Spring 5-1-2010

The Effect of Exogenous Estrogens on Primordial Follicle Assembly in Vivo

Jenna Rose Karavan

The Effect of Exogenous Estrogens on Primordial Follicle Assembly *in Vivo*

A Capstone Project Submitted in Partial Fulfillment of the Requirements of the Renée Crown University Honors Program at Syracuse University

Jenna Rose Karavan

Candidate for B.S., Biochemistry
and Renée Crown University Honors

August 2010

Honors Capstone Project in _____Biochemistry_____

Capstone Project Advisor: _____
(Dr. Melissa Pepling)

Honors Reader: _____
(Dr. John Belote)

Honors Director:_____
Samuel Gorovitz

Date:_____

Abstract

During embryogenesis in the mouse, primordial germ cells develop, move to the genital ridge of the embryo, and form germline cysts as the ovary develops. The cells in these cysts are linked by intercellular bridges. The cysts then undergo a breakdown process which ultimately results in primordial follicles, each of which consists of a single oocyte surrounded by somatic cells called granulosa cells. During this cyst breakdown process, approximately one-third of the original oocytes become enclosed in primordial follicles, the amount of which is representative of the number of eggs a female will have available to her during her reproductive life, while the other oocytes die. It has already been evidenced that introducing environmental estrogens into the female mouse during one of three critical time periods in development can alter oocyte development, thus limiting the female's number of primordial follicles that can be used to reproduce. Because the cyst breakdown mechanism is conserved in other mammals; the mouse can be used as a model for studying germ cell development in humans, thus providing valuable insight into female reproductive disorders that may be caused by follicle depletion, such as primary amenhorrea and premature ovarian insufficiency. My area of research is to determine whether exogenous estrogens have an effect on cyst breakdown during the perinatal period. In order to accomplish this, neonatal mice were injected subcutaneously with two concentrations of three different estrogenic compounds on post-natal days 1-4 (PND1-4). These mice were dissected for their ovaries on PND5. After dissection, the ovaries were fixed, stained with an oocyte marker, and observed using a laser-scanning confocal microscope. The images were analyzed to characterize and quantify the follicles within the ovaries, and the results were compared to see if there was a difference between the amount of cyst breakdown and the progression of follicle development between the experimental groups and the control group. Additionally, materials that were used to prepare the injections of exogenous estrogens were separately tested on mice in order to test the effectiveness of the injection materials.

TABLE OF CONTENTS

Acknowledgments

There are many individuals and programs without whose support the completion of this project would not have been possible.

Firstly, I would like to thank Dr. Melissa Pepling for her faith in me to design and complete such a challenging task and also for her guidance and patience in the actual completion of my thesis. Her astute knowledge and diligence are truly admirable.

Secondly, I would like thank Dr. John Belote not only for serving as my second reader, but also—in collaboration with Dr. Larry Wolf—for helping me to develop my public speaking abilities and overall professionalism.

I would also like to extend my gratitude toward The Renée Crown University Honors Program, the Department of Biology, and the Department of Chemistry at Syracuse University for their continuous support and helpful advice.

Finally, I would like to thank the Ruth Meyer Scholarship Program for its generous financial support, which has sustained my project and made it a reality.

Introduction

One crucial area of study within developmental biology is reproduction. Research within the realm of mammalian reproduction is especially significant since it pertains to reproductive disorders in human beings, the incidence of which has increased significantly among women in recent years in the United States: from 6.7 million in 1995 (Stephen and Chandra, 1995) to 7.3 million in 2002 (Chandra *et al.,* 2006). Scientific research to thoroughly understand biological mechanisms of mammalian female reproduction is still in progress, and these research attempts include further investigation of germ cell development. Recently, the key components of oocyte development have been identified. Through their research on mice (*Mus musculus),* Pepling and Spradling determined that the mechanism of germline cyst formation, in which synchronously dividing cysts form germ cells that are connected by intercellular bridges, is conserved in mice, as it has already been observed in invertebrates (Pepling and Spradling, 1998). Through a combination of cell separation and germ cell death, or apoptosis, one-third of the oocytes in these germ line cysts survive to become enclosed in primordial follicles, each of which consists of a single oocyte surrounded by a single layer of flattened granulosa cells. This follicle pool is incredibly important for fertility because its population represents the total amount of oocytes that will be available to a female during her reproductive lifetime. Because this population is determined at birth, understanding the biological mechanisms for primordial follicle assembly is critical to understanding mammalian reproduction. In addition to elucidating the

components involved in normal follicle assembly, it is important to determine if and how external influences affect this process since some female reproductive disorders involve the depletion of this pool of follicles. Two such female reproductive disorders are primary amenorrhea and premature ovarian insufficiency (POI) (Pepling, 2006). Primary amenorrhea is defined as an absence of menarche in females of at least 16 years of age who have developed normal secondary sex characteristics (Master-Hunter and Heiman, 2006). Primary ovarian insufficiency is marked by a premature depletion of ovarian follicles or arrested folliculogenesis (menopause) in females age 40 and younger (Beck-Peccoz and Persani, 2006). Hopefully, continued research on primordial follicle assembly and cyst breakdown will shed light on the prevention and treatment of reproductive disorders caused by follicle depletion.

The primordial follicle stage represents the first phase of mammalian folliculogenesis, the process by which the ovarian follicle matures. After the primordial follicle pool is established, follicular growth progresses. The primordial follicle is stimulated by lutenizing hormone (LH) to begin the maturation process. The granulosa cells surrounding the oocyte then develop into cuboidal granulosa cells. When an oocyte is entirely surrounded by these cuboidal cells, it is in a primary follicle. The oocyte of the primary follicle enlarges, and the cuboidal granulosa cells undergo mitosis to produce multiple layers of granulosa cells, which characterize a secondary follicle. In this stage, the oocyte is enveloped by the zona pellucida, an extracellular matrix containing special proteins which allow subsequent penetration by a sperm. Also, a layer of

theca cells forms from interstitial stroma cells and surrounds the proliferating granulosa cells. This theca layer is vascularized by a network of capillary vessels which deliver influential endocrine factors to the follicle. (van der Hurk and Zhao, 2005). Due to apoptosis of either the granulosa cells or the oocyte, most of the follicles undergo atresia, or follicle death, at this point (Hsu and Hsueh, 2000). A follicular antrum full of fluid then forms in the follicle, causing this antral follicle to significantly increase in size. This antrum contains growth-supporting proteins and hormones. At this point in folliculogenesis, the antral follicle's development is reliant on follicle-stimulating hormone (FSH). The antral follicle continues to grow and develop until a surge of lutenizing hormone (LH) during the preovulatory stage. Stimulation by LH causes the fully grown preovulatory follicle to resume meiosis and pause again in metaphase II. Then ovulation occurs: the follicle ruptures, and the oocyte is released from the ovary, where it waits to be fertilized. Sperm penetration triggers an activation stimulus that instigates the completion of meiosis and the beginning of embryonic development. The granulosa cells remaining in the empty follicle become the corpus luteum, which secretes the estrogen and progesterone needed to maintain the endometrium of the uterus during pregnancy. If fertilization does not occur within fourteen days, the corpus luteum degenerates into the corpus albicans, which may leave scar tissue in the ovary (van der Hurk and Zhao, 2005). Abnormalities in follicle development may also contribute to female reproductive disorders, such as polycystic ovary syndrome (PCOS). In PCOS, an excessive amount of follicles exhibit prolonged survival and enter the growth phase of

folliculogenesis but arrest in the antral stage, thus causing anovulatory infertility, a large accumulation of unovulated follicles in the ovary, and hormonal imbalance (Franks and Hardy, 2010). Thorough knowledge of follicle development could provide insight into follicle depletion and follicle arrest and continues to be an active area of scientific research.

Figure 1 describes a timeline of primordial follicle assembly. Primordial follicles form from primordial germ cells (PGCs), or oocyte precursors, through the process of cyst breakdown. At approximately 10.5 days post coitum (dpc), PGCs move to the genital ridge from the embryo's external and then divide mitotically until approximately 13.5 dpc. At this point, the germ cells are called oogonia, and in the mouse ovary they colonize in clusters called germ line cysts. Like the germ cells of invertebrate females, female mouse germ cells have been found to divide synchronously and connect through intercellular bridges that form as a result of incomplete cytokinesis. Next, these connected germ cell clusters begin to undergo meiosis until around 17.5 dpc, when meiosis for these germ cells—now called oocytes—comes to a pause at the diplotene stage of prophase I, during which the synaptonemal complex, a protein structure which facilitates the synapsis of homologous chromosomes, disbands and the chromosomes separate slightly. The germ cell clusters separate through a process called cyst breakdown during the perinatal period. Primordial follicle assembly is accomplished through a combination of cell separation and apoptosis within these smaller germ cell clusters until single oocytes remain.

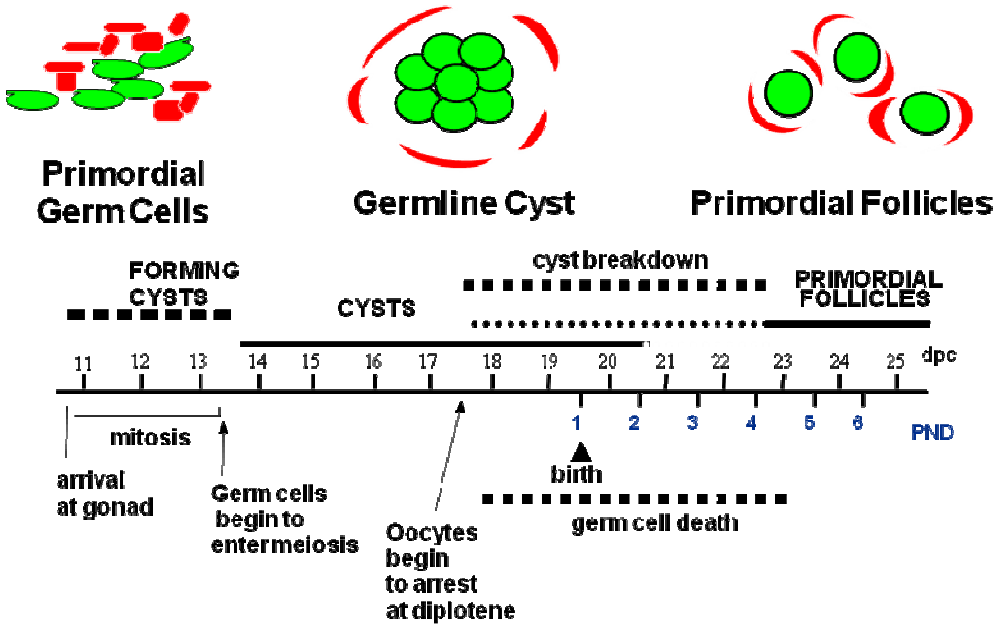

Figure 1. Timeline of mouse germ cell development (Pepling, 2006). Oocytes are labeled in green, and granulosa cells are labeled in red.

During the perinatal period, the apoptosis of germ cells accompanies cyst breakdown, and nearly two-thirds of the initial germ cell population dies. Figure 2 describes the incorporation of apoptosis in cyst breakdown. The function of this systematic cell death is still unknown and requires more research. It is possible that it is a required process for the completion of cyst breakdown (Pepling and Spradling, 2001).

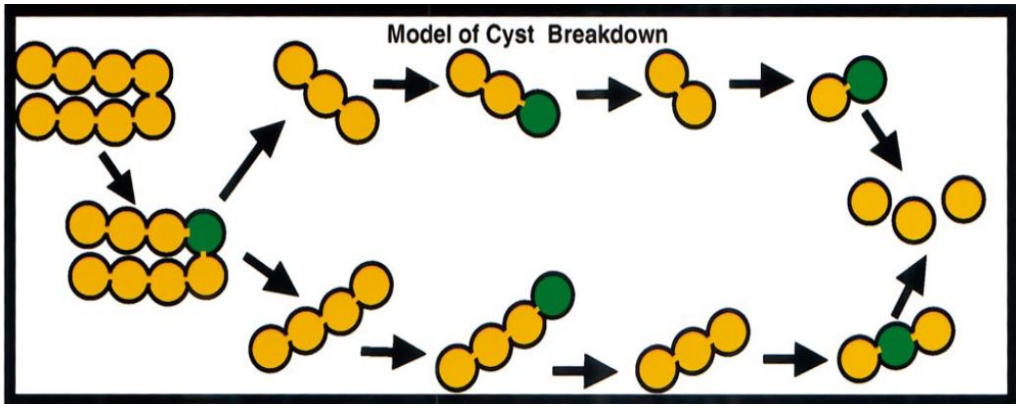

Figure 2. Model of germ line cyst breakdown (Pepling and Spradling, 2001). Surviving oocytes are labeled in yellow, and oocytes undergoing cell death are labeled in green. Apoptosis results in the breaking apart of the cyst. Ultimately, the process results in primordial follicles, single oocytes each surrounded by one layer of granulosa cells (not pictured here).

