Environmental (anti-)androgenic chemicals affect germinal vesicle breakdown (GVBD) of *Xenopus laevis* oocytes in vitro

Shan Cao \(^a\,^b\), Wei Xu \(^a\,^b\), Qin-Qin Lou \(^b\), Yin-Feng Zhang \(^b\), Ya-Xian Zhao \(^b\), Wu-Ji Wei \(^a\,^*\), Zhan-Fen Qin \(^b\,^*\)

\(^a\) Nanjing University of Technology, Nanjing 21009, China
\(^b\) State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

**Abstract**

Progesterone-induced germinal vesicle breakdown (GVBD) of *Xenopus* oocytes in vitro was used to study endocrine disrupting activity of chemicals in previous studies. In this study, we investigated for the first time effects of environmental androgens on oocyte maturation and effects of anti-androgens on androgen-induced oocyte maturation, using *Xenopus* GVBD *in vitro*. Trenbolone and nandrolone, two environmental androgens, were found to induce *Xenopus* GVBD at low concentrations. The potential of trenbolone to induce GVBD was approximately 100-fold lower than that of testosterone, while nandrolone had a several-fold lower potential than testosterone. Our findings have aroused new concerns for effects of environmental androgens on amphibian oocyte maturation at environmentally relevant concentrations, and suggested that *Xenopus* GVBD can be used to test androgenic activity of suspicious environmental androgens. Androgen receptor (AR) antagonist flutamide at 10 μM only exhibited a weakly inhibitory effect on androgen-induced GVBD, while another known AR antagonist vinclozolin had no effect even at high concentrations. The results show that *Xenopus* GVBD is not sensitive to AR-mediated environmental anti-androgens. In contrast to flutamide and vinclozolin, methoxychlor (a weaker AR antagonist) inhibited dramatically androgen-induced GVBD, suggesting that androgen-induced *Xenopus* GVBD can be used to study non-AR-mediated effects of chemicals on oocyte maturation.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

*Xenopus laevis* oocytes have been used for many decades to study steroid-induced maturation due to large size and relative abundance (Hammes, 2004). Historically, progesterone was considered as the physiological mediator of *Xenopus* oocyte maturation, which is accompanied by germinal vesicle breakdown (GVBD), spindle formation, and extrusion of the first polar body (Bayaa et al., 2000; Ferrell, 1999). However, recent observations have proven that androgens rather than progesterone are the physiological regulators of oocyte maturation. *In vitro* experiments have shown testosterone as well as progesterone can induce GVBD of *Xenopus* oocytes, and effective concentrations of testosterone (~50 nM) are lower than those of progesterone (submicromolar) (Goascogne et al., 1985; Lutz et al., 2001, 2003). *In vivo* experiments, a dramatic increase in androgen levels but not in progesterone levels was found in serum of female frogs with the injection of human chorionic gonadotropin (HCG), which can induce oocyte maturation and ovulation (Lutz et al., 2001). Furthermore, it has been demonstrated that androgen-induced *Xenopus* oocyte maturation is mediated by classical androgen receptors (ARs), and AR antagonist flutamide can inhibit androgen-triggered maturation (Lutz et al., 2001, 2003).

