Exposure to formaldehyde at therapeutic levels decreases peripheral blood lymphocytes and hematopoietic progenitors in the pronephros of tilapia *Oreochromis niloticus*

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ABSTRACT: Formaldehyde (HCHO) was recently detected at concentrations above the cancer benchmark in 90 % of 60 000 surveyed United States census tracks. Formaldehyde leaches into and mixes with water extremely well, exposing aquatic life. Further, formaldehyde is used therapeutically in aquaculture to remove external protozoa and other parasites from fish. The present study was undertaken to determine if sub-acute HCHO causes immunologic changes in tilapia *Oreochromis niloticus*. Fish were exposed to 0, 50, or 150 ppm of HCHO. Immune parameters examined included blood hematology, spleen/body weight ratio, spleen and pronephros total cellularity, leukocyte blastogenesis, and natural killer cell cytotoxicity. Organ/body weight ratios and total cellularity were not different from controls. Similarly, mitogen response and natural killer cell function were unchanged. Peripheral blood lymphocytes decreased as HCHO exposure increased. Formaldehyde exposure also decreased the number of progenitor cells in the fish pronephros. These observations suggest possible immunosuppressive effects of HCHO in fish.

KEY WORDS: Formaldehyde · Immune system · Fish · Tilapia

INTRODUCTION

Formaldehyde (HCHO) is an industrial chemical known to be an inhalational toxicant (McNary & Jackson 2007), dermal contact irritant (Hilton et al. 1996), mutagen (Odeigah 1997), and carcinogen (McCarthy et al. 2009). Exposure to HCHO has been shown to exert deleterious effects in both laboratory animals and humans, with the precise effect varying based on concentration, exposure route, and duration of exposure. Despite these concerns, HCHO is nonetheless widely used in numerous applications, with some of its uses being indispensable in the health field (Balamurugan et al. 2004).

Normal husbandry practices in the commercial aquaculture industry include therapeutic immersion of fish in formalin solutions to remove external parasites, including protozoa (Bodensteiner et al. 1993, MacLean et al. 2006, Lahnsteiner & Weismann 2007). Formaldehyde also has numerous product manufacturing uses, playing an important role in production of foam insulation, carpeting, pressed wood products (Maddalena et al. 2009), as well as the more commonly known use in the preparation of laboratory specimens for anatomical study. Such broad use of HCHO in numerous venues has resulted in widespread environmental contamination with this chemical. A 1998 report from the United States Environmental Protection Agency (US EPA) indicated that HCHO was not only detected, but also found at levels exceeding the cancer benchmark concentration in approximately 90 % of the 60 000 census tracks

(geographic study regions) measured throughout the United States (Woodruff et al. 1998). Formaldehyde is also a common aquatic contaminant that mixes with water extremely well (Murai et al. 2008). Contamination of aquatic environments and the resulting exposure of aquatic life to this chemical is thus a concern.

The immune response of fish is becoming increasingly characterized in the laboratory. Though the humoral response of the piscine immune system relies more heavily on IgM-like immunoglobin than other isotypes of antibody, the immune response in fish has been shown to be similar to that of mammals in many regards (Smith et al. 1999). Indeed, recent studies have indicated that fish may be a good alternate species to rodents for evaluating chemical-induced immunotoxicity (Gogal et al. 1999, Müller et al. 2009, Iwanowicz et al. 2009). Regarding environmental exposure, fish have been shown to respond in a recognizable manner to exposure to many chemicals, including various water-borne contaminants. A study on metallic aquatic pollutants has demonstrated exposure-related changes in cell-mediated, humoral, and innate immunity (Zelikoff et al. 1995). Another study showed that lymphoproliferative responses of fish found in sites contaminated with polycyclic aromatic hydrocarbons (PAH) decreased (Faisal & Huggett 1993).

Because HCHO, among chemicals, is known to contaminate various bodies of water, possible immunotoxicant effects of HCHO on the piscine immune system are of interest from the perspective of water contamination. Despite diverse indications that HCHO has some effect on the immune system, results of studies investigating numerous immune parameters including functional tests (Dean et al. 1984) have indicated that effects of inhalant HCHO exposure is not overtly immunotoxic in mice. Indeed, in that study, an apparent immunostimulatory effect was observed in the form of an increase in resistance to bacterial challenge. Given that many similarities between fish and mammals have been demonstrated in response to chemicals that are known immunotoxicants (Gogal et al. 1999), the question also arises whether formaldehyde has an immunomodulatory effect in fish. Examination of possible effects of HCHO exposure on the immune system of fish is of interest from each of these perspectives. Moreover, completion of such an investigation would also further the characterization of the piscine immune response as either fundamentally similar to or different from that of mammals. The present study investigated the effects of HCHO exposure on the piscine immune system, employing tests from the National Institute of Environmental Health Sciences' (NIEHS) National Toxicology Program (NTP)-approved testing battery (Dean 2004) in a laboratory environment. Nile tilapia

Oreochromis niloticus were selected for study as a high-production, relevant, and economically important species (Gogal et al. 1999).

