Estradiol Uptake, Toxicity, Metabolism, and Adverse Effects on Cadmium-Treated Amphibian Embryos

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The exposure of *Bufo arenarum* embryos to 25 μmol/L 17β-estradiol (E_2) resulted in 100% lethality within 48 hr, whereas 10 μmol//L E_2 was the no observed effect concentration value for short-term chronic (7 days) exposure. The toxicity profile curves show that lethal effects were proportional to the E_2 concentration and the time of exposure. The E_2 uptake resulted in 20.1 ng E_2/mg embryo at 8 hr posttreatment, but 67.3% of this value was achieved during the first 30 min of incubation with this estrogen. Regarding metabolism, the embryos synthesize estrone (E_1) from E_2 by means of 17β-hydroxysteroid dehydrogenase. Simultaneous treatments of *Bufo arenarum* embryos with 1 mg/L Cd^{2+} and 0.1, 1, or 10 μmol/L E_2 enhanced the lethality exerted by cadmium in 76.7, 80, and 83.3% of embryos, respectively. The results indicate that estrogenic endocrine disruptors could have an adverse effect on amphibian embryos and enhance the toxic effect of Cd on amphibian embryos. This study points to the possibility of using the AMPHITOX test as a screening method for potential endocrine disruption as well as the combined effects of chemical mixtures. *Key words:* amphibian embryos, AMPHITOX, endocrine disruption, estradiol toxicity, estradiol uptake, estradiol–cadmium synergism. *Environ Health Perspect* 112:862–866 (2004). doi:10.1289/ehp.6647 available via *http://dx.doi.org/* [Online 10 March 2004]

Environmental estrogens include a variety of very different synthetic chemicals such as polychlorinated biphenyls (PCBs), organochlorine pesticides, alkylphenols, phthalates, and food antioxidants, which may have hormonelike activity in whole-animal studies and *in vitro* assays (Bevan et al. 2003; Birnbaum and Fenton 2003; Colborn et al. 1993; Jobling et al. 1995; McLachlan and Korach 1995; Soto et al. 1995; Toppari et al. 1996; Zava et al. 1997). Heavy metals, with a large number of adverse effects on the biota, could also exert xenoestrogenic activities (Baccarelli 1999; Colborn et al. 1993) and promote human diseases such as cancers, cognitive impairments, and sexual abnormalities (Baccarelli 1999; Schantz and Widholm 2001). Estrogenic compounds typically bind estrogen receptors (ERα and ERβ), mimicking the estrogenic actions (Hammond et al. 1979; Krishnan et al. 1993; White et al. 1994) and induce transactivation of estrogen-responsive genes/reporter genes. By means of estrogen equivalents, the relative estrogenic potencies for individual compounds and mixtures can be reported (Gaido et al. 1998; Verdeal and Ryan 1979). Although their potencies are usually 1,000-fold lower than those observed for estradiol (E_2) and both synthetic estrogens and phytoestrogens are weakly estrogenic (Hammond et al. 1979; Krishnan et al. 1993; White et al. 1994), all of these compounds induce distinct patterns of ER agonist/antagonist activities that are dependent on cell context and promoters, suggesting that these compounds could induce time-specific *in vivo* ER agonistic or antagonistic activities. These results suggest that other receptors, such as the aryl hydrocarbon receptor, that also bind structurally diverse ligands may have unique effects *in vivo* that are not predicted by standard *in vitro* bioassays (Safe et al. 2002). Moreover, the acute cytotoxicity of E_2 in cultured MCF-7 breast cancer cells is clearly not mediated by nuclear ERs (Welshons et al. 2003).

