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Effects of Chlorocresol (4-Chloro-2-methyl Phenol) Administered During the Fertilization and Cleavage Phases of *Xenopus laevis*

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The Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) is a bioassay that makes use of embryos of *Xenopus laevis*; it has been applied to evaluate the embryo toxicity of xenobiotics (i.e., mortality, teratogenicity and growth inhibition). The assay entails the continuous exposure of *Xenopus* embryos from the blastula to the free-swimming larvae. The original methodology (Dumont *et al.* 1983; Bantle *et al.* 1989) was modified with the addition of *in vitro* fertilization (i.v.f.) (Bernardini *et al.* 1994) and modFETAX was applied to ecotoxicological studies (Vismara *et al.* 1993; Presutti *et al.* 1994). One of the advantage of i.v.f. is the possibility to study the effects of toxicants on the fertilization and on the early stages of development, the fertilization and the cleavage. In this paper, we have extended the study of the effect of a toxicant on those phases not covered by FETAX. We have tested 4-chloro-2 methyl phenol, a molecule present as contaminant in the commercial MCPA pesticide (4-chloro-2-methyl phenoxyacetic acid) and present in the environment as its soil metabolite (Räsänen *et al.* 1977; Sattar 1982).

MATERIALS AND METHODS

Adult *Xenopus* females were injected with 700-1000 I.U. of human chorionic gonadotropin (Sigma Chemical Co., St. Louis, Missouri). About 16 hr later, females were made to lay eggs in sterile plastic Petri dishes; eggs were immediately inseminated with sperm suspension as previously described (Bernardini *et al.* 1994) in 1-2 mL of cold control FETAX solution, whose composition (in mg/L) was: NaCl 625, $NaHCO_3$ 96, KCl 30, $CaCl_2$ 15, $CaSO_4$-$2H_2O$ 60 and $MgSO_4$ 70

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(Dawson *et al.* 1987).

The treatment during the fertilization phase was started 1 min post fertilization (p.f.) and was suspended 40-50 min later. At the beginning, 10 mL of FETAX solution were added to the control groups while 10 mL of two different concentrations of chlorocresol (40 and 80 mg/L in FETAX solution) were added to the first and second treated groups, respectively. At the end of the exposure, all solutions were replaced with FETAX solution. The fertilization success was detected 6 hr p.f.; the embryos not showing any sign of cleavage were considered unfertilized.

We have utilized for the treatment during the cleavage phase the same concentrations of chlorocresol used in the fertilization study (40 and 80 mg/L of chlorocresol in FETAX solution). The treatment was started 1.25 hr p.f., just before the first cleavage and it was suspended 8 hr p.f., when embryos were at the stage of normal blastulae, onset of gastrulation. At the end of the exposure time, all the solutions were replaced with FETAX solution that was renewed every day for the following 5 days. Each day dead embryos were removed and their number was noted. Embryos were kept in a thermostatic chamber at $23°C \pm 0.5$. At 120 hr p.f., end of organogenesis, all experimental groups were evaluated for the mortality rate while the surviving embryos were anaesthetized and evaluated for malformations.

Histological examination was performed on the embryos treated during the cleavage phase. Control and treated groups of embryos were fixed in Bouin's fluid at 8 hr and 24 hr p.f.; paraffin sections were stained with hematoxylin and eosin.

The number of fertilized eggs versus the total number of eggs, the number of dead embryos versus the total number of embryos and the number of malformed embryos versus the survived embryos were analyzed by the chi-square test. The significance level was $p<0.05$ and $p<0.01$.

RESULTS AND DISCUSSION

Chlorocresol at the concentrations of 40 and 80 mg/L did not affect the rate of fertilization (Table 1).

Table 1. Exposure to chlorocresol during the fertilization phase.

	Control	40 mg/L	80 mg/L
Total eggs	237	406	289
fertilized eggs	161	253	213
% fertilization	68	62	73

Table 2. Exposure to chlorocresol during the cleavage phase.

	Control	40 mg/L	80 mg/L
Total embryos	228	236	59
Dead embryos	110	185	57
Live embryos	118	51	2
Malformed embryos	13	11	2
% mortality	48	78*	97*
% malformed embryos	11	22	100*

* $p \leq 0.01$

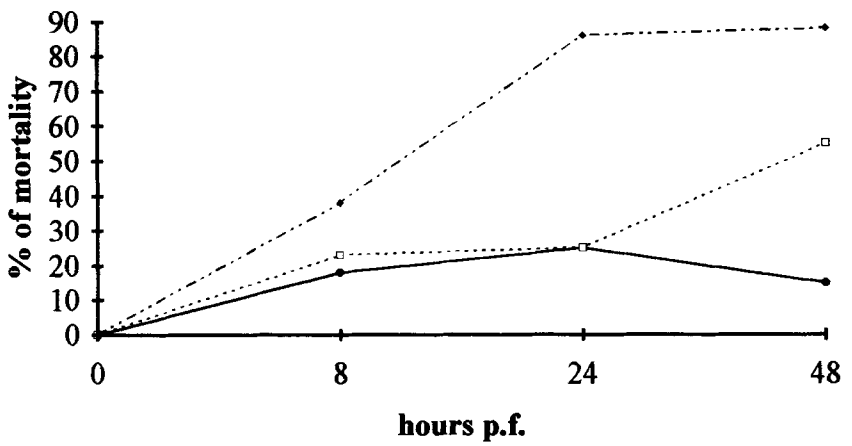

Figure 1. Embryo mortality plotted versus embryo age, after exposure to chlorocresol during the cleavage phase (filled dots, control; empty squares 40 mg/L; triangles, 80 mg/L)

