

EQUOL INHIBITS GROWTH, INDUCES ATRESIA, AND INHIBITS STEROIDOGENESIS
OF MOUSE ANTRAL FOLLICLES

BY

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THESIS

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ABSTRACT

Equol is a non-steroidal estrogen metabolite produced by microbial conversion of daidzein, a major isoflavone phytoestrogen in soybean, in the gut of some humans and many animal species. Previous studies indicate that isoflavones and their metabolites can affect endogenous estradiol production, action, and metabolism via several mechanisms, which could in turn influence ovarian follicle growth. However, no studies have examined the direct effects of equol on intact antral follicles, which are responsible for sex steroid synthesis and further development into ovulatory follicles. The present study tested the hypothesis that equol inhibits antral follicle growth, increases follicle atresia, and inhibits steroidogenesis in the adult mouse ovary. Ovarian antral follicles from adult CD-1 mice were cultured with vehicle control or equol (600 nM, 6 μ M, 36 μ M, 100 μ M) for 48 and 96 h. Follicle diameters were measured every 24 h to monitor follicle growth, and at 48 and 96 h, follicles were subjected to gene expression analysis and media were subjected to measurement of hormone levels. Follicles were also subjected to western blot analysis of certain steroidogenic enzymes, as well as histological evaluation of atresia following 96 h of culture. Equol (100 μ M) inhibited follicle growth and induced follicle atresia. Further, equol decreased the levels of estradiol, testosterone, androstenedione, and progesterone, and decreased mRNA levels of cholesterol side-chain cleavage, steroid 17- α -hydroxylase, and aromatase compared to control. Collectively, these data suggest that equol alters antral follicle function by inhibiting growth, increasing atresia, and inhibiting steroidogenesis.

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CHAPTER 1

OVERVIEW

Equol is a non-steroidal estrogen metabolite produced by microbial conversion of daidzein, a major isoflavone phytoestrogen in soybean, in the gut of some humans and many animal species (Atkinson *et al.*, 2005; Axelson *et al.*, 1984; Khan *et al.*, 2011; Lundh, 1995; Setchell *et al.*, 2005; Setchell *et al.*, 2002; Setchell and Cole, 2006). In addition, equol is commercially available as a dietary supplement for its potential beneficial effects against aging, skin conditions, hair loss, prostate cancer, obesity, hot flashes, menopause, osteoporosis, heart disease, and neurologic conditions (Setchell *et al.*, 2001; Yee *et al.*, 2008). Based on studies measuring plasma and urine equol concentrations, the level of human exposure through diet and supplement use is estimated to be between 400 nM and 5 μ M (Setchell *et al.*, 2001; Setchell *et al.*, 2009; Setchell *et al.*, 1997; Vedrine *et al.*, 2006). Dietary exposure in animals occurs due to the common inclusion of soybean as a protein source in animal feed (Lindsay and Kelly, 1970; Lundh, 1995; Woclawek-Potocka *et al.*, 2013; Woclawek-Potocka *et al.*, 2008). Substantial exposure to equol can also occur in domestic animals consuming large quantities of isoflavones while grazing on phytoestrogen-rich pastures. This has been previously associated with reproductive disorders in cattle and sheep (Adams, 1995; Dusza *et al.*, 2006; Kallela *et al.*, 1984; Shutt and Braden, 1968; Shutt *et al.*, 1970; Woclawek-Potocka *et al.*, 2005b).

Unfortunately very few studies have investigated the potential adverse effects of equol on the female reproductive system, and in particular the ovary. The ovary is a heterogeneous organ with

follicles at different stages of development. The follicle is the functional unit of the ovary, and contains the oocyte surrounded by granulosa and theca cells. The least developed stage is the primordial follicle, and the female is born with a finite number of these follicles. The primordial follicle undergoes gradual development into primary follicle, preantral follicle, and finally an antral follicle, which consists of a fluid filled space called the antrum, in addition to the oocyte, granulosa cells, and theca cells (Hirshfield, 1991). Antral follicles are the only follicles in the ovary capable of undergoing further development into ovulatory follicles. Antral follicles are also the major source of sex steroid hormones in the ovary. Healthy antral follicles are therefore essential for normal female reproduction. Potential damage to antral follicles can lead to infertility, inhibition of sex steroid synthesis, and premature ovarian failure (Patel *et al.*, 2015). Negative effects of ovarian damage can also extend to non-reproductive function, including cardiovascular, neurologic, and bone health (Bagur and Mautalen, 1992; Cooper and Sandler, 1998; Everson *et al.*, 1995; Hu *et al.*, 1999).

The ovarian antral follicle is an important target tissue for equol because it contains numerous estrogen receptors (both alpha and beta subtypes), which can bind to equol. However, the direct effects of equol on intact antral follicles are unknown. Thus, the goal of my research was to examine the effects of a broad range of concentrations of equol on ovarian antral follicles using an *in vitro* culture system. To achieve this goal, I tested the hypothesis that equol inhibits antral follicle growth, increases follicle atresia, and inhibits steroidogenesis in the adult mouse ovary by altering the expression of enzymes required for estradiol biosynthesis. To test this hypothesis, I completed the following specific aims.

Specific Aim 1: Determine if equol inhibits the growth of antral follicles *in vitro*

To complete this aim, I cultured antral follicles from the ovaries of adult CD-1 mice with vehicle control (dimethylsulfoxide, DMSO) or equol (600 nM, 6 μ M, 36 μ M, and 100 μ M) for 96 hours. I measured follicle diameters at every 24 hour time point to assess the percent change in follicle diameter over 96 hours of culture. The data indicate that equol exposure inhibited follicle growth at 72 and 96 hours.

Specific Aim 2: Determine if equol induces atresia of antral follicles *in vitro*

To complete this aim, I cultured antral follicles from the ovaries of adult CD-1 mice with vehicle control (dimethylsulfoxide, DMSO) or equol (600 nM, 6 μ M, 36 μ M, 100 μ M) for 48 and 96 hours. Following these two time points, I examined the follicle *Bax* to *Bcl2* mRNA ratio, a useful measure of apoptosis occurring in the ovary. The data indicate that equol exposure increased this ratio at 96 hours. To confirm that equol increased apoptosis, I then examined antral follicles histologically following 96 hours of culture to detect the presence of apoptotic bodies in the granulosa cells. The data indicate that follicles treated with equol showed a higher level of atresia, indicated by a greater abundance of apoptotic bodies. Overall, these data suggest that equol increases follicle atresia potentially via dysregulation of the apoptotic pathway.

Specific Aim 3: Determine if equol inhibits steroidogenesis of antral follicles *in vitro*

To complete this aim, I cultured antral follicles from the ovaries of adult CD-1 mice with vehicle control (dimethylsulfoxide, DMSO) or equol (600 nM, 6 μ M, 36 μ M, 100 μ M) for 48 and 96 hours. Following these two time intervals, I examined the levels of sex steroid hormones in the culture media as well as the steroidogenic enzyme expression of antral follicles. Further, I investigated the protein levels of certain steroidogenic enzymes following 96 hours of culture. The data indicate that equol exposure decreased the levels of estradiol, testosterone, androstenedione, and progesterone at 96 hours. In addition, equol inhibited the expression of *Cyp11a1* and *Cyp17a1* at 48 and 96 hours, and *Cyp19a1* at 96 hours. However, western blot analysis of CYP17A1 and CYP19A1 at 96 hours revealed that equol did not affect the protein levels of these two enzymes at this time point. Collectively, these data suggest that equol inhibits sex steroid production of antral follicles, potentially through disruption of upstream steroid hormones and steroidogenic enzymes. However, the exact mechanisms behind inhibition of steroidogenesis need further investigation.

In summary, Chapter 1 described the overview of my Master's research work. Chapter 2 provides a background on phytoestrogens and equol in particular, as well as their potential ovotoxic effects. Chapter 3 details the methods I used to test my hypothesis, and Chapter 4 describes the results of the present study. Chapter 5 provides a detailed discussion of the results obtained, and Chapter 6 summarizes the findings of my research and outlines future directions. Finally, Chapter 7 provides the data in tables and figures.

CHAPTER 2

INTRODUCTION

Phytoestrogens are non-steroidal phenolic plant compounds that have agonistic, partial agonistic, and antagonistic interactions with estrogen receptors, as well as other biological targets involved in endogenous estrogen synthesis, action, and metabolism (Whitten and Patisaul, 2001). People are exposed to these botanical compounds through the diet and in the form of supplements taken for cardiovascular, renal, neurologic health, reproductive health, bone health, weight loss, menopausal symptoms, and chemoprevention (Andres *et al.*, 2011; Bhathena and Velasquez, 2002; Ho and Jie, 2007; Khan *et al.*, 2011; Kurzer, 2003; Setchell and Lydeking-Olsen, 2003; Shibata, 2000). Wildlife and domestic animals are exposed to phytoestrogens through grazing on phytoestrogen-rich pastures and through diets containing soybean, a commonly used protein source in animal feed (Lindsay and Kelly, 1970; Lundh, 1995; Setchell *et al.*, 1987; Shutt and Braden, 1968; Whitten and Patisaul, 2001; Woclawek-Potocka *et al.*, 2013; Woclawek-Potocka *et al.*, 2008).

Isoflavones, found predominantly in legumes like soy and red clover, represent an important class of phytoestrogens, and include genistein, daidzein, glycitein, and formononetin. Equol is a metabolite of daidzein and is produced by the action of intestinal microflora in 20-60% of adults consuming soy foods or isoflavones, and has also been identified in the plasma and urine of many animal species consuming isoflavones in the diet (Atkinson *et al.*, 2005; Axelson *et al.*, 1984; Khan *et al.*, 2011; Lundh, 1995; Setchell *et al.*, 2005; Setchell *et al.*, 2002; Setchell and

Cole, 2006). In addition, equol is commercially available as a dietary supplement and has been developed as a nutraceutical agent for the treatment of a variety of medical conditions (Setchell *et al.*, 2001; Yee *et al.*, 2008). Equol occurs as two enantiomeric forms, S-(−)equol and R-(+)equol, with the S-(−)equol enantiomer being the form specifically produced by intestinal bacteria (Setchell *et al.*, 2005). Equol binds both estrogen receptor alpha and beta, though it has been found to have higher affinity for estrogen receptor beta (Morito *et al.*, 2001; Setchell *et al.*, 2005). It has greater biological activity than its parent isoflavone (daidzein), and has been shown to exert estrogenic activity in E-SCREEN assays using breast cancer and ovarian cell lines (Muthyala *et al.*, 2004; Schmitt *et al.*, 2001). Further, equol may have anti-androgenic activity by binding to and blocking the effects of dihydrotestosterone (Lund *et al.*, 2004).

