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In Vitro Toxicity of Methyl Mercury to Fathead Minnow Cells

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Mercury pollution is widespread, in part due to a global cycling of mercury through both the biotic and abiotic portions of the biosphere (Gagnon and Fisher 1997). A variety of environmental mechanisms are able to efficiently convert inorganic forms of elemental mercury to highly toxic organic forms like methyl mercury (MeHg) (Fitzgerald and Mason 1997, Mottet et al 1997). MeHg as well as inorganic forms of mercury are readily bioconcentrated in ecosystems and partitioned into fish tissue (Bodaly et al. 1997, Post et al. 1996). Fish consumption in turn, represents a major source of MeHg for human populations (Bloom 1992).

Whereas mammalian cells in culture have often been shown to mimic *in vivo* responses to toxic or carcinogenic agents, little data is available on the effect of methyl mercury (MeHg) on cell lines derived from teleostean fishes (Dierickx and Bredael-Roxen 1996). There are currently more than 70 poikilotherm cell lines available for in vitro studies (Fryer and Lannan 1994). One species, with such a cell line, the fathead minnow *(Pimephales promelas),* has been used extensively both as a bioindicator species and as a model organism in toxicity studies (Devlin et al 1996).

The present study was designed to examine the action of MeHg on CCL-42 cells, a cell line derived from the fathead minnow (FHM). Cells were exposed to a graded series of concentrations of MeHg under controlled conditions and a number of specific toxicological endpointswere examined. These included uptake, cell survival, cell morphology, total protein and induction of 70 kDa proteins.

MATERIALS AND METHODS

Fathead minnow cells (CCL-42) were obtained from the American Type Culture Collection, CCL-42 cells were grown at 34C under normal atmosphere in CRCM-30 media (Sigma Chemical) supplemented with 10% calf serum supplemented with pen/strep/neomycin at 50 units/ml (Sigma P-9032). Cells for microscopic analysis were plated with 4 replicates/dose at 5,000 cells/ml on Lab-Tek chamber slides. Live cells were examined and photographed with an Olympus IX70 flourescent phase contrast inverted photomicroscope with a Princeton Instruments digital camera and Universal Imaging's Metamorph/Metafluor software.

Cell survival data were derived from cultures grown in T-25 tissue culture flasks at 34C. Cells were plated at 5,000 cells/ml and allowed to attach for 4 hours. They were then dosed, with 3 replicates/dose, with a graded series (10-1000 ug/l) of MeHg (Methylmercury (II) hydroxide, Alpha 13395) continuously for 96 hours, which represents about two cell cycles at 34C. Cell numbers were determined using both a model ZBF Coulter counter and a hemocytometer. To determine the relationship between coulter counter data and true cell viability cells were stained with trypan blue and counted using dye-exclusion procedures.

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Cell survival is reported as an EC50 value or the effective concentration that decreases the number of cells by 50%. EC50s were determined using the PROBIT software program developed by the US EPA (1994) that relies on the probit analysis of toxicity data to estimate the EC50 and its 95% confidence interval (Cl). The calculated Cl represents the range in which the true EC50 mean will fall 95% of the time.

MeHg uptake studies utilized 5 x 10^4 cells/ml grown in 3 replicates/dose in T-75 tissue culture flasks. Cells were plated and allowed to grow for 24 hours, then exposed to 200 ug/l in the culture media which was replaced daily throughout the six-day experiment. Cells were harvested, counted, pelleted and rinsed 3X in Hanks buffer. Total mercury in the rinsed, pelleted cells was determined using the cold vapor technique with a Buck Scientific Model 400 mercury analyzer.

