EFFECTS OF EXPOSURE TO THE PHTHALATE SUBSTITUTE ACETYL TRIBUTYL CITRATE IN FEMALE CD-1 MICE

by

Lindsay Marie Rasmussen Vance

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DEDICATION

I lovingly dedicate this thesis to my family, who supported me every step of the way. You mean the world to me; I will never be able to thank you enough.
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CHAPTER 1 - INTRODUCTION

Endocrine disruptors

Endocrine disrupting chemicals (EDCs) are exogenous substances that interfere with the endocrine system by causing tumors, birth defects, reproductive disorders, and other developmental disorders (Magnusson & Persson. 2015). There are many reports of adverse effects on reproduction in multiple species including humans. These chemicals have been shown to have negative effects on spermatogenesis (Veeramachaneni. 2008), cryptorchidism (Virtanen & Adamsson. 2012), onset of puberty (Magnusson & Ljungvall. 2014), fetal development (McLachlan, et al. 2012), sexual behavior (Frye, et al. 2012), and antral follicle growth and development (Craig, et al. 2013), (Craig, et al. 2014). EDCs include a wide variety of chemicals that are used for industrial manufacturing (Jenardhanan, et al. 2016), agriculture, and cattle feeding operations (Bartelt-Hunt, et al. 2012).

Plasticizers

Plasticizers are usually low-melting solids or high-boiling liquids which are used to impart flexibility and durability to a plastic product (Lioy, et al. 2015). Worldwide, more than six million tons of plasticizers are produced and consumed each year (Lioy, et al. 2015). Phthalates are a class of chemicals used widely in industry as a plasticizer or solvent (Ohta, et al. 2003),(Kavlock, et al. 2002c). Phthalates and plasticizers like bisphenol A (BPA) are found in many consumer products, such as beauty products (nail polish, perfume, soaps) (CDC, 2005), oral medications (Hernandez-Diaz, et al. 2009), medical devices (disposable
gloves, blood bags) (Department of Health and Human Services 1998), dental sealants, food and beverage containers (Dodson, et al. 2012), and children’s care products and toys (Jahnke, et al. 2000), (Kavlock, et al. 2002a, Kavlock, et al. 2002b, Kavlock, et al. 2002c). The excessive worldwide production and presence of plasticizers pose an immediate risk for exposure through ingestion, inhalation, intravenous, and dermal contact (CDC, 2005). Epidemiological studies have shown evidence which supports continual daily exposure in humans through detectable levels of phthalates in more than 75% of spot urine samples (CDC, 2005). Over 95% of adult urine samples tested showed detectable estrogenic activity induced by BPA (Calafat, et al. 2005). Women are reported to have higher urinary phthalate levels than men (CDC 2005), while other studies have shown an association to phthalate exposure and increased miscarriage rates (Aldyreva, et al. 1975) and increased pregnancy complications (Tabacova. 1986). Therefore, it is important to not only remove these chemicals from products, but also replace them with safer alternatives.

**Acetyl tributyl citrate**

Citric acid is widely used in food, beverages, household cleaners, pharmaceuticals, and the plasticizer industry (Lioy, et al. 2015). When citric acid is combined with alcohols of varying lengths, the resulting citrate esters can be used as solvents and plasticizers. One of the resulting citrate esters is acetyl tributyl citrate (ATBC), which is a commercially available and approved plasticizer for food contact substances by the FDA (CDC, 2005), (Goulas, et al. 2007). ATBC is commonly used as a plasticizer in food-grade polyvinyl chloride (PVC) cling-
film (Goulas, et al. 2007), (Heath & Reilly. 1981). ATBC is a clear, colorless, high-boiling liquid (172 C at 1 mm Hg) which is miscible in most organic solvents (Heath & Reilly. 1981). Over the last ten years the use of ATBC as an alternative to phthalates in children’s toys, cosmetic products, and plastics has increased dramatically (Lioy, et al. 2015), (Takeshita, et al. 2011). Even though ATBC commercial use has greatly increased, there is very limited research available on the female reproductive effects of ATBC. There are currently no studies that have reported the low-dose reproductive effects on the ovary from oral exposure to ATBC. It is important to understand how exposure to ATBC could directly affect the ovary, an organ crucial for normal reproductive function in females.

**Infertility**

Ovaries are the female reproductive organs which age most quickly when compared to other reproductive tissues like the uterus, pituitary gland, or mammary glands (Amanvermez & Tosun. 2016). As age-specific decline occurs, both the quality and number of oocytes in the ovaries decrease. As this progressive decrease occurs, a point is reached at which no more viable offspring may be produced and ovarian steroid production ceases; this stage in a woman’s life is known as menopause (Amanvermez & Tosun. 2016). Age-related decline in female fertility is associated with a reduced chance of conception and an increased probability of spontaneous abortion (Guyton & Hall. 2006), (Hall & Guyton. 2011). Along with aging, exposure to exogenous agents like EDCs, can cause infertility. EDCs have the ability to interfere with normal reproductive function by interfering with hormone signaling and altering the availability of
ovarian hormones (Craig, et al. 2011), thus any substance destined to replace EDCs should be devoid of this activity.

**Ovary**

The paired endocrine glands known as the ovaries, contain germinal epithelium, which form female gametes. The ovary functions as both an endocrine gland and gonad. The ovaries consist of five layers: the external layer known as the epithelium, which covers the tunica albuginea, followed by the theca externa cells, theca interna cells, and finally, granulosa cells (Guyton & Hall. 2006). During the female reproductive lifespan ovarian follicles mature, produce ovarian sex steroids, and become candidates for ovulation (Brown & Stubbs. 1983a).

**Primordial germ cell development**

The functional units of the mammalian ovaries are ovarian follicles ([Figure 1.1](#)), each individual follicle consists of an ovum (egg) surrounded by epithelioid granulosa cells, which form a primordial follicle (Brown & Stubbs. 1983a). Before birth, primordial follicles are produced from a pool of primordial germ cells (PGCs), which can progress through mitotic divisions with incomplete cytokinesis, resulting in interconnected oogonia (Brown & Stubbs. 1983a). After mitotic divisions cease, the germ cells enter meiosis I and progress through the first few stages of prophase I before undergoing cell arrest. The cohorts of germ cells then begin to breakdown, resulting in most of the oocytes being lost through
apoptosis, which is programmed cell death (Espey. 1994). The remaining oocytes are surrounded by a layer of somatic pre-granulosa cells, the cells then become primordial follicles during mid-gestation (Guyton & Hall. 2006). Females are born with a non-renewable primordial follicle ovarian reserve which contains about 500,000 follicles in each ovary, decreasing in number to about 300,000 total follicles by sexual maturity as a result of cell death known as atresia (Brown & Stubbs. 1983a).

