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# Effects of Methoxychlor Pre-Exposure on Sea Urchin Gametes

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**Potential adverse effects of environmental pesticides on embryo and early fetal development have been of considerable concern in the past decade. One commonly used organochlorine pesticide is methoxychlor (MXC), a structural analog of DDT. Methoxychlor is used as a broad-spectrum insecticide on crops in home gardens and dairy barns for horsefly control (Reuber 1979). Several organochlorine compounds are found up to 10 ppm in the environment (Simonich and Hites 1995) and MXC concentrations up to 100 ppm have been reported in the environment (IARC 1979). Studies have documented the deleterious effects of MXC on reproduction and sexual development in mammals. These effects which include testicular carcinomas (Reuber 1980), anomalous fetuses (Khera *et al.* 1978) and abnormalities of the reproductive system of mice (Martinez and Swartz 1991, 1992) have been ascribed to the estrogenicity of MXC (Bulger and Kupfer 1983). A study of the influence of organochlorine pesticides on sea urchin embryonic development showed that MXC resulted in abnormal plutei, and significantly reduced the level of DNA synthesis (Bresch and Arendt 1977).**

**A previous study on the effects of MXC on early sea urchin development (Green *et al.* 1997) showed that exposure of fertilized eggs at 15 min post-insemination (PI) to MXC for 30, 60 or 90 min resulted in delay of cleavage and abnormal gastrulation. At gastrula stage the MXC-exposed embryos displayed either stunted guts or no signs of gut formation. Since exposure of embryos to pesticides during early development results in abnormal morphogenesis, a question arose as to whether exposure of gametes to MXC *before* fertilization would result in similar developmental defects. The aim of this study, therefore, was to assess the developmental effects when gametes are exposed to MXC before fertilization. The questions we address in this communication are as follows: 1) Do gametes retain their developmental competence if they are exposed briefly to environmentally relevant concentrations of MXC? and 2) If any embryos develop from exposed gametes, do they develop normally?**

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## MATERIALS AND METHODS

Artificial sea water (ASW) was prepared according to Marine Biological Laboratory formulae (Cavanaugh 1956) and buffered with 10 mM TAPS (tris [hydroxymethyl] methylaminopropane sulfonic acid; Sigma) to a final pH of 8.0 (Green *et al.* 1990). Sea urchins *(Strongylocentrotus purpuratus)* were purchased (Kim Siewers, Santa Cruz, CA) and kept in a salt water (30 parts per thousand salinity) aquarium at 14-15°C. Gamete shedding was induced by intracoelomic injection of 0.55M KCI. Eggs were collected by inverting the females over beakers of ASW. Semen was collected "dry" by pipet from the gonopores of males and stored undiluted at 4°C (Green *et al.* 1990) Sperm aliquots were adjusted to give approximately 95% fertilization.

Methoxychlor (98% grade, Sigma), being relatively insoluble in water (≈ 0.1 ppm at 25°C; Richardson and Miller 1960), was dissolved in 0.5% dimethyl sulfoxide (DMSO, Sigma) immediately before use. The addition of MXC did not change the pH (8.0) of the ASW.

For spermiotoxicity experiments, 25 µL undiluted sperm was added to 10 mL 3 ppm of MXC in 0.5% DMSO in ASW (experimentals) or to 0.5% DMSO in ASW (controls). After exposure for 10, 20 or 30 min, 1 mL of the sperm suspension was mixed with 30 mL of a 2% suspension of unexposed eggs in ASW (residual [MXC]≈0.1 ppm). Eggs were considered fertilized if a normal fertilization envelope (FE) elevated. A normal FE is one that has elevated symmetrically around the egg and appears smoothly rounded. Normal elevation is completed within one minute. Fertilization was scored by counting in triplicate (N = 300) the percentage of fertilized eggs at 10 min PI. After the eggs had settled the overlying ASW was aspirated to remove MXC. Embryos were then resuspended in 10 volumes of ASW (residual [MXC]≈ 0.01 ppm) and allowed to develop. Previous studies showed no deleterious effects on development at these low concentrations of MXC (Green *et al. 1997).*

A similar procedure was used to assess the effect of MXC on unfertilized eggs. A 2% suspension of unfertilized eggs was exposed to MXC (or the DMSO control) for 30 min before the eggs were washed with fresh ASW. The eggs were then inseminated with freshly diluted sperm, checked for normal FE elevation and the resulting embryos were allowed to develop.

