

Research Project Technical Completion Report

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EFFECTS OF CADMIUM SALTS ON THE REPRODUCTIVE POTENTIAL OF
MALE RAINBOW TROUT AS DETERMINED BY INVIVO AND INVITRO TECHNIQUES.

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INTRODUCTION

The physiologic effect of Cd salts on fish has yet to be clearly shown. Most chemosterilant studies involving Cd have used mammalian species. Chemosterilant activity of Cd in mammals can occur at the sublethal concentration of 5 mg/kg body weight, primarily due to involvement of male gonadal tissue in mammals having descended testes (Gunn, et al., 1970, and Chiquoine et al., 1965).

The mechanism of chemosterilization is not yet clear and may differ with the species involved. Chiquoine et al. (1965) have shown that Cd induces testicular necrosis in mice while Hodgen et al. (1969) found that chemosterilization in rats is a result of interference with testis vascular endothelium as measured by carbonic anhydrase inhibition.

Two mechanisms have been presented in the literature to explain the mechanism of Cd toxicity in fish. Cd has been shown to act on specific metabolic pathways without circulatory involvement. Levels of 0.59 mg CdCl₂/l have been shown to inhibit Bluegill liver mitochondrial succinic oxidase (Hiltibran, 1971). Alkaline phosphatase activity from Fundulus is shown to have 80% inhibition in the presence of 18 mg CdCl₂/l (Jackim et al., 1970). Brook trout testicular steroid synthesis inhibition has been demonstrated by placing testis in media ranging from 1 to 100 mg CdCl₂/l (Sangalang et al., 1972). In contrast, histologic changes in fish testis have been observed which indicate that Cd may cause circulatory changes leading to testis degradation. CdCl₂ injected intraperitoneally into goldfish at levels of 7 to 10 mg CdCl₂/kg body weight caused histologic changes in testis with levels

of 10 and 20 mg/kg body weight giving an LD₅₀ of 96 h (Tafanelli, 1972). Sublethal environmental concentrations of 0.010 mg/l CdCl₂ induced testicular hemorrhagic necrosis in Brook trout (Sangalang and O'Halloran, 1972). I present further evidence of CdCl₂ inducing a metabolic effect on fish gonad cells without the influence of circulatory involvement. This was accomplished by determining the dose sensitivity of the rainbow trout gonad cell line (RTG-2) doubling time to various levels of CdCl₂ added to tissue culture medium.

MATERIALS AND METHODS

RTG-2 cells were obtained from the American Type Culture Collection in passage 60 and grown in pH 7.2 Leibovitz L-15 medium containing 2mM L-glutamine, 10% fetal calf serum, and 100 IU penicillin and 100 μ g Streptomycin per ml, at 20° C in Falcon no. 3012 (25 cm²) plastic tissue culture flasks.

