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Toxic Effects of Cadmium and Mercury in Rainbow Trout (*Oncorhynchus mykiss*): A Short-Term Bioassay

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Heavy metals, particularly cadmium, and mercury represent part of the major aquatic pollutants since they are present throughout the ecosystem and are detectable in critical amounts in many parts of the world. Not only environmental organizations, such as EPA and UNEP but also the public itself is concerned for the possible adverse consequences of such pollutants to aquatic biota and, indirectly, to humans.

The IARC Monographs on the Evaluation of the Carcinogenic Risks to Humans, for example has categorized, cadmium as a possible carcinogen (Group 2) (UNEP\FAO\IAEA, 1995). This categorization was based only on results of epidemiological studies and/or of carcinogenicity bioassays, proposed from the NCI (National Cancer Institute) that were conducted on animals, particularly on rodents (IARC, 1987;Snow 1992; Waalkes et al. 1992; Oberdorster & Cox, 1992).

Even though heavy metals are considered as an emerging class of carcinogens, there has been a lot of debates on their carcinogenic action. For example, available data for some metals such as arsenic and selenium suggest that there is sufficient evidence for its carcinogenicity in humans, but according to rodent carcinogenicity, bioassays have not clearly suggested any direct carcinogenic action (Kazantzis and Lilly 1986; Norseth, 1988). The opposite evaluation has been suggested for cadmium. Although the carcinogenicity of different cadmium compounds after several routes of administration has been demonstrated in rats and mice (IARC, 1987; Waalkes et al., 1992), it is still debated as to whether cadmium is capable to act as a carcinogenic agent in humans (Kazantzis et al., 1988). For mercury, there are so far very few studies that have extensively investigated this metal for carcinogenesis and there has been no direct evidence of excess neoplasia epidemiologically and experimentally (Woo and Arcos 1981; Clarkson 1989; Waalkes et al., 1992; Snow 1992).

It is therefore anticipated that the use of an alternative animal model in metal toxicopathological testing is required. Actually, the rainbow trout has been proven to be sensitive to a number of carcinogenic chemicals (Metcalfe 1989). Given that neoplasms have been induced in trout by using several different experimental protocols and that fish respond to carcinogens in much the same

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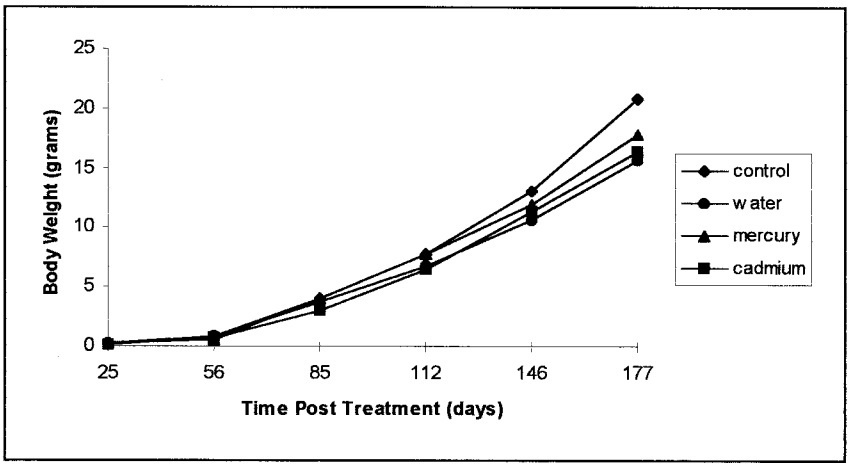

Figure 1. The rainbow trout growth rate in a six month test period for the Control, Water and metal treated groups (Cd-Hg).

manner as mammalian species, it is estimated that fish can be front-line indicators of suspected aquatic pollutants such as metals (Masahito et al., 1988; Kotsanis and Metcalfe, 1991; UNEP\FAO\IAEA,1995; Bailey et al.,1996).

In the present study short-term fish bioassays were conducted according to the rainbow trout microinjection bioassay by two common aquatic toxic metal pollutants, cadmium and mercury. The purpose of this study was i) to investigate *in vivo* their toxic impact, starting the exposure from a concentration usually measured in the aquatic environment, ii) to observe in detail the toxicopathological symptoms over a 6 month test period, iii) to report separately their toxic effects since these pollutants are components of complex mixtures undergoing a variety of interactions both in the aquatic environment and the host organisms in which they are bioaccumulated and iv) to provide new data for further research in metal carcinogenicity that would help predicting the toxicological hazards to aquatic life and humans.

