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Effects of Heavy Metals on the *in vitro* Follicular Steroidogenesis in Amphibians

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Abstract: Heavy metals are well known as important environmental pollutants and also considered as endocrine disrupters. This study was performed to evaluate the direct effects of heavy metals such as cadmium (Cd), zinc (Zn), mercury (Hg), lead (Pb), cobalt (Co), and arsenic (As) on the various steroidogenic enzymes in frog ovarian follicles. Ovarian follicles from *Rana catesbeiana* were isolated and cultured for 18 hours in the presence of frog pituitary homogenate (FPH, 0.05 gland/ml) or various steroid precursors with or without heavy metals (0.01-100 μM), and steroid levels in the follicle or culture medium were measured by radioimmunoassay (RIA). Thus, the steroidogenic enzyme activities were indirectly evaluated by measuring the converted steroid levels from the added precursor steroid. Among heavy metals, Hg, Cd and Zn significantly inhibited FPH-induced pregnenolone (P_5) production by the follicles (EC_{50}, 4.0 μM, 25.6 μM and 5.7 μM, respectively), and also suppressed the conversion of testosterone (T) to estradiol 17β (E_2) (EC_{50}, 4.2 μM, 7.5 μM and 80.0 μM) while Pb, Co and As are not or less effective in the inhibition. Other enzymes such as C_{17-20} lyase and 17β-hydroxysteroid dehydrogenase (17β-HSD) were suppressed only in the high concentration of Hg, Cd and Zn. Taken together, these data demonstrate that cytochrome P450 side chain cleavage (P450scc) and aromatase are much more sensitive to heavy metals than other steroidogenic enzymes and Hg, Cd and Zn show stronger toxicity to follicles than other heavy metals examined.

Keywords: Amphibians, ovarian follicles, endocrine disruptors, steroidogenesis, steroidogenic enzyme

Heavy metals are dispersed throughout the modern environment mainly as a result of pollution from a variety of sources. High concentration of heavy metals in soil and water resulted in the accumulation of them in foods such as meat, fish, and eventually exposed to humans. Prolonged exposure to heavy metals provides a large number of adverse effects to humans. Some of heavy metals could exert xenoestrogenic activities and some others promote disease such as cancers and sexual abnormalities (Baccarelli, 1999; Schantz and Widholm, 2001).

Various effects of heavy metals on animal reproduction also have been described. For example, lead was known to inhibit both basal and human chorionic gonadotrophin (hCG) - stimulated testosterone production in rat leydig cell (Thoreux-Manlay et al., 1995). Lead was also known to suppress the steroidogenesis of mouse Leydig tumor cells (MA-10) by inhibiting steroidogenic enzymes such as P450 side-chain cleavage (P450scc) or 3β-hydroxysteroid dehydrogenase (3β-HSD) (Liu et al., 2001). Similarly, Cd was known to inhibit progesterone synthesis in cultured granulosa cells from rat and human (Pasky et al., 1997; Piasek and Lasky, 1994; Piasek and Lasky, 1999). Cd was found to suppress the progesterone synthesis in cultured human placental trophoblast cells by inhibiting steroidogenic enzyme activity such as P450scc (Kawai et al., 2002). Heavy metals such as Hg, Cd and Zn also appeared to suppress steroidogenesis in the dispersed interrenal cells of fish (Leblond and Hontela, 1999). They showed that cortisol synthesis in the interrenal steroidogenic cells of rainbow trout was suppressed strongly by heavy metals in the order of Hg > Cd > Zn (Leblond and Hontela, 1999).

However, there were no systematic studies on the effect of heavy metals or other endocrine disrupters on the follicular steroidogenesis *in vitro* or *in vivo*. Because frog ovarian follicles are big enough to handle and contain all the steroidogenic pathways from cholesterol to estradiol, they may provide a useful model for this study.

By the use of frog ovarian follicle culture system, present study was carried to find out the enzymatic step(s) that is

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sensitive to heavy metals in the steroidogenic pathways and to compare the toxicity of heavy metals on the steroidogenesis, and finally to develop a screening procedure for potential endocrine disrupters using this amphibian follicle culture system.