Studies indicate that compounds originating from plants, including phytoestrogens, may have beneficial effects and could be potentially useful in treating many disease conditions (Barnes, 1998; Cassidy, 1996; Tham *et al.*, 1998). The isoflavones are potent antioxidants, and the metabolite equol in particular, has been shown to possess superior antioxidant activity compared to daidzein and genistein (Arora *et al.*, 1998; Hwang *et al.*, 2003; Mitchell *et al.*, 1998; Rufer and Kulling, 2006). Equol is marketed as a nutraceutical agent for its potential protective effects against aging, skin conditions, hair loss, prostate cancer, obesity, hot flashes, menopause, osteoporosis, heart disease, and neurologic conditions (Setchell *et al.*, 2001; Yee *et al.*, 2008). However, the potential side effects of botanical chemicals including isoflavones and their metabolites are less clear. Of particular concern are the potential adverse effects that botanical compounds may have on the ovary. The ovary is an important target tissue for botanical compounds because it contains numerous estrogen receptors (both alpha and beta subtypes),

which can bind to estrogenic botanical compounds. Chemicals that damage ovarian function have the potential to reduce fertility, inhibit estradiol synthesis, and cause premature ovarian failure (Patel *et al.*, 2015). Reduced estradiol levels are associated with reduced fertility, depression, bone loss, cardiovascular disease, and menopausal symptoms (Armamento-Villareal *et al.*, 1992; Bush *et al.*, 1987; Christiansen, 1993; Cooper and Sandler, 1998; Dennerstein *et al.*, 1999; Everson *et al.*, 1995; Hu *et al.*, 1999; Mosca, 1998). Premature ovarian failure is associated with an increased risk for neurological disorders, osteoporosis, and cardiovascular disease (Bagur and Mautalen, 1992; Cooper and Sandler, 1998; Everson *et al.*, 1995; Hu *et al.*, 1999). Thus, botanical compounds that target the ovary have the potential to negatively impact both reproductive and non-reproductive function.

Unfortunately, very few studies have examined the effects of equol on the female reproductive system, and its effects on ovarian function are virtually unknown. Equol has been associated with infertility in sheep and cattle grazing on isoflavone-rich pastures or fed high soybean diets (Adams, 1995; Kallela *et al.*, 1984; Shutt and Braden, 1968; Shutt *et al.*, 1970; Woclawek-Potocka *et al.*, 2005b). Equol was also found to disrupt the secretory activity of bovine endometrium at different stages of the estrous cycle and during pregnancy (Woclawek-Potocka *et al.*, 2005a; Woclawek-Potocka *et al.*, 2005b; Woclawek-Potocka *et al.*, 2006; Woclawek-Potocka *et al.*, 2005c). It also altered ovarian development and delayed oocyte maturation in Japanese medaka fish (Kiparissis *et al.*, 2003). It also inhibits rainbow trout ovarian aromatase activity, required for conversion of testosterone to estradiol (Pelissero *et al.*, 1996). However, to our knowledge, no studies have examined the effects of equol on intact antral follicles, which are the functional units of the ovary and are responsible for steroid hormone synthesis and further

development into ovulatory follicles. Thus, the goal of this study was to test the hypothesis that equol inhibits antral follicle growth, increases follicle atresia, and inhibits steroidogenesis in the adult mouse ovary by altering the expression of enzymes required for estradiol biosynthesis.

CHAPTER 3

MATERIALS AND METHODS

Animals

Cycling female CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA) and acclimatized for 24-72 hours in the College of Veterinary Medicine Animal Facility at the University of Illinois at Urbana–Champaign. The mice were housed in groups of four, in a controlled environment (22 ± 1 °C, 12-hour light–dark cycles) and provided food and water ad libitum. All procedures involving animal care, euthanasia, and tissue collection were approved by the Institutional Animal Use and Care Committee at the University of Illinois at Urbana–Champaign.

Antral follicle culture and chemical treatment

Female CD-1 mice were euthanized on postnatal days (PND) 32-35 and their ovaries were collected aseptically. Antral follicles were manually isolated from the ovaries based on relative size (225-400 μ m), and cleaned of interstitial tissue using watchmaker forceps. Approximately 20-40 follicles were obtained from each mouse. Isolated antral follicles were randomly and individually plated in wells of a 96-well tissue culture plate, using sterile technique, such that each treatment group contained 5-18 follicles. A minimum of three separate cultures for each experiment was performed. Cultures were carried out for a total period of 48 hours and 96 hours to evaluate experimental endpoints including follicle growth, hormone levels, and enzyme expression.

S-(–)equol was supplied by the Botanical Estrogen Research Center, University of Illinois at Urbana-Champaign, courtesy of Dr. William Helferich. It was synthesized at the National Center for Natural Products Research, University of Mississippi and the chemical and optical purity was confirmed to be greater than 98% by liquid chromatography/time-of-flight/mass spectrometry, nuclear magnetic resonance, optical rotation and chiral high-performance liquid chromatography. Stock solutions of equol in various concentrations (0.19, 1.94, 11.63, and 32.30 mg/mL) were prepared using dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) as the vehicle. This allowed for an equal volume of each stock to be added to the culture wells to control for vehicle concentration (0.75 μ L per mL of medium). This translated to final working concentrations of 0.14, 1.45, 8.72, and 24.23 μ g of equol per mL of culture medium, which is equivalent to 600 nM, 6 μ M, 36 μ M, and 100 μ M, respectively (molecular weight of equol: 242.27 g/mol). A broad range of concentrations of equol was chosen for our study because it is not known exactly how much of the equol ingested from supplements or as a metabolite from soy in the diet reaches the ovaries and antral follicles. The level of environmental exposure in humans, based on studies measuring plasma and urine equol concentrations, is similar to the lower-range concentrations used in our study (600 nM and 6 μ M) (Setchell *et al.*, 2001; Setchell *et al.*, 2009; Setchell *et al.*, 1997; Vedrine *et al.*, 2006). The level of exposure in domestic animals may be greater than that in humans, given the widespread use of soybean as a protein source in animal feeds (Lindsay and Kelly, 1970; Lundh, 1995; Woclawek-Potocka *et al.*, 2013; Woclawek-Potocka *et al.*, 2008). Further, equol has been reportedly associated with adverse reproductive effects in sheep and cattle grazing on phytoestrogen-rich pastures (Adams, 1995; Dusza *et al.*, 2006; Kallela *et al.*, 1984; Shutt and Braden, 1968; Shutt *et al.*, 1970; Woclawek-Potocka *et al.*, 2005b). The concentrations of equol tested in our study were also based on those used in previous *in vitro* and

in vivo studies published in the literature (Choi, 2009; Ju *et al.*, 2006; Liang *et al.*, 2014; Matulka *et al.*, 2009; Pelissero *et al.*, 1996). Reports of other soy isoflavones such as genistein showing harmful effects on female reproduction used a similar range of doses (Gregoraszczuk *et al.*, 1999; Jefferson *et al.*, 2002; Whitehead and Lacey, 2000).

The five treatment groups (DMSO or vehicle control, equol at 600 nM, 6 μ M, 36 μ M and 100 μ M) were prepared in supplemented α -MEM, containing 1% ITS (10 ng/mL insulin, 5.5 ng/mL transferrin, 5.5 ng/mL selenium, Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO), 100 mg/mL streptomycin (Sigma-Aldrich, St. Louis, MO), 5 IU/mL human recombinant follicle-stimulating hormone (FSH; Dr. A. F. Parlow, National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA), and 5% fetal calf serum (Atlanta Biologicals, Lawrenceville, GA) as described previously (Gupta *et al.*, 2006; Miller *et al.*, 2005). Each follicle was cultured in 150 μ L of medium for either 48 hours or 96 hours in an incubator at 37 °C and 5% CO₂.

Follicle growth analysis

To assess antral follicle growth over time, individual follicle diameters were measured (in micrometers) on perpendicular axes at every 24 hour time point for each culture, with an inverted microscope equipped with a calibrated ocular micrometer. The individual diameters were averaged among the five treatment groups for each 24 hour interval. The average diameters for each 24 hour interval were then divided by the initial average measurement (0 hour) of each of the respective treatment groups to calculate the percent change in follicle diameter over time.

This percent change in antral follicle diameter was used for statistical analysis. Images of the follicles were taken using a digital microscope camera (ProgRes CT3 model; Jenoptik) and captured using image acquisition software (ProgRes CapturePro; Jenoptik).

Enzyme linked immunosorbent assays

After every 48 and 96 hour culture, media from each well were collected and pooled according to treatment. They were stored at – 80 °C until subjected to enzyme linked immunosorbent assays (ELISAs) for measurement of the levels of progesterone, androstenedione, testosterone, and 17 β -estradiol. These sex steroid hormones were chosen based on their necessity for normal female reproduction. The levels of these hormones were measured using ELISA kits purchased from Diagnostics Research Group (DRG, Springfield, NJ) following manufacturer's instructions. The analytical sensitivity of each kit was 0.1 ng/mL for progesterone, 0.019 ng/mL for androstenedione, 0.083 ng/mL for testosterone, and 9.71 pg/mL for estradiol. Samples were run in duplicate, and had intra-assay coefficients of variability of less than 15%. Samples were diluted as needed to match the dynamic range of each ELISA kit, and read using Multiskan Ascent software (Thermo Scientific). The mean values for each sample were used for statistical analysis. Cross reactivity of equol at the higher concentrations (6, 36, and 100 μ M) was checked in the estradiol assay, and when background was present, it was subtracted from sample means before statistical analysis.

RNA isolation and quantitative real-time polymerase chain reaction

After each 48 and 96 hour culture, antral follicles were pooled according to treatment group, snap frozen in liquid nitrogen, and stored at -80°C until subjected to quantitative real-time polymerase chain reaction (qPCR) analysis. Total RNA was extracted from the follicles using the RNeasy Micro Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol, and quantified using Nanodrop spectrophotometer. Total RNA (100 ng per sample) was then reverse transcribed to complementary DNA (cDNA) using the iScript RT kit (Bio-Rad Laboratories, Inc., Hercules, CA) following the manufacturer's protocol. Each cDNA sample was diluted 1:4 using nuclease-free water prior to qPCR. qPCR was conducted using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA) and accompanying CFX Manager Software according to the manufacturer's protocol.

All qPCR reactions were done in triplicate using 1 μl cDNA, forward and reverse primers (5 pmol) for steroidogenic acute regulatory protein (*Star*), cytochrome P450 cholesterol side-chain cleavage (*Cyp11a1*), 3 β -hydroxysteroid dehydrogenase 1 (*Hsd3b1*), cytochrome P450 steroid 17 α -hydroxylase 1 (*Cyp17a1*), 17 β -hydroxysteroid dehydrogenase 1 (*Hsd17b1*), cytochrome P450 aromatase (*Cyp19a1*), bcl2-associated X protein (*Bax*), B cell leukemia/lymphoma 2 (*Bcl2*), or beta-actin (*Actb*), in addition to SsoFastEvaGreen Supermix for a final reaction volume of 10 μl . Specific qPCR primers (Integrated DNA Technologies, Inc., Coralville, IA) for the target genes of interest as well as the reference gene, beta-actin (*Actb*) are listed in Table 1. The genes tested were chosen because they are regulators of ovarian steroidogenesis (*Star*, *Cyp11a1*, *Hsd3b1*, *Cyp17a1*, *Hsd17b1*, and *Cyp19a1*) (Falck, 1959; Hanukoglu, 1992; Penning, 1997; Richards, 1980) and apoptosis (*Bax*, *Bcl2*) (Flaws *et al.*, 2001;

Greenfeld *et al.*, 2007; Hengartner, 2000). The CFX96 machine quantifies the amount of PCR product generated by measuring SsoFastEvaGreen dye (Bio-Rad Laboratories, Inc., Hercules, CA) that fluoresces when bound to double-stranded DNA. The qPCR program protocol consisted of an enzyme activation step (95 °C for 1 min), a denaturation and annealing step (40 cycles of 95 °C for 10 s, 60 °C for 10 s), an extension step of 72 °C for 5 min, a melt curve (65 °C–95 °C heating 0.5 °C/s with continuous fluorescence readings), and a final step at 72 °C for 5 min. Standard curves were generated from five to seven serial dilutions of a combination of samples to obtain PCR efficiency. *Actb* was used as a reference gene because its expression did not differ between the 5 treatment groups. Expression data were generated using the Pfaffl method for relative quantification, and the calculated relative fold changes were used for statistical analysis.