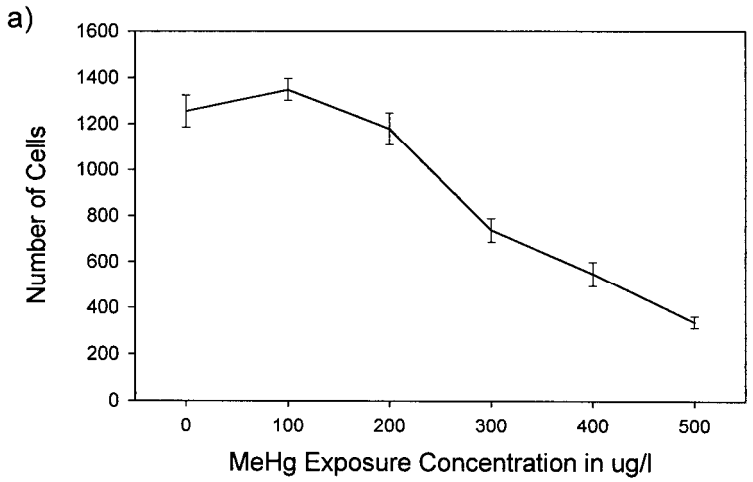
Cells for total protein analysis were plated with 9 replicates/dose at 5 x 10^4 cells/ml in T-25 culture flasks. Cells were allowed to grow for 24 hours and then exposed to 200 ug/l MeHg in their media. Three replicates were selected at 48 hour intervals, harvested, counted, pelleted, rinsed 3X in Hanks and disrupted using a ultrasonically at 0C. The total protein in each sample was determined using standard calorimetric procedures (Bio-Rad).

Proteins were prepared for SDS PAGE by rinsing cells 3X in Hanks buffer, trypsinizing cells, pelleting cells, 3x washing in Hanks buffer, and finally determining total cell number. Cells were resuspended in 0.75 molar SDS and sonicated. Sample buffer was added and 80 ul of protein sample and standard MW protein solutions were loaded onto gels. Gels ran for 8 hours in a cold room at 20 milliamps. Gels were stained with Coomassie Brilliant Blue. Band intensity was quantified using a Hoffer GS300 Scanning Densitometer and MacLab software using standard procedures (Hanes and Rickwood 1981). Integration of the area under the curve corresponding to the stained bands was taken as an indication of the relative intensity of the bands from the control and MeHg-exposed cells.

RESULTS AND DISCUSSION

A series of experiments were conducted to determine the effect of a range of MeHg concentrations on cell survival. The normal pattern of growth of a nonconfluent culture of CCL-42 cells is a short lag phase followed by a log growth phase with a doubling time of about 48 hours at 34 C. At lower MeHg concentrations (100-200 ug/l) the cells show a similar pattern of growth of the culture. However, cultures exposed to very low MeHg concentrations (below 100 ug/l) consistently exhibited increases in the numbers of cells over control cells. It should be noted that in most cases the increases in cell number with low MeHg exposure concentrations were not significant, yet most of our data show a rise in cell number at low MeHg levels. A similar stimulatory effect of MeHg on neuroblastoma cell proliferation was noted by Repetto et al (1993).

With increasing MeHg exposure concentrations the rate of growth decreases. At higher MeHg concentrations cell growth became cytostatic and eventually decreased at the highest exposure concentrations, The 96-hour EC50 of MeHg was calculated to be 360 ug/l with a 95% Cl of 320-420 ug/l (figure 1 a). A range of in vitro sensitivities to MeHg have been reported. Repetto et al. (1993) found inhibition of cell proliferation at 320 ug/l MeHg in neuroblastoma cells. In contrast, RTG-2, a cell line derived from the rainbow trout is extremely sensitive to MeHg exposure with an EC50 of 2.7 ug/l (Devlin and Mottet 1992). Uptake studies demonstrated the ability of cells to rapidly bioaccumulate MeHg. Cells were able to effectively partition most of the MeHg from the exposure media into the rinsed pelleted cells within 24 hours. Previous unpublished studies (Devlin) did not renew the MeHg on a daily basis. These