**Folliculogenesis**

The maturation of ovarian follicles from primordial into pre-ovulatory follicles is known as folliculogenesis (Figure 1.1). Each follicle consists of an oocyte surrounded by granulosa cells and theca cells (Guyton & Hall. 2006). Follicular stage is characterized by the number of layers of granulosa cells surrounding the oocyte and the overall size of the follicle. Primordial follicles, the smallest and most immature of all ovarian follicles, contain an oocyte arrested in meiosis, are surrounded by a single layer of flattened granulosa cells, and make up the female ovarian reserve (Brown & Stubbs. 1983b). After recruitment, the oocyte secretes growth differentiation factor 9 (GDF-9), which signals the proliferation of granulosa cells (Dong, et al. 1996). The signaling mechanism for primordial follicle recruitment into folliculogenesis is not well understood, but granulosa cell proliferation is crucial for follicular growth, development, and steroid production (Dong, et al. 1996). The subsequent stages of folliculogenesis are categorized as either primary, secondary, or antral follicles. A primary follicle is characterized by a complete layer of cuboidal granulosa cells enclosed by a
basal lamina (Pedersen & Peters. 1968). Secondary follicles have two or more layers of granulosa cells and the initial deposition of zona pellucida material.

Antral follicles are the largest and most mature of all ovarian follicles categorized by the fluid-filled cavity called the antrum (Aerts & Bols. 2010), (Pedersen & Peters. 1968). Antral follicles are the only follicles which can ovulate during the later stages of maturation and are often referred to as pre-ovulatory follicles (Brown & Stubbs. 1983b).
Figure 1.1: Ovarian follicular development. Primordial follicles, the most immature follicle, contain an oocyte (yellow) surrounded by a single layer of squamous granulosa cells (red). Oocytes are arrested in meiosis I and are finite in number; a female is born with her lifetime supply. Primordial follicles are recruited for follicular development, they then grow and progress through folliculogenesis. During growth, the oocyte increases in size and acquires multiple layers of granulosa cells as well as theca cells (green) which are found in the outermost layers of the follicle. The vast majority of recruited follicles die by atresia. In mono-ovulatory animals a single follicle becomes the dominant follicle and the only candidate for ovulation.
Hypothalamic-pituitary-gonadal axis

The hypothalamic-pituitary-gonadal axis (HPG axis) refers to the hypothalamus, pituitary gland, and gonadal glands working in conjunction as if they were a single entity. The endocrine glands work together to control the fluctuations in hormones that assert developmental and regulatory effects on the body’s reproductive system. Gonadotropin-releasing hormone (GnRH) is secreted by GnRH expressing neurons in the hypothalamus (Brown & Stubbs. 1983b). Following secretion from the hypothalamus, GnRH travels down to the anterior pituitary via the hypophyseal portal system and binds a gonadotropin-releasing hormone receptor (GNRHR). This action stimulates (Guyton & Hall. 2006) the anterior pituitary to release luteinizing-hormone (LH) and follicle-stimulating hormone (FSH) into the bloodstream which then cause profound changes in developing ovarian follicles which also influences 17β-estradiol production (Tng. 2015). When the threshold of estradiol is reached, the LH surge then occurs which triggers ovulation. LH is also responsible for causing luteinization which causes the residual granulosa and theca cells that remain after ovulation to form a corpus luteum (Guyton & Hall. 2006). FSH regulates the recruitment and maturation of ovarian follicles (Brown & Stubbs. 1983a) and is a major factor in preventing apoptosis in maturing ovarian follicles. FSH is enhanced by the protein complex activin and suppressed when the dominant recruited follicle produces enough estradiol and inhibin to lower serum FSH levels (Guyton & Hall. 2006).
Monthly ovarian cycle

The normal female reproductive years are characterized by monthly rhythmic changes in sex steroid secretion and the corresponding physiological changes in the ovaries and other sexual organs, referred to as the menstrual cycle (Guyton & Hall. 2006). The menstrual cycle duration averages 28 days; some variation in cycle length is normal, but regular abnormalities in cycle length are often associated with decreased fertility. The menstrual cycle is divided into two phases: the follicular phase, which includes follicle growth and ovulation, and the luteal phase, which encompasses luteinization of the granulosa and theca cells of the ruptured follicle followed by the regression of the corpus luteum (Guyton & Hall. 2006). Ovulation is the rupture of a pre-ovulatory follicle resulting in the release of an oocyte from the ovaries and is essential for mammalian reproduction. For ovulation to occur, normal ovarian tissue must rupture to allow the expulsion of the oocyte (Boots & Jungheim. 2015). The changes that take place in the ovary rely on the gonadotropic hormones, FSH and LH, which are secreted and regulated by the anterior pituitary gland through a feedback mechanism involving hormones secreted by the ovary (Brown & Stubbs. 1983a). In a normal menstrual cycle a single ovum is released from the ovaries on the 14th day of the cycle, an event which marks the end of the follicular phase. During the luteal phase the oocyte will either be fertilized by spermatozoa or be expelled along with the shedding of the uterine lining known as menses (Brown & Stubbs. 1983b). The shedding of the uterine lining marks the end of the luteal phase, the follicular phase then begins again. The menstrual cycle continues in a cyclical fashion until the female goes through menopause, which results from a lifelong
continuous process of follicular atresia and a lack of remaining primordial follicles (Jones & DeCherney. 2005).

In rodents, the reproductive cycle is called the estrous cycle which lasts approximately 4-5 days (Caligioni. 2009). Rodent’s short cycle length makes them an ideal animal model for investigating changes that occur during the reproductive cycle. Vaginal smear cytology (Figure 1.2) is used to determine the current stage of the estrous cycle through observing types of cells present in the vaginal smear (Long & Evans. 1922). Three types of cells observed in vaginal smears are nucleated epithelial cells, cornified epithelial cells, and leukocytes. The full estrous cycle in mice can be divided into four stages: proestrus, estrus, metestrus, and diestrus. During proestrus, the cytological smear is dominated by nucleated epithelial cells, which may appear in clusters or individually (Walmer, et al. 1992). Proestrus corresponds to the pre-ovulatory day, when estradiol concentrations are increasing, with the LH and FSH surge following and triggering ovulation (Parkening, et al. 1982). During estrus, metestrus, and diestrus, plasma concentrations of LH and FSH remain low (Parkening, et al. 1982). Estrus is characterized by the dominant presence of cornified epithelial cells with no visible nucleus, which appear in clusters. The shape of the cells is irregular with granular cytoplasm. During estrus, estradiol remains elevated but falls back to basal levels towards the end of the phase (Walmer, et al. 1992). Vaginal cytology during metestrus shows a mix of the three cell types with a predominance of leukocytes. This phase is characterized by low plasma estradiol concentrations (Walmer, et al. 1992). During diestrus, the cytological smear is dominated by leukocytes which signal the increase in progesterone and the
decrease in estradiol concentration (Walmer, et al. 1992). The leukocytes may be clustered or individually spaced during diestrus.

