Anim. Reprod., v.5, n.3/4, p.83-89, Jul./Dec. 2008

Reproductive and toxicological effects of isoflavones on female offspring of rats exposed during pregnancy

V. Romero, C. Dela Cruz, O.C.M. Pereira[1]

Department of Pharmacology, Institute of Biosciences, São Paulo State University (UNESP), Botucatu, SP, Brazil.

Abstract

We investigated the actions of isoflavones (daidzin + daidzein 22.7%, daidzein 16.9%, genistin + genistein 16.3%, genistein 13.1%) in pregnant rats, assessing possible repercussions regarding the development of female conceptus. Doses of isoflavones, 10 mg/kg or 100 mg/kg, were administered to pregnant rats by oral gavage, from the sixth day of pregnancy until its completion. The pregnant rats exposed to isoflavones at these doses when laparotomized showed alterations in the number of live fetuses, lysed fetuses, number of resorption sites, and implantation sites. In the pregnant rats, no maternal toxicity was observed, despite presenting a significant anticipation of the offspring birth date. The rats belonging to the isoflavones 10 mg/kg and 100 mg/kg groups also gave birth to a smaller number of offspring. In female offspring, the parameters for presenting pelage, eyes opening, ears unfolding, and age for onset of puberty occurred early. At adult life, despite not observing any difference in the number of estruses, irregularities in estrous cycle including prolongation of the estrus phase in hours were observed in the group exposed to 10 mg/kg of isoflavones. These results indicate that isoflavones, despite presenting weak estrogenic activity, were able to interfere in sexual and bodily development, resulting in reproductive alterations. Extensive studies should be done, aiming to evaluate the possible effects of isoflavones in this critical development period.

Keywords: estrogenic activity, isoflavones, phytoestrogens, pregnancy, reproductive aspects.

Introduction

Soy isoflavone has structures similar to estrogen and have received much attention for the prevention of postmenopausal symptoms such as osteoporosis (Fujioka *et al*., 2004). Isoflavones are added to glucose, forming glucosides. In the soy germ, the main glycosides found are daidzein, genistein, and glycitein; the latter two being the most common, with genistein predominating, corresponding to 75% of the total. Daidzein, genistein, and equol are the main phytoestrogens found in the blood and urine of humans and animals (Morais and Silva, 2000). The biological effect of isoflavonoids varies according to the female biological phase. In pre-menopause, when the concentration of circulating hormones is high, the estrogen receptors are active and phytoestrogens compete for these sites. As they present less biological activity than the endogenous estrogen, the result is only a weak anti-estrogenic action. In post-menopausal females, the concentration of circulating endogenous estrogen is reduced by 60%; the receptors will be more available, favoring the weak estrogenic action of flavonoids (Chambô-Filho *et al*., 2000).

According to Clapauch *et al.* (2002), the estrogenic and anti-estrogenic properties of phytoestrogens depend on their concentration, the concentration of endogenous sex steroids, and on the specific target organ involved in the interaction with the estrogen receptors (ER). This effect can be explained through the existence of two types of ER: α and β. The ER-α are the main receptors found in the breast and the uterus, with ER-β predominating in the bone and cardiovascular system. Estradiol binds to both receptors, while the isoflavones are more selective for ER-β, in the proportion 1/20 for α, and 1/3 for β (Claupach *et al*., 2002).

Studies show that women who consume high quantities of products rich in soy isoflavones present a large concentration of isoflavone in their urine, reducing the risk and the probability of breast cancer (Duncan *et al.*, 1999). Epidemiological studies, such as the diet of asian countries, show that their diet with phytoestrogens propitiates protection against some forms of cancer, particularly those that are hormone-dependent, as the cancer of prostate (Adlercreutz *et al*., 2000). Thus, the consumption of isoflavones is also recommended for its possible cancer prevention properties. There is also evidence that isoflavones reduce the intensity and frequency of vasomotor symptoms in menopausal women. The majority of these observations regarding the use of phytoestrogens are epidemiologic, many of them based on studies done in regions where the consumption of soy is high. It has been noted that less than 20% of japanese women experienced hot flashes, compared with 80% of european women. This difference is attributed, in part, to the diet of japanese women, rich in soy derivatives (Anelli *et al.*, 2003). Soy contains the isoflavonoid phytoestrogen daidzin, that following metabolism to daidzein has a positive estrogenic effect on hot flashes which is immediate (Murkies *et al.*, 1995). In double-blind studies, with a placebo control, it was discovered that 60 g/day

