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A Fish Model as an Indicator for Teratogenic Substances

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A recent expansion has occurred in development of *in vivo* methods for teratogenesis (Birge *et al.*, 1983; Whitby and Flynn, 1987). This effort has focused on a number of potential nonmammalian systems, and fish have received considerable attention (Laale and Lerner, 1981; Collins, 1987; Weis and Weis, 1987; Solomon and Faustman, 1987). We have developed a fish embryo model as an indicator for teratogenic substances. Our procedure utilizes naturally spawned embryonic inland silversides, *Menidia beryllina*. This euryhaline fish is ubiquitous in estuaries, coastal rivers and numerous lakes in the southeastern United States and is also found in the Mississippi River basin (Sisk and Stephens, 1964; Gomez and Lindsay, 1972; Johnson, 1975; Chernoff *et al.*, 1981).

Embryo Acquisition

Adult *M. beryllina* are easily maintained in the laboratory. A population of 25 males and 25 females collected from a low salinity (0-5 $^{0}/_{00}$) estuarine site in early April (20°C) and maintained at $5^{0}/_{00}$ 25°C produced 300 to 2000 embryos daily for approximately 5 months (Middaugh *et al.*, 1985). Thereafter a new population of young adults collected in late September, produced embryos throughout the winter. Holding tank configuration, environmental and nutritional requirements for brood stock adults have been summarized by Middaugh *et al.* (1986).

Test Procedure

Natural unpolluted seawater used in all tests is treated by pre-filtration through a 20 µm polypropylene filter. It is then passed through a 6 µm filter, diluted to $5^{0}/_{00}$ salinity with deionized water and autoclaved. Stock solutions are prepared by diluting the analytical grade test chemical in deionized water, acetone, or another appropriate solvent.

A range finder test may be employed using a reduced number of embryos and a wide range of concentrations to bracket the potential teratogenic response levels. It is preferable that a concentration range be chosen that limits the lethal response at the highest treatment levels while enhancing the teratogenic response at the lower treatment levels.

Just prior to beginning a test, aliquots of stock solution are added to 1.0 litre of autoclaved saline water ($5^{0}/_{00}$) to yield the desired nominal exposure concentrations. We generally use 5 concentrations of the suspected teratogen plus a seawater control. If

an organic solvent such as acetone is employed, an additional carrier control must be maintained with the concentration of the solvent equal to that in the highest exposure concentration of teratogen. The quantity of solvent added to each treatment should be equal to that in the highest exposure concentration of toxicant and in the carrier-control; and should not exceed concentrations recommended by ASTM (1980).

Exposures are conducted in borosilicate glass tissue culture tubes 16 x 93 mm (window size 11 x 55 mm). Twenty tubes per concentration are used, including 20 controls and 20 carrier controls (if required). Numbered tubes are randomized prior to addition of embryos.

Early blastula embryos, rinsed 3 times with autoclaved $5^0/_{00}$ water, are placed in a crystallization dish under a dissecting scope (15-20x). Single embryos are drawn into a pasteur pipette and placed in an empty tube with ~ 0.1 ml of $5^0/_{00}$ water from the crystallization dish. The randomized numbered tubes, each containing a single embryo, are then quickly reordered (1-20, 21-40, 41-60, 61-80, 81-100, 101-120, 121-140) and 6 ml of respective exposure solutions (or clean $5^0/_{00}$ saline water for controls) are added to each tube. Tubes, each with an airspace of 7 cc, are sealed with teflon-lined caps, placed in racks and stored in a horizontal position in an incubator at 25± 1°C with a 14L:10 D photoperiod. Cool-white fluorescent lamps provide 500 lux illumination during the light phase.

Dissolved oxygen (D.O.) and pH should be checked in 3 to 5 control(s) and respective treatment tubes at the end of each test. A Lazar Model DO 166* oxygen probe and Orion Model SA 520 pH meter, or equivalent, are suitable for measurements. In conducting ~ 50 tests with a variety of teratogens, including aliphatic and aromatic hydrocarbons, measured pH values have ranged from 6.1 to 7.3 and D.O. from 5.1 to 7.6 mg/l. Maintenance of adequate D.O. concentrations is essential since low D.O. can also cause terata.

Quantification of Responses

Individual control and chemically-exposed embryos are examined daily for 7 days until death or hatching occurs. The criterion for a successful test is 85% or greater hatching in the control and carrier control. A Zeiss Invertoscope D Microscope, or equivalent, is used to categorize responses. Observed terata are scored each day using a numerical severity index for craniofacial (CR), cardiovascular (CV), and skeletal (SK) defects. Numerical scores (Table 1) are based upon classification schemes devised by Weis and Weis (1977), Weis *et al.* (1981), and Weis and Weis (1982).

