INTRODUCTION

Daidzein belongs to isoflavone family, existing mostly in soybean and clover. Some studies demonstrated that long-term intake of daidzein as well as some other isoflavones could interfere with animal reproduction and cause estrous disorders and irregular ovary and genital development (Kaladas, 1989; Nwannenna et al., 1995; Odum et al., 2001; Mitchell, 2001). Other studies suggested its stimulatory effects on growth performance in broiler, beef, sheep and pig (Wang and Han, 1994; Han, 1999; Payne et al., 2001). Neuro-endocrine and molecular evidences showed that daidzein could modulate secretary patterns of GH, LH, PRL, estradiol and testosterone (Parvizi et al., 1998; Wang et al., 1999, 2000; Weber et al., 1999, 2001), down-regulate estrogen receptor beta in hypothalamus and stimulate fetal growth, accompanied by higher IGF-I receptor gene expression in skeletal muscle (Ren et al., 2001). Great attentions have been also paid in daidzein and other isoflavones because many studies in recent years revealed that they could inhibit or prevent some cardioavascular diseases, estrogen-related human cancers and metabolic disorders, which might be associated with immune system (Kelly, 1988; Zhang et al., 1993; Wojtowicz, 1997; Yan et al., 1997; Setchell and Cassidy, 1999; Clifton-Bligh et al., 2001). Since previous studies have been mostly concentrated on mature female animal or human and less emphasis was laid on intact premature males especially in their immune response, this study was aimed at furthering our understandings of daidzein on its roles in growth, cellular immune reaction and functional development of immune system in the intact male piglets.

MATERIALS AND METHODS

Animals

12 intact male piglets (Large White×Meishan) at 5-6 weeks old with similar start body weight were randomly assigned into the experimental and control groups. They were pre-fed ad libitum with basal complete pellet for a week under natural housing condition in the Spring (around 20°C-25°C). Because of blocks of blood catheters during the experiment, 2 animals were culled and data from them were excluded from statistics. There were 5 animals in each group throughout the whole experimental period.

Treatments

After 1 week pre-feeding, the animals in the experimental group were injected intro-muscularly with 0.5 mg daidzein emulsified in peanut-oil per kilogram start body weight at the 1st day. The injection volume was 2 ml. The same treatment was repeated once every 3 days continuously for 8 injections. The control animals received...
the injection only with same volume of peanut oil. All the animals were weighted with normal scale (minimum measurement 100 g) at 08:00 h before the morning feeding on days 1, 14, 28.

**Blood sampling**

On day 15 after the treatments, all the animals were anesthetized and an indwelling silicone catheter was inserted surgically into the right jugular vein of each animal, and maintained in the normal way. Because of block of the catheters, 2 animals were culled and all the data from them were excluded from statistics. At 09:00-12:00 h on days 18, 21 and 25, 3 blood samples at 1 h interval were obtained from all the animals and sera were separated and stored in -20°C for IGF-I assay. For analysis of blood parameters, 2 ml blood sample from each animal was collected into the sterilized tubes infused with heparin between 09:00-10:00 hour on days 20, 22, 24, 26 and 28, respectively. All the animals were killed through jugular artery bleeding and the thymus and spleen were surgically separated and weighted with electric scale (minimum detection 0.1 mg).

**Blood preparation for IGF-I assay**

For removing IGF-binding proteins, IGF-I was extracted from the blood with acid-ethanol extraction solvents (21.6 ml concentrated hydrochloric acid+103.4 ml de-ionic H2O+875 ml ethanol, AEES) according to Breier et al. (1991): 0.1 ml serum sample and 0.9 ml AEES were incubated at the room temperature for 30 min, then centrifuged at 1,500 rpm, 4°C for 30 min. 0.2 ml supernatant with 0.2 ml Tris Base (103.54 g Tris dissolved into 1 L de-ionic water, 0.855 M, pH 11.0) was re-incubated at the room temperature for 30 min, and then centrifuged again in the same condition. Finally 0.2 ml supernatant was taken and mixed with 0.8 ml PBS, stored in 2-8°C for IGF-I assay within 2 weeks.

**IGF-I RIA** : Determined in duplicate in the Key Laboratory of Agricultural Ministry for Animal Physiology and Biochemistry in Nanjing Agricultural University according to the literatures (Breier et al., 1991; Bauer and Parvizi, 1996, modified). IGF-I standard and IGF-I antibody were kindly provided by professor N. Parvizi in Institut fuer Tierzucht und Tierverhalten, FAL, Germany. 125I-IGF-I (tracers) was labeled in Shanghai Institute of Biotechnology. To detect IGF-I, 100 µl IGF-I standards or extracted sample, 100 µl IGF-I antibody, 100 µl tracer and 200 µl assay buffer were incubated at the room temperature for 18 h. Then 100 µl 2nd antibody were added into each tube and incubated for another 18 h before centrifugation at 4°C, 3,500 rpm for 20 min. Radio activities were detected with γ-counter. The minimum detection rate was 0.06 ng/ml, intra- and interassay coefficients of variation were 4.8% and 8.2%.