[1]Corresponding author: pereira@ibb.unesp.br
Fax: +55(14)3815-3744
Received: November 12, 2007
Accepted: June 17, 2008

of isolated soy protein (food supplement) was superior to the placebo, reducing vasomotor symptoms by 50% (Albertazzi *et al.*, 1998). Other assays in human beings have registered that isoflavones, isolated or associate to the soy protein, attenuates the bone loss that occurs during and after menopause (Alekel *et al.*, 2000; Morabito *et al.*, 2002). Considering the advantages referenced above, the addition of isoflavones found in soy germ has been recommended for inclusion in the diet of both men and women. In this way, the development of products rich in soy derivatives would be an alternative to increase the presence of these substances in the diet. However, a great difficulty in modifying the dietary habits of western women is observed. In this way, one of the often-utilized methods is the administration of the active substance – isoflavones – taken orally. Although interpretation of the role of individual components of the diet is difficult from epidemiologic and dietary studies, it is recognized that there are many plant-derived bioactive non-nutrients as isoflavones that can confer significant health benefits (Setchell, 1998). Nevertheless, the occurrence of possible adverse side effects through continued use of isoflavones, because of its estrogenic activity, will continue to be measured and/or better studied. On the basis of these considerations, the aim of the present study was to determine the action of isoflavones (daidzin + daidzein 22.7%, daidzein 16.9%, genistin + genistein 16.3%, genistein 13.1%) in pregnant rats, assessing its repercussions regarding the development of female conceptus.

Materials and Methods

Animal maintenance

Wistar rats (Rattus norvegicus) were obtained from the stock of the University of Sagrado Coração and kept under controlled conditions under a constant 12 h light-dark cycle at room temperature around 25 $\pm$ 1ºC, humidity of 55 $\pm$ 5% and had free access to regular lab chow (estrogen-, soy-, and soy-derivative free) and tap water. This study was conducted in accordance to the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and was approved by the Institute of Biosciences/UNESP-Botucatu Ethical Committee for Animal Research (Protocol number: 037/05).

Substances and treatment

Isoflavones - dry extract compound with daidzin + daidzein 22.7%, daidzein 16.9%, genistin+genistein 16.3%, genistein 13.1% - were supplied by Galena Chemistry and Pharmaceutical Ltd. Brazil (São Paulo/SP). The chemical was dissolved in 50% dimethyl sulfoxide (DMSO) plus 50% propylene glycol (PPG). Female rats (around 100 days old) were mated overnight at the proportion of three females to each male. Vaginal smears were collected daily and examined for the presence of sperm. The day of sperm detection in vaginal smears was considered day 0 of pregnancy. The mating procedure was repeated until a sufficient number of pregnant rats were obtained (20/group). Thus, these pregnant female rats were randomly assigned to three groups, one of which served as a vehicle control (50% dimethyl sulfoxide – DMSO - plus 50% propylene glycol; PPG), while the other two were treated with two different dose-regimens of isoflavones (10 mg/Kg and 100 mg/Kg). The dams were treated daily by oral gavage with 10 mg/kg or 100 mg/kg of isoflavone beginning on the sixth day of gestation up to its conclusion, while the body weights were measured from day 0 to day 19 of pregnancy. The doses of 10 mg/kg or 100 mg/kg were contained in 0.15 ml of vehicle.

Exploratory laparotomy

Between days 19 and 20 of pregnancy, 20 rats from each group, Control, and Isoflavones were killed by an overdose of ketamine hydrochloride, followed by a cut of large cervical vas. Immediately after, they were laparotomized to analyze the ovaries and uterine horns. After removing the ovaries and uterine horns, the rate of pregnancy and the numbers of implantation sites, live fetuses, dead fetuses, resorption sites, and corpora lutea were recorded. When necessary, the technique for coloration of the uterine horns was used with the Salewsky reactant, a preparation of 10% ammonium sulfate (Salewsky, 1964), that allowed visualization of the occurrence of resorptions in the initial stages of implantation.