MATERIALS AND METHODS

Rainbow trout were obtained from a commercial supplier (Giannetas S.A., Trout hatchery Station, Ioannina, Greece) at the eyed stage of development and were held in the laboratory at 10-11°C for approximately one week until the completion of hatching. Once all the trout were in the sac-fry stage of development they were separated into five groups: (1) A control group where no treatment was applied, (2) A water-injected group where fish were treated only with deionized water, (3) A $CdCl_2$+water and $HgCl_2$+water injected group where fish were treated with these compounds dissolved in deionized water and (4) A sham-injected group where fish were only inocculated with the needle itself. All five groups were placed seperately into hatchery boxes for the microinjection period. After the single injection in the sac-fry, the fingerlings

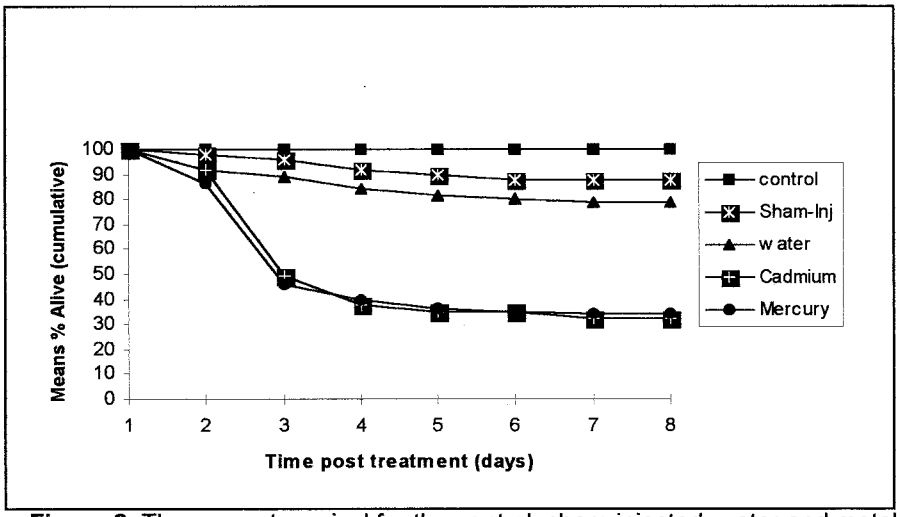

Figure 2. The percent survival for the control, sham-injected, water and metal treated groups (Cd-Hg).

were placed back into the raceways and raised at a water temperature of 12-15°C for as long as 6months, reaching a weight of approximately 20 grams (Fig., 1.). The density of the tanks was kept constant. Fish were fed ad libitum with a commercial trout diet (Trouvit Co, IOANNINA, GREECE). The diet contained 53% crude protein, 13% crude fat, 0.6% crude fibber, 12% Ash, 9% moisture. Fish were raised in 95% recycled fresh water (pH 7.3-7.8, hardness 45mg $CaCO_3$/l, temperature 19-22°C).

During development and growth fish were weighed, counted and grossly examined for possible external abnormalities every 30 days post treatment for 6 months. At the end of the experiment fish were sacrificed by an overdose of Isoamyl Alcohol. All fish tissues were preserved in Bouin's fixative. The liver, kidney, spleen, stomach and gastric caeca were histologically examined. The specimens were embedded in paraffin, sectioned at a thickness of 4-5 ìm and stained with hematoxylin and eosin (H&E).

Cadmium chloride ($CdCl_2$) and mercury chloride ($HgCl_2$), was purchased from Merck Chemical Co. Fish were anaesthetised, before being weighed, using Isoamyl Alcohol (Carlos Erba).

Once all rainbow trout embryos had reached the sac-fry stage and as soon as the temperature and the laboratory environment was suitable for the procedure, a batch of approximately 10 sac-fry was taken and anaesthetised with isoamyl alcohol. The microinjections with metals were done using the Hamilton push-button repeating dispenser model (BP 2000). Once the fry had resumed normal activity, they were placed back into their hatchery boxes. The concentration of the injective solution for every metal was 0.5mg Cd/kg and 0.5mg Hg/kg.

The statistical analysis was performed with the aid of the computer program SPSS (V.8). Analysis of Variance (ANOVA), x^2 test and student's *t* test were applied (Zarr, 1984).

RESULTS AND DISCUSSION

The use of the microinjection technique is known to be a rapid procedure and offers plenty of advantages (Kotsanis and Iliopoulou-Georgudaki, 1999).Mortalities were observed, approximately, for a week after the microinjection procedure. Actually, the majority of the mortalities among the inoculated fry were observed during the first 24-48 hours post treatment in the deionized water-injected group (0.5 μl/sac-fry) while in the metal groups during the first 24-72 hours. Control fish (no injection) showed no mortality during the microinjection phase, while the sham-injected group, which were kept only for the microinjection phase, had a 12% mortality (Fig 2).

