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Equol inhibits growth, induces atresia, and inhibits steroidogenesis of mouse antral follicles *in vitro*

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Abstract

Equol is a non-steroidal estrogen metabolite produced by microbial conversion of daidzein, a major soy isoflavone, in the gut of some humans and many animal species. Isoflavones and their metabolites can affect endogenous estradiol production, action, and metabolism, potentially influencing ovarian follicle function. However, no studies have examined the effects of equol on intact ovarian antral follicles, which are responsible for sex steroid synthesis and further development into ovulatory follicles. Thus, the present study tested the hypothesis that equol inhibits antral follicle growth, increases follicle atresia, and inhibits steroidogenesis in the adult mouse ovary. To test this hypothesis, antral follicles isolated from adult CD-1 mice were cultured with vehicle control (dimethyl sulfoxide; DMSO) or equol (600 nM, 6 μ M, 36 μ M, 100 μ M) for 48 and 96 h. Every 24 h, follicle diameters were measured to monitor growth. At 48 and 96 h, the culture medium was subjected to measurement of hormone levels, and the cultured follicles were subjected to gene expression analysis. Additionally, follicles were histologically evaluated for signs of atresia after 96 h of culture. The results indicate that equol (100 μ M) inhibited follicle growth, altered the mRNA levels of bcl2-associated X protein and B cell leukemia/lymphoma 2, and induced follicle atresia. Further, equol decreased the levels of estradiol, testosterone, androstenedione, and progesterone, and it decreased mRNA levels of cholesterol side-chain cleavage, steroid 17- α -hydroxylase, and aromatase. Collectively, these data indicate that equol inhibits growth, increases atresia, and inhibits steroidogenesis of cultured mouse antral follicles.

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Keywords

equol; ovary; antral follicle; atresia; steroidogenesis

Introduction

Phytoestrogens are 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). 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 non-steroidal estrogen metabolite of daidzein and is produced by the action of intestinal microflora in 20–60% of adults consuming soy foods (Setchell and Cole, 2006). In addition, equol is marketed as a nutraceutical agent, and numerous people consume equol in supplement form 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). Substantial exposure to equol can occur in wildlife and domestic animals consuming large quantities of isoflavones while grazing on phytoestrogen-rich pastures, and dietary exposure also occurs due to the common inclusion of soybean as a 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). Therefore, equol is a relevant estrogenic compound in the diet of both humans and animals.

Although botanical chemicals are widely used for their potential benefits, the side effects of these compounds and their metabolites are less clear. Of particular concern are their potential adverse effects on the ovary because the ovary contains numerous estrogen receptors (both alpha and beta subtypes), which can bind to estrogenic botanical compounds. Chemicals that damage ovarian function can 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; Cooper and Sandler, 1998; Everson *et al.*, 1995). Premature ovarian failure is associated with an increased risk for neurological disorders, osteoporosis, and cardiovascular disease (Bagur and Mautalen, 1992; 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. Specifically, no studies have examined the effects of equol on intact antral follicles, which are the functional units of the ovary 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 mRNA levels of enzymes required for estradiol biosynthesis.

Materials and methods

Animals

Cycling, adult, female CD-1 mice purchased from Charles River Laboratories (Wilmington, MA) were 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

Cycling, adult, female CD-1 mice were euthanized on postnatal days (PND) 32–35. Early antral follicles were manually isolated from their ovaries based on relative size (225–400 μm) and cleaned of interstitial tissue using watchmaker forceps. Approximately 20–40 follicles were obtained from each mouse. Isolated antral follicles were pooled from five to six different mice for each experiment, and randomly and individually plated in wells of 96-well tissue culture plates, using sterile technique, such that each treatment group contained 5–18 follicles. Experiments were replicated on a minimum of three separate occasions. Cultures were carried out for total periods of 48 hours and 96 hours to evaluate experimental endpoints, including follicle growth, hormone levels, and enzyme mRNA levels. *S*-(–)equol, the biologically active form of 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 μL per mL of medium). This translated to final working concentrations of 0.14, 1.45, 8.72, and 24.23 μg of equol per mL of culture medium, which is equivalent to 600 nM, 6 μM , 36 μM , and 100 μM , 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 human exposure, based on studies measuring plasma and urine equol concentrations, is similar to the lower-range concentrations used in our study (600 nM and 6 μM) (Setchell *et al.*, 2001; Setchell *et al.*, 2009; Setchell *et al.*, 1997; Veldrine *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). The concentrations of equol tested in our study were also based on those used in previously published *in vitro* and