Many questions remain regarding this mechanism. Currently, it is uncertain as to why only one-third of oocytes are selected to live while the others die and how the survivor oocytes are chosen. However, it is possible that two-thirds of oocytes die in order to nurse the remaining oocytes and ensure their survival (Pepling and Spradling, 2001). Additional areas of research within this realm are whether the process of cyst breakdown is susceptible to external influences and if so, when cyst breakdown is most vulnerable to their effects. In mammalian oogenesis, three critical time windows during which exogenous estrogen exposure may adversely affect development have already been identified: the initiation of meiosis in the fetal ovary, perinatal formation of follicles, and oocyte growth and maturation (Hunt and Hassold, 2008).

In order to answer these questions, it is key to understand the mechanism of folliculogenesis' regulators. One such regulator may be estrogen. Since neonates' exposure to estrogen drastically changes during the perinatal period, it is possible that cyst breakdown could be a product of this adjustment (Pepling, 2006).

Upon their diffusion into the cell, estrogenic compounds bind specific receptor proteins, estrogen receptor alpha (ERα) or estrogen receptor beta (ERβ). The ligand-receptor complex then binds as a dimer to an estrogen response element (ERE), a segment of DNA in the upstream region of the target gene. Then, the necessary coactivators are recruited, and transcription begins when chromosome structure is modified (Dahlman-Wright *et al.,* 2006). Insightful information has been gained through extensive research on some exogenous estrogens, synthetic and naturally-occurring materials in the environment that mimic estrogens in their hormonal function and interrupt the function of natural hormones. Examples of exogenous estrogens which signal through this mechanism include: bisphenol-A (BPA), diethylstilbestrol (DES), ethinylestradiol (EE), genistein, dioxin, dichlorodiphenyldichloroethylene (DDE), dichlorodiphenyltrichloroethane (DDT), and polychlorinated biphenyls (PCB's) (see Figure 3).

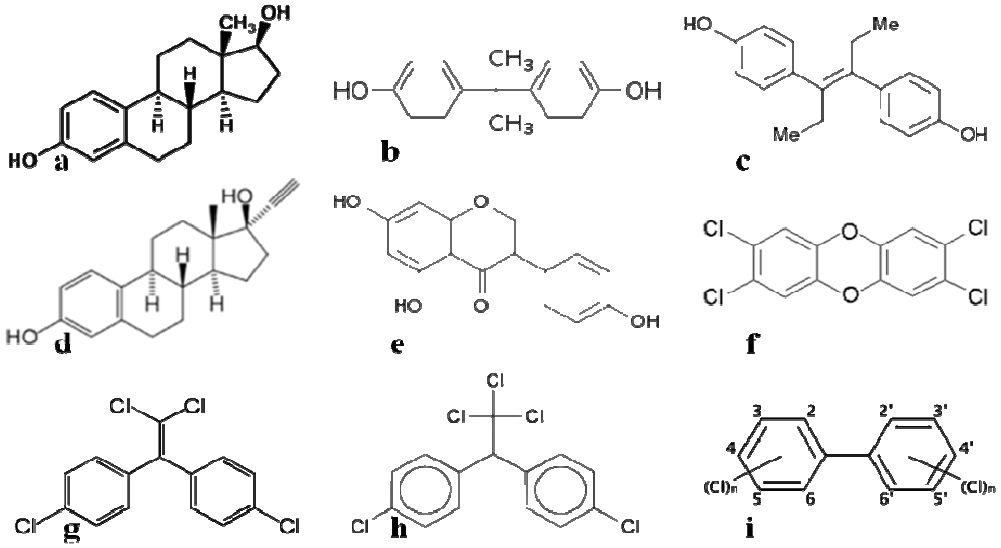

Figure 3. Molecular structures of estradiol (a) and some exogenous estrogens (b-i): bisphenol A (b), diethylstilbestrol (c), ethinylestradiol (d), genistein (e), dioxin (f), dichlorodiphenyldichloroethylene (g), dichlorodiphenyltrichloroethane (h), and polychlorinated biphenyls (i).

Exogenous estrogen exposure has varying effects on development expanding beyond the mammalian realm. Claims have been made that pesticides, especially DDT, have adverse effects on shorebird populations. This was a serious concern considering the increased integration of pesticides into the diet of Americans (Carson, 1962). The importance of Carson's claim was realized when some species of birds became endangered due to their eggshells becoming increasingly fragile after these birds had been exposed to DDT (Cooke, 1973). Additional problems within animal populations have been linked with exogenous estrogen exposure, such as the occurrence of intersex fish, or male fish that develop female reproductive organs (Scholz and Klüver, 2009) and the decline in the Florida alligator population due to a decreased birth rate and the reduced size of male genitalia (Guillette *et al.,* 1994).

Three extensively-researched exogenous estrogens are DES, EE, and BPA. Many pregnant women were treated with DES in their first trimester during the 1940s-1970s in order to prevent miscarriage or spontaneous abortion. Thus far, this exposure has had a multigenerational effect. The first DES generation, which consists of the women who ingested DES while pregnant, had an increased risk of breast cancer (Steiner and Klubert, 2008). Teratogen-related defects in the second DES generation coined the term "DES daughters" and "DES sons" in reference to children of women who consumed DES during pregnancy. In DES daughters, there was no significant increase in risk of breast cancer development; however, these women did exhibit a significantly higher risk of clear cell adenocarcinoma (CCA) of the cervix and vagina (Verloop *et al.,* 2010) and

pregnancy complications including preeclampsia (Troisi *et al.,* 2007). DES daughters were also more likely to have spontaneous pregnancy losses, ectopic pregnancies, and pre-term deliveries (Kaufman *et al.,* 2000). DES sons experienced an increased risk of cryptorchidism, epididymal cysts, and testicular inflammation and infection, and these risks increased with earlier exposure (Palmer *et al.,* 2009). Research on transgenerational effects in the third DES generation, children of individuals with prenatal DES exposure, has just recently begun since these individuals are still relatively young. Thus far, preliminary findings have determined that members of the third DES generation may have an increased risk of ovarian cancer (Titus-Ernstoff *et al.,* 2008) and hypospadias (Klip *et al.,* 2002); however, the severity of these risks is still under debate (Brouwers *et al.,* 2006; Palmer *et al.,* 2005; Titus-Ernstoff *et al.,* 2008). Ethinylestradiol is one of the main active ingredients in many contraceptive pills and can leave the body through the urine and feces as a xenoestrogen. In fish downstream from a wastewater treatment plant effluent that contained estrogenic compounds, there was a significantly higher incidence of reduced gonad size, modified sex ratios, and intersex fish (Vajda *et al.,* 2008). Like DES, neonatal BPA exposure may have a multigenerational effect. Deformities found in the oocytes of BPA-exposed fetal mice (second generation) were the abnormalities of incomplete synapsis and end-to-end associations of chromosomes. Incomplete synapsis is the absence of synapsis during the pachytene stage of meiosis I. End-to-end associations are synapses between the ends of nonhomologous chromosomes, and this 2007 study may be the first to report such a meiotic defect.

Because normal meiotic chromosomal activity is disrupted, oocytes in the BPA group had a much higher incidence of hyperploidy. In the third generation, similar levels of hyperploidy were observed in the embryos of the BPA-treated group (Susiarjo *et al.,* 2007). Exposure to bisphenol A may increase obesity (Ebobeid and Allison, 2008). BPA is also a thyroid disruptor as it binds thyroid hormone receptors, which interrupts thyroid hormone signaling, an integral developmental pathway (Zoeller, 2007), and it is associated with elevated levels of some liver enzymes, heart disease, and diabetes (vom Saal and Myers, 2008). Perniatal exposure to low BPA doses can increase breast cancer risk and alter breast development (Brisken, 2008). Additionally, studies in rodents and in humans have indicated that BPA exposure increases the risk of prostate cancer and alters fetal prostate development (Nagel *et al.,* 1997; Timms *et al.,* 2005; Ho *et al.,* 2006; Richter *et al.,* 2007). The breadth of these effects is understandable considering the prevalence of BPA in polycarbonate plastics and food and beverage cans.

Exposure to environmental estrogens during one of Hunt and Hassold's three critical time periods may have multiple effects on follicle development. These can include an arrest or a delay in follicle development and a decrease in germ cell apoptosis. For instance, the exposure of neonatal mice to genistein, a phytoestrogen found in soybeans, resulted in an increased incidence of multiple oocyte follicles (MOFs), or follicles containing more than one oocyte, which could possibly be part of a germ line cyst that never fully underwent cyst breakdown to become a group of single oocytes individually surrounded by

granulosa cells (Jefferson *et al.,* 2006). In mice, this is problematic because the prescence of MOF's decreases their fertility (Pepling, 2006). Also, a larger number of oocytes survived cyst breakdown, indicating that less apoptosis of germ cells occurred. If these dying germ cells do, in fact, serve a nursing function, then the surviving oocytes may be malnourished (Jefferson *et al.,* 2006). A second study shows that *in vivo* and *in vitro* perinatal exposure to estrogen, progesterone, and genistein, respectively, inhibits oocyte nest breakdown and the formation of primordial follicles, but does not affect oocyte number (Chen *et al.,* 2007). This study aims to determine whether perinatal exposure to other exogenous estrogens has an effect cyst breakdown, germ cell apoptosis, and follicle development in the mouse.

Materials and Methods

Animals

The mouse strain used in these experiments was an outbred strain called CD-1, which was obtained from Charles River Laboratories. CD-1 females were mated with males and checked daily for vaginal plugs. When a plug was noticed in a female, she was isolated and the date at which the plug was observed was known as 0.5 days post-coitum (dpc). Birth usually occurred at approximately 19.5 dpc, and the day of birth became known as post-natal day (PND) 1. Ovaries were collected from CD-1 neonates at PND 5. Using a dissecting microscope, these ovaries were collected via dissection in 1x phosphate-buffered saline (PBS).

Whole-Mount Antibody Staining of Neonatal Ovaries

In order to perform analysis on the CD-1 ovaries, the mice were dissected, and their ovaries were transferred to tubes containing 600 μL of a fixative mixture. The mixture contained a final concentration of 0.67x PBS and 5.3% formaldehyde (200 μL of 16% formaldehyde from Ted Pella Inc. and 400 μL of PBS). The tubes containing the ovaries were labeled with the age of the ovaries, the mouse strain, the treatment they received, and the date of collection. These tubes were left to nutate overnight at 4°C. The next day, the ovaries were washed twice quickly with 1 mL of PT (1x PBS/0.1% Triton X-100), and for the third wash in PT they were placed on a nutator for at least 30 minutes at room temperature. The ovaries were then washed in 1 mL of PT+5% bovine serum albumin (BSA) for 60 minutes on a nutator at room temperature. Ovaries were either processed immediately or stored at 4°C in 1 mL of PT+5% BSA on a nutator.

Next, 1 μL of the primary antibody STAT-3 (C20) (Santa Cruz Biotechnology), which serves as an oocyte marker (Murphy, *et al.* 2005), in 500 μL of PT+5% BSA was added to the ovaries, which were incubated overnight at 4°C on a nutator. In a second tube, 2.5 μL of the secondary antibody, goat anti-rabbit Alexa 488 (Molecular Probes), was added to 500 μL of PT+5% BSA. A pinch of embryo powder (ground pellet isolated from homogenized PND12.5-14.5 mouse embryos incubated in ice-cold acetone and centrifuged at 10,000 g) was added to the mixture, and this tube was labeled accordingly, wrapped in aluminum foil, and incubated on a nutator overnight at 4°C for use the following

day. The solution containing the secondary antibody was centrifuged briefly before it was added to ovaries.

