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Soy isoflavones administered to rats from weaning until sexual maturity affect ovarian follicle development by inducing apoptosis

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ABSTRACT

Twenty-one-day-old female Wistar rats were treated daily with orally administered soy isoflavones (SIFs) at concentrations of 50, 100, or 200 mg/kg body weight from weaning until sexual maturity (3 mo.), and ovarian follicle development was evaluated. At the end of the treatment period, the ultrastructure of the ovarian granulosa cells was examined by transmission electron microscopy. The apoptotic cell death of ovarian granulosa cells was detected using TUNEL staining. The mRNA expression levels of caspase-3, caspase-8, caspase-9, Bcl2, Bax, and Fas were determined by real-time quantitative PCR. The protein expression levels of caspase-3, Bcl2, Bax, and Fas were determined by western blotting. Our data showed that exposure to SIFs resulted in morphological changes consistent with ovarian granulosa cell apoptosis. The percentage of TUNEL-positive granulosa cells was increased. The mRNA expression levels of the apoptosis-related genes caspase-3, caspase-8, caspase-9, Bax, and Fas increased significantly. The protein levels of Bax, Fas, and cleaved caspase-3 were also increased. These results indicate that the exposure of rats to modest doses of SIFs from weaning until sexual maturity can affect ovarian follicle development by inducing apoptosis. The mechanism of SIF-induced alterations in ovarian follicle development may involve the activation of Fas-mediated and Bcl2/Bax-mediated apoptotic signaling pathways.

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1. Introduction

Soy isoflavones (SIFs) are a group of phytochemicals that include the most commonly found phytoestrogens that are present in soy and other legumes. SIFs are also found in soy-based products such as tofu, soy milk, soy infant formula, and some over-the-counter dietary supplements. Epidemiological and experimental studies show that SIFs have many beneficial health effects, such as the prevention of cancer and cardiovascular disease and the prevention of osteoporosis in menopausal and postmenopausal women (Chen and Anderson, 2002; Cooke, 2006; Kurzer, 2008; Ma et al., 2008; Xiao, 2008). In experimental studies, ovariectomized animals are generally used to avoid the potentially complicating effects of endogenous estrogens (Chang et al., 2013; Lee et al., 2012; Ramos et al., 2012). However, whether SIFs influence the function of the ovaries has not been intensively studied. SIFs are capable of binding to estrogen receptors, which may adversely affect estrogen-sensitive target tissues such as the uterus, oviduct, vagina, and mammary gland (Jefferson et al., 2009; Kuiper et al., 1998; Newbold et al., 2001; Nikaido et al., 2005, 2004; Padilla-Banks et al., 2006). However, despite there is an abundant literature on whether the ovary, as an estrogen-producing tissue, is affected by SIFs in a variety of species including rats, mice and pigs, further studies should be designed to explore the exact mechanism.

During certain periods of life, exposure to xenoestrogens may have adverse effects on the female reproductive system (Pryor et al., 2000; Sharpe and Irvine, 2004). Although the fetal and neonatal periods are the most sensitive to hormonal perturbations (Atanassova et al., 2005), exposure to SIFs is usually limited during these periods. Studies show that the concentrations of the SIFs genistein and daidzein in human breast milk are very low (5–15 ng/ml), and although these concentrations can increase up to 10-fold when the lactating mother consumes soy-based foods (Franke and Custer, 1996), the daily intake of phytoestrogens by the infant from human milk is only 0.005–0.01 mg (Setchell et al., 1997). In contrast, exposure to SIFs is greatly increased during childhood. Children may be exposed to high levels of SIFs through soy foods,

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soy-based infant formulas, and other commercialized, over-the-counter SIF supplements that are often specifically marketed for them (Franke et al., 1998; Setchell et al., 1998). Such products are not closely regulated, and the potentially dangerous effects of SIF exposure are of particular concern. Furthermore, children may be exposed to higher levels of SIFs than adults. Studies show that infants who consume a diet of soy-based formula are exposed to 6–9 mg per kg per day of SIFs, whereas adults who consume a diet with modest amounts of SIFs may be exposed to only 1 mg per kg per day (Setchell et al., 1997). In addition, due to low estrogen levels and the immature development of the hypothalamic–pituitary–ovarian axis, children are more sensitive to exogenous estrogenic compounds than adults (Olea et al., 1999). Therefore, it is possible that SIFs may pose a significant hazard to the developing reproductive system; thus, public concern over SIF exposure has recently increased.

Our previous studies have shown that modest doses of SIFs affect ovarian follicle development in rats when administered from weaning until sexual maturity, as demonstrated by decreases in serum estradiol levels and increases in both ovarian follicle atresia and corpus luteum number (Wang et al., 2013). Metabolomic analysis (High-performance liquid chromatography and mass spectrometry, HPLC–MC) has demonstrated SIF treatment-related metabolic alterations in follicular fluid. This study identified 24 metabolites that were significantly altered, including primary sex hormones, amino acids, fatty acids, and metabolites involved in energy metabolism (Wang et al., 2013). Studies show that apoptosis contributes to follicular atresia, which can occur in any follicular developmental stage. The acceleration of follicular atresia is the main contributor to premature ovarian failure (Fukaya et al., 1997; Palumbo and Yeh, 1994). In addition, given that the mitochondria are the main sites of energy metabolism, the disruption of energy metabolism noted in our previous study indicates that SIFs may harm the structure and function of the mitochondria, which also serve as initial sites of apoptotic signaling pathways (Verdin et al., 2010). Furthermore, recent studies have shown that daidzein and genistein can inhibit cancer cell growth by inducing apoptosis (Rajah et al., 2012; Szliszka and Krol, 2011; Tang et al., 2013). However, whether SIFs can affect ovarian follicle development by inducing apoptosis remains unclear.

Based on these observations, we conducted this study to determine whether SIFs administered to rats from weaning to sexual maturity can affect ovarian follicle development by inducing apoptosis. An additional objective of this study was to determine which apoptotic signaling pathways regulate ovarian cell apoptosis. The results provide experimental evidence further elucidating the ovarian toxicity of SIF.

2. Materials and methods

2.1. Animals

Eighty 21-day-old female Wistar rats weighing 45 ± 5 g were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. The animals were housed in a controlled laboratory environment (25 °C, 50% humidity), and the lighting schedule was maintained at 12 h of light per day. The animals were allowed to have free access to a pelleted soy- and alfalfa-free diet and drinking water. The experimental protocol was approved by the Animal Care and Use Committee of Fujian Medical University (No: 2013-036). The experimental procedures were performed in accordance with international guidelines for the care and use of laboratory animals.

2.2. Chemicals

Soy isoflavones (SIFs, a mixture of genistein, daidzein, and glycetin, accounting for 60%, 20%, and 13% of the total isoflavones, respectively) were obtained from Zhengzhou Lion Biological Technology Co. Ltd. (Henan, China). Genistein and daidzein standards were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The soy- and alfalfa-free diet was formulated at the Laboratory Animal Centers of Fujian Medical University. The doses of SIFs used in this study were selected based on our previous studies and in accordance with other previous reports (McCarver et al., 2011; Michael McClain et al., 2006; Setchell et al., 1997; Wang et al., 2013; Zhang et al., 2009). Based on these studies, the SIFs exposure levels of children range from 1 to 9.3 mg/kg per day (the equivalent dosage for rats is 7–65 mg/kg per day) and the no observed adverse effect level (NOAEL) of SIFs to rats is 200 mg/kg per day and the no observed effect level (NOEL) of genistein to rats is 5 mg/kg per day. Therefore, 200 mg/kg per day of SIFs was selected as the high dose and 100 and 50 mg/kg per day were set as the medium and low doses respectively. The TUNEL apoptotic cell detection kit was obtained from Nanjing KeyGEN Biotech. Co. Ltd. (Nanjing, China). Protease K was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade.