in vivo studies (Choi, 2009; Ju *et al.*, 2006; Liang *et al.*, 2014; Matulka *et al.*, 2009; Pelissero *et al.*, 1996).

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. FSH was used in the cultures because follicles fail to grow at a normal rate and undergo atresia in the absence of FSH *in vitro* (Spears *et al.*, 1998).

Follicle growth analysis

To assess antral follicle growth over time, individual follicle diameters were measured on perpendicular axes at every 24 hour time point for each culture, with an inverted microscope equipped with a calibrated ocular micrometer. Follicles having diameters of 225–400 μ m were considered to be antral follicles (Cortvriendt and Smits, 2002), and this correlates with the histological appearance of these follicles. The individual diameters were averaged within each of the five treatment groups for every 24 hour interval. The average diameters for each time 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.

Enzyme linked immunosorbent assays

After every 48 and 96 hour culture, medium from each well was collected and pooled according to treatment, before being 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 hormone levels were measured using ELISA kits purchased from Diagnostics Research Group (DRG, Springfield, NJ) following manufacturer's instructions. Samples were run in duplicate, and had intra-assay and inter-assay coefficients of variability of less than 10%. 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 and subjected to quantitative real-time polymerase chain reaction (qPCR) analysis. Total RNA was extracted using the RNeasy Micro Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol, and quantified using a 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. 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 μ L cDNA, 5 pmol gene specific primers (Integrated DNA Technologies, Inc., Coralville, IA; Table 1), and 5 μ L SsoFast EvaGreen Supermix (Bio-Rad laboratories, Hercules, CA) for a final reaction volume of 10 μ L. The qPCR program protocol was similar to that used in previous studies (Hannon *et al.*, 2015) and the primers were validated for use with qPCR in previous studies (Hannon *et al.*, 2015). Standard curves were generated from five to seven serial dilutions of a combination of samples to obtain PCR efficiency. Beta-actin (*Actb*) was used as a reference gene because its mRNA level did not differ among the 5 treatment groups. Expression data were generated using the Pfaffl method for relative quantification, and the calculated relative fold changes used for statistical analysis.

The mRNAs tested were chosen because they are regulators of ovarian steroidogenesis (Hanukoglu, 1992) and apoptosis (Flaws *et al.*, 2001; Greenfeld *et al.*, 2007). In the ovarian estradiol biosynthesis pathway, the key steroidogenic enzymes namely 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), and cytochrome P450 aromatase (CYP19A1) convert cholesterol to estradiol in a highly coordinated and step-wise manner. Apoptosis in the ovary is partly regulated by the pro-apoptotic factor bcl2-associated X protein (BAX) and the anti-apoptotic factor B cell leukemia/lymphoma 2 (BCL2), with BAX promoting atresia and BCL2 promoting 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).

Histological evaluation of atresia

After 96 hours of culture, antral follicles were collected and individually 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 μ m were mounted on glass slides and stained with Lee's methylene blue-basic fuchsin staining protocol. Five sections of each follicle were randomly selected and 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 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 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 $p = 0.05$.