**Red blood cell count (RBC) and white blood cell count (WBC, Zhu, 1992)**

In brief, heparin-infused blood was diluted with saline and the suspension of the blood cells was pipetted to blood cell counting meter, the number was counted under the microscope and calculated according to the dilution rate.

**Peripheral T-lymphocytes counting**

ANAE Complex Contrast Dying Method (Jiang and Chen, 1983; Tao and Zhang, 1993) : 1 drop of heparin-infused blood was smeared on glass slides, wind-dried and dyed in ANAE dye for 3 h. The slides were then washed and re-dyed in ANAE dye for 2 h, washed and dried. The lymphocytes were counted under the microscope according to the color of nuclear granules. The cells with red-brown granules were defined ANAE positive cells (T-lymphocytes), and the other cells without colored granules defined ANAE negative cells (non-T-lymphocytes).

**Transformation of T-lymphocytes**

Micro Whole Blood Method (Yu et al., 1982) : 0.2 ml phytohemagglutinin (PHA, Sigma) was mixed with 4 ml 199 cell culture medium (CCM) in the sterilized tubes before 0.5 ml heparin-infused blood was pipetted into them. The tubes were then tightly capped, shaken for several times and incubated at 37°C for 72 h. During the incubation period, the tubes were also shaken up once a day. After incubation, they were centrifuged at 2,000 rpm for 15 min and the supernatant was decanted. The cells were re-suspended with 2 ml 199 CCM and re-centrifuged at the same condition as before. The supernatant was decanted and smeared glass slides were prepared in duplicate with the sediments, dried in air and dyed with Giemsa-Ruite. The cells were then counted under the microscope. All T-lymphocytes (ATL) were defined into 4 different stages according to their color, shape and nuclear: mature lymphocytes (ML), transferring lymphocytes (TL), lymphoblasts (LB) and reticular lymphocytes (RL). Totally 200 cells were counted in each slide and the transformation rate was calculated according to following formula:

\[ \text{T-lymphocytes transformation rate}=\frac{(\text{TL}+\text{LB})}{\text{ATL}}\times 100\% \]

**Statistics**

All data was analyzed by t-test in SAS, and expressed in mean±standard difference (x±SD). p<0.05 was defined significant and p<0.01 very significant.

**RESULTS**

**Effect of daidzein on the growth and serum IGF-I level**

The body weight and average daily gain (ADG) were not significantly different between the experimental and
control groups from days 1-14 and 1-28. But ADG between days 14-28 was higher in the experimental group than in the control (p<0.05, table 1). IGF-I levels in the experimental group were 20.53% (p<0.05), 15.92% (p<0.05) and 23.46% (p<0.05) higher than in the control group on day 18, 21 and 25, respectively (table 2).

**Effect of daidzein on the weights of thymus and spleen**

The weights of thymus and spleen in the experimental group on day 28 after the treatment were 15.83% and 15.42% higher than in the control group but without significant differences. The ratios of the weight of thymus and spleen to the body weight were also not different (p>0.05, table 3).

**Effect of daidzein on RBC and WBC**

There was no significant difference in the number of red blood cells between the experimental and control groups (p>0.05). The white blood cell count in the experimental group reached the apex on day 24, significantly higher than in the control and also higher than its own levels on day 20, 22 (p<0.05 or p<0.01). They remained in higher levels on days 26 and 28 (p=0.058, figure 1).

**Effect of daidzein on the ratios of T-lymphocytes and T-lymphocyte transformation**

The percentage of T-lymphocyte to all the white blood cells in the experimental group rose steadily from day 22 after the injection with daidzein, which was coordinated with the changes of WBC and significantly higher than those in the control on day 24, 26 (p<0.01 or p<0.05). The ratio of T-lymphocyte transformation did not significantly vary between the groups at different sampling time (figures 2 and 3).

**DISCUSSION**

The rising interest in daidzein in animal production is most probably based on the hope that it might be used as feed additive for improving growth performance of meat animals because it has weak estrogenic effect but has no residuary problem, although it may have adverse impacts on reproduction (Lundh, 1995; Whitten et al., 1995; Han, 1999; Bayer et al., 2001). Some studies presented positive results that daidzein promoted animal growth rates (Wang and Han, 1994; Han, 1999; Payne et al., 2001), gave a higher birth weight in male piglets by enhancing fetal growth (Ren et al., 2001). This study also exhibited some stimulatory effect of daidzein on the growth, probably by its weak estrogenic character, which could compensate the deficiency of estrogen in premature male pig. But it seems that the male piglets could react only at later stages of the treatment. Since we used premature pigs to minimize the effect of gonadal steroids and only one single dose in the study, the rules of daidzein on growth performance in male pigs in other doses or other growth periods remain to be known.