Maternal and reproductive outcome data

The female rats were monitored daily throughout pregnancy and lactation, always 10 rats/group. The remote possibility of clinical manifestation of intoxication such as salivation, tremors, and seizures were also monitored throughout the treatment period. Variables including length of pregnancy and litter size were also assessed. The pups were born naturally and left undisturbed together with their mothers until weaning, always 8 newborns/dam. The pups were culled to 5 females and 3 males to ensure the presence of both sexes in the litters.

Female offspring

After natural birth, the body mass of each female offspring was recorded on day 1, 7, 14, and 21 after birth. For each experimental group, a maximum of three female siblings was taken from each litter in order to avoid “litter effects”. Each pup was examined daily to record the variables of general development: the days on

which pelage appeared, ears unfolded, and eyes opened. From the day 30 after birth the female offspring were observed by looking at the time the vagina opened. The day of the complete vaginal ostium opening was then recorded and estimated as the age of puberty onset (Pereira *et al*., 1997; Marty *et al*., 1999; Piffer and Pereira, 2004).

Estrous cycle study

At the age of 75 days, the same female rats used for observation of puberty onset in both experimental groups were employed in a study of estrous cycle. The vaginal secretion was collected once a day, at the same hour, and smeared on a plate, and observed through an optical microscope. The characterization of each phase is based on the proportion among types of cells observed in the vaginal smear: epithelial cells, cornified cells, and leukocytes. Thus, the phases of the estrous cycle and the number of estruses observed were recorded for 15 consecutive days.

Statistical analyses

The results were analyzed prior to a two-way analysis of variance (ANOVA). Then, the Tukey-Kramer or Kruskal-Wallis-Dunn tests were employed, with the results considered significant if $P < 0.05$.

Results

Exploratory laparotomy

The pregnant rats exposed to 100 mg/kg of isoflavones showed a smaller number of corpora lutea; while those exposed to isoflavones at doses of 10 mg/kg and 100 mg/kg produced a smaller number of live fetuses. The number of fetuses in lyses was larger in the isoflavones 100 mg/kg group. The pregnant rats exposed to this dose also demonstrated a larger number of resorption sites and a smaller number of implantation sites (Table 1).

Table 1. Parameters of exploratory laparotomy: number of corpora lutea, live fetuses, lysed fetuses, resorption sites, and implantation sites.

Parameters in numbers	Groups		
	Control (n = 20)	Isoflavone 10 mg/kg (n = 20)	Isoflavone 100 mg/kg (n = 20)
Corpora Lutea	10.35 ± 0.15^{A}	10.10 ± 0.12^{A}	9.60 ± 0.29^{B}
Live fetuses	10.00 ± 0.13^{A}	7.70 ± 0.48^{B}	5.25 ± 1.05^{B}
Lysed fetuses	0.05 ± 0.05^{A}	1.85 ± 0.46^{B}	3.15 ± 0.88^{C}
Resorption sites	0.35 ± 0.15^{A}	0.25 ± 0.12^{A}	0.45 ± 0.23^{B}
Implantation sites	10.00 ± 0.12^{A}	$7.00 \pm 0.71^{A, B}$	4.80 ± 1.16^{B}

Values expressed as means ± SEM of 20 female rats/group.
^{A,B,C}Different superscript letters indicate significant difference ($P < 0.05$, test of Tukey-Kramer) within rows.

The dams treated with doses of 10 mg/kg and 100 mg/kg of isoflavones did not show any sign of maternal toxicity (such as salivation, tremors, and seizures). However, there was a significant reduction in the maternal mass gain along the gestational period in rats treated with 100 mg/kg of isoflavone (control group 38.42 ± 2.90^{A} g; isoflavones 10 mg/kg 46.69 ± 2.93^{A} g; isoflavones 100 mg/kg 27.96 ± 1.45^{B} g; $n = 10$/group; $P < 0.001$, Tukey-Kramer test); while both doses of isoflavones reduced significantly the gestational period in days (Table 2). The birth of the offspring occurred naturally in all groups. Pregnant rats (10/group) exposed to isoflavones at doses of 10 mg/kg and 100 mg/kg presented a reduced number of pups in relation to control group: Control 102^{A} (52 males plus 50 females); Isoflavones 10 mg/kg 57^{B} (28 males plus 29 females); Isoflavones 100 mg/kg 51^{B} (25 males plus 26 females; $P < 0.001$, Tukey-Kramer test).

