ELSEVIER

Aquatic Toxicology 77 (2006) 250–256

AQUATIC TOXICOLOGY

www.elsevier.com/locate/aquatox

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

Lin Cong [a,b], Zhan-Fen Qin [a,*], Xiang-Ning Jing [b], Lei Yang [a,b], Jing-Ming Zhou [a], Xiao-Bai Xu [a]

[a] *The State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Enviromental Sciences, The Chinese Academy of Sciences, Beijing, China*

[b] *Beijing Forestry University, Beijing, China*

Received 26 February 2005; received in revised form 5 December 2005; accepted 5 December 2005

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 (E_2) 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.

Keywords: *Xenopus laevis*; Reproductive toxicity; Phytoestrogen; Quercetin; Feminization

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 experimental 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

* Corresponding author at: P.O. Box 2871, Research Center for Eco-Environmental Sciences, The Chinese Academy of Sciences, Beijing 100085, China. Tel.: +86 10 62919177; fax: +86 10 62923563.

E-mail address: zhfqin@yahoo.com (Z.-F. Qin).

0166-445X/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquatox.2005.12.014

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 alterative 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). Villalpando 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 (E_2) 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 E_2 (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 E_2 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). Ovotestes occurred among E_2-exposed individuals (16.7%). Exposure of