Bull. Environ. Contam. Toxicol. (1998) 61:577-582

Bulletin of Environmental Contamination and Toxicology

Estradiol Disrupts Sea Urchin Embryogenesis Differently from Methoxychlor

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Received: 24 September 1998/Accepted: 28 October 1998

The environmental proestrogen methoxychlor (MXC), a widely used pesticide, has adverse effects on reproduction and development (Bal 1984; Martinez and Swartz 1991). In mammals cytochrome P450 enzymes metabolize MXC into metabolites with estrogenic activity (Levin *et al.* 1968; Kupfer *et al.* 1990). The observed deleterious effects following MXC exposure have been attributed to its estrogenic metabolite, 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) (reviewed by Cummings 1997). We have reported abnormal gastrulation in sea urchins when gametes and zygotes had been pulsed with MXC for 30 min starting at 10 min after fertilization (Green *et al.* 1997; Mwatibo and Green 1997, 1998).

Since MXC is a proestrogen and there is much recent interest in the effects of estrogenic compounds in the environment (Cummings 1997) we were interested in learning if the MXC-induced developmental defects we observed in sea urchin embryos could be ascribed to its estrogenic activity. In our previous experiments, gametes and zygotes were exposed to, then removed from MXC, well before gastrulation. Since P450 cytochrome activity is not appreciable until the gastrula stage (Bresch and Arendt 1977) we reasoned that the breakdown of MXC into its estrogenic metabolites within the sea urchin embryo was not likely to have occurred and, therefore, have caused the results of our experiments. Accordingly, the aim of this brief study was to ascertain whether zygote exposure to the pure estrogen 17β-estradiol would result in developmental anomalies similar to those we have reported for MXC.

MATERIALS AND METHODS

Sea *urchins, Strongylocentrotus purpuratus,* were purchased from Kim Siewers (Santa Cruz, CA) and maintained in an aerated and refrigerated laboratory aquarium at 14-15° C. Gamete shedding was induced by intracoelomic injection of 0.55*M* KC1 and gametes were collected in artificial seawater (ASW). ASW was prepared according to Marine Biological Laboratory formulae (Cavanaugh 1956) and buffered with 10 m*M* TAPS (tris [hydroxy-methyl] methyl aminopropane sulfonic acid) to a final pH of 8.0. Semen was pipetted "dry" from the gonopores of males and stored undiluted at 4° C until use. Before use, eggs were washed in three changes of ASW to remove debris and coelomic fluid from

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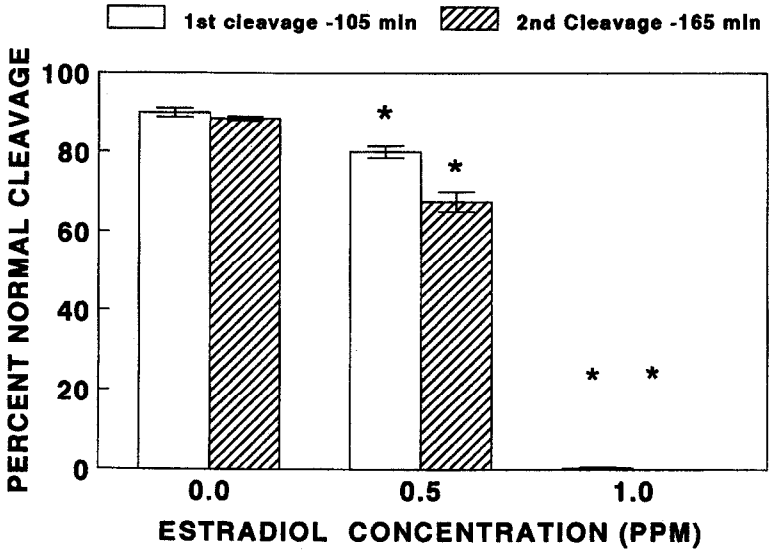


Figure 1. Embryos of *S. purpuratus* undergoing normal first and second cleavages under the influence of 17β-estradiol. The decreases in percentages of treated embryo cleavage rates are statistically significant (*). Standard error bars (s.e.m.) are displayed.

the suspension. A 2% suspension of eggs was fertilized by the addition of semen diluted to effect 95 to 100% fertilization. After insemination and swirling of the egg-sperm mixture to thoroughly mix the gametes, the fertilized eggs were allowed to settle. The overlying ASW was aspirated to remove excess sperm and the embryos were resuspended in fresh ASW. Fertilized eggs elevate fertilization envelopes (FE) which harden by 10 min postinsemination (PI). Elevated FE were counted to determine the rate of fertilization and only egg batches that achieved 95% or more fertilization were used for experiments. Normal first cleavage (i.e., division into two symmetrical blastomeres) was monitored to check for polyspermy. Batches of eggs that did not achieve at least 90% normal first cleavage were discarded. The embryos were allowed to develop at 15° C with gentle stirring at 60 rotations per minute using motor-driven paddles. With these culture conditions, synchronous division and development of embryos was obtained. Development was monitored by periodic microscopic examination. 17β-estradiol (Sigma) was dissolved in DMSO (similarly to MXC in previous experiments). Fertilized eggs were exposed to final concentrations of 1.0 or 0.5 ppm estradiol in 0.5% DMSO/ASW beginning at 10 min PI. Controls were allowed to develop in 0.5% DMSO/ASW. The final concentration (0.5%) of DMSO in ASW was previously shown not to interfere with normal development (Green *et al.* 1997). Aliquots of embryos were taken at the following developmental stages: 2-cell, gastrula and pluteus. Experiments were terminated at the pluteus stage, after which time further development requires external food sources. These samples were fixed in neutral buffered 10% formalin in ASW.

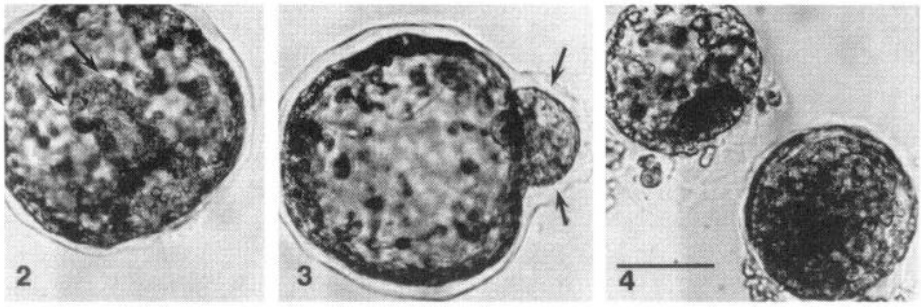
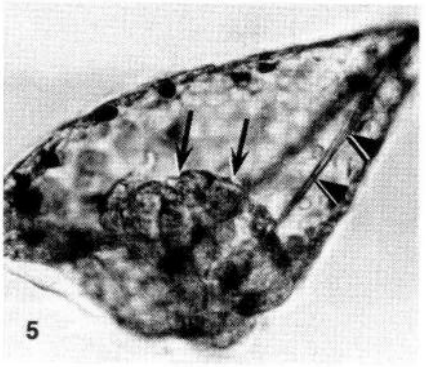


Figure 2. Control 36-hr gastrula with normal gut (arrows) developing in 0.5% DMSO/ASW. **Figure 3.** 36-hr gastrula exposed to 0.5 ppm estradiol with exogut (arrows). **Figure 4.** Moribund embryos after exposure to 1.0 ppm estradiol. Scale bar represents 50 pm.

One hundred embryos were scored in triplicate by light microscopic examination. Statistical analysis was by the Chi-Square test at the 95% confidence limit. Representative embryos were photographed.

RESULTS AND DISCUSSION

Embryos were evaluated at the normal time for controls to complete first and second cleavages, at 105 min and 165 min respectively. Zygotes in 1.0 ppm estradiol had not cleaved by the time of normal first and second cleavages (Fig. 1). Chronic exposure of embryos to 0.5 ppm 17β-estradiol starting at 10 min PI reduced the percentages of embryos completing normal first cleavage from 90% to 80%, a significant reduction of 11%. Second cleavage was reduced from 88% to 68%, a significant reduction of 23% compared to control embryos (Fig. 1). Abnormal cleavage may be attributed to either polyspermy [supernumerary sperm contribute extra centrioles which divide and form abnormal numbers of mitotic spindles, thereby resulting in unequal numbers of chromosomes segregated in the daughter cells (Boveri, 1902 as described in Gilbert 1997)] or to estradiol (Agrell 1954). Polyspermy was ruled out as the cause for the following reasons. 1) The concentration of sperm used did not result in polyspermy in the controls; and 2) the permanent (physical) block to polyspermy (*i.e.,* the elevation of the fertilization envelope) occurs within 1 to 2 min PI (Ray and Shapiro 1985). This was well before the addition of estradiol at 10 min PI. Therefore, these eggs would not have been susceptible to penetration by supernumerary sperm. It is apparent that the delay of cleavage was induced by the estradiol treatment. The effect of chronic exposure to 17β-estradiol was monitored further through periodic assessments of the embryo culture at different stages of development. At gastrula stage (36 hr PI), ≥ 99% of control gastrulae had normally developing guts, extending across the blastocoel (Fig. 2). However, among the gastrulae



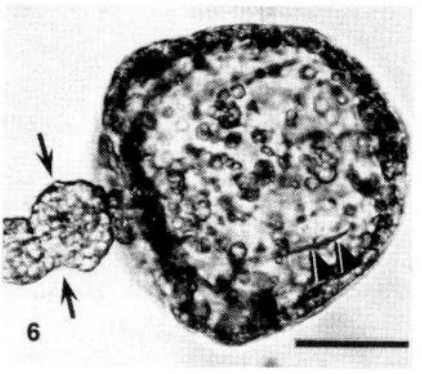


Figure 5. Control 72-hr pluteus with typical tripartite gut (arrows) and well-developed spicules (arrowheads). **Figure 6.** 72-hr pluteus after 0.5 ppm estradiol exposure with differentiated exogut (arrows) and spicules (arrowheads). Scale bar represents 50 µm.

exposed to estradiol, 9% appeared normal, 37% showed no gut development, and 54% had evaginated guts, *i.e.*, they were exogastrulae (Fig. 3). This compares with ≤ 1% exogastrulae in the control group. Altogether, 91% of the developing gastrulae exposed to 0.5 ppm 17β-estradiol displayed abnormal gut development. At the time of gastrula stage in the control embryos, those exposed to 1 ppm 17β-estradiol were moribund (Fig. 4).

At pluteus stage (72 hr PI) control embryos had undergone further differentiation of the gut into the typical tripartite structure (foregut, midgut, hindgut) and a skeleton imparting a tetrahedral shape on the larvae (Fig.5). Embryos exposed to 0.5 ppm estradiol exogastrulated with tripartite exoguts (Fig. 6). Thus, although exposure to estradiol resulted in exogastrulation, it did not inhibit further differentiation of the exogut. Skeletal spicules were present in these exogastrulae, but were not as robust as those of controls.

During the prehatching stages of embryonic development in the sea urchin chronic exposure to 17β-estradiol delayed early cleavage. Presumably, this delay was due to estradiol inducing abnormal mitotic spindles (Agrell 1954), and/or its inhibition of DNA synthesis (Agrell and Perrson 1956; Jolley et al. 1962). Bresch and Arendt (1977) reported that MXC inhibited DNA synthesis.

Although both MXC and estradiol delayed cleavage in our hands (Green *et al.* 1997; this report), the chronic exposure of embryos to 17β-estradiol had a different overall effect on gastrulation when compared to MXC exposure. Using the highest concentrations of estradiol or MXC that supported development to the pluteus stage, estradiol induced exogastrulation in more than 50% of the gastrulae. MXC induced both stunted guts and abnormal spicules (Green *et al.* 1997; Mwatibo and Green 1997; 1998), but not exogastrulae. Even though

exoguts were produced under estradiol influence, they did differentiate into a tripartite structure similar to controls. While little is known about the factors that govern directionality of gut development, the observed effects of estradiol are morphologically similar to those of exogenously applied exogastrula-producing peptides (EGIP) (Ishihara *et al.* 1982; Suyemitsu *et al.* 1989). EGIPs cause exogastrulae by affecting spindle microtubules at early gastrula stage (Fujita *et al.* 1993). It is probable that estradiol acts in a similar fashion.

Since the developmental effects of MXC and estradiol on gastrulation in the sea urchin differ dramatically, it is unlikely that the effects of MXC can be ascribed to its estrogenic metabolites. Most likely, they should be ascribed to a non-specific toxicity of the parent compound. These results should be kept in mind when putative estrogenic activities of other environmental xenobiotics on various organisms are being investigated.

Acknowledgments. This research was supported in part by the Department of Cell Biology and Anatomy, LSU Medical Center-New Orleans.

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