Table 2. Gestational duration.

Gestational Time	Groups		
	Control (n = 10)	Isoflavone 10 mg/kg (n = 10)	Isoflavone 100 mg/kg (n = 10)
Days	21.30 ± 0.15^{A}	19.50 ± 0.16^{B}	20.40 ± 0.16^{B}

Values expressed as means ± SEM. of 10 female rats/group.
^{A,B}Different superscript letters indicate significant difference ($P < 0.05$, test of Tukey-Kramer) within rows.

Female offspring

On the first day after natural birth, the female offspring from the isoflavone 10 mg/kg group showed a body mass significantly larger when compared to the offspring in the control group. At 7 days post-birth, the three experimental groups showed similar body masses; while on postnatal day 14, the

female offspring from the isoflavone 100 mg/kg group showed a significant lower gain in body mass. At weaning, on day 21 following birth, the female offspring from the 10 mg/kg and 100 mg/kg groups showed a significant lower gain in body mass in relation to the control group (Table 3).

Table 3. Postnatal corporal development (weight) of female pups from birth until postnatal day 21.

Parameters	Groups		
	Control (n = 27)	Isoflavone 10mg/kg (n = 22)	Isoflavone 100mg/kg (n = 29)
Weight (g) postnatal day 1	6.77 ± 0.11^{A}	7.48 ± 0.08^{B}	$7.18 \pm 0.07^{A, B}$
Weight (g) postnatal day 7	11.44 ± 0.26^{A}	11.19 ± 0.25^{A}	11.77 ± 0.37^{A}
Weight (g) postnatal day 14	21.96 ± 0.44^{A}	$20.77 \pm 0.34^{A, B}$	19.55 ± 0.45^{B}
Weight (g) postnatal day 21	42.57 ± 1.05^{A}	37.31 ± 0.30^{B}	33.43 ± 0.94^{C}

Values expressed as means ± SEM.
^{A,B,C}Different superscript letters indicate significant difference ($P < 0.05$, test of Tukey-Kramer) within rows.

The female offspring from the isoflavone 100 mg/kg group showed a significantly early appearance of pelage growth; while the average time for the unfolding of the ears was found to be significantly shorter in the female offspring from the isoflavone 10 mg/kg and 100 mg/kg groups. The average time for the opening of the eyes was significantly larger in the female offspring from the isoflavones 10 mg/kg and 100 mg/kg groups. The female offspring from the isoflavone 10 mg/kg group showed a significantly shorter average time for the opening of the vaginal canal in relation to control ones. Meanwhile, no significant difference was observed in body weights (Table 4).

Table 4. Age at which pelage appeared, eyes opened, ears unfolded, and vagina opened.

Parameters (days)	Groups		
	Control (n = 27)	Isoflavone 10mg/kg (n = 22)	Isoflavone 100mg/kg (n = 29)
Pelage appearing	8.29 ± 0.11^{A}	$8.00 \pm 0.20^{A, B}$	7.68 ± 0.19^{B}
Ears unfolding	8.55 ± 0.09^{A}	7.59 ± 0.12^{B}	7.58 ± 0.13^{B}
Eyes opening	14.29 ± 0.3^{A}	15.50 ± 0.17^{B}	15.55 ± 0.15^{B}
Vaginal opening	38.29 ± 0.13^{A}	37.59 ± 0.14^{B}	$37.93 \pm 0.16^{A, B}$

Values expressed as means ± SEM.
^{A,B}Different superscript letters indicate significant difference ($P < 0.05$, test of Kruskal Wallis – Dunn) within rows.