Developing organisms are particularly susceptible to environmental insult, resulting in anomalies or death as well as increased susceptibility to cancer. In fact, a large number of xenoestrogenic effects can be exerted by means of estrogen treatments during early life stages, as has been reported in mammals (Greco et al. 1993) and, based on epidemiologic studies, also in humans (Gaido et al. 1998). For instance, prenatal exposure to natural and synthetic estrogens is associated with increases in breast and vaginal tumors in humans as well as uterine tumors in animals, whereas prototypical endocrine-disrupting compounds could be related to endocrine-related cancers or susceptibility to cancers (Birnbaum and Fenton 2003). Amphibian embryos, because of their high susceptibility to physicochemical agents, have been successfully used for hazard assessment of a large number of single physicochemical agents as well as complex mixtures (e.g., Ankley et al. 2002; Herkovits and Pérez-Coll 1990; Herkovits et al. 1996). By means of AMPHITOX, a standardized test customized for acute, short-term chronic, chronic, and early life stages toxicity assessment, the hazard of a single agent or complex mixtures can be reported according to the more convenient end point in each case (Herkovits et al. 1996, 1997, 2002; Herkovits and Pérez-Coll 2003). It has recently been reported that environmental estrogens could increase amphibian embryo mortality, induce

deformations, increase apoptosis, and alter the deposition and differentiation of melanocytes derived from the neural crest (Bevan et al. 2003; Fridman et al. 2002). On the other hand, based on laboratory and field evidence, even 0.1 ppb atrazine, acting as an endocrine disruptor, could induce hermaphroditism in northern leopard frogs (*Rana pipiens*) (Hayes et al. 2003). Recent evidence that atrazine also potentiates parasitic infections in amphibians (Kiesecker 2002) points out the complex adverse effects exerted by physicochemical agents on the amphibian population, a fact probably related to the decline of many amphibian species (Kiesecker et al. 2001).

Cadmium is among a large number of substances that exert adverse effects on ecosystems and human health. The large amount of this heavy metal employed by industry and agriculture produces a gradual increase of Cd in water, soils, and food (Landis and Yu 1999). This metal is embryotoxic, causing different kinds of malformations and lethality in mammals (Belmonte et al. 1989; Fein et al. 1997) as well as amphibians (Herkovits et al. 1996; Pérez-Coll et al. 1986; Soukupova and Dostal 1991); a stage-dependent susceptibility to this heavy metal has been reported (Herkovits et al. 1997). Because Cd toxicity can be affected by several factors, including the presence of other metals (Herkovits and Pérez-Coll 1990), and taking into account the large number of xenoestrogenic substances in the environment (Birnbaum and Fenton 2003; Colborn et al. 1993; McLachlan and Korach 1995), it would be of interest to explore the potential effect of estrogen on Cd toxicity. The combination of Cd and xenoestrogens could be of particular concern in urban/industrial areas and watersheds. In this study we report on the toxicity of E_2 (the most potent natural estrogen), its

uptake and metabolism, and the effect of E_2 on Cd toxicity in early life stages of amphibian organisms. The results point out the potential adverse effects of endocrine disruptors on the toxicity of environmental pollutants and indicate the possibility of evaluating these adverse effects by means of studies conducted with amphibian embryos.

Materials and Methods

Animals. We chose to use the South American toad, *Bufo arenarum*, as the standard test animal for the AMPHITOX test. The adult toads, which are commonly found in Argentina, were captured in Lobos, Buenos Aires Province. We induced ovulation of female South American toads by intraperitoneal injection of homologous hypophysis (Pisanó 1956). Oocytes were fertilized *in vitro* with a sperm suspension in AMPHITOX solution (AS; 14.75 mg/L Na+, 0.26 mg/L K+, 0.36 mg/L Ca2+, 22.71 mg/L Cl−, 1.45 mg/L HCO3 (Herkovits et al. 2002). Embryos were maintained in AS until the end of embryonic development, that is, the complete operculum stage (stage 25) (Del Conte and Sirlin 1951).

Chemicals. We purchased unlabeled crystalline E_2 from Sigma Chemical Co. (St. Louis, MO, USA), and [2,4,6,7-³H(N)] E_2 (71.0 Ci/mmol) from New England Nuclear Research Products (Boston, MA, USA). All chemicals and solvents were of analytical grade.