The next day, the ovaries were washed in 1 mL of PT+1% BSA for 30 minutes at room temperature. In order to remove the RNA from the ovaries, they were then washed with 10 µL of 10 mg/ml RNase in 1 mL of PT+1% BSA for 30 minutes at room temperature. After that, the ovaries were incubated in 10 µL of 0.5 mg/mL propidium iodide (PI) (Molecular Probes) in 1 mL PT+1% BSA (to give a final concentration of 5 µg/mL) on a nutator for 20 minutes at room temperature. After the PI solution was added, the tubes were wrapped entirely in aluminum foil and remained wrapped in the foil for the rest of the staining procedure in order to prevent fluorescence loss. Excess PI was removed from the ovaries by washing them in 1 mL of PT+1% BSA for 30 minutes on a nutator at room temperature. Next, the preabsorbed secondary antibody mixture was added to the ovaries, and the foil-covered tube was placed on a nutator for 2-4 hours at room temperature. Then, the ovaries were washed at room temperature in 1 mL of PT+1% BSA three times with each wash having a duration of 30 minutes. One quick wash with 1 mL of 1x PBS was applied to the ovaries, and then the PBS was removed. Approximately 100 µL of Vectashield (Vector Laboratories) was added to the ovaries, and the ovaries were left to sit in the Vectashield for 15 minutes. Finally, the ovaries were carefully mounted on a slide (Fisher Finest) and covered with a coverslip (Corning), sealed with nail polish, and stored at -20°C.

Confocal Microscopy

Indirect immunofluorescence was used to view the stained PND5 ovaries using a Zeiss LSM 710 confocal microscope. Quantifications of oocyte number, follicle development, and cyst breakdown were obtained using images obtained from confocal microscopy. The confocal pictures were taken by imaging two separate areas in each ovary, with four planar images 20 μm apart in each area to give a total of eight sections per ovary. The number of oocytes was determined by counting the number of oocytes in each image. If an image plane did not contain any oocytes, then its data was omitted from the average of the data from all the image planes. The amount of cyst breakdown that had occurred was analyzed by counting the number of single oocytes and comparing this amount to the number of oocytes that were still in cysts (or unassembled). In order to determine whether oocytes were in cysts or not, above and below the section analyzed, 1 μm apart, a ten-image stack was taken for each of the eight sections. Since each image stack had five images above and below the section being analyzed, this allowed one to see whether an oocyte was part of a germline cyst above or below the plane of focus. Finally, follicle development was quantified in each of the eight sections by observing each oocyte, determining its stage in follicle development, and quantifying the number of total number of oocytes in each stage.

The oocytes were classified as unassembled if they were not entirely surrounded by granulosa cells and if they were observed as being in clusters (Figure 4a). In order for an oocyte to be considered a single oocyte, it was

required that it not touch another oocyte. Single oocytes were enclosed in follicles which fell into three categories: primordial, primary, or secondary. A single oocyte surrounded by flattened, crescent-shaped granulosa cells defined a primordial follicle (Figure 4b). A primary follicle (Figure 4d) was a single oocyte completely enclosed in one layer of cuboidal granulosa cells. A secondary follicle (Figure 4e) was a larger single oocyte surrounded by multiple layers of cuboidal granulosa cells.

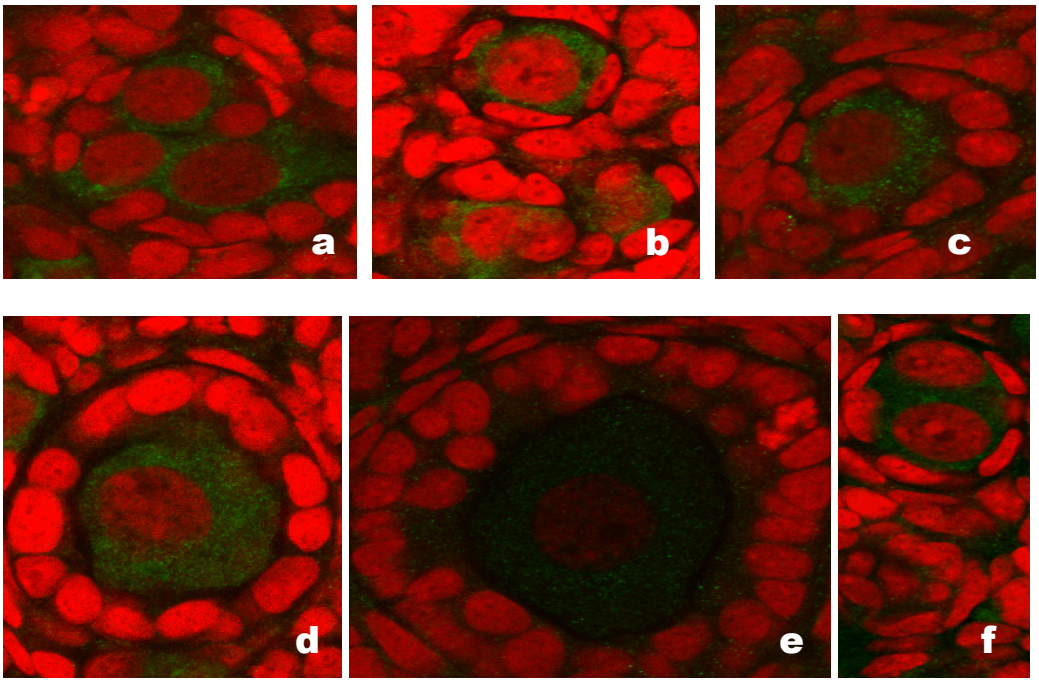

Figure 4. Stages of follicle development. Ovaries are stained with PI (red) and STAT-3 (C20) (green) and imaged at 63x. Oocytes in cysts that had connecting cytoplasm are considered unassembled (a). Single oocytes surrounded by one layer of flattened granulosa cells were classified as primordial follicles (b). A transitional follicle (c) is surrounded by a single layer of granulosa cells, part of which are flattened with the rest being cuboidal in shape. Single oocytes, which were surrounded by a single layer of cuboidal granulosa cells, were classified as primary follicles (d). A secondary follicle (e) was characterized by a single oocyte surrounded by multiple layers of cuboidal granulosa cells. A multiple oocyte follicle (MOF) consists of multiple oocytes surrounded by granulosa cell layers (f).

Preparation and Delivery of Estrogenic Compound Injections

CD-1 neonates were injected with a total volume of 50 μL of either plain peanut oil (Wegman's) or a mixture consisting of an estrogenic compound dissolved in peanut oil. Three different estrogenic compounds were dissolved, respectively, into peanut oil: diethylstilbestrol (DES) (Acros), ethinylestradiol (EE) (Sigma-Aldrich), and bisphenol-A (BPA) (Sigma-Aldrich). Two different mixtures were made for each estrogenic compound that was tested to yield a total of six different injection mixtures containing estrogenic compounds. The mixture with the lower concentration had a final concentration of 5 mg per kg of body weight per day (mg/kg/day) when injected into each neonate, thus exposing each neonate to 10 μg of the estrogenic compound on each day of injection. The mixture with the higher concentration had a final concentration of 50 mg/kg/day when injected into each neonate, thus exposing each neonate to 100 μg of the estrogenic compound on each day of injection.

One injection was made for each neonate. Prior to making the injections the amount of peanut oil and estrogenic compound were carefully determined according to the desired concentration. The pre-measured amount of the estrogenic compound was added to 0.5 mL of absolute ethanol (Pharmco-AAPER) (Jordan *et al.,* 1983) in a 20 mL beaker. A small, magnetic stirrer was added to the 20 mL beaker, which was left to stir on a magnetic stirring plate until the estrogenic compound was dissolved. When it had fully dissolved, the peanut oil from the 100 mL beaker was poured into the 20 mL beaker. The contents of this beaker were stirred for one minute. Then the total contents of this beaker

were transferred back to the 100 mL beaker, which was left to stir on the magnetic stirrer plate for one hour. Once all of the ethanol had evaporated from the beaker, 1-mL syringes (Kendall Monoject) were filled slightly past the 0.05 mark. The syringes were capped with 26-gauge needles (Becton-Dickinson), and the syringe's plunger was pushed to the 0.05 mL mark to remove air from the syringe and needle. The syringes were labeled according to any estrogenic compounds and the concentrations that they contained.

The injection of neonates began on PND1. On PND1, the entire litter of neonates was removed from its cage and placed in a small plastic container with an open top. After each neonate was injected subcutaneously with the syringe's contents, a small portion of its tail was clipped off using a small pair of sterilized scissors. This allowed the observer to see whether or not each neonate had been injected on the subsequent post-natal days of injection. Then, the neonate was carefully placed back in its cage in order to ensure that its mother would recognize and continue to feed it. This procedure, with the exception of the tail-clippings, was repeated once a day on PND2-PND4. On PND5, the neonate's ovaries were collected via dissection and placed into the dilute fixative, the first step of the antibody staining procedure.

Statistical Analyses of Cyst Breakdown, Total Number of Oocytes, and Follicle Development

Statistical significance was determined by conducting one-way ANOVA tests with *P* values less than 0.05 considered significant. One-way ANOVA was

implemented in order to investigate treatment effects on cyst breakdown, total number of oocytes, and follicle development.

Results

Examination of Mice Injected with Estrogenic Compounds

Ovaries from mice injected with estrogenic compounds dissolved in peanut oil were collected at PND5 and investigated for defects in oocyte development and were compared to ovaries from mice injected with plain peanut oil. From these ovaries, cyst breakdown (Figures 5, 6 and 7), total number of oocytes (Figure 8), and follicle development (Figure 9) were analyzed.

Cyst Breakdown in Mice Injected with Estrogenic Compounds

In order to determine whether or not exogenous estrogen exposure may play a role in cyst breakdown during the perinatal period, neonatal mice that had been exposed to either 10 μg/day or 100 μg/day of DES, EE, or BPA dissolved in peanut oil or were injected with plain peanut oil during PND1-4 were analyzed at PND5. For the evaluation of cyst breakdown, the number of single oocytes was compared to the number of oocytes in cysts. Mice injected with peanut oil had 84.2% single oocytes. In mice treated with 10 μg DES per day, the percentage of single oocytes decreased significantly to 55.3%. While the percentage of single oocytes decreased slightly to 50.8% in mice treated with the higher concentration of DES, in comparison to those treated with the lower concentration of DES this difference was not significant (Figure 5).

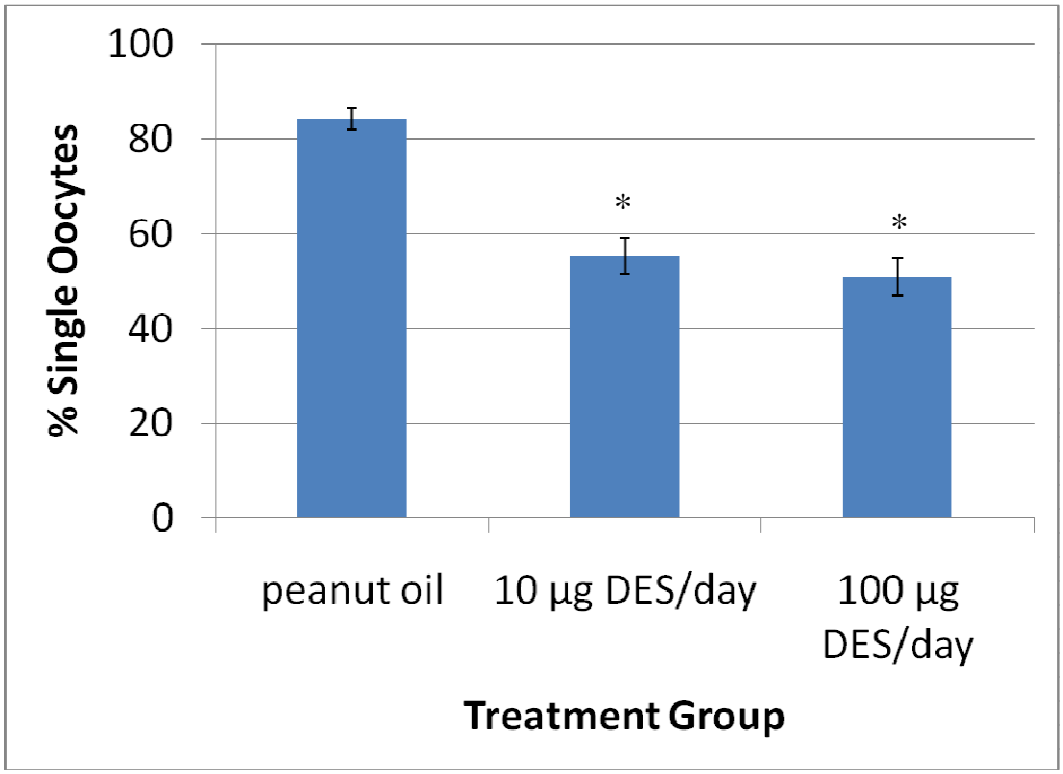

Figure 5. Cyst breakdown in the control group and in mice treated with DES. Significant difference between percentage of single oocytes at the same stage of development with or without neonatal exposure to DES (one way ANOVA, $P < 0.05$; n = 10-12 ovaries per group). Asterisks indicate significance in comparison to mice injected with peanut oil.