MATERIALS AND METHODS

Animal

Female frogs were collected from fields in the Chunbuk area from spring to fall and kept in a water tank in room temperature. Frogs were used in a week after capture.

Hormones and reagents

Various steroid precursors (pregnenolone, P_5; progesterone, P_4;17α- hydroxy progesterone, 17α-OHP; androstenedione, AD; testosterone, T; and estradiol 17α, E_2) were purchased from Sigma (St. Louis, Mo, USA). Cadmium chloride hemi (penta-hydrate) ($CdCl_2 \cdot 2.5H_2O$), mercuric chloride ($HgCl_2$), cobalt chloride hexahydrate ($CoCl_2 \cdot 6H_2O$) and sodium arsenate dibasic heptahydrate ($Na_2HAsO_4 \cdot 7H_2O$) were also purchased from Sigma (St. Louis, Mo) and zinc chloride ($ZnCl_2$) and lead (II) acetate trihydrate ($(CH_3COOP)_2Pb \cdot 3H_2O$ were purchased from Junsei Chemical Co. (Japan). Steroid precursors and $ZnCl_2$ were dissolved in vehicle composed of ethanol and propylene glycol (1 : 1). $CdCl_2$, $(CH_3COO)_2Pb$, Na_2HAsO_4 and $CoCl_2$ were dissolved in deionized water and $HgCl_2$ were dissolved in demehtyl sulfoxyde (DMSO). The final concentration of DMSO and vehicle in the medium was below 1% v/v and was not found to influence on the steroid secretion (data not shown). Frog pituitary homogenate (FPH) was obtained from frogs. Pituitary Glands were homogenized in Amphibian Ringer solution (AR) with a ultrasonic homogenizer (Ultrasonic W-380, USA) at 4°C. The homogenate were centrifuged (4°C, 10,000 rpm, 15 min) to remove debris, and supernatant was frozen (−20°C) in aliquots until use (Kwon et al., 1993; Kwon and Ahn, 1994).

Follicle culture

Immediately after animal sacrifice by decapitation, ovaries were removed and placed in Amphibian Ringer solution (AR) (Kwon and Schuetz 1985). Full grown follicles were isolated from ovaries and ten follicles were cultured in each well of culture dishes (24well/dish; Nunc, Denmark). Cultures were treated with heavy metals of various doses (0.01 μM, 0.1 μM, 1 μM, 10 μM, 100 μM) in the presence of FPH (0.05 gland/ml) or various steroid precursors (100 ng/ml) in a shaker incubator (25°C) at 110 oscillations for 18 hr. At the end of incubation, medium were saved and kept in a deep freezer (−40°C) until use for measuring steroid production by steroid radioimmunoassay (RIA). Because P_5 was not secreted into medium, it was extracted from follicles by the procedure described elsewhere (Kwon and Schuetz, 1985).

Steroid radioimmunoassay

Steroid concentration (P_4, 17α-OHP, AD, T, and E_2) in the culture medium were measured by radioimmunoassay (RIA). General assay procedures were adapted from those described previously (Kwon et al., 1993; Kwon and Ahn, 1994). Labled P_5 ([7-^{3}H (N)]-pregnenolone; 25 Ci/mmol) was purchased from Perkln Elmer Life science (Boston, MA). Labeled P_4 (1,2,6,7-^{3}H-progesterone; 99Ci/mmol), 17α-OHP([1,2,6,7,-^{3}H])-hydroxy progesterone; 58.5 Ci/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, less than 3% with cholesterol, 17α-OHP, androstenedione, T and DHT. The P_4 antiserum cross-reacted 4% with corticosterone, 3% with 11-OHP, 1.5% with 17α-OHP and less than 0.1% with other steroids. 17α-OHP antiserum cross-reacted 0.25% with P_4, less than 0.1% with 21-deoxycortisol, less than 0.01% of corticosterone and 0.05% with 5α-pregnanedione. The AD antiserum cross-reacted 6% with dihydroepiandrostenedione, 4.5% with T, less than 0.001% with 17-Estradiol and P_4 and less than 0.1% with other steroids. The T antiserum cross-reacted 3.3% with 11β-hydroxy testosterone, less than 0.1% with 17α-methyl testosterone, E_2, P_4 and other steroids. The E_2 antiserum cross-reacted 14% with oestrone, 5% with oestriol, 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 standards were included in each assay (5-2,000 pg). Steroid concentrations were calculated on a microcomputer using SecuRIA software (Packard, Downners Grove, IL). 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%, for 17α-OHP, 6.2% and 6.5%, for AD, 5.6% and 6.9%, for T, 6.3% and 7.9% and for E_2, 6.6% and 7.7%, respectively.