2.3. Experimental design

After acclimatization, animals were randomly divided into four groups of twenty rats each. Group I (control) rats received distilled water (2 ml/kg body weight, once per day) intragastrically throughout the course of the study. Group II, Group III, and Group IV rats were treated daily by the intragastric administration of SIFs at a dose of 50, 100, or 200 mg/kg body weight, respectively, until they reached sexual maturity (3 mo.). During the experimental period, all animals were fed a soy- and alfalfa-free diet, and weight gain was measured daily. After an interval of 12 h from the final SIF treatment, serum samples were collected to measure the total concentrations of genistein and daidzein. Subsequently ten animals of each group were killed by decapitation during the estrus phase of the estrous cycle; ovarian samples were harvested and stored at −80 °C until use. After an interval of 48 h from the final SIF treatment, the other ten animals in each group were subjected to superovulation experiments.

2.4. Serum genistein and daidzein concentrations

Sera were separated from blood by centrifugation and frozen until analysis. Total genistein and daidzein concentrations were measured using high-performance liquid chromatography and mass spectrometry (HPLC-MS) (Fritz et al., 1998). Briefly, serum samples were incubated with β-glucuronidase/sulfatase (St. Louis, MO, USA), and acetic ether was added to the samples and then vortexed, followed by centrifugation. The supernatant was collected and evaporated under nitrogen and redissolved in 80% aqueous methanol. A 15 μl aliquot of the supernatant was injected into a 2.1 × 150 mm Agilent Zorbax SB-C18 5 μm column, using a Dionex Ultimate 3000 HPLC for LC-MS (Dionex, USA). Acetonitrile/0.01% acetic acid was used as the mobile phase. Mass spectral analysis was performed using an MS 3200 Q TRAP (ABI, USA), operating in positive ion electrospray mode. Samples were spiked with biochanin A as an internal standard. Genistein and daidzein concentrations were quantified by a comparison of peak areas with those of the standard curves. The limit of detection was 5 nM.

2.5. Transmission electron microscopy

Three left ovaries per group were chosen randomly, and one-half of each ovary was fixed for 48 h in a solution containing 3% glutaraldehyde and 1.5% paraformaldehyde. The samples were then fixed with 1% osmium and 1.5% potassium ferrocyanide, dehydrated by successive passes through a series of ethyl alcohol-acetone baths, and embedded in epoxy resin. Fifteen sections of each ovarian sample (70–80 nm thick) (taking one section from every 50 sections) were prepared using an ultramicrotome and stained with uranyl acetate and lead citrate. Cells were observed and imaged using a transmission electron microscope (EM 208 type, Netherlands' Philips Ltd., Netherlands).

2.6. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining and follicle counting

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining for detecting the apoptotic cell death of ovarian granulosa cells was performed as described by our previous study (Weng et al., 2014). Briefly, ten left ovaries per group were selected, and one-half of each ovary was fixed and dehydrated by successive passes through a series of ethyl alcohol-water baths, cleaned in xylene, and embedded in paraffin. Ten sections (5–6 μm thick) of each ovarian sample (taking one section from every 30 sections) were prepared using a rotary microtome and stained using a TUNEL staining kit according to the manufacturer's instructions. The nuclei of apoptotic cells were stained brown and were observed and imaged using the OLYSIA-BioReport imaging software (BH 200, Japan's Olympus Optical Co., Ltd., Japan). The extent of apoptosis was calculated as the percentage of apoptotic cells among all granulosa cells in the ovarian follicles at different phases using the Image-Pro Plus 4.5 image analysis software. The overall follicle numbers in the above ovary samples (seven intact left ovaries per group, namely the seven left ovarian halves not used in the TEM studies and the corresponding seven left ovarian halves used in the TUNEL experiments) were also counted using light microscopy. The data are reported as total follicles counted per ovary. Follicles were classified as primordial, primary, antral, and corpora lutea.

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