Western blot analysis

After 96 hours of culture, antral follicles were pooled according to treatment group, snap frozen in liquid nitrogen, and stored at – 80 °C until subjected to western blotting for analysis of protein levels of the steroidogenic enzymes CYP17A1 and CYP19A1. The protein levels of these enzymes were tested based on the results of our gene expression experiments at 96 hours. Antral follicles were homogenized using T-Per (Thermo Fisher Scientific, Rockford, IL) containing protease inhibitors (Roche Diagnostics, Mannheim, Germany). After homogenization, the protein concentration in the lysate was determined using the BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, IL). Electrophoresis and immunoblotting were performed using XCell SureLock Mini-Cell Blot Module Kit and recommended reagents as per manufacturer's protocol (Invitrogen, Carlsbad, CA). The protein lysate (10 µg for CYP17A1 and 20 µg for CYP19A1) was loaded on precast 4–12% bis-tris sodium dodecyl sulfate-polyacrylamide gels

(SDS-PAGE; Invitrogen, Carlsbad, CA), followed by wet transfer of the proteins to a blot at 4 °C. The blots were incubated overnight at 4 °C with the primary antibodies (Santa Cruz Biotechnology Inc, Dallas, TX) anti-CYP17A1 (1:500) or anti-CYP19A1 (1:100), followed by incubation with secondary horseradish peroxidase (HRP)-linked antibodies (1:5000 for CYP17A1; Abcam, Cambridge, MA and 1:1000 for CYP19A1; Santa Cruz Biotechnology Inc, Dallas, TX), for one hour at room temperature. To ensure uniform loading of the proteins, the blots were stripped and incubated with anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1000; EnCor Biotechnology Inc., Gainesville, FL) for two hours at room temperature, followed by incubation with an HRP-linked secondary antibody (1:5000; Sigma-Aldrich) for one hour at room temperature. The immune complexes on the blots were visualized using an enhanced chemiluminescence detection kit (Cell Signaling Technologies, Danvers, MA) and captured on X-ray film. Scanning densitometry using the Image J software downloaded from the NIH website (<http://rsb.info.nih.gov/ij/>) was used to compare the protein levels. Densitometric units of CYP17A1 and CYP19A1 were normalized to GAPDH for quantification, and the relative density was used for statistical analysis.

Histological evaluation of atresia

After 96 hours of culture, antral follicles were collected and processed for histological evaluation of atresia. Follicles were fixed for at least 24 hours in Dietrich's solution, then dehydrated through ascending grades of alcohol. Follicles were embedded using the Technovit 7100 plastic embedding system (Electron Microscopy Science), and serial sections of 2 µm were mounted on glass slides and stained with Lee's methylene blue-basic fuchsin staining protocol. Five sections of each follicle were observed for apoptotic bodies in the granulosa cell layers, the presence of

which is a morphological sign of atresia. Based on the extent of apoptotic bodies, the follicles were scored in a blinded fashion on a scale of 1-4 as previously described, with minor modifications (Gupta *et al.*, 2006; Miller *et al.*, 2005). A rating of 1 indicated the presence of apoptotic bodies encompassing 0-3% of total area of the follicle, a rating of 2 indicated the presence of apoptotic bodies encompassing 4–10% of total area of the follicle, a rating of 3 indicated the presence of apoptotic bodies encompassing 11–30% of total area of the follicle, and a rating of 4 indicated the presence of apoptotic bodies encompassing > 31% of the total area of the follicle. Follicles with a rating of 1 or 2 were considered to be healthy or undergoing early atresia, whereas those with a rating of 3 or 4 were considered to be undergoing mid to late atresia. Atresia ratings of each follicle were reported based on the average of all 5 ratings for the different sections of that follicle, and the averaged ratings were used for statistical analysis. Images of the follicles were taken using a digital microscopy camera (model DFC290; Leica Microsystems) and captured with image acquisition software (ImagePro; Leica).

Statistical analyses

All data were analyzed using SPSS statistical software (SPSS, Inc., Chicago, IL). Data were expressed as means $\pm$ standard error of the means (SEM), and at least three separate experiments were conducted for each treatment group prior to data analysis. Data were checked for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test for homogeneity of variance. Multiple comparisons between normally distributed experimental groups for continuous data were made using the one-sided Dunnett's test when variances were equal or the Welch test followed by the Games-Howell test for post-hoc comparison when variances were unequal (Haseman *et al.*, 2001). Multiple comparisons between non-normally

distributed experimental groups or for ordinal data were made using the Kruskal–Wallis test, followed by the Mann-Whitney test for post-hoc comparison. Statistical significance was assigned at $p \leq 0.05$.

CHAPTER 4

RESULTS

Effect of equol on antral follicle growth

Follicles were cultured with the vehicle control (DMSO) or different concentrations of equol (600 nM, 6 μ M, 36 μ M, 100 μ M), and percent change in follicle growth was analyzed every 24 hours for a total period of 96 hours. Follicles cultured with the vehicle control (DMSO) showed significant growth over time (Figure 1). Exposure to equol at the 100 μ M concentration inhibited follicle growth at 72 and 96 hours of culture when compared to the vehicle control group (Figure 1; $n=3-10$, $p\leq 0.05$). Exposure to equol at the lower concentrations (600 nM, 6 μ M, 36 μ M) did not significantly inhibit follicle growth compared to the vehicle control group at any time point (Figure 1).

Effect of equol on antral follicle-produced sex steroid hormone levels

Follicles were cultured with the vehicle control (DMSO) or different concentrations of equol (600 nM, 6 μ M, 36 μ M, 100 μ M), and the levels of four sex steroid hormones normally produced by growing antral follicles were determined in the culture media at 48 hours and 96 hours. Exposure of the follicles to equol at 600 nM, 6 μ M, 36 μ M, and 100 μ M did not affect levels of estradiol, testosterone, androstenedione, or progesterone at 48 hours when compared to DMSO (Figures 2-5). However, equol at 6 μ M, 36 μ M, and 100 μ M significantly decreased production of estradiol (Figure 2; $n=3-10$, $p\leq 0.05$) and androstenedione (Figure 4; $n=3-7$, $p\leq 0.05$) by 96 hours when compared to vehicle control.

Equol at the 100 μ M concentration also decreased the levels of testosterone (Figure 3; n=3-10, $p \leq 0.05$) and progesterone (Figure 5; n=3-10, $p \leq 0.05$) by 96 hours when compared to vehicle control.

Effect of equol on antral follicle mRNA expression of steroidogenic enzymes

Our data indicate that equol decreased the levels of estradiol and its metabolic pre-cursors testosterone, androstenedione, and progesterone at 96 hours of culture (Figures 2-5). Therefore, we investigated if equol inhibits sex steroid production of antral follicles by altering the gene expression of steroidogenic enzymes important in estradiol biosynthesis. Specifically, we examined the effects of different concentrations of equol (600 nM, 6 μ M, 36 μ M, or 100 μ M) on the expression of the following steroidogenic enzymes at 48 and 96 hours of culture.

Steroidogenic acute regulatory protein (Star)

STAR is a transport protein that regulates cholesterol transfer into the mitochondria and is a rate-limiting step for steroidogenesis (Miller and Strauss, 1999; Strauss *et al.*, 1999). Our results indicate that equol did not affect the expression of *Star* at either 48 or 96 hours of culture when compared to the control group (Figure 6A).

Cytochrome P450 cholesterol side-chain cleavage (Cyp11a1)

CYP11A1 is another rate-limiting step for steroidogenesis and metabolizes cholesterol to pregnenolone, which is the pre-cursor for progesterone (Miller and Strauss, 1999; Payne and Hales, 2004). We found that the expression of *Cyp11a1* was significantly decreased at 48 hours and showed a trend towards decrease at 96 hours in the 100 μ M treatment group when compared

to the vehicle control group (Figure 6B; n=3-4, $p \leq 0.05$). The lower concentrations of equol did not affect *Cyp11a1* expression at either time point (Figure 6B).

3 β -hydroxysteroid dehydrogenase 1 (Hsd3b1)

HSD3B1 metabolizes and converts $\Delta 5$ -steroids (pregnenolone and dehydroepiandrosterone) into $\Delta 4$ -steroids (progesterone and androstenedione) (Payne and Hales, 2004; Readhead *et al.*, 1983). Our data indicate that equol did not affect the expression of *Hsd3b1* at either 48 or 96 hours of culture when compared to the vehicle control (Figure 6C).

Cytochrome P450 steroid 17- α -hydroxylase 1 (Cyp17a1)

CYP17A1 is important for the synthesis of dehydroepiandrosterone and androstenedione from pregnenolone and progesterone, respectively (Conley and Bird, 1997; Payne and Hales, 2004). Equol at 100 μ M significantly decreased the expression of *Cyp17a1* at both 48 and 96 hours of culture when compared to control (Figure 6D; n=3-4, $p \leq 0.05$). The expression of this enzyme was not affected by exposure to the lower concentrations of equol at either time point (Figure 6D).

17 β -hydroxysteroid dehydrogenase 1 (Hsd17b1)

HSD17B1 is responsible for conversion of androstenedione to testosterone as well as estrone to estradiol (Armstrong, 1968; Payne and Hales, 2004). Equol did not affect the expression of *Hsd17b1* compared to control at either 48 or 96 hours of culture (Figure 6E).

Cytochrome P450 aromatase (Cyp19a1)

CYP19A1 converts androstenedione to estrone as well as testosterone to estradiol (Armstrong, 1968; Payne and Hales, 2004). Equol at 100 μ M caused a significant decrease in *Cyp19a1* expression at 96 hours (Figure 6F; $n=3-7$, $p\leq 0.05$), but this effect was not seen at 48 hours (Figure 6F). Further, the lower concentrations of equol (600 nM, 6 μ M, 36 μ M) did not affect *Cyp19a1* expression at either time point (Figure 6F).

Effect of equol on antral follicle protein levels of steroidogenic enzymes

Our data indicate that the mRNA expression of *Cyp17a1* and *Cyp19a1* was downregulated at 96 hours of culture in the equol (100 μ M) treated follicles (Figure 6). Therefore, we investigated if equol decreases the antral follicle protein levels of these two important steroidogenic enzymes. Specifically, we examined CYP17A1 and CYP19A1 levels of follicles exposed to three different concentrations of equol (6 μ M, 36 μ M, or 100 μ M) at 96 hours of culture. Equol did not affect the protein levels of CYP17A1 and CYP19A1 when compared to the vehicle control group (Figure 7).

