

Effects of Daidzein on Body Weight Gain, Serum IGF-I Level and Cellular Immune Function in Intact Male Piglets

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ABSTRACT : 10 male piglets at 5-6 weeks old with similar body weight (BW) were randomly assigned into the experimental (EXP) and control (CON) groups. The animals in EXP received intro-muscular injection with daidzein (DA) at the dose of 0.5 mg DA per kg start BW on day 1. The same procedures were repeated once every 3 days for eight times. The animals in CON received the injection only with same volume of control peanut oil. The animals were weighted on day 14 and 28 and the blood samples were obtained at different stages of the treatment for determining IGF-I levels and blood parameters. At the end of the experiment, the thymus and spleen from all the animals were surgically taken out and weighted. The results showed that BW and average daily gain (ADG) were not significantly different between the groups in term of the whole period, but ADG between days 14-28 was higher in EXP than in CON ($p < 0.05$). On days 18, 21 and 25, IGF-I levels in EXP group were 20.53% ($p < 0.05$), 15.92% ($p > 0.05$) and 23.46% ($p < 0.05$), respectively, higher than those in CON. The weights of thymus and spleen, the ratios of their weights to BW and red blood count (RBC) did not significantly differ between the groups at all stages. White blood count (WBC) in EXP steadily increased from day 22, reached its apex on day 24, which was higher than in CON ($p < 0.05$) and its own levels on day 20 and 22 ($p < 0.01$ or $p < 0.05$), and remained higher on the later time ($p = 0.058$). The results of percentage of T-Lymphocytes also demonstrated similar trend to WBC, but T-Lymphocyte transformation rate (%) appeared no significant change between the groups. In conclusion, Daidzein could stimulate male piglet growth and elevate serum IGF-I levels at certain stages of the treatment. It could also increase WBC and T-Lymphocyte rates, but had no significant impacts on RBC and T-Lymphocyte transformation rate. (*Asian-Aust. J. Anim. Sci.* 2002. Vol 15, No. 7 : 1066-1070)

Key Words : Daidzein, IGF-I, T-Lymphocytes, Male Piglets

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 secretory 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 cardiovascular 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

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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:

T-lymphocytes transformation rate=(TL+LB)/ATL×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 mediators (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 concanavallin 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 1. Effect of daidzein on body weight and average daily gain in the piglets

Group	Body weight (kg)			Average daily gain (g)		
	1 d	14 d	28 d	1-14 d	1-28 d	14-28 d
Control (n=5)	9.39±0.99	14.05±0.73	19.7±0.26	333±23	368±28	404±27
Experiment (n=5)	9.86±0.89	14.95±1.04	21.2±1.30	364±48	405±38	446±39*

* $p < 0.05$ vs. the control at the same period, d-day.

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

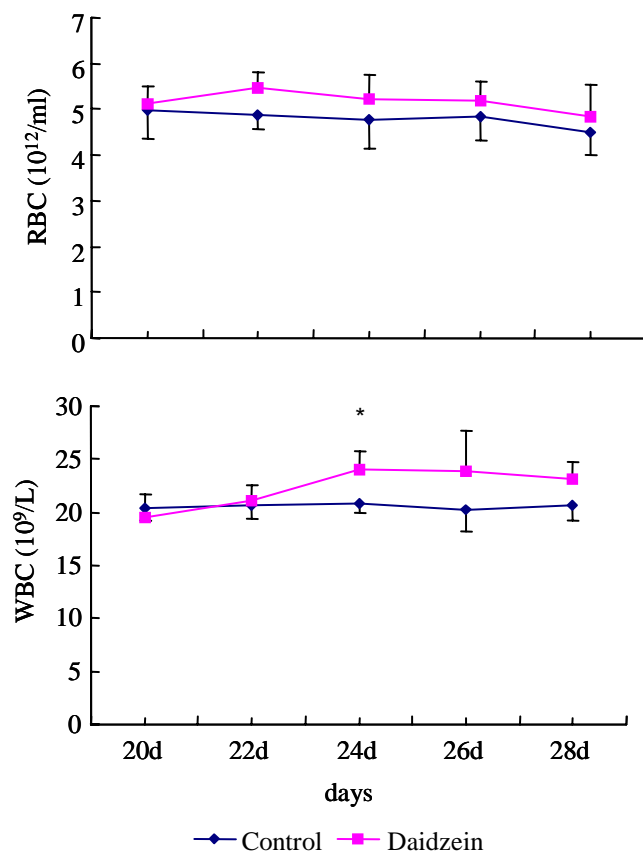
Group	IGF-I level (ng/ml serum)		
	18 d	21 d	25 d
Control (n=5)	141.39±18.25	145.48±11.72	143.86±10.78
Experiment (n=5)	170.42±21.95*	168.64±24.18	177.61±19.24*

* p<0.05 vs. the control at the same period, d-day.

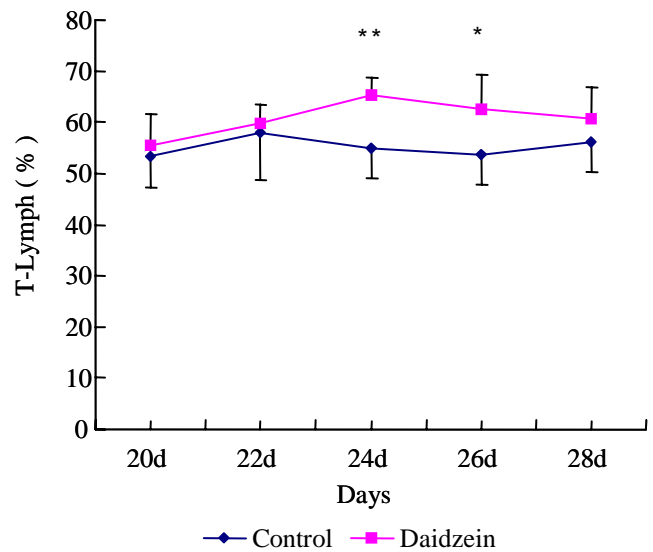
Table 3. Effects of daidzein on the weights of thymus and spleen in the piglets

Group	Thymus wt. (g)	Thymus/BW (g/kg)	Spleen wt. (g)	Spleen/BW (g/kg)
Control (n=5)	18.57±5.98	1.01±0.24	49.53±17.37	2.88±1.51
Experiment (n=5)	21.51±6.40	1.08±0.26	57.17±27.64	3.00±1.67

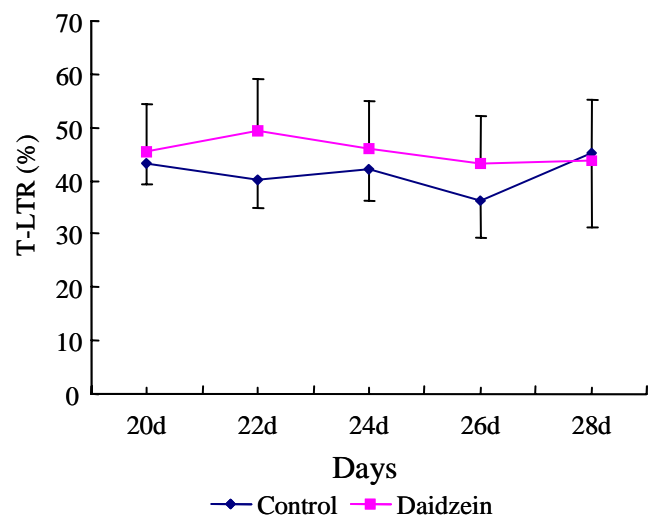
wt.-Weight, BW-Body weight, Thymus/BW and Spleen/BW refer to ratios of weights of thymus and spleen to body weight.



*Daidzein=the experimental group; Control=the control group.

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

*Daidzein=the experimental group; Control=the control group.

