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ORIGINAL ARTICLE

The developmental effects of isoflavone aglycone administration on early chick embryos

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ABSTRACT

Soybeans contain the isoflavone aglycone, an endocrine disrupter. To determine the effects of small amounts of isoflavones on developmental processes, we administered 6.25, 62.5, or 625 µg isoflavone per egg to early stage (stage 10) developing chick embryos via the yolk just beneath the embryo. Eggs were kept at 37±0.5 °C and >80% relative humidity, with one rotation per hour for 48 hrs. The embryos were observed under a stereomicroscope for morphological abnormalities and number of somites. Relative to control eggs, there were no significant differences in the average number of somites in eggs administered isoflavone aglycone. Isoflavone, however, had a dose associated effect on abnormal embryogenesis. Embryos treated with isoflavone aglycone showed developmental arrest not reaching somitegenesis, dysmorphology of the neural tube, and shortening of entire embryos.

KEY WORDS: isoflavone aglycone; estrogen receptor; chick embryo; somites; abnormality

Introduction

The Japanese population consumes large quantities of soy products, including tofu, miso, and fermented soybeans, which contain isoflavones. In women, soy isoflavone is associated with reduced incidence of breast cancer and menopausal disorders (Kokubo *et al.,* 2007; Iwasaki *et al.,* 2008; Messina *et al.,* 2006; Nagata, 2010).

Isoflavone is a flavonoid classified as a polyphenol and is present in many leguminous plants, including soybeans. Isoflavone aglycone contains genistein, daidzein, and glycitin, and glycosides include daidzin, glycitin, and genistin. The effects of isoflavone are similar to those of estrogen (Kroon *et al.,* 2004). Components of isoflavone, called phytoestrogens, bind to estrogen receptors and act as an estrogen agonist. Soy isoflavone has been reported effective in reducing menopausal disorders and type 2 diabetes mellitus (Anderson *et al.,* 1999; Jayagopal *et al.,* 2002).

Estrogen has profound effects on menstruation and pregnancy. Following menopause, the secretion of estrogen decreases. This reduction, resulting in a lack of estrogen binding to osteoblasts and an increase in activity of osteoclasts, may frequently lead to the development of osteoporosis in postmenopausal women. Since estrogen acts to regulate the amount of calcium in bone (Popat *et al.,* 2009; Lacroix *et al.,* 2008; Hisa *et al.,* 2008), the phytoestrogen isoflavone may be useful for maintaining the health of postmenopausal women (Mann *et al.,* 2007; Reinwald *et al.,* 2006).

In recent years, the consumption of soybean containing foods has decreased in Japan. Many dietary supplements in Japan include soy isoflavone. It is unclear, however, whether the consumption of isoflavone in dietary supplements is equivalent to its consumption in soy containing foods. Although the biological effects of isoflavone have been studied in Japan, a clear conclusion has not been reached. The Japanese Food Safety Commission recommended an upper limit of soy isoflavone aglycones of 70–75 mg/day (The Japanese Food Safety Commission, 2006) . The effects of these large quantities of isoflavone on embryo development are not known. We have developed a toxic experimental method using early chick embryos (Saito *et al.,* 2012). It thus seems that attention should be paid to the teratogenicity of isoflavone, which is of emphasized merit. We therefore assessed the biological effects of the isoflavone aglycone administration on early chick embryos.

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Materials and methods

The Japanese Food Safety Commission has established an upper limit of soy isoflavone aglycone as 75 mg/day, or 1.25 µg/g. The average weight of a fertilized egg is 50 g, thus the basic quantity of isoflavone administered per egg was 62.5 µg.

Isoflavone aglycones were purchased from Wako Pure Chemical Co. Ltd (Tokyo). Stock solutions of 125 µg/µl were prepared in dimethyl sulfoxide (DMSO) and diluted into phosphate buffered saline (PBS, pH7.0, Wako) to concentrations of 12.5 µg/µl and 1.25 µg/µl.