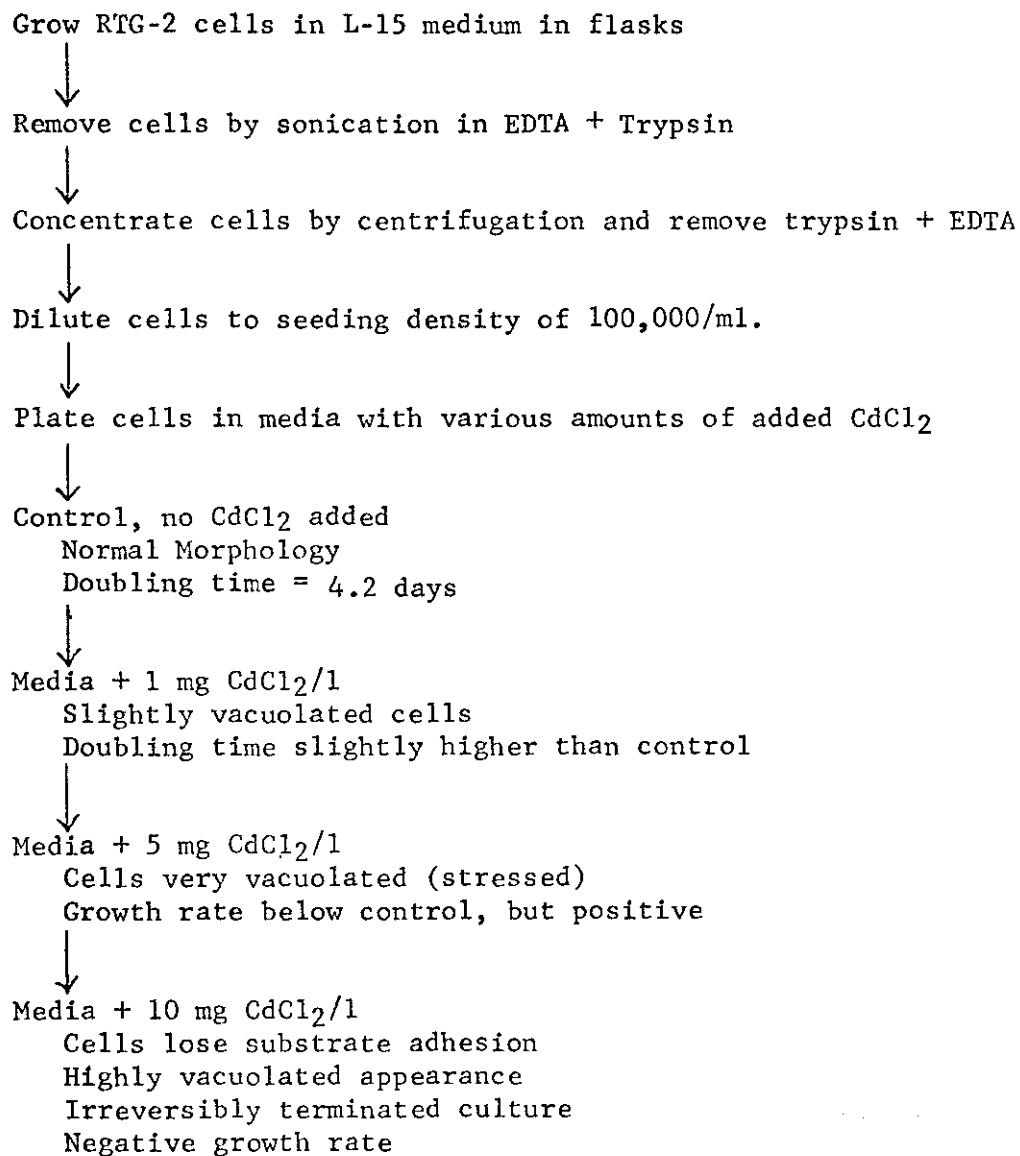
Attempts to remove these cells from the flasks by the technique of Plumb and Wolf (1971) employing 500 mg of 1:250 trypsin and 200 mg disodium ethylenediaminetetraacetate (EDTA) per liter in Puck's modified solution A at a temperature of 20°C for a period of 10 min resulted in incomplete removal of the cells from the flask surface. However, the use of this trypsin and EDTA solution removes sufficient cells to allow replating of cells to initiate new cultures, the cells present being in the form of clumps of 10 to 75 cells, making cell counts very difficult.

We have found that the replacement of L-15 medium in the flask with 3 ml of 500 mg of 1:250 trypsin and 200 mg EDTA per liter in modified Puck's solution A (Gibco Cat. No. 530), immediately followed by sonication by immersion of the trypsin containing sealed flasks in a 100 watt output Branson model 220 ultrasonic cleaner filled with 2 cm of water for 1 to 1.5 min at ambient temperature, resulted in the abrupt removal of all cells from the plastic surface. The temperature of the ultrasonic bath water should not exceed 25°C, as this temperature has been shown to be near the upper limit of viability for this RTG-2 cell line (Wolf and Quimby, 1962).

Sterile 25 cm² (Falcon no. 3012) plastic tissue culture flasks containing RTG-2 cells in 3 ml of medium with or without cadmium were incubated at 20°C with loosened caps to maintain a pH of 7.2.