Statistical analysis

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

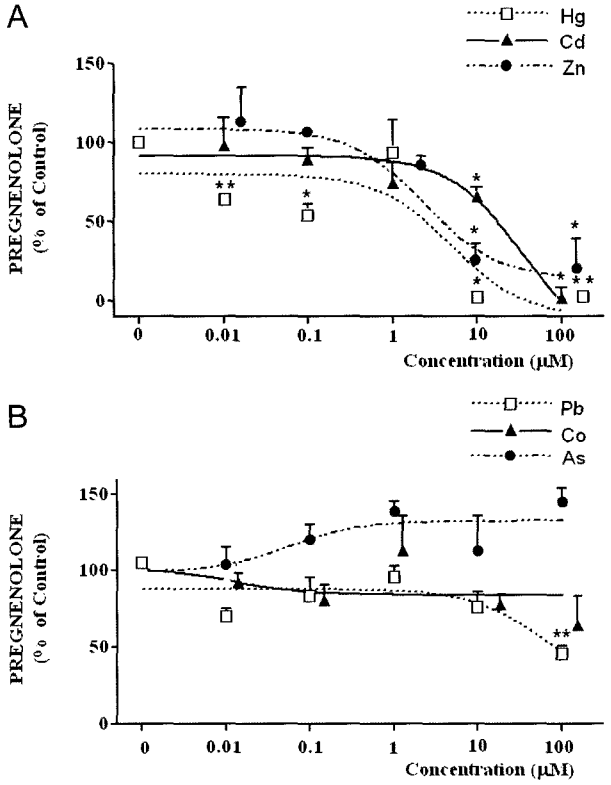

Fig. 1. Effects of heavy metals on FPH-induced pregnenolone synthesis by frog ovarian follicles. Isolated full-grown follicles were cultured for 18 hours in the presence of FPH (0.05 gland/ml) and/or various concentrations of heavy metals, Hg, Cd, or Zn (A) and Pb, Co or As (B). Each point in the figure represents relative P_5 levels (mean SEM, % of control). $*p < 0.05$ and $**p < 0.01$, when compared to control by paired student *t*-test. (n = 3, 3 animals).

