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Effects of isoliquiritigenin on ovarian antral follicle growth and steroidogenesis

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ABSTRACT

Isoliquiritigenin is a botanical estrogen used as a dietary supplement. Previous studies show that other botanical estrogens affect ovarian estradiol synthesis, but isoliquiritigenin's effects on the ovary are unknown. Thus, this study tested the hypothesis that isoliquiritigenin inhibits ovarian antral follicle growth and steroidogenesis. Antral follicles from CD-1 mice were cultured with vehicle control (dimethyl sulfoxide; DMSO) or isoliquiritigenin (0.6 μM, 6 μM, 36 μM, and 100 μM) for 48–96 h. During culture, follicle diameters were measured daily to assess follicle growth. After culture, media were collected for hormone assays and follicles were collected for gene expression analysis of steroidogenic enzymes. Isoliquiritigenin inhibited antral follicle growth and altered estradiol, testosterone, and progesterone levels. Additionally, isoliquiritigenin altered the mRNA levels of cytochrome P450 steroid 17-α-hydroxylase 1, aromatase, 17β-hydroxysteroid dehydrogenase 1, and steroidogenic acute regulatory protein. These data indicate that exposure to isoliquiritigenin inhibits growth and disrupts steroid production in antral follicles.

1. Introduction

Phytoestrogens are phenolic compounds found in plants that can interact with estrogen receptors and other targets in endogenous estrogen production and action [1]. Although potential benefits have been identified with the use of phytoestrogens [2], their adverse effects are less understood. Isoliquiritigenin is a flavonoid phytoestrogen extracted from the roots of *Glycyrrhiza*, a type of licorice. The U.S. Food and Drug Administration (FDA) classifies licorice and licorice extracts/derivatives as generally recognized as safe (GRAS) for use in foods (21 CFR 184.1408) and animal feeds (21 CFR 582.10; 582.20). They are also FDA-approved for use in certain over-the-counter drugs (21CFR 310.528; 310.544; 310.545) [3]. Licorice is used as a flavoring agent in candy, gum, tobacco, toothpaste, cough mixtures, herbal teas and other beverages. It is also frequently found in skin care products [4]. Licorice has been used extensively in traditional Asian and European medicine to treat conditions ranging from peptic ulcers, pharyngitis, and abdominal pain, to asthma, insomnia, malaria, and other infections [5]. Licorice may also be effective in weight loss and metabolic syndrome [5,6]. Dietary supplements containing licorice are popular among women for relief from symptoms associated with premenopausal syndrome and menopause [5,7].

Isoliquiritigenin, one of the bioactive components of licorice, is often used as an anti-inflammatory, antimicrobial, anti-diabetic, and anti-tussive agent [5,8]. Moreover, isoliquiritigenin has been found to inhibit growth and aromatase activity of breast cancer cells and thus, has potential to be used as a chemotherapeutic agent in breast cancer [9–11]. It may also have potential utility as a therapy in other cancers, as it has been found to inhibit mouse colon cancer and proliferation of prostate cancer cells [12,13]. Isoliquiritigenin reduces contraction in the mouse uterus, indicating that it may be useful for treating uterine pain due to excessive contraction [14,15]. Thus, exposure to isoliquiritigenin commonly occurs through various licorice-containing foods and products, as well as through the clinical use of isoliquiritigenin and licorice supplements for a wide variety of medical conditions. Average daily human exposure to isoliquiritigenin through the diet and/or supplements is estimated to be 1–2 mg/kg [6]. Chronic exposure is possible with high intake of licorice-flavored tobacco and in individuals consuming licorice tablets or capsules as a heath product [3]. The female population

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may be more likely to be exposed to isoliquiritigenin, especially postmenopausal women seeking alternatives to traditional hormone replacement therapy [6,7].

Unfortunately, to our knowledge, the effects of isoliquiritigenin on the ovary have not been published. Chemicals that target the ovary may disrupt ovarian function, resulting in decreased fertility, reduced estradiol synthesis, and premature ovarian failure [16]. The ovary contains numerous estrogen receptors and thus, it is an important target organ for phytoestrogens such as isoliquiritigenin, which can bind to estrogen receptors [7,17,18]. This, coupled with the fact that isoliquiritigenin has broad clinical and commercial applications, makes it imperative to understand how this botanical compound affects the ovary. Additionally, understanding the effects of isoliquiritigenin on the ovary may have applications in veterinary medicine for production animals that graze on *Glycyrrhiza,* and might be compromising their reproductive value.