Data Analysis

Upon completion of a test embryo deaths are categorized based on percentage mortality and analysed, by either probit analysis to derive an LC50 or by a one-way analysis of variance (ANOVA) and Duncans multiple range test, using arcsine transformed data to

*Mention of tradenames does not imply endorsement by the U.S. Environmental Protection Agency.

indicate differences between control(s) and treatment responses (Finney, 1978; Steel and Torrie, 1960; Sokal and Rohlf, 1969). It is preferable that a concentration range be chosen that limits the lethal response at the highest treatment level while enhancing the teratogenic response.

Analysis of the total teratogenic response is performed as follows: for each tube, CR, CV, and SK values (Table 1) are summed to provide a total daily score which can range from 0 for a normally developing embryo to 13 for a severely deformed embryo (i.e., CR 3 + CV 5 + SK 5 = 13). If an embryo dies before the end of the test, a value of 14 is assigned to the tube daily from the date of death until test termination. The daily scores are then summed to generate a total severity index for each embryo. By assigning a value of 14 to exposed and control embryos that die, the uncertainty of causal effect for death is accounted for without biasing the total severity-indices for terata (Middaugh *et al.*, 1988). To test for differences in terata between control(s) and treatment concentrations, nonparametric Van der Waerden normal scores tests (SAS, 1985) and Van der Waerden post-hoc multiple comparison procedures, $\alpha = 0.05$ (Marascuilo and McSweeney, 1977) are conducted, using total severity-indices.

Analysis of individual response categories, CR, CV and SK (Table 1), may be conducted as follows: for each embryo, the daily score for the CR index could range from 0 to 3; for CV, 0 to 5; and for SK, 0 to 5. If an embryo dies before the end of the test, a daily value of 4 is assigned to the CR index; the CV and SK indices are assigned a value of 6. As with total severity-indices, assignment of a daily value for embryo death in individual categories enables one to account for mortalities without introduction of bias by mortalities of unknown cause (Middaugh *et al.*, 1986). Respective categorical indices are then analysed using Van der Waerden normal scores tests (SAS, 1985) and post-hoc multiple comparison procedures (Marascuilo and McSweeney, 1977).

With some compounds, the teratogenic expression is limited to one dysmorphic response, e.g., skeletal defect. In this case, the data analysis is greatly simplified. The teratogenic response is analysed by calculating the percentage of embryos that elicit a response at each treatment level. The data can then be presented graphically or arcsine transformed and analysed by one-way ANOVA and post-hoc procedures, if appropriate. If categories based on the severity of the response can be distinguished with the single morphogenic response, then the analysis is the same as described above for individual response categories (CR, CV and SK).

Utilization of the system described above provides for quantification of teratogenic/toxic effects of chemicals during embryonic development. Because the exposure tubes are sealed for the duration of the test, the risk of human exposure to the test compounds is greatly reduced. Furthermore, embryos from other species such as the Japanese medaka, *Oryzias latipes*, and zebra fish, *Brachydanio rerio*, probably could be tested in this system.

Table 1. *Menidia beryllina*. Synopsis of observed teratological responses in embryos and numerical severity-index for craniofacial (CR), cardiovascular (CV), and skeletal (SK) defects. Adapted in part from Weis and Weis (1977), Weis *et al.* (1981), and Weis and Weis (1982).

Craniofacial		Cardiovascular		Skeletal	
Value	**Effect**	**Value**	**Effect**	**Value**	**Effect**
0	none observed	0	none observed	0	none observed
1	slight defect in structure or size	1	slight defect in structure or function including reduced circulation	1	slight bend or kink including scoliosis, lordosis
2	moderate defect in structure or size including synophthalmia	2	tube heart, beating with or without circulation	2	major bend or kink (greater than 90° angle or more than one bend)
3	severe defect in structure or size including microphthalmia, anophthalmia, or anencephaly	3	tube heart, not beating	3	stunted
		4	beating tissue, but no heart structure	4	very stunted, but axis discernible
		5	no discernible heart	5	no axis discernible

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16. ABSTRACT

A fish model, suitable for use as an indicator for teratogenic substances, is described. Individual blastula stage embroys of the inland silverside, Menidia beryllina, are exposed to teratogens in sealed tissue culture tubes containing 6 mℓ of saline test media, 5°/°° salinity and 25 + 1° C. Individual embryos are examined daily and tetra enumerated using a system that ranks craniofacial (CR), cardiovascular (CV) and skeletal (SK) responses. Procedures for statistical analysis of data are described.

17. KEY WORDS AND DOCUMENT ANALYSIS

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