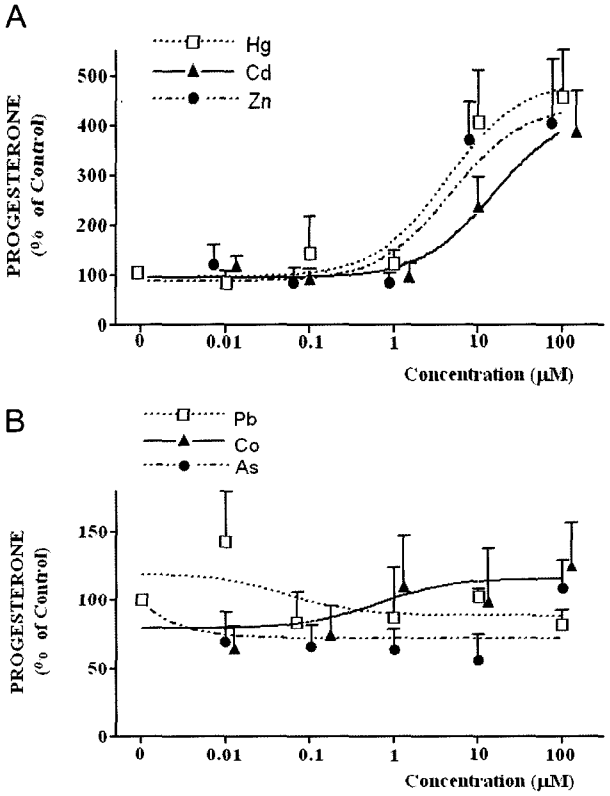

Fig. 2. Effects of heavy metals on the conversion of P_5 to P_4 by frog ovarian follicles. Isolated full-grown follicles were cultured for 18 hours in the presence of P_5 (100 ng/ml) and/or various concentrations of heavy metals, Hg, Cd, or Zn (A) and Pb, Co or As (B). Each point in the figure represents relative P_4 levels (mean SEM, % of control). $*p < 0.05$, $**p < 0.01$, when compared to control by paired student *t*-test. (n = 3, 3 animals).

RESULTS

Effects of heavy metals on FPH stimulated pregnenolone synthesis in frog ovarian follicles

Initially, to determine the effects of heavy metals on the first step of the steroidogenic pathway, the conversion of cholesterol to P_5 by P450scc, ovarian follicles were isolated and cultured in the presence of cholesterol precursor (100 ng/ml) and its conversion to P_5 was examined. However, the cholesterol was not converted to P_5 (data not shown), As the exogenous cholesterol could not be utilized by the follicles for some unknown reasons, the follicles were stimulated to produce P_5 with FPH (0.05 gland/ml) in the presence of various heavy metals (0.01 μM~100 μM). As shown in Fig. 1, considerable amount of P_5 were produced (854 pg/follicle, n = 6) and heavy metals reduced the P_5 production. Particularly, Hg suppressed the conversion at very low dose (EC_{50}, 4.0 μM), Zn and Cd also strongly suppressed the conversion at relatively low dose (EC_{50}, 5.7 μM and EC_{50}, 25.6 μM, respectively) (Fig. 1A). Thus, Hg, Cd and Zn appeared to suppress P450scc very effectively. In contrast, Pb, Co and As did not suppress the production of P_5. Only Pb (100 μM) exhibited a partial suppression of P_5 production at high dose (Fig. 1B).

Thus the data showed that the follicular P450scc enzyme was very sensitive to some heavy metals and the enzyme activity induced by frog gonadotropins was suppressed dramatically in exposure to Hg, Cd, and Zn. Interestingly, however, the FPH-stimulated enzyme activity was not affected by metals such as Co and As even at high dose (100 μM).

Effects of heavy metals on the conversion of P_5 to P_4 by frog ovarian follicles in vitro

To determine whether heavy metals affect the 3β-HSD activity in the follicles, frog ovarian follicles were isolated and cultured in medium containing P_5 (100 ng/ml) as steroid precursor. After culture, the conversion of exogenous P_5 to P_4 by the follicles was examined. Fig. 2 showed that Hg, Zn and Cd at high concentration (10 or 100 μM) rather increased P_4 accumulation three or four times to that of control (Fig. 2A). Interestingly, Pb. Co and As did not influence on the conversion of P_5 to P_4 by the follicles when compared to control (Fig. 2B).