MATERIALS AND METHODS

Fish. Pathogen-free Nile tilapia *Oreochromis niloticus* were hatched, reared, and maintained at the Aquatic Medicine Laboratory at the Virginia-Maryland Regional College of Veterinary Medicine. All experiments were approved prior to initiation by the Virginia Tech Animal Care and Use Committee. Throughout the study, all fish were then cared for and maintained in accordance with Virginia Tech institutional guidelines.

The fish were fed a commercial pelleted diet (Zeigler Brothers) that was administered once daily at approximately 3% of body weight. Water quality analysis was performed daily, and included the values of ammonia and nitrite levels, pH, and temperature. During rearing, fish were maintained in tanks provided with continuous filtration and aeration, with water temperature maintained at $28 \pm 4.0°C$ and lighting maintained automatically on a 12 h light: 12 h dark cycle. Sample size for each experiment consisted of 3 fish. Range-finding studies for selecting HCHO exposure levels utilized fish within a specified weight range (65 ± 18 g); these were arbitrarily selected and transferred to 80 l tanks that had been partitioned to individually accommodate 3 fish per tank. Larger fish were necessary for the immune-related experiments in order to yield adequate numbers of cells. These larger fish (n = 9 per control or treatment group) were again of uniform size and weight (150 ± 35 g), arbitrarily selected, and transferred to 300 l tanks that also had been partitioned to individually accommodate 3 fish per tank. Following transference, monitoring of water quality values, as well as maintenance of filtration and the light/dark cycle, was performed as previously described. Fish were allowed to acclimate to the study tanks for 14 d prior to exposure to formalin.

Exposure to formalin. Limited data were available regarding the toxic effects of HCHO in tilapia; thus, initial range-finding studies with 3 fish per group were conducted with exposure to 0, 50, 100, 150, and 250 ppm. Limited signs of overt toxicity were observed at 250 ppm HCHO, primarily as a reduction in motion. Based on the results of these range-finding studies, for the immunologic evaluations, only 0, 50, and 150 ppm exposures were used, with 9 fish per group. In all treatments, fish were placed in a HCHO tank for 30 min every other day for a total of 2 treatments. The fish were sampled 48 h after the last exposure.

Peripheral blood leukocyte collection and enumeration. In this part of the study, 3 replicates were run (thus summed n = 9 fish per dose group). Fish were anesthetized using tricaine methane sulfonate (MS-222, Sigma). Blood was collected from the caudal tail vein using a 21-gauge needle and a 1 ml syringe. Blood was transferred to a heparin-treated pediatric tube, reserving sufficient blood to prepare duplicate blood smears from each fish. The slides were air dried, fixed in 95% methanol, and stained with modified Wright's stain (Sigma). Differential leukocyte counts (lymphocytes, basophils, eosinophils, monocytes, and heterophils) were performed at 100× under oil immersion. To determine total blood cellularity, an aliquot of the heparin-treated blood sample from the pediatric tube was serially diluted and stained with Natt-Herrick stain and enumerated with a hemacytometer. Total cellularity per milliliter was reported.

Spleen and head kidney (pronephros) collection and enumeration. In this part of the study, 3 replicates were run (thus summed n = 9 fish per dose group). On completion of venipuncture, each fish was euthanized by blunt cranial trauma while still anesthetized. The body weight of the fish was determined and recorded. The spleen and head kidney (pronephros) were then isolated from each fish, the spleens weighed, and each organ (spleen and head kidney) individually placed in a 60 × 15 mm Petri dish containing incomplete RPMI-1640 (RPMI 1640 containing 2 mmol l^{-1} glutamine, 25 mmol l^{-1} HEPES, 20 mmol l^{-1} glucose, and 25 µg ml^{-1} gentamicin). Spleens were dissociated over a Sieves screen (Sigma, Chemical Co.) using curved forceps. Splenic cell suspensions were then washed twice by centrifugation (200 × g, 5 min; 22°C) in culture medium, resuspended in 1 ml of culture medium, and counted using an electronic cell counter (CASY 1; Cell Tools). Pronephric cell suspensions were prepared in a manner identical to that described for splenic cells.

Cyto-centrifugation (cytospin) phronephros leukocyte differentials. A 200 µl aliquot of isolated pronephric leukocytes (1.0 × 10^6 cells) was placed into a cyto-centrifugation chamber (Cyto-TEK) containing 250 µl phosphate-buffered saline and 50 µl of 15% BSA (Sigma). The cells were spun at 50 × g for 5 min at 23°C. The slides were then fixed with 95% methanol and stained with modified Wright's stain (Sigma). Slides were covered with a coverslip and sealed with Permount (Fisher Scientific). Cells were examined under oil immersion at 100×. The number of leukocytes counted was reported as a percentage of total counts, with 100 cells counted per cytospin slide.