Eco-toxicological effects and human health risk of endocrine-disrupting chemicals (EDCs), such as environmental (anti-)estrogens and (anti-)androgens, have attracted more attention for several decades (Meeker, 2012; Schug et al., 2011; Softer and Tyler, 2012; Vandenberg et al., 2012). Amphibians are susceptible to EDCs due to their permeable skins, biphasic life cycle and some biological characteristics (Van der Schalie et al., 1999). Numerous studies have described effects of EDCs on gonadal differentiation, the secondary sex characteristic development, and metamorphic process of amphibians (Crump et al., 2002; Hayes et al., 2002, 2010; Porter et al., 2011; Qin et al., 2003). Also, some of EDCs, such as ethinyl estradiol, atrazine, 2,4-dichlorophenoxyacetic acid and methoxychlor, have been shown to affect oocyte maturation in amphibians, suggesting potential adverse effects on reproductive function of amphibians (Fort et al., 2002; Ghodageri and Katti, 2013; Pickford and Morris, 1999; Stebbins-Boaz et al., 2004). Given that androgens can induce oocyte maturation and anti-androgen flutamide can inhibit androgen-induced oocyte maturation, we propose that environmental androgens and anti-androgens could disrupt oocyte maturation of amphibians.
Androgenic-anabolic steroids (AAS) used in human and animals are released into the environment and become environmental androgens. Trenbolone, one of AAS, is widely used as a growth promoter in animal agriculture. Some studies investigated the levels of trenbolone in wastewater, soil and solid waste in feedlots (Khan et al., 2008; Qu et al., 2012). Trenbolone was also found in the receiving environment of feedlot wastewater and general environment (Gall et al., 2011; Khan and Lee, 2012; Liu et al., 2011). Generally, the concentrations of trenbolone in water are at the level of ng/L (Liu et al., 2012), but the μg/L level of trenbolone was also reported in some water samples (Khan and Lee, 2012). Nandrolone as a doping agent is widely abused to improve athletic performance and body image (Hemmersbach and Große, 2010; Lumia and McGinnis, 2010). Nandrolone is also used medically as a treatment for anemia and other diseases (Cederholm and Hedström, 2005; Deicher and Hör, 2005). Several studies reported the presence of nandrolone in wastewater, and even the level exceeded 60 ng/L (Backe et al., 2011; Sun et al., 2010). Recently, two studies described effects of trenbolone on the growth, survival, and sexual differentiation of X. laevis (Olmstead et al., 2012; Finch et al., 2013). Overall, available information on adverse effects of AAS on amphibians has been very limited.

In addition, some chemicals have been demonstrated to exhibit AR-mediated anti-androgenic activity. Several fungicides, such as vinclozolin, procymidone, and prochloraz, are well-known AR antagonists (Alt-Aissa et al., 2010; Kille et al., 2011; Wilson et al., 2008). Methoxychlor was shown to have a very weak AR-antagonistic activity (Maness et al., 1998). However, whether these environmental anti-androgens affect androgen-mediated oocyte maturation of amphibians has never been studied. In this study, our aim was to investigate effects of environmental androgens (trenbolone and nandrolone) on amphibian oocyte maturation, and effects of environmental anti-androgens (vinclozolin and methoxychlor) on androgen-induced oocyte maturation using Xenopus GVB D in vitro.

2. Materials and methods

2.1. Chemicals

Testosterone (99.5%), trenbolone (98.0%), nandrolone (99.0%) and vinclozolin (99.0%) were purchased from Dr. Ehrenstorfer (Germany). Methoxychlor (98.9%) was purchased from Accustandard (USA). Flutamide (96.0%) was purchased from ChromaDex (USA). Dimethyl sulfoxide (DMSO) and 3-aminobenzoic acid ethyl ester (MS-222) were from Sigma-Aldrich (USA). All chemicals above were dissolved in DMSO and stored at 4 °C. Collagenase I was purchased from Gibco (USA). Trichloroacetic acid, sodium pyruvate, and 4-((2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Biotop (China). Commercial amphibian diet was obtained from Totoro Supplies (HongKong, China). Other reagents were purchased from Beijing Chemical Reagents (Beijing, China).