E_2 toxicity. E_2 was dissolved in ethanol, and the final alcohol concentration in the maintaining medium was never higher than 1%. To determine E_2 toxicity, we placed 10 embryos in glass Petri dishes (in triplicate) in 40 mL AS containing E_2 at nominal concentrations of 3.7, 10, 15, 20, 25, 30, and 36.5 μmol/L for 6 days. Triplicate groups of 10 individuals maintained in AS without E_2 served as controls. Control embryos were maintained in AS containing a range of ethanol concentrations between 0.1% and 1%. We evaluated lethality of embryos every 24 hr.

E_2 uptake. To determine E_2 uptake and bioconcentration, we treated batches of 10 embryos with 10 μmol/L E_2 plus 2 μCi ³H-E_2 and determined radioactivity in the experimental animals at 0, 0.5, 1, 2, 4, and 8 hr. Each group of embryos was washed with 50 mL AS, dried with filter paper, homogenized, and placed in vials containing scintillation liquid. The incorporated radioactivity was measured in a Beckman LS 7800 liquid scintillation spectrometer (Beckman Instruments, Irvine, CA, USA). For time zero of exposition, animals were introduced into the solution and immediately withdrawn and washed; the radioactivity measured in these controls was subtracted from counts measured in embryos exposed to the five experimental conditions.

Activity of 17β-hydroxysteroid dehydrogenase. We determined the activity of

17β-hydroxysteroid dehydrogenase (17β-HSD) by a modification of the procedure used by Fridman and Gurpide (1980). Embryos were exposed in Petri dishes to 25 μmol/L E_2 plus 1 μCi ³H-E_2 in AS for 3 hr. Groups of 20 embryos, after withdrawal from the dishes, were washed with 50 mL AS, dried with filter paper, and homogenized in methanol containing nonradioactive E_2 and estrone (E_1; 100 μg each). The lipid fractions were extracted from the homogenates four times with 2 mL methanol. The methanolic homogenate was centrifuged to separate precipitated proteins and nucleic acids, and the extracts were evaporated to dryness under nitrogen at 37°C. The residues were chromatographed on silica gel thin-layer plates using the system chloroform–ethyl acetate [4:1 vol/vol; Rf (quotient between the E_2 or E_1 spots and the solvent front in the thin-layer chromatography): E_1 0.75 μmol/L, E_2 0.40 μmol/L]. The ultraviolet-absorbing zones corresponding to E_1 and E_2 in the developed plates were eluted with methanol and rechromatographed in a chloroform–methanol (97:1 vol/vol) system (Rf: E_1 0.50 μmol/L, E_2 0.25 μmol/L). Aliquots of the purified estrogens eluted from the plates were used for measurements of radioactivity and for the spectrometric estimation of the amounts of E_1 and E_2 recovered in order to calculate the concentration of the labeled estrogens in the original samples. Recrystallization of E_1 to constant specific activity was carried out as a final identification by the addition of approximately 5 mg E_1 according to Axelrod et al. (1965). We used methanol–water as the solvent system.

Effect of E_2 on Cd toxicity. Cd²⁺ solutions were prepared with AS, and E_2 solutions were prepared from a standard solution of 1 mg E_2/mL in ethanol. We evaluated the interaction of E_2 and Cd²⁺ by maintaining triplicate groups of 10 embryos in 40 mL AS with 1 mg/L cadmium chloride (CdCl₂) plus 0.1, 1, or 10 μmol/L E_2. Embryo survival was evaluated daily up to 168 hr of exposure (7 days). Dead embryos were removed.

Statistics. We used PROBIT analysis to obtain and plot the E_2 concentrations representing LC₁₀ (lethal concentration for 10% of the test subjects), LC₅₀, and LC₉₀ from 24 to 144 hr as toxicity profile (TOPs) curves. All values are reported as mean ± SD. We performed analysis of variance (ANOVA) using Statistica software (StatSoft, Tulsa, OK, USA). A *p*-value ≤ 0.05 was considered significant.