Effect of equol on antral follicle atresia

The inhibition of antral follicle growth and sex steroid hormone production that we observed in the equol treated follicles could be due to increased apoptosis of the follicles. Therefore, we investigated the effects of equol exposure on the gene expression of bcl2-associated X protein (*Bax*) and B cell leukemia/lymphoma 2 (*Bcl2*), two major regulators of apoptosis in the ovary. *Bax* is a pro-apoptotic factor that promotes atresia, whereas *Bcl2* is an anti-apoptotic factor that promotes follicle survival. The *Bax* to *Bcl2* ratio is a useful measure of apoptosis, with an

increase in the ratio suggesting an increase in apoptosis (Chao and Korsmeyer, 1998). Exposure of the follicles to equol at 600 nM, 6 μ M, 36 μ M, and 100 μ M did not affect the *Bax* to *Bcl2* mRNA ratio at 48 hours of culture (Figure 8C). However, equol at 100 μ M significantly increased the *Bax* to *Bcl2* mRNA ratio at 96 hours of culture when compared to the vehicle control (Figure 8C; n=3-4, $p \leq 0.05$).

Based on these results, we further investigated follicle atresia at 96 hours by subjecting antral follicles to morphological assessment of atresia as described in methods. Representative images of a DMSO treated follicle and an equol 100 μ M treated follicle are shown in Figure 8A-B. Our data indicate that exposure to equol at 100 μ M significantly increased the number of apoptotic bodies, represented by a higher atresia rating, when compared to the vehicle control, DMSO (Figure 8D; n=3, $p \leq 0.05$). However, the histological analysis did not reveal significant differences in atresia in the follicles exposed to the lower concentrations of equol (6 μ M and 36 μ M) when compared to the vehicle control (Figure 8D).

CHAPTER 5

DISCUSSION

We utilized an ovarian antral follicle culture system to show that equol inhibits growth and increases atresia of intact mouse antral follicles. We also have shown that equol inhibits steroidogenesis of antral follicles and downregulates the gene expression of some steroidogenic enzymes. These are novel findings on possible mechanisms of toxicity of equol on the ovary.

Exposure to equol at 100 μ M inhibited antral follicle growth at 72 and 96 hours of culture (Figure 1). The underlying process by which equol inhibits follicle growth is unclear, but potential mechanisms could include disruption of cell cycle regulators, reduced levels of sex steroid hormones or growth factors, and increased follicle atresia (Cain *et al.*, 1995; Chaffin and Vandervoort, 2013; Channing *et al.*, 1980; Drummond, 2006; Findlay *et al.*, 2001; Hannon *et al.*, 2015; Miller *et al.*, 2005; Rosselli *et al.*, 2000). Although to our knowledge, no previous studies have examined the effects of equol on growth of antral follicles, equol at 100 μ M was found to inhibit proliferation of rainbow trout primary myocytes by inhibition of DNA abundance and protein synthesis (Cleveland, 2014). Equol also inhibited proliferation of human hepatocellular carcinoma cells in a concentration-dependent manner by causing S-phase cell cycle arrest through p21 upregulation and cyclin A2 downregulation (Liang *et al.*, 2014). Equol may also bring about its negative effects on cell growth through inhibition of enzymes involved in cell cycle regulation, such as MAP kinase (Dubey *et al.*, 1999). Other phytoestrogens such as the soy isoflavone genistein have been shown to similarly inhibit cell proliferation potentially via mechanisms including inhibition of tyrosine kinase activity and enzymes required for DNA

replication (Okura *et al.*, 1988; Whitehead and Lacey, 2000). Other endocrine disrupting chemicals such as di(2-ethylhexyl)phthalate and methoxychlor have been shown to inhibit antral follicle growth by altering the levels of cell cycle regulators as well as apoptotic factors of the Bcl-2 family (Gupta *et al.*, 2009; Hannon *et al.*, 2015; Miller *et al.*, 2005).

Our data indicate that equol at 100 μ M decreased the levels of estradiol and its precursor hormones testosterone, androstenedione, and progesterone produced by the antral follicles at 96 hours of culture (Figure 2-5). Additionally, equol at 6 μ M and 36 μ M decreased the levels of estradiol (Figure 2) and androstenedione (Figure 4) at 96 hours. Antral follicles are the major source of sex steroids in the female, and normal levels of these hormones not only regulate their own growth within the ovary, but are also essential for maintenance and function of other reproductive tissues. (Channing *et al.*, 1980; Drummond, 2006; Findlay *et al.*, 2001; Lunenfeld *et al.*, 1975). These hormones also have important non-reproductive functions in the body (Munoz-Cruz *et al.*, 2011; Priest and Pfaff, 1995). Estradiol is produced by converting precursor hormones in the theca and granulosa cells of the follicles (Armstrong, 1968). Thereby, adequate levels of the precursor hormones are needed for adequate estradiol synthesis (Falck, 1959; Hirshfield, 1991; Richards, 1980). The low androstenedione levels that we observed in follicles exposed to equol at 6 and 36 μ M could in turn have led to a decrease in estradiol production by these follicles. This is a similar mechanism as other endocrine disruptors such as dioxin, which was found to inhibit estradiol secretion in human luteinized granulosa cells by decreasing androstenedione levels (Heimler *et al.*, 1998). The estradiol levels in the follicles treated with equol at 100 μ M could potentially be low due to insufficient availability of testosterone, androstenedione, and progesterone. Our findings are consistent with a few previous studies that

have found potential links between equol and suppressed steroidogenesis. High concentrations of equol in plasma and corpora lutea (CL) of cattle on a soybean diet were associated with lower concentrations of progesterone in their plasma and CL, compared to cattle on a standard diet (Piotrowska *et al.*, 2006). In another study, low dose supplementation of feed of postpartum dairy cows with a commercial soybean meal resulted in high plasma equol concentrations that were negatively correlated with the area occupied by steroidogenic cells in the CL, though this did not affect plasma progesterone levels (Cools *et al.*, 2014). Exposure of Leydig cells of geese to equol at 5 and 50 μ M reduced their ability to produce testosterone potentially through alterations in the steroidogenic pathway (Opalka *et al.*, 2012). Additionally, studies show that daily consumption of isoflavone-containing diets decreased plasma estradiol and progesterone levels in ewes during the luteal phase of their estrous cycle and in women over their entire menstrual cycle (Lu *et al.*, 2000; Obst and Seamark, 1970, 1975). The soy isoflavone genistein has also been found to reduce estradiol and progesterone levels in several in vitro and in vivo studies (Basini *et al.*, 2010; Lacey *et al.*, 2005; Nynca and Ciereszko, 2006; Nynca *et al.*, 2015; Ryokkynen *et al.*, 2006; Whitehead *et al.*, 2002; Zin *et al.*, 2013).

A feasible mechanism for the equol-induced inhibition of hormones in the present study is an alteration in the availability of steroidogenic enzymes responsible for sex steroid synthesis in the antral follicles. In the estradiol biosynthesis pathway, STAR transports cholesterol from the outer membrane to the inner membrane of the mitochondria (Miller and Strauss, 1999; Strauss *et al.*, 1999). Cholesterol then is converted to pregnenolone by CYP11A1 (Payne and Hales, 2004). Pregnenolone is converted to estradiol via two pathways. In the first pathway, pregnenolone is converted to dehydroepiandrosterone by CYP17A1 (Conley and Bird, 1997).

Dehydroepiandrosterone is further converted to androstenedione by HSD3B1 (Armstrong, 1968; Readhead *et al.*, 1983). Androstenedione is converted to either estrone by CYP19A1 or testosterone by HSD17B1 (Armstrong, 1968; Payne and Hales, 2004). Finally, CYP19A1 converts testosterone to estradiol (Armstrong, 1968; Payne and Hales, 2004). In the second pathway, pregnenolone is converted to progesterone by HSD3B1 (Readhead *et al.*, 1983). Progesterone is converted to androstenedione by CYP17A1 (Conley and Bird, 1997), then testosterone, and finally estradiol (Armstrong, 1968; Payne and Hales, 2004).

Our data indicate that equol at 100 μ M significantly decreased the expression of *Cyp11a1*, a rate-limiting enzyme in the estradiol biosynthesis pathway at 48 hours of culture (Figure 6B). We also observed a trend towards decreased expression of this enzyme at 96 hours (Figure 6B). Since CYP11A1 is responsible for conversion of cholesterol to pregnenolone, and pregnenolone is the steroid precursor of all downstream hormones in the pathway, downregulation of *Cyp11a1* as early as 48 hours could be a likely explanation for diminished production of progesterone, androstenedione, testosterone, and estradiol in the follicles at 96 hours. Additionally, equol (100 μ M) decreased the expression of *Cyp17a1* at both 48 and 96 hours (Figure 6D), which could further contribute to low levels of androstenedione at 96 hours in this treatment group, due to suboptimal conversion from progesterone. We also found that equol (100 μ M) decreased the expression of *Cyp19a1*, required for conversion of testosterone to estradiol, at 96 hours, but not at 48 hours (Figure 6F). This could be another possible factor in the decreased production of estradiol by the antral follicles observed at 96 hours. Our data on these effects of equol at 100 μ M are consistent with studies on other endocrine disrupting chemicals such as bisphenol-A and di(2-ethylhexyl)phthalate, which found they inhibit estradiol, testosterone, androstenedione, and

progesterone secretion of cultured mouse antral follicles by altering steroidogenic enzyme expression (Hannon *et al.*, 2015; Peretz *et al.*, 2011).

Although exposure of antral follicles to equol at 6 and 36 μM inhibited their production of estradiol and androstenedione at 96 hours, we did not find any alterations in the gene expression of steroidogenic enzymes in these two treatment groups at either 48 or 96 hours (Figure 6). Additionally, when we examined the protein levels of CYP17A1 and CYP19A1 at 96 hours, we found no alterations in protein levels (Figure 7) despite a significant decrease in the mRNA levels of these two enzymes at 96 hours in the 100 μM treatment group.

A plausible explanation is that there is often not a strong correlation between proteins and their associated mRNA levels, and this is highly variable depending on the system and the individual protein (Vogel and Marcotte, 2012). It is possible that different concentrations of equol have differential effects on mRNA and protein levels, or that equol is inhibiting the activity of the enzymes, rather than affecting mRNA or protein levels. Previous studies have shown that certain endocrine disruptors, including bisphenol A, produced dose-dependent inhibition of CYP19A1 activity without significant effects on mRNA levels (Nativelle-Serpentini *et al.*, 2003; Sanderson *et al.*, 2002). Further, phytoestrogens are known to inhibit human CYP19A1 activity (Kao *et al.*, 1998; Kellis and Vickery, 1984), and one study that examined the effects of various phytoestrogens on steroidogenic enzymes in human granulosa-luteal cells found inhibition of CYP19A1 and HSD3B1 activity without a change in protein levels of these enzymes (Lacey *et al.*, 2005). Additionally, equol (IC_{50} 793 μM) has been previously shown to inhibit rainbow trout

ovarian CYP19A1 activity (Pelissero *et al.*, 1996). Hence, future studies should determine whether equol alters enzyme activity in antral follicles, so as to more fully understand its effects at different doses on follicle steroidogenesis. Another conceivable mechanism for inhibition of estradiol levels without a change in upstream steroidogenic enzymes is that equol increases metabolism of estradiol (Rosselli *et al.*, 2000). Thus, further studies are needed to elucidate if equol induces cytochrome P450 1A1 (CYP1A1) and cytochrome P450 1B1 (CYP1B1), thereby affecting estradiol metabolism.