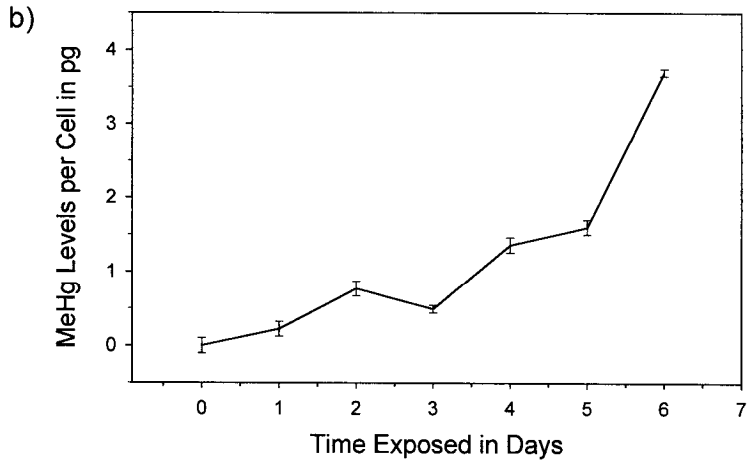


Figure 1. a). Effect of MeHg on CCL-42 survival. Three replicates were exposed to a range of MeHg concentrations for 96 hours. Note that at lower MeHg concentrations, there appears to be a stimulatory effect of MeHg on cell number. **b).** Uptake of MeHg by CCL-42 cells over time. Three replicate T-75 flasks were exposed to 200 ug/l MeHg. Throughout the 6 days exposure the total cell numbers decreased but the load of mercury per cell continued to increase over time. The error bars represent 95% confidence intervals around the mean values.

studies demonstrated that mercury levels leveled off quickly with no subsequent increase in uptake. The current study utilized fresh media with daily renewals of 200 ug/l MeHg in the culture media throughout the six-day experiment. As would be expected, at this exposure concentration, cell numbers were lowerthan controls. When the total mercury readings in the rinsed pellet were adjusted for cell numbers, there was a clear pattern of continuing uptake of MeHg over time (figure 1 b). MeHg may be partitioned in number of cellular compartments. For example, it exhibits a strong binding to membrane lipids that is electrostatic in nature (Girault et al 1997) and is known to have a high affinity for thiol groups (Trombetta and

Kromidas 1992). As in the uptake experiment, the total protein in the rinsed, pelleted sample of cells was adjusted for cell number. This procedure demonstrated an increase in total protein per cell with increasing MeHg concentration (figure 2. a). This may be the result of hypertrophy of cells or the result of induction of specific protein synthesis. Microscopic examination revealed MeHg exposed cells were the same size or somewhat smaller than control cells. Therefore it is likely that there is the induction of increased protein synthesis in similarly sized cells. It is interesting to note that a decrease in protein synthesis resulting from MeHg exposure has been demonstrated *in vivo* in both fish (Sharma and Davis 1980) and mammals (Syversen 1982) and *in vitro* in HeLa cells (Gruenwenel et al 1981). Decreased protein synthesis noted in these studies may be due to the higher exposure concentration of MeHg used and its accompanying cytotoxic effects.

Following exposure to a number of stressors, both physical and chemical, many cells begin the increased production of a group of constitutively expressed proteins called heat shock proteins (Bauman et al 1993). In many instances the induction of heat shock proteins is a sensitive measure of toxic stress (Saunders 1993). The induction of specific families of proteins in response to MeHg exposure was investigated with SDS PAGE. The results of the electrophoresis appeared to indicate that as MeHg exposure concentrations increase, so does the production of a 70 kDa protein thought to be HSP70. A number of other bands appeared to have enhanced with MeHg exposure, included 82, 78, 40 and 39 kDa bands, But the intensity of these scanned bands were very similar to the intensities of the corresponding bands of the control cells.

The induction of a similar 70 kDa protein following MeHg exposure has been reported by others. For example, MeHg has been shown to increase both HSP70 and HSP90 production in a variety of mammalian systems including the mouse and human (Bauman et al 1993, Yamada and Koizumi 1993). A number of heat shock proteins including HSP70 have also been reported in the fathead minnow tissues (Dyer et al 1991, Sanders 1993) and in a number of other fish species including the catfish, medaka and the rainbow trout (Abukhalaf et al 1994, Arai et al 1994, Currie and Tufts 1997).