White Leghorn eggs, fertilized within 24 hrs after laying, were purchased from the Nippon Institute for Biological Science. Twelve eggs were used in the control group or the experimental (isoflavon administered) group. Using a Hamilton syringe, we injected each egg yolk with 5µl isoflavone aglycones (125, 12.5 or 1.25 µg/µl) or DMSO, also diluted in PBS (negative control), through a pin hole made on the shell. Therefore, the amount of isoflavone aglycone administered per embryo was 0, 6.25, 62.5 or 625 µg. It was difficult to define the exact site of administration relative to the embryo, except that it was just underneath the embryo. A hole 2 cm in diameter was cut into each shell to allow to visualize the embryos at stage 10, and sealed with cover silicon gum (KE3475T, Shin-Etsu Chemical Co. Ltd., Tokyo) adhesive with polyethylene film. The eggs were placed in the incubator (model 05, Showa Furanki Ltd.) and incubated for 48 hr at 37±0.5 °C and >80% relative humidity, with one rotation per hr.

The embryos were subsequently excised from the shells and fixed in 70% alcohol for >4 days. The external appearance of each embryo was examined under a stereomicroscope (S8APO, Leica, Germany) and the number of somites was counted. Developmental stage was scored as described (Hamburger *et al.,* 1951).

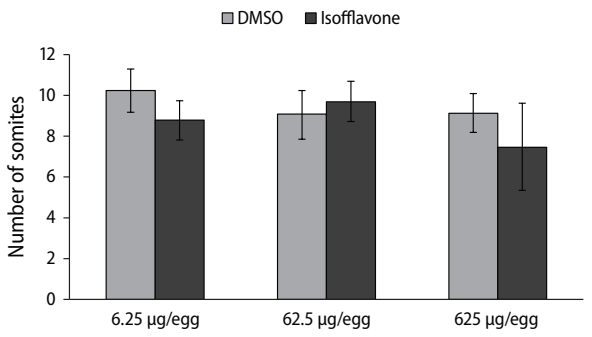

Figure 1. Mean±SEM number of somites in chick embryos treated with isoflavone. The numbers of somites in embryos administered 6.25 µg isoflavone and controls were 9.33±0.94 (n=12) and 10.31±1.02 (n=13), respectively; the numbers in embryos administered 62.5µg isoflavone and their controls were 9.71±1.00 (n=14) and 9.10±1.24 (n=10), respectively; and the numbers in embryos administered 625 µg isoflavone and their controls were 10.20±0.96 (n=10) and 9.27±1.56 (n=11), respectively. None of these differences was statistically significant.

Statistical analysis

The mean numbers of somites in groups of embryos were compared using Student's t test and the incidence of abnormalities was compared using the chi square test. Statistical significance was defined as $p < 0.01$ or $p < 0.05$.

Results

The mean ± SEM number of somites was similar in embryos treated with the 3 concentrations of isoflavone and each set of control embryos (Figure 1). While control chick embryos developed normally (*e.g.* Figure 2), those treated with isoflavone aglycone were malformed, with end neural tube dysmorphology and cylindrical heads (*e.g.* Figure 3). Stasimorphia chick embryos were also observed, with a head and neural tube, but no somite (Figure 4). Other embryos showed head dysplasia and end neural tube dysmorphology (Figure 5). Some embryos showed heteroplasia of the head and neural tube dysmorphology, with an open end of the neural tube (Figure 6) or a plate-shaped head (Figure 7).

Altogether, malformations were observed in 58.3%, 71.4%, and 90.9% of embryos administered 6.25 µg, 62.5 µg and 625 µg isoflavone, respectively (Figure 8). In comparison, malformations were observed in 15.4%, 20%, and 0% of embryos administered 1:10, 1:100, and 1:1000 fold dilutions of DMSO, respectively. The incidence of malformations was significantly higher in each of the isoflavone group than in its control group ($p<0.05$).

Discussion

The female sex hormones estrogen and progesterone are secreted periodically and maintain various physical functions. Isoflavones are phytoestrogens that interact with estrogen receptors and function as weak estrogens. Genistein binds more strongly to estrogen receptors than daidzein. Imbalances in female sex hormone may result in various disorders.

The Japanese Food Safety Commission has set the upper limit of intake of soy isoflavone aglycones as 70–75 mg/day, the Italian Ministry of Health recommended that isoflavone supplements should not exceed 80mg/day, and the French Food Safety Agency set the quantity of isoflavone aglycone at 1 mg/kg/day (Setchell *et al.,* 1997). The risks of phytoestrogen have also been assessed by the U.S. FDA (Food and Drug Administration) and the AHRQ (Agency for Healthcare Research and Quality).

