Xenopus laevis is a potential alternative model animal species to study reproductive toxicity of phytoestrogens

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Abstract

This study investigated effects of phytoestrogen quercetin on the gonadal development in *Xenopus laevis*. *X. laevis* at Nieuwkoop and Faber stage 46/47 were exposed to 50, 100 and 200 µg/L quercetin till 1 month postmetamorphosis. Gonads from frogs at 1 and 3 months postmetamorphosis were examined in gross morphology and histology. The highest dose of quercetin as well as estradiol (E₂) significantly increased the percentages of phenotypic females. Exposure to quercetin at all doses induced abnormal testes with certain ovarian characteristics to some degree in gross morphology, including ovotestes. The abnormality rate exceeded 10% in each quercetin treatment. Histologic examination revealed that some abnormal testes exhibited intersexuality with testicular structure and ovarian structure or oocytes interspersed in testicular structure at 1 month postmetamorphosis. At 3 months postmetamorphosis, testicular abnormalities were more obvious, such as necrosis or apoptosis of spermatogonia, occurrence of developed or undeveloped oocytes, delay of the development of seminiferous tubes without or less late stage spermatocytes. The results have shown that quercetin cannot only feminize but also impair testicular development of *X. laevis*, i.e. *X. laevis* is sensitive to phytoestrogen. It is suggested that *X. laevis* might be an alternative model species to study reproductive toxicity of phytoestrogens.

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1. Introduction

Phytoestrogens are plant-derived components with estrogen-like structure and activities (Setchell, 1998; Adlercreutz, 2002). Since phytoestrogens in alfalfa and clovers were found to affect the fertility of sheep about 50 years ago (Bennetts et al., 1946), reproductive toxicity of phytoestrogens as a kind of endocrine disruptor has aroused considerable attention. A myriad of experimental studies were performed to explore effects of phytoestrogens on the structures and functions of the reproductive system and related mechanisms. To date, there is evidence that phytoestrogens (such as genistein, coumestrol, quercetin), given in high doses or at critical stages of development in experi-

mental animals can result in severe reproductive tract disorders (Lamartiniere et al., 1995; Strauss et al., 1998; Tou et al., 1999), and temporary infertility syndromes in domestic animals have been related to high phytoestrogens consumption in grazing (Adams, 1995).

Most of the current understanding of reproductive toxicity of phytoestrogens is based on studies in rodent model system, especially in rats. Rats are conventional model animals for reproductive biology and endocrinology, and they have being used to study toxicity of toxicants. The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) recommended the laboratory rat as the species for screening and testing endocrine disruptors (U.S. EPA, 1998). However, many studies involving endocrine disruptors (including phytoestrogens) with rats lack reproducibility and also show inter-laboratory inconsistencies. In addition to the complexity of the endocrine system, some researchers think that different sensitivities of model animals and variable levels of phytoestrogen in diets should be responsible for the variable results (Everitt and Foster, 2004; Naciff et

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al., 2004; Thigpen et al., 2004). Stokes suggests that it is critical to select appropriate animal models and diets for endocrine disruptor studies (Stokes, 2004). Consequently, we considered whether there is an alternative animal species, whose diets do not contain phytoestrogens, to study reproductive toxicity of phytoestrogens, especially effects on the gonadal development.

Amphibians might represent potential sentinels for assessing adverse effects of environmental factors because of their permeable skins and biphasic life-history strategies (van der Schalie et al., 1999). *Xenopus laevis* is an amphibian species used widely as a model for developmental biology in laboratory studies. In recent years, *X. laevis* has attracted interest of environmental scientists because of the potential sensitivity to endocrine disruptors. In previous studies, the sensitivity of the gonadal development in *X. laevis* to estrogens has been demonstrated in vivo and in vitro (Witschi and Allison, 1950; Gallien, 1953; Chang and Witschi, 1956; Miyata and Kubo, 2000). Vilalpando and Merchant-Larios reported that 100 µg/L estradiol benzoate induced complete sex-reversal in genetic male *X. laevis* (1990). Miyata et al. treated *X. laevis* with 100 µg/L estradiol (E₂) and obtained 100% females (1999). There is increasing evidence that endocrine disruptors with estrogenic activities also can induce gonadal feminization in *X. laevis* and affect sexual hormone homeostasis (Kloas et al., 1999; Hayes et al., 2002; Qin et al., 2003; Levy et al., 2004). In our previous study, *X. laevis* was suggested to be good model animal for assaying endocrine disruptors (Qin et al., 2003). Furthermore, in a pre-experiment on the sensitivity of gonadal development, we found that quercetin might have feminizing effects on the gonadal development of *X. laevis*. Considering the disadvantages of rats as model animals to study phytoestrogens, therefore, we suppose whether *X. laevis* might be used as an alternative species if the gonadal development of *X. laevis* is sensitive to phytoestrogens. In this study we further examined the effects of quercetin, which is a natural flavonol widely distributed in plant diets and which can exert various biological effects, and determined the sensitivity of the gonadal development of *X. laevis* to this phytoestrogen.