The morphology and behavior of CCL-42 cells responded in a predictable manner with increasing exposure concentration to MeHg. The normal morphology of CCL-42 cells is fibroblastic with a highly branched cytoplasmic extensions (figure 3 a). Under normal conditions they are contact inhibited at higher cellular densities and appear to be less highly branched. At MeHg concentrations below 100 ug/l cells are mostly normal in appearance. At slightly elevated concentrations they often form clumps in non-confluent cultures (figure 3 b.).

At higher concentrations, the cells appear to withdraw their cytoplasmic extensions and become more spherical in form. MeHg exposure seems to inhibit the cells ability to adhere to the sides of the culture flask which results in larger than normal numbers of floating cells. At MeHg concentrations above 250 ug/l larger scale cellular damage occurs. Damage is in the form of a number of vesicles or bodies forming under the nuclear membrane. The plasma membrane also becomes compromised and leaky as shown by the increase in the number of cells that accumulate trypan blue under elevated exposure concentrations.

In the present study FHM cells in culture exhibited a 36-hour EC50 to MeHg of 360.5 ug/l, although low MeHg levels appear to have a stimulatory effect of cell numbers. FHM cells were shown to continuously bioaccumulate MeHg and the total protein per cell as well as the intensity of a 70 kDa SDS PAGE band increased with increasing levels of MeHg exposure. Cellular morphology exhibited a predictable dose-related pattern in response to MeHg exposure. Current studies are underway to characterize the effect of MeHg *in vivo* using embryos of fathead minnows with the intent of comparing the *in vitro* and *in vivo* responses to MeHg exposure.

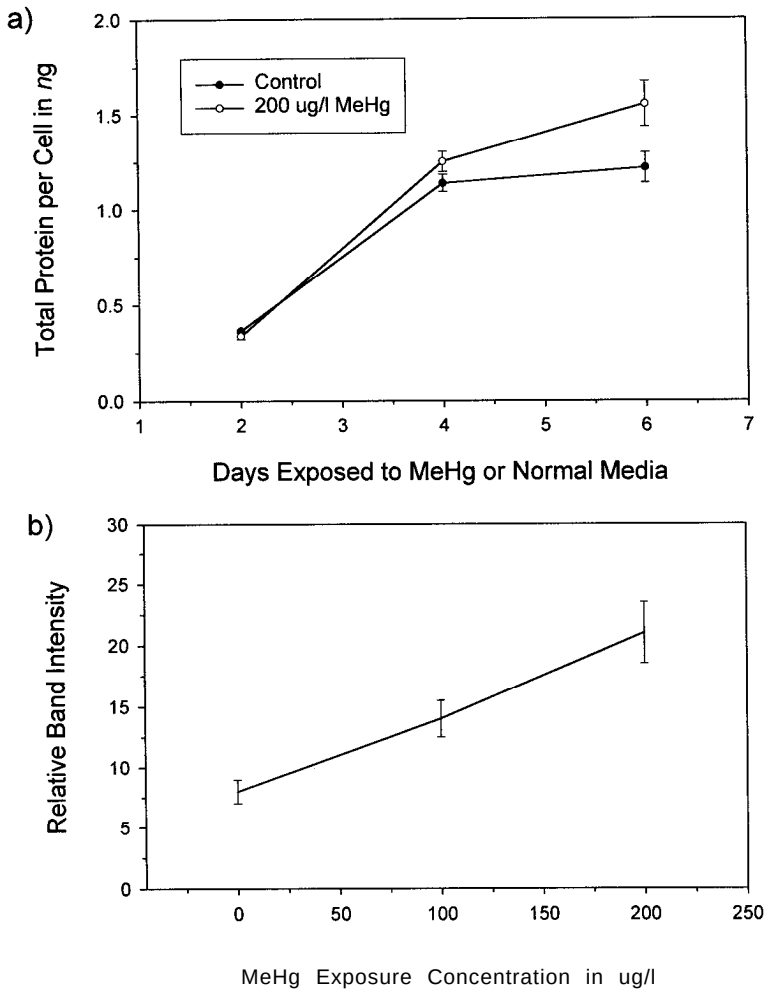


Figure 2. a). Total protein per cell in both control and MeHg-exposed CCL-42 cells. Note the increase in the protein per cell in the MeHg-exposed cells, This may be due to the induction of specific stress proteins like the 70 kDa band proteins seen in (b). **b).** Increase in 70 kDa band intensity. A linear increase in band intensity of a 70 kDa protein is seen in response to MeHg exposure in CCL-42 cells. At higher MeHg levels, cellular damage is widespread which results in lower 70 kDa band intensities on the gels, The error bars represent a 95% confidence interval around the mean values.

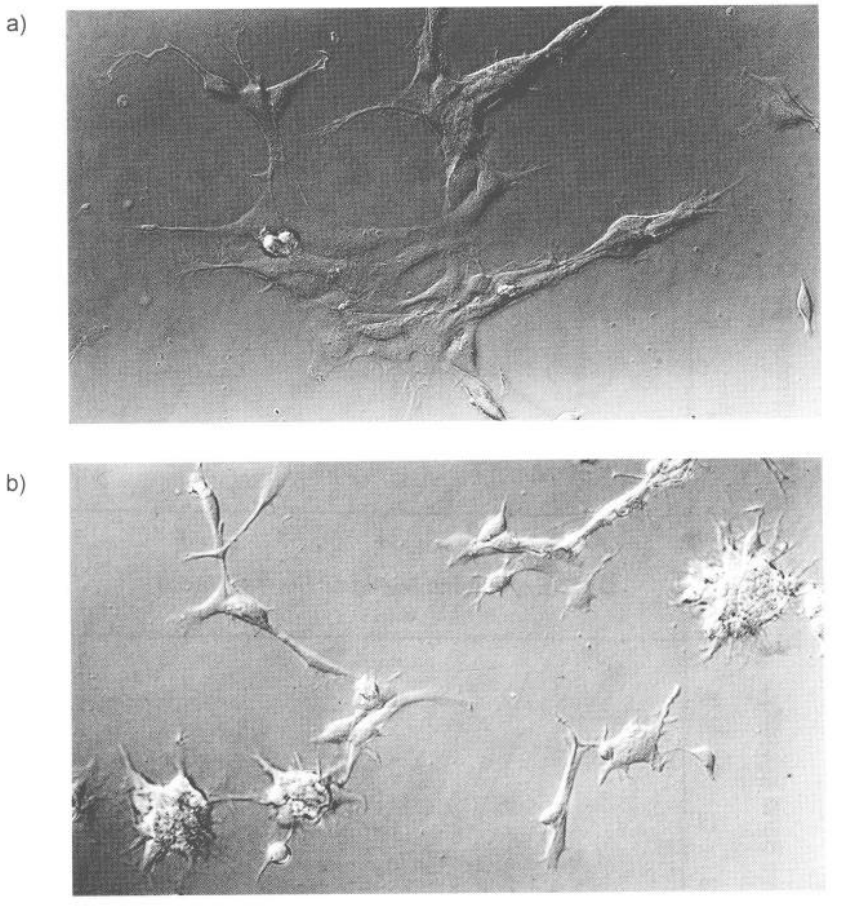


Figure 3. a). Control CCL-42 cells. Note the normal fibroblastic morphology of the cells with their long cytoplasmic extensions. **b).** MeHg treated CCL-42 cells. There are a number of morphologies of CCL-42 cells in response to MeHg exposure. Shown are the formation of a number of clumps of cells with shortened cytoplasmic extension resulting from exposure to lower MeHg concentrations. There appears to be a disruption of the cytoskeleton.

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