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Effect of Endocrine Disruptors on the Oocyte Maturation and Ovulation in Amphibians, *Rana dybowskii*

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Abstract: Recently, we have shown that some endocrine disruptors, heavy metals, organotins and azoles suppressed steroidogenic enzymes such as P450 side-chain cleavage enzyme (P450scc) and aromatase in bullfrog ovarian follicles. In the present study, by using an amphibian ovarian follicle culture system, we examined the effects of these endocrine disruptors on maturation and ovulation of oocytes from *Rana dybowskii in vitro*. Ovarian fragments or isolated follicles were cultured for 24 h in a medium containing frog pituitary homogenate (FPH) or progesterone (P_4) with or without endocrine disruptors, and oocyte maturation (germinal vesicle breakdown, GVBD) and ovulation were examined. Among the organotins, tributyltin (TBT) strongly inhibited both FPH- and P_4-induced oocyte maturation (ED_{50}: 0.6 and 0.7 μM, respectively); however, tetrabutyltin (TTBT) and dibutyltin (DBT) showed only partial suppression, while monobutyltin (MBT) showed no inhibitory effect. All of the organotins suppressed P_4-induced oocyte ovulation very effectively at a low concentration, and TBT and DBT exerted an inhibitory effect on FPH-induced ovulation. Among the heavy metals, mercury (Hg), cadmium (Cd) and cobalt (Co) were very effective in inhibiting FPH-induced oocyte maturation and ovulation, while lead (Pb), arsenite (As) and zinc (Zn) were less effective. However, all of the heavy metals suppressed FPH-induced oocyte ovulation at a high dose (100 μM). Among the azoles, itraconazole (ICZ), ketoconazole (KCZ) and clotrimazole (CTZ) effectively inhibited FPH-induced oocyte maturation and ovulation, while econazole (ECZ), miconazole (MCZ) and fluconazole (FCZ) were considerably less effective. These results demonstrated that the abovementioned endocrine disruptors exhibited differential effects on oocyte maturation and ovulation in amphibian follicles and that the frog ovarian culture system could be used as an effective experimental tool to screen and evaluate the toxicity of various endocrine disruptors *in vitro*.

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Among the various environmental pollutants, endocrine disruptors are known to exhibit the most serious effects on animal reproduction and human health (de Solla et al., 1998; Fry, 1995). In general, endocrine disruptors have been reported to interfere with steroid hormone production or steroid-mediated signaling in the gonads and eventually lead to serious damage of the reproductive organs such as the ovary, vagina, oviduct, prostate or testis (Cocco and Benichou, 1998; Paulozzi, 1999; Sugawara et al., 2002). Some organotin compounds, azoles and heavy metals are known to adversely affect animal reproduction and are considered as representatives of endocrine disruptors.

For many years, organotins have been used as agricultural fungicides, rodent repellents and biocides in antifouling paints for ships and fishing nets. Furthermore, organotin residues have been found in many plants and animals, such as seaweeds, marine invertebrates, fish and birds (Strand and Jacobsen, 2005). Recently, it was found that these organotins suppressed various steroidogenic enzymes in different animal tissues (Schoenfelder et al., 2003; Ohno et al., 2005; Yamazaki et al., 2005). Furthermore, they were reported to suppress the activity of natural killer cells in humans (Whalen et al., 1999). Other chemicals classified as azoles are also known to exhibit the characteristics of endocrine disruptors. Azoles have been widely used as antifungal agents because they inhibit the biosynthesis of ergosterol that is essential for the normal function of fungal cytoplasmic membrane. Most of the azole agents are also known to suppress adrenal or gonadal steroidogenesis by inhibiting P450 side-chain cleavage enzyme (P450scc) activity. Ketoconazole (KCZ), one of the azoles, was observed to suppress ovulation and follicular progesterone

(P_4) production in rats (Tsafriri et al., 1998).

Heavy metals, the other type of endocrine disruptors, are the most typical environmental contaminants generated from various chemical industries. It is well known that heavy metals in soil and water can accumulate in foods such as meat, fish and vegetables and eventually result in human exposure. Various effects of heavy metals on animal reproduction have been reported by many investigators. For example, lead was observed to inhibit P450scc or 3β-hydroxysteroid dehydrogenase (3β-HSD) in rats (Thoreux-Manlay et al., 1995) or mouse Leydig cells (Liu et al., 2001). Similarly, cadmium was observed to suppress P_4 synthesis in cultured human placental trophoblast cells by inhibiting P450scc activity (Kawai et al., 2002).