Results

Estradiol toxicity and TOP curves. Figure 1 shows that after 48 hr of exposure 25, 30, and 36.5 μmol/L E_2 exerted 23.3, 60, and 50% of lethality, respectively. At 96 hr, lethality reached 100% in all these exposure conditions, whereas 3.7, 10, 15, and 20 μmol/L E_2 exerted 0, 0, 7, and 60% mortality, respectively.

Exposure to 15 and 20 μmol/L E_2 produced 40 and 77% of lethality at 120 hr and 57 and 87% at 144 hr, respectively. Below 10 μmol/L E_2, no mortality was registered. The LC₁₀, LC₅₀, and LC₉₀ values corresponding to E_2 toxicity in *Bufo arenarum* embryos during 22–144 hr of treatment are shown in Figure 2. The LC₅₀ values for E_2 diminished from 33.69 to 15.13 μmol/L E_2, respectively. A similar pattern occurred with the values corresponding to LC₁₀ (from 27.43 to 11.69 μmol/L E_2) and LC₉₀ (from 41.37 to 19.57 μmol/L E_2). Therefore, E_2 toxicity increased very considerably (about 2.23 times) by expanding the exposure period from 24 to 144 hr. Table 1 shows that 95% confidence intervals (CI) of LC₅₀ are not overlapping with those of LC₁₀ and LC₉₀ except for its higher value at 24 hr of exposure. The no observed effect concentration (NOEC) value was approximately 10 μmol/L E_2 during the whole period, whereas the LC₁₀₀ was between 48.91 and 24.14 μmol/L E_2. Control *Bufo arenarum* embryos treated with ethanol up to 1% did not exhibit lethal effects.

Estradiol uptake. The uptake of E_2 at 0.5, 1, 2, 4, and 8 hr (expressed as nanograms E_2 per milligram of embryo) were 13.52 ± 0.20, 17.15 ± 0.13, 19.17 ± 1.24, 19.38 ± 0.55, and 20.10 ± 0.12, respectively (Figure 3). These values represent bioconcentration factors of 4.94, 6.30, 7.04, 7.11, and 7.38, respectively. The results show that at 0.5 hr,
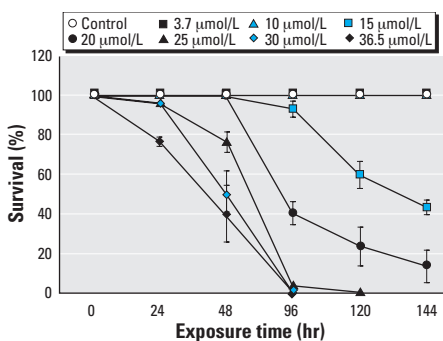
Figure 1. Concentration–response relationships in *Bufo arenarum* embryos treated with E_2. Error bars indicate SE; no error bars are shown for values of 0 and 100%.
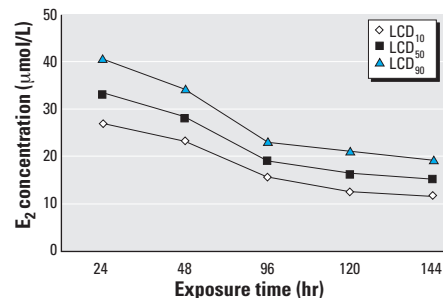
Figure 2. Lethal concentrations of E_2 in *Bufo arenarum* embryos by PROBIT analysis.

the embryos incorporated 69% of the E_2 measured in the plateau.

Metabolism of E_2. Based on chromatographic mobility in two different solvent systems and recrystallization of E_1 to constant specific activity *Bufo arenarum* embryos biosynthesize E_1 from E_2. Table 2 shows that after three recrystallizations with methanol–water as the solvent system, the specific activity of E_1 achieved constant values.