2. Materials and methods

2.1. Breeding and housing

Mature female and male *X. laevis* were maintained separately in glass tanks containing dechlorinated water at 22 ± 2 °C with a 12-h light/12-h dark cycle and fed once a week on chopped pork liver. Breeding was induced by injection of human chorionic gonadotrophin. After eggs were laid, the females and the males were taken out of the breeding tank. Fertilized eggs were incubated at 22 ± 2 °C with a 12-h light/12-h dark cycle. On day 5 after fertilization, tadpoles were fed on *Daphnia* and *Artemia* daily.

2.2. Exposure to chemicals

Quercetin (purity 98%, National Research Center for Certified Reference Materials) and E₂ (purity 98%, Merck) were dissolved in ethanol to produce stock solutions. The experiment

water was prepared by adding the stock solution to dechlorinated water. The final concentration of E₂ as positive control in water was 100 µg/L. Quercetin exposure was performed in three doses, 50, 100 and 200 µg/L. The control group received the same amount of ethanol used as solvent for the highest quercetin dose. On day 6 after fertilization, healthy tadpoles at NF stage 46/47 among the offspring of a pair of parental frogs were randomly selected for the exposure experiment (Nieuwkoop and Faber, 1956). Each treatment in duplicate consisted of 30 tadpoles in a glass tank containing 18 L water. Exposure stopped at 1 month postmetamorphosis. All tanks were the same in size and shape (30 cm × 20 cm × 25 cm). The experiment water was renewed twice weekly.

2.3. Gonadal identification and histologic examination

The survival rate of each group was examined at 1 month postmetamorphosis, and approximately two thirds of frogs were sacrificed to determine the sex. One third of frogs was maintained to 3 month postmetamorphosis and examined. The sex ratios in each group were measured based all examined frogs. The sex of all individuals was determined based on gross gonadal morphology using a dissecting microscope. Normal ovaries are characterized by greater length, lobed structure, and melanin granules. Normal testes are characterized by ellipsoid/cylinder, smooth surface, and without melanin granules. In our experiment, frogs with typical ovaries were distinguished as females, while frogs with typical testes were distinguished as normal males. The other frogs that had abnormal testes with certain ovarian characteristics to some degree were determined to be abnormal males. Histologic analysis was conducted on all abnormal gonads and some normal gonads. Gonads attached to kidneys were fixed with Bouin's fixative, then embedded in paraffin, sectioned at 6 µm, and stained with hematoxylin and eosin. Sections were examined with a light microscope. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

2.4. Statistical methods

Data were analyzed by using the *t*-test. A value of $\alpha = 0.05$ was chosen to detect significant difference.

3. Results

The survival rates of 50, 100 and 200 µg/L quercetin-exposed groups were 71.7% (43/60), 71.7% (43/60), and 76.7% (46/60), respectively, while the positive control and the control were 80% (48/60) and 73.3% (44/60). The survival rate had no difference among each group. The percentage of females in the 200 µg/L treatment group (67.4%, 31/46) as well as that in the positive control group (83.3%, 40/48) was significantly higher than in the control (48.8%, 20/41), while there was no significant difference between the 50 or 100 µg/L quercetin-exposed groups (55.3%, 21/38; 60%, 24/40) and the control (Fig. 1). Ovarytes occurred among E₂-exposed individuals (16.7%). Exposure of