Frog follicular oocytes arrested in meiotic prophase can be induced to mature and ovulate *in vitro* following treatment with frog pituitary homogenate (FPH) or P_4 during ovarian follicle or ovarian fragment culture (Kwon et al., 1992). In the present study, by using a frog ovarian follicle culture system, we investigated the effects of three different types of endocrine disruptors (butyltin, heavy metals and azoles) on hormone-induced oocyte maturation and ovulation of frog follicles *in vitro*. Our study data suggested that the frog ovarian follicle culture system could serve as a useful experimental model to screen or identify potential endocrine disruptors.

MATERIALS AND METHODS

Animals

Hibernating frogs (*Rana dybowskii*) were collected during winter (January) from streams in the Chonbuk area. The animals were housed in a cold room maintained in darkness at 4°C; they were kept (10-20 days) in a state of artificial hibernation in plastic boxes containing tap water (Ahn et al., 1993).

Hormones and reagents

Frog pituitary homogenate was prepared from pituitary glands obtained during the experimental period. The pituitary glands were dissected from the brain and homogenized in amphibian Ringer's solution (AR) by using an ultrasonic homogenizer (Ultrasonic W-380, USA) at 4°C. The homogenate was centrifuged (4°C, 10000 rpm, 15 min) to remove debris, and the supernatant was frozen (–20°C) in aliquots until required (Kwon et al., 1993; Kwon and Ahn, 1994). Progesterone (Sigma, St. Louis, MO, USA) was dissolved in vehicle composed of ethanol and propylene glycol (1 : 1). Butyltins {monobutyltin (MBT), dibutyltin (DBT), tributyltin (TBT) and tetrabutyltin (TTBT)} and heavy metals (cadmium, $CdCl_2$; mercury, $HgCl_2$; cobalt, $CoCl_2$; and arsenate, Na_2HAsO_4) were purchased from Sigma. Zinc ($ZnCl_2$) and lead [$(CH_3COOP)_2Pb$] were purchased from Junsei Chemical Co. (Japan). Butyltins and $ZnCl_2$ were dissolved in vehicle composed of ethanol and propylene glycol (1 : 1). $CdCl_2$, $(CH_3COOP)_2Pb$, Na_2HAsO_4, and $CoCl_2$ were dissolved in deionized water. The compounds $HgCl_2$ and azoles {(KCZ, clotrimazole (CTZ), miconazole (MCZ), econazole (ECZ), fluconazole (FCZ), and itraconazole (ICZ)} were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO and vehicle in the medium was below 1% v/v, and this concentration was not found to affect oocyte maturation and ovulation (data not shown).

Ovarian follicle culture

The animals were killed by decapitation, and the ovaries were removed immediately and placed in AR. Fresh ovaries were used for each experiment. When oocyte maturation was examined, individually isolated follicles from the ovary were cultured, and oocyte germinal vesicle breakdown (GVBD) was examined after culture. When oocyte ovulation was examined, ovarian fragments containing 10 follicles were cultured, and oocytes liberated from the ovarian fragments were counted after culture and regarded as ovulated oocytes (Kwon et al., 1992). Individual follicles or ovarian fragments were isolated from the ovaries using watchmaker's fine forceps under a stereomicroscope. Routine *in vitro* cultures were carried out using multiple 24-well culture dishes (Falcon, NJ, USA) in a 25°C shaking incubator (100~120 oscillations/min). Ten individual follicles or a fragment containing 10 follicles were cultured for 24 h in 1 ml of AR/well in the presence of FPH (0.05 gland/ml) or P_4 (100 ng/ml) and various doses (0.01~100 μM) of butyltins (MBT, DBT, TBT, or TTBT), heavy metals (Cd, Hg, Zn, Co, As, or Pb), or azoles (KCZ, CTZ, MCZ, ECZ, FCZ or ICZ). After the culture, the oocytes or fragments were fixed with 5% trichloroacetic acid and examined for oocyte GVBD or ovulation. The occurrence of oocyte GVBD or ovulation was expressed as the percentage of oocytes without any intact germinal vesicle or oocytes liberated from the fragment.

Statistical analysis

All experiments were repeated three to five times using sister follicles in the ovaries obtained from three animals. The differences between control and treated groups were analyzed by paired Student's t test using Prism statistical software. P values of less than 0.05 were considered significant.

RESULTS

Effects of butyltins on FPH- or P_4-induced oocyte maturation

Initially, experiments were carried out to determine whether

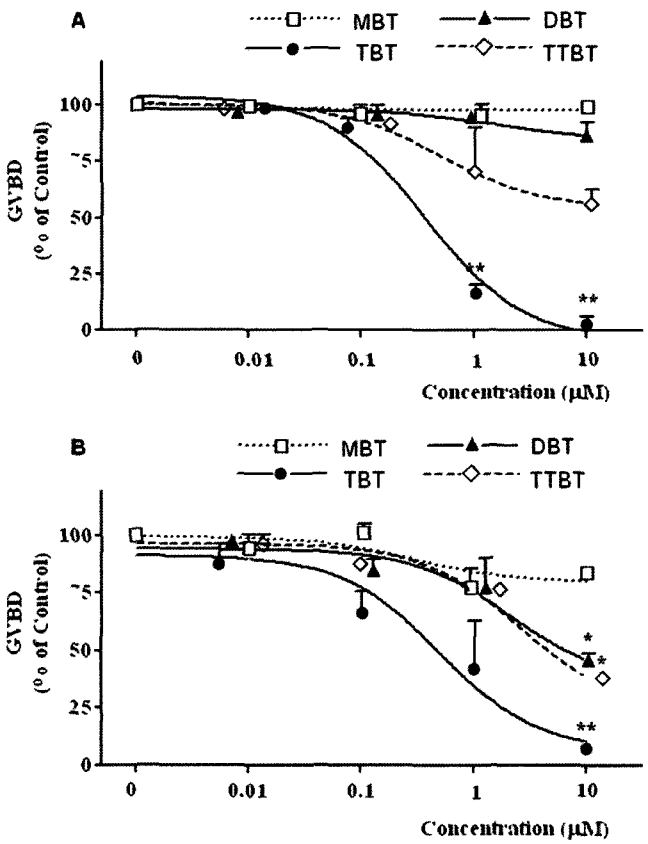

Fig. 1. Effect of butyltins on FPH or P_4-induced oocyte maturation *in vitro*. Isolated follicles were cultured for 24 h in the presence of FPH (0.05 gland/ml) or P_4 (100ng/well) and various concentrations of MBT, DBT, TBT and TTBT. After the culture, oocyte GVBD was examined. Each point in the figure represents average % of GVBD (mean ± SEM) based on data obtained from 3 animals. Effect of butyltins on FPH-induced oocyte maturation was depicted in A and that on P_4 in B. $*p < 0.05$, $**p < 0.01$, when compared to the control by paired Student's t-tests (n = 3).

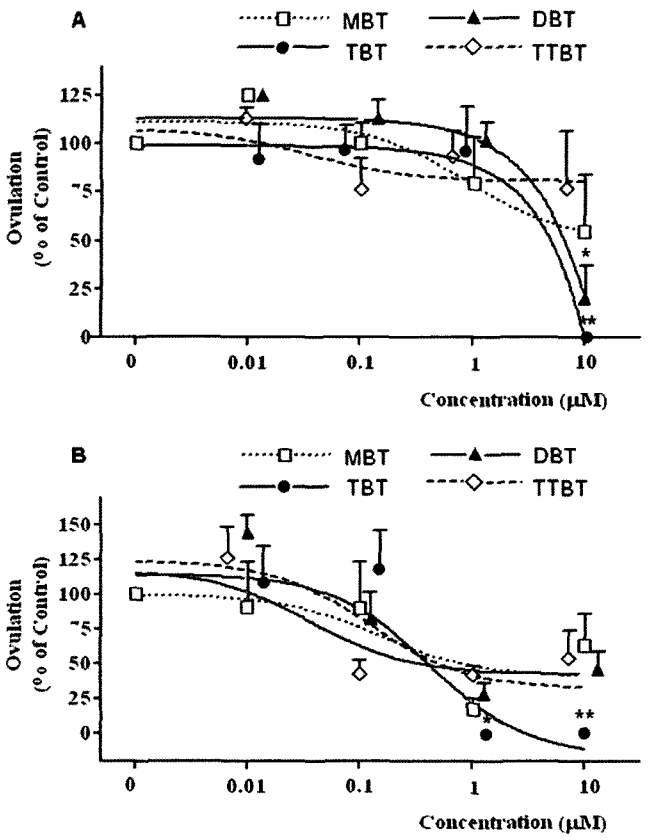

Fig. 2. Effect of butyltines on FPH or P_4-induced oocyte ovulation *in vitro*. Ovarian fragments containing approximately 10 follicles each were cultured for 24 h in the presence of FPH (0.05 gland/ml) or P_4 and various concentrations of butyltins MBT, DBT, TBT and TTBT. After the culture, number of oocytes liberated from fragment was counted. Each point in the figure represents average % of ovulation (mean ± SEM) based on data obtained from 5 animals. Results obtained from FPH induced ovulation was depicted in A and those from P_4 in B. $*p < 0.05$, $**p < 0.01$, when compared to the control by paired Student's t-tests (n = 5).

butyltins - well-known endocrine disruptors - might have any effects on hormone-induced oocyte maturation. Isolated follicular oocytes were incubated for 24 h in the presence of FPH (0.05 gland/well) or P_4 (100 ng/well) and various concentrations (0.01~100 μM) of butyltins (TTBT, TBT, DBT or MBT). Oocyte GVBD was examined after the culture. As shown in Fig. 1A, TBT significantly inhibited FPH-induced oocyte maturation at 1 μM (EC_{50}: 0.6 μM); in contrast, other butyltins (TTBT, DBT and MBT) exhibited considerably less or negligible inhibitory effects on FPH-induced oocyte maturation. Interestingly, TTBT and DBT partially suppressed P_4-induced oocyte maturation (approximately 50% inhibition), while MBT showed no effect (Fig. 1B). Thus, the toxic potential of these organotin compounds on FPH- or P_4-induced oocyte maturation appeared to be in the order of TBT > TTBT > DBT > MBT.

Effects of butyltins on FPH- or P_4-induced oocyte ovulation *in vitro*

In order to determine whether butyltins might suppress FPH- or P_4-induced oocyte ovulation *in vitro*, frog ovarian fragments were incubated in the presence of FPH (0.05 gland/well) or P_4 (100 ng/well) and various concentrations of butyltins. As shown in Fig. 2A, TBT and DBT significantly inhibited FPH-induced oocyte ovulation at a high concentration (10 μM; EC_{50}: 7.1 and 8.5 μM, respectively). However, MBT and TTBT had a negligible inhibitory effect on FPH-induced oocyte ovulation. In the case of P_4-induced oocyte ovulation, only TBT showed significant inhibition from 1 μM (EC_{50}: 0.7 μM), while the other compounds (TTBT, DBT, and MBT) exhibited marginal suppression (Fig. 2A and 2B). Taken together, these results demonstrated that

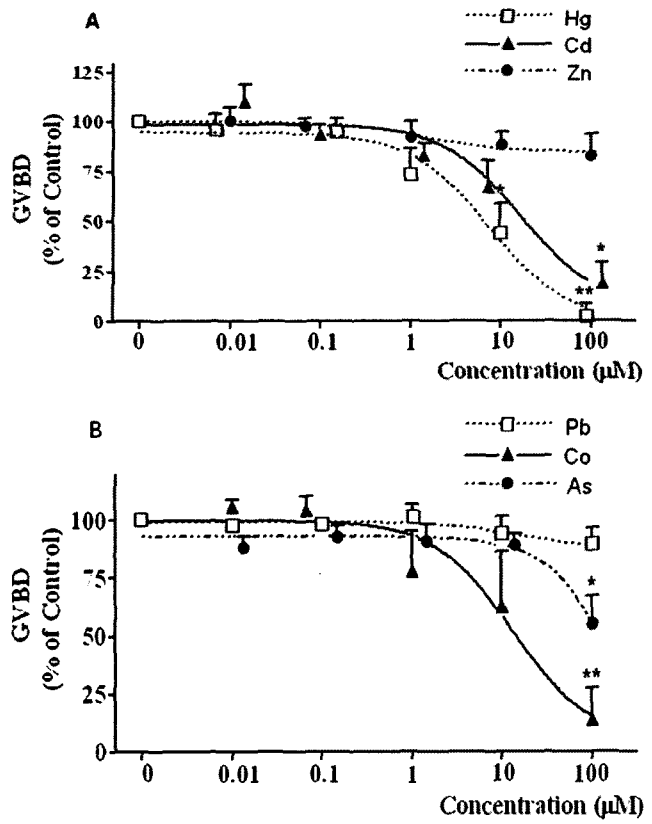

Fig. 3. Effect of heavy metals on FPH-induced oocyte maturation *in vitro*. Isolated follicles were cultured for 24 h in the presence of FPH (0.05 gland/ml) and various concentrations of Cd, Zn, Hg, Pb, Co or As. After the culture, oocyte GVBD was examined. Each point in the figure represents average % of GVBD (mean ± SEM) based on data obtained from 4 animals. Results of Hg, Cd and Zn were depicted in A and those of Pb, Co and As in B. $*p < 0.05$, $**p < 0.01$, when compared to the control by paired Student's t-tests (n = 4).

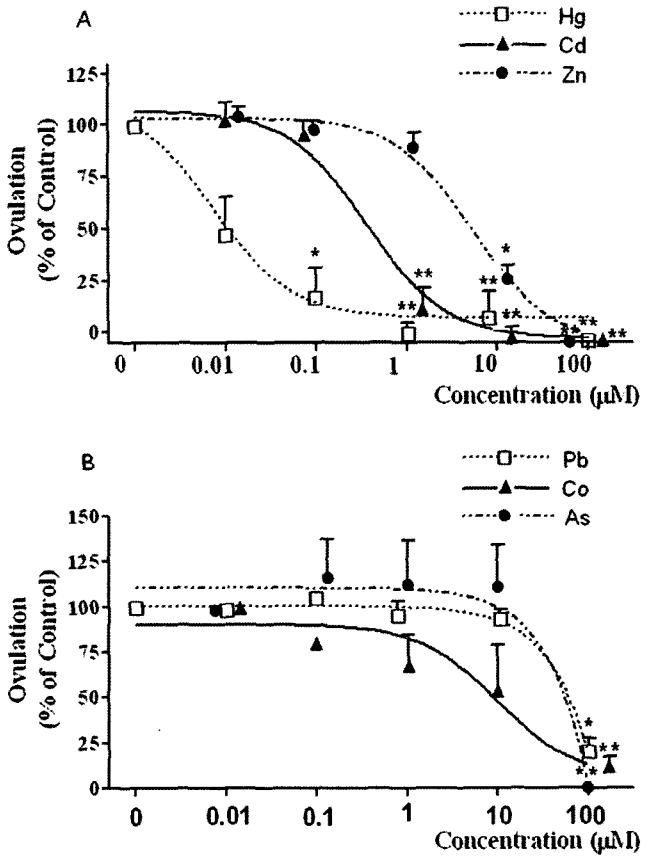

Fig. 4. Effect of heavy metals on FPH- or P_4-induced oocyte ovulation *in vitro*. Ovarian fragments containing approximately 10 follicles each were cultured for 24 h in the presence of FPH (0.05 gland/ml) and various concentrations of Cd, Zn, Hg, Pb, Co or As. After the culture, oocytes liberated from fragments were counted. Each point in the figure represents average % of ovulation based on data obtained from 5 animals. Results of Hg, Cd and Zn were depicted in A and those of Pb, Co and As in B. $*p < 0.05$, $**p < 0.01$, when compared to the control by paired Student's t-tests (n = 5).

only TBT had a strong inhibitory effect on FPH- or P_4-induced oocyte ovulation, and the other butyltins were considerably less effective. Overall, the toxic potential of the butyltins in the oocyte ovulation model was similar to that observed in the oocyte maturation model.

Effects of heavy metals on FPH-induced oocyte maturation *in vitro*

In order to ascertain whether heavy metals might have any effects on hormone-induced oocyte maturation in frogs, isolated follicular oocytes were incubated for 24 h in the presence of FPH (0.05 gland/well) or P_4 (100 ng/well) and various concentrations (0.01~100 μM) of heavy metals (Hg, Cd, Zn, Pb, Co, and As). Oocyte GVBD was examined after the culture. As shown in Fig. 3, among the heavy metals tested, only Hg, Cd and Co strongly inhibited FPH-induced oocyte maturation in a dose-dependent manner (EC_{50}: 8.3, 28 and 17 μM, respectively) from 10 μM concentrations only partial suppression was achieved at a high concentration (100 μM) however Zn and Pb failed to suppress FPH-induced oocyte maturation. Thus, it was evident that Hg, Cd and Co could be strong endocrine disruptors in nature. It was also interesting to note that Zn and Pb, the metals known to suppress steroidogenesis in fish and mammals, did not affect oocyte maturation.

Effects of heavy metals on FPH-induced oocyte ovulation *in vitro*

To determine whether heavy metals might inhibit FPH-induced oocyte ovulation, ovarian fragments of *R. dybowskii* were cultured for 24 h in the presence of FPH (0.05 gland/well) and various concentrations of heavy metals (0.01~100 μM). Oocytes ovulated from the ovarian fragment were counted after the culture. As shown in Fig. 4, Hg most strongly suppressed FPH-induced ovulation (EC_{50}: 0.01 μM) in a dose-dependent manner from very low concentrations (0.1 μM, $p < 0.05$). Similarly, Cd (EC_{50}: 0.6 μM), Zn (EC_{50}: 7.0 μM) and Co (EC_{50}: 9.5 μM) suppressed oocyte

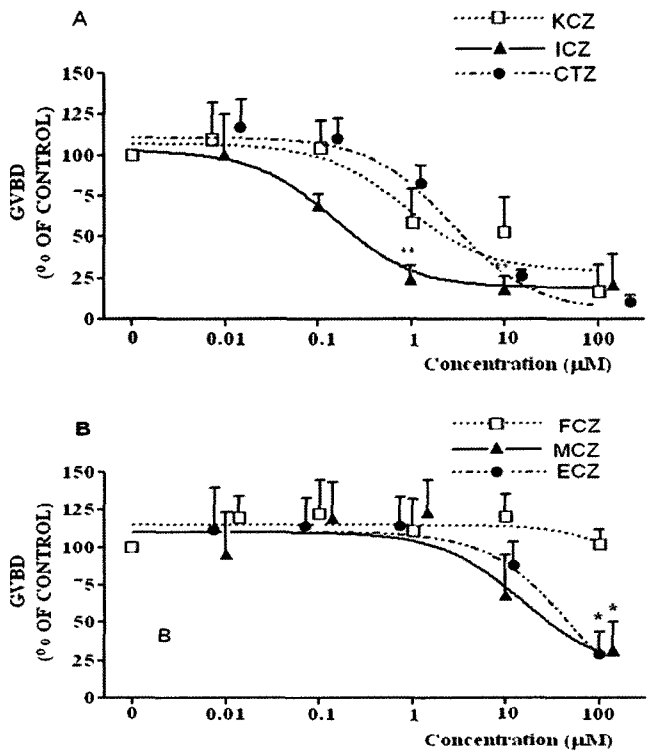

Fig. 5. Effects of azoles on FPH-induced oocyte maturation *in vitro*. Isolated follicles were cultured for 24 h in the presence of FPH (0.05 gland/ml) and various concentrations of KCZ, ICZ, CTZ, FCZ, MCZ or ECZ. After the culture, oocyte GVBD was examined. Each point in the figure represents average % of GVBD (mean ± SEM) based on data obtained from 3 animals. Results of KCZ, ICZ and CTZ were depicted in A and those of FCZ, MCZ and ECZ in B. $*p < 0.05$, $**p < 0.01$, when compared to the control by paired Student's t-tests (n = 3).

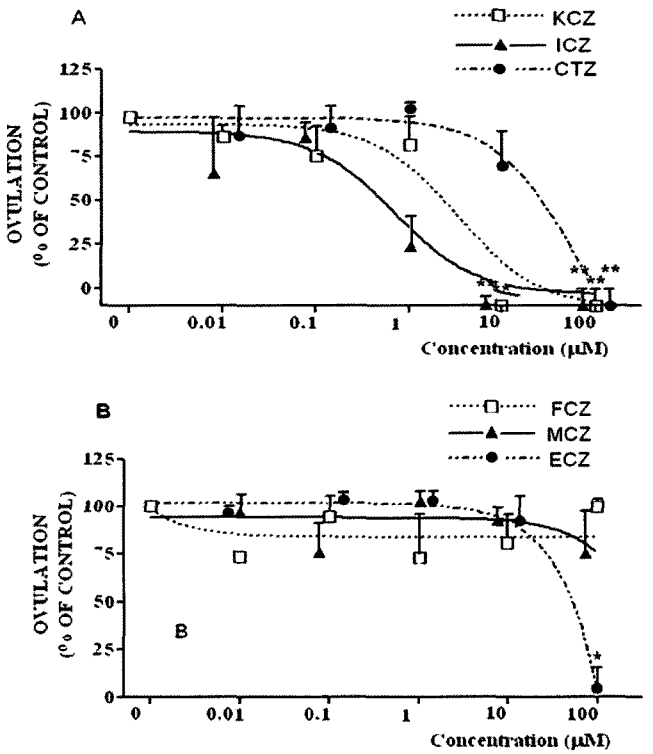

Fig. 6. Effects of azoles on FPH-induced oocyte ovulation *in vitro*. Ovarian fragments containing approximately 10 follicles each were cultured for 24 h in the presence of FPH (0.05 gland/ml) and various concentration of KCZ, ICZ, CTZ, FCZ, MCZ or ECZ. After the culture, the oocytes liberated from fragments were counted. Each point in the figure represents average % of ovulation (mean ± SEM) based on data obtained from 3 animals. Results of KCZ, ICZ and CTZ were depicted in A and those of FCZ, MCZ and ECZ in B. $*p < 0.05$, $**p < 0.01$, when compared to the control by paired Student's t-tests (n = 3).