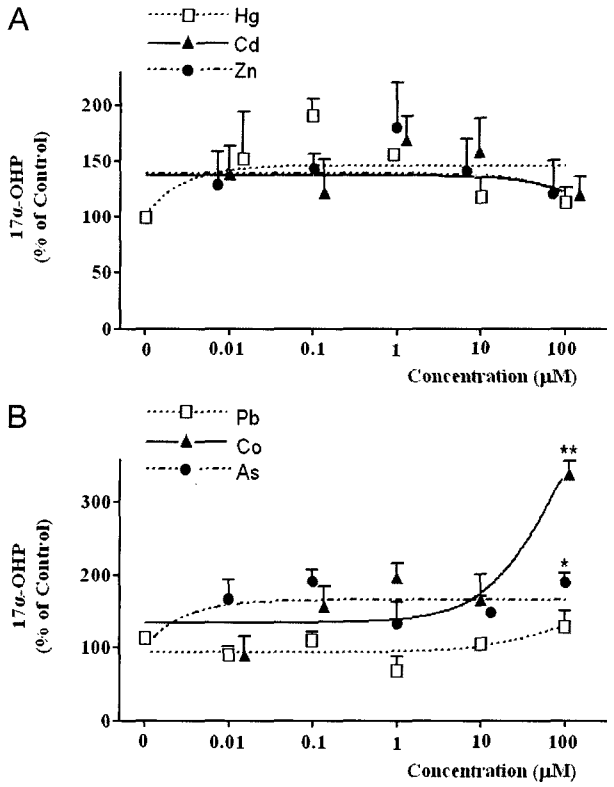

Fig. 3. Effects of heavy metals on the conversion of P_4 to 17α-OHP in by frog ovarian follicles. Isolated full-grown follicles were cultured for 18 hours in the presence of P_4 (100 ng/ml) and/or various concentrations of heavy metals, Hg, Cd, or Zn (A) and Pb, Co or As (B). Each point in the figure represents relative 17α-OHP levels (mean SEM, % of control). *$p < 0.05$ and **$p < 0.01$, when compared to control by paired student *t*-test. (n = 3, 3 animals).

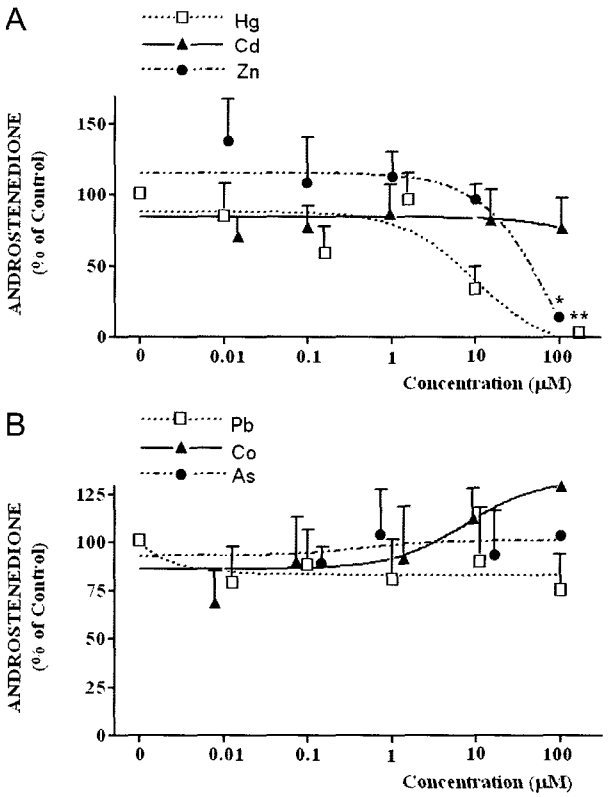

Fig. 4. Effects of heavy metals on the conversion of 17α-OHP to AD by frog ovarian follicles. Isolated full-grown follicles were cultured for 18 hours in the presence of 17α-OHP (100 ng/ml) and/or various concentrations of heavy metals, Hg, Cd, or Zn (A), and Pb, Co or As (B). Each point in the figure represents relative AD levels (mean SEM, % of control). *$p < 0.05$ and **$p < 0.01$, when compared to control by paired student *t*-test. (n = 3, 3 animals).

Effects of heavy metals on the conversion of P_4 to 17α-OHP by frog ovarian follicles in vitro

To determine whether heavy metals affect 17α-HSD activity, frog ovarian follicles were isolated and cultured in medium containing P_4 (100 ng/ml). After 18 hours of culture, conversion of P_4 to 17α-OHP by the follicles was examined. As Fig. 3 shows, Cd, Zn and Hg in medium did not affect the level of 17α-OHP, but rather slightly increased the level of 17α-OHP (Fig. 3B). The heavy metals such as Pb, Co, and As also showed similar result. At high dose (100 μM), Co significantly increased the accumulation of 17α-OHP (Fig. 3B). Thus, it was evident that the heavy metals in medium did not directly suppress the follicular 17α-OHP enzyme activity.