Estradiol plus Cd treatment. The treatment with 1 mg/L Cd^{2+} exerted 40% of lethality within 24 hr of exposure (Figure 4). By means of simultaneous treatment with Cd^{2+} and E_2, we observed an additional and significant adverse effect on embryo survival between 24 and 72 hr. However, at 168 hr of exposure, this tendency was maintained only at the higher E_2 concentration.

The interaction of Cd^{2+} at a concentration exerting approximately 50% of mortality in acute exposure conditions and E_2 in three different concentrations (0.1, 1, and 10 µmol/L) was evaluated. E_2 at 10 µmol/L did not produce lethality even after 168 hr of exposure; 1 mg/L Cd^{2+} exerted 40, 56.7, and 83.3% of mortality at 24, 48, and 168 hr, respectively. We observed a dose-dependent increase on embryonic mortality exerted by Cd^{2+} plus E_2 at the three concentrations. Figure 4 summarizes the effect of E_2–Cd^{2+} interactions on *Bufo arenarum* embryonic survival. Low E_2 concentrations (e.g., 0.1 µmol/L) had a clear-cut effect on enhancing Cd^{2+} toxicity within the first 24 hr of exposure; this tendency was maintained until the third day of exposure. This adverse effect was transient because at day 7, we found no differences between the embryos treated with Cd plus E_2 and those treated with Cd alone.

Discussion

The LC_{50} values for E_2 in *Bufo arenarum* embryos at 48 and 96 hr of exposure diminished from 33.69 to 15.13 µmol/L E_2. In other organisms, it has been also shown that under certain conditions E_2 is embryotoxic. For example, in *Xenopus laevis* embryos, different natural and synthetic estrogens induce malformations of the head and abdomen, suppress organogenesis, and exert lethality (Nishimura et al. 1997). In *Fundulus heteroclitus*, 0.01 and 1 µmol/L E_2 in the maintaining medium induces death of embryos and fry, malformations, sex reversal, and incomplete

ossification of vertebrae and cranial bones, which would result in shorter body and head lengths and in malformed vertebrae, leading to a hunchback condition (Urushitani et al. 2002). Kishida et al. (2001) reported that the treatment of zebrafish embryos (*Danio rerio*) with 0.1–10 µmol/L E_2 between 2 and 72 hr postfertilization provoked dose–response effects on mortality and hatching and induced the "curved tail down" phenotype characteristically similar to mutants with defects in the central nervous system. According to the authors, these effects could be mediated by the P450 aromatase enzyme (Kishida et al. 2001). The acute toxic effect of E_2 in *Bufo arenarum* embryos reported in this study occurs at similar concentrations as in zebrafish. According to Welshons et al. (2003), these lethal effects, which occur at very high E_2 concentrations in MCF-7 human breast cancer cells, do not depend on ERs. Therefore, although it is generally accepted that estrogenic chemicals act via binding to the ER, based on present results and the study of Welshons et al. (2003), E_2 toxicity occurs at concentrations several orders of magnitude above the receptor saturation level. In mammals, including humans, the xenoestrogenic effects of several synthetic compounds such as pesticides, herbicides, or industrial products, alone or in combination, may promote human diseases (Auger et al. 1995). Xenoestrogens bind and activate the ER, thus increasing the estrogen-dependent transcription of the target genes and promoting undesirable estrogenic effects such as the decreased quantity and quality of human and animal semen and the increasing incidence of testicular cancer in men and breast cancer in women (Davis et al. 1993; Forman and Moller 1994; Newbold et al. 1986; Weinberg 1996). Xenotoxic molecules can also be formed from endogenous sources such as the estrogen catechols produced by breast epithelium (Yager and Liehr 1996). These compounds derived from the hydroxylation of the natural estrogens E_1, E_2, and estriol can be oxidized to quinones, which are putative tumor initiators (Cavalieri et al. 1997).