Exposure of antral follicles to equol (100 μ M) induced follicle atresia at 96 hours, as evident by histological evaluation and increased *Bax* to *Bcl2* mRNA ratio (Figure 8). Atresia is the natural process by which follicles undergo death via apoptosis (Hirshfield, 1988). Increased atresia negatively impacts the health of the antral follicle, and may have long-lasting effects on reproductive and non-reproductive function (Hannon *et al.*, 2015). Follicle atresia in the ovary is primarily dependent on the balance of pro-apoptotic and anti-apoptotic factors belonging to the BCL-2 family (Flaws *et al.*, 2001; Greenfield *et al.*, 2007; Hengartner, 2000). An increase in the ratio of pro-apoptotic to anti-apoptotic factors drives atresia (Chao and Korsmeyer, 1998). Our finding that equol increases the mRNA ratio of *Bax* (pro-apoptotic) to *Bcl2* (anti-apoptotic) at 96 hours suggests that alterations in the expression of the BCL-2 family of proteins may be responsible for the equol-induced follicle atresia. Equol has been previously associated with infertility in sheep grazing on isoflavone-rich pastures (Adams, 1995; Shutt and Braden, 1968), and one study found that the ovaries of these sheep developed significantly increased numbers of small and medium sized follicles undergoing atretic changes (Adams, 1977). Additionally, equol (20, 50, 100 mmol/L) induced death of human hepatocellular carcinoma cells partly through

upregulation of BAX and downregulation of BCL-2, thus further supporting our findings on potential mechanisms of equol-induced apoptosis (Liang *et al.*, 2014). Previous *in vivo* studies in rats exposed to genistein, another soy isoflavone, have also been associated with increased ovarian follicle atresia (Medigovic *et al.*, 2012; Zin *et al.*, 2013).

The overall finding of ovarian toxicity in the present study is thereby consistent with several *in vivo* and *in vitro* studies which suggest that equol may have adverse reproductive effects in multiple species (Adams, 1995; Cline *et al.*, 2004; Kallela *et al.*, 1984; Shutt and Braden, 1968; Woclawek-Potocka *et al.*, 2005b). However, one study examining the developmental and reproductive toxicity of perinatal exposure to equol in Sprague-Dawley rats did not find obvious adverse effects on the ovary, although equol reportedly increased uterine weights and caused prolonged uterine hyperplasia (Brown *et al.*, 2011). This discrepancy could possibly be attributed to species differences and differing study design, including doses of equol and *in vitro* versus *in vivo* methods (Murkies *et al.*, 1998; Rosselli *et al.*, 2000; Thompson *et al.*, 1984). Furthermore, Sprague-Dawley rats have been reported to be less sensitive to estrogenic effects of endocrine disrupting chemicals such as phytoestrogens than CD-1 mice (Thigpen *et al.*, 2007).

In conclusion, we have used an antral follicle culture system to investigate direct effects of equol on follicle growth, atresia and steroidogenesis in the adult mouse ovary. Our results suggest that equol inhibits follicle growth, increases atresia potentially through dysregulation of the apoptotic pathway, and inhibits steroidogenesis potentially through inhibition of upstream precursor steroid hormones and expression of steroidogenic enzymes. These effects of equol could be a concern if they lead to negative impacts on reproductive outcomes including fertility, ovulation,

reproductive senescence, initiation and maintenance of pregnancy, sexual behavior and secondary sex characteristics, puberty, estrous cyclicity, maintenance and function of the reproductive tract, as well as non-reproductive health (Bagur and Mautalen, 1992; Bush *et al.*, 1987; Christiansen, 1993; Cooper and Sandler, 1998; Daston *et al.*, 1997; Dennerstein *et al.*, 1999; Everson *et al.*, 1995; Findlay *et al.*, 2001; Hu *et al.*, 1999; Hu *et al.*, 2010; Mosca, 1998). However, the ovotoxic effects in this study are primarily seen at a high concentration of equol (100 μ M), with the lower concentrations (600 nM, 6 μ M, 36 μ M) having no or minor effects. This is in general agreement with studies on other endocrine-active chemicals whose *in vitro* actions often require concentrations higher than typically seen in plasma (Whitten and Patisaul, 2001). Human exposure through the diet and supplement use is close to the lower-range of concentrations used in our study (Setchell *et al.*, 2001; Setchell *et al.*, 2009; Setchell *et al.*, 1997; Vedrine *et al.*, 2006), although it is unknown as to what percentage of equol circulating in plasma actually reaches the ovary, or whether equol accumulates in follicular fluid. Our findings of potential ovarian toxicity with exposure to high concentrations of equol may be more relevant to wildlife and livestock populations that are likely to be exposed to such levels through isoflavone-rich pastures and commercial feeds using soybean as a protein source (Lindsay and Kelly, 1970; Lundh, 1995; Woclawek-Potocka *et al.*, 2013; Woclawek-Potocka *et al.*, 2008). This may be of further importance given that equol has been previously associated with reproductive disorders in animals (Adams, 1995; Dusza *et al.*, 2006; Kallela *et al.*, 1984; Setchell *et al.*, 1987; Shutt and Braden, 1968; Shutt *et al.*, 1970; Woclawek-Potocka *et al.*, 2005b; Woclawek-Potocka *et al.*, 2013).

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

The goal of the present study was to determine the effects of the isoflavone metabolite equol on mouse ovarian antral follicles using an *in vitro* culture system. Specifically, we examined the effects of equol on antral follicle growth, atresia, and steroidogenesis.

The growth of antral follicles exposed to equol at 100 μ M is inhibited starting at 72 hours, and remains suppressed at 96 hours. Follicles exposed to equol at 100 μ M also show an increase in atresia at 96 hours, as evident by histological evaluation. A high *Bax* to *Bcl2* mRNA ratio in these follicles suggests a dysregulation of the apoptotic pathway as one of the potential mechanisms of increased atresia. The ability of antral follicles to produce the sex steroid hormones estradiol, testosterone, androstenedione, and progesterone at 96 hours of culture is diminished by exposure to equol at 100 μ M. Additionally, follicles treated with equol at 6 and 36 μ M show decreased synthesis of estradiol and androstenedione at 96 hours. Equol may therefore be causing low estradiol production in antral follicles via depletion of precursor steroid hormones. Equol at 100 μ M downregulates the expression of *Cyp11a1* (48 and 96 hours), *Cyp17a1* (48 and 96 hours), and *Cyp19a1* (96 hours); however the protein levels of the latter two steroidogenic enzymes remain unchanged at 96 hours.

In conclusion, although the present study has provided evidence that equol alters antral follicle function in several ways, further studies are needed to elucidate its mechanisms of action on the

ovary in detail. For instance, future studies to evaluate the levels of cell cycle regulators in equol treated follicles could help in better understanding equol-induced follicle growth inhibition. The mechanisms behind equol-induced increased atresia should be further investigated by analyzing both upstream and downstream regulators of apoptosis at the mRNA and protein level. Future studies should also determine if equol alters steroidogenic enzyme activity in antral follicles, in order to more fully understand the inhibition of sex steroid hormones. Another possible mechanism that could be examined is whether equol induces estradiol metabolism. Since the present study only assessed different endpoints at 48 or 96 hours, it is important to conduct time course experiments to determine the exact timeline of the observed effects of equol.

CHAPTER 7

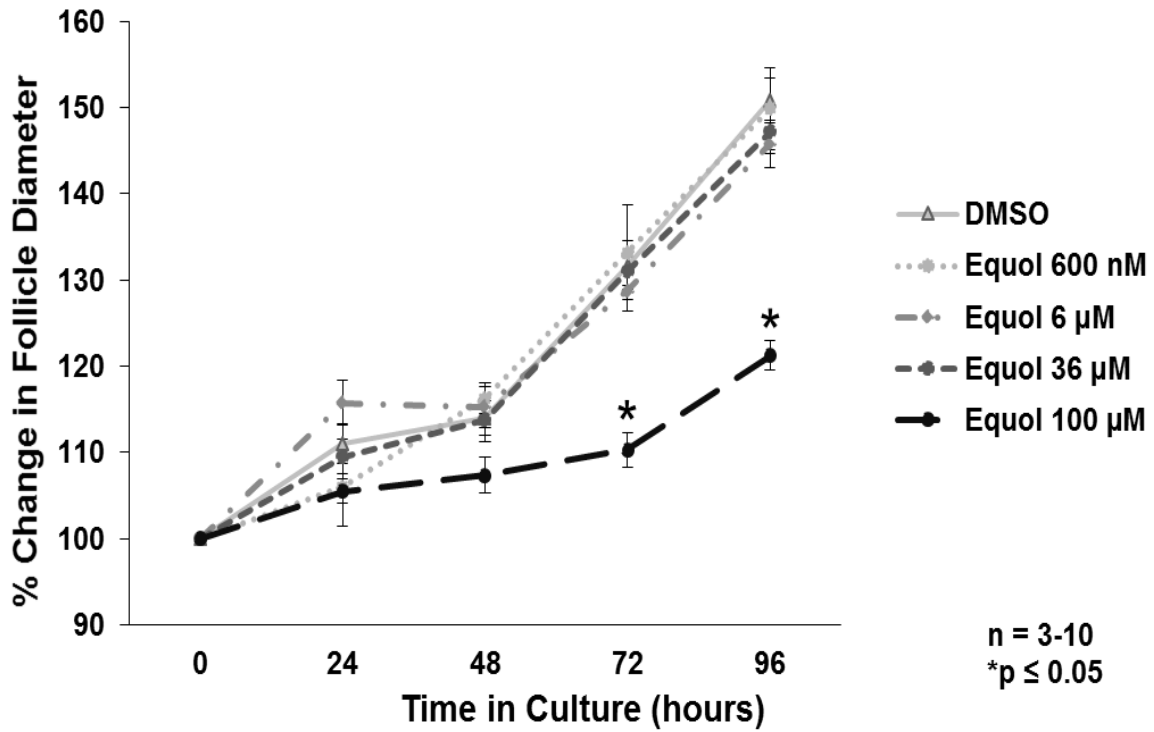
TABLES, FIGURES, AND LEGENDS

Table 1. Sequences of primer sets used for gene expression analysis

Gene Name	Gene symbol	Accession No.	Forward primer	Reverse primer
Beta-actin	<i>Actb</i>	NM_007393	GGGCACAGTGTGGGTGAC	CTGGCACCACACCTTCTAC
Steroidogenic acute regulatory protein	<i>Star</i>	NM_007810	CAGGGAGAGGTGGCTATGCA	CCGTGTCTTTTCCAATCCTCTG
Cytochrome P450 cholesterol side-chain cleavage	<i>Cyp11a1</i>	NM_008293	AGATCCCTTCCCCTGGCGACAATG	CGCATGAGAAGAGTATCGACGCATC
Cytochrome P450 steroid 17- α -hydroxylase 1	<i>Cyp17a1</i>	NM_007809	CCAGGACCCAAGTGTGTCT	CCTGATACGAAGCACTTCTCG
3 β -Hydroxysteroid dehydrogenase 1	<i>Hsd3b1</i>	NM_008293	CAGGAGAAAGAACTGCAGGAGGTC	GCACACTTGCTTGAACACAGGC
17 β -hydroxysteroid dehydrogenase 1	<i>Hsd17b1</i>	NM_010475	ACTGTGCCAGCAAGTTTGGC	AAGCGGTTTCGTGGAGAAGTAG
Cytochrome P450 aromatase	<i>Cyp19a1</i>	NM_007810	CATGGTCCCGAAACTGTGA	GTAGTAGTTGCAGGCACTTC
Bcl2-associated X protein	<i>Bax</i>	NM_007527	TGAAGACAGGGGCCTTTTGG	AATTCGCCGGAGACACTCG
B cell leukemia/lymphoma 2	<i>Bcl2</i>	NM_009741	ATGCCTTTGTGGAAGTATATGGC	GGTATGCACCCAGAGTGATGC