Effects of heavy metals on the conversion of 17α-OHP to AD by frog ovarian follicles in vitro

To determine whether heavy metals affect $C_{17\text{-}20}$ lyase activity, frog ovarian follicles were isolated and cultured in medium containing 17α-OHP (100 ng/ml). After 18 hours of culture, conversion of 17α-OHP to AD by the follicles was examined. As Fig. 4 shows, Hg and Zn at high dose strongly suppressed the secretion of AD (EC_{50}, 8 μM, 67 μM, respectively), but Cd failed to suppress the AD secretion (Fig. 4A). However, Pb, Co and As did not affect the secretion of AD even at high concentration (Fig. 4B). Thus it was clear that 17α-OHP enzyme activity was suppressed only by Hg and Zn but not by other heavy metals.

Effects of heavy metals on the conversion of AD to T and T to E2 by frog ovarian follicles in vitro

To determine whether heavy metals affect 17β-hydroxysteroid dehydrogenase (17β-HSD) activity, frog ovarian follicles were cultured in medium containing androstenedione (100 ng/ml). After 18 hours of culture, conversion of AD to T by the follicles was examined. As shown in Fig. 5, only Hg and Cd at high concentrations (10 or 100 μM) exerted a partial suppression, but other metals did not decrease the level of T (Fig. 4). Thus it was evident that 17β-HSD in follicles was not sensitive to heavy metals.

In order to assess the effect of the heavy metals on the aromatase activity in frog ovarian follicles, the follicles were cultured for 18 hours in the presence of T (100 ng/ml)

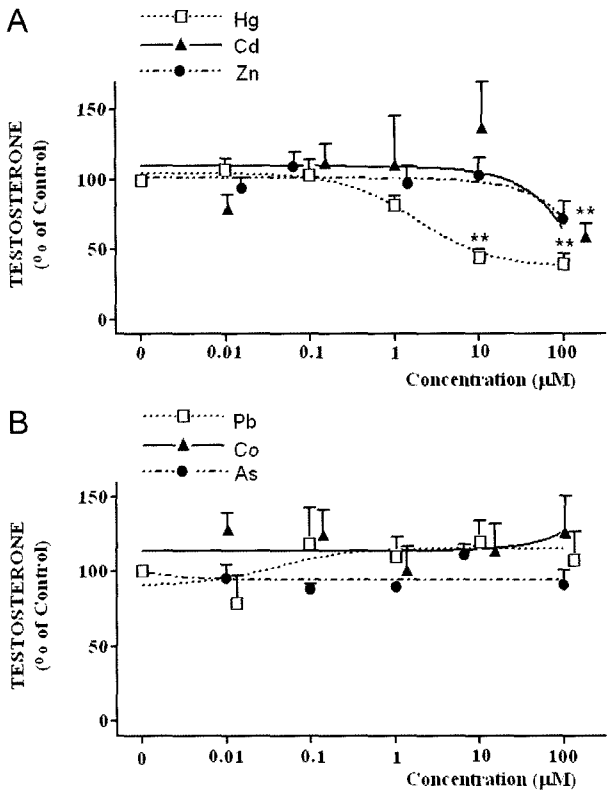

Fig. 5. Effects of heavy metals on the conversion of AD to T by frog ovarian follicles. Isolated follicles were cultured for 18 hours in the presence of AD (100 ng/ml) and/or various concentrations of heavy metals Hg, Cd, or Zn (A) and Pb, Co or As (B). Each point in the figure represents relative T levels (mean SEM, % of control). $*p < 0.05$ and $**p < 0.01$, when compared to control by paired student *t*-test. (n = 5, 5 animals).