Animal growth is not only controlled by somatotrophic axis, but also mediated by IGF-I and some other mediates (Breier et al., 1988; Bauer and Parvizi, 1996). Daidzein could elevate GH and/or IGF-I levels in the serum or colostrum of sow, sheep or rat (Zhang et al., 1995; Liu, 1996; Han, 1999). Ren et al. reported that daidzein stimulated fetal growth and promoted the IGF-I receptor gene expression in male pigs (Ren et al., 2001). This study proved that daidzein could increase serum IGF-I level at certain stage, which was also correspondingly agreed with ascendant average daily gain of the piglets between days 14-28. However, we could only cautiously conclude the positive correlation of IGF-I to male growth since this study only tested the response of premature pigs at single dose and in short period of the growth. We didn’t either detect IGF-I level at early stages of the treatment, i.e. between day 1 and day 17.

Another reason why daidzein highlighted the research fields is because of its potential inhibition to some estrogen-related cancers, cardiac-vascular diseases and other metabolic problems in human beings, which may be associated with improvement of the immune function. Daidzein could enhance the phagocytosis of ventral macrophages, peripheral blood lymphocyte transformation induced by PHA in mouse, IL-2 and IL-3 in vitro secretion by T-lymphocytes induced by concanavalin A (ConA), and in vitro multiplication of spleen lymphocytes induced by ConA (Zhang et al., 1993; Wang et al., 1997; Wang, 2000). It also has anti-inflammatory and anti-allergic activities (Chang et al., 2000). This preliminary study showed that daidzein had no influence in the counting of total erythrocytes, but did affect the counting of total leucocytes. After the treatment, the numbers of leucocytes tended to rise, so did T-lymphocytes although transformation rate of T-lymphocytes did not demonstrate significant changes. These results seemed to suggest that

<table>
<thead>
<tr>
<th>Table 1. Effect of daidzein on body weight and average daily gain in the piglets</th>
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<tr>
<td>Group</td>
</tr>
<tr>
<td></td>
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<tr>
<td>Control (n=5)</td>
</tr>
<tr>
<td>Experiment (n=5)</td>
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</tbody>
</table>

* p<0.05 vs. the control at the same period, d-day.
EFFECTS OF DAIDZEIN ON PIGLETS

Daidzein could stimulate the leucocyte-genesis, especially T-lymphocytes-genesis, which was resulted not from the changes of development of thymus but presumably from the increasing of cytogenesis ability. This study did not prove the differences of transformation rate of T-lymphocytes between the daidzein treated group and the control, which suggested that daidzein in this case could not affect the maturity and function of T-lymphocytes. But it gave an evidence that daidzein do affect cellular immune system.

Table 2. Effect of daidzein on serum IGF-I level in the piglets

<table>
<thead>
<tr>
<th>Group</th>
<th>18 d IGF-I level (ng/ml serum)</th>
<th>21 d IGF-I level (ng/ml serum)</th>
<th>25 d IGF-I level (ng/ml serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>141.39±18.25</td>
<td>145.48±11.72</td>
<td>143.86±10.78</td>
</tr>
<tr>
<td>Experiment</td>
<td>170.42±21.95*</td>
<td>168.64±24.18</td>
<td>177.61±19.24*</td>
</tr>
</tbody>
</table>

(n=5) * p<0.05 vs. the control at the same period, d-day.

Table 3. Effects of daidzein on the weights of thymus and spleen in the piglets

<table>
<thead>
<tr>
<th>Group</th>
<th>Thymus wt. (g)</th>
<th>Thymus/BW (g/kg)</th>
<th>Spleen wt. (g)</th>
<th>Spleen/BW (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18.57±5.98</td>
<td>1.01±0.24</td>
<td>49.53±17.37</td>
<td>2.88±1.51</td>
</tr>
<tr>
<td>Experiment</td>
<td>21.51±6.40</td>
<td>1.08±0.26</td>
<td>57.17±27.64</td>
<td>3.00±1.67</td>
</tr>
</tbody>
</table>

(n=5) wt.-Weight, BW-Body weight, Thymus/BW and Spleen/BW refer to ratios of weights of thymus and spleen to body weight.

Figure 1. Effect of Daidzein on RBC and WBC in the piglets.

Figure 2. Effect of daidzein on percentage of T-lymphocyte in the piglets.

Figure 3. Effect of daidzein on the transformation ratio of T-lymphocyte (T-LTR) in the piglets.
although the mechanism of this role needs further explanation, which may inspire further interests in the field.

REFERENCES


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