Figure 6 shows the significant decrease in percentage of single oocytes in mice treated with both concentrations of EE, respectively. Mice treated with the higher concentration of EE had a slightly larger percentage of single oocytes at 62.6%; however, this difference was not significant when compared to mice treated with the lower concentration of EE, which had 61.2% single oocytes

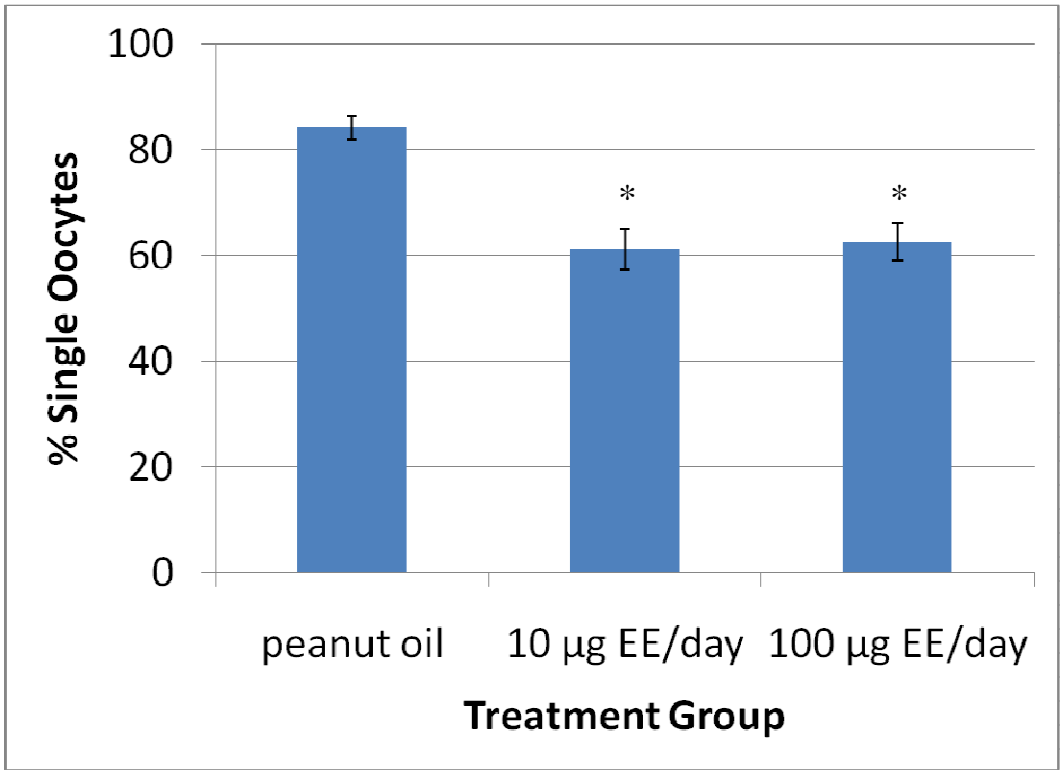

Figure 6. Cyst breakdown in the control group and in mice treated with EE. Significant difference between percentage of single oocytes at the same stage of development with or without neonatal exposure to EE (one way ANOVA, $P < 0.05$; n = 8-12 ovaries per group). Asterisks indicate significance in comparison to mice injected with peanut oil.

Figure 7 displays the significant difference between the control group and mice injected with the higher concentration of BPA. The percentages of single oocytes decreased in a manner that was directly proportional to the concentration of BPA that was injected; however, the differences between the control group and mice injected with the lower concentration of BPA and between the two treatment groups were not significant with 75.4% single oocytes for the lower concentration and 68.6% single oocytes for the higher concentration of BPA.

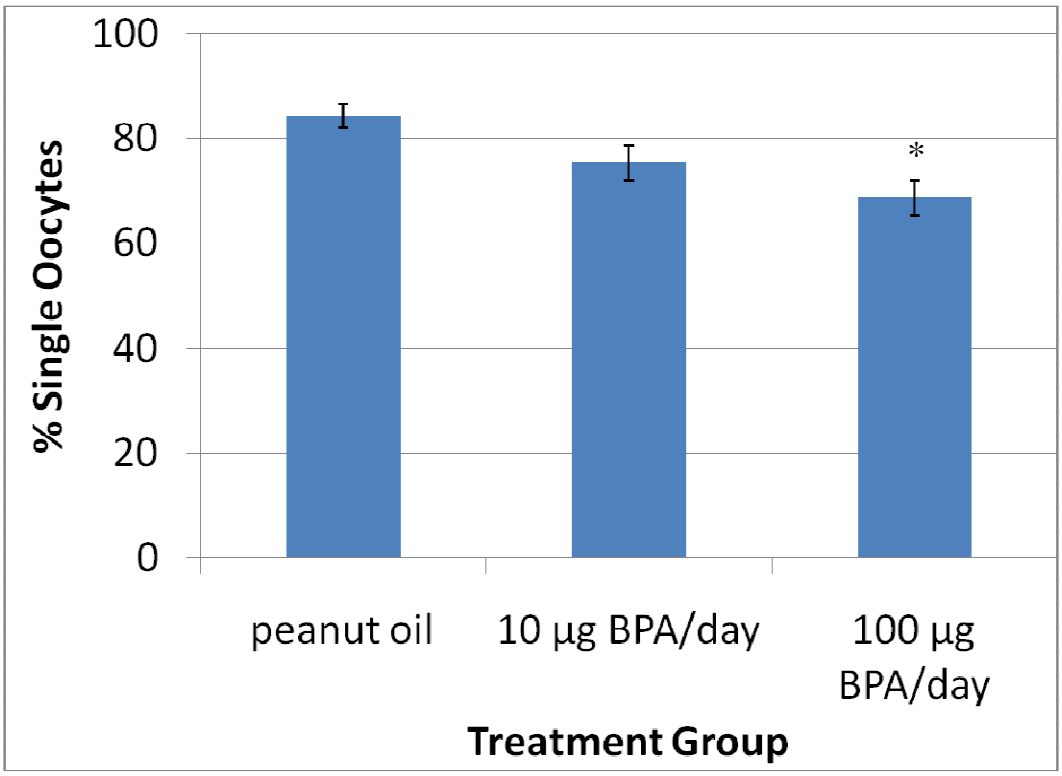

Figure 7. Cyst breakdown in the control group and in mice treated with BPA. Significant difference between percentage of single oocytes at the same stage of development with or without neonatal exposure to BPA (one way ANOVA, $P < 0.05$; n = 8-12 ovaries per group). Asterisks indicate significance in comparison to mice injected with peanut oil.

Total Number of Oocytes in Mice Injected with Estrogenic Compounds

The total number of oocytes per confocal section was determined for the mice injected with plain peanut oil and for mice injected with two different concentrations of DES, EE, or BPA dissolved in peanut oil, respectively, in order to determine whether exogenous estrogen exposure has a role in oocyte survival. It is expected that two-thirds of oocytes undergo apoptosis during the perintal period. Figure 8 displays a trend that increased exposure to DES, EE, and BPA does inhibit germ cell death during the perinatal period. These treatment groups had 12.3, 12.5, 13.0, and 15.8 oocytes per confocal section, respectively. The only insignificant differences belonged to the groups injected with 10 µg DES/day and

10 µg EE/day, respectively, which had 8.8 and 10.2 oocytes per section. Mice injected with peanut oil had an average of 8.0 oocytes per section.

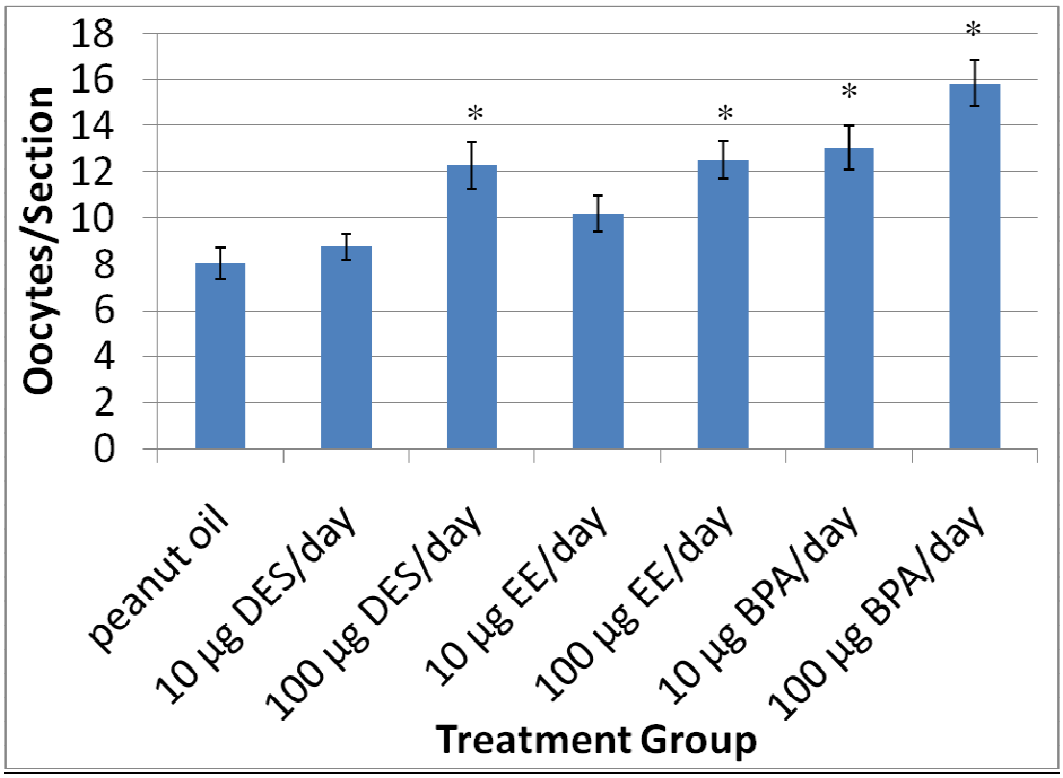

Figure 8. Total number of oocytes in mice injected with plain peanut oil and with two concentrations of DES, EE, and BPA, respectively, dissolved in peanut oil. Significant difference between the number of oocytes per confocal section at the same stage of development with or without neonatal exposure to DES, EE, or BPA (one way ANOVA, $P < 0.05$; n = 8-12 ovaries per group). Asterisks indicate significance in comparison to mice injected with peanut oil.

Follicle Development in Mice Injected with Estrogenic Compounds

In order to determine whether exogenous estrogen exposure had a significant role in follicle development, oocytes from mice injected with plain peanut oil and from mice injected with two different concentrations of DES, EE, or BPA dissolved in peanut oil, respectively, were assessed using confocal microscopy. The single oocytes in each confocal section were classified as primordial follicles, primary follicles, or secondary follicles. Mice injected with

peanut oil had 62.3% primordial follicles, 27.9% primary follicles, and 9.7% secondary follicles.