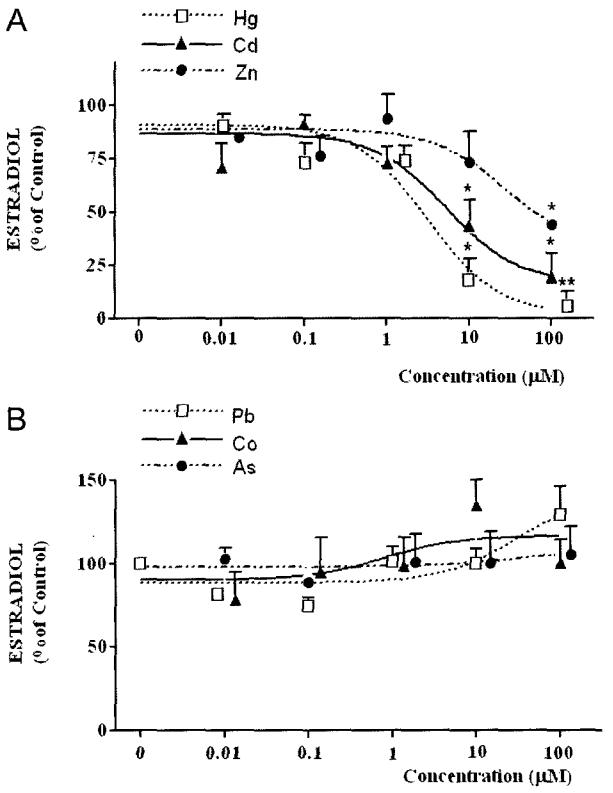

Fig. 6. Effects of heavy metals on the conversion of T to E2 by frog ovarian follicles. Isolated middle sized follicles were cultured for 18 hours in the presence of T (100 ng/ml) and/or various concentrations of heavy metals, Hg, Cd, or Zn (A) and Pb, Co or As (B). Each point in the figure represents relative E_2 levels (mean SEM, % of control). $*p < 0.05$ and $**p < 0.01$, when compared to control by paired student *t*-test.) (n = 3, 3 animals).

and measured the level of E_2 in the medium after culture. As shown in Fig. 6, Hg, Cd, and Zn in medium strongly suppressed the conversion of T to E_2 in a dose dependent manner (EC_{50}, 4.2 μM, 7.5 μM, and 80 μM, respectively, Fig. 6B). Interestingly, however, Pb, Co and As did not influence on the conversion of T to E_2 at all (Fig. 6B).

DISCUSSION

The data presented here demonstrated that 1) among the heavy metals tested, mercury, cadmium exhibited stronger toxic effects than other metals on the follicular steroidogenesis, 2) among the follicular enzymes in the steroidogenic pathway (cholesterol → estradiol), P450scc and aromatase are much more sensitive to the toxicity of heavy metals than other enzymes, and 3) in general, the data presented here are in good agreement with those of others obtained from the studies in fish or mammals. Thus, present study raised the possibility that amphibian follicle culture model can be developed as a practical screening method for potential endocrine disrupters like heavy metals.

As frog ovarian follicles could not utilize exogenous cholesterol as precursor for P_5 production (Petrino and Schuetz, 1987), we could not assess the direct effect of heavy metals on the P450scc activity, the first enzyme, in the steroidogenic pathway in the follicles. However, the frog follicles could produce considerable amount of P_5 or P_4 in response to frog pituitary homogenates (FPH) (Kwon and Ahn, 1994). Thus, it is evident that frog ovarian follicles contained enough endogenous cholesterol and the enzyme P450scc that can be stimulated with FPH. Therefore, treatment of FPH has the same effect as to add exogenous cholesterol for P_5 production in the follicles. As shown in Fig. 1, FPH-induced P_5 production was suppressed by Hg, Cd, and Zn in a dose dependent manner whereas Pb, Co, and As were not effective in the suppression. Similarly, Hg, Cd, and Zn in the medium strongly suppressed the conversion of T to E_2 (aromatase activity) in a dose dependent manner while other metals are not effective in the suppression (Fig. 6). Thus the potential toxicity of heavy metals on the follicular P450scc or aromatase are in the order of Hg > Cd, Zn > Pb > Co, AS.

Interestingly, however, other steroidogenic enzymes such

as 3β-HSD, 17α-hydroxylase, C_{17-20}-lyase and 17β-HSD in the follicles are not suppressed by the metals at all or partially suppressed by certain heavy metals at high concentration (Figs. 2, 3, 4 and 5). In Fig. 2, heavy metals appear to increase, rather than decrease, the accumulation of P_4 in the presence of exogenous P_5 by the follicles. But this seems to be due to the prevention of P_4 metabolism rather than stimulation of P_4 synthesis.