Estrous cycle study

The estrous cycle of the rats from the Control group, isoflavone 10 mg/kg group, and isoflavone 100 mg/kg group followed for 15 consecutive days showed no difference in the number of estruses. However, irregularities in estrous cycles were observed in females of the isoflavones 10 mg/kg group, including prolongation of the estrus phase in hours (Control 24.40 ± 0.40^{A}; isoflavones 10 mg/kg 26.80 ± 0.93^{B}; Isoflavones 100 mg/kg $25.33 \pm 0.72^{A,B}$; n = 15/group; $P < 0.01$, Tukey-Kramer test).

Discussion

In the present study, despite the absence of signs of maternal toxicity, the exploratory laparotomy of the pregnant rats treated with isoflavones at doses of 10 mg/kg and 100 mg/kg showed a significant reduction in the number of live fetuses. In addition, the pregnant rats that received isoflavones at a dose of 100 mg/kg produced a larger number of fetuses in lyses and resorption sites. In a toxicological assessment approach, agents acting on reproductive parameters may be identified as potentially able to offer risk to the reproductive system (Zenick and Clegg, 1989). Thus, despite the non-existence of maternal toxicity, these alterations show a reproductive injury induced by isoflavones, probably by a potential embryotoxic action. The variation in gestation time can also indicate an effect of the drug on the gestation or birth process. In this sense, a reduction in weight of the offspring treated with isoflavones at doses of 10 mg/kg and 100 mg/kg was expected, since the date of birth was anticipated. However, the female offspring showed an increase in weight at doses of 10 mg/kg and 100 mg/kg. Therefore, the body mass of the pup at birth was influenced not only by the length of pregnancy but also, probably, by the intrauterine growth resultant from the treatment of the pregnant rats with isoflavones. These results might indicate that the development of the offspring in the uterus seems to be favored by the presence of isoflavones. It was also observed in this study that pregnant rats that received isoflavones at doses of 10 mg/kg and 100 mg/kg showed litters with a reduced number of offspring when compared to rats of the control group. It is known that litters with a large number of offspring, in general, have offspring of a

smaller mass in relation to those coming from litters of few offspring. The size of the litter is, then, influenced by the number of viable ova for fertilization, by the levels of fertilization and implantation, and by the proportion of implanted embryos that survive until birth. The increase in body mass of the female offspring, whose dams received isoflavones, was probably not due to the small number of offspring/litter. If it was, reduction in mass would not have occurred in the treated group whose birth was anticipated, when there was a smaller number of viable offspring per litter. In a study (McClain *et al.,* 2007) some effects were also noted at the low dose of genistein 20 mg/kg/day including, increased pup mortality, decreased pup body weights, and decreased pup milk uptake, however at the mid-dose of 150 mg/kg/day, no adverse effects on the pups were noted.

Agents capable of interfering with sexual development can also affect the external sexual characteristics of offspring, altering the ratio of females to males in a litter. Administering isoflavones at the studied doses in the present study also did not alter the ratio of females to males in the litter of different groups (data not shown). The maternal mortality, which is also a sign of toxicity, did not occur in the present study. However, other variables can be indicative of subtler adverse effects, such as alterations during the treatment in body mass and the pregnancy parameters mentioned by the U. S. Environmental Protection Agency (US EPA; 1991, 1996). Therefore, additional studies are necessary to determine if the use of isoflavones at doses that have not shown maternal toxicity would be able to provoke external, visceral, or skeletal malformations in conceptus. It is worthwhile to highlight that agents that induce toxicity during development, without producing maternal toxicity, generate a concern not only for the toxic potential, but also for the possibility of interfering with the later development of the individual, for example in cases of endocrine disrupters (Pereira, 2003). Besides this, independent of the origin of the adverse effect on the descendents, these effects if presented in the mother can be reversed, while in the offspring they can be permanent or detectable only in adult reproductive life.

In this study, the rats descended from dams submitted to treatment with isoflavones, at a dose of 100 mg/kg, showed a smaller gain in body mass on day 14 after birth compared to control rats. Female offspring from the isoflavones 10 mg/kg and 100 mg/kg groups showed a smaller body mass gain on day 21 after birth compared to the offspring of the control groups. The smaller gain in body mass from offspring may be a consequence of a systemic toxicity (Zenice and Clegg, 1989; US EPA, 1996) of the isoflavones exposure. Isoflavones administered during the gestational period probably had compromised the quantity and/or quality of the milk of the first breast feedings as well as may have influenced the metabolic system of the offspring.