Exposure to the lower concentration of DES did not significantly alter the percentage of primordial follicles at 62.2%; however, exposure to the higher concentration of DES increased the percentage of primordial follicles significantly to 82.6%. Treatment with the lower concentration of DES increased the number of primary follicles to 37.8% and decreased the number of secondary follicles to 1.4%, and treatment with the higher concentration of DES decreased the percentage of primary follicles to 16% and the percentage of secondary follicles to 1.4%. However, these changes were not significant. A similar trend was observed for both concentrations of EE, respectively. Mice injected with the lower concentration of EE had 64.6% primordial follicles, 34.6% primary follicles, and 0.8% secondary follicles. Mice injected with the higher concentration of EE had 82.7% primordial follicles, 16.9% primary follicles, and 0.4% secondary follicles. Exposure to both concentrations of BPA significantly increased the percentage of primordial follicles to 81.0% for the lower concentration and 89.5% for the higher concentration. While treatment with both concentrations did lower the percentage of primary follicles, this effect was significant only for the higher concentration of BPA, which had 10.5% primary follicles. Treatment with both concentrations of BPA also lowered the percentage of secondary follicles to 0.8% and 0.0%, but this effect was insignificant in both instances (Figure 9).

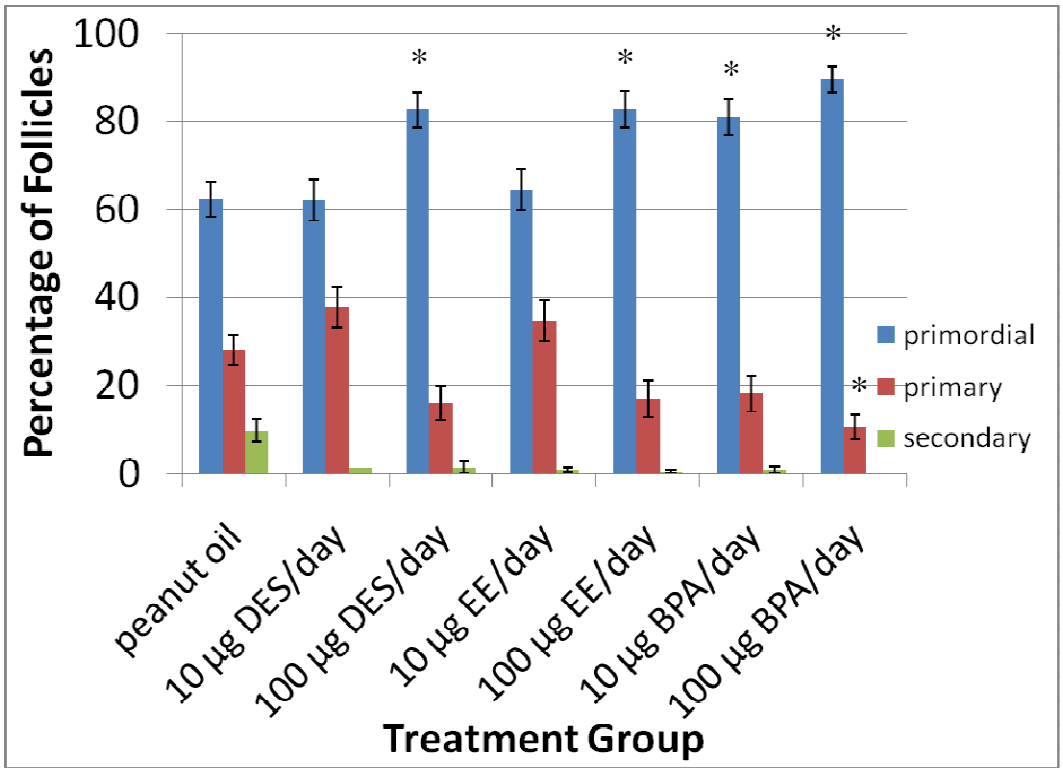

Figure 9. Follicle development in mice injected with plain peanut oil and with two concentrations of DES, EE, and BPA, respectively, dissolved in peanut oil. Significant difference between percentages of follicles at the same stage of development with or without neonatal exposure to DES, EE, or BPA (one way ANOVA, $P < 0.05$; n = 8-12 ovaries per group). Asterisks indicate significance in comparison to mice injected with peanut oil.

Evaluation of media used to prepare injections of estrogenic compounds

Two experiments were completed in order to determine whether the media used in the preparation of estrogenic compound injections had affected any changes resulting from exogenous estrogen exposure. The first experiment investigated the effects of using absolute ethanol in injection preparation by collecting and analyzing ovaries from two treatment groups. The first treatment group consisted of mice that received injections of 10 µg estradiol/day prepared without dissolving the estradiol (Acros) in absolute ethanol. The second treatment group consisted of mice that received injections of 10 µg estradiol/day prepared by dissolving the estradiol in the absolute ethanol and adding peanut oil

to this mixture. All ovaries were collected at PND5. Ovaries from both groups of mice treated with estradiol were investigated for defects in oocyte development by comparing them to ovaries from mice injected with peanut oil. From these ovaries, cyst breakdown (Figure 10), total number of oocytes (Figure 11), and follicle development (Figure 12) were analyzed. The second experiment investigated the effects of the type of oil used in injection preparation by collecting and analyzing ovaries from two additional treatment groups. In this experiment untreated ovaries served as the control group, and ovaries from mice injected with 50 μL of plain peanut oil and corn oil, respectively, comprised the treatment groups. All ovaries were collected at PND5. Ovaries from mice injected with oil were investigated for defects in oocyte development by comparing them to ovaries from untreated mice. From these ovaries, cyst breakdown (Figure 13), total number of oocytes (Figure 14), and follicle development (Figure 15) were analyzed.

The Effect of Absolute Ethanol in Injection Preparation on Mouse Ovary Cyst Breakdown

In order to determine whether using absolute ethanol in the preparation of the injections of exogenous estrogens may have affected the amount of cyst breakdown during the perinatal period, neonatal mice were exposed to 10 μg estradiol/day that was either dissolved in absolute ethanol before being added to peanut oil or added directly to the peanut oil. Mice received injections of estradiol prepared with or without absolute ethanol during PND1-4, and their

ovaries were collected and analyzed at PND5. Exposure to estradiol administered via injections prepared without absolute ethanol significantly decreased the amount of cyst breakdown to 69.6%. While only 64.6% single oocytes were observed for the mice which received estradiol injections prepared with absolute ethanol, this change was not significant in comparison to mice which received estradiol injections prepared without ethanol (Figure 10).

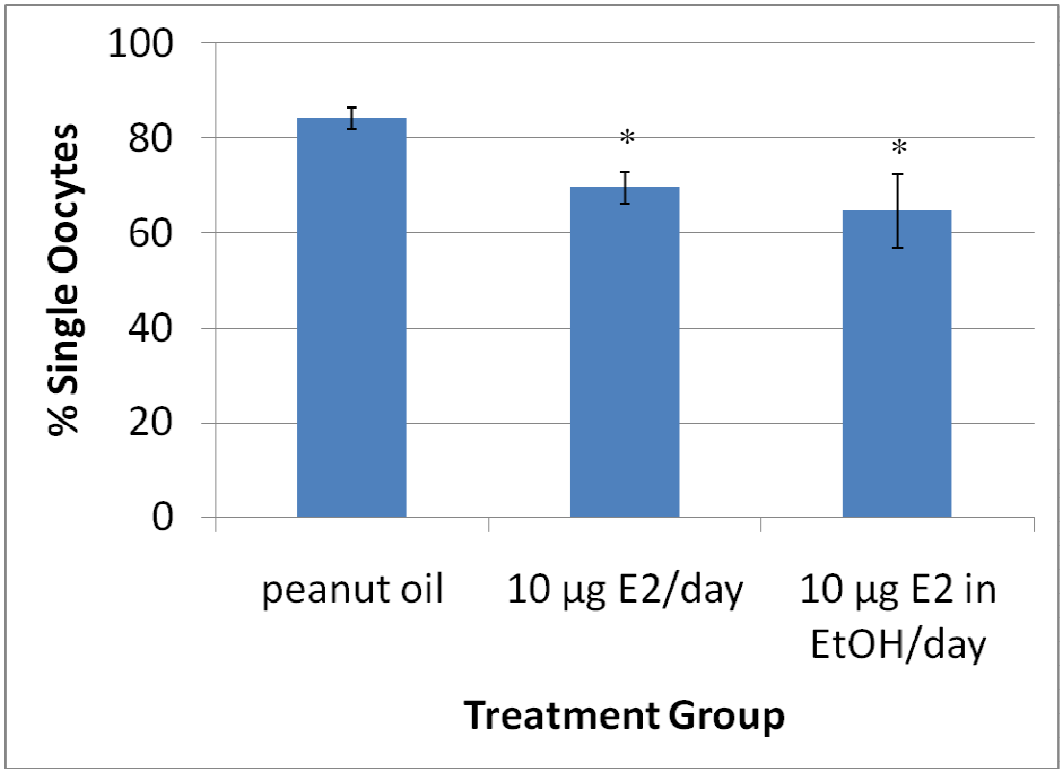

Figure 10. Cyst breakdown in mice injected with plain peanut oil and with 10 µg estradiol/day in peanut oil prepared with or without absolute ethanol. Significant difference between percentage of single oocytes at the same stage of development with or without absolute ethanol used in injection preparation (one way ANOVA, $P < 0.05$; n = 8-12 ovaries per group). Asterisks indicate significance in comparison to mice injected with peanut oil.

The Effect of Absolute Ethanol in Injection Preparation on the Total Number of Oocytes

The total number of oocytes per confocal section was determined for mice injected with plain peanut oil and for mice receiving injections of peanut oil containing 10 μg estradiol/day prepared with or without absolute ethanol in order to determine whether using absolute ethanol in injection preparation played a role in oocyte survival. Figure 11 shows that ovaries from mice that received estradiol injections prepared without absolute ethanol had a significantly larger amount of oocytes per confocal section at 11.4 oocytes per section. Ovaries from mice that received estradiol injections prepared with absolute ethanol had 14.1 oocytes per section; however, this increase was not significant relative to the ovaries from mice which received injections prepared without absolute ethanol.

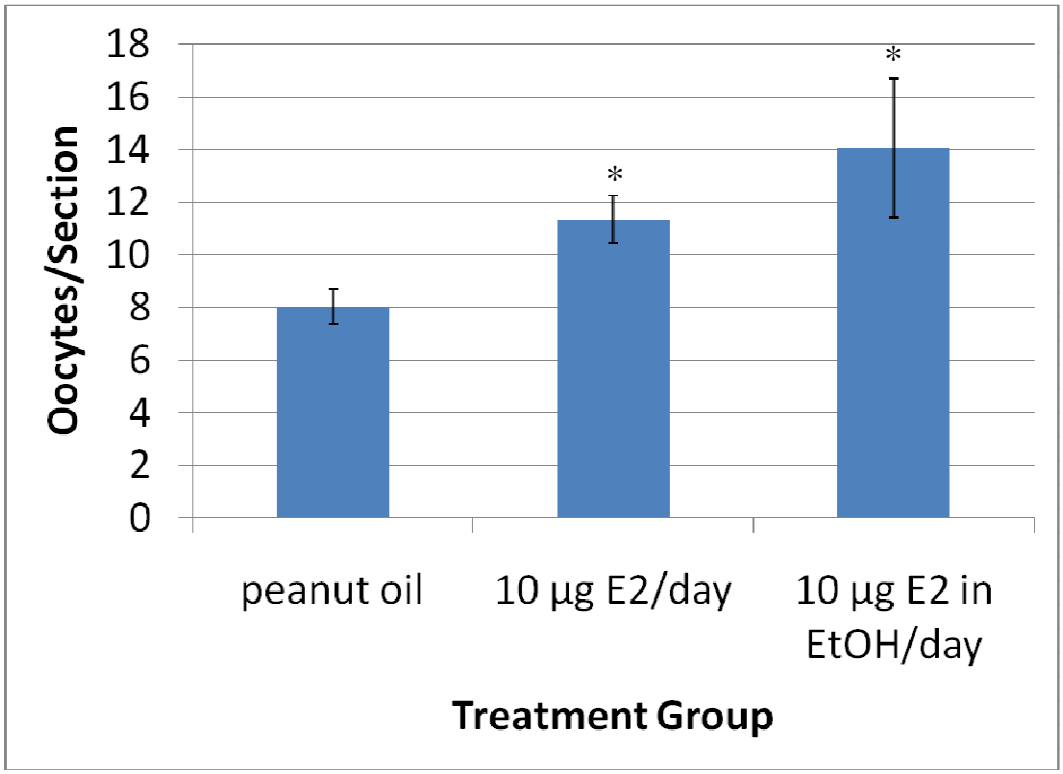

Figure 11. Total number of oocytes in mice injected with plain peanut oil and with 10 µg estradiol/day in peanut oil prepared with or without absolute ethanol. Significant difference between the number of oocytes per confocal section at the same stage of development with or without absolute ethanol in estradiol injection preparation (one way ANOVA, $P < 0.05$; n = 8-12 ovaries per group). Asterisks indicate significance in comparison to mice injected with peanut oil.