5.6 µl of swinnex Millipore-filtered cadmium chloride was added as an isotonic solution (18.3 mg CdCl₂/ml H₂O) to 9.98 ml L-15 medium to give a stock solution of 100 mg CdCl₂/l. Dilutions of the stock solution with Cd-free complete L-15 were made to get the concentrations of 1, 5, and 10 mg CdCl₂/l. Concentrations were verified by analysis of CdCl₂ containing L-15 medium on a Perkin Elmer atomic absorption spectrophotometer using a heated graphite atomizer standardized against aqueous CdCl₂ standards. Experiments were performed in duplicate for each dose level. All experiments were initiated by seeding cultures from a common pooled source into four flasks for each dose level. Two flasks from each dose level were harvested and counted on day 6, with the balance of the flasks harvested and counted on day 9. Cell culture density was determined by counting four samples of suspended cells from each flask by hemocytometer. Cells were harvested for counting by using sonication in trypsin (500 mg 1:250/1) and disodium ethylenediaminetetraacetate (200 mg EDTA/1) in Puck's modified solution A (Sanford, 1974, in press). Photomicrographs were made using a Wild M-40 phase contrast inverted microscope and a Nikon AFM semi-automatic 35 mm format camera assembly. Cells were viewed and counted at room temperature, usually 21-25°C.

Flow Chart - Experimental Procedure



The appearance of the cells prior to removal from the substrate is shown in Fig. 1. After 15 sec of ultrasonic treatment, the cells could be seen to be releasing their points of adhesion from the substrate (Fig. 2). This was followed by the total release from the substrate, resulting in predominantly monodisperse cells. This is in contrast to the large clumps of cells removed by using trypsin and EDTA alone, without sonication. The suspended monodisperse cells removed by sonication appeared spherical and similar in appearance to those present in clumps removed by trypsin and EDTA alone.

When clumps of cells are present after sonication, the clumps usually contained less than four cells per clump (Fig. 3). Longer sonication might remove all the clumps, but we have noted a tendency for the cells to begin to disrupt after more than 1.5 min of sonication. Older cultures seem to be more susceptible to sonic degradation than those more recently planted. After removal from the flask surface by sonication in trypsin and EDTA, cells in L-15 medium reattached to the flask surface within 1 to 2 min (Fig. 4), and pseudopods formed within 5 min (Fig. 5). The time required for adhesion and pseudopod formation was the same as observed in cultures treated with trypsin and EDTA without sonication. Viability of the cells after this treatment was 94% as determined by trypan blue exclusion (Merchant et. al., 1964). Application of pancreatin and EDTA to cultured RTG-2 cells yield cells having 95% viability (Plumb and Wolf 1971). Very little suspended debris remained after the cells were plated out in new flasks. By sonication removal, 100% released of the cells was possible, allowing reliable counts to be made. No morphological changes were noted in the cells

Table 1. Cell counts from Cd-treated RTG-2 cells with standard error of the means.

CdCl ₂ level (mg/l)	Day 6		Day 9	
	pooled means (cells/ml x 10 ³)	s _{x̄} (x10 ³)	pooled means (cells/ml x 10 ³)	s _{x̄} (x10 ³)
0	182	8.54	290.5	10.15
1	222.5*	5.30	346.5*	11.1
5	155*	6.06	173.0**	8.40
10	68**	3.39	20.0**	1.93

* Not significantly different than value at 0 mg CdCl₂/l

** Significant at 95% level from control value

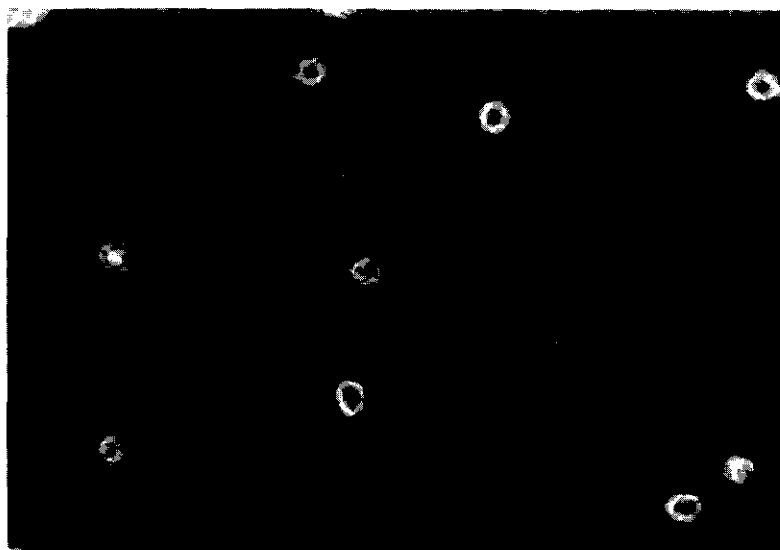


Fig. 4. RTG-2 cells in L-15 medium immediately after 75 sec sonication in trypsin + EDTA. Note lack of aggregates.