The effects of soy isoflavone have been assessed in fetuses and babies. The concentrations of plasma genistein and daidzein were significantly higher in 4-month-old babies who consumed 900–1000 ml soybean milk than in 4-month-old babies who consumed cow's milk and breast milk (Xu *et al.,* 1994). The concentration of soy isoflavone is lower in the breast milk of a woman who eats soybean containing foods than in soybean. As yet, however, there are no guidelines related to the safe consumption for

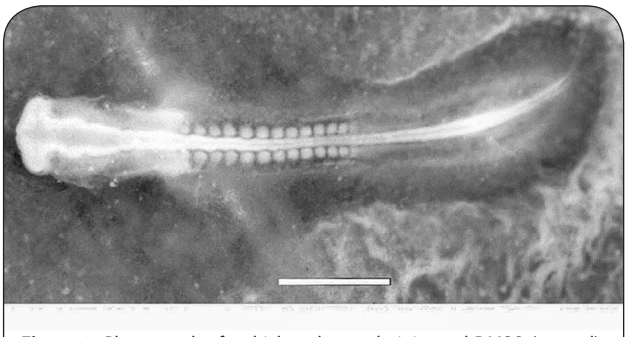

Figure 2. Photograph of a chick embryo administered DMSO (control), showing normal development. A white bar represents 1 mm.

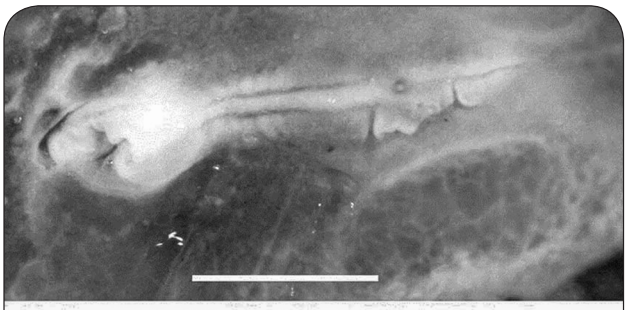

Figure 3. Photograph of a chick embryo administered 6.25 µg isoflavone aglycone, showing heteroplasia of the head and neural tube dysmorphology. A white bar represents 1 mm.

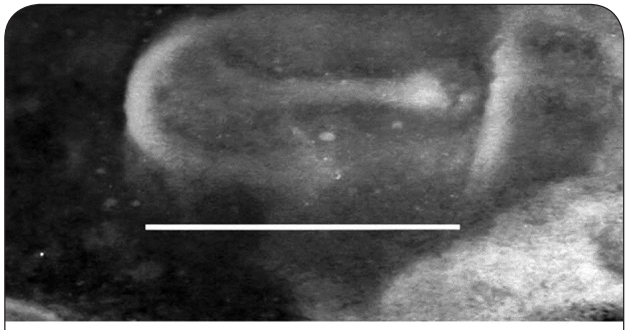

Figure 4. Photograph of a chick embryo administered 625 µg isoflavone aglycone, showing neural tube dysmorphology. A white bar represents 1 mm.

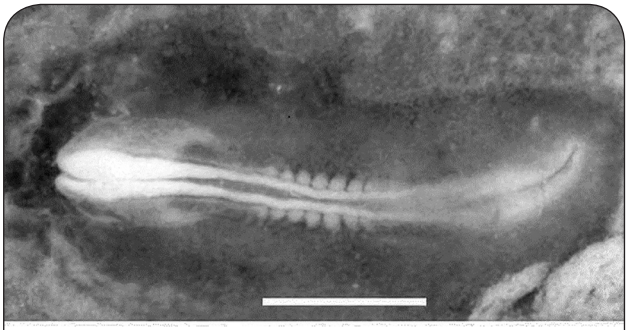

Figure 5. Photograph of a chick embryo administered 625µg isoflavone aglycone, showing heteroplasia of the head and neural tube dysmorphology. A white bar represents 1 mm.