2.2. Experimental animals

Adult female X. laevis frogs (three years old) were raised in glass tanks in charcoal-dechlorinated water at 22 ± 2 °C. The water quality was as follows: chlorine concentration <5 μg/L, iodine concentration 2.14–3.92 μg/L, pH 6.5–7.0, the dissolved oxygen concentration >5 mg/L, and water hardness (CaCO3) approximately 150 mg/L. The water was changed completely twice weekly after feeding with chopped pork liver: commercial amphibian diet (1:1). Fluorescent lighting provided a photoperiod of 12 h light: 12 h dark with a light intensity ranging from 100 to 200 lux at the water surface. Each frog was injected by 100 IU human chorionic gonadotropin (HCG) two weeks before surgery. Female frogs were anaesthetized by submersion in 100 mg/L MS-222 buffered with 200 mg/L of sodium bicarbonate about 15 min until their reflexes disappeared. In every independent experiment, the female frog was placed on an ice bed and 2–4 ovary lobes were pulled out carefully through a small ventral incision (0.8–1 cm). Then, the incision was closed in two layers with absorbable gut for the muscle layer and nylon suture for the skin closure. Finally, the frog was put in the water. Xenopus husbandry and all animal procedures complied with the Xenopus guideline edited by Green (2010), and all animal procedures also accorded with Regulations for the Administration of Affairs Concerning Experimental Animals (State Science and Technology Commission of the People’s Republic of China, 1988).

2.3. Preparation and culture of Xenopus oocytes

Ovary lobes were cut into small strips, and rinsed several times in OR-2 solution (NaCl 82.5 mM, KCl 2.5 mM, MgCl2 1.0 mM, HEPES 5.0 mM, pH 7.6), then digested by 0.15% (w/v) collagenase I in OR-2 for 2 h at a shaker (TY10QB-128, Kylin-Bell, China) until blood vessels and follicle cell layers were removed from the oocytes. The oocytes were rinsed 4–5 times in fresh OR-2 and ND-96 solution (NaCl 96.0 mM, KCl 2.0 mM, CaCl2 1.8 mM, MgCl2 1.0 mM, HEPES 5.0 mM, sodium pyruvate 2 mM, pH 7.6), respectively. Then, the oocytes were transferred to a disposable 100-mm petridish containing fresh ND-96 solution. Follicle cell-free oocytes at stage V–VI (diameter 1.2–1.3 mm) were picked out using Pasteur pipette under a stereomicroscope for experiments.

2.4. GVB D assay for testosterone, trenbolone, nandrolone

The oocytes at stage V–VI were plated (20/well) in 12-well polystyrene culture plates (Costar Corporation, Cambridge, MA) with 2 mL ND-96 solution containing series of concentrations (10 nM–10 μM) of testosterone, trenbolone, and nandrolone. Each treatment group consisted of three replicate wells. DMSO concentration was 0.01% (v/v) in the solvent control. The oocytes were then incubated in an incubator (MIR-153, Sanyo, Japan) at 22 °C. After 4h-incubation, GVB D was determined visually by the emergence of a white Roux spot at the animal pole under a stereomicroscope (Pickford and Morris, 1999). Culture plates were replaced into the incubator for a prolonged 4h-incubation. At the end of the whole 8h-incubation, the oocytes were fixed in 5% (w/v) trichloroacetic acid, and GVB D was verified by breakdown of germinal vesicles in opened oocytes (Pickford and Morris, 1999). The GVB D percentage was calculated by counting the number of mature oocytes of 20 oocytes in each well. The experiment for each test compound was repeated three times using oocytes derived from different X. laevis.

2.5. GVB D assay for anti-androgenic compounds

According to the method described above, the oocytes were exposed to series of concentrations of flutamide or vinclozolin or methoxychlor in the presence or absence of 10 nM testosterone. After 4h-incubation, GVB D was determined visually by the emergence of a white Roux spot and verified by breakdown of germinal vesicles in trichloroacetic acid-fixed oocytes. The GVB D percentages were calculated by counting the number of mature oocytes of 20 oocytes in each well. The experiment for each test chemical was repeated two times using oocytes derived from different X. laevis.
2.6. Statistical analysis

Statistical analysis was performed using SPSS software version 18.0 (SPSS, Chicago, IL, USA). The effective concentration that stimulates 50% GVB (EC50) was calculated by Probit analysis. The data on GVB percentages were expressed as mean ± SD. Significant differences in GVB percentages between treatment groups and the control were tested by one-way analysis of variance (ANOVA). Statistical significance was considered as P < 0.05.