To score for abnormal development, embryos that developed from gametes exposed to MXC before fertilization were observed at prism stage (≈48 hr PI) for the presence of morphologic abnormalities. The criteria for scoring an embryo as abnormal were a stunted gut and/ or incomplete spicules. At least 100 embryos were scored in duplicate and

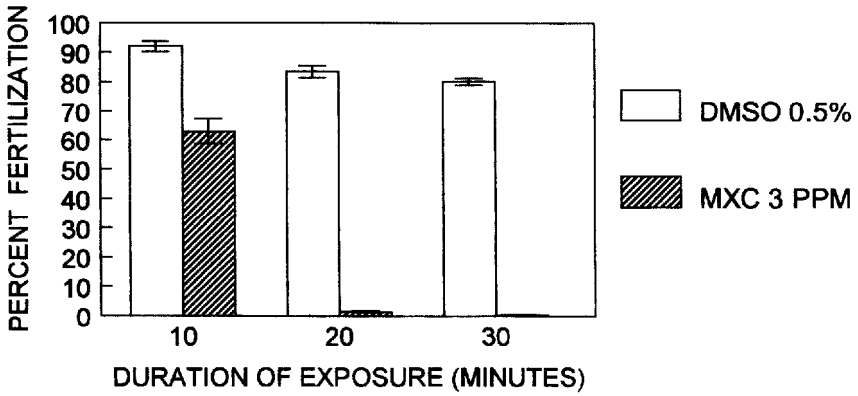


**Figure 1.** Fertilization efficiency of sperm exposed to MXC for 10, 20 and 30 min before fertilization.

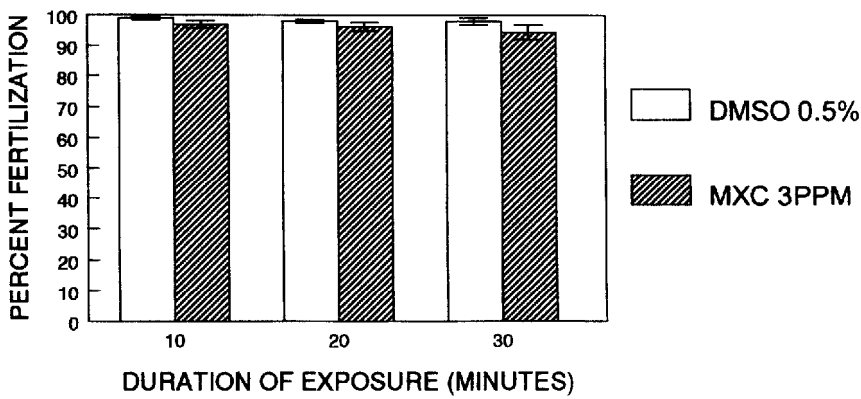


**Figure 2. Fertilization of eggs exposed to MXC before fertilization.**

**results expressed as the percentage of normal development. Statistical analysis was performed using the Chi-square test.**

## RESULTS AND DISCUSSION

**When sea urchin sperm were exposed to 3 ppm MXC for 10 min, there was a ≈30% reduction in fertilization (Fig. 1). Exposure to MXC for 30 min was spermiotoxic since such an exposure resulted in virtually no fertilization (<1% compared to 80% in controls). Between 10 and 30 min, exposure to DMSO caused a 13% decrease in fertilization efficiency. However, this decrease was not comparable to the effect of MXC, which reduced fertilization from 63% after a 10 min exposure to**

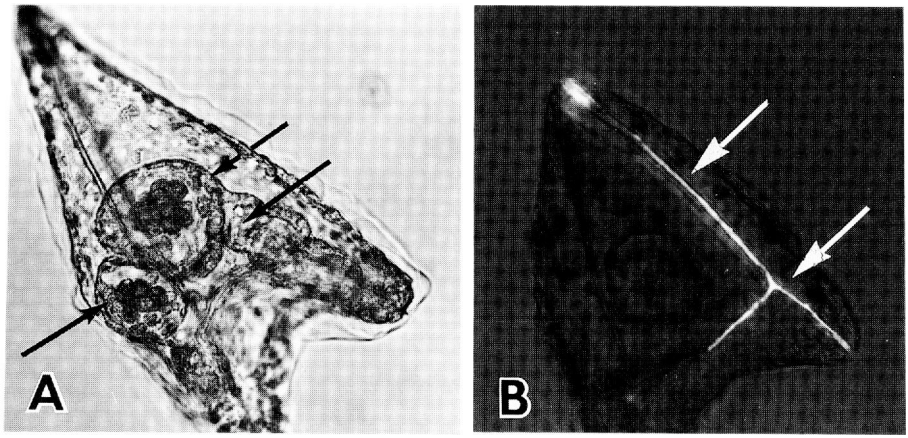


**Figure 3. Pluteus stage (DMSO controls). Embryos were tetrahedral in shape and displayed a tripartite gut (arrows in A) and (B) a complete skeleton (arrows) as seen with polarized light.**