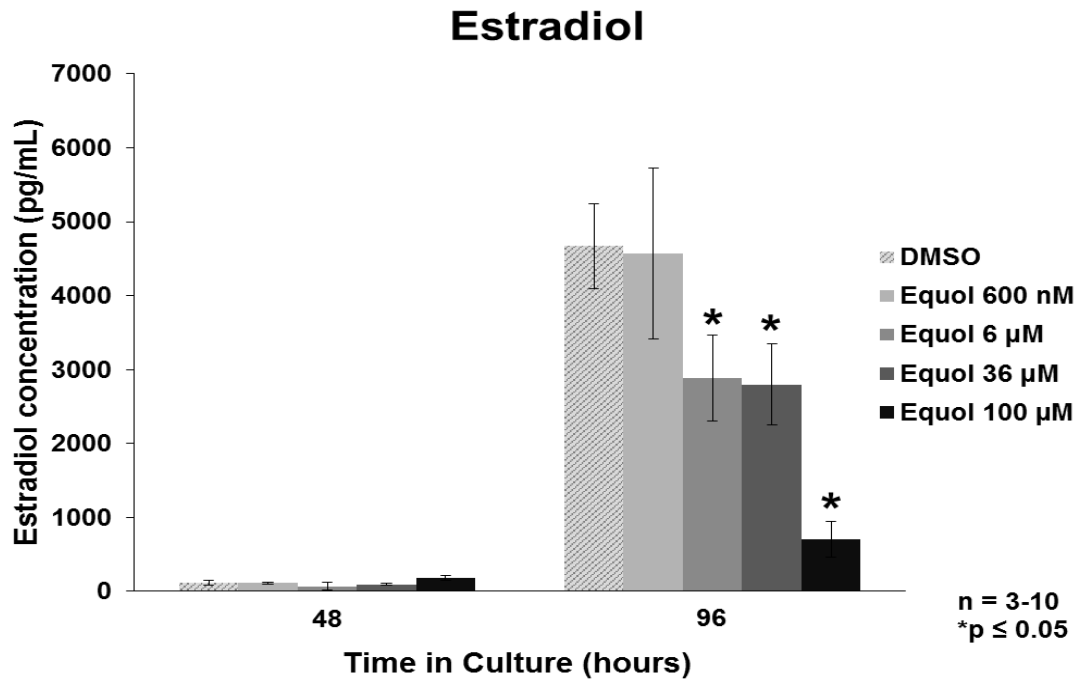
Specific primer sequences used for qPCR analysis of gene expression.

Figure 1. Effect of equol on follicle growth over time



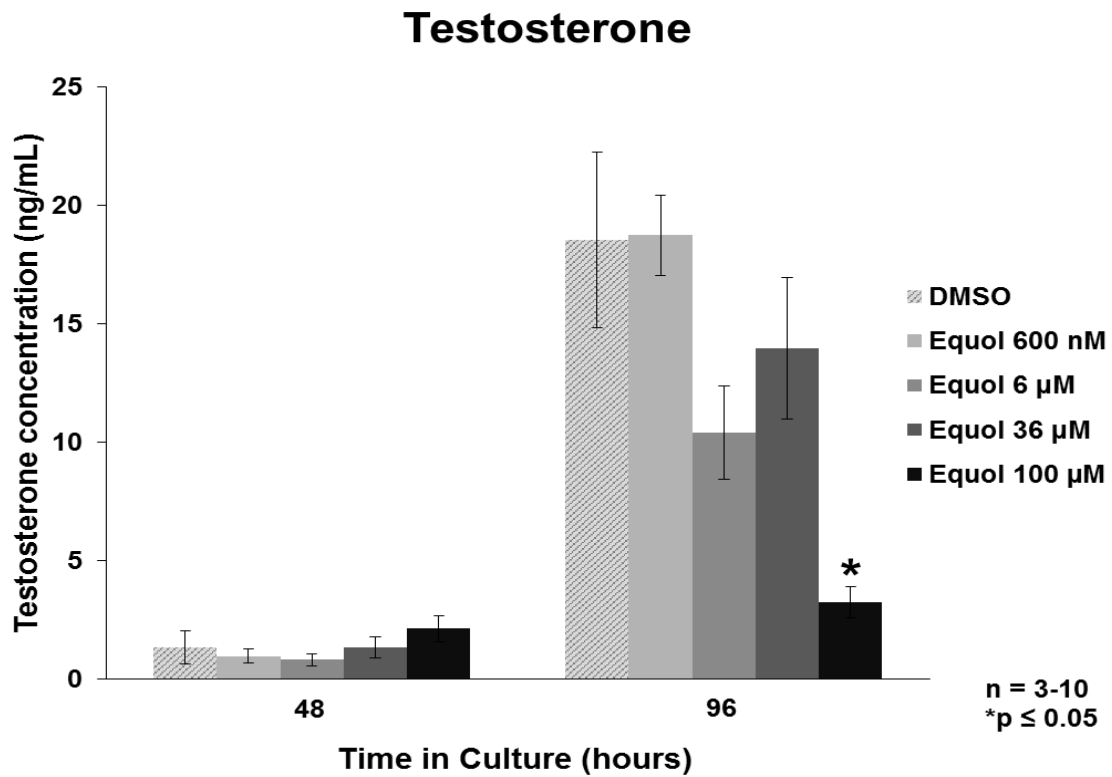
Antral follicles were isolated from adult CD-1 mice and cultured with vehicle (DMSO) or equol for 96 h. Follicle diameters were measured daily along perpendicular axes and percent change in growth was determined over 96 h. The graph represents the means $\pm$ SEM of percent change in follicle growth from 3 to 10 separate experiments. Asterisks (*) represent a significant difference between vehicle control and equol ($p \leq 0.05$).

Figure 2. Effect of equol on follicle estradiol production



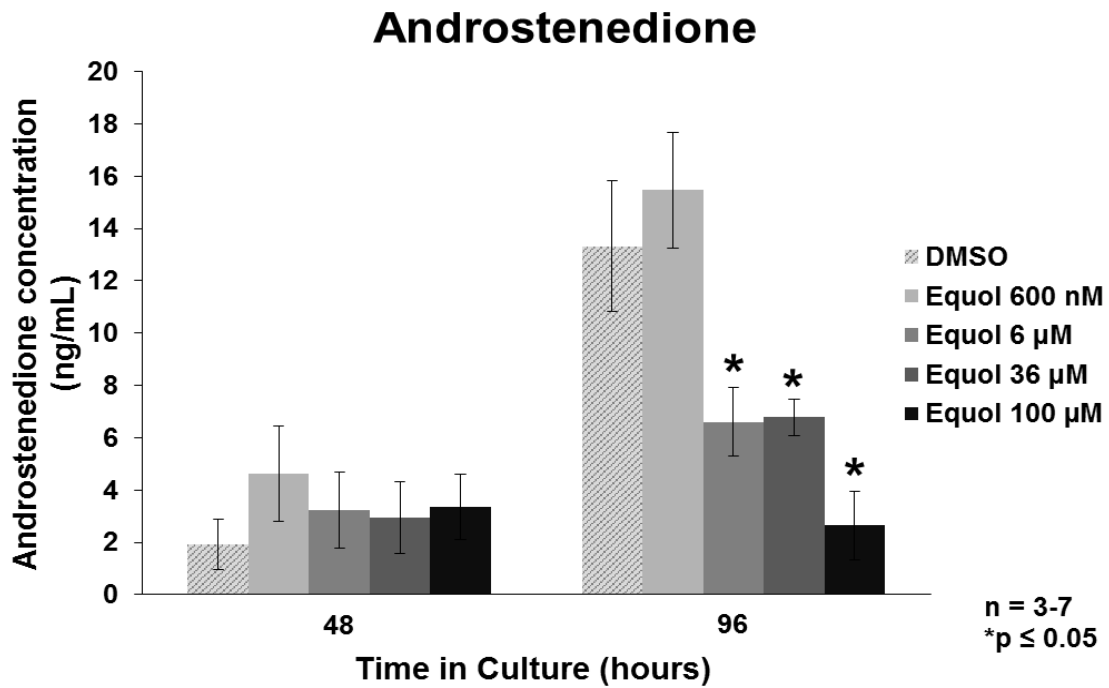
Antral follicles were isolated from adult CD-1 mice and cultured with vehicle (DMSO) or equol for 48 and 96 h. Media then were pooled by treatment group and subjected to enzyme-linked immunosorbent assays for estradiol. The graph represents the means $\pm$ SEM from 3 to 10 separate experiments. Asterisks (*) represent a significant difference between vehicle control and equol ($p \leq 0.05$).