The female offspring from the isoflavones 100 mg/kg group showed an early time for pelage appearance, while in the offspring from both isoflavones-treated groups there was a retardation of the times at which eyes opened and ears unfolded, showing a probable long-term participation of the extract revealed in the postnatal development of these animals. In addition, the female offspring from the isoflavones 10 mg/kg group showed early vaginal opening when compared to those of the control group, demonstrating an estrogenic activity of isoflavones in this parameter. The opening of the vaginal canal, a non-invasive indicator of female sexual development, depends on the secretion of estrogen. Therefore, the early opening of the vaginal canal can be provoked by substances that show estrogenic activity (Marty *et al.,* 1999). Rats exposed to estradiol during the prenatal period also revealed an early puberty (Pereira *et al*., 1997).

In a study (Delclos *et al*., 2001) now using Sprague Dawley rats exposed to the isoflavone genistein, no significant effect was observed on the onset of male puberty, as assessed by preputial separation, but did show a significant trend toward acceleration of vaginal opening. The dietary genistein produced effects in multiple estrogen-sensitive tissues in males and females that were generally consistent with its estrogenic activity. In this sense, isoflavones, particularly genistein, are the active components contributing to (or responsible for) the beneficial effects of soy (Delclos *et al*., 2001). These data agree with those from the current study in which isoflavones were used in Wistar rats.

Isoflavones and their components – genistein, daidzein, and glycitein – are absorbed rapidly in the diets of rodents (King, 1998; Chang *et al.,* 2000; Degen *et al.,* 2002) and act as pharmacological estrogens both *in vivo* and *in vitro* via estrogen receptors (Farmakilidis and Murphy, 1984; Farmakilidis *et al.,* 1985; Markiewicz *et al*., 1993; Song *et al.,* 1999). Thus, exposure to these agents can act, in long term, on the reproductive and functional development of rats (Boettger-Tong *et al.*, 1998; Brown and Setchell, 2001; Odum *et al.*, 2001). Therefore, substances that destabilize the hormonal balance can interfere in the process of starting puberty, accelerating it or slowing it. Besides the opening of the vaginal canal and the uterus mass development, other measures to evaluate the estrogenic activity are recommended. Among these measures we find the presence of cornified vaginal cells, indicating an estrus phase. The induction of persistent estrus by dietylestylbestrol (DES) in ovariectomized rats, is still one of the most used methods in the test of estrogenic activity *in vivo* by the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC; 1998). Until now, little has been studied about the action of isoflavones on the estrous cycle of rats. In this study, adult rats, exposed *in utero* to isoflavones at doses of 10 mg/kg and 100 mg/kg did

not show notable alterations in the number of observed estruses in 15 consecutive days of the estrous cycle. Despite this, there was a significant increase in the duration of estrus in rats of the isoflavones 10 mg/kg group. The alteration of the estrous cycle with prolongation of estrus can be a response to exposure to an agent with estrogenic properties or an agent able to block ovulation (US EPA, 1996). However, the normality of the estrous cycle, despite being an indication of good functioning of the reproductive neuroendocrine system and of the ovaries, does not characterize the effect of a substance on female fertility. In addition, a regular vaginal cycle does not necessarily indicate that ovulation has occurred. On the other hand, substances can induce alterations in the estrous cycle at doses that do not compromise fertility (US EPA, 1996).