E_2 metabolism in *Bufo arenarum* embryos was evaluated by the presence of 17β-HSD, the enzyme that regulates the relative concentrations of potent 17β-hydroxy and biologically weak 17-keto forms of estrogens and androgens locally at the target tissue level. The identification of the biosynthesized ^3H-E_1

from ^3H-E_2 was performed by mean of two successive thin-layer chromatographies and recrystallization to constant specific activity (Table 2). In the stage-25 embryo after 3 hr of incubation, radioactive E_1 had metabolized from the radioactive E_2 used as tracer. 17β-HSD, with mainly oxidative activities, tends to decrease the potency of estrogens and consequently may protect tissues from excessive hormone action. Other enzymes, such as P450 aromatase, estrogen sulfatase, and estrogen sulfotransferase, also modulate estrogens. Hence, the concentration of biologically active steroid hormone in a cell is dependent on the transfer of hormones and the actions of different enzyme types in the cell. Inactivation of E_2 by its oxidation to E_1 is essential for induction and maintenance of secretory endometrium (Tseng and Gurpide 1975). The first enzyme shown to be involved in this process was 17β-HSD type 2, the isoform that catalyzes the oxidative pathway (Casey et al. 1994). The toxic effects produced by high concentrations of estrogens as well as endocrine disruptors could be related to the disruption of physiologic steroidogenic pathways and/or estrogenic mechanisms. It is noteworthy that E_2 bioaccumulation in the amphibian embryo could reach endogenous levels more than seven times greater than the concentration in the medium; therefore, lethality was caused by internal estrogen concentrations several orders of magnitude higher than the physiologic concentrations of this hormone. Moreover, this high E_2 level in the amphibian embryos was achieved within the initial 4 hr of exposure, whereas lethality increased slightly over the whole acute and short-term chronic exposure period.

The evaluation of the acute toxicity of a chemical is typically reported as individual statistics, for example, the LC_{50} at 48 hr, the most commonly used threshold in environmental toxicology (Hoekstra 1993). Because the toxicity exerted by a substance depends on the concentration and time of exposure,
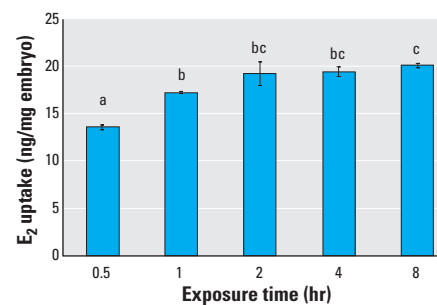
Figure 3. Estradiol uptake in *Bufo arenarum* embryos calculated as a function of the radioactive E_2 incorporated from the AS. Using ANOVA and Tukey highest significant difference test, we identified the groups with significantly different means ($p < 0.05$). Bars with different letters are significantly different. Error bars indicate SE.

Table 1. Mean (95% CI) lethal concentrations of estradiol toxicity in *Bufo arenarum* embryos.[a]

Hours of treatment	LC_{10}	LC_{50}	LC_{90}
24	27.43 (21.83–29.51)	33.69 (30.78–67.07)	41.37 (34.83–189.98)
48	23.40 (20.55–24.91)	28.57 (27.14–30.82)	34.90 (31.96–42.77)
96	15.67 (13.95–16.82)	19.07 (17.98–20.13)	23.22 (21.81–25.59)
120	12.56 (10.76–13.79)	16.33 (15.13–17.45)	21.25 (19.68–23.88)
144	11.69 (9.98–12.85)	15.13 (13.98–16.19)	19.57 (18.09–22.06)

[a]Note that the overlapping of the 95% CI occurs only in the case of LC_{50} and LC_{90} at 24 hr exposure.