Mitogen stimulation assay. Using 96-well round-bottom tissue culture plates (Corning), 100 µl aliquots of 5.0 × 10^6 cells ml^{-1} Lymphoprep-enriched pronephric leukocytes were added to triplicate wells containing 100 µl of either RPMI-1640 medium alone or medium plus phytohemagglutinin (PHA, Sigma), phorbol 12-myristate 12-acetate (PMA, Sigma) and ionomycin (Ion, Sigma). The cells were incubated overnight in a humidified incubator at 28°C and 5% CO$_2$. After 24 h of incubation, 20 µl of Alamar Blue dye (Accumed International) was added to each well and the plates were returned to the incubator. Differences in the specific absorbance of the oxidized form of this dye (600 nm) reflect the level of proliferation (Ahmed et al. 1994). Then, 48 h after the dye was added, the plates were removed from the incubator and the absorbance was measured at 570 and 600 nm with a kinetic microplate reader (Molecular Devices). The specific absorbance of the unstimulated cells (in medium alone) was subtracted from the specific absorbance of the PHA-stimulated cells to yield a change in specific absorbance.

Chromium-release assay. Splenic natural killer cell cytotoxicity against target tumor cells was measured using the standard chromium-release assay. Briefly, lymphoprep-separated splenic cells (10.0 × 10^6 cells ml^{-1}) were aliquoted into quadruplicate wells of a 96-well tissue culture plate. The cells were serially diluted such that the ratio of effector to target cells was 50:1, 25:1, and 12.5:1 when 2.0 × 10^5 cells ml^{-1} of ^{51}Cr-tagged YAC-1 tumor cells were added to each well. The plates were incubated overnight at 28°C and 5% CO$_2$. The cells were harvested with the skatron technique as previously described (Gogal et al. 1999) and counted on a Packard Cobra II gamma counter. Values were reported as the percentage of ^{51}Cr release (CPM) defined by the equation (Hudson & Hay 1989):

Percent specific release =
$$100 \times (CPM_{exp} - CPM_{min})/(CPM_{max} - CPM_{min})$$

where 'exp' represents experimental, 'min' represents minimum or spontaneous release, and 'max' represents maximum or lysed cell release.

Statistical analysis. Data were expressed as the arithmetical mean ± SEM. Analysis of variance (ANOVA) was used with Dunnett's t-test to compare means of treatment groups. Results described as different show a significant difference from the control, $p < 0.05$.

RESULTS

Peripheral blood leukocyte collection and enumeration

Total peripheral blood leukocyte cellularity was unchanged at any HCHO exposure level (Fig. 1). Cell types measured included lymphocytes, basophils, eosinophils, monocytes, and heterophils. Among these, only the percent of peripheral blood lymphocytes was significantly decreased by 150 ppm HCHO exposure (Fig. 2).
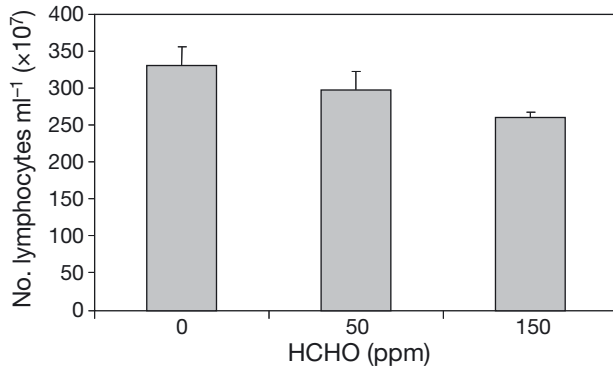
Fig. 1. *Oreochromis niloticus*. Peripheral blood leukocyte cellularity (cells ml^{-1}) in fish exposed to 0, 50, or 150 ppm formaldehyde in water for 30 min, every other day for 2 treatments

Spleen/body weight ratio and splenic and pronephric cellularity

The spleen/body weight ratio showed a high level of variability in all experiments (Fig. 3) and was not significantly changed by HCHO exposure in any replicate experiment. Similarly, neither splenic nor pronephric cellularity were significantly changed at any HCHO dosage (Fig. 4A,B).

Cyto-centrifugation leukocyte differentials

Fish exposed to HCHO demonstrated a significant decrease in the percentage of progenitor cells in the pronephros (Table 1). The total number of progenitor cells in each experimental group (i.e. the product of total organ cellularity × percentage of progenitors) was 40.5×10^6 cells in control fish, 37.6×10^6 cells at 50 ppm, and 28.2×10^6 cells at 150 ppm, showing similar decrease after HCHO. However, pronephros cellularity determination is naturally subject to somewhat high variability (Hