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# Effects of Azoles on the *In vitro* Follicular Steroidogenesis in Amphibians

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**Abstract:** Azoles are widely used antifungal agents, which inhibit the biosynthesis of fungal cell-membrane ergosterol. In this study, using an amphibian follicle culture system, the effects of azoles on follicular steroidogenesis in frogs were examined. Itraconazole (ICZ), clotrimazole (CTZ) and ketoconazole (KCZ) suppressed pregnenolone ($P_5$) production by the follicles ($ED_{50}$; 0.04 μM, 0.33 μM, and 0.91 μM, respectively) in response to frog pituitary homogenates (FPH). However, fluconazole (FCZ), miconazole (MCZ) and econazole (ECZ) were not effective in the suppression of $P_5$ production. Not all the azoles examined suppressed the conversion of exogenous $P_5$ to progesterone ($P_4$) (by 3β-HSD) or $P_4$ to 17α-hydroxyprogesterone (17α-OHP) (by 17α-hydroxylase), or androstenedione (AD) to testosterone (T) (by 17β-HSD). In contrast, CTZ, MCZ and ECZ in medium partially suppressed the conversion of 17α-OHP to AD (by $C_{17-20}$ lyase) ($ED_{50}$; 0.25 μM, 4.5 μM, and 0.7 μM, respectively) and CTZ, KCZ, ECZ and MCZ strongly suppressed the conversion of exogenous T to estradiol ($E_2$) (by aromatase) ($ED_{50}$; 0.02 μM, 8 μM, 0.07 μM, 0.8 μM, respectively). These results demonstrated that some azole agents strongly suppress amphibian follicular steroidogenesis and particularly, P450scc and aromatase are more sensitive to azoles than other steroidogenic enzymes.

**Key words:** Azole agents, amphibians, ovarian follicle, endocrine disrupter, steroidogenesis

Azoles are broadly used antifungal agents for the treatment of systemic infection for human (Bodey, 1992; Georgopapadakou, 1998). According to their chemical structure, azole compounds are classified into imidazoles or triazoles (Zarn et al., 2003). The imidazole group is comprised of ketoconazole (KCZ), clotrimazole (CTZ), miconazole (MCZ) and econazole (ECZ). The triazole group include fluconazole (FCZ) and itraconazole (ICZ). However, the antifungal activity of both groups is due to the same molecular mechanism and the main function is to inhibit the activity of P450scc and sterol 14α-demethylase in fungi and yeast (Aoyama et al., 1996), thereby blocking the biosynthesis of ergosterol, an essential sterol component in the membranes of fungi and yeast (Espinel-Ingroff 1997; Georgopapadakou 1998; Joseph-Horne and Hollomon 1997). Thus, it is suggested that the azole class of antifungal agents may have some toxic effects on human reproduction and that those agents can disrupt endocrine function.

Recently, however, it was found that azoles inhibit fish ovarian steroidogenesis by inhibition of P450scc enzymes (Pedro et al., 2000) and was also reported that azole fungicides affect mammalian steroidogenesis by inhibiting sterol 14α-demethylase and aromatase in mammals, raising the possibility that human health may be influenced by the fungicides (Zarn et al., 2003). One of the azoles, KCZ, was observed to suppress ovulation and follicular $P_4$ production in the rat (Tsafriri et al., 1998). Sterol 14α-demethylase is crucial for the production of meiosis-activating sterols which are known to modulate germ cell development and aromatase is responsible for the physiologic balance of androgens and estrogens in mammals (Conley and Hinshelwood, 2001; Zarn et al., 2003). Thus, it is suggested that the azole class of antifungal agents may have some toxic effects on the vertebrate reproductive system and that those agents can act as endocrine disrupters.

As amphibian ovarian follicles contain all kinds of steroidogenic enzymes that are necessary to produce steroid hormones (Ahn and Kwon, 1996), they provide a useful tool to examine steroidogenesis *in vitro*. In this study, the effects of various azoles on follicular steroidogenesis in frog ovarian follicles were systematically analyzed to confirm the toxicity of the azoles, to determine which enzymatic steps are sensitive to azoles in the process of steroidogenesis,

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and finally, to assess the possibility of utilizing this culture system to screen for potential endocrine disrupters.

## MATERIALS AND METHODS

### Animals

Female bullfrogs (*Rana catesbeiana)* were collected from a field in the Chunbuk area from spring to fall, maintained at room temperature, and sacrificed immediately prior to the experiment. In general, full grown follicles were isolated from frog ovaries and used for *in vitro* culture, but small sized follicles were used for $E_2$ study. Isolated follicles were cultured in the presence of FPH (0.05 gland/ml) or various precursor steroids (100 ng/ml) and azole agents at different concentrations (0.01~100 μM).

### Hormones and reagents

Steroids (Sigma, St. Louis, MO, USA) were dissolved in a solution composed of ethanol and propylene glycol (1 : 1). All azole compounds were dissolved in a solution of dimethylsulfoxide (DMSO). Different concentrations of regents were prepared by diluting the stock solution with various amounts of DMSO. The effects of gonadotropin were examined using frog pituitary homogenate (FPH). Pituitary glands were obtained from female frogs collected during the experimental period. Pituitaries were homogenized in Amphibian Ringer (AR) solution at 4°C with a glass homogenizer. The homogenate was centrifuged (4°C, 10,000 rpm, 15 min) to remove debris, and the supernatant was maintained frozen (−20°C) until needed (Kwon et al., 1993; Kwon and Ahn, 1994).

### Follicle culture

Ovaries from adult female frogs were surgically removed and placed immediately in AR solution (Kwon and Schuetz, 1985). Full grown follicles or small sized follicles were isolated from ovaries using watchmaker's fine forceps. 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 per min). Ten follicles were cultured in 1 ml of AR per well in the presence or absence of FPH (0.05 gland/ml) or various steroid precursors (100 ng/ml), and azole compounds in various doses (0.01~100 μM). After culture, product steroids in the follicle ($P_5$) or medium (other steroids) were measured by radioimmunoassay (RIA).

### Follicle extraction and sample preparation for RIA

As $P_5$ was not secreted into the culture medium, $P_5$ was extracted from follicles by a procedure previously described by Kwon and Schuetz (1985). After culture, follicles were extracted in the culture well using methanol (GR, Merck, 1 ml methanol per 10 follicles per well) and shaken for 15 min (100 oscillations per min). Methanol extracts were dried by vacuum using a centrifugal lyophlizer (Savent SVC-100; Hickville, USA), and reconstituted using 0.05 M gelatin phosphate buffered saline (GPBS, pH 7.2). Extracts in GPBS were assayed without further purification. For other steroids, the culture medium was directly used for measuring the steroids by RIA.

### Steroid radioimmunoassay (RIA)

Steroids secreted by the ovarian follicle into the medium during culture were measured by RIA. General assay procedures were adapted from those described by previous studies (Kwon et al., 1993; Kwon and Ahn, 1994). Labeled $P_5$ ([7-$^3$H (N)]-pregnenolone; 25 Ci/mmol) was purchased from Perkin Elmer Life science (Boston, MA, USA). Labeled $P_4$ (1,2,6,7-$^3$H-progesterone; 99Ci/mmol), 17α-OHP([1,2,6,7,-$^3$H])-hydroxy progesterone; 58.5Ci/mmol), labeled AD ([1,2,6,7-$^3$H]-androstenedione; 86.1 Ci/mmol, T([1,2,6,7-$^3$H]-testosterone; 98 Ci/mmol) and $E_2$ [2,4,6,7-$^3$H]-estradiol, 108 Ci/mmol) were obtained from Amersham (Buckinghamshire, England). The steroid antiserum against AD was purchased from Sigma (St. Louis, MO, USA) and those against $P_5$, $P_4$, 17α-OHP, T and $E_2$ were obtained from Biogenesis (England). The $P_5$ antiserum cross-reacted 19% with $P_4$, and less than 3% with 17α-OHP, AD, and T. The $P_4$ antiserum cross-reacted 4% with T, 2% with 17α-OHP and less than 0.1% with other steroids. The 17α-OHP antiserum cross-reacted 0.25% with $P_4$, and less than 0.1% with other steroids. The AD antiserum cross-reacted 4.5% with T, and less than 0.1% with other steroids. The T antiserum cross-reacted 3.3% with 11β-hydroxy testosterone, and less than 0.1% with $E_2$, $P_4$ and other steroids. The $E_2$ antiserum cross-reacted 5% with oestriol, and less than 0.01% with $P_5$, $P_4$, 17α-OHP and T. Each sample was quantified for tritium using a Packard Tri-Carb 2900TR liquid scintillation analyzer. Routinely, duplicate steroid standards were included in each assay (5~2,000 pg). The between and within assay coefficients of variation (CV) for $P_5$ were 9.2% and 9.3%, respectively. The CVs for $P_4$ were 11.5% and 6.7%, 6.2% and 6.5% for 17α-OHP, 5.6% and 6.9% for AD, 6.3% and 7.9% for T, and 6.6% and 7.7% for $E_2$, respectively.

### Statistical analysis

Differences between control and treated groups were evaluated by Student's t-test using Prism statistical software. P-values less than 0.05 were considered significant.

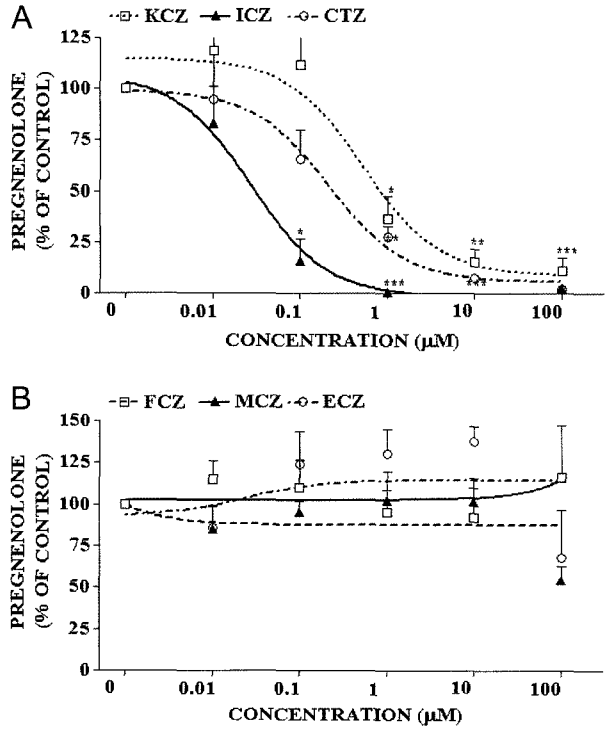


**Fig. 1.** Effects of azoles on the FPH-induced pregnenolone synthesis by frog ovarian follicles *in vitro*. Isolated full-grown follicles were cultured for 18 h in the presence of FPH (0.05 gland/ml) with or without various concentrations of azoles such as KCZ, ICZ, CTZ (A), and FCZ, MCZ, ECZ (B). Each point in the figure represents relative $P_5$ levels (mean SEM, % of control). $*p < 0.05$, $**p < 0.01$, when compared to the control by paired Student's t-tests (n = 3, 3 animals).

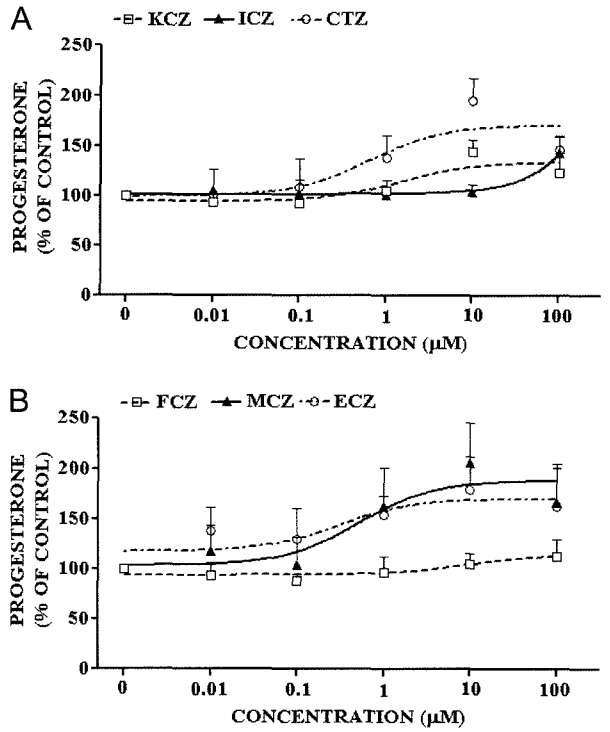


**Fig. 2.** Effects of azoles on the conversion of $P_5$ to $P_4$ by frog ovarian follicles *in vitro*. Isolated full-grown follicles were cultured for 18 h in the presence of $P_5$ (100ng/ml) with or without various concentrations of azoles such as KCZ, ICZ, CTZ (A), and FCZ, MCZ, ECZ (B). Each point in the figure represents relative $P_4$ levels (mean SEM, % of control). (n = 4, 4 animals).

## RESULTS

### Effects of azoles on the production of pregnenolone ($P_5$) in the presence of FPH by frog ovarian follicles

Initially, the effects of azoles on the conversion of cholesterol to pregnenolone ($P_5$) by P450scc were examined. As it was known that the exogenous cholesterol could not be utilized by the follicles (Petrino and Schuetz, 1987), FPH was used to stimulate $P_5$ production utilizing an endogenous precursor. Thus, the activity of P450scc was estimated from the amount of $P_5$ produced by the follicles. Isolated follicles were cultured for 18 h in the presence of FPH (0.05 gland/ml) and/or azoles at various concentrations (0.01~100 μM). After culture, the amount of $P_5$ in the follicles was measured by RIA. A considerable amount of $P_5$ was produced by the follicles in response to FPH (999~1531 pg/follicle, n = 6). As shown in Fig. 1A, ICZ strongly inhibited FPH induced $P_5$ synthesis in a dose-dependent manner ($ED_{50}$, 0.04 μM) when represented as a percent of the control. KCZ and CTZ also inhibited steroid synthesis significantly from 1 μM dose-dependently ($ED_{50}$; KCZ, 0.91 μM and CTZ, 0.33 μM). However, FCZ, MCZ and ECZ did not inhibit $P_5$ production (Fig. 1B). Thus, the toxicities of azoles with regard to P450scc were in the order of ICZ > CTZ > KCZ > FCZ, MCZ and ECZ.

### Effects of azoles on the conversion of $P_5$ to $P_4$ by frog ovarian follicles

To assess the effects of azoles on 3β-hydroxysteroid dehydrogenase (3β-HSD), which converts pregnenolone ($P_5$) to progesterone ($P_4$) in the follicles, ovarian follicles were cultured in medium containing $P_5$ (100 ng/ml) as a precursor. After culture for 18 h, the conversion of exogenous $P_5$ to $P_4$ by the follicles was examined. The amount of $P_4$ converted from $P_5$ in plain medium (control) ranged from 416~1153 pg/follicle (n = 8). Without the addition of $P_5$, a very low level of $P_4$ (1~107 pg/follicle, n = 8) was produced by the follicles. The results showed that most of the azoles tested did not suppress the activity of 3β-HSD, rather, $P_4$ seemed to be accumulated in medium at high concentrations of azoles (10 μM or 100 μM) (Figs. 2A and B). However, this seemed to be due to the inhibition of steroid metabolism, rather than the stimulation of 3β-HSD by the azoles. Thus, the azoles tested did not seem to affect 3β-HSD activity directly.

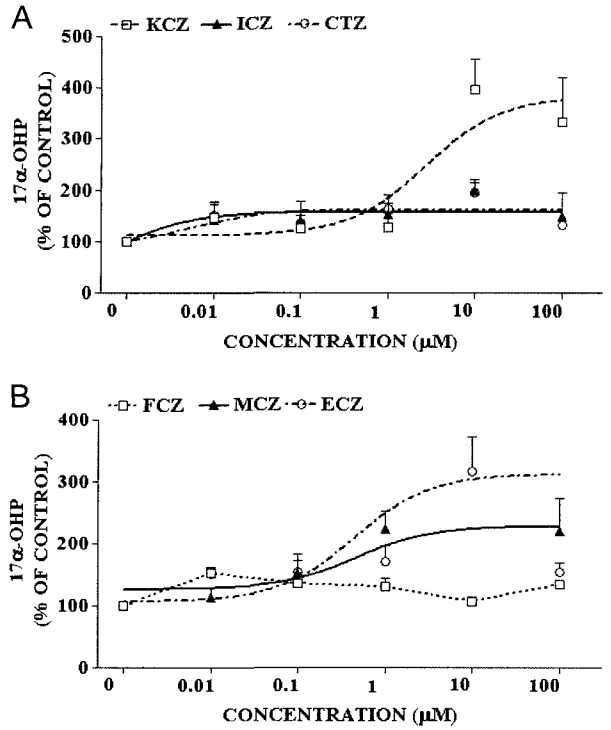


**Fig. 3.** Effects of azoles on the conversion of $P_4$ to 17α-OHP by frog follicles *in vitro*. Isolated full-grown follicles were cultured for 18 h in the presence of $P_4$ (100 ng/ml) with or without various concentrations of azoles such as KCZ, ICZ, CTZ (A), and FCZ, MCZ, ECZ (B). Each point in the figure represents relative 17α-OHP levels (mean SEM, % of control) (n = 3, 3 animals).

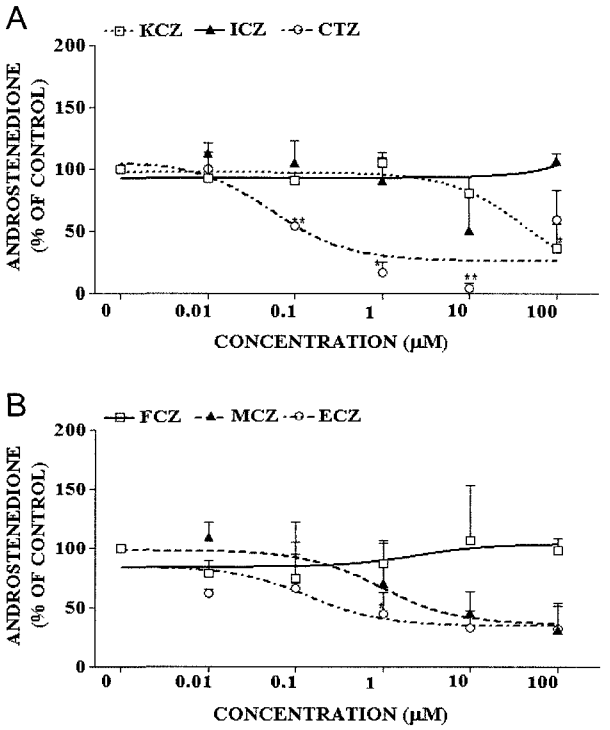


**Fig. 4.** Effects of azoles on the conversion of 17α-OHP to AD by frog follicles *in vitro*. Isolated full-grown follicles were cultured for 18 h in the presence of 17α-OHP (100 ng/ml) with or without various concentrations of azoles such as KCZ, ICZ, CTZ (A), and FCZ, MCZ, ECZ (B). Each point in the figure represents relative AD levels (mean SEM, % of control). $*p < 0.05$, $**p < 0.01$, when compared to the control by paired Student's t-tests (n = 4, 4 animals).

### Effects of azoles on the conversion of $P_4$ to 17α-OHP by frog ovarian follicles

To determine whether azoles have any effect on the activity of 17α-hydroxylase, which converts $P_4$ into 17α-OHP in the follicles, frog ovarian follicles were cultured in medium containing $P_4$ (100 ng/ml), and the conversion of the exogenous $P_4$ to 17α-OHP by the follicles was examined. The amounts of 17α-OHP converted from $P_4$ (100 ng/well) during culture in control follicles was in the range of 43~294 pg/follicle (n = 6). As shown in Fig. 3, the amount of 17α-OHP increased by several times the value observed for the control in high doses (10 μM or 100 μM). The accumulation of 17α-OHP seems to be due to the inhibition of steroid metabolism rather than the stimulation of 17α-hydroxylase by the azoles. Like 3β-HSD, 17α-hydroxylase activity may not be affected by azoles in medium.

### Effects of azoles on the conversion of 17α-OHP to AD by frog ovarian follicles

To ascertain whether azoles have any effect on the activity of $C_{17\text{-}20}$ lyase, which converts 17α-OHP into AD in the follicles, ovarian follicles were isolated and cultured in medium containing 17α-OHP (100 ng/ml) for 18 h, and the conversion of exogenous 17α-OHP to AD by the follicles was examined. The amount of AD secreted by the control follicles ranged from 111~626 ng/follicle (n = 8). Without addition of 17α-OHP, low levels of AD (0~39 pg/follicle, n = 8) were produced by the follicles. As shown in Figure 4, CTZ significantly inhibited conversion from a very low dose ($ED_{50}$; 0.25 μM, 55% inhibition at 0.1 μM), while KCZ inhibited conversion only at high concentrations (100 μM) (Fig. 4A). MCZ (46% inhibition at 10 μM, $ED_{50}$, 4.5 μM) and ECZ (45% inhibition at 1 μM) showed a partial, but significant inhibition of conversion (Fig. 4B). ICZ and FCZ did not affect the conversion at all (Figs. 4A and B). Thus, the toxicities of azoles on $C_{17\text{-}20}$ lyase were in the order of CTZ > ECT > MCZ > KCZ > ICZ and FCZ.

### Effects of azoles on the conversion of AD to T by frog ovarian follicles

In order to examine the effect of azoles on 17β-hydroxysteroid dehydrogenase (17β-HSD), which converts AD to T, isolated follicles were cultured in medium containing AD (100 ng/ml) and the conversion of AD to T by the follicles was examined. The amounts of T in the control group ranged from 35~438 pg/follicle (n = 6). As shown in Fig. 5, most of the azoles tested here had no effect on the conversion of

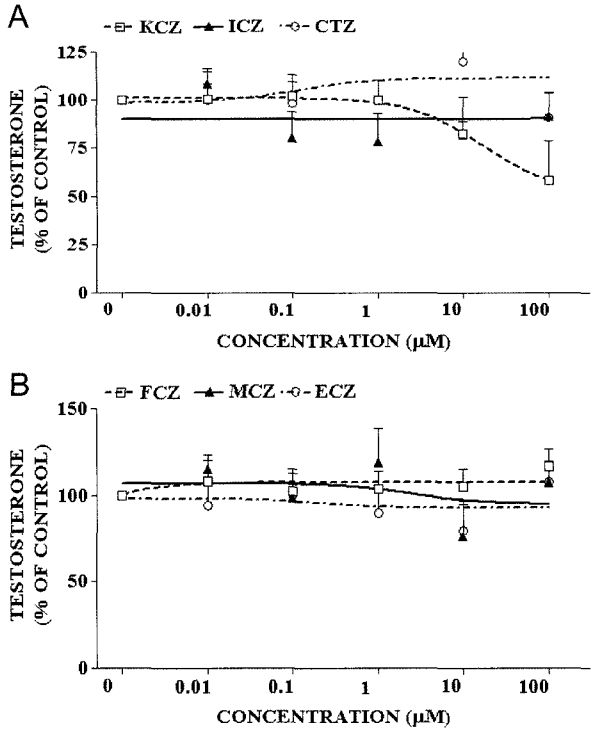


**Fig. 5.** Effects of azoles on the conversion of AD to T by frog follicles *in vitro*. Isolated full-grown follicles were cultured for 18 h in the presence of AD (100 ng/ml) with or without various concentrations of azoles such as KCZ, ICZ, CTZ (A), and FCZ, MCZ, ECZ (B). Each point in the figure represents relative T levels (mean SEM, % of control) (n = 3, 3 animals).