It must be emphasized that after the microinjection phase mortalities ceased for almost a week but continued throughout the "swim up" stage, attributed to the shift from endogenous to exogenous feeding, a stage characterized as 'the critical period''. Thus, after 28 days post treatment the overall mortality was 71.6% and 70% in the $CdCl_2$+water and $HgCl_2$+water groups respectively, while a 29.4% mortality was observed in the water injected group and a 12% mortality in the control fish.

The test concentration for each metal was chosen after an extensive preliminary test assay at which the sac-fry were weighed prior the treatment and showed to have approximately a constant weight of 0.095 grams (±0.003gr) at a temperature of 10°C. The LD_{50} (96h) obtained in this assay for the microinjection of $CdCl_2$ was 0.57mg Cd/kg (0.09-3.6mg Cd/kg) and for $HgCl_2$ was 0.51mg Hg/kg (0.02-20.2mg Hg/kg). It should be emphasized that for the estimation of the LD_{50} in the preliminary test we had subtracted the mortalities recorded in the control-water injected group, which was approximately 20-22%.

Table 1. Incidence of kidney and liver lesions of control, water and metallic treated groups of rainbow trout.

Treatment		Visual Survey		Final Survey	
		Kidney	Liver	Kidney	Liver
Control[a]	(n=40)	0/40 0 %	0/40 0 %	0/40 0 %	0/40 0 %
Water[a]	(n=40)	0/40 0 %	0/40 0 %	0/40 0 %	0/40 0 %
$CdCl_2$	(n=39)	3/39 7.7%[a]	0/39 0 %	5/39 12.6%[a]	1/39 2.6%[a]
$HgCl_2$	(n=38)	0/38 0%	0/38 0 %	1/38 2.6%[a]	0/38 0%

a : Incidence was shown to be statistically different.

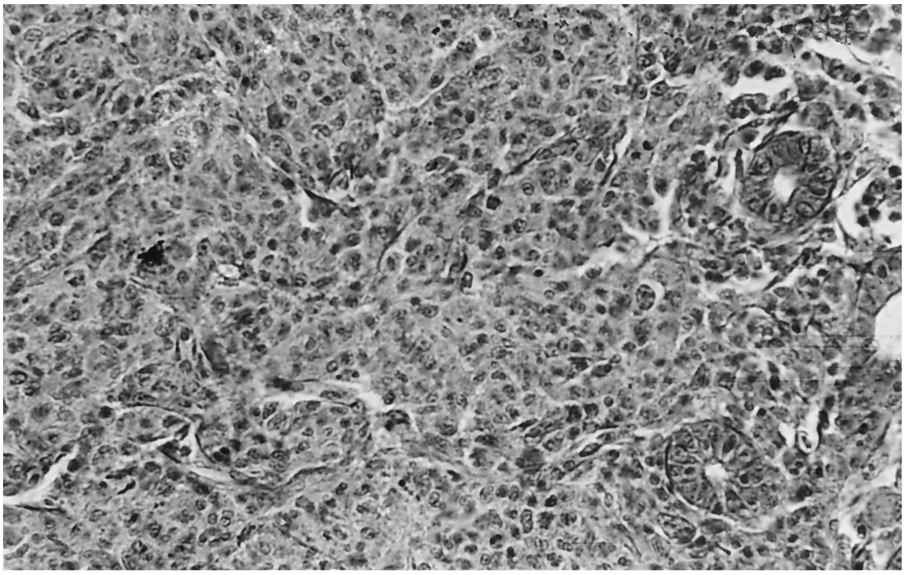

Figure 3. Light microscopy of an area of kidney fibrosis of a 6 months rainbow trout treated initially with 0.5mgCd/Kg, (H&E, x40)

At the end of the six month test period, the animals were sacrificed. At the necropsy, kidney lesions were observed grossly in the cadmium group 3/39 (7.7%) (Table 1). No lesions were observed grossly in the liver, spleen, stomach and gastric caeca from all groups. The kidney lesions were usually observed at the posterior part of the kidney. These lesions, on the kidney were round, white and their size was relatively large 5-10mm. It should be mentioned that multiple lesions were observed grossly on some kidneys. Grossly-visible lesions were not noticed in the control, water-injected and mercury-injected groups (Table 1).

The histopathological analysis in the cadmium group (Table 2) showed that only one kidney lesion was observed, which was interpreted as a fibromas. It was a large area of fibrosis characterized by an extensive proliferation of fibrotic tissue and a regenerative proliferation of glomerular cells (Fig. 3).

In addition to this lesion, granulomas (four) were also observed. They were basically multifocal lesions in liver and mostly in kidney, consisting of several cell types, but primarily histiocyte-like cells with large, foamy cytoplasm and a small vesicular nucleus distributed among a network of fibrocytes and capillaries (Table 2). Necrosis was also noticed, rarely, in the liver and extensively in the kidney, involving principally the second proximal segments. Obviously the cellular alterations in the liver were not as many as in the kindey.

On the contrary the histopathological analysis performed in the mercury group (Table 2) showed that the only lesion observed was in the kidney and was interpreted as a granulomas similar to the above description.

Table 2. Histopathological results of control, water and metallic treated groups of rainbow trout.

Histopathological Interpretation	Treatment			
	Control n=40	Water n=40	Cadmium n=39	Mercury n=38
Fibromas in Kidney	0/40 0%	0/40 0%	1/39 2.6%[a]	0/38 0%
Granulomas in Kidney	0/40 0%	0/40 0%	4/39 10.5%[a]	1/38 2.6%
Granulomas in Liver	0/40 0%	0/40 0%	1/39 2.6%[b]	0/38 0%

a:The incidence was not shown to differ statistically from the mercury group ($p=0.13$).
b: Incidence were shown to be statistically different from the control & mercury treated groups($p<0.05$).

In the present short term chronic toxicity bioassay we saw that a single injection of nanogram quantities of a metal solution into the yolk-sac of the rainbow trout was capable of inducing cellular alterations. Actually kidney fibrosis (cadmium group) and cytolethalic alterations (mercury group) were only observed, without any evidence of inducing lesions evolving to preneoplasia as it has been proven in the case of arsenic (Kotsanis and Iliopoulou-Georgudaki, 1999). Thus, our experimentation resulted only in the induction of degenerative and inflammatory lesions.

In addition, the purpose of using deionized water as carrier instead of DMSO (dimethyl sulfoxide), which has been used in similar bioassays, was: a) to provide additional data comparing the results with those observed in assays using DMSO and b) to simulate our test to the natural process given that one way for the bioavailability of metals to fish is through the water basin (Black, 1988; Metcalfe et al.,1988;Kotsanis and Metcalfe, 1991; Kotsanis and Iliopoulou-Georgudaki, 1997).

According to our results the deionized-water injections of this study were responsible for approximately 20-22% mortality while those of DMSO showed a mortality of 17-19% (Kotsanis and Metcalfe, 1991; Kotsanis and Iliopoulou-Georgudaki, 1997). Probably this difference must be attributed to the clotting of the fish's yolk provoked by the water. Moreover other studies that used deionized water as a carrier showed a 40% mortality (Metcalfe and Sonstegard, 1984). Generally the high rate of mortality in such bioassays is usual. According to the literature, the mortalities are attributed primarily to the sensitivity of the embryos to xenobiotics as well as to the stress caused by the procedure (Black, 1988; Metcalfe, 1989)

Generally very few studies are known concerning the toxicopathological effects of cadmium to chronic exposure on fish while most fish assays conducted were usually acute toxicity testing (Ramade 1987; Mance 1990; Kotsanis and

Iliopoulou-Georgudaki, 1997)). The trout were found to be more sensitive to cadmium than non-salmonid species and was shown to be very useful for immunotoxicological studies, whereas developmental studies showed that larvae were consistently more sensitive than the embryos and showed diminished growth (Weis and Weis 1991; Zelikoff et al., 1995). Field studies revealed that cadmium could contribute to various liver diseases, such as neoplasia when significantly elevated concentrations of it were found in sediments (Malins et al., 1987). On the contrary, it should be mentioned that field studies conducted on the toxicity of mercury have not shown evidence of any difference in the sensitivity between salmonids and nonsalmonids species. However, the data published vary widely even though the same experimental procedures were used. This was probably due to metal loss, sample storage or because mercury is a relatively volatile metal (Mance,1990). In general, the literature supports that organo-mercury compounds do not indicate any greater toxicity from those of inorganic mercury (Mance, 1990; Hellawell, 1990).). Mercuric chloride was shown to be acute toxic in rainbow trout to as low as 10μgHg/l to as high as 1 mgHg/l (Boetius 1960; McLeod and Pessah, 1973; Hale, 1977).

Fish *in vivo* toxicopathological studies are of great scientific interest due to the fact that fish species have shown to be suitable test organisms for assessing a chemical's potential adverse effects on health. Thus, the use of a fish protocol such as the rainbow trout, is considered very informative and useful because of the deleterious toxicological impact of metals (U.S./EPA, 1980; UNEP/FAO/IAEA, 1995).

According to our findings, a single exposure of rainbow trout to Cd and Hg revealed minor toxicopathological activity, which in any case are potentially reversible. Hopefully, this study expands our knowledge on metal activity in fish protocols promoting further experimentation since these metals are tested in such *in vivo* fish bioassays for the first time.

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