Toxicology and Applied Pharmacology 269 (2013) 280–289

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

# Metabolomic changes in follicular fluid induced by soy isoflavones administered to rats from weaning until sexual maturity

Wenxiang Wang [a], Wenchang Zhang [b,*], Jin Liu [b], Yan Sun [c], Yuchen Li [b], Hong Li [b], Shihua Xiao [b], Xiaohua Shen [b]

[a] Department of Nutrition and Health Care, School of Public Health, Fujian Medical University, Fuzhou, Fujian, PR China
[b] Department of Occupational and Environmental Health, School of Public Health, Fujian Medical University, Fuzhou, Fujian, PR China
[c] Center for Reproductive Medicine, Teaching Hospital of Fujian Medical University, Fujian Maternity and Child Health Hospital, Fuzhou, Fujian, PR China

ARTICLE INFO

*Article history:*
Received 9 November 2012
Revised 6 January 2013
Accepted 11 February 2013
Available online 20 February 2013

*Keywords:*
Soy isoflavones
Follicular fluid
Follicle
Metabolomics
Phytoestrogens

ABSTRACT

Female Wistar rats at 21 days of age were treated with one of three concentrations of soy isoflavones (SIF) (50, 100 or 200 mg/kg body weight, orally, once per day) from weaning until sexual maturity (3 months) in order to evaluate the influence of SIF on ovarian follicle development. After treatment, the serum sex hormone levels and enumeration of ovarian follicles of the ovary were measured. The metabolic profile of follicular fluid was determined using HPLC-MS. Principal component analysis (PCA) and partial least-squares-discriminant analysis (PLS-DA) was used to identify differences in metabolites and reveal useful toxic biomarkers. The results indicated that modest doses of SIF affect ovarian follicle development, as demonstrated by decreased serum estradiol levels and increases in both ovarian follicle atresia and corpora lutea number in the ovary.
SIF treatment-related metabolic alterations in follicular fluid were also found in the PCA and PLS-DA models. The 24 most significantly altered metabolites were identified, including primary sex hormones, amino acids, fatty acids and metabolites involved in energy metabolism. These findings may indicate that soy isoflavones affect ovarian follicle development by inducing metabolomic variations in the follicular fluid.


## Introduction

Phytoestrogens are a group of non-steroidal, estrogenic compounds that display estrogen-like properties. Soy isoflavones (SIF) are the most common phytoestrogen and are found in many food products, especially soy-based products such as tofu, soy milk, soy infant formula and some over-the-counter dietary supplements. Epidemiological evidence and experimental studies have repeatedly linked the consumption of soy isoflavones or soy products to a variety of beneficial health effects, such as the prevention of cancer and cardiovascular disease, as well as osteoporosis in menopausal and postmenopausal women (Chen and Anderson, 2002; Cooke, 2006; Kurzer, 2008; Ma et al., 2008; Xiao, 2008). In these experimental studies, ovariectomized animal models were consistently used to avoid the effects of endogenous estrogen. However, whether soy isoflavones influence the function of the ovaries has seldom been intensively studied. Soy isoflavones are capable of binding to estrogen receptors due to the fact that they have a similar structure to estradiol. The binding of soy isoflavones to estrogen receptors may adversely affect estrogen-sensitive target tissues, including the uterus, oviduct, vagina and mammary gland (Jefferson et al., 2009; Kuiper et al., 1998; Newbold et al., 2001; Nikaido et al., 2004, 2005; Padilla-Banks et al., 2006). However, whether the ovary, especially the ovarian follicle, which as an endogenous estrogen-producing tissue, is affected by soy isoflavones remains unclear.

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 most sensitive developmental periods are the fetal and neonatal periods (Atanassova et al., 2005), exposure to soy isoflavones during these developmental periods tends to be limited. For example, the concentrations of the soy isoflavones genistein and daidzein in human breast milk are very low (5 to 15 ng/ml), and although they 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 to 0.01 mg (Setchell et al., 1997). In contrast, the odds of exposure are greatly increased during childhood. Children may be exposed to high levels of phytoestrogens through soy-based infant formulas, foods that are often specifically marketed for them, and other commercialized over-the-counter soy isoflavones supplements (Franke et al., 1998; Setchell et al., 1998). Such products are not closely regulated, and the potentially dangerous effects of soy isoflavones exposure are concerning. Furthermore, children may be exposed to higher levels of soy isoflavones than adults. It is estimated that infants who consume a diet of soy-based formula are exposed to 6–9 mg per kg per day of soy isoflavones, whereas adults who consume a diet with modest amounts of soy isoflavones may only be exposed to approximately 1 mg per kg per day (Setchell et al., 1997). In

* Corresponding author. Fax: +86 59122862023.
*E-mail address:* wenchang2002@sina.com (W. Zhang).

0041-008X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.taap.2013.02.005

addition, unlike adults, children have low estrogen levels and the development of hypothalamic–pituitary–ovarian axis is immature, which makes them more sensitive to exogenous estrogenic compounds. Therefore, it is reasonable to assume that soy isoflavones may pose a greater hazard to the developing reproductive system in children, and public concern about this issue has recently increased.

Follicular fluid is a complex, semi-viscous, yellow extracellular fluid that begins to accumulate in the antrum of the ovarian follicles during the early stages of follicular development. Follicular fluid is essential for oocyte maturation and fertilization, as well as granulosa cell proliferation and differentiation (Pinero-Sagredo et al., 2010). Its components originate from two sources: blood and ovarian cells (granulosa and thecal cells). Although the ovarian vascular and follicular antrum do not communicate directly, nutrients from the blood plasma can pass through the basal lamina to enter the antrum. Ovarian cells also produce and secrete a number of soluble factors, including steroids and growth factors, into the follicular fluid (Fortune et al., 2004). Disruption of these components can lead to abnormal metabolic activity of the ovarian cells and can alter the physiological status of the follicle, ultimately affecting ovulation and luteinization. However, to our knowledge, the disruptive effects of phytoestrogens (e.g., soy isoflavones) have not been studied.