Results

Effect of equol on antral follicle growth

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 hours ($F(4,32) = 16.55$, $p = 0.05$) and 96 hours ($F(4,32) = 27.36$, $p = 0.05$) when compared to the vehicle control group (Figure 1). 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

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 ($F(4,30) = 8.59$, $p = 0.05$; Figure 2) and androstenedione ($F(4,19) = 6.96$, $p = 0.05$; Figure 4) by 96 hours when compared to the vehicle control group. Equol at the 100 μ M concentration also decreased the levels of testosterone (Welch's $F(4,9.6) = 20.3$, $p = 0.05$; Figure 3) and progesterone ($H(4) = 15.52$, $p = 0.05$; Figure 5) by 96 hours when compared to vehicle control.

Effect of equol on levels of mRNA for steroidogenic enzymes in antral follicles

Equol did not affect the mRNA level of *Star* when compared to the control group (Figure 6A). However, the mRNA level of *Cyp11a1* was significantly decreased at 48 hours ($F(4,10) = 7.69$, $p = 0.05$) and showed a trend towards decrease at 96 hours ($F(3,12) = 2.46$, $p = 0.055$) in the 100 μ M treatment group when compared to the vehicle control group (Figure 6B). The lower concentrations of equol did not affect the level of mRNA of

Cyp11a1 at either time point (Figure 6B). Equol also did not affect the mRNA level of *Hsd3b1* at either time point when compared to the vehicle control (Figure 6C). However, equol at 100 μ M significantly decreased the mRNA level of *Cyp17a1* at both 48 hours (*Welch's F*(4,4.86) = 7.08, p = 0.05) and 96 hours (*F*(3,12) = 2.25, p = 0.05) when compared to control (Figure 6D). The mRNA level of this enzyme was not affected by exposure to the lower concentrations of equol (Figure 6D). Further, equol did not affect the mRNA level of *Hsd17b1* compared to control (Figure 6E). Equol at 100 μ M, however, caused a significant decrease in *Cyp19a1* mRNA level at 96 hours (*F*(4,20) = 4.01, p = 0.05), although 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 the level of mRNA of *Cyp19a1* at either time point (Figure 6F).

Effect of equol on antral follicle atresia

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 7C). 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 (*F*(3,12) = 2.84, p = 0.05; Figure 7C). Further, histological analysis of antral follicles indicates that exposure to equol at 100 μ M significantly increased the number of apoptotic bodies (Figure 7A–B), represented by a higher atresia rating, when compared to the vehicle control, DMSO (*H*(3) = 8.1, p = 0.05; Figure 7D). However, there were no significant differences in atresia in the follicles exposed to equol at 6 μ M and 36 μ M when compared to the vehicle control (Figure 7D).

Discussion

We utilized an *in vitro* ovarian follicle culture system to show that equol is directly toxic to antral follicles, which are essential for normal female reproduction. Specifically, the growth of mouse antral follicles exposed to equol at 100 μ M was inhibited starting at 72 hours, and remained suppressed at 96 hours of culture. Follicles exposed to equol at 100 μ M also showed an increase in atresia at 96 hours, as evident by histological evaluation. A high *Bax* to *Bcl2* mRNA ratio in these follicles suggested 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 over 96 hours of culture was diminished by exposure to equol at 100 μ M. Additionally, follicles treated with equol at 6 and 36 μ M showed decreased synthesis of estradiol and androstenedione at 96 hours. Our data further indicate that equol at 100 μ M downregulated the steady-state levels of mRNA for *Cyp11a1* (48 and 96 hours), *Cyp17a1* (48 and 96 hours), and *Cyp19a1* (96 hours), therefore suggesting that equol inhibited steroidogenesis of antral follicles through disruption of steroid hormones and steroidogenic enzymes upstream of estradiol (Figure 8). These are novel findings on the mechanisms of action of equol on the ovary, which could partly explain the adverse reproductive effects that have been associated with equol in multiple species.