Figure 3. Effect of equol on follicle testosterone production



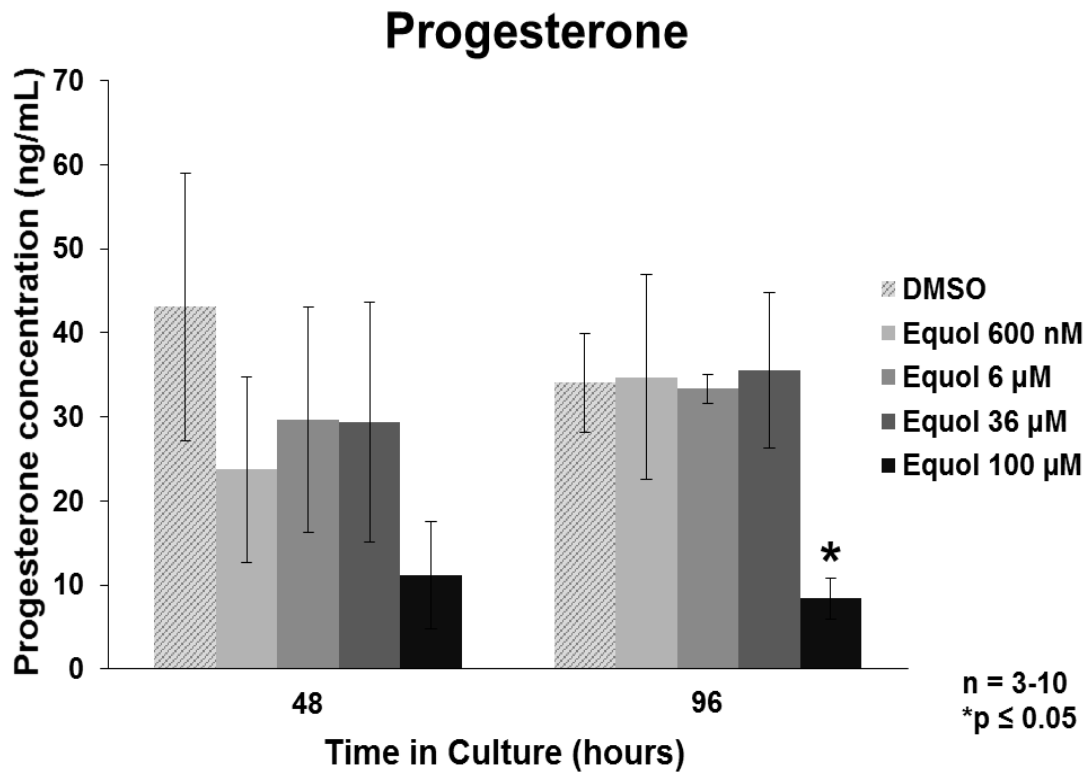
Antral follicles were isolated from adult CD-1 mice and cultured with vehicle (DMSO) or equol for 48 and 96 h. Media then were pooled by treatment group and subjected to enzyme-linked immunosorbent assays for testosterone. The graph represents the means $\pm$ SEM from 3 to 10 separate experiments. Asterisk (*) represents a significant difference between vehicle control and equol ($p \leq 0.05$).

Figure 4. Effect of equol on follicle androstenedione production



Antral follicles were isolated from adult CD-1 mice and cultured with vehicle (DMSO) or equol for 48 and 96 h. Media then were pooled by treatment group and subjected to enzyme-linked immunosorbent assays for androstenedione. The graph represents the means $\pm$ SEM from 3 to 7 separate experiments. Asterisks (*) represent a significant difference between vehicle control and equol ($p \leq 0.05$).

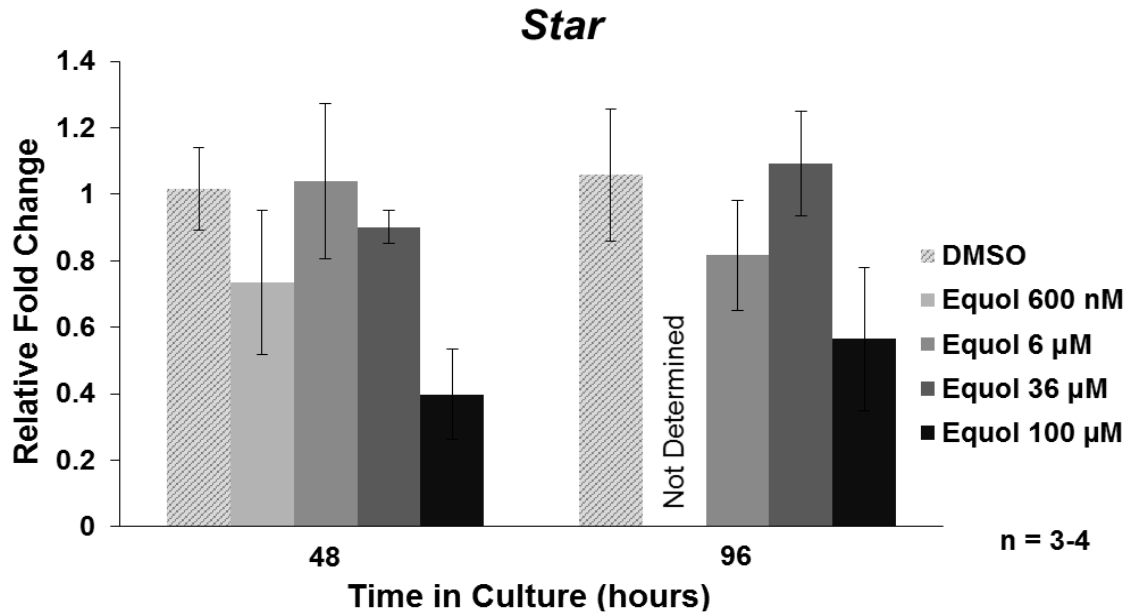
Figure 5. Effect of equol on follicle progesterone production



Antral follicles were isolated from adult CD-1 mice and cultured with vehicle (DMSO) or equol for 48 and 96 h. Media then were pooled by treatment group and subjected to enzyme-linked immunosorbent assays for progesterone. The graph represents the means $\pm$ SEM from 3 to 10 separate experiments. Asterisk (*) represents a significant difference between vehicle control and equol ($p \leq 0.05$).

Figure 6. Effect of equol on follicle mRNA expression of steroidogenic enzymes

A



B

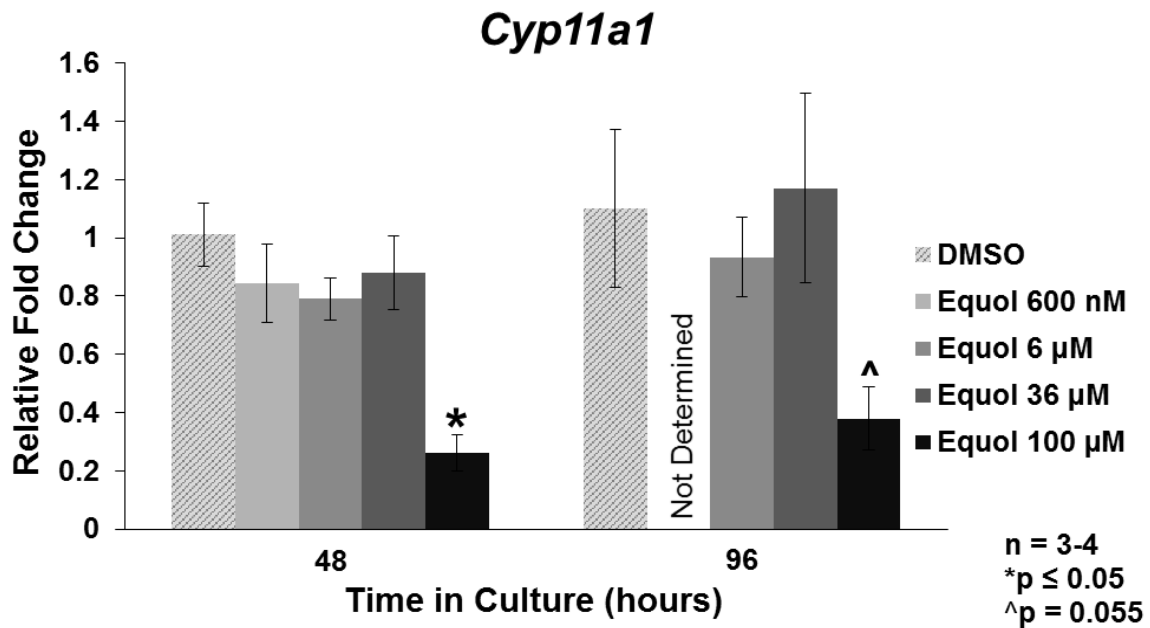
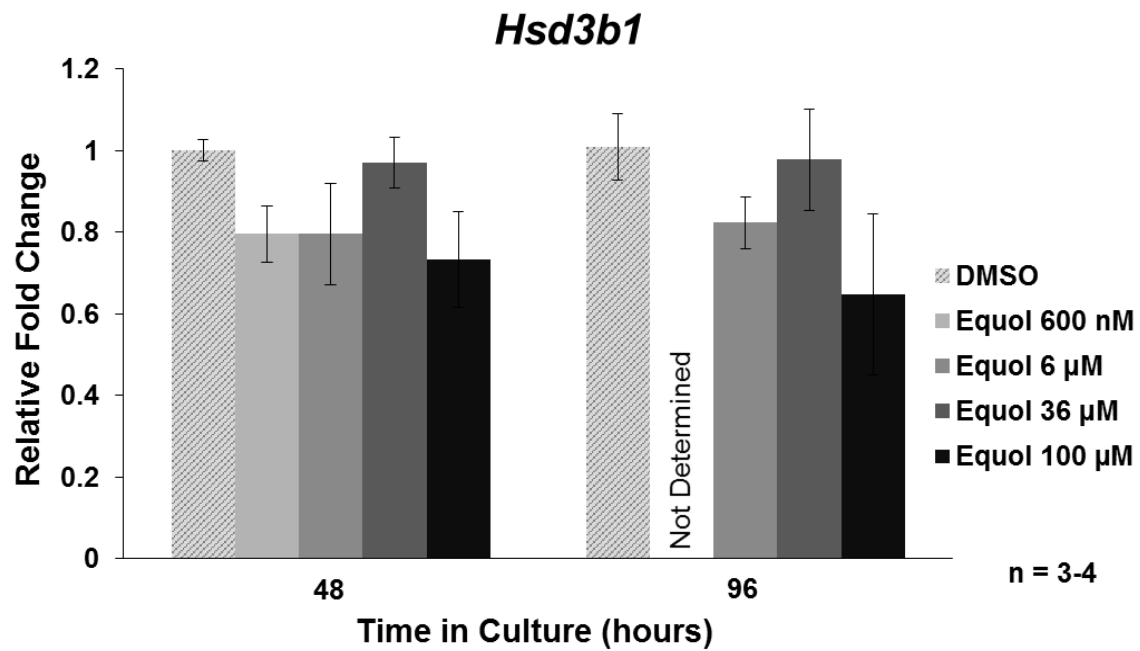


Figure 6 (cont.)