Thus, the present study examined the effects of isoliquiritigenin on ovarian antral follicles, which are responsible for sex steroid production and further development into ovulatory follicles. Specifically, the present study tested the hypothesis that isoliquiritigenin inhibits antral follicle growth and sex steroid synthesis in the adult mouse ovary.

2. Materials and methods

2.1. Animals

Adult, cycling, female CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA) and acclimatized for 24–72 h 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 h 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.

2.2. Chemicals

Isoliquiritigenin (98% pure as ascertained by high-performance liquid chromatography) was supplied by the Botanical Estrogen Research Center, University of Illinois at Urbana-Champaign. Dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) was used as the vehicle to dissolve the isoliquiritigenin and to prepare treatment concentrations of 0.6 μM, 6 μM, 36 μM, and 100 μM, which are approximately equivalent to *in vivo* doses of 0.1 mg/kg, 1.6 mg/kg, 9.6 mg/kg, and 26.6 mg/kg respectively. To our knowledge, data are not available on the plasma isoliquiritigenin levels in women consuming licorice and licorice root supplements or on the potential toxicity of isoliquiritigenin on the ovary. Taking into account this paucity of information, a broad range of concentrations was chosen for the present study. The doses of 0.6 μM and 6 μM fall within the range of estimated human exposure to isoliquiritigenin (1–2 mg/kg) [6]. These doses are also representative of the plasma isoflavone levels that have been found in humans for other phytoestrogens such as daidzein and genistein [1,19]. Previous studies have found genistein to be a toxicant for granulosa cells at concentrations of 50 μM, while daidzein, another phytoestrogen, was not toxic at dosages as high as 100 μM [20]. These varying results to different exposures of different phytoestrogens warrant testing isoliquiritigenin at concentrations ranging from 0.6 μM to 100 μM. The isoliquiritigenin concentrations tested in the present study are also similar to those used in previously published *in vitro* and *in vivo* studies investigating its effects on glioma cells, cervical cancer cells, and heart muscle [21–23].

2.3. Antral follicle culture

Adult, cycling, female CD-1 mice were euthanized on postnatal days (PND) 32–35, and based on relative follicle size (225–400 μm), antral follicles were manually isolated from the ovaries and cleaned of interstitial tissue using watchmaker forceps. The age selection of the mice in the current study is based on previous studies using the same follicle culture method [24,25]. Follicles were pooled from four to five different mice for each experiment, with 20–40 follicles obtained from each mouse. The follicles were individually plated in wells of 96-well tissue culture plates, so that each treatment group contained 10–18 follicles. The different treatment groups (DMSO or vehicle control, isoliquiritigenin at 0.6 μM, 6 μM, 36 μM, and 100 μM) 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 [25]. Various stock concentrations of isoliquiritigenin (0.2, 2.05, 12.29, and 34.16 mg/mL) were prepared using DMSO, which 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 0.15, 1.54, 9.22, and 25.62 μg of isoliquiritigenin per mL of culture medium, which is equivalent to 0.6 μM, 6 μM, 36 μM, and 100 μM, respectively (molecular weight of isoliquiritigenin: 256.25 g/mol). The cultures were carried out for total periods of 48, 72, and 96 h in an incubator supplying 5% CO_2 at 37 °C. The experimental endpoints of follicle growth, sex steroid hormone levels, and mRNA levels of selected regulators of steroidogenesis were evaluated at these time-points. The cultures were performed for up to 96 h because previous studies indicate that non-treated follicles and vehicle control (DMSO) follicles continue to grow and remain viable up to 96 h without medium changes [25,26].

2.4. Follicle growth analysis

The growth of antral follicles over time was assessed by measuring follicle diameters on perpendicular axes at every 24 h time point, with an inverted microscope equipped with a calibrated ocular micrometer. Follicles with diameters of 225–400 μm were considered to be antral follicles, which correlates with the histological appearance of these follicles [27]. The individual diameters were averaged within treatment group for each 24 h interval, and the average values were divided by the initial average measurement (0 h) 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.

2.5. Hormone assays

After each 48, 72, and 96 h culture, medium from each well was collected and pooled according to treatment group, and then subjected to enzyme linked immunosorbent assays (ELISAs) for measuring the levels of progesterone, testosterone, and 17β-estradiol. These sex steroid hormones were chosen because they are essential for normal female reproductive function. The levels of these hormones were measured using ELISA kits purchased from Diagnostics Research Group (DRG, Springfield, NJ) according to manufacturer's instructions. Samples were run in duplicate, and had inter-assay and 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 with Multiskan Ascent soft-