INTRODUCTION

Isoflavones are a subgroup of flavonoids that are found in plants such as soybeans, clover, and bluegrass (Reinli and Block, 1996). Two of the primary isoflavones found in soybeans and soybean feed products are genistein and daidzein. Daidzein can bind to the estrogen receptor (ER), affect estrogen-regulated gene expression (Totta et al., 2005), and display both estrogen and anti-estrogen properties (Kurzer and Xu, 1999). Isoflavones are known to affect reproductive function in many animal species including humans. Daidzein has been found in plasma and urine of humans, rats, ruminants and pigs and indicates the possibility of indirect or direct action on ovarian cells. Daidzein up-regulated mRNA expression of gonadotropin receptors and P450arom to improve the development of preovulatory follicles in white silky fowls after the peak laying period (Liu et al., 2007). Study suggested that genistein and daidzein decreased pig ovarian follicle cell apoptosis in a dose-responsive manner, and daidzein was more potent than both estradiol and genistein (Suttner et al., 1998).

Bone morphogenetic proteins (BMPs), which belong to the transforming growth factor β (TGF-β) superfamily, form dimers that are interconnected by seven disulfide bonds (Knight and Glister, 2006). Members of each subgroup (BMP-2, BMP-4, BMP-6, BMP-7, BMP-15, and growth differentiation factor-9(GDF-9)) have been shown to affect ovarian folliculogenesis, follicular growth and differentiation, cumulus expansion, ovulation and luteinization (Knight and Glister, 2006). BMPs transmit their signals through specific receptors in the granulosa cell membrane. Recent studies demonstrated that interaction of BMPRIB and BMPRII elicits BMP15 biological activity (Moore et al., 2003), and ALK-5 and BMPRII are essential for GDF-9 signaling in granulosa cells (Vitt et al., 2002a). Protein and mRNA expression for type I and II BMP receptors has been demonstrated in sheep ovarian somatic...
and germ cells (Souza et al., 2001). The BMP receptor pathway is very important for ovarian function, since mutations in BMPRIB are associated with increased ovulation rate in ewes (Mulsant et al., 2001; Souza et al., 2001; Jia et al., 2005), and impaired follicular development in mice (Yi et al., 2001). Receptors for BMPs may be precisely controlled in a stage- and hormone-dependent manner during follicular development in the mammalian ovary (Jayawardana et al., 2006). Many systemic factors, such as E2, glucocorticoids and vitamin D, have been shown to affect activity of BMPs (Jia et al., 2003; Jayawardana et al., 2006). Daidzein is an estrogenic compound that can bind to the estrogen receptor (Kurzer and Xu, 1999). Whether daidzein effect the mRNA expression of BMP receptor genes in ovine granulosa cells remain to be elucidated.

In vitro studies concerning action of isoflavones on reproduction were performed mostly on granulosa cells. Cultured granulosa cells have been widely studied for examination of the effects of hormones and other chemicals on the female reproductive system (Vinze et al., 2004). Ovarian granulosa cells play a complex and fundamental role in the development of the ovarian follicle (Im et al., 1995).

Therefore, in order to get an insight into the possible reproductive-related consequences of daidzein, this study was undertaken to evaluate the effect of daidzein on proliferation and steroidogenesis of ovine granulosa cells. Moreover, there have been few reports on the influence of expression of BMPRII, BMPRIB and ALK5 by daidzein in ovine granulosa cells. We studied the effect of daidzein on characteristics of mRNA expression of these genes in ovine granulosa cells.