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

*Daidzein=the experimental group; Control=the control group

Figure 3. Effect of daidzein on the transformation ratio of T-lymphocyte (T-LTR) in the piglets.

daidzein could stimulate the leucocyte-gensis, especially T-lymphocytes-gensis, 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

although the mechanism of this role needs further explanation, which may inspire further interests in the field.

REFERENCES

- Bauer, M. and N. Parvizi. 1996. Pulsatile and diurnal secretion of GH and IGF-I in the chronically catheterized-pig fetus. *J. Endocrinol.* 149:125-133.
- Bayer, T., T. Colnot and W. Dekant. 2001. Disposition and biotransformation of the estrogenic isoflavone daidzein in rats. *Toxicol. Sci.* 62:205-211.
- Breier, B. H., B. W. Gallaher and P. D. Gluckman. 1991. Radioimmunoassay for insulin-like growth factor-I: solutions to some potential problems and pitfalls. *J. Endocrinol.* 128:347-357.
- Breier, B. H., P. D. Gluckman and J. J. Bass. 1988. The somatotrophic axis in young steers: influence of nutritional status and estradiol-17 β on hepatic high and low-affinity somatotrophic binding sites. *J. Endocr.* 116:169-177.
- Chang, C. Y., L. J. Huang, J. P. Wang, C. M. Teng, S. C. Chen and S. C. Kuo. 2000. Synthesis and anti-platelet, anti-inflammatory and anti-allergic activities of methoxyisoflavanquinone and related compounds. *Chem. Pharm. Bull (Tokyo).* 48:964-973.
- Clifton-Bligh, P. B., R. J. Baber, G. R. Fulcher, M. L. Nery and T. Moreton. 2001. The effect of isoflavones extracted from red clover (Rimostil) on lipid and bone metabolism. *Menopause.* 8:259-265.
- Han Zhengkang. 1999. An isoflavone phytoestrogen-daidzein: its effect on growth and some endocrine in male animals. *Animal Husb. Vet. Med.* 31:1-2.
- Jiang Shibo and Chen Zehong. 1983. Complex Contrast Dying Method of T lymphocytes ANAE. *Chinese J. Medi. Tests.* 16:234-237.
- Kaladas, R. S. 1989. Reproduction and general metabolic effects of phytoestrogens in mammals. *Reproductive Toxicology Review.* 3:81-89.
- Kelly, K. W. 1988. Cross-talk between the immune and endocrine system. *J. Anim. Sci.* 66:2095-2108.
- Liu, G. T. 1996. Isoflavone-daidzein: its effect on animal growth and lactation and neuro-endocrine mechanism involved. Ph.D. dissertation, Nanjing Agricultural University.
- Lundh, T. 1995. Metabolism of estrogenic isoflavones in domestic animals. *Proc. Soc. Exp. Bio. Med.* 208:33-39.
- Mitchell, J. H., E. Cawood, D. Kinniburgh, A. Provan, A. R. Collins and D. S. Irvine. 2001. Effect of a phytoestrogen food supplement on reproductive health in normal males. *Clin. Sci.* 100:613-618.
- Nwannenna, A. I., T. J. Lundh, A. Madej, G. Fredriksson and G. Bjornhag. 1995. Clinical changes in ovariectomized ewes exposed to phytoestrogens and 17 β -estradiol implants. *Proc. Soc. Exp. Bio. Med.* 208:92-97.
- Odum, J., H. Tinwell, K. Jones, J. P. Van Miller, R. L. Joiner, G. Tobin, H. Kawasaki, R. Deghenghi and J. Ashby. 2001. Effect of rodent diets on the sexual development of the rat. *Toxicol. Sci.* 61:115-127.
- Parvizi, N., G. L. Wang and El-Bolking. 1998. Effects of an estrogenic and a non-estrogenic xenobiotic on LH secretion and reproductive performance in the pig and in the rat. *Reprod. Dom. Anim.* 33:209-211.