The Effect of Absolute Ethanol in Injection Preparation on Mouse Follicle Development

In order to determine whether using absolute ethanol in injection preparation had a significant role in follicle development, oocytes from mice injected with plain peanut oil and mice which received estradiol injections prepared with or without absolute ethanol were assessed using confocal microscopy. The single oocytes were classified as primordial, primary, or secondary.

A significant increase in the percentage of primordial follicles was observed between mice injected with peanut oil and both groups of mice injected

with estradiol. Mice that received estradiol injections prepared with absolute ethanol did not have a significantly larger increase than those that received injections prepared without absolute ethanol, with 87.8% versus 79.5% primordial follicles. For both treatment groups the percentages of primary follicles descreased to 16.5% and 11.5%, respectively; however, this change was significant for estradiol injections prepared with ethanol only. Decreases in the percentages of secondary follicles were also observed for both treatment groups, but at 4% and 0.5%, these differences were not significant (Figure 12).

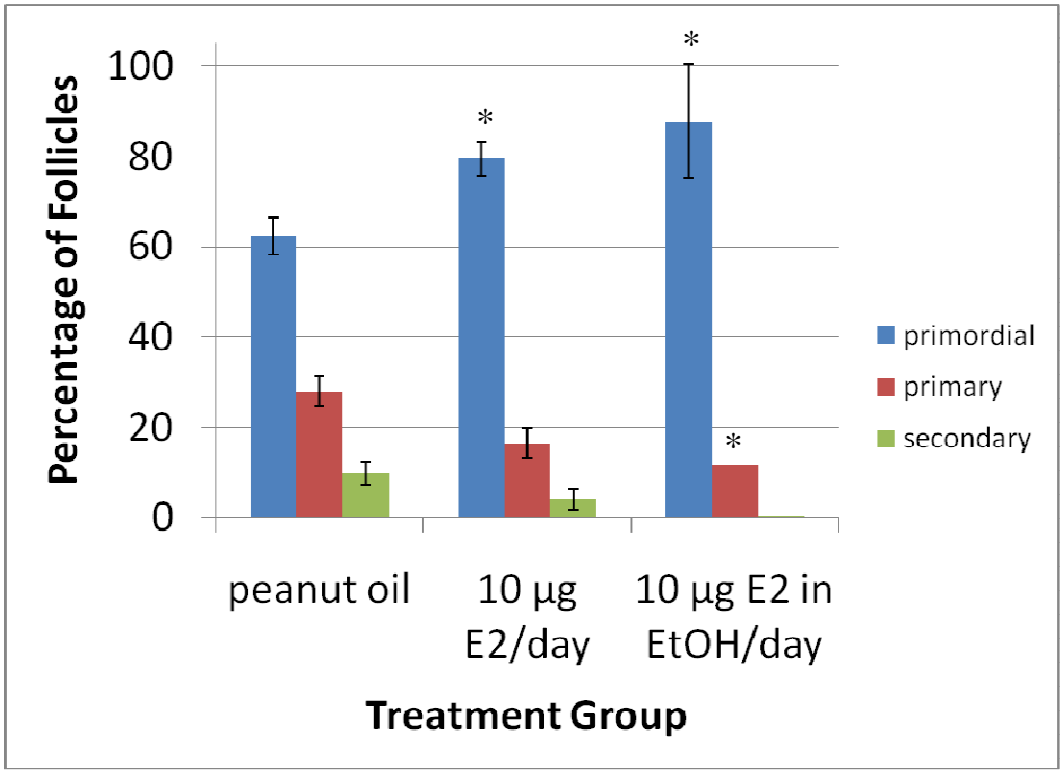

Figure 12. Follicle development in mice injected with plain peanut oil and with 10 µg estradiol/day in peanut oil prepared with or without absolute ethanol. Significant difference between percentages of follicles at the same stage of development with or without using absolute ethanol in estradiol injection preparation (one way ANOVA, $P < 0.05$; n = 8-12 ovaries per group). Asterisks indicate significance in comparison to mice injected with peanut oil.

The Effect of Peanut Oil as a Delivery Medium on Mouse Ovary Cyst Breakdown

In order to determine whether or not using peanut oil as a delivery medium affected the cyst breakdown resulting from exogenous estrogen exposure during the perinatal period, neonatal mice were injected with plain peanut oil or corn oil, respectively, during PND1-4. Ovaries from untreated mice and mice injected with corn oil and peanut oil were collected and analyzed at PND5. Figure 13 shows that less cyst breakdown occurred in ovaries from mice injected with corn oil and peanut oil at 81.5% and 84.2%, respectively; however, these differences were not significant relative to ovaries from untreated mice. While slightly more cyst breakdown was observed in mice injected with peanut oil than in those injected with corn oil, this difference was also insignificant.

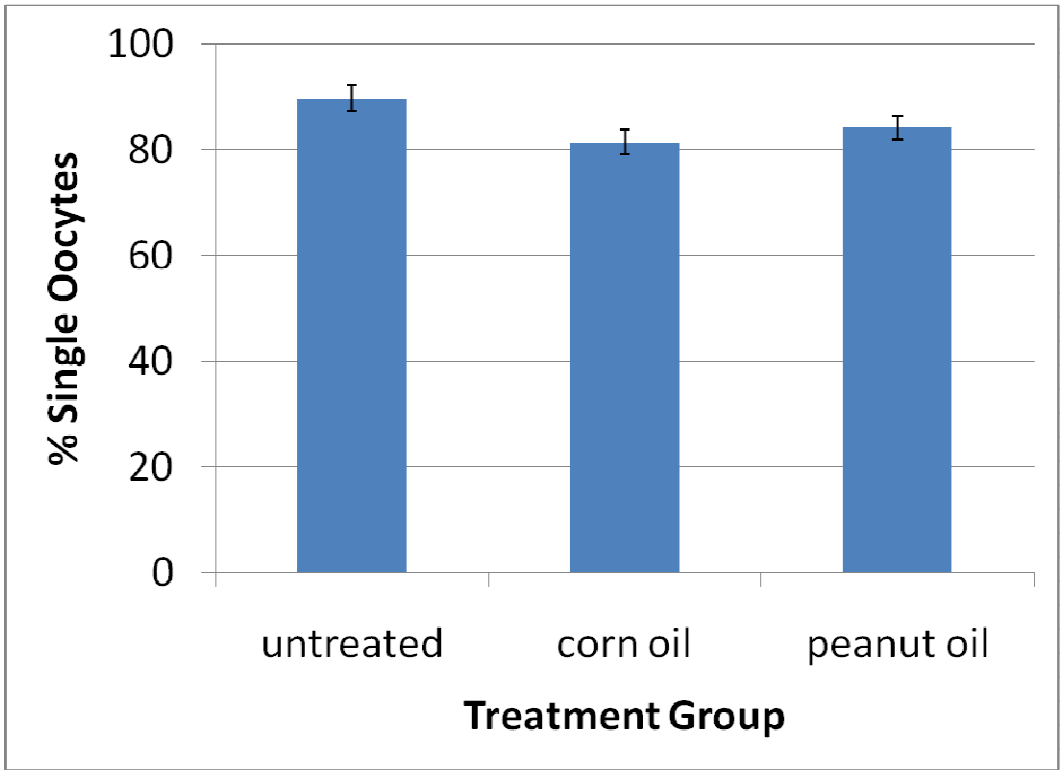

Figure 13. Cyst breakdown in untreated mice and mice injected with corn oil and peanut oil, respectively. Significant difference between percentage of single oocytes at the same stage of development with or without oil injection (one way ANOVA, $P < 0.05$; n = 8-12 ovaries per group). Asterisks indicate significance in comparison to untreated mice.

The Effect of Peanut Oil as a Delivery Medium on the Total Number of Oocytes

The total number of oocytes per confocal section was determined for untreated mice, mice injected with corn oil, and mice injected with peanut oil in order to determine whether using peanut oil as an exogenous estrogen delivery medium affected the number of oocytes per section that resulted from exogenous estrogen exposure. Figure 14 shows that ovaries from mice injected with corn oil had a significantly larger amount of oocytes (14.2) per confocal section than both those from untreated mice and mice injected with peanut oil, which had 6.1 and 8.0 oocytes per section, respectively. The difference in the total number of

oocytes between untreated mice and mice injected with peanut oil was not significant.

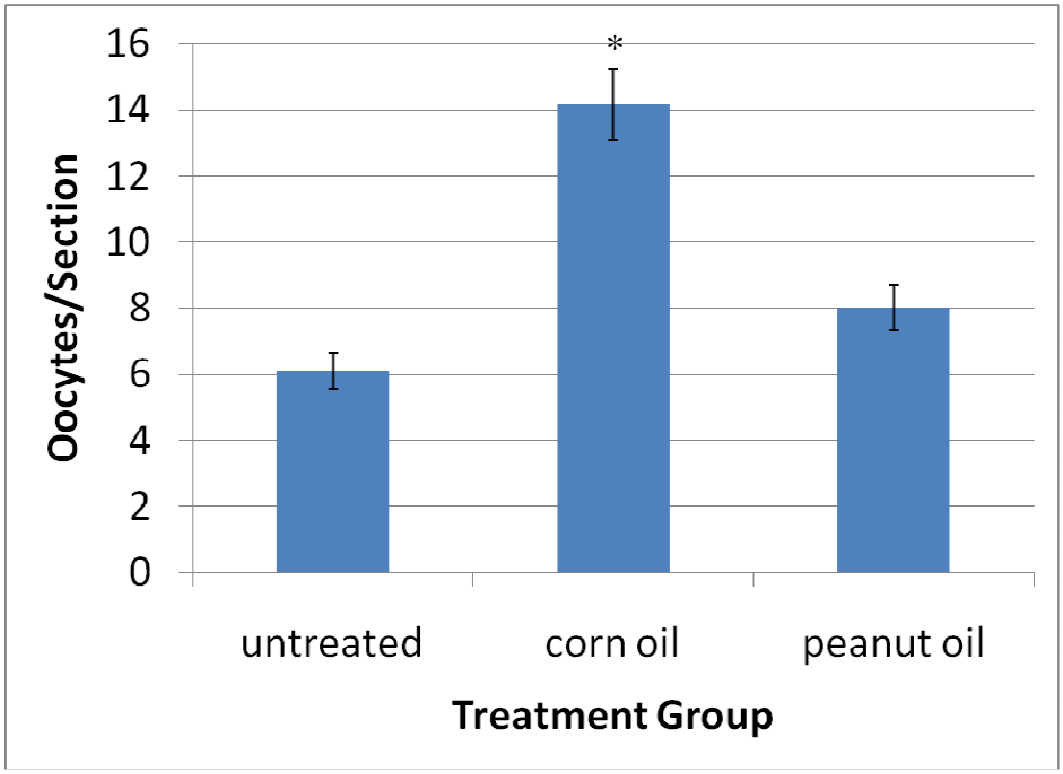

Figure 14. Total number of oocytes in untreated mice and mice injected with corn oil and peanut oil, respectively. Significant difference between the number of oocytes per confocal section at the same stage of development with or without oil injection (one way ANOVA, $P < 0.05$; n = 8-12 ovaries per group). Asterisks indicate significance in comparison to untreated mice.