MATERIAL AND METHODS

Isolation and culture of granulosa cell

Unless specified otherwise, all regents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The experiments were conducted during the breeding season from August to October 2007 in Zhejiang Province, China. Granulosa cell culture was performed according to the method described by Campbell et al. (1996).Ovaries were collected at a local slaughterhouse, and were transported to the laboratorv at 37 °C. Granulosa cell suspension was collected. After centrifugation at 800 g for 5 min, the cell pellet was resuspended in culture medium (McCoy’s 5a containing bicarbonate, supplemented with Hepes (20 nmol/L), penicillin (100,000 IU/L), streptomycin (0.1 μg/L), L-glutamine (3 mmol/L), BSA (0.1%, w/v), androstenedione (10^{-7} mol/L), transferrin (2.5 mg/L), long R3 insulin-like growth factor (LR3- IGF, 10 μg/L), selenium (4 μg/L)). The number of living cells in each suspension was estimated with a hemocytometer and trypan blue exclusion. Cells were seeded at 7.5×10^4 cells/well in 96-well plates and 10^6 cells/well in 24-well plates (Costar, Corning Inc., USA) for proliferation assay and mRNA isolation, respectively. The plates were incubated in a humidified atmosphere with 5% CO2 in air at 37°C.

Treatment of granulosa cells with daidzein

After 24 h subculture, daidzein effects on proliferation and mRNA expression of BMP receptor genes were examined in ovine granulosa cells. Daidzein was initially dissolved in dimethylsulfoxide (DMSO) at 1 μg/ml and diluted with medium immediately prior to each experiment, to give a final concentration of 1-1,000 ng/ml. The final concentration of DMSO in the medium was ≤0.1%. The control received only the vehicle. At the end of the culture period, the number of cells per well was estimated after trypsinization with a hemacytometer under a phase contrast microscope. Treatments were terminated by aspirating medium and rinsing cells twice with D-PBS, and stored in Trizol reagent (Invitrogen, Life technologies) at -80°C until used for RNA extraction. Each treatment was added to a minimum of three wells. A total of three separate experiments were performed.

MTT assay

The MTT assay [reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoli um um bromide to a purple formazan product] was used to estimate cell viability. Cells were incubated with 0.5 mg/ml of MTT in the last 4hr or the culture period tested. The medium was then decanted, formazan salts were dissolved with 200 μl of DMSO, and the absorbance was determined at 490 nm using a plate reader (Microplate reader, model 680, Bio-Rad Laboratories, Inc. Hemel Hempstead, UK).

Reverse transcription and Real-time PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. Aliquots (2 μg) of total RNA were reverse transcribed to cDNA using random primers and M-MLV reverse transcriptase (Promega, Madison, WI, USA). The gene-specific primers were designed from ovine sequence in Genbank if available, or devised from the
conserved regions of human genes according to the mouse, human, and bovine sequence alignment (Table 1).

Genes for BMPRII, BMPRIB, ALK-5 and GAPDH were quantified by real-time PCR with an iCycler iQ (Bio-Rad, Inc., Hercules, CA, USA) using a commercial kit (TaKaRa). The generated cDNA was used as a template for PCR in a 25 μl reaction mixture for 40 cycles. Each cycle was held at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. The relative expression between the samples was calculated based on the threshold cycle (Ct) value, and standardized to the amount of a housekeeping gene (GAPDH) using the formula

\[ x = 2^{-\Delta\Delta C_t} \]

where

\[ x = \text{fold difference in amount of starting material between two treatment groups (experimental vs. control)} \]

\[ \Delta\Delta C_t = \Delta C_{\text{exp}} - \Delta C_{\text{GAPDH,exp}} - \Delta C_{\text{control}} + \Delta C_{\text{GAPDH,control}} \]

“Ct” stands for the amount of amplified target which reaches a fixed threshold product (Livak and Schmittgen, 2001). After amplification, each sample was applied to a 2% agarose/ethidium bromide gel for electrophoresis.

Hormone assay

E2 and progesterone (P4) amounts in the culture media from each experiment were measured by double-antibody enzyme immunoassay (EIA) using 96-well ELISA plates (Corning Glass Works, Corning, NY, USA), as described previously (Purinton and Wood, 2002; Wood and Giroux, 2003). Standard curves were made for 0-2,000 pg/ml E2 and 0-40 ng/ml P4.

Statistical analysis

All data are expressed as means±SEM. Statistical differences were analyzed using ANOVA, followed by Waller Duncan’s multiple range test (p<0.05) using the one-way ANOVA procedure of SPSS13.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Cell proliferation

The proliferation effects of different concentrations of daidzein on ovine granulosa cells are presented in Figure 1. Granulosa cells in daidzein (1 ng/ml) were not significantly higher than in the control group. At levels of 10-100 ng/ml, daidzein induced a statistically significant increase in cell proliferation (p<0.05). High concentrations of daidzein (1,000 ng/ml) inhibited proliferation of granulosa cells, compared with control and low concentration (10 and 100 ng/ml) values.