**Figure 1.2: Vaginal cytology.** Proestrus (P) is characterized by the predominance of nucleated epithelial cells. Estrus (E) is characterized by the cornified squamous epithelial cells throughout the slide. Metestrus (M), also known as diestrus 1 consists of a mixture of the three cells types. Diestrus (D) is dominantly leukocytes.
CHAPTER 2 – IN VITRO EXPOSURE TO ATBC

Abstract

Some plasticizers are endocrine-disrupting chemicals that cause reproductive toxicity in both males and females. Several chemicals already approved by the Food and Drug Administration have been proposed as substitutes for some of these plasticizers, one example is acetyl tributyl citrate (ATBC). However, no studies have tested whether ATBC causes direct toxicity to the ovary. Ovarian antral follicles are essential for female fertility because they are the major producers of ovarian steroids and are the only follicle type that can ovulate in response to gonadotropin stimulation. Previous studies have used in-vitro ovarian follicle culture as a screening tool to demonstrate that EDCs can cause direct ovarian toxicity. Therefore, we designed this study to test whether exposure to in vitro treatment with ATBC causes ovarian toxicity in CD-1 mice. We mechanically isolated antral follicles from the ovaries of adult CD-1 mice (35-39 days old) and individually exposed them (n=5 cultures with n≥8 follicles per treatment) to supplemented media alone (NT), dimethyl sulfoxide (DMSO, vehicle for ATBC), and ATBC (0.001-100 µg/mL) for 24-72 h. Follicle growth and survival were monitored by measuring follicle diameter and cytotoxicity (compromised membrane integrity; CellTox Green) every 24 h, and assessing number of metabolically active cells (ATP concentration; Promega CellTiterGlo) at the end time point. The DNA synthesis inhibitor hydroxyurea (HU, 100 mM) was used as a positive control for the viability assays. Exposure to ATBC did not affect the ability of antral follicles to increase their diameter over time at all concentrations tested. When stratified by growth pattern, there was not a significant difference in the proportion of follicles growing normally, growing slowly, or not growing following ATBC exposure at all concentrations tested.
ATBC treatment did not cause compromised membrane integrity and did not inhibit ATP production at any of the concentrations tested. The positive control, HU, inhibited follicle growth (24-72 h), decreased follicle cell membrane integrity (72 h), and inhibited ATP production (24-72 h).
MATERIALS AND METHODS

Reagents

Acetyl tributyl citrate (ATBC, CAS 77-90-7), dimethyl sulfoxide (DMSO), ITS (insulin, transferrin, selenium), penicillin/streptomycin, hydroxyurea (HU), were obtained from Sigma-Aldrich (St. Louis, MO). Alpha-minimal essential media (α-MEM) was obtained from Life Technologies (Grand Island, NY). Human recombinant follicle-stimulating hormone (rFSH) was obtained from Dr. A.F. Parlow from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA) and charcoal-stripped fetal bovine serum (cFBS) was obtained from Life Technologies (Carlsbad, CA).

Animals

Animals were obtained from Charles River Laboratories (Charles River, CA). Animals were housed in single-use BPA-free Innovive Disposable Rodent Caging (IVC) at the University of Arizona Central Animal Care Facility. Animals were subjected to 12L: 12D cycles, water and food were provided ad libitum, and temperature was maintained at 22±1°C. Animals were allowed to acclimate for at least 24 h before handling. The animals were used for in vitro ovarian follicle culture. All experiments and methods involving animals conformed to protocols approved by The Institutional Animal Care and Use Committee (IACUC) at the University of Arizona. 58-75

For the in vitro ovarian follicle culture experiment cycling female CD-1 mice (age 28-34 days) were euthanized by carbon dioxide inhalation followed by
cervical dislocation. The ovaries were excised and antral follicles were mechanically isolated and subjected to culture as described.
Antral follicle culture

Ovaries were removed, trimmed of fat, and antral follicles were mechanically isolated based on relative size (diameter ≥ 200 µm) using watchmaker forceps and placed in culture (Craig et al. 2013). Follicles were isolated from 3-4 mice per culture with approximately 15–18 antral follicles obtained from each ovary. Each follicle culture experiment contained a minimum of 8 follicles per treatment. Following isolation, antral follicles were placed individually in Costar® Assay Plate 96 Well White with Clear Flat Bottom Tissue Culture Treated plates with 75 µL of unsupplemented α-MEM prior to treatment. Treatment groups included six concentrations of ATBC (0.001, 0.01, 0.1, 1, 10 and 100 µg/mL), a vehicle control consisting of DMSO (0.075%), and a positive control of Hydroxyurea (HU 100 mM).

Figure 2.1: Antral follicle culture system. Ovaries are excised from female CD-1 mice age 35-39 days old and antral follicles (220-350µm) are collected and individually plated in a 96 well plate in either vehicle (DMSO), 0.001-100µg/mL ATBC, or the positive control (HU). Follicles are then measured under an inverted light microscope and membrane integrity is evaluated every 24 h. ATP production assay is run at the end time point.
ATBC treatments

A stock solution of ATBC was prepared using dimethyl sulfoxide (DMSO) as a solvent. Various stock concentrations (133.33, 13.33, 1.33, .133, .0133, and .00133 µg/mL) were prepared to ensure that an equal volume would be added to culture wells for each treatment group to control for solvent concentration. Final concentrations of ATBC in culture were 0.001, 0.01, 0.1, 1, 10, 100 µg/mL. These doses were selected for this study due to the insufficient research on low dose ATBC exposure and also to develop a dose-response. All dosing solutions were prepared individually in supplemented α-MEM, an equal volume of chemical was added to each dose to control the amount of vehicle in each preparation. Supplemented α-MEM contained 5% FBS, 1% ITS (10 ng/mL insulin, 5.5 ng/mL transferrin, 5.5 ng/mL selenium), 100 U/mL penicillin, 100 mg/mL streptomycin, and 5 IU/mL human rFSH. For treatment, unsupplemented media was replaced with 150 µL supplemented α-MEM containing vehicle or ATBC at the various concentrations. Follicles were then incubated for 24, 48, and 72 h at 37°C in 5% CO₂.