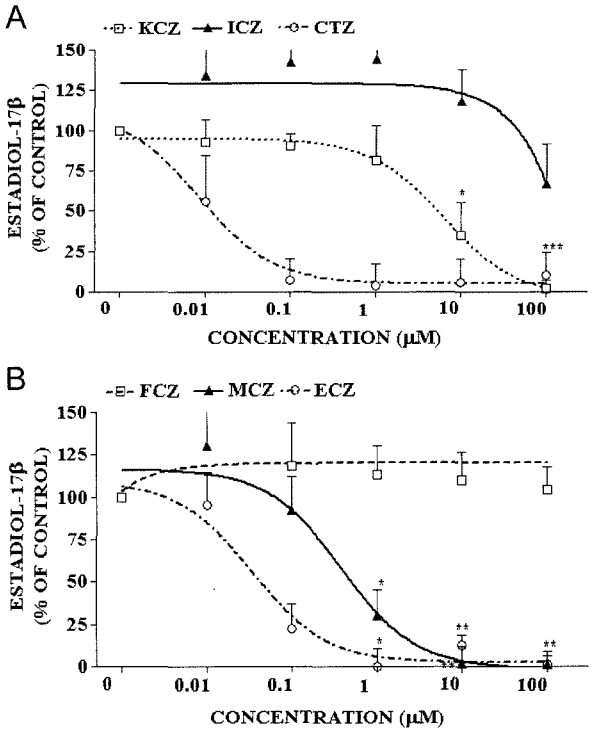


**Fig. 6.** Effects of azoles on the conversion of T to $E_2$ by frog follicles *in vitro*. Isolated small sized follicles were cultured for 18 h in the presence of T(100ng/ml) with or without various concentrations of azoles such as KCZ, ICZ, CTZ (A), and FCZ, MCZ, ECZ (B). Each point in the figure represents relative $E_2$ levels (mean SEM, % of control). $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, when compared to the control by paired Student's *t*-tests (n = 4, 4 animals).

AD to T. Only KCZ showed a slight inhibition of conversion, and then only at the highest dose (100 μM). Thus, it is evident that azoles do not affect 17β-HSD activity in frog ovarian follicles.

### Effects of azoles on the conversion of T to $E_2$ by frog ovarian follicles

To examine whether azoles affected the aromatase, which converts T to $E_2$ in the follicles, growing ovarian follicles (small size) were isolated and cultured in medium containing testosterone (100 ng/ml) and the conversion of T to $E_2$ by the follicles was examined. Our previous study showed that only growing follicles could produce $E_2$ (Ahn et al., 1999). The amounts of T levels in the control group ranged from 120~414 pg/follicle (n = 6). Figure 6. shows that CTZ and ECZ in medium very strongly suppressed the conversion in a dose-dependent manner ($ED_{50}$; 0.02 μM and 0.07 μM, respectively). MCZ and KCZ also exhibited a dose-dependent inhibition on the conversion of T to $E_2$ ($ED_{50}$; 0.8 μM and 8 μM, respectively), however, FCZ and ICZ did not affect this conversion significantly (Fig. 6). Thus, the toxicities of azoles on aromatase were in the following order, CTZ > ECZ > MCZ > KCZ > ICZ and FCZ. The data indicate that most azoles suppressed aromatase very effectively in comparison with other enzymes.

## DISCUSSION

Recent reports demonstrate that many currently-used antifungal agents have the capacity to disrupt reproductive function in animals. Although this reproductive dysfunction is typically characterized by alterations in serum steroid hormone levels, disruptions in spermatogenesis, and loss of fertility, the specific mechanisms involved in antifungal agent-induced infertility remain unclear. Usually reproductive dysfunction is correlated with disrupted regulation of steroid hormone synthesis by the key regulators; steroidogenic enzymes (Walsh LP et al., 2000a, b). The biosynthesis of all steroid hormones begins with cleavage of the side chain of cholesterol to form pregnenolone, which is catalyzed by P450scc. As a subsequent event, each hormone produced is converted into another form by the action of a corresponding enzyme in the steroidogenic pathway (Ahn et al., 1999; Walsh et al., 2000a, b; Payne and Hales, 2004). Therefore, if some agents negatively affect steroidogenic enzyme function, impaired regulation of steroid hormone synthesis and further reproductive dysfunction may occur.

Azole agents exhibit broad antifungal activity and are widely used in clinical therapies for various infections. Because they also suppress estrogen production, they are also used in the management of advanced estrogen-responsive breast tumors in postmenopausal women (Murray 2001). The actual target enzyme of azole agents is expressed in many species, including humans. In previous research, several azole agents were shown to inhibit the activity of aromatase and to disturb the balance of androgen and estrogen *in vivo* and further certain anti-fungal agents have toxicity in steroidogenesis and the reproductive system (Pedro et al., 2000; Zarn et al., 2003). Until now, however, it has not been clear whether each azole agent has specificity for a particular steroidogenic enzyme. In this study, in order to screen the effects of azole agents as disrupters of steroidogenesis, and to identify the target molecules, we used a frog follicle culture system with many kinds of enzyme involved in the synthesis of steroid hormones.

In the present study, we showed that itraconazole ($ED_{50}$; 0.04 μM), ketoconazole ($ED_{50}$; 0.91 μM) and clotrimazole ($ED_{50}$; 0.33 μM) strongly suppressed FPH-induced $P_5$ production in a dose-dependent manner, while fluconazole, miconazole, and econazole did not affect steroid production (Fig. 1). Thus, it is evident that ICZ, KCZ and CTZ strongly inhibited P450scc activity in the follicle whereas FCZ, MCZ and ECZ were not effective inhibitors of P450scc activity. Interestingly, not all the azoles examined inhibited follicular conversion of exogenous pregnenolone to progesterone, progesterone to 17α-hydroxyprogesterone and androstenedione to testosterone during culture (Figs. 2, 3 and 5), indicating that the azoles in medium did not suppress 3β-HSD, 17α-hydroxylase and 17β-HSD activity in the follicles. In some cases, the added azoles even stimulated the accumulation of steroid produced by the follicles during culture (Figs. 2 and 3) and appeared to stimulate corresponding steroidogenic enzymes in the follicles. However, this is probably not the case. Rather, the accumulation of steroid may have been caused by the suppression of the metabolism of product steroid by the azoles. If the enzymes in the downstream of the steroidogenic pathway, such as $C_{17\text{-}20}$ lyase or aromatase, are suppressed by the azoles, intermediate metabolite steroids such as $P_4$ and 17α-OHP will be accumulated in medium. In fact, our data showed that CTZ ($ED_{50}$; 0.25 μM), ECZ ($ED_{50}$; 0.6 μM) and MCZ ($ED_{50}$; 4.5 μM) suppressed the conversion of 17α-OHP to AD by inhibiting $C_{17\text{-}20}$ lyase (Fig. 4). Furthermore, CTZ ($ED_{50}$; 0.02 μM), KCZ ($ED_{50}$; 8 μM), ECZ ($ED_{50}$; 0.07 μM) and MCZ ($ED_{50}$; 0.8 μM) in medium strongly suppressed the conversion of T to $E_2$, suggesting that these azoles strongly inhibited aromatase in follicles (Fig. 6). These data demonstrated that P450scc and aromatase are more sensitive to exposure to azoles than other steroidogenic enzymes in the follicles, such as 3β-HSD, 17α-hydroxylase and 17β-HSD. It is also of interest that the toxicity of azoles appeared to be different among enzymes. For example, ICZ and CTZ were most effective in the inhibition of P450scc whereas CTZ and ECZ were most effective in the inhibition of aromatase. In contrast, ECZ and MCZ were very effective in the inhibition of aromatase, but did not affect P450scc activity at all (Figs. 1 and 6).

Taken together, these results demonstrate that some azole agents, irrespective of class, have adverse effects on steroidogenesis and that they have different properties which may act on different specific steps of steroid metabolism, even though they share nearly the same antifungal activity with the same molecular mechanisms. Moreover, our findings suggest that if we can use this amphibian follicle culture system as an effective experimental tool to identify and investigate endocrine disrupters or environmental toxins, and to determine an environmentally safe dosage of therapeutic agents in clinical use. Based on the clear effects on steroidogenic enzymes *in vitro*, the structural properties of each of the azole agents with regard to their inhibitory activity against steroidogenic enzymes should be studied.

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