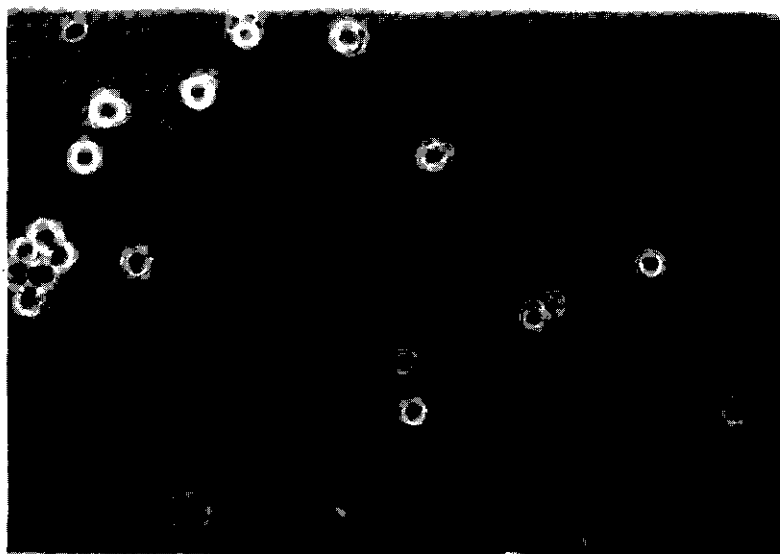


Fig. 5. RTG-2 cells in L-15 medium 10 min after 75 sec ultrasonic treatment in trypsin EDTA. Cells adherent to plastic and flattening out.