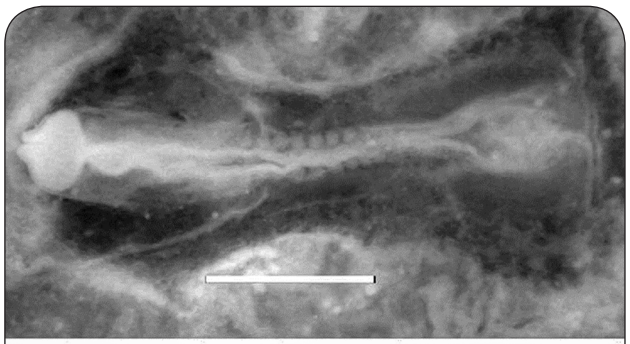

Figure 6. Photograph of a chick embryo administered 625µg isoflavone aglycone, showing heteroplasia of the head and neural tube dysmorphology. A white bar represents 1 mm.

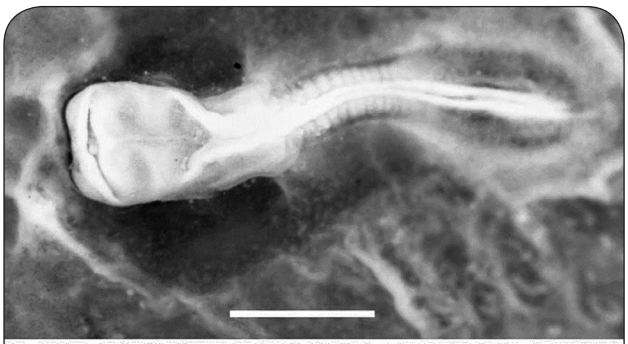

Figure 7. Photograph of a chick embryo administered 625µg isoflavone aglycone, showing heteroplasia of the head and neural tube dysmorphology. A white bar represents 1 mm.

infants of soy isoflavone in soybean milk (Chen *et al.,* 2004). Moreover, the effects of maternal consumption of soy isoflavone on the estrogen synthesis pathway in fetuses have not yet been determined.

Among the soy isoflavone aglycones are genistein, daidzein and glycitin. Genistein treatment resulted in malformation of zebrafish embryos, including spinal kyphosis (Kim *et al.,* 2009), suggesting that excess genistein is a teratogen in exposed zebrafish embryos. Female mice treated neonatally with genistein (50 mg/kg/day) have ovarian follicles and are infertile even after superovulation (Jefferson *et al.,* 2009).

Exposure of pregnant animals to high concentrations of soy isoflavone aglycones may have deleterious effects on fetal health (Newbold *et al.,* 2001; Wisniewski *et al.,* 2003).

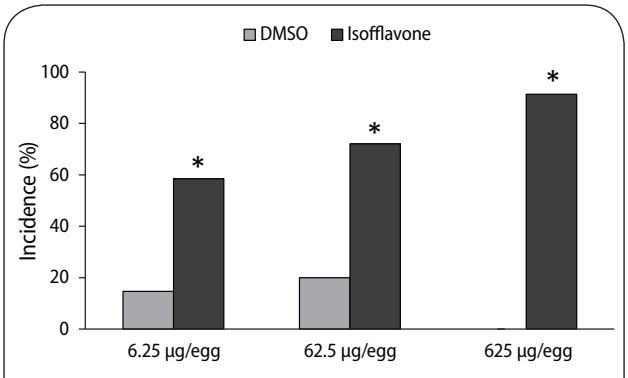

Figure 8. Incidence of abnormalities in chick embryos administered 6.25, 62.5, or 625 µg isoflavone aglycone or DMSO. *$p<0.05$ compared with DMSO by the chi-square test.

The results of animal experiments cannot be extrapolated to human fetuses. To date, little is known about risks to fetuses from maternal consumption of large amounts of soy isoflavone aglycones.

We found that administration of isoflavone aglycones influenced the development of early stage chick embryos. The incidence of embryo malformation was associated with the dose of isoflavone aglycones.

Conclusions

We observed that exposure of developing chick embryos to soy isoflavone aglycones resulted in adverse effects in a dose-responsive manner. Although the number of somites did not differ significantly between embryos administered isoflavone aglycones and control embryos, the incidence of malformations was significantly higher in groups administered each dose of isoflavone aglycones than in its respective control group. The abnormal embryogenesis associated with soy isoflavone aglycones included dysmorphology of the neural tube, heteroplasia of the head, and plate-shaped heads.

The presented study suggests that it may be desirable to restrict or prohibit administration of isoflavone at least for females in the reproductive generation.

It is eagerly awaited that the clinical etiology of teratogenicity of isoflavone will be elucidated in the future.

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