Analysis of follicular growth and viability

Antral follicles were cultured as described above for 24, 48, and 72 h. Follicles were examined at 24 h intervals under an inverted light microscope equipped with a calibrated ocular micrometer. Follicle growth was assessed by measuring the average of the horizontal and vertical diameter of each follicle as described previously (Craig et al. 2013). Antral follicles were considered as those having average diameters of 220 µm or greater (Cortvrindt and Smitz, 2002).
which correlates with the histological appearance of these follicles. At least five separate culture experiments were performed for each chemical treatment. Follicle diameter measurements were converted to percent change relative to 0 h and averaged among treatment groups. The resulting data were compared between the chemical treatments over time. Inhibition of growth by HU (positive control) was used as a positive control for the assay.

**Cytotoxicity Assay**

Cytotoxicity was evaluated using commercially available assays which measure any change in membrane integrity that allows fluorescent dye to enter dead cells. The dye was added to each individual well at the beginning of the experiment (0 h) and follicle toxicity was measured every 24 h using the Promega CellTox™ Green Cytotoxicity Assay\(^{(a)}\) per manufacturer’s instructions. An endpoint fluorescence detection protocol for 96-well plates was performed using a Biotek Synergy H\(_1\) microplate monochromator reader with an excitation of 480 nm and emission of 520 nm from above at a read height of 7 mm.

**Cell Viability Assay**

The Promega CellTiter-Glo® Luminescent Cell Viability Assay was used to determine the number of viable cells in culture based on quantitation of ATP present signaled by luminescence. The assay requires cell lysis to produce a luminescent signal proportional to the amount of ATP present in culture, due to required cell lysis the CellTiter-Glo® Luminescent Cell Viability Assay was performed at each culture plate endpoint (24, 48, or 72 h) per the manufacturer’s instructions. The assay was performed using the Biotek Synergy H\(_1\) microplate monochromator reader from above at a read height of 1 mm with an integration time of 1.00 second.
**Statistical analysis**

Growth pattern, growth rate, follicle cytotoxicity, and follicle viability data were analyzed using SPSS Statistics 22 software (IBM, Chicago, IL). Growth pattern analysis data was analyzed using a Chi-square nonparametric test. Growth rate and follicle cytotoxicity data were analyzed using a General Linear Model (Repeated Measures) test. Follicle viability data were analyzed using a General Linear Model (Univariate) test. For all comparisons, statistical significance was assigned at \( p \leq 0.05 \) and marked using asterisks (*) on their respective graphs.
RESULTS

Effect of ATBC treatment on isolated antral follicle growth

The purpose of this experiment was to analyze isolated antral follicle growth in culture following treatment with varying concentrations of ATBC. We hypothesized that in vitro treatment with ATBC will not inhibit antral follicle growth in culture. Follicles treated with vehicle (DMSO) and ATBC at 0.001, 0.01, 0.1, 1, 10, and 100 µg/mL grew normally throughout each culture period without significant differences in growth over time (P≤0.05; Figure 2.2). Follicles treated with 100 mM HU were unable to grow over time and were significantly smaller than vehicle-treated (DMSO) and ATBC treated follicles for all cultures 24-72 h (Figure 2.2). A growth pattern analysis was used to determine individual follicle growth and categorize growth as a normal increase in growth, decreased growth, or no growth when compared to follicles treated with the control (Figure 2.3). The analysis confirms there was not an over-compensation of healthy follicles keeping the mean growth percent changes normal. The growth pattern analysis provides insight on the individual follicle’s growth pattern behavior. The growth pattern analysis indicated that all concentrations of ATBC did not differ significantly in number of normal, decreased growth, and no growth follicles from the vehicle (DMSO). The positive control (HU) was significantly decreased for normal growth follicles and significantly increased for no growth follicles when compared to vehicle (DMSO) and all ATBC treatments.
**Figure 2.2: Follicle growth.** Follicle growth data were normalized to diameter at 0 hours and are expressed as mean percent change ± SEM (n=5 separate experiments with ≥8 follicles per treatment). Data were analyzed using General Linear Model Repeated Measures tests with significance set at p≤0.05. Asterisks (*) indicate significantly different from control (p≤0.05).

**Figure 2.3: Growth pattern analysis.** Growth pattern analysis data were normalized to follicle diameter and are expressed as a proportion of total follicles per treatment (n=5 separate experiments with ≥8 follicles per treatment) at 72 h. Data were analyzed using Chi-square test with significance set at p≤0.05. Asterisks (*) indicate significantly different from control (p≤0.05).
**Effect of ATBC treatment on isolated antral follicle cytotoxicity**

The purpose of this experiment was to determine whether exposure to ATBC induces cytotoxicity by altering mouse antral follicle membrane integrity. Following culture, each follicle was individually subjected to measurement of cytotoxicity using the CellTox™ Green Cytotoxicity Assay (a) as described in the Materials and Methods section. Antral follicles treated with vehicle (DMSO) and ATBC (0.001-100 µg/mL) did not show a significant increase in cytotoxicity for any of the time points tested (**Figure 2.4**). Follicles treated with 100 mM HU showed significant increase in cytotoxicity at 72 hours (**Figure 2.4**).

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**Figure 2.4: Follicle cytotoxicity.** Cell membrane integrity data were normalized to follicle diameter and are expressed as mean normalized fluorescence ± SEM (n=4 separate experiments with ≥8 follicles per treatment). Data were analyzed using General Linear Model Repeated Measures tests with significance set at p≤0.05. Asterisks (*) indicate significantly different from control (p≤0.05).
**Effect of ATBC treatment on isolated antral follicle viability**

Quantitation of ATP present was used as an indicator of number of viable cells in culture. Following culture, each follicle was individually subjected to measurement of metabolically active cells using the CellTiter-Glo® Luminescent Cell Viability Assay as described in the *Materials and Methods* section. Follicles treated with vehicle (DMSO) and ATBC (0.001-100 µg/mL) showed no significant decrease in ATP production at all time points tested (**Figure 2.5**). Follicles treated with 100 mM HU showed a significant decrease in ATP production at 24, 48, and 72 hours (**Figure 2.5**).