Fig. 1. Sexual percentages based on gonadal examination using microscope in quercetin treatments compared with the control (E) and the positive control (E_2). The percentages of females in 200 $\mu\text{g/L}$ treatment as well as that in E_2 was significantly higher compared to the control, while it had no significant difference between 50 and 100 $\mu\text{g/L}$ treatments with the control ($p < 0.05$).

quercetin at all doses induced abnormal testes with certain ovarian characteristics to some degree in gross morphology, including ovotestes (Fig. 2). The abnormality rate exceeded 10% in each quercetin treatment with no obvious abnormality in the control. The results indicate that quercetin has induced feminization of *X. laevis*. Histologic examination revealed that some abnormal testes exhibited inter-sexuality with testicular structure and ovarian structure or oocytes interspersed in testicular structure at 1 month postmetamorphosis (Fig. 3). At 3 months postmetamorphosis, normal testes consist of well-developed seminiferous tubes with multi-stages spermatocysts and spermatozoa. At least

five testes were histologically examined in each group, and all showed varying degrees of testicular abnormalities such as necrosis or apoptosis of spermatogonia, occurrence of developed or undeveloped oocytes, or delay of the development of seminiferous tubes without or less late stage spermatocytes (Fig. 4). The histologic results have shown that quercetin cannot only feminize but also impair testicular development of *X. laevis*.

4. Discussion

Gonadal differentiation in amphibians corresponds to their genetic sex constitution (Hayes, 1998). Natural intersexuality exists among some juvenile amphibians, but it does not occur in *X. laevis* (Gallien, 1974). However, estrogen-treatment during embryonic development in *X. laevis* can produce gonadal sex-reversal in genetic males (Gallien, 1953; Villalpando and Merchant-Larios, 1990). In 1999, Kloas et al. reported that bisphenol A and 4-nonylphenol caused a significantly higher number of female phenotypes compared to control *X. laevis*, which is the first evidence that endocrine disruptor can induce feminization in *X. laevis* (1999). Since then, there has been increasing data on feminizing effects of endocrine disruptors on *X. laevis*, such as atrazine, polychlorinated biphenyls and bisphenol A (Hayes et al., 2002; Qin et al., 2003; Levy et al., 2004) despite contrary results reported as well (Pickford et al., 2003; Carr et al., 2003). In this study, we for the first time report that the phytoestrogen quercetin also has influence on the gonadal development in *X. laevis*. The sex ratio towards females has shown that quercetin has feminizing effect on genetic male *X. laevis*. Histologic intersexuality has further demonstrated feminizing effect of quercetin. Undevelopmental seminiferous tubes with early stage spermatocytes and necrosis or apoptosis of spermatogonia

Fig. 2. Abnormal testes induced by quercetin compared with control gonads. (a) Control ovaries (O) distinguished by greater length, lobed structure, and melanin granules. (b) Control testes (T) distinguished by ellipsoid/cylinder, smooth surface, and no melanin granules. (c–d) Abnormal testes caused by quercetin. (c) There were melanin granules and goffer in the end of testis (arrow). (d) Constrictions (arrow) occurred in some sites so that testes tended to be divided into lobed structures.

Fig. 3. Photomicrographs of gonadal sections from frogs at 1 month postmorphosis except for (c). (a) Normal ovarian tissue with numerous oocytes. (b) Normal testicular tissue with numerous spermatogonia. (c) Normal ovarian tissue from a tadpole at stage 58. Cavities occur in the medullary region in the process of ovarian development. (d) Intersexuality induced by E_2 . Testicular structure and ovarian structure exist in a gonad. (e–f) Intersexuality induced by quercetin. Testicular structure and ovarian structure exist in a pair of gonads, or oocytes are interspersed in testicular structure.

have indicated that quercetin cannot only feminize but also impair testicular development.

Quercetin can exert various biological effects, including anti-carcinogenic, antioxidant and (anti-) estrogenic effects, and modulation of sex hormone homeostasis (Schmitt and Stopper, 2001). Estrogenic and anti-estrogenic effects of phytoestrogens involving sex determination are complex and controversial. In this study, quercetin exhibited estrogenic effects on gonadal development in *X. laevis*. The mechanism of feminizing effects of quercetin has been unknown despite several studies suggesting that quercetin possibly exerts (anti-) estrogenic effects through modulation of sex hormone homeostasis by affecting aromatase, a key enzyme in the synthesis of estrogens from androgens (Campbell and Kurzer, 1993; Papiez et al., 2002; Sanderson et al., 2004).