Many studies have previously used ovarian follicle culture to investigate folliculogenesis and oogenesis *in vitro* (Cortvrindt and Smitz, 2002; Miller *et al.*, 2005; Nayudu and Osborn, 1992; Spears *et al.*, 1994). Further, using antral follicles which represent the intact

functional units of the ovary rather than isolated granulosa or theca cells for our experiments more closely resembles *in vivo* conditions. This experimental model has also allowed us to measure changes in growth of single follicles over time in response to chemical exposure, which would have been difficult to do with *in vivo* techniques.

The inhibition of growth that we observed in follicles treated with 100 μ M equol for 96 hours could be attributed to altered levels of apoptotic factors of the Bcl-2 family. Our data indicate that the follicle mRNA ratio of *Bax* to *Bcl2* as well as morphological signs of atresia were increased in this treatment group. Equol-induced atresia may therefore be a contributing factor to follicle growth inhibition in the present study. Further, antral follicle growth is partly dependent on normal levels of ovarian sex steroid hormones (Drummond, 2006; Lunenfeld *et al.*, 1975). Thus, reduction of estradiol, testosterone, androstenedione, and progesterone over 96 hours in the 100 μ M equol-treated follicles may have also played a role in growth inhibition. Although no previous studies to our knowledge 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). Disruption of cell cycle regulators is therefore a potential mechanism by which equol inhibits follicle growth and so future studies should investigate whether equol alters the levels of cell cycle regulators in antral follicles.

Our data indicate that equol at 100 μ M decreased the levels of estradiol, testosterone, androstenedione, and progesterone over 96 hours. Additionally, equol at 6 μ M and 36 μ M decreased the levels of estradiol and androstenedione over 96 hours. Antral follicles are the major source of sex steroids in the female, and normal levels of these hormones are essential for maintenance and function of reproductive tissues. (Drummond, 2006; Lunenfeld *et al.*, 1975). Estradiol is produced from precursor hormones in the theca and granulosa cells of the follicles. Therefore, adequate levels of the precursor hormones are needed for adequate estradiol synthesis (Falck, 1959). 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 mechanism is similar to that of 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 previous studies that have found 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). Additionally, daily consumption of soy decreased plasma steroid hormone levels in healthy, regularly cycling women over the entire menstrual cycle (Lu *et al.*, 2000).

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. Our data indicate that equol at 100 μM significantly decreased the mRNA level of *Cyp11a1*, a rate-limiting enzyme in the estradiol biosynthesis pathway at 48 hours of culture. We also observed a trend towards decreased mRNA level of this enzyme at 96 hours. 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 μM) decreased the mRNA level of *Cyp17a1* at both 48 and 96 hours, 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 μM) decreased the mRNA level of *Cyp19a1*, required for conversion of testosterone to estradiol at 96 hours, but not at 48 hours. Thus, these findings may also explain the decreased production of estradiol by the antral follicles at 96 hours. Our data on these effects of equol at 100 μM are consistent with studies on other endocrine disrupting chemicals such as bisphenol A and di(2-ethylhexyl)phthalate, which found that they inhibit estradiol, testosterone, androstenedione, and progesterone secretion of cultured mouse antral follicles by altering mRNA levels of steroidogenic enzymes (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 mRNA levels of steroidogenic enzymes in these two treatment groups at either 48 or 96 hours. A plausible explanation is that equol at these lower concentrations is inhibiting the protein levels or activity of the enzymes, rather than affecting mRNA levels. Previous studies using human tissues have shown that phytoestrogens are capable of inhibiting steroidogenic enzyme activity and this may not necessarily be associated with a change in protein levels (Kao *et al.*, 1998; Lacey *et al.*, 2005). Additionally, equol (IC_{50} 793 μM) was found to inhibit ovarian CYP19A1 activity in rainbow trout (Pelissero *et al.*, 1996). Hence, future studies should determine whether equol alters the protein levels or enzyme activity in antral follicles, so as to more fully understand its effects at different doses on follicle steroidogenesis.