threshold values taking into account these parameters may be of high value for skillfully customized hazard/risk assessment. For this purpose, toxicity data could be easily extended to a concentration–response relationship; by plotting LC_{90}, LC_{50}, and LC_{10} at regular intervals of time (e.g., 24 hr), the TOP curve can be obtained, providing exposure and concentration thresholds for a given substance or sample (Herkovits et al. 1996, 2002). In addition, conducting the toxicity test with embryos and extending the exposure period to at least 7 days, it is possible to derive a good predictive correlation between survival data and independent ecologic parameters, especially species richness and diversity [U.S. Environmental Protection Agency (EPA) 1991]. The TOP curves for E_2 in *Bufo arenarum* embryos (Figure 2) illustrate a significant decrease in the concentrations exerting lethal effects during the first 96 hr of exposure (around two times for concentrations exerting LC_{10}, LC_{50}, and LC_{90}), whereas from that time onward, the concentration threshold for lethality varies very slightly. As a whole, the E_2 toxicity for *Bufo arenarum* embryos increases 2.23 times from 24 to 144 hr of exposure. This result is different from the toxicity pattern of some metals such as lead (Herkovits et al. 1996), copper (Herkovits and Helguero 1998), aluminum (Herkovits et al. 1997), and Cd (Herkovits and Pérez-Coll 1996) on *Bufo arenarum* embryos, in which similar LC values were reported from 24 hr onward even up to 7 days (short-term chronic) of exposure. Other concentration thresholds such as LC_{90} and LC_{10} seem also to be meaningful for hazard assessment of E_2 because their 95% CIs in most cases do not overlap those of LC_{50} (Table 1). In fact, LC_{50} values do not inform about concentrations that exert a negligible effect or the ones that cause a very severe effect. In the case of *Bufo arenarum* embryos, TOP curves provide this information as well as the range of variation in the susceptibility between the most sensitive and more resistant individuals, resulting in 38.91 μmol/L E_2 for the first 24 hr of exposure and 14.14 μmol/L E_2 at 144 hr. The values obtained by the TOP curves were a guide for the simultaneous treatments of E_2 in sublethal doses with Cd in order to explore the potential effect of endocrine disruptors on toxicity of this heavy metal.

Table 2. Final identification of biosynthesized E_1 in *Bufo arenarum* embryos: specific activities during the recrystallization of E_1 using methanol–water as the solvent system.

Crystallization	cpm/mg crystals
1	1,222
2	775
3	572
4	565
5	550

In previous studies, we reported that amphibian embryos treated with Cd exhibit a stage-dependent susceptibility for teratogenic and lethal effects that could be related to some extent to changes in bioaccumulation features (Herkovits et al. 1996). The effect of E_2 on Cd^{2+} toxicity was evaluated by using simultaneous treatments of E_2 and Cd^{2+}. The presence of E_2 in all the concentrations evaluated enhanced Cd^{2+} toxicity. This augmented response could be related to the Cd^{2+} action on steroidogenic enzymes. Androgen levels and the activity of 17β-HSD in cultured Leydig cells obtained from testes of male inbred KP mice after a single injection of $CdCl_2$ showed a marked decrease of activity of dehydrogenase; also, the androgen level decreased after Cd^{2+} administration (Godowicz and Bilinska 1981). Biswas et al. (2001) reported that in adult Sprague-Dawley male rats, Cd^{2+}-induced stress adversely affected testicular activity by decreasing testicular Δ(5)-3β-HSD and 17β-HSD activities as well as serum testosterone and accessory sex organ weight. Mukherjee et al. (1992) observed that isolated ovarian tissues from the common carp, *Cyprinus carpi*, incubated with $CdCl_2$ were impaired by gonadotropin-induced fish ovarian steroidogenesis; 17β-HSD activity was particularly affected. Ghosh et al. (1987) reported that a single injection of $CdCl_2$ (0.5 mg/toad, *Bufo melanostictus*) resulted in reduced 17β-HSD activity in the testis and decreased serum testosterone 7 days later; they also observed an increase in thumb pad glycogen content. The injection of $CdCl_2$ in the same toad increased both Δ(5)-3β-HSD and 17β-HSD activities in Bidder's organ, a rudimentary ovary attached to the testis (Ghosh et al. 1984). In contrast, in an estrogen-dependent breast cancer cell line, Cd^{2+} mimicked the effects of E_2 through the activation of ERα, probably interacting with the steroid-binding domain (Stoica et al. 2000). This mechanism represents a direct action of Cd^{2+} enhancing the cell response to

Figure 4. Dose–response enhancement of Cd toxicity by E_2 treatment. The most significant adverse effects occur from 24 to 72 hr of exposure. Error bars indicate SE; no error bars are shown for values of 0 and 100%.

E_2. An indirect effect can be based on Cd^{2+} stimulating the synthesis of metallothioneins (MTs) in *Bufo arenarum* adults and embryos (Pérez-Coll et al. 1997, 1999). These proteins bind heavy metals as a detoxification mechanism. A deficiency in the expression of MTs could augment Cd^{2+} toxicity. Olsson et al. (1995) have reported this effect in the salmon, *Oncorhynchus mykiss*, in which the combined treatment with E_2 and Cd^{2+} caused an inhibition of transcription and translation of MT mRNA in the liver. This was also observed in mammals; in ovariectomized mice, E_2 reduces MT-I mRNA expression induced by Cd^{2+} (Sogawa et al. 2001). The eventual inhibition of MT synthesis by E_2 in *Bufo arenarum* embryos could be the mechanism by which estrogens enhance the toxic effects of Cd^{2+}.

In rats the results are contradictory. Blazka and Shaikh (1991) observed that E_2 induces a higher and fast uptake of Cd^{2+} by the liver, together with a major induction of MT synthesis in liver and kidney. However, also in rats, Nishiyama et al. (1988) observed that pretreatment with E_2 directly increased the accumulation of Cd^{2+} into the renal cells without inducing the synthesis of MTs. In females of the squirrelfish family (Holocentridae), E_2 did not directly alter the levels of zinc or MT mRNA in the liver (Thompson et al. 2001). However, the hepatic MT protein concentration increased differentially in the nuclear fraction. Thus, E_2 is probably responsible for the association of MT with the nuclear fraction previously observed in untreated mature female squirrelfish. Other steroids also regulate the synthesis of MTs. In rat hepatocyte primary cell lines, the glucocorticoid corticosterone induces the synthesis of MTs, and the synthetic glucocorticoid dexamethasone is a more potent MT inductor than is Zn^{2+} and Cd^{2+} (Bracken and Klaassen 1987). Taking into account the above-mentioned studies and our results, we can conclude that the effects of E_2 on development are complex, depending on the mechanisms of toxicity of other substances as well the susceptibility of certain cell differentiation processes such as the neural-crest–derived melanocytes reported in the case of *Xenopus laevis* embryos (Bevan et al. 2003). Because developing organs can be extremely sensitive to the toxic effect of pollutants, it is increasingly evident that the period of development during which exposure occurs can be more crucial than the total quantity of exposure to pollutants (Axelrod et al. 2001). The present results confirm the possibility of evaluating endocrine disruption effects *in vivo* by means of toxicity tests in amphibian embryos as well as the eventual adverse effects of endocrine disruptors at subtoxic levels on the toxicity of other substances (e.g., metals), a fact that could be relevant for environmental scenarios and human health.
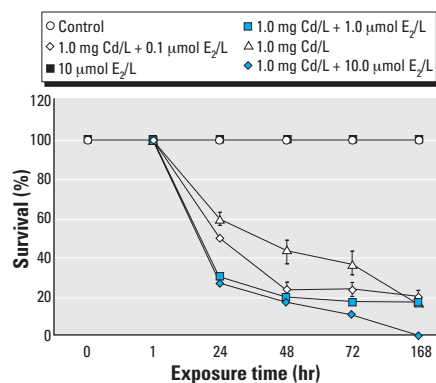
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