The Effect of Peanut Oil as a Delivery Medium on Mouse Follicle Development

Percentages of primordial follicles increased significantly to 82.0% and 62.3% in ovaries from mice treated with corn oil and peanut oil, respectively. The percentage of primordial follicles in mice treated with corn oil was significantly larger than the percentage of primordial follicles in mice injected with peanut oil. While the percentages of primary follicles decreased in mice injected with corn oil and peanut oil to 16.7% and 27.9%, respectively, this was

only significant for mice injected with corn oil. Although the percentages of secondary follicles decreased in both mice injected with corn oil (1.3%) and peanut oil (9.7%), these differences were not significant (Figure 15).

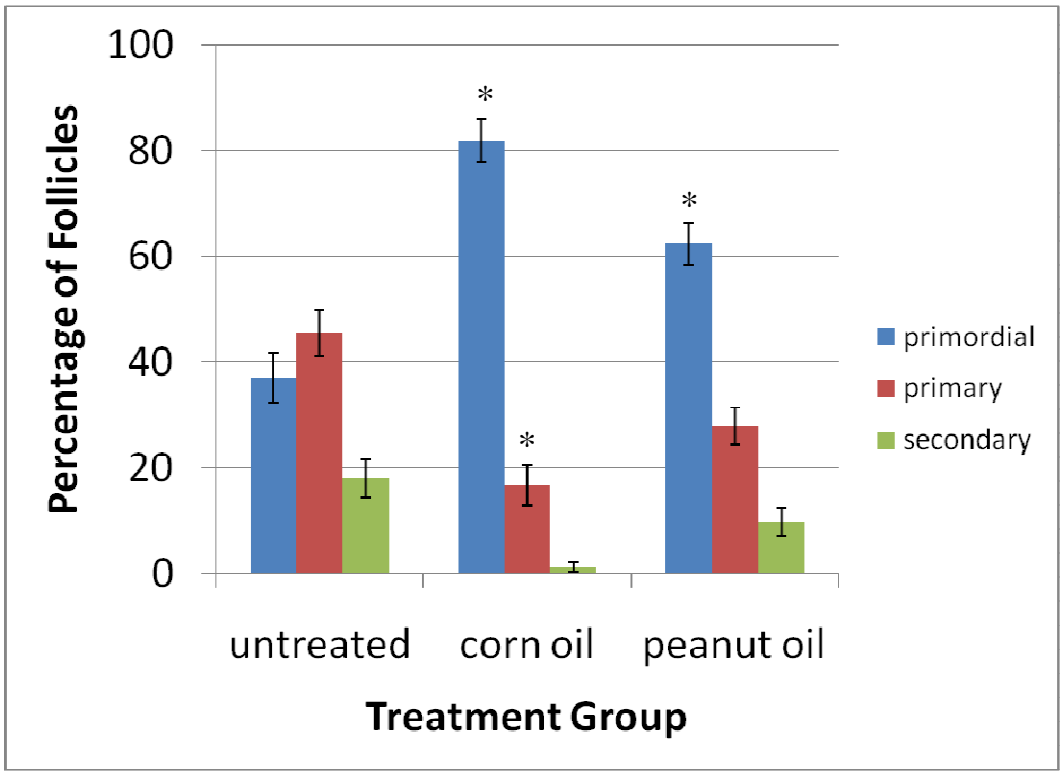

Figure 15. Follicle development in untreated mice and mice injected with corn oil and peanut oil, respectively. Significant difference between the percentages of follicles at the same stage of development with or without oil injection (one way ANOVA, $P < 0.05$; n = 8-12 ovaries per group). Asterisks indicate significance in comparison to untreated mice.

Discussion

Evidence shows that exposure to exogenous estrogens can inhibit germline cyst breakdown in mouse. Since infertility affects such a large population in the United States, understanding the relationship between exogenous estrogen exposure and primordial follicle assembly is imperative. A simultaneous occurrence of cyst breakdown and apoptosis contributes to primordial follicle assembly and approximately one-third of germ cells survive to become primordial

follicles (Pepling and Spradling, 2001). Since some female reproductive disorders, such as primary amenorrhea and primary ovarian insufficiency, result from follicle depletion, understanding the role of exogenous estrogens in primordial follicle assembly is integral to the preservation of a female's pool of primordial follicles and thus, the enhancement of her reproductive capacity. Thorough knowledge of this mechanism would allow further investigation of preventative measures through which a female's pool of primordial follicles could be maximized.

Estrogens play many roles in the female body, including the regulation of metabolism, reproduction, behavior (Boon *et al.*, 2010), and the skeletal system (Frenkel *et al.,* 2010). It has been suggested that the exposure of fetal oocytes to maternal estrogen could maintain fetal germline cysts and that cyst breakdown during the perinatal period is initiated by the decline in maternal estrogen levels at birth (Pepling, 2006). The effects of exogenous estrogen exposure on the mouse ovary have been previously researched. Neonatal exposure to DES increases the occurrence of MOF's in the ovaries of mice at PND10-34 (Iguchi *et al.,* 1986). Also, neonatal exposure to estradiol, progesterone, and genistein was shown to inhibit cyst breakdown in the ovaries of mice at PND4 (Chen *et al.,* 2007). In this study, we wanted to determine whether neonatal exposure to DES, EE, or BPA would have an effect on primordial follicle assembly that would be visible in the mouse ovary at PND5. Additionally, we investigated whether an increase in the concentration of these exogenous estrogens would have a significant effect on primordial follicle assembly. We observed that DES and EE significantly

decreased the percentage of single oocytes in the mouse ovary with there being no significant effect due to concentration increase. The results of BPA were unique in that exposure to the lower concentration did not significantly decrease the percentage of single oocytes, while the higher concentration did. The lower concentrations of DES and EE were sufficient for cyst breakdown inhibition; however, they were not sufficient to affect the total number of oocytes. Only the higher concentrations of DES and EE significantly increased the total number of oocytes while both concentrations of BPA had this effect. The significant increases in the percentages of primordial follicles resulting from exogenous estrogen exposure (Figure 9), which exhibit a pattern similar to that in Figure 10, could suggest that inhibition of germ cell death and arrest during follicle development proceed through similar pathways for DES, EE, and BPA or that a certain threshold amount exists for each of these in the mouse.

The second part of this study was to evaluate the roles of absolute ethanol in injection preparation and peanut oil as a delivery medium. Using absolute ethanol in the preparation of estradiol injections had no significant effect on cyst breakdown or the total number of oocytes. The only significant difference observed between mice that received estradiol injections prepared with or without absolute ethanol, respectively, was that a significantly lower percentage of primary follicles was observed in mice given estradiol prepared with absolute ethanol. This suggests that using absolute ethanol in the preparation may have additional inhibitory effects regarding progression through follicle development.

Neither corn oil nor peanut oil affected cyst breakdown; however, mice injected with corn oil had a significantly higher amount of oocytes per confocal section in comparison to untreated mice and mice injected with peanut oil. This suggests that corn oil may itself contain a germ cell death inhibitor, which makes it undesirable as an injection delivery medium since effects of the delivery medium should mimic untreated mice as closely as possible so that the effects of the estrogenic compound in transit are not unnecessarily masked or enhanced. Considering this criterion, both corn oil and peanut oil may seem unappealing as delivery media due to their effects on follicle development: injection with both oil types, respectively, significantly increased the percentages of primordial follicles, and injection with corn oil significantly decreased the percentages of primary follicles. While exposure to neither oil yielded a follicle development pattern that resembled that of untreated mice, it would be more beneficial to use peanut oil as a delivery medium since its results are closer to those of untreated mice. However, this does not eliminate the usage of other oils or substances as delivery media, and future research could find a model delivery medium which would not affect cyst breakdown, the total number of oocytes, and follicle development.

While DES, EE, and BPA are important and prevalent exogenous estrogens in the environment, they most certainly are not the only ones. Considering the significant results of this study and previous research, additional investigation of other exogenous estrogens should continue in order to gain

extensive knowledge regarding how these endocrine disruptors affect primordial follicle assembly, and thus, female fertility.

Works Cited

Beck-Peccoz, P. and L. Persani. "Premature ovarian failure." Orphanet Journal of Rare Diseases. 1 (2006) 9: 1-5.

Boon, W.C., J.D. Chow, and E.R. Simpson. "The multiple roles of estrogens and the enzyme aromatase." Progress in Brain Research. 181 (2010): 209-232.

Brisken, C. "Endocrine Disruptors and Breast Cancer." CHIMIA: International Journal for Chemistry. 62 (2008) 4: 406-409.

Brouwers, M.M., W.F. Feitz, L.A. Roelofs, L.A. Kiemeney, R.P. de Gier, and N. Roeleveld. "Hypospadias: a transgenerational effect of diethylstilbestrol?" Human Reproduction. 21 (2006) 3: 666-669.

Carson, R. Silent Spring. Boston: Houghton Mifflin, 1962.

Chandra, A., G.M. Martinez, W.D. Mosher, J.C. Abma, and J. Jones. "Fertility, family planning, and reproductive health of U.S. women: data from the 2002 National Survey of Family Growth." Vital Health Statistics. 23 (2005) 25: 1-174.

Chen, Y., W. Jefferson, R. Newbold, E. Padilla-Banks, and M. Pepling. "Estradiol, Progesterone, and Genistein Inhibit Oocyte Nest Breakdown and Primordial Follicle Assembly in the Neonatal Mouse Ovary *in Vitro* and *in Vivo*." Endocrinology. 148 (2007) 8: 3580-3590.

Cooke, A.S. "Shell thinning in avian eggs by environmental pollutants." Environmental Pollution. 4 (1973): 85-152.

Dahlman-Wright, K., V. Vavailles, S. A. Fuqua, V. Craig Jordan, J.A. Katzenellenbogen, K.S. Korach, A. Maggi, M. Muramatsu, M.G. Parker, and J. Gustafesson. "Internation Union of Pharmacology. LXIV. Estrogen Receptors. Pharmacological Reviews. 58 (2006): 773-781.

Ebobeid, M.A. and D.B. Allison. "Putative environmental-endocrine disruptors and obesity: a review." Current Opinion in Endocrinology, Diabetes, and Obesity. 15 (2008) 5: 403-408.

Franks, S. and K. Hardy. "Aberrant follicle development and anovulation in polycystic ovary syndrome." Ann Endocrinol (Paris). 7 (2010) 3: 228-230.

Frenkel, B., A. Hong, S.K. Baniwal, G.A. Coetzee, C. Ohlsson, O. Khalid, and Y. Gabet. "Regulation of adult bone turnover by sex steroids." Journal of Cell Physiology. E-publication ahead of print, 2010.

Guillette, L.J., T.S. Gross, G.R. Masson, J.M. Matter, H.F. Perceival, and A.R. Woodward. "Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida." Environmental Health Perpectives. 102 (1994) 8: 680-688.

Ho, S.M., W.Y. Tange, J. Belmonte de Frausto, and G.S. Prins. "Developmental exposure to estradiol and bisphenol A increases susceptibility to prostate carcinogenesis and epigenetically regulates phophodiesterase type 4 variant 4." Cancer Research. 66 (2006) 11: 5624-5632.

Hsu, S.Y. and A.J.W. Hsueh. "Tissue-specific Bcl-2 protein partners in apoptosis: an ovarian paradigm." Physiological Reviews. 80 (2000): 593-614.

Hunt, P. and T. Hassold. "Human female meiosis: what makes a good egg go bad?" Cell Press. 24 (2008) 2: 86-93.

Iguchi, T., N. Takasugi, H.A. Bern, and K.T. Mills. "Frequent Occurrence of Polyovular Follicles in Ovaries of Mice Exposed Neonatally to Diethylstilbestrol." Teratology. 34 (1986): 29-35.

Jefferson, W., R. Newbold, E. Padilla-Banks, and M. Pepling. "Neonatal genistein alters ovarian differentiation in the mouse: inhibition of oocyte nest breakdown and increased oocyte survival." Biology of Reproduction. 74 (2006) 1: 161-168.

Jordan, V. C., R. Bain, R.R. Brown, B. Gosden, and M.A. Santos. "Determination and Pharmacology of a New Hydroxylated Metabolite of Tamoxifen Observed in Patient Sera during Therapy for Advanced Breast Cancer." Cancer Research. 43 (1983): 1446-1450.