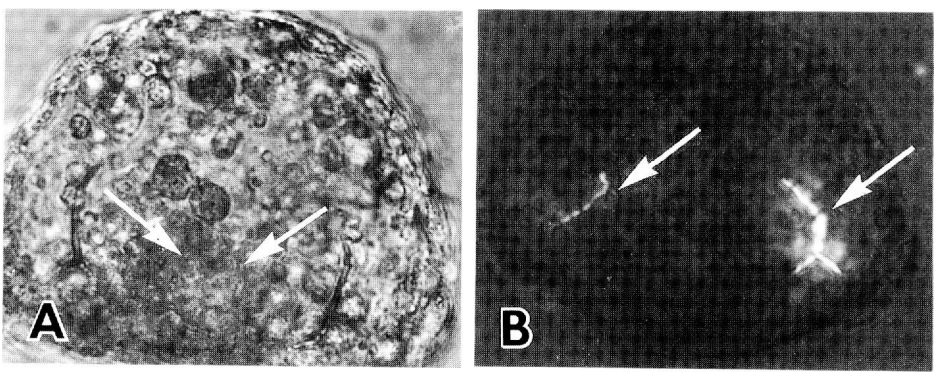


**Figure 4. Pluteus stage (MXC-exposed). Embryos were spherical in shape and displayed a stunted gut (arrows in A) and (B) an incomplete skeleton (arrows) as seen with polarized light.**

**$\leq$1% after a 30 min exposure, a decrease of 98%. On the other hand, eggs exposed to 3 ppm MXC for 30 min before insemination had a mean fertilization rate of 95%, similar to controls exposed only to DMSO (Fig. 2) and to those in normal ASW.**

**When gametes were exposed to MXC for 10 min (sperm) or 30 min (eggs) before fertilization, the resulting embryos were allowed to develop. At the prism stage (48 hr) they were evaluated for the presence of morphologic abnormalities. At this stage, control (DMSO) embryos had a normal appearing gut tube extending across the blastocoel. By pluteus stage (72 hr) the gut had differentiated into fore-,**

mid- and hind-gut (Fig. 3a). The embryonic spicules were normally formed (Fig. 3b). Approximately 3.5% and 5.5% of those embryos (Table 1) that developed from MXC-exposed sperm and eggs, respectively, displayed an abnormal, stunted gut or had only a thickened vegetal plate epithelium with disorganized clusters of mesenchymal cells. These abnormalities persisted until the pluteus stage (Fig. 4a). The spicule primordia had been secreted in the normal sites within the blastocoel, but the spicules were malformed, *ie.,* they either had short rods or abnormally branched rods (Fig. 4b). In addition to the malformed skeleton, the MXC-exposed embryos were spherical rather than the usual tetrahedral shape characteristic of embryos at this stage (compare Fig. 3a and 4a).

There was a small, but significant increase ($p < 0.05$) in the percentage of abnormal prisms from those eggs that were fertilized after pre-exposure to MXC compared to DMSO controls (Table 1). Exposure of sperm did not result in a significant increase in abnormal prisms ($p > 0.05$).

Table 1. Percentage of abnormal prisms at 48 hrs.

| | |
|---|---|
| Sperm pre-exposed to 0.5% DMSO for 10 min | 2.5± 0.5* |
| Sperm pre-exposed to 3 ppm MXC for 10 min | 3.5± 0.5 |
| Eggs pre-exposed to 0.5% DMSO for 30 min | 2.0± 1.0 |
| Eggs pre-exposed to 3 ppm MXC for 30 min | 5.5± 0.5 |

(N= 200; * Mean % ± S.E.M.)

Green *et al.* (1997) found that sea urchin zygotes exposed to relatively short time pulses of MXC beginning at 15 min PI underwent a delay in cleavage and disruption of gastrulation. At the gastrula stage these embryos displayed either stunted guts or showed no signs of gut formation. Since exposure of gametes to environmental contaminants may lead to abnormal development, we sought to determine whether exposure of sea urchin gametes to MXC before fertilization would result in similar developmental defects. This is pertinent since many aquatic organisms are external fertilizers which shed their gametes into waters containing putative environmental contaminants such as MXC.