![ATP Production Chart](image)

**Figure 2.5: Follicle ATP production.** ATP production data are expressed mean normalized luminescence ± SEM (n=5 separate experiments with ≥8 follicles per treatment). Data were analyzed using General Linear Model Univariate test with significance set at p≤0.05. Asterisks (*) indicate significantly different from control (p≤0.05).
CHAPTER 3 - *IN VIVO ORAL EXPOSURE TO ATBC*

Abstract

The purpose of this experiment is to evaluate the effects of oral exposure to ATBC in female CD-1 mice. For the *in vivo* experiments, the female mice (n=22; PND 81) were randomly divided into treatment groups and dosed according to daily body weight with one of the following treatments: corn oil (vehicle, n=7), 5 mg/kg/day ATBC (n=8), or 10 mg/kg/day (n=7) ATBC for 15 consecutive days. Vaginal smears were performed and analyzed daily to measure any change in estrous cyclicity. After the 15th day of dosing the female mice were introduced into an individually housed proven breeder male's cage. Daily body weight measurements continued and plug checks were performed every morning. Pregnancy and time to conception data did not statistically differ from the vehicle (oil) for all ATBC treatments (days to conception: vehicle 2.43 ± 0.65, 5 mg/kg/day ATBC 2 ± 0.33, 10 mg/kg/day ATBC 2.5 ± 0.22; gestation length: vehicle 19.71 ± 0.18, 5 mg/kg/day ATBC 20 ± 0.19, 10 mg/kg/day ATBC 20 ± 0.00). On the day of parturition the dams and pups were sacrificed; organ weight and gross morphology data was collected for: uterus, kidneys, adrenals, spleen, liver, and ovaries. The data analyzed includes: estrous cyclicity, pre-dosing body weight % gain, dosing body weight % gain, pregnant body weight % gain, organ weight, gestation length, litter size (live vs. dead), litter weight, implantation sites, time to conception, and pup sex ratio. Interestingly, there was an increase in spleen weight at the 5 mg/kg/day treatment when compared to vehicle control-treated spleen weights (spleen weight, Oil: n=7; 5 mg/kg ATBC: n= 8). Treatment with 5 mg/kg/day ATBC caused a significant decrease in average estrous cycle length in days compared to the pre-dosing average estrous cycle length. Animals exposed orally to 10 mg/kg/day ATBC showed a
significant decrease in total follicle number (10 mg/kg/day ATBC, 313 ± 20.37) when compared to vehicle (oil) treated mice (vehicle, 433.71 ± 34.85). Also treatment with 10 mg/kg/day ATBC resulted in significant reduction in secondary (101.67 ± 5.7) and late antral (4.17 ± 1.45) follicle numbers when compared to the vehicle-treated mice (secondary follicles: 141.14 ± 14.79, late antral follicles: 7.00 ± 1.54). ATBC treatment with 10 mg/kg/day showed a significantly decreased mean body weight percent gain during pregnancy on days 3, 5, and 14 when compared to animals treated with vehicle (oil), while animals treated with 5 mg/kg/day ATBC showed a significant decrease in weight gained on only day 13 when compared to the vehicle (oil). These novel findings show that ATBC could disrupt ovarian function in mice when exposed to low-dose ATBC.
MATERIALS AND METHODS

Reagents

Acetyl tributyl citrate (ATBC, CAS 77-90-7, phosphate buffered saline (PBS), and sodium hydroxide (NaOH) were obtained from Sigma-Aldrich (St. Louis, MO). Tocopherol-stripped corn oil was obtained from MP Biomedicals (vehicle, Solon, OH). Xylene, clarifier, hematoxylin, eosin, and permount were obtained from Fisher Scientific (Hanover Park, IL).

Animals

Animals were obtained from Charles River Laboratories (Charles River, CA). Animals were housed in single-use BPA-free Innovive Disposable Rodent Caging (IVC) at the University of Arizona Central Animal Care Facility. Animals were subjected to 12L: 12D cycles, water and food were provided ad libitum, and temperature was maintained at 22±1°C. Animals were allowed to acclimate for at least 24 h before handling. Three groups of animals were used to perform this experiment including males for breeding, naïve females for proving male fertility, and females used for in vivo oral exposure to ATBC and fertility assessment. All experiments and methods involving animals conformed to protocols approved by The Institutional Animal Care and Use Committee (IACUC) at the University of Arizona.

Prior to assessing the effects of ATBC exposure on female fertility, we performed an independent breeding study with the males to confirm their fertility status. To assess male fertility, male CD-1 mice (n=22, age 60 days) and cycling female CD-1 mice (n=22, age 60 days) were used. Males were housed with one
cycling female per cage, with pregnancy used as a marker to assess male fertility. After pregnancy was confirmed through plug checks and weight gain, the females used for proving male fertility were separated out on day 18 of gestation and housed individually. Following parturition the pups were euthanized by decapitation and dams were euthanized by carbon dioxide inhalation followed by cervical dislocation.

To assess the effects of oral exposure to ATBC on fertility cycling female CD-1 mice (n=22, age 60 days) were used for dosing and housed up to five mice per cage (separated by treatment group) prior to breeding at the University of Arizona Central Animal Care Facility. Post dosing females were then individually housed with a proven male breeder for breeding and until day 18 of gestation when they were separated out and housed individually. Animals were euthanized within 24 h of parturition (age 118-124 days) by decapitation under isoflurane sedation. The uterus, ovaries, kidneys, adrenals, spleen, and liver were collected from the adult dams.
ATBC dosing

On postnatal day 88, animals (n=22) were randomly assigned to receive tocopherol-stripped corn oil (vehicle; MP Biomedicals, Solon, OH) or ATBC (dissolved in vehicle, 99.6% purity, Sigma-Aldrich, St. Louis, MO) at 5 or 10 mg/kg/day. Animals were weighed and dosed daily for 15 consecutive days. All doses were administered orally by placing a pipette tip containing the dosing solution into the mouth past the incisors and into the cheek pouch. Doses were selected to approximate the migration from plasticized films into foods previously reported for ATBC (Castle et al. 1988) (Figure 3.1).

**Figure 3.1: In vivo experimental design.** Body weight and estrous cyclicity were monitored in female CD-1 mice (n=22; PND 81) for 20 days prior to dosing. Animals were then separated into three dosing categories: vehicle (oil), 5 mg/kg/day ATBC, and 10 mg/kg/day ATBC. Animals were dosed orally for 15 consecutive days, and then immediately placed with a proven male. Plug checks were performed for the first 10 days after being placed with a male while body weight was measured daily throughout the study. On the day of parturition the dams and pups were euthanized and data collected.
Body Weights

Daily body weight was monitored throughout the experiment and divided into three different categories: pre-dosing, dosing, and gestational. Body weight was measured daily in grams and used to assess overall animal health. Body weight percent gain was analyzed for each category and expressed as a percent change normalized to the first day in that category (Figure 3.1).

Estrous Cyclicity

Estrous cyclicity was monitored by daily vaginal cytology starting on postnatal day 60 and throughout the study (Figure 3.1). Vaginal cytology was assessed as previously reviewed [22], with minor modifications (Figure 1.2). Briefly, animals were restrained gently and 20µL of sterile-filtered PBS was used to perform a vaginal washing. Vaginal washings were placed on microscope slides and evaluated unstained under an inverted microscope without knowledge of treatment. Percentage of days in proestrus, estrus and metestrus/diestrus were determined by dividing the total number of days spent in each stage by the total number of days in the study period and multiplying that number by 100.