In conclusion, based on the results obtained in the current study, using a soy derivative isoflavones, it can be seen that doses of 10 mg/kg and 100 mg/kg show estrogenic activity in rats. However, no alterations in gestational parameters were observed, thus indicating absence of maternal toxicity. Conversely, there was a significant reduction in the duration of pregnancy, at 10 mg/kg of isoflavone. The analysis of fetuses at the end of gestation revealed that this natural product could also result in some non-demonstrable effects, such as embryofetal-toxicity. Prenatal exposure to isoflavones also compromised the later development of the offspring, accelerated the onset of puberty in female rats, and increased the duration of the estrous phase. Thus, despite the indiscriminate recommendation of the use of soy and its derivatives, the results of this study also show that this attitude may be not totally free from undesirable effects. Caution must be taken until the completion of more extensive studies that evaluate the possible undesirable effects of the consumption of soy and its derivatives, especially isoflavones at critical developmental periods. Therefore, the use of valid protocols by regulatory agencies and scientific communities to better evaluate the possible risks that this natural product can cause has become imperative.

Acknowledgments

We are grateful to Isabel C. Meneghetti and Geraldo Rosa-Junior for help in the exploratory laparotomy. This work constituted part of the MSc Thesis, presented to São Paulo State University (UNESP), in 2007, by Valeria Romero.

References

Adlercreutz H, Mazur W, Bartels P, Elomaa V, Watanabe S, Wahala K, Landstrom M, Lundin E, Bergh A, Damber JE, Aman P, Widmark A, Johansson A, Zhang JX, Hallmans G. 2000. Phytoestrogens and prostate disease. *J Nutr*, 130:658S-659S.

Albertazzi P, Pansini F, Bonaccorsi G, Zanotti L, Forini E, Aloysio DD. 1998. The effect of dietary soy supplementation on hot flushes. *Obstet Gynecol*, 1:6-11.

Alekel DL, Germain AS, Peterson CT, Hanson KB, Stewart JW, Toda T. 2000. Isoflavone-rich soy protein isolate attenuates bone loss in the lumbar spine of perimenopausal women. *Am J Clin Nutr*, 72:844-852.

Anelli A, Gimenez LD, Rocha AP, Abreu CM, Freitas HC. 2003. Hormonal replacement therapy and the risk of breast cancer: evaluation of attitudes in patients previously treated by breast cancer. *Rev Hosp Clin*, 2:91-96.

Boettger-Tong H, Murthy L, Chiappetta C, Kirkland JL, Goodwin B, Adlercreutz H, Stancel GM, Mäkelä S. 1998. A case of a laboratory animal feed with high estrogenic activity and its impact on in vivo responses to exogenously administered estrogens. *Environ Health Perspect*, 106:369-373.

Brown NM, Setchell KD. 2001. Animal models impacted by phytoestrogens in commercial chow: implications for pathways influenced by hormones. *Lab Invest*, 81:735-747.

Chambô-Filho A, Chambô D, Chambô F. 2000. The soy as functional food in gynecology. *Rev Bras Nutr*, 15:326-329.

Chang HC, Churchwell MI, Delclos KB, Newbold RR, Doerge KB. 2000. Mass spectrometric determination of genistein tissue distribution in diet-exposed Sprague-Dawley rats. *J Nutr*, 130:1963-1970.

Clapauch R, Meirelles RMR, Julião MASG, Loureiro CKC, Giarodoli PB, Pinheiro SA, Harrigan AR, Spritzer PM, Pardini DP, Weiss RV, Athayde A, Russo LA, Póvoa LC. 2002. Phytoestrogens: positioning of the Department of Feminine Endocrinology of the Brazilian Society of Endocrinology and Metabolism (SBEM). *Arq Bras Endocrinol Metab*, 46:679-695.

Degen GH, Janning P, Diel P, Bolt HM. 2002. Estrogenic isoflavones in rodent diets. *Toxicol Lett*, 128:145-157.

Delclos KB, Bucci TJ, Lomax LG, Latendresse JR, Warbritton A, Weis CC, Newbold RR. 2001. Effects of dietary genistein exposure during development on male and female CD (Sprague-Dawley) rats. *Reprod Toxicol*, 15:647-663.

Duncan AM, Merz BE, Xu X, Ángel TC, Phipps WR, Kurzer MS. 1999. Soy isoflavones exert modest hormonal effects in premenopausal women. *J Clin Endocrinol Metab*, 84:192-197.

Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC). 1998. *Final report, EPA/743/R-98/003*. Washington, DC: US Environmental and Protection Agency.