3. Results

As expected, testosterone at low concentrations induced GVB of Xenopus oocytes, while no GVB was found in the control group during 8h-incubation (Fig. 1). Overall, testosterone-induced GVB exhibited a concentration-dependent manner in the range from 5 nM to 50 nM, although the trend of the increase in GVB with increasing concentrations became weak due to a high GVB induction by 5 nM testosterone. With the same testosterone concentrations, 8h-exposure induced higher GVB percentages than 4h-exposure, showing that testosterone-induced GVB was dependent on exposure time in a relatively short time range. There was a large difference in the sensitivity to testosterone among oocytes from different individuals. In three independent repeated experiments, the 4h-EC50 values of testosterone for GVB varied from 3.7 to 20.2 nM, with a range of 1.0–7.3 nM for 8h-EC50 (Table 1).

In accordance with testosterone, trenbolone induced GVB of Xenopus oocytes in a concentration-dependent manner (Fig. 2). In three independent repeated experiments, the 4h-EC50 values of trenbolone for GVB varied from 232.5 to 2315.7 nM, with a range of 32.2–1045.4 nM for 8h-EC50 (Table 1). Nandrolone exhibited a stronger potential to induce GVB than trenbolone, but lower than testosterone (Fig. 3). In three independent repeated experiments, the 4h-EC50 values of nandrolone for GVB ranged 13.6–31.1 nM, with a range of 9.2–29.0 nM for 8h-EC50 (Table 1). Because the oocytes for each independent repeated experiments for testosterone, trenbolone and nandrolone were from the same three frogs, their potentials to induce GVB can be compared roughly by EC50 values. The potential of trenbolone to induce GVB was approximately 100-fold lower than that of testosterone, while nandrolone had a several-fold lower potential than testosterone.

Based the above results, we chose 4h-exposure to 10 nM testosterone to study effects of anti-androgenic chemicals on GVB of Xenopus oocytes. Exposure to 10 nM testosterone for 4 h induced 40–60% GVB in the experiments for anti-androgenic chemicals. In the absence of testosterone, none of flutamide, vinclozolin and methoxychlor induced GVB. In the concentration range of 10–10,000 nM, only 10,000 nM methoxychlor significantly inhibited testosterone-induced GVB in two independent repeated experiments (Fig. 4A). Vinclozolin at all test concentrations (10–10,000 nM) had no inhibitory action on testosterone-induced GVB in two independent repeated experiments (Fig. 4B). Both 100 nM and 1000 nM methoxychlor significantly inhibited testosterone-induced GVB in one independent experiment, whereas 10 nM besides two higher concentrations had a significantly inhibitory action on testosterone-induced GVB in another independent experiment, exhibiting a concentration-dependent manner (Fig. 4C).

4. Discussion

In the present study, the 4h-EC50 values for testosterone-induced GVB of Xenopus oocytes varied from 3.7 to 20.2 nM. The EC50 values are comparable with the range from 5 nM to 10 nM of EC50 for testosterone-induced GVB of Xenopus oocytes in a previous study (Lutz et al., 2001). These data show that oocyte maturation of amphibians is very sensitive to androgens, although there is a large difference in the sensitivity to androgen among oocytes derived from different individuals. The individual variability in the sensitivity to hormones has been well described in the literature, including Xenopus data (Lutz et al., 2008; Pickford and Morris, 1999; Williams, 2008). It is generally believed that the individual variability in the sensitivity to hormones might be related to hormone levels and their receptor levels in animals and humans (Opitz et al., 2006; Vandenberg et al., 2012). We found that serum levels of testosterone varied in a large range among adult X. laevis (unpublished data), which could explain individual variability in the sensitivity of oocyte mature to testosterone. Therefore, it is necessary to use different individuals to repeat GVB experiment.