Metabolomics is a robust, high throughput and rapid post-genomic technology for pattern recognition analyses of biological samples (Goodacre, 2004). The technology analyzes low molecular weight (less than 1000 Da) metabolites, which represent the end products of cellular regulatory processes, can reveal the response of biological systems to a variety of genetic, nutritional or environmental influences. Metabolomic analysis has been applied to nutrition studies, toxicological investigations, disease diagnoses and drug discovery (Evans Alison et al., 2002; Hirai et al., 2004; Holmes et al., 2000; Keun and Athersuch, 2007; Lin et al., 2010; Pasikanti et al., 2010; Wilcoxen et al., 2010). Recently, several studies have used this technology to analyze variations in the components of follicular fluid in an effort to predict oocyte developmental competency and subsequent embryo quality (Revelli et al., 2009), however, comprehensive metabolic profiling of follicular fluid is limited.

Based on these observations, the purpose of this study was to determine the influence of the administration of soy isoflavones from weaning to sexual maturity on ovarian follicle development in rats. We also investigated the metabolomic variations in follicular fluid using HPLC-MS and uncovered useful toxic biomarkers. These findings may indicate that soy isoflavones can affect ovarian follicular development by inducing metabolomic variations in follicular fluid.

## Materials and methods

*Animals.* Eighty female 21 day old Wistar rats weighing $45 \pm 5$ g were obtained from Shanhai 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. The experimental procedures were carried out in accordance with international guidelines for the care and use of laboratory animals.

*Chemicals.* Soy isoflavones (SIF, a mixture of genistein, daidzin and glycetin, accounting for 60, 20 and 13%, respectively) were obtained from Zhengzhou Lion Biological Technology Co. LTD. (Henan, China). The soy- and alfalfa-free diet was formulated at the Laboratory Animal Centers of Fujian Medical University. The doses of SIF used in this study were selected based on our preliminary experiments and in accordance with previous reports (McCarver et al., 2011; Setchell et al., 1997; Shen et al., 2011). Serum estradiol and progesterone test kits were obtained from the Beijing DiaCha Biological Engineering Co. LTD. (Beijing, China). All other reagents were of analytical grade.

*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 intragastric administration of SIF (50, 100 or 200 mg/kg body weight, respectively) until they reached sexual maturity (3 months). During the experimental period, all animals were fed a soy- and alfalfa-free diet, and weight gain was measured every day. After the treatment period, the animals were killed by decapitation at the estrus of estrous cycle, and blood, ovarian and uterine samples were harvested and stored at 4 °C until use.

*Determination of serum estradiol (E2) and progesterone (P4) levels.* Serum was collected from the blood samples by centrifugation at 2000 rpm for 15 min. The samples were stored at −20 °C prior to measurement. Chemiluminescent immunoassays (CLIA) to measure serum estradiol and progesterone levels were carried out using commercially available kits (Beijing DiaCha Biological Engineering Co. LTD. Beijing, China), according to the manufacturer's instructions. Luminous intensity (relative light units, RLU) was detected using the BHP9504 microplate luminometer at Fujian Medical University, China. All samples were run in duplicate in a single assay to reduce inter-assay variability.

*Enumeration of ovarian follicles.* For enumeration of ovarian follicles analysis, the ovarian samples were fixed for 48 h in a 10% formalin–saline solution, dehydrated by successive passing through a series of ethyl alcohol-water baths, cleaned in xylene and embedded in paraffin. Tissue sections (5–6 μm thick) were prepared using a rotary microtome, stained with hematoxylin and eosin, and subsequently placed in a neutral deparaffinized xylene (DPX) medium for microscopic evaluation. Follicles were counted in every 30th section to avoid double counting of small preantral follicles. Data are reported as total follicles counted per ovary. Follicles were classified as primordial, primary, antral, atretic and corpora lutea.

*Follicular fluid collection and preparation.* Follicular fluid was collected from all of the antral follicles (400–500 μm in diameter) of the ovary using a microinjector under an inverted microscope. Follicular fluid collected from the left ovaries of three rats from the same group were pooled, up to a total volume of 15 μl, followed by deliquating with deionized water to 50 μl. The follicular fluids were then prepared by centrifugation at 15,000 rpm for 15 min and the supernatant was collected for analysis. Methanol (150 μl) was added to 50 μl of the upper follicular fluid and then spiral mixed for 2 min, followed by centrifugation at 15,000 rpm at 4 °C for 5 min.

*High-performance liquid chromatography and mass spectrometry (HPLC-MS).* A 5 μl aliquot of the supernatant was injected into a $2.1 \times 150$ mm Agilent Zorbax SB-C18 5 μm column, using a Dionex Ultimate 3000 HPLC for LC-MS (Dionex, USA). The column was maintained at 30 °C and eluted with a linear gradient of 10–95% B at a flow rate of 0.75 ml/min (where A = water and B = acetonitrile) for 0–20 min. After holding the solvent content at 90% acetonitrile for 7 min, the column was returned to the starting conditions. The column elution was split to the ESI-MS.

Mass spectral analysis was performed using an MS 3200 Q TRAP (ABI, USA), operating in positive ion electrospray. Argon was used as the collision gas, while nitrogen was used as the nebulizing gas. The ionspray voltage was set at 5.5 kV. The injection voltage and cone voltage were optimized at 10 V and 50 V, respectively. The source temperature was set at 580 °C. The curtain gas, ion source