Exposure of antral follicles to equol (100 μM) induced follicle atresia at 96 hours, as evident by histological evaluation and increased *Bax* to *Bcl2* mRNA ratio. Atresia is the natural process by which follicles undergo death via apoptosis. 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; Greenfeld *et al.*, 2007). 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 levels 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).

Equol has been previously shown to affect the reproductive health of several species including humans (Adams, 1995; Cassidy *et al.*, 1994; Lu *et al.*, 2000; Woclawek-Potocka *et al.*, 2005). Equol is implicated in clover disease of sheep, characterized by infertility, early embryonic loss, abortion, dystocia, uterine prolapse, and metritis. In addition, equol-induced subclinical infertility in sheep is an extensive problem causing reduced rates of ovulation and conception, and permanent infertility with prolonged exposure (Adams, 1995; Shutt and Braden, 1968). Equol has also been associated with cystic ovaries, irregular estrus, anestrus, and conception failure in cows grazed on isoflavone pasture (Adams, 1995). Further, it was found to disrupt reproductive efficiency in cows by altering the secretory activity of the endometrium at different stages of the estrous cycle and during pregnancy (Woclawek-Potocka *et al.*, 2005). Mice that were fed diets containing isoflavones in concentrations that mimic human exposure showed pronounced reproductive effects attributed to daidzein and its metabolite, equol (Cline *et al.*, 2004). Studies in healthy, regularly cycling women showed that daily isoflavone consumption altered their hormonal profiles, prolonged their cycles, and decreased plasma steroid hormone levels (Cassidy *et al.*, 1994; Lu *et al.*, 2000). The findings of the present study are significant because they can potentially explain those equol-induced reproductive abnormalities associated with impaired ovarian function.

In conclusion, the results of the present study indicate that equol inhibits the growth of mouse antral follicles, increases atresia likely via dysregulation of the apoptotic pathway, and inhibits steroidogenesis by suppressing upstream precursor steroidogenic enzymes *in vitro*. It should be noted that the toxic effects on the ovarian follicles 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 require concentrations higher than typically seen in plasma (Whitten and Patisaul, 2001). However, our findings are relevant to wildlife and livestock populations that are exposed to high levels of equol through isoflavone-containing pastures and commercial diets (Lindsay and Kelly, 1970; Lundh, 1995; Woclawek-Potocka *et al.*, 2013; Woclawek-Potocka *et al.*, 2008). Further, although human dietary exposure is close to the lower-range of concentrations used in our study (Setchell *et al.*, 2001; Setchell *et al.*, 2009; Setchell *et al.*, 1997), exposure through equol

supplements may be higher. Moreover, it is unknown as to what percentage of equol circulating in plasma actually reaches the ovary, or whether equol accumulates in follicular fluid. This, coupled with increased soy consumption and the rising number of women taking phytoestrogen supplements, makes it important to understand the implications of equol toxicity for both humans and animals.

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Highlights

- Equol exposure inhibits antral follicle growth
- Equol exposure increases follicle atresia
- Equol exposure inhibits sex steroid hormone levels
- Equol exposure inhibits mRNA levels of certain steroidogenic enzymes

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 within experiment 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 within experiment 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. Asterisks (*) represent 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 within experiment 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 within experiment 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. Asterisks (*) represent a significant difference between vehicle control and equol ($p < 0.05$).

Figure 6. Effect of equol on levels of mRNA for steroidogenic enzymes in antral follicles
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 within experiment 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 atresia

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 within experiment 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 $\pm$ SEM from 3 to 4 separate experiments. Asterisks (*) represent a significant difference between vehicle control and equol ($p < 0.05$).

Figure 8. Effect of equol on follicle steroidogenesis

This schematic of the ovarian steroidogenic pathway shows how estradiol is produced from cholesterol through the action of steroidogenic enzymes in the theca and granulosa cells of the antral follicle. Also summarized are the effects of equol on the hormones and enzymes of the estradiol biosynthesis pathway.

Table 1

Sequence of Primers used for Gene Expression Analysis

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