Fertility, gestation, and pup count

Fertility was determined through the ability to become pregnant and carry to term. Gestation data included days to conception, uterine implantation sites, and gestation length. Days to conception were determined by subtracting plug date from days accompanied by male. Gestation Length was determined as the
time from plug date to parturition. Implantation sites were determined using a 2% NaOH stain which allowed easy identification of uteri implantation sites. Live pup count and litter data were collected for all litters. Pup count is number of live pups born. Litter weight is the total weight of all pups.

**Follicle and corpora luteal counts**

Following euthanasia, two ovaries per animal used for the *in vivo* oral dosing study (n = 14-16 ovaries per treatment) were fixed and processed for histological classification and enumeration of ovarian follicles and corpora lutea. Briefly, ovaries were fixed in 4% formalin (2 hour fixation), transferred to 70% ethanol, and embedded in paraffin. Paraffin-embedded ovaries were serially sectioned at 5 µm thickness, mounted on glass slides, and processed for hematoxylin and eosin staining. Oocyte containing follicles with visible nuclear material and corpora lutea were counted on every 20th section without knowledge of treatment by two experienced individuals using criteria previously described [23]. Specifically, follicles were classified as primordial if they consisted of a single oocyte surrounded by a single layer of squamous granulosa cells, primary if the oocyte was surrounded by a single layer comprised of ≥50% cuboidal granulosa cells, secondary if the oocyte was surrounded by two or more layers of cuboidal granulosa cells and a theca layer, and antral if the oocyte was surrounded by multiple layers of cuboidal granulosa cells, theca cells and contained an antrum.
Gross organ morphology and organ weight

Gross organ morphology consisted of visual examination of organs upon euthanasia, consisting of uterus, kidneys, adrenals, liver, spleen, and ovary. The organs were then weighed and normalized to body weight on the day of euthanasia and compared as a mean percent of total body weight.

Statistical analysis

Organ weight, body weight, gestational and pup, fertility data, estrous cyclicity, follicle, and CL count data were analyzed using SPSS Statistics 22 software (IBM, Chicago, IL). Body weight (pre-dosing, dosing, and gestational), and estrous cyclicity data were analyzed using a General Linear Model (Repeated Measures) test. Organ weight, gestational and pup, fertility, follicle, and CL count data were analyzed using a One-way ANOVA test. Post hoc comparisons between vehicle and ATBC-treated groups were conducted using Dunnett’s or Mann–Whitney non-parametric test as appropriate. For all comparisons, statistical significance was assigned at p ≤ 0.05 and marked using asterisks (*) on their respective graphs.
RESULTS

Effect of oral exposure to ATBC on body weight

To determine if ATBC exposure causes any changes in body weight gain, we analyzed daily body weight as a percent change normalized from beginning time period body weight. The experiment was divided into three body weight time periods: prior to dosing with ATBC, dosing with ATBC, and gestational body weight percent gain. There were no differences in the percentage of weight gained during the pre-dosing or dosing between mice treated with oil (vehicle), 5 mg/kg/day ATBC, and 10 mg/kg/day ATBC (Figures 3.2 and 3.3). ATBC treatment with 10 mg/kg/day showed a significantly decreased mean body weight percent gain during pregnancy on days 3, 5, and 14 when compared to animals treated with vehicle (oil), while animals treated with 5 mg/kg/day ATBC showed a significant decrease in weight gained on only day 13 when compared to the vehicle (oil) (Figure 3.4). Interestingly, total body weight gain during gestation (day 20) did not differ from that of the control for all ATBC doses tested.

**Figure 3.2: Pre-dosing body weights.** Pre-dosing body weight data were normalized to each animal’s day 1 pre-dosing body weight and are expressed as mean body weight percent change ± SEM (n=22 with ≥7 animals per treatment). Data were analyzed using General Linear Model Repeated Measures test with significance set at p≤0.05.
Figure 3.3: Dosing body weights. Dosing body weight data were normalized to each animal’s day 1 dosing body weight and are expressed as mean body weight percent change ± SEM (n=22 with ≥7 animals per treatment). Data were analyzed using General Linear Model Repeated Measures test with significance set at p≤0.05.

Figure 3.4: Gestational body weights. Gestational body weight data were normalized to each animal’s day 1 gestation (plug date) body weight and are expressed as mean body weight percent change ± SEM (n=22 with ≥7 animals per treatment). Data were analyzed using General Linear Model Repeated Measures test with significance set at p≤0.05. Asterisks (*) indicate significantly different from control (p≤0.05).
Effect of oral exposure to ATBC on estrous cyclicity

To determine if oral ATBC exposure disrupts estrous cyclicity, we analyzed vaginal cytology daily and compared the average percentage of time that animals spent in each stage of the estrous cycle (proestrus, estrus, and diestrus) between treatments. There were no differences in the percentage of time spent in proestrus, estrus, and diestrus between mice treated with vehicle (oil), 5 mg/kg/day ATBC, and 10 mg/kg/day ATBC (Table 3.2). A significant difference between pre-dosing and during dosing average estrous cycle length was observed in mice treated with ATBC at 5 mg/kg/day. (Table 3.1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pre-Dosing</th>
<th>During Dosing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=7)</td>
<td>5.65 ± 0.34</td>
<td>5.17 ± 0.64</td>
</tr>
<tr>
<td>5 mg/kg ATBC (n=8)</td>
<td>5.97 ± 0.49</td>
<td>4.55 ± 0.27 *</td>
</tr>
<tr>
<td>10 mg/kg ATBC (n=7)</td>
<td>6.37 ± 0.61</td>
<td>5.26 ± 0.63</td>
</tr>
</tbody>
</table>

Table 3.1 - Average estrous cycle length in days

Estrus cycle phases are expressed as treatment group mean percent ± SEM. Data were analyzed using One-Way ANOVA and General Linear Model (Repeated Measures) tests with significance set at p≤0.05. Asterisks (*) indicate significantly different from control (p≤0.05).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pre-Dosing Proestrus Estrus Diestrus</th>
<th>During Dosing Proestrus Estrus Diestrus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=7)</td>
<td>22.68% ± 1.4% 11.97% ± 1.58% 52.91% ± 3.17%</td>
<td>21.9% ± 2.8% 13.33% ± 4.11% 50.48% ± 5.61%</td>
</tr>
<tr>
<td>5 mg/kg ATBC (n=8)</td>
<td>21.35% ± 2.65% 14.49% ± 2.87% 56.06% ± 1.9%</td>
<td>25.0% ± 3.7% 13.33% ± 2.18% 52.5% ± 5.41%</td>
</tr>
<tr>
<td>10 mg/kg ATBC (n=7)</td>
<td>21.86% ± 1.09% 10.84% ± 1.91% 56.68% ± 4.24%</td>
<td>22.42% ± 2.04% 7.62% ± 2.27% 54.29% ± 2.85%</td>
</tr>
</tbody>
</table>

Table 3.2 - Percent of time spent in each phase of the estrous cycle

Estrus cycle phases are expressed as treatment group mean percent ± SEM. Data were analyzed using One-Way ANOVA and General Linear Model (Repeated Measures) tests with significance set at p≤0.05.
Effect of oral exposure to ATBC on fertility

The purpose of this experiment was to determine whether oral exposure to ATBC affects overall female fertility. To measure effects of ATBC, we compared days to conception, gestation length, implantation sites, pup count, and litter weight as described in the Materials and Methods section. ATBC treatment at all dosages tested did not cause any statistical difference in days to conception, gestation length, implantation sites, pup count, and litter weight (Table 3.3-3.4).