Although trenbolone and nandrolone have been found in surface water, effects of the two environmental androgens on amphibian oocyte maturation have never been reported in the literature.

**Table 1**

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Xenopus laevis 1</th>
<th>Xenopus laevis 2</th>
<th>Xenopus laevis 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 h</td>
<td>8 h</td>
<td>4 h</td>
</tr>
<tr>
<td>Testosterone</td>
<td>6.0</td>
<td>3.6</td>
<td>20.2</td>
</tr>
<tr>
<td>Trenbolone</td>
<td>580.5</td>
<td>586.0</td>
<td>2315.7</td>
</tr>
<tr>
<td>Nandrolone</td>
<td>25.6</td>
<td>24.6</td>
<td>31.1</td>
</tr>
</tbody>
</table>

**Fig. 1.** GVB induced by 4h-exposure (A) and 8h-exposure (B) to testosterone in three independent experiments, with a female Xenopus laevis for each experiment. Data (mean ± SD) are expressed as percentages. Each data point represents the mean percentage from three replicate culture wells, with 20 oocytes per well. Statistically significant differences (P < 0.05) between treatment groups and the control are tested by one-way analysis of variance (ANOVA) and shown as a, b, c.
To our knowledge, this is the first study to investigate effects of trenbolone and nandrolone on amphibian oocyte maturation. We found that trenbolone even at the low concentration of 10 nM (2.7 µg/L) can induce amphibian oocyte maturation (Fig. 2), despite the potential of trenbolone to induce GVBD was approximately 100-fold lower than that of testosterone. Given the µg/L levels of trenbolone in some water samples (Khan and Lee, 2012), our result has aroused new concerns for effects of trenbolone on oocyte maturation at environmentally relevant concentrations. Compared to trenbolone, nandrolone had higher potential to induce GVBD of Xenopus oocytes, in spite of lower potential than testosterone. Even, 10 nM nandrolone induced 50% GVBD of Xenopus oocytes with higher sensitivity to androgens (Fig. 3). It is reasonable to believe that nandrolone of lower concentrations than 10 nM could induce GVBD of Xenopus oocytes. Thus, effects of nandrolone on amphibian oocyte maturation should receive much more attention. In particular, co-existence of multiple AASs in environmental water, such as trenbolone, methyl testosterone, boldenone, and dihydrotestosterone (Gall et al., 2011; Liu et al., 2012), warrants a further study on the combined effect of these AAS on oocyte maturation of amphibians or aquatic wildlife.

Previous studies have shown that androgen-induced Xenopus oocyte maturation is mediated by classical ARs (Lutz et al., 2001). Given the high sensitivity of amphibian oocyte maturation to environmental androgens, we suggest that Xenopus GVBD can be used to test androgenic activity of suspicious environmental androgens. Several in vitro AR transactivation assays, such as yeast transactivation assay, are used to assay androgenic activity of endocrine disruptors (Blake et al., 2010; Christen et al., 2010; Gee et al., 2008; Sun et al., 2007). Effective concentrations of androgens as positive reference substances in these in vitro assays generally ranged 1–1000 nM. In the present study, the 4h-EC50 and 8h-EC50 values of testosterone for GVBD ranged 3.7–20.2 nM and 1.0–7.3 nM, respectively. In term of effective concentrations of androgens, the sensitivity of GVBD test using Xenopus oocytes is comparable to these in vitro assays in the literature. Although Xenopus GVBD is very sensitive to androgenic chemicals, one chemical cannot be determined whether or not to have androgenic activity by alone Xenopus GVBD test because progestin chemicals can also induce Xenopus GVBD (Fort et al., 2002). Then, it is necessary to combine other specific methods when determine the androgenic activity of chemicals using Xenopus GVBD.