Farmakalidis E, Murphy PA. 1984 Oestrogenic response of the CD-1 mouse to the soya-bean isoflavones genistein, genistin and daidzein. *Food Chem Toxicol*, 22:237-239.

Farmakalidis E, Hathcock JN, Murphy PA. 1985. Oestrogenic potency of genistin and daidzin in mice. *Food Chem Toxicol*, 23:741-745.

Fujioka M, Uehara M, Wu J, Adlercreutz H, Susuki

K, Kanazawa K, Takeda K, Yamada K, Ishimi Y. 2004. Equol, a metabolite of daidzein, inhibits bone loss in ovariectomized mice. *J Nutr*, 134:2623-2627.

King, RA. 1998. Daidzein conjugates are more bioavailable than genistein conjugates in rats. *Am J Clin Nutr*, 68:1496S-1499S.

Markiewicz L, Garey J, Adlercreuctz H, Gurpide E. 1993. *In vitro* bioassays of non-steroidal phytoestrogens. *J Steroid Biochem Mol Biol*, 45:399-405.

Marty MS, Crissman JW, Carney EW. 1999. Evaluation of the EDSTAC female pubertal assay in CD rats using 17 beta-estradiol, steroid biosynthesis inhibitors, and a thyroid inhibitor. *Toxicol Sci*, 52:269-277.

McClain RM, Wolz E, Davidovich A, Edwards J, Bausch J. 2007. Reproductive safety studies with genistein in rats. *Food Chem Toxicol*, 45:1319-1332.

Morabito N, Crisafulli A, Vergara C, Gaudio A, Lasco A, Frisina N, D'anna R, Corrado F, Pizzoleo MA, Cincotta M, Alta Villa D, Ientile R, Squadrito F. 2002. Effects of genistein and hormone-replacement therapy on bone loss in early postmenopausal women: a randomized double-blind placebocontrolled study. *J Bone Min Res*, 176:1904-1912.

Morais AAC, Silva AL. 2000. Nutritional and functional value of the soy. *Rev Bras Nutr Clin*, 15:316-315.

Murkies AL, Lombard C, Strauss BJG, Willcox G, Burger HG, Morton MS. 1995. Dietary flour supplementation decreases postmenopausal hot flushes: effect of soy and wheat. *Maturitas*, 21:189-195.

Odum J, Tinwell H, Jones K, Van Miller JP, Joiner RL, Tobin G, Kawasaki H, Deghenghi R, Ashby J. 2001. Effect of rodent diets on the sexual development of the rat. *Toxicol Sci*, 61:115-127.

Pereira OCM, Carvalho NFS, Carlos CP. 1997. Perinatal estrogen exposure: later repercussion on the fertility of rats. *Comp Biochem Physiol*, 118:241-245.

Pereira OCM. 2003. Endocrine disruptors and hypotalamic sexual differentiation. *Annu Rev Biomed Sci*, 5:87-94.

Piffer RC, Pereira OCM. 2004. Reproductive aspects in female rats exposed prenatally to hydrocortisone. *Comp Biochem Physiol Part C*, 139:11-16.

Salewsky E. 1964. Farbemethode zum markroskopishen nachweis von implantat cosstellen am uterus de ratter naunyn schmuderbeys. *Arch Pharmacol*, 247:367. (abstract).

Setchell KD. 1998. Phytoestrogens: the biochemistry, physiology and implications for human health of soy isoflavones. *Am J Clin Nutr*, 68:1333S-1346S.

Song TT, Hendrich S, Murphy PA. 1999. Estrogenic activity of glycitein, a soy isoflavone. *J Agric Food Chem*, 47:1607-1610.

U.S. Environmental Protection Agency (US EPA). 1991. Guidelines for developmental toxicity risk assessment, EPA/600/fr-91/001. *Fed Regist*, 56(234):63798-63826.

U.S. Environmental Protection Agency (US EPA). 1996. Guidelines for reproductive toxicity risk assessment, EPA/630/r-96/009. *Fed Regist*, 61(212):56274-56322.

Zenick H, Clegg ED. 1989. Assessment of male reproductive toxicity: a risk assessment approach. *In*: Principles and Methods of Toxicology. New York: Raven Press. pp. 275-309.