similar estrogen inhibitors in conjunction with BPA would provide an indication of the pathways used by BPA to exert the effects reported in this thesis.

It is important to consider the diet of an animal when conducting experiments investigating EDCs, as physiologically active compounds commonly found in rodent diets may influence the results. Phytoestrogens, with similarities in structure to 17β-estradiol and the capacity to bind to estrogen receptors, can influence the effectiveness of exogenously-administered hormones and endocrine-disrupting chemicals such as BPA (Miyake *et al.*, 2009; Muhlhauser *et al.*, 2009). Unpublished work conducted in the deCatanzaro laboratory has shown that the dose-response curves established in Chapter 2 and 3 were not influenced by removing phytoestrogens from the diet of inseminated females. It remains unknown whether placing females on a phytoestrogen-free diet would have a greater effect on the minimum oral dose required to disrupt pregnancy.

As mentioned above, the administration of higher doses of BPA often led to the development of skin irritation that could have induced a stress response in inseminated females. Diverse stressors are known to have deleterious effects on the establishment of pregnancy (see review by deCatanzaro and MacNiven, 1992). One method that could be applied to investigate this variable would be to assay corticosterone levels noninvasively in conjunction with BPA administration. A wide range of oral and subcutaneous doses would be administered and urine from females would be collected and assayed for corticosterone levels. This would provide an indication whether stress-induced hormonal events correlate with the observed results.

The results of the experiments in this thesis showed variability of number of implantation sites, hormone secretion, and protein expression within groups administered the same concentration of BPA. Endogenous hormonal levels may play a role in influencing the sensitivity to exogenously-administered steroids or steroid-mimicking substances. The influence of variations in adult hormonal cycling and baseline concentrations could also play a role in creating variability in sensitivity to endocrine-disrupting chemicals. By monitoring hormonal variability it could be determined whether individuals with greater natural variation in hormonal levels on a daily or cyclical basis show more or less sensitivity. Altering hormonal levels during development by chronic administration of low concentrations of steroid hormones or endocrine-disrupting substances in addition to manipulating social environments may provide insight into the mechanisms of the observed variability. This research would provide further insight into

the mechanisms of between- and within-individual variability and potentially explain how some individuals are more susceptible to the influence of different chemical toxicants.

The experiments reported in this thesis investigated a small subset of variables known to be imperative for successful implantation. Intrauterine blastocyst implantation is a highly complex process with numerous other factors that play critical roles which could also be examined. For instance, the expression of leukemia inhibitory factor (LIF), a member of the interleukin-6 family of cytokines, is known to be regulated by estradiol (Chen *et al.*, 2000) and has been found to be essential for implantation (see review by Cheng *et al.*, 2002). Endocrine-disrupting chemicals such as PCBs and phyto-estrogens have been previously shown to influence LIF synthesis in human and bovine oviduct cells (Reinhart *et al.*, 1999), however the influence of BPA on LIF synthesis during the peri-implantation period in inseminated female mice has yet to be fully investigated. Other molecular factors that have been associated with successful implantation, such as the production of prostaglandins via the COX pathway (see review by Dey and Lim, 2006) and expression of integrins and HOX genes (Lessey *et al.*, 1994; Taylor *et al.*, 1994), could also be explored to establish the mechanisms by which BPA exposure leads to implantation failure.

Conclusion

The data presented in this thesis provide support for the weak estrogenic properties of BPA. For the first time, BPA exposure solely during the peri-implantation period was shown to have detrimental effects on pregnancy outcome, even following a

single exposure. BPA was also found to alter hormonal output, uterine morphology and steroid receptor expression. Doses at or below the calculated acceptable exposure level of 50 μg/kg/day did not produce any measureable effects. These data suggest that BPA is exerting its effects by binding to nuclear estrogen receptors. However, the results from the protein expression experiments imply that the BPA-bound estrogen receptor may not be inducing similar increases in gene transcription typically associated with the binding of estradiol. Continued investigation into the mechanisms of the observed effects of peri-implantation exposure to BPA is needed as humans and wildlife continue to be exposed through its use in commercial and consumer goods.

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