Effect of daidzein on the expression of BMP I and II receptor mRNA

To determine whether daidzein regulated expression of BMPRII, BMPRIB and ALK-5 transcriptionally, granulosa cells were treated with 1-100 ng/ml of daidzein for 48 h. Total RNA was isolated, and mRNA expression was examined by quantitative real time RT-PCR. In Figure 2, the PCR for detection of BMPRII, BMPRIB and ALK-5 is depicted, the densitometric data being relative to GAPDH mRNA expression. Daidzein induced mRNA expression of BMPRII, BMPRIB and ALK-5 in a dose-dependent manner. Daidzein treatment led to an increase of BMPRII, BMPRIB and ALK-5 expression. When the daidzein concentration was 10 ng/ml, the BMPRII, BMPRIB and ALK-5 mRNA were significantly increased, and the increase was maintained at 100 ng/ml (Figure 2A-C).

Table 1. Primer pairs used for detection of mRNAs

<table>
<thead>
<tr>
<th>Gene</th>
<th>PCR primera</th>
<th>EMBLb</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>For 5'-CTGACCTGCCCGCTTGAGAAG -3'</td>
<td>U39557</td>
<td>Mulsant et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>Rev 5'-GGTGACCTGGCTGATGTTGTT -3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMPRII</td>
<td>For 5'-CAAGAGTTGGCTGATGTTGTT -3'</td>
<td>A J53490</td>
<td>Jayawardana et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>Rev 5'-ATGACCTGGCTGATGTTGTT -3'</td>
<td>AF312016</td>
<td></td>
</tr>
<tr>
<td>BMPRIB</td>
<td>For 5'-GGTGACCTGGCTGATGTTGTT -3'</td>
<td>A J53490</td>
<td>Jayawardana et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>Rev 5'-ATGACCTGGCTGATGTTGTT -3'</td>
<td>AF312796</td>
<td></td>
</tr>
<tr>
<td>ALK-5</td>
<td>For 5'-CAAACCACGCTGACCTGGCTGATGTTGTT -3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rev 5'-ATGACCTGGCTGATGTTGTT -3'</td>
<td></td>
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For = Forward; Rev = Reverse. EMBL accession number.
Effects of daidzein on hormone production

The effect of daidzein on E2 and P4 production of ovine granulosa cells is shown in Figure 3. Addition of daidzein at any concentration had no significant effect on E2 production (Figure 3A), but 100 ng/ml of Daidzein inhibited P4 production (Figure 3B).

**DISCUSSION**

Recently, isoflavonoids have received much more attention since more and more studies on humans, animals and cell culture systems have suggested that isoflavonoids play an important role in the prevention of oxidative...
damage, menopausal symptoms, osteoporosis, cancer, heart disease (Kurzer and Xu, 1999). Previous study has demonstrated that the isoflavonoids may have a significant effect on the promotion of primordial germ cell proliferation (Tang et al., 2006). Genistein was found to inhibit basal and stimulated P4 production by human, rat and bovine granulosa cells as well as porcine theca and luteal cells (Makarevich et al., 1997; Nynca et al., 2006). Moreover, to prevent reproduction disorders in domestic animals elicited by environmental pollutants, a diet containing isoflavonoids has been regarded as an important part of a feeding strategy (Kotwica et al., 2006).

The effect of daidzein constituents on proliferation and differentiation of sheep germ cells has been scarcely investigated. The present study systematically analyzed the differentiation of sheep germ cells has been scarcely investigated. The present study systematically analyzed the β-granulosa cells by mediation of estrogen receptor-β (ER-β) (Duszał et al., 2006). However, results of studies conducted on laboratory animals as well as on large farm animals have shown that effects of phytoestrogens vary depending on species, sex, routes of administration, dose and exposure time (Merkies et al., 1998). The effects of individual phytoestrogens can vary considerably, and the effects are species- as well as tissue-dependent (Rosselli et al., 2000).