Table 3.3 - Gestational and pup data

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days to Conception</th>
<th>Gestation Length</th>
<th>Litter Weight</th>
<th>Pup Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=7)</td>
<td>2.43 ± 0.65</td>
<td>19.71 ± 0.18</td>
<td>20.91 ± 1.18</td>
<td>12.29 ± 0.84</td>
</tr>
<tr>
<td>5 mg/kg ATBC (n=8)</td>
<td>2 ± 0.33</td>
<td>20 ± 0.19</td>
<td>22.09 ± 0.67</td>
<td>10.88 ± 1.46</td>
</tr>
<tr>
<td>10 mg/kg ATBC (n=7)</td>
<td>2.5 ± 0.22</td>
<td>20 ± 0.00</td>
<td>21.27 ± 1.28</td>
<td>12.71 ± 0.78</td>
</tr>
</tbody>
</table>

Gestational and pup data are expressed as treatment group mean ± SEM. Data were analyzed using One-Way ANOVA tests with significance set at p≤0.05.

Table 3.4 - Fertility data

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Corpora Lutea</th>
<th>Implantation Sites</th>
<th>Pup Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=7)</td>
<td>14 ± 0.87</td>
<td>13.29 ± 0.71</td>
<td>12.29 ± 0.84</td>
</tr>
<tr>
<td>5 mg/kg ATBC (n=8)</td>
<td>13 ± 0.76</td>
<td>13.25 ± 0.49</td>
<td>10.88 ± 1.46</td>
</tr>
<tr>
<td>10 mg/kg ATBC (n=7)</td>
<td>12.5 ± 1.12</td>
<td>13.43 ± 0.65</td>
<td>12.71 ± 0.78</td>
</tr>
</tbody>
</table>

Fertility data are expressed as treatment group mean ± SEM. Data were analyzed using One-Way ANOVA tests with significance set at p≤0.05.
Effect of oral exposure to ATBC on ovarian morphology: follicle enumeration

The purpose of this experiment was to identify any changes in ovarian morphology in animals orally dosed with ATBC. We classified and counted the ovarian follicles present in the ovaries of vehicle (oil) and ATBC treated mice to determine whether ATBC caused any disruptions in folliculogenesis and ovulation. Ovaries from vehicle (oil) and all ATBC treated groups had statistically similar overall percentages of follicles in each stage of folliculogenesis: primordial, primary, secondary, early antral, and late antral. Furthermore, ATBC treatment did not alter the number of corpora lutea present in the ovaries of treated mice (Table 3.5 and Figure 3.5). Interestingly, there was a significant reduction in secondary and late antral follicle number in animals treated with 10 mg/kg/day ATBC when compared to vehicle-treated mice. The reduction in secondary and late antral follicles lead to an overall decrease in total follicle number in mice treated with 10 mg/kg/day ATBC (313 ± 20.37) when compared to vehicle-treated (433.71 ± 34.85).

Table 3.5 - Ovarian follicular stage percentages

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Primordial</th>
<th>Primary</th>
<th>Secondary</th>
<th>EarlyAntral</th>
<th>LateAntral</th>
<th>Total Follicle Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil</td>
<td>26.05% ± 13.14%</td>
<td>35.47% ± 11.76%</td>
<td>32.54% ± 10.48%</td>
<td>4.31% ± 31.24%</td>
<td>1.61% ± 22.04%</td>
<td>433.71 ± 34.85</td>
</tr>
<tr>
<td>5 mg/kg ATBC</td>
<td>24.19% ± 12.8%</td>
<td>36.78% ± 15.04%</td>
<td>34.92% ± 12.62%</td>
<td>3.02% ± 23.37%</td>
<td>1.09% ± 30.19%</td>
<td>377.25 ± 40.08</td>
</tr>
<tr>
<td>10 mg/kg ATBC</td>
<td>25.61% ± 10.66%</td>
<td>36.37% ± 14.40%</td>
<td>32.48% ± 5.53%</td>
<td>4.21% ± 18.23%</td>
<td>1.33% ± 34.73%</td>
<td>313 ± 20.37 *</td>
</tr>
</tbody>
</table>

Ovarian follicular stage percentages data are expressed as treatment group mean ± SEM. Data were analyzed using One-Way ANOVA tests with significance set at p≤0.05.
Figure 3.5: Follicular stage count. Ovarian follicular stage count data are expressed as treatment group mean ± SEM. Data were analyzed using One-Way ANOVA and Mann-Whitney non-parametric tests with significance set at p≤0.05. Asterisks (*) indicate significantly different from control (p≤0.05) while hashtag (#) indicates a trend of p≤0.1.
Effect of oral exposure to ATBC on organ weight

To determine if oral exposure to ATBC causes adverse effects on organ weight, we examined and weighed each major organ during necropsy. The overall gross organ morphology showed no visible differences at any of the tested ATBC concentrations. Organ weight was normalized to body weight on the day of necropsy and compared as a mean percent of total body weight. No significant differences were seen in uterus, kidney, adrenals, liver, and ovary weight at 5 mg/kg/day and 10 mg/kg/day ATBC. There was a statistically significant increase in spleen weight for animals treated with 5 mg/kg/day ATBC which was not observed in vehicle (oil) or 10 mg/kg/day ATBC (Table 3.6).