In previous studies, AR antagonist flutamide was reported to inhibit partly androgen-induced GVBD of Xenopus oocytes (Lutz et al., 2001, 2003). In accordance with previous studies, we also found that only 10,000 nM flutamide inhibited weakly androgen-induced GVBD of Xenopus oocytes. However, vinclozolin, another well-known AR antagonist, had no effect on androgen-induced GVBD of Xenopus oocytes in this study. In the literature, there are inconsistent results in term of anti-androgenic activity of vinclozolin compared with flutamide. Several authors reported that vinclozolin and flutamide had similar anti-androgenic activity (Kjærstad et al., 2010; Kolle et al., 2011), whereas other studies demonstrated higher anti-androgenic activity of vinclozolin than flutamide (Freyberger et al., 2010) or in reverse (Jolly et al., 2009). In terms of the induction of GVBD, our results show that flu-
Tamamide seems to have higher anti-androgenic activity than vinclozolin. In the literature, flutamide and vinclozolin were reported to have high AR antagonistic activity and can inhibit androgen-induced effects at low concentrations (0.1 \( \mu \)M) in some assays (Freyberger et al., 2010; Kjærstad et al., 2010). In the present study, however, the inhibitory effect of flutamide only occurred at the highest concentration (10 \( \mu \)M), suggesting Xenopus GVBD is less sensitive to AR antagonists compared with other assay systems for anti-androgenic chemicals. Evalul et al. (2007) explained the weak antagonistic effect of flutamide on Xenopus GVBD by a proposal that androgens could induce signal pathways of Xenopus GVBD by binding to progesterone receptors (PRs) as well as ARs. In other words, androgens could still induce Xenopus GVBD by PRs, when ARs are antagonized by AR antagonists. Our finding that vinclozolin lacked AR antagonistic activity on androgen-induced GVBD provides a support for the proposal by Evalul et al. (2007).

Generally, methoxychlor is considered as a weaker AR antagonist than flutamide and vinclozolin (Maness et al., 1998). In this study, however, methoxychlor exhibited a dramatic inhibitory effect on androgen-induced GVBD of Xenopus oocytes (Fig. 4C). In accordance with our findings, Pickford and Morris (1999) reported that methoxychlor inhibited significantly progesterone-induced GVBD of Xenopus oocytes, and suggested that the inhibitory action of methoxychlor on progesterone-induced GVBD was not mediated by PRs. Given the fact that methoxychlor has a weaker AR antagonistic activity than flutamide and vinclozolin, we suggest that the inhibitory effect of methoxychlor on androgen-induced GVBD is not mediated directly by ARs but by some of downstream pathways of oocyte maturation. The mechanism of the inhibitory action of methoxychlor on androgen-induced GVBD may be similar to its mechanism for inhibiting progesterone-induced GVBD.

5. Conclusions

We for the first time reported stimulatory effects of trenbolone and nandrolone on Xenopus oocyte maturation, and demonstrated the sensitivity of Xenopus oocyte maturation to AAS. Our results have aroused new concerns for effects of AAS on oocyte maturation at environmentally relevant concentrations. Given the high sensitivity of amphibian oocyte maturation to environmental androgens, we suggest that Xenopus GVBD can be used to test androgenic activity of suspicious environmental androgens. The results on flutamide and vinclozolin show that Xenopus GVBD is not sensitive to AR antagonists. The inhibitory action of methoxychlor on androgen-induced GVBD demonstrates that androgen-induced Xenopus GVBD is a good model to study non-AR mediated effects of some chemicals on oocyte maturation.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

This work was supported by Grants from Hi-Tech Research and Development Program of China (863 Plan) (2012AA06A302), Public Welfare Research project (201109048, 201110250), and National Natural Science Foundation of China (21077125).

Appendix A. Supplementary material

Transparency Documents associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tiv.2013.12.003.

References