C



D

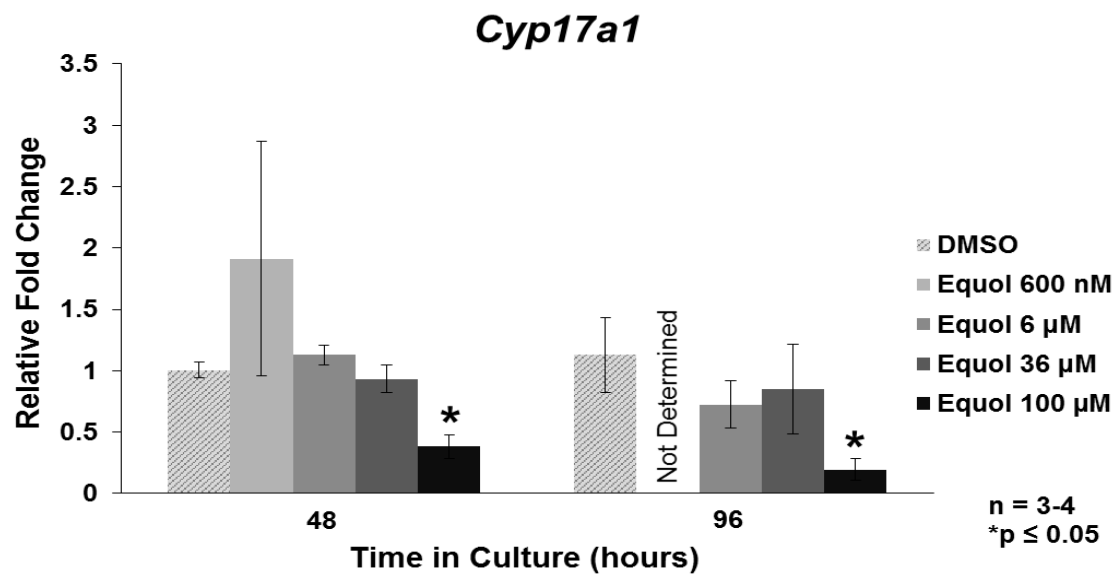
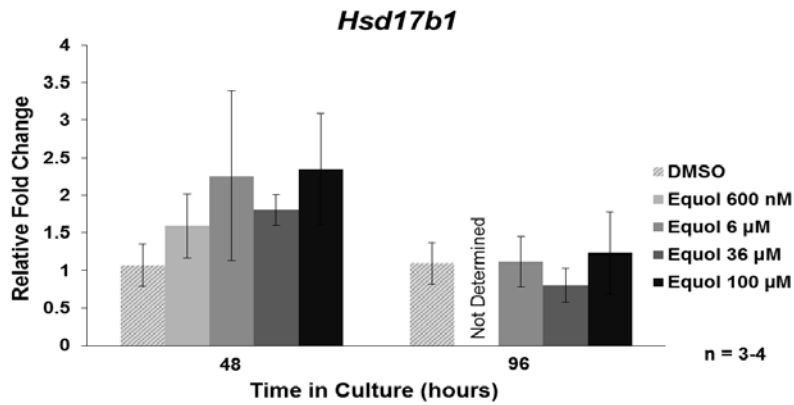
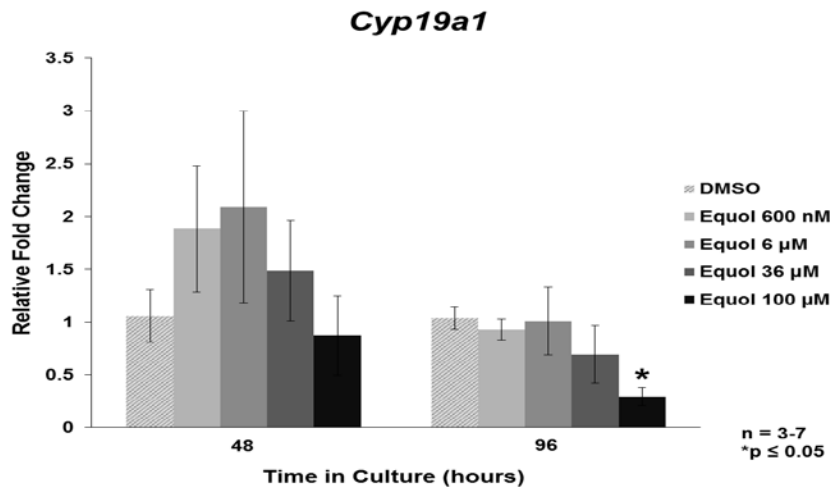


Figure 6 (cont.)

E



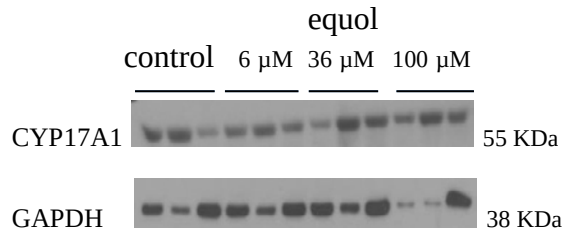
F



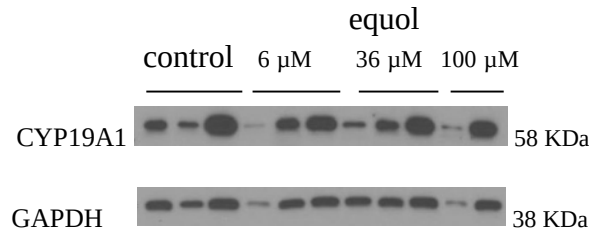
Antral follicles were isolated from adult CD-1 mice and cultured with vehicle (DMSO) or equol for 48 and 96 h. Follicles then were pooled by treatment group and subjected to quantitative polymerase chain reaction for *Star* (panel A), *Cyp11a1* (panel B), *Hsd3b1* (panel C), *Cyp17a1* (panel D), *Hsd17b1* (panel E), and *Cyp19a1* (panel F). All values were normalized to *Actb*. The graphs represent the means $\pm$ SEM from 3 to 7 separate experiments. Asterisks (*) represent a significant difference between vehicle control and equol ($p \leq 0.05$).

Figure 7. Effect of equol on follicle protein levels of steroidogenic enzymes

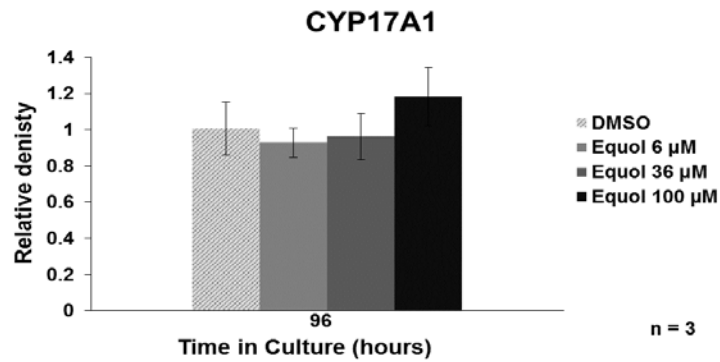
A. CYP17A1 western blot



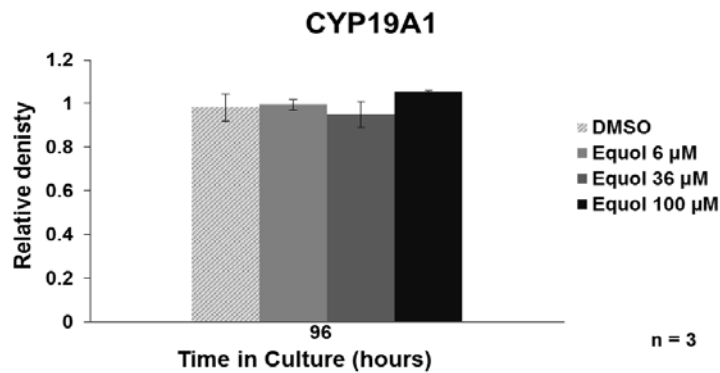
B. CYP19A1 western blot



C.



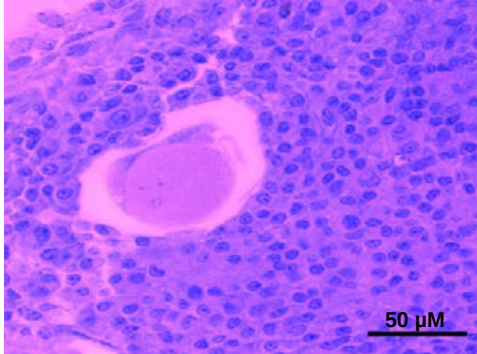
D.



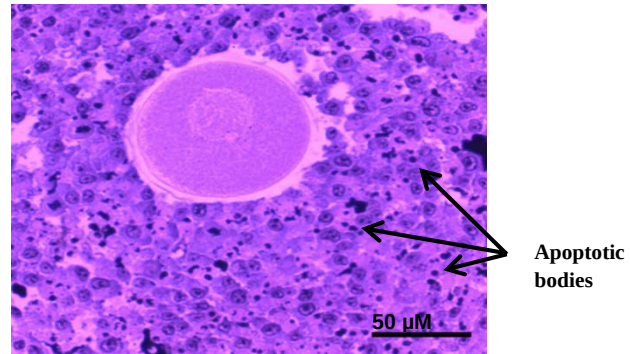
Antral follicles were isolated from adult CD-1 mice and cultured with vehicle (DMSO) or equol for 96 h. Follicles then were pooled by treatment group and subjected to western blot analysis for CYP17A1 (panel A) and CYP19A1 (panel B). All values were normalized to GAPDH. The graphs represent the means $\pm$ SEM from 3 separate experiments. Note that for CYP19A1 analysis in the equol 100 μ M treatment group, only 2 separate experiments were performed due to insufficient sample in this group, and their average value was considered for an n of 3.

Figure 8. Effect of equol on follicle atresia

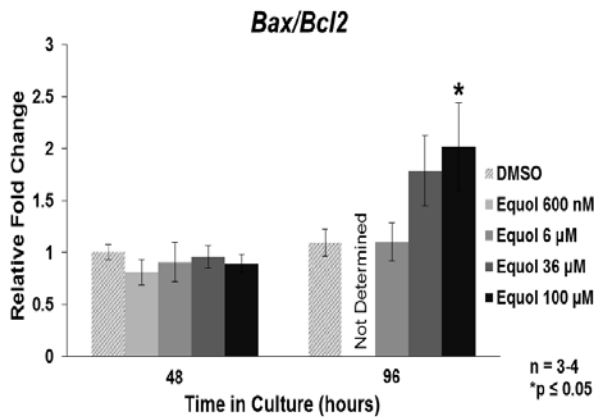
A. DMSO treated follicle



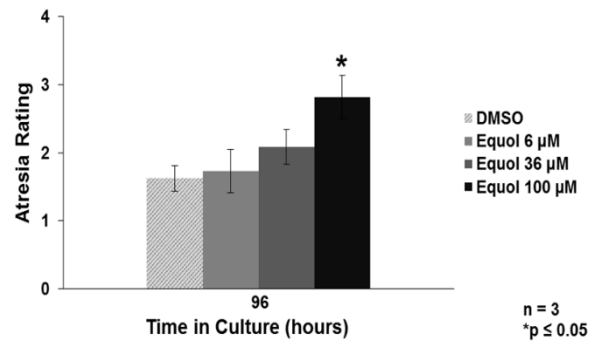
B. Equol 100 μM treated follicle



C. *Bax* to *Bcl2* mRNA ratio



D. Atresia rating



Antral follicles were isolated from adult CD-1 mice and cultured with vehicle (DMSO) or equol for 48 and 96 h. Follicles then were pooled by treatment group and subjected to quantitative polymerase chain reaction for measurement of *Bax* to *Bcl2* ratio (panel C). Following 96 h, follicles were also processed for histological evaluation of atresia. A representative image of a DMSO treated follicle is found in panel A. A representative image of an equol-treated follicle (100 μM) is found in panel B. Atresia ratings were assigned based on the presence of apoptotic bodies. A rating of 1 indicated the presence of apoptotic bodies encompassing 0-3% of the follicle section, a rating of 2 indicated the presence of apoptotic bodies encompassing 4-10% of the follicle section, a rating of 3 indicated the presence of apoptotic bodies encompassing 11-30% of the follicle section, and a rating of 4 indicated the presence of apoptotic bodies encompassing >31% of the follicle section. The graphs represent the means ± SEM from 3 to 4 separate experiments. Asterisks (*) represent a significant difference between vehicle control and equol ($p \leq 0.05$).

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