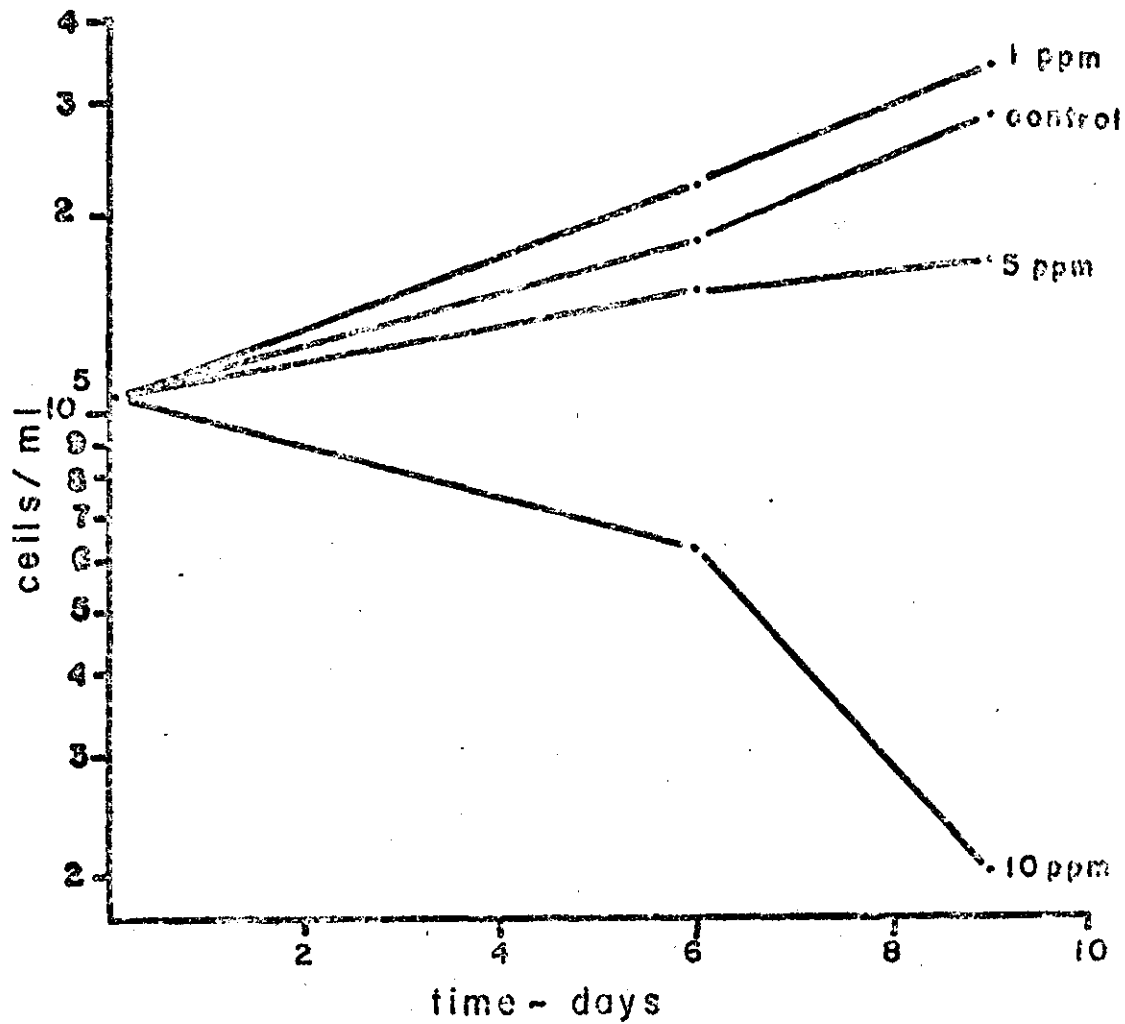


Fig. 6 . Growth rates of RIG-2 cells in the presence of 0, 1, 5, and 10 mg CdCl_2/l in L-15 culture medium. Data points represent average of means of two replicate experiments, each replicate representing four counts of cell density. Seed density was 103,000 cells/ml.

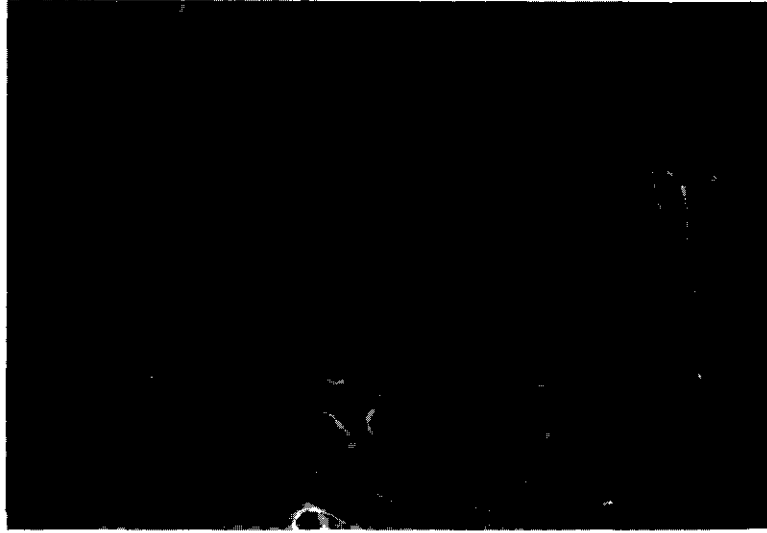


Fig. 9. 5.0 mg CdCl₂/l. Note appearance of filamentous, granular cell projections



Fig. 10. 10.0 mg CdCl₂l. Cells highly vacuolated, showing reduced adhesion

measurements of accumulated Cd within the testis were given. If selective Cd binding sites exist within the testis, an increase in Cd concentration in the testis above environmental levels would result in dose levels equivalent to those active on RTG-2 cells, and the 10 mg CdCl₂/kg levels inhibiting androgen synthesis in minced incubated trout testis (Sangalang and O'Halloran, 1972).

It should be recognized that the RTG-2 gonad cell is a fibroblast type cell in tissue culture - a dedifferentiated state limiting the metabolic expression of the genetic potential and therefore exhibiting different levels of Cd tolerance than the differentiated cells found in whole fish. However, the RTG-2 cells are sensitive to a lower level of CdCl₂ than demonstrated in androgen synthesis inhibition in minced trout testis (Sangalang and O'Halloran, 1972). Thus, the 5 mg CdCl₂/l sensitivity becomes significant as a measure of gonadal cell inhibition if the trout is capable of concentrating CdCl₂ from sub-lethal environmental concentrations of less than 1 mg CdCl₂/l.