A complete BMP signaling system is present in ruminant ovaries from fetal to adulthood stage. Elements of the BMP system play a key role in regulation of the number of ovari al follicles (Monge et al., 2002; Evans, 2003). In the TGF-β subfamily, GDF-9 and BMP-15 are essential for ovarian follicular growth (Juengel et al., 2004), and cooperate to regulate proliferation and gonadotropin-induced differentiation of granulosa cells in sheep (McNatty et al., 2005). GDF-9 and BMP-15 have been shown to signal through known TGF-β superfamily receptors, such as BMPRII, BMPRIB, and ALK-5, to activate the SMAD intracellular cascade for transmitting their mitogenic actions in granulosa cells. The cooperative effect of GDF-9 and BMP-15 on granulosa cell function is modulated primarily through BMPRII (Edwards et al., 2008). The expression of BMP-15, GDF-9, and BMPRIB has been observed in sheep ovary. BMPRIB is expressed by granulosa cells and oocytes from the primary to the late antral follicle stages. Among the family of BMP receptors, BMPR-IB and BMPRII receptor are expressed mainly in the granulosa cells of primary to late antral follicles in sheep ovary (Souza et al., 2002). The present study showed that mRNA of BMP receptor genes increased in the cultured granulosa cells in response to daidzein in the culture medium.

The synthesis of BMPs can be modulated by some systemic factors (Groeneveld and Burger, 2000). The gonadotrophins are critically important for follicular growth and differentiation in ruminants. During antral growth of nonatretic follicles up to 3 mm diameter, follicular concentration of E2 rapidly increases in sheep. Previous study showed that estradiol regulated the expression of BMPRII and ALK-5 genes in bovine granulosa cells (Jayawardana et al., 2006). Daidzein is an estrogenic compound that can bind to the ER, which may regulate the production of one or more of the BMPs (Jia et al., 2003). Diadzein promoted proliferation of cultured ovarian germ cells by estrogenic action (Liu et al., 2006). Moreover, daidzein has a direct stimulatory effect on bone formation in cultured osteoblastic cells in vitro, which may be mediated by increased production of BMPs in osteoblasts (Jia et al., 2003). BMPs are recognized as multifunctional paracrine/autocrine regulators of mammalian follicle function, evidenced by the ability of BMPs to control proliferation and differentiation of granulosa cells (Shimasaki et al., 2004). In vitro experiments on isolated granulosa cells have revealed modulatory effects of BMPs on steroidogenesis (Shimasaki et al., 1999). With rat and bovine granulosa cells, BMP-4,-6, and -7 were shown to suppress basal and gonadotropin-stimulated progesterone secretion (Glider et al., 2004). In cellular physiology, daidzein acts by regulation of the synthesis of local factors (including BMPs) which exert autocrine and paracrine action in the formation and development of ovarian follicles. Although the phytoestrogens were frequently studied for both beneficial and deleterious effects on different cell types, their mechanism of action largely remains unclear and no studies on phytoestrogens in regulating the expression of BMPs receptor genes in ovine granulosa cells have been reported. To confirm whether BMP expression is influenced by the presence of daidzein, we examined the expression of the BMP receptor genes using real-time PCR. The results indicated that daidzein (10-100 ng/ml) caused a significant increase in BMPRII, BMPRIB and ALK-5 mRNA. Therefore, this also supports the idea that low daidzein stimulated ovine granulosa cell proliferation and differentiation due to the effects on expression of BMPs receptor-mediated signaling pathway.

The current results indicated that low concentrations of
daidzein did not adversely impact proliferation of granulosa cells and may in fact improve granulosa cell proliferation at certain concentrations by promoting the expression of BMP receptor genes. Further studies would begin to provide some information on the role of isoflavones on bone morphogenetic protein and its receptor gene expression in the sheep follicle.

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REFERENCES


Nynca, A. and R. E. Ciereszko. 2006. Effect of genistein on steroidogenic response of granulosa cell populations from...