Table 3.6 - Organ weights as a percentage of body weight

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Uterus</th>
<th>Kidneys</th>
<th>Adrenals</th>
<th>Spleen</th>
<th>Liver</th>
<th>Ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=7)</td>
<td>2.43% ± 0.13</td>
<td>0.95% ± 0.03</td>
<td>0.02% ± 0.002</td>
<td>0.35% ± 0.01</td>
<td>5.34% ± 0.24</td>
<td>0.07% ± 0.01</td>
</tr>
<tr>
<td>5 mg/kg/day ATBC (n=8)</td>
<td>2.39% ± 0.18</td>
<td>1.04% ± 0.04</td>
<td>0.02% ± 0.003</td>
<td>0.45% ± 0.03*</td>
<td>5.6% ± 0.22</td>
<td>0.07% ± 0.01</td>
</tr>
<tr>
<td>10 mg/kg/day ATBC (n=7)</td>
<td>2.27% ± 0.11</td>
<td>0.98% ± 0.04</td>
<td>0.024% ± 0.003</td>
<td>0.35% ± 0.01</td>
<td>5.69% ± 0.16</td>
<td>0.06% ± 0.01</td>
</tr>
</tbody>
</table>

Organ weight percentages data are expressed as treatment group mean ± SEM. Data were analyzed using One-Way ANOVA tests with significance set at p≤0.05.
CHAPTER 4 - DISCUSSION

Using in vitro methods followed by an in vivo oral dosing study we examined the effects of low-dose ATBC exposure in CD-1 mice. The in vitro antral follicle culture system used to analyze the effects of ATBC did not show a significant decrease in growth rate, percentage of growing follicles, membrane integrity, and ATP production. During the in vivo oral dosing we exposed adult female CD-1 mice to varying concentrations of ATBC, evaluated estrus cyclicity, fertility, time to conception, gestational characteristics, pup count, litter weight, body weight gain, ovarian follicle count, major organ weight and gross morphology. Specifically, we show that a significant decrease in secondary and late antral follicles was accompanied by a reduced number of total follicles in animals treated with 10 mg/kg/day ATBC. Treatment with 10 mg/kg/day ATBC showed a significantly decreased mean body weight percent gain during pregnancy on days 3, 5, and 14 when compared to animals treated with vehicle (oil), while animals treated with 5 mg/kg/day ATBC showed a significant decrease in percentage of weight gained on only day 13 when compared to the vehicle (oil). A significant increase in spleen weight was observed in animals treated with 5 mg/kg/day ATBC.

Although this is the first study evaluating the effects of low-dose ATBC on antral follicle growth and viability in vitro, other studies have reported detrimental effects in reproductive tissues when exposed to endocrine disruptors. In mice, endocrine disruptors like di-n-butyl phthalate (DBP), mono(2-ethylhexyl) phthalate (MEHP), di-2-ethylhexyl phthalate (DEHP), and BPA have been shown to disrupt cell cycle and apoptotic signaling in mouse antral follicles (Craig, et al. 2013), (Craig, et al. 2014), (Huo, et al. 2015), (Inada, et al. 2012). Previously
shown, exposure to brominated BPA in cell lines leads to decreased cell viability, alterations in MAPK signaling pathways, and G₂/M cell cycle arrest (Strack, et al. 2007). In the present studies, we used a novel approach by analyzing the effects of ATBC using growth pattern analysis, CellTox™ Green Cytotoxicity Assay (a), and CellTiter-Glo® Luminescent Cell Viability Assay. The in vitro antral follicle culture system used to analyze the effects of 0.001-100µg/mL concentrations of ATBC did not show a significant decrease in growth rate, percentage of growing follicles, membrane integrity, and ATP production at any of the concentrations tested. The positive control hydroxyurea (HU) caused significantly decreased antral follicle growth and viability in vitro.

The purpose of the in vivo experiment was to determine if oral ATBC exposure causes any changes in female CD-1 mouse folliculogenesis and fertility. We analyzed, classified, and counted ovarian follicles present in the ovaries of vehicle and ATBC treated mice. Surprisingly, we detected a significant decrease in number of secondary (101.67 ± 5.62) and late antral follicles (4.17 ± 1.44), which resulted in a total ovarian follicle count decrease in animals treated with 10 mg/kg ATBC (313 ± 20.38) when compared to the vehicle (433.71 ± 34.85). These findings suggest that oral dosing of ATBC interferes with folliculogenesis and total follicle count in CD-1 mice. The in vitro results and the in vivo results suggest ATBC toxicity targets different stages of folliculogenesis. It is likely that ATBC at 10 mg/kg/day causes disruptions in folliculogenesis by decreasing follicle numbers in secondary and late antral follicle stages.

A significant increase in spleen weight was observed in animals treated with 5 mg/kg/day ATBC when compared to animals treated with the vehicle (oil). Stress often occurs during toxicity studies which results in catecholamine release
and an increase in serum glucocorticoid concentrations. One of the downstream effects of these neuroendocrine signals includes altered spleen organ weight (Everds, et al. 2013). While no other studies have reported the effects of low-dose oral ATBC treatment on increased spleen weight, some studies have reported a dose-dependent inhibition of mammalian cell growth at concentrations as low as 30µg/mL ATBC (Mochida, et al. 1996). *In vitro* exposure to ATBC has been shown to have an ID$_{50}$ of 44.7±2.99 µg/mL in KB cells, 39.9±2.02 µg/mL in Vero cells, and 42.1±2.02 µg/mL in MDCK cells (Mochida, et al. 1996). ATBC has also been shown to induce CYP3A4 mRNA levels in human intestinal cells, which may alter metabolism of endogenous steroid hormones (Takeshita, et al. 2011). Other research shows that ATBC does not exert sex-hormonal activity when given orally or subcutaneously at 0.5 or 500 mg/kg ATBC (Ohta, M.). Perhaps, the susceptibility and response to ATBC insult varies greatly between the tissue, species, and system challenged. No changes were observed in pre-dosing and dosing animal body weight in all treatments of ATBC. There was a significant decrease in body weight percent change in animals treated with 10 mg/kg/day ATBC on days 3, 5, and 14 and animals treated with 5 mg/kg/day ATBC on day 13 in body weight during pregnancy as compared to vehicle-treated animals. The mice treated with 5 and 10 mg/kg/day ATBC overall body weight percent gain during pregnancy did not differ significantly from the control, meaning the animals were able to recover the difference of weight gained by the end of pregnancy.

The present study constitutes the first report of ATBC-induced effects on *in vivo* ovarian follicle numbers, and it is consistent with previous studies reporting disruptions in normal ovarian function following exposure to BPA
(Peretz, et al. 2011), DEHP (Gupta, et al. 2010), MEHP (Craig, et al. 2014), and DBP (Craig, et al. 2013). Together, the present findings suggest that ATBC may cause disruptions in folliculogenesis in the mouse ovary by altering the number of secondary and late antral follicles available for maturation. Future studies should aim to understand ATBC’s follicular stage selection and as well as alterations in spleen morphology and physiology. It is essential to understand the mechanisms by which this endocrine and follicular disruption is occurring, as it may provide insight which could aid in the treatment of infertility and other infertility associated diseases in women.
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