In general, the relative toxicity of heavy metals (Hg > Cd and Zn > Pb, Co and As) observed in this study was similar to those obtained from other types of cells. Adrenal cells were known to produce corticosteroid in response to ACTH. Some investigators used this adrenal cell culture system to evaluate the toxicity of heavy metals. They found that $HgCl_2$ was more toxic than $CdCl_2$, which in turn was more toxic than $ZnCl_2$ in suppressing the corticosteroid production in response to ACTH (Ng and Liu, 1990). The same ranking in the toxicity among the heavy metals was also observed in testosterone production by rat Leydig cells in response to human chorionic gonadotropin (Laskey and Phelps, 1991). A similar study with fish cell line obtained from bluegill sunfish (Babich et al., 1986), fathead minnow (Dierickx and Bredael-Rozen, 1996), and interrenal cells of rainbow trout (Leblond and Hontela, 1999) also showed the same relative toxicity among heavy metals observed in this study.

Our study showed that, in frog ovarian follicles, P450scc and aromatase appeared to be much more sensitive to Hg, Cd or Zn than other steroidogenic enzymes in the follicles (Figs. 1 and 6). In contrast, 3β-HSD ($P_5 \rightarrow P_4$) and 17α-hydroxylase ($P_4 \rightarrow$ 17α-OHP) were not affected at all by the heavy metals even at high dose (100 μM) (Figs. 2 and 3). Interestingly, $C_{17,20}$-lyase (17α-OHP → AD) were suppressed at high concentration (100 μM) by Hg and Zn (Fig. 4), and 17β-HSD (AD → T) were partially suppressed by Hg and Cd (Fig 5). Taken together, the sensitivity of the enzymes to heavy metals were in the order of P450scc, aromatase > $C_{17,20}$-lyase, 17β-HSD > 3β-HSD, 17α-hydroxylase. This is the first study to show the relative sensitivity of steroidogenic enzymes to heavy metals in animal cells.

There have been many studies indicating that certain metals suppressed a particular enzyme in the steroidogenic pathway in different animal models. Pasky et al. (1997) showed that Cd (16 μM) inhibited gonadotropin (FSH) stimulated progesterone production by 60% in cultured human granulosa cells and Kawai et al (2002) showed that in cultured human trophoblasts, Cd (20 μM) significantly inhibited progesterone production in the presence of 25 hydroxycholesterol (added as precursor). Interestingly, they showed that Cd suppressed P450scc activity but not 3β-HSD activity in the cultured trophoblasts. These reports are in good agreement with ours showing that P450scc enzyme is very sensitive to heavy metals while 3β-HSD is not. In mouse Leydig cells, 22 hydroxy cholesterol or P_5 stimulated progesterone production was known to reduce to 30-40% by the treatment of lead (Liu et al., 2001). Thus mammalian Leydig cells seem to be more sensitive to lead than amphibian follicles cells observed in this study.

The present study demonstrated that frog ovarian follicle culture system could be utilized as a screening method for endocrine disrupters like heavy metals. In practical viewpoint, this model has several advantages when compared with other animal cell culture models. Typically, frog ovarian follicle contains follicular oocyte enclosed with a layer of follicle cells and thecal layer without antrum. As frog ovarian follicles can be easily isolated from ovary under a dissecting microscope and can be cultured at organ level, follicle cells are not needed to be separated or isolated from oocyte for cell culture. Furthermore, during follicle culture, follicle cells can utilize rich nutrients from oocytes and thus, they can produce considerable amount of various steroids without any precursors in a simple Ringer solution (AR) in response to FPH. The data presented here also showed that sensitivity of the follicles to heavy metals are very similar to or even lower than those observed from various cell lines from mammals or fish.

Thus this bio-assay system provides a technically simple and economic way to examine the toxic effect of environmental pollutants or endocrine disrupters on animals including humans.

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