In past decades, most studies showed that phytoestrogens given in high dose or at critical stages of development in rodents can result in severe reproductive system disorders, such as inhibition of LH secretion, decrease of plasma testosterone concentrations and prostate weight (Wanichacheewa et al., 2001; Wisniewski et al., 2003; Opalka et al., 2004). Simultaneously, some authors reported that there are no significant effects of phytoestrogens in rodents (Roberts et al., 2000; Mitchell et al., 2001; Lamartiniere et al., 2002). One of the explanations for these inconsistent results is that commercial rodent diets all contain high and variable levels of abundant phytoestrogens, which could affect results of studies on reproductive toxicity of test phytoestrogens (Odum et al., 2001; Strohecker et al., 2003; Naciff et al., 2004; Thigpen et al., 2004). In this study, *X. laevis* tadpoles were fed *Daphnia* and *Artemia*, and frogs were

Fig. 4. Photomicrographs of gonadal sections from frogs at 3 month postmorphosis except for (c). (a) Normal ovarian tissue with numerous oocytes. (b) Normal testicular tissue with well-developed seminiferous tubes with multi-stages of spermatocysts and spermatozoa. (c) Testicular tissue from a frog exposed E₂. Some spermatogonia seem to be necrotizing or apoptosing (*). Some spermatogonia have the appearance of oocytes (arrows). (d) Testicular tissue from a frog exposed quercetin. It exhibits the same abnormality as (c). (e–f) Intersexuality induced by quercetin. Oocytes are interspersed in testicular structure. There are not well-developed seminiferous tubes. There are more early spermatocytes and less late spermatocytes.

fed pork liver. These diets do not contain phytoestrogens, and did not contain some endocrine disruptors such as DDT, polychlorinated biphenyls (unpublished data). Thus, the studies on phytoestrogens using *X. laevis* as a model animal species can avoid the confounding effect of phytoestrogens in the diet.

Recently, several research groups used fish to study reproductive toxicity of phytoestrogens. Zhang et al. reported that genistein increased the estradiol production of ovaries and decreased testosterone production from ex vivo testis and a comparable reduction in circulating testosterone level in Japanese medaka (Zhang et al., 2002). The phytoestrogen beta-sitosterol was reported to alter the reproductive endocrine status of goldfish (MacLachy and Van Der Kraak, 1995). These studies showed that fish are sensitive species for assaying reproductive toxicities of phytoestrogens. However, there is no evidence that phytoestrogen can induce intersexuality or sex reversal in fish.

In this report, feminizing effects of low dose quercetin-exposure, including intersexuality, were observed at 3 months

postmetamorphosis. In the rodent model, reproductive toxicity of phytoestrogens can be found generally at ppm dose levels (Awoniyi et al., 1998; Weber et al., 2001). However, intersexuality like that induced by quercetin in *X. laevis* has not been reported in rodents. In other words, *X. laevis* might be more sensitive to phytoestrogens than rodents. In a previous study, we have suggested that *X. laevis*, a well-developed animal model for developmental biology, might be an alternative animal model for studying endocrine disruptors because of their sensitivity to endocrine effects and a rich literature on reproduction and development (Qin et al., 2003). The sensitivity of *X. laevis* to endocrine disruptors has attracted attention (Kloas, 2002; Klann et al., 2005), and increasingly, researchers are beginning to use *X. laevis* to study endocrine disruptors (Hayes et al., 2002; Pickford and Morris, 2003; van Wyk et al., 2003). Based on our results, here, we suggest *X. laevis* might be alternative model animal to study reproductive toxicity of phytoestrogens involving endocrine disruption. Our study also responds

to the proposal of EDSTAC that amphibian development and reproduction are promising endpoints for evaluating endocrine disruptors (U.S. EPA, 1998). In conclusion, this study revealed feminizing effects of phytoestrogen quercetin on the gonadal development in *X. laevis*, and suggests that *X. laevis* might be a useful alternative model species to study reproductive toxicity of phytoestrogens.

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