The present study shows that a brief, 10 min exposure of sperm to 3 ppm MXC before fertilization caused a 30% reduction in fertilization. Moreover, exposures of 20 and 30 min resulted in a drastic decrease in

**fertilization (< 1% at 30 min), indicating that such exposures were lethal to sperm. Since exposure of eggs to MXC before fertilization resulted in no noticeable effect on fertilization, it is evident that sperm are more susceptible to MXC.**

**However, there was a small increase in the frequency of developmental abnormalities of prisms derived from either pre-exposed gamete. The frequency of abnormalities was slightly higher for pre-exposed eggs than sperm. This suggests a minor but transmissible effect of MXC on the gametes that is expressed later in development. The overall effect in the environment is likely to be considerable because of the propensity of runoff MXC to bioaccumulate (USA EPA, 1983). At fertilization each gamete provides essential components and signals that are important for normal embryonic development. In many organisms, the egg has large stores of maternal mRNA and proteins synthesized before fertilization (Davidson, 1986), while the sperm supplies factors which initiate the developmental program. Exposure of gametes to environmental toxicants may result in alteration of the essential components contributed by either gamete, resulting in failure of fertilization and/or defective development. While the mechanisms of MXC toxicity on sea urchin development remain to be ascertained, potential targets of its toxicity could include pH perturbation, protein synthesis, respiration, etc.**

**Although pH shifts (especially decreases) have been shown to induce developmental defects (Pagano *et al.* 1985), the abnormalities we observed resulting from MXC-exposure were probably not pH-related in regard to the external environment, since dissolution of MXC in the embryo culture medium (0.5% DMSO/ASW) did not alter the pH of the medium. At the present time, we cannot rule out the effects of changes in the internal cellular PH. The small increase in the percentage of abnormal prisms may be due to MXC-induced alterations in such early embryonic events as synthesis of proteins essential for cell division, cell migration, cell-cell interactions, *etc.* This is a distinct possibility, since all the mRNAs essential for gastrulation are usually synthesized by late blastula (Guidice et al. 1968).**

**Exposure to MXC for 20 to 30 min was spermiotoxic since there was virtually no fertilization following this duration of sperm exposure. This spermiotoxicity, together with the fact that embryos exposed to MXC after fertilization develop abnormally (Green *et al. 1997),* raises significant concern regarding the effect of run-off MXC contamination on the reproductive success and development of aquatic organisms.**

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## REFERENCES


Bresch H, Arendt U (1977) Influence of different organochlorine pesticides on the development of the sea urchin. Environmental Res 13:121-128.

Bulger WM, Kupfer D (1983) Estrogenic action of DOT analogs. American J Ind Med 4:163-173.

Cavanaugh GM (ed) (1956) Formulae and Methods of the Marine Biological Laboratory Chemical Room. Marine Biological Laboratory, Woods Hole, MA.

Davidson EH (1986) Gene activity in early development, 3rd ed. Academic Press, Orlando, FL.

Green JD, Glas PS, Cheng SD, Lynn JW (1990) Fertilization envelope assembly in sea urchin eggs inseminated in chloride-deficient sea water. II. Biochemical effects. Molec Reprod Develop 25:177-185.

Green JD, Mwatibo JM, Swartz WJ (1997) The effects of methoxychlor on early sea urchin development. Environ Res 72 (in press).

Guidice G, Mutolo V, Donatuti G (1968) Gene expression in sea urchin development. Wilhelm Roux's Arch 116:118-128.

IARC (1979) Methoxychlor. Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans 20:259-281.

Khera KS, Whalen C, Trivett A (1978) Teratogenicity studies on linuron, malathion and methoxychlor in rats. Toxicol Appl Pharm 45:435-41.

Martinez EM, Swartz WJ (1991) Effects of methoxychlor on the reproductive system of the adult female mouse, 1. Gross and histologic observations. Reprod Toxicol 5:139-147.

Martinez EM, Swartz WJ (1992) Effects of methoxychlor on the reproductive system of the adult female mouse. 2. Ultrastructural observations. Reprod Toxicol 6:93-98.

Pagano G, Maria C, Corsale G, Esposito A, Ragucci E, Giordano GG (1985) pH-induced changes in mitotic and developmental patterns in sea urchin embryogenesis. 1. Exposure of embryos. Terat Carcin Mut 5: 101-112.

Reuber MD (1980) Carcinogenicity and toxicity of methoxychlor. Environ Health Perspect 26:205-219.

Reuber MD (1979) Carcinomas of the liver in Osborne-Mendel rats in ingesting methoxychlor. Life Sci 24:1367-1372.

Richardson LT, Miller DM (1960) Fungitoxicity of chlorinated hydrocarbon insecticides in relation to water volubility and vapor pressure. Can J Bot 38:163-175.

Simonich S, Hites R (1995) Global distribution of persistent organochlorine compounds. Science 269:1851-1854.

US EPA (1983) Occurrence of pesticides in drinking water, food and air. US EPA Office of Drinking Water, Washington DC.