Chlorocresol exposure during the cleavage phase caused a concentration-dependent increase of mortality; 40 mg/L caused a 78% of mortality and with 80 mg/L mortality reached the value of 97% (measured at 120 hr p.f.); the difference between control and treated

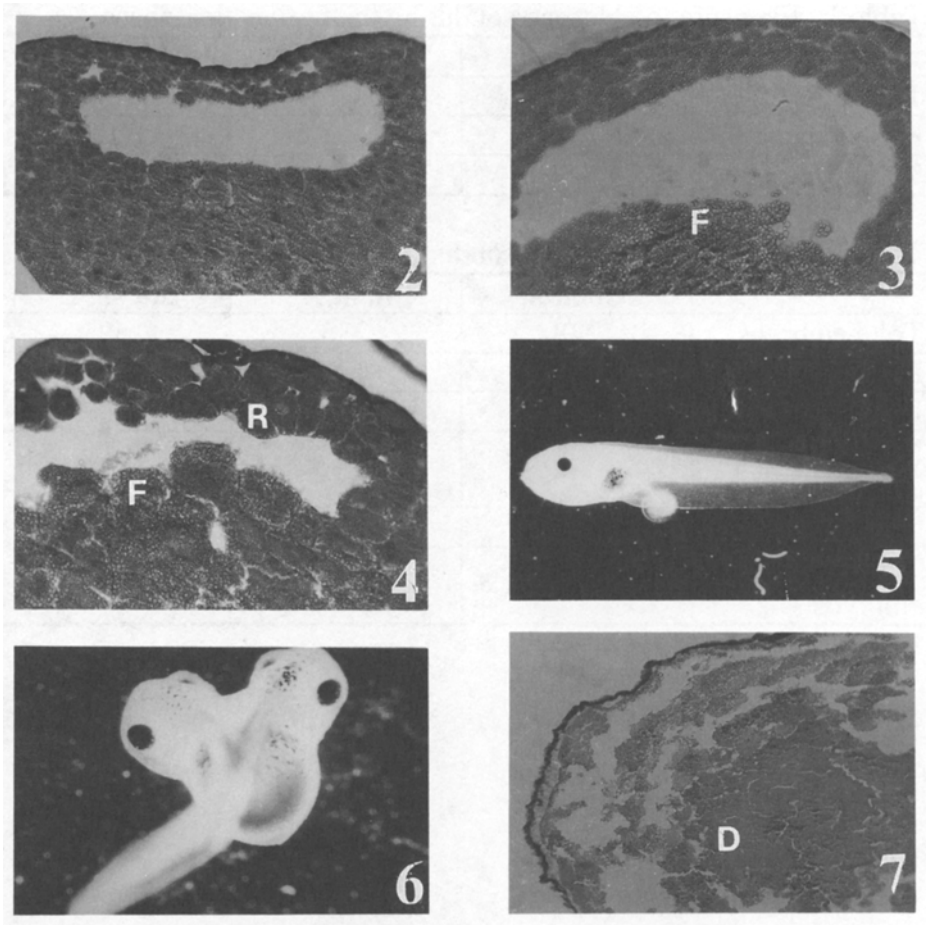

Figure 2. Control blastula (x 100). Figure 3. Blastula of embryo exposed to 40 mg/L of chlorocresol; blastocoel showing an alteration of floor (F) (x 100). Figure 4. Blastula of embryo exposed to 80 mg/L of chlorocresol; blastocoel showing an heavy alteration of roof (R) and floor (F) (x 100). Figure 5. Control embryo at 120 hr p.f. (x 6). Figure 6. Embryo exposed to 40 mg/L of chlorocresol during the cleavage phase: stage 39, *duplicitas anterior* (x 12). Figure 7. Blastula of embryo exposed to 80 mg/L of chlorocresol during the cleavage phase; blastula do not shows any sign of cellular organization (D) (24 hr p.f.) (x 100).

groups was highly significant ($p \leq 0.01$) (Table 2). The concentration of 40 mg/L caused the 22% of malformed embryos (at 120 hr p.f.); this value was not significantly different from the control; the only 2 surviving embryos exposed to 80 mg/L of chlorocresol were severely

malformed ($p \leq 0.01$) (Table 2). Chlorocresol concentration of 40 mg/L caused a peak of mortality within 48 hr p.f.; this was due to an high frequency of severe malformations that did not allow embryos to survive. Almost all the embryos exposed to of 80 mg/L of chlorocresol died within 24 hr p.f.; they were not able to start gastrulation phase (Fig. 1). Therefore, since one of the aim of this study was to verify the embryotoxic effects showed during the organogenesis after treatment on the cleavage phase, the group treated with 80 mg/L of chlorocresol was discarded because of the high mortality rate.

Blastulae of embryos exposed to 40 mg/L showed an alteration of blastocoel floor (Figs. 2, 3); the following gastrulation was completed, but the development occurred abnormally, with many cases of *duplicitas anterior* and *posterior* (Figs. 4, 5). Blastulae of embryos exposed to 80 mg/L of chlorocresol exhibited an heavy alteration both in the blastocoel roof and floor (Fig. 6), later on embryos failed to start the gastrulation; 24 hr p.f. most embryos were dead showing signs of decomposition (Fig. 7).

Most teratologists believe in fact that in mammals, after the exposure to exogenous insults during the pre-implantation period, the embryo either dies or survives unharmed to term (Wilson 1965; Giavini *et al.* 1981); in Xenopus, on the contrary, teratogenic effects can be induced also by an exposure during the cleavage phase.

In conclusion, we believe that an evaluation of the effects of the aquatic toxic compounds on the life cycle of an organism should include the exposure from the fertilization to the end of organogenesis. The results must be integrated in a test battery for quality control of water, for the evaluation of risks to human health as well as the protection of aquatic ecosystem.

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