- Payne, R. L., T. D. Bidner, L. L. Southern and J. P. Geaghan. 2001. Effects of dietary soy isoflavones on growth, carcass traits, and meat quality in growing-finishing pigs. *J. Anim. Sci.* 79:1230-1239.
- Ren, M. Q., G. Kuhn, J. Wegner, G. Nurnberg, J. Chen and K. Ender. 2001. Feeding daidzein to late pregnant sows influences the estrogen receptor beta and type 1 insulin-like growth factor receptor mRNA expression in newborn piglets. *J. Endocrinol.* 170:129-135.
- Setchell, K. D. and A. Cassidy. 1999. Dietary isoflavones: Biological effects and relevance to human health. *J. Nutr.* 129:758S-767S.
- Tao Yixun and Zhang Gusheng (chief editors). 1993. *Clinical Immune Tests.* Shanghai Science & Technology Publishing House. 1st edition: 44-47.
- Wang Genlin, Chen Jie, Chen Weihua and Han Zhaoyu. 1999. Effect of daidzein on secretion of GnRH, LH and inhibin in gilts. *J. Jiangsu Agric. Sci.* 15:26-29.
- Wang, G. L., Z. H. Zhao, W. H. Chen and J. Chen. 2000. Effect of daidzein intramuscular injection on GnRH receptor binding in the pituitary in the piglets. *J. Jiangsu. Agric. Sci.* 16:233-236.
- Wang Guojie and Han Zhengkang. 1994. Effects of red-clover isoflavones on growth and serum testosterone in male chicken. *Res. Anim. Sci.* 15:65-69.
- Wang Hui. 2000. Functional regulation of T-lymphocytes by growth hormone. *Chinese J. Immuno.* 16:480-484.
- Wang, W., C. M. Higuchi and R. Zhang. 1997. Individual and combinatory effects of soy isoflavones on the in vitro potentiation of lymphocyte activation. *Nutr. Cancer.* 29:29-34.
- Weber, K. S., N. A. Jacobson, K. D. Setchell and E. D. Lephart. 1999. Brain aromatase and 5 α -reductase, regulatory behaviors and testosterone levers in adult rats on phytoestrogen diet. *Pro. Soc. Exp. Bio. Med.* 221:131-135.
- Weber, K. S., K. D. Setchell, D. M. Stocco and E. D. Lephart. 2001. Dietary soy-phytoestrogens decrease testosterone levels and prostate weight without altering LH, prostate 5 α -reductase or testicular steroidogenic acute regulatory peptide levels in adult male Sprague-Dawley rats. *J. Endocrinol.* 170:591-599.
- Whitten, P. L., C. Lewis, E. Russell and F. Naftolin. 1995. Potential adverse effects of phytoestrogens. *J. Nutr.* 125:771S-776.
- Wojtowicz, S. P. Reversal of tumor-induced immunosuppression: a new approach to cancer therapy. *J. Immunotherapy.* 1997, 20:165.
- Yan, X. H., J. F. Gu and C. P. Sun. 1997. Progress in anticancer mechanisms of soybean isoflavones. *Sheng Li Ke Xue Jin Zhan (Advance of Physiology)* in Chinese. 28:362-364.
- Yu Huo, Xie Shaowen and Yang Guizhen (chief editors). 1982. *Clinical Immune Techniques.* Shanghai Science & Technology Publishing House. 1st edition:314-320.
- Zhang Rongqing, Han Zhengkang, Chen Jie. 1993. Impact of isoflavone phytoestrogen on immune function in the mouse. *J. Nanjing Agricultural University.* 16:64-68.
- Zhang Rongqing, Han Zhengkang and Chen Jie. 1995. Effect of daidzein on immune function and levels of serum and colostrum GH, PRL and SS in sows. *J. Zoology.* 41:201-206.
- Zhu Zhongyong (chief editor). 1992. *Practical Medical Test.* Renmin Military Surgeon Press. 1st edition:6-12.

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