Kaufman, R.H., E. Adamn, E.E. Hatch, K. Noller, A.L. Herbst, J.R. Palmer, and R.N Hoover. "Continued follow-up of pregnancy outcomes in diethylstilbestrol-exposed offspring." Obstetrics and Gynecology. 96 (2000) 4: 483-489.

Klip, H., J. Verloop, J.D. van Gool, M.E. Koster, C.W. Burger, F.E. van Leeuwen, and the OMEGA Project Group. "Hypospadias in sons of women exposed to diethylstilbestrol in utero: a cohort study." The Lancet. 359 (2002) 9312: 1102-1107.

Master-Hunter, T. and D.L. Heiman. "Amenorrhea: Evaluation and Treatment."

American Family Physician. 73 (2006) 8: 1374-1382.

Murphy K., L. Carvajal, L. Medico, and M. Pepling. "Expression of Stat3 in germ cells of developing and adult mouse ovaries and testes." Gene Expression Patterns. 5 (2005) 4: 475-482.

Nagel, S.C., F.S. vom Saal, K.A. Thayer, M.G. Dhar, M. Boechler, and W.V. Welschons. "Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative in vivo bioactivity of the xenoestrogens bisphenol A and octylphenol." Environmental Health Perspectives. 105 (1997): 570-572.

Palmer, J.R., A.L. Herbst, K.L. Noller, D.A. Boggs, R. Troisi, L. Titus-Ernstoff, L. Hatch, L.A. Wise, W.C. Strohsnitter, and R.N. Hoover. "Urogenital abnormalities in men exposed to diethylstilbestrol in utero: a cohort study." Environmental Health. 8 (2009) 37: 1-6.

Palmer, J.R., L.A. Wise, S.J. Robboy, L. Titus-Ernstoff, K.L. Noller., A.L. Herbst., R. Troisi, and R. N. Hoover. "Hypospadias in sons of women exposed to diethylstilbestrol in utero." Epidemiology. 16 (2005) 4: 583-586.

Pepling, M.E. "From primordial germ cell to primordial follicle: mammalian female germ cell development." Genesis. 44 (2006): 622-632.

Pepling, M.E., Spradling, A.C. "Female mouse germ cells form synchronously dividing cysts." Development. 125 (1998): 3323-3328.

Pepling, M.E. and A.C. Spradling. "The mouse ovary contains germ cell cysts that undergo programmed breakdown to form follicles." Developmental Biology 234 (2001): 339-351.

Richter, C.A., J.A. Taylor, R.L. Ruhlen, W.V. Welshons, and F.S. Vom Saal. "Estradiol and Bisphenol A stimulate androgen receptor and estrogen receptor gene expression in fetal mouse prostate mesenchyme cells." Environmental Health Perspectives. 116 (2007) 6: 902-908.

Scholz, S. and N. Klüver. "Effects of endocrine disrupters on sexual, gonadal development in fish." Sexual Development. 3 (2009) 2-3: 136-151.

Steiner, E., and D. Klubert. "Assessing Breast Cancer Risk in Women." American Family Physician.78 (2008) 12: 1361-1366.

Stephen, E.H. and A. Chandra. "Use of Infertility Services in the United States: 1995." Family Planning Perspectives. 32 (2000) 3: 132-137.

Susiarjo, M., T.J. Hassold, E. Freeman, and P.A. Hunt. "Bisphenol A Exposure In Utero Disrupts Early Oogenesis in the Mouse." PLoS Genetics.

3 (2007) 1: 63-70.

Timms, B.G., K.L. Howdeshell, L. Barton, S. Bradley, C.A. Richter, and F.S. vom Saal. "Estrogenic chemicals in plastic and oral contraceptives disrupt development of the fetal mouse prostate and urethra." Proceedings of the National Academy of Science of the United States of America. 102 (2005) 19: 7014-7019.

Titus-Ernstoff, L. R. Troisi, E.E. Hatch, M. Hyer, L.A. Wise, J.R. Palmer. R. Kaufman, E. Adam, K. Noller, A.L. Herbst, W. Strohsnitter, B.F. Cole, P. Hartge, and R.N. Hoover. "Offspring of women exposed in utero to diethylstilbestrol (DES): a preliminary report of benign and malignant pathology in the third generation." 19 (2008) 2: 251-257.

Troisi, R., L. Titus-Ernstoff, M. Hyer, E.E. Hatch, S.J. Robboy, W. Strohsnitter, J.R. Palmer, B. Øglaend, E. Adam, R. Kaufman, A.L. Herbst, R.N. Hoover. "Preeclampsia risk in women exposed in utero to diethylstilbestrol." Obstetrics and Gynecology. 110 (2007) 1: 113-120.

Vajda, A.M., L.B. Barber, J.L. Gray, E.M. Lopez, J.D. Woodling, and D.O. Norris. "Reproductive disruption in fish downstream from an estrogen wastewater effluent." Environmental Science and Technology. 42 (2008) 9: 3407-3414.

van den Hurk, R. and J. Zhao. "Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles." Theriogenology. 63 (2005) 1717-1751.

Verloop, J., F.E. van Leeuwn, T.J. Helmerhorst, H.H. van Boven, and M.A. Rookus. "Cancer risk in DES daughters." Cancer, Causes, and Control. E-publication ahead of print, 2010.

vom Saal, F.S. and J.P. Myers. "Bisphenol A and risk of metabolic disorders." Journal of the American Medical Association. 300 (2008) 11: 1303-1310.

Zoller, R.T. "Environmental chemicals impacting the thyroid: targets and consequences." Thyroid. 17 (2007) 9: 811-817.

Summary of Capstone Project

Since infertility is a growing issue for human beings, more research must be done in order to thoroughly understand mammalian reproduction. Some female reproductive disorders are the result of an insufficient amount of eggs within the ovaries or a defect in the process by which these eggs mature and become ready for ovulation. Studying the development and maturation of eggs in genetically similar animals can provide insight into human reproduction, and the mouse has been used as a model organism for such studies. Recent studies in mice have shown that during the perinatal period, or the period around and including birth, large clusters of interconnected eggs undergo a series of division and programmed cell death and separation until a smaller amount of individual eggs form in the fetal ovary. The amount of single eggs that result from this process represents the entire amount of eggs that will be available to a female during her reproductive lifetime; therefore, it is imperative that flaws are eliminated from this mechanism. Based on several studies, it has been theorized that the removal of newborn mice from the maternal high-estrogen environment may initiate the process by which individual eggs form. Considering this hypothesis, recent research has shown that perinatal exposure to estrogen and to compounds that mimic estrogen in their signaling pathways in the cell can inhibit this breakdown process, thus reducing the number of individual eggs that can form and diminishing the female's reproductive capacity. The experiments described in this project aimed to describe any significant changes in the breakdown process by investigating three estrogenic substances whose effects had

not previously been evaluated during the perinatal period in mice: diethylstilbestrol (DES), ethinylestradiol (EE), and bisphenol A (BPA).

In order to expose baby mice to these three substances, injections were prepared. First, the appropriate amounts of peanut oil and the estrogenic compound were measured. The estrogenic compound was dissolved in ethanol, and this solution was added to the oil. The oil was slowly mixed until all of the ethanol evaporated to leave a solution of the estrogenic compound dissolved in peanut oil. Small and equal amounts of this solution were then loaded into sterile syringes, which were capped with thin sterile needles. Any air was squeezed out of the needle. On the day of birth, which is designated post-natal day one (PND1), each newborn mouse received an injection of the solution just below its skin. After each mouse was injected, a small portion at the end of its tail was clipped off. Tail removal ensured that the mice injected on the first day would be distinguishable on subsequent days of injection. Mice with clipped tails received an injection of the same estrogenic substance once a day for the next three days to give a total of four injections for each mouse. On PND5, or four days after the day of birth, these mice were dissected for their ovaries, which were then placed into a preservative solution. The ovaries soaked in the preservative solution overnight. The next day, the preservative was washed out of the ovaries, and the ovaries were soaked in solutions that removed all ribonucleic acid (RNA) from the ovaries' cells and stained the egg cells green and the nuclei in the ovary cells red. The stained ovaries were then mounted onto clear glass slides in a medium which preserved their color stains. A smaller glass slide was placed over the

ovaries and mounting medium, which were sealed between the two glass slides with nail polish. This was completed for a total of six treatment groups: DES, EE, and BPA at two different concentrations each.

These ovaries were imaged under a confocal microscope, which used a laser to scan through eight equidistant optical planes in each ovary. These images were analyzed in order to determine the total number of eggs in each section, the number of single eggs versus the number of eggs still in interconnected clusters, and the developmental stage of each egg. These numbers were compared to those from the ovaries of mice injected with plain peanut oil. The breakdown of the cluster was significantly inhibited by both concentrations of DES and EE and by the higher concentration of BPA; therefore, fewer single eggs were present in these ovaries. The number of eggs in each optical plane increased significantly in ovaries treated with the higher concentrations of DES and EE, respectively, and the ovaries treated with both concentrations of BPA, respectively. Once single eggs form, they must mature through a few developmental stages before they are ready for ovulation and fertilization. Classifying single eggs according to their developmental stages determined whether treatment with an estrogenic substance had an effect on the eggs' developmental progress. The number of single eggs in the first developmental stage increased significantly in ovaries from mice treated with the high concentrations of DES and EE and both concentrations of BPA, respectively. The number of single eggs in the next developmental stage decreased significantly in ovaries from mice treated with the high concentration of BPA. These findings suggest that perinatal exposure to estrogenic substances

may cause eggs to arrest in the first developmental stage or possibly slow down the process of maturation. Either way, this could lead to a loss of ovulation or irregular ovulation, thus leading to infertility.

In order to determine whether the preparation method and/or delivery medium had any effects on these results, additional experiments were performed in order to evaluate the usage of ethanol in injection preparation and the effectiveness of peanut oil as a delivery medium. In order to determine whether using ethanol in injection preparation affected the results, the same analyses that were performed on ovaries from mice injected with peanut oil containing estradiol—a naturally-occurring estrogen found in the body—prepared with ethanol and on ovaries from mice injected with peanut oil containing estradiol that was prepared without ethanol. The results were compared those of mice which did not receive any injections or treatment. The breakdown of interconnected clusters and programmed cell death were significantly inhibited in both treatment groups, and there was no significant difference for either cyst breakdown or the number of oocytes per optical plane between these two groups. Although there was no significant difference in cyst breakdown and in total number of oocytes between the two groups that were injected with estradiol, egg development may have been affected by using ethanol in the preparation of the estradiol injections: while ovaries from mice that received both types of estradiol injections had significantly larger amounts of single eggs in the first developmental stage, only mice injected with estradiol injections prepared with ethanol had a significantly lower amount of single eggs in the second developmental stage. This suggests

that ethanol could activate estradiol in such a way that the inhibition of these eggs' developmental progress is enhanced. It is imperative to understand what activates substances that mimic estrogen signaling pathways considering the abundance of exogenous estrogens in the environment and in everyday items, such as aluminum cans and plastic water bottles. In order to evaluate the effectiveness as peanut oil as a delivery medium for exogenous estrogens, ovaries were isolated from mice injected with plain corn oil and peanut oil, respectively, and were analyzed and compared to ovaries from mice which received no injections. No significant differences in the breakdown of interconnected clusters were observed in ovaries from mice injected with corn oil and peanut oil, respectively; however, the number of eggs per section increased significantly only in mice that were injected with corn oil. Additionally, while mice injected with peanut oil and corn oil both had significantly higher amounts of single eggs in the first developmental stage, only mice injected with corn oil also had a significantly lower amount of single eggs in the second developmental stage. This suggests that corn oil or something that it contains may inhibit the progression of egg development and cell death, also.

The early years are imperative to healthy development, and this experiment supplies additional evidence of this. Influences that may affect an organism's reproductive capacity are especially important to monitor because they can cause infertility. This is especially important within the realm of mammalian reproduction since millions of human beings struggle with infertility and invest in expensive treatments. These experiments not only show the effects

that external influences can have on mammalian reproduction, but also show that the effects of these substances may be enhanced by other materials with which they come in contact.