ovulation in a dose-dependant manner; however, they were less effective than Hg. Pb and As inhibited ovulation only at the highest concentration tested (100 μM). Thus, the toxic potential of these heavy metals on FPH-induced oocyte ovulation was in the order of Hg > Cd > Zn (EC_{50}: 7.0 μM) > Co (EC_{50}: 9.5 μM) > As (EC_{50}: 73.4 μM) > Pb (EC_{50}: 78.3 μM).

Effects of azoles on FPH-induced oocyte maturation *in vitro*

The azole group of antifungal agents has been reported to exhibit some adverse effects on mammalian reproduction. To investigate whether azoles might inhibit oocyte maturation in amphibians, frog follicular oocytes were incubated for 24 h in the presence of FPH (0.05 gland/well) and various concentrations (0.01~100 μM) of azoles (KCZ, ICZ, MCZ, FCZ, CTZ and ECZ). Oocyte GVBD was examined after the culture. As shown in Fig. 5, ICZ, KCZ and CTZ inhibited FPH-induced oocyte maturation in a dose-dependent manner and significantly suppressed maturation from low concentrations (1 or 10 μM). The effective doses of ICZ, KCZ and CTZ that resulted in 50% suppression (ED_{50}) were 0.4, 4.5 and 6 μM, respectively (Fig. 5A). However, FCZ did not inhibit oocyte maturation (Fig. 5B), while MCZ and ECZ inhibited oocyte maturation only at a high concentration (100 μM). Thus, the toxic potential of azoles on FPH-induced oocyte maturation was in the order of ICZ (EC_{50}: 0.4 μM) > KCZ (EC_{50}: 4.5 μM) > CTZ (EC_{50}: 6 μM) > ECZ (EC_{50}: 46.6 μM) > MCZ (EC_{50}: 63.6 μM) > FCZ.

The above data demonstrated that azoles have differential effects on the maturation of amphibian oocytes and that ICZ and KCZ are particularly toxic to amphibian follicles.

Effects of azoles on FPH-induced oocyte ovulation *in vitro*

To determine whether azoles might also affect oocyte ovulation, frog ovarian fragments were incubated for 24 h in the presence of FPH (0.05 gland/well) and various concentrations of azoles (KCZ, ICZ, MCZ, FCZ, CTZ, and ECZ). Oocyte GVBD was examined after the culture. As shown in Fig. 6A, ICZ and CTZ strongly suppressed FPH-induced oocyte ovulation in a dose-dependent manner.

KCZ and ECZ also suppressed ovulation but only at a high concentration (100 μM) (Fig. 6). In contrast, MCZ and FCZ did not exhibit any suppressive effect on ovulation (Fig. 6B). Thus, the toxic potential of azoles on FPH-induced oocyte ovulation was in the order of ICZ (EC_{50}: 0.8 μM) > KCZ (EC_{50}: 4.4 μM) > CTZ (EC_{50}: 44 μM) > ECZ (EC_{50}: 72.2 μM) > MCZ and FCZ. Taken together, these results suggested that the azole compounds exhibited similar inhibitory effects on oocyte maturation and ovulation.

DISCUSSION

The present data demonstrated that some endocrine disruptors (butyltins, azoles and heavy metals) very effectively inhibited hormone-induced frog oocyte maturation and ovulation in a dose-dependent manner. The effective doses of the endocrine disruptors that inhibited frog oocyte maturation and ovulation were similar to those observed in other animal models. Thus, the present study raised the possibility that the amphibian follicle culture system could be used as an effective experimental tool to screen and evaluate the toxicity of potential endocrine disruptors.

Many investigators have reported that organotins, heavy metals, and azoles exhibit the characteristics of endocrine disruptors and can disrupt steroidogenesis by inhibiting enzyme activities (Schoenfelder et al., 2003; Ohno et al., 2005; Yamazaki et al., 2005). Our previous studies also showed that some butyltins (TBT, DBT, and TTBT), heavy metals (Cd, Zn, and Hg), and azoles (ICZ, KCZ, and CTZ) effectively disrupted ovarian follicular steroidogenesis in bullfrog by suppressing the activities of enzymes such as P450scc and aromatase (Choi et al., 2006; Kim et al., 2006). Based on the abovementioned results, in this study, we examined whether these endocrine disruptors affected FPH- or P_4-induced oocyte maturation or ovulation in the frog *R. dybowskii in vitro* by using a follicle and ovarian fragment culture system. This system was developed in our previous studies to examine oocyte maturation and ovulation in the Korean frog *R. dybowskii* (Kwon et al., 1992; Ahn et al., 1993; Chang et al., 1997).

Among the butyltins tested, TBT suppressed oocyte maturation and ovulation most effectively, while the other butyltins were less effective. Thus, the various butyltins tested in this study exerted differential effects on oocyte maturation and ovulation. The effective doses of butyltins that inhibited oocyte maturation and ovulation were similar to those that inhibited follicular steroidogenesis in bullfrog. Many investigators have previously shown that butyltins exert various adverse effects on animal cells in culture (Whalen, 1999; Yu et al., 2000; Heidrich et al., 2001). To date, it is unclear how butyltins inhibit oocyte maturation and ovulation. Some investigators suggested that amphibian oocyte maturation is sensitive to xenobiotics exhibiting estrogenic activity that have an ability to disrupt sex steroid signaling (Pickford and Morris, 1999), while other investigators reported that TBT showed a strong estrogenic activity (Choe et al., 2003). Further studies are required to understand the action mechanism of butyltins on oocyte maturation.

Studies on heavy metals revealed that various metals exhibit differential effects on oocyte maturation and ovulation. Among the heavy metals tested, Hg, Cd and Co significantly inhibited FPH-induced oocyte maturation and ovulation in a dose-dependent manner, while Zn, As and Pb were less effective (Fig. 3 and 4). Our previous studies also showed that Hg and Cd most effectively suppressed FPH-induced follicular steroidogenesis in bullfrog (Choi et al., 2006). Interestingly, Zn did not show any adverse effects on frog follicles, despite the fact that it strongly suppressed follicular steroidogenesis in fish (Ng and Liu, 1990) and mammals (Laskey and Phelps, 1991). Our previous study also showed that Zn suppressed follicular steroidogenesis in bullfrog (Choi et al., 2006). Thus, it is evident that the sensitivity of oocytes and follicle cells to heavy metals is not always identical.

Several investigators have reported that $HgCl_2$ (1.5 mg/kg body weight (BW)) could damage the ovarian function and reduce the number of superovulated oocytes in mice (Shen et al., 2000). Administration of $CdCl_2$ inhibited ovulation and decreased serum progesterone levels in golden hamster (Saksena and Salmonsen, 1983). Cd treatment was also found to inhibit the cumulus expansion of oocytes that is necessary for ovulation and follicle-stimulating hormone (FSH)-induced progesterone secretion by oocyte-cumulus complexes in pigs (Vrsanska et al., 2003). Thus, it is evident that Hg and Cd exert a serious adverse effect on the reproduction of amphibians and mammals.

The present data showed that among the azoles tested, ICZ, KCZ and CTZ strongly inhibited FPH-induced oocyte maturation and ovulation, whereas KCZ, CTZ and MCZ were considerably less effective in inhibiting both maturation and ovulation (Fig. 5 and 6). Interestingly, ICZ, KCZ and CTZ were also considerably more effective than other azoles in inhibiting follicular steroidogenesis in bullfrog (Kim et al., 2006). These findings demonstrate that some azoles have adverse effects on oocyte maturation, ovulation and follicular steroidogenesis in frogs. Lu et al. (2000) showed that ketoconazole - one of the azoles - significantly suppressed FSH-induced GVBD in cumulus cell-enclosed oocytes in mice; furthermore, Tsafriri et al. (1998) showed that *in vivo* treatment of rats with ketoconazole suppressed ovulation by 40%. Thus, it is evident that some azoles exert adverse effects on the reproduction of amphibians and mammals.

In summary, the present study demonstrated that the well-known endocrine disruptors such as butyltins, heavy metals and azoles differentially affect frog oocyte maturation

and ovulation *in vitro*. Some of the endocrine disruptors suppressed maturation and ovulation in a dose-dependent manner from very low to high doses; other endocrine disruptors suppressed maturation and ovulation only at the highest dose; some did not exhibit suppression. The effective doses of the endocrine disruptors that inhibited frog oocyte maturation and ovulation were similar to those observed in experiments with other animal models. These findings raised the possibility that the frog oocyte maturation model, a simple bioassay system, could be used as a practical tool to screen and evaluate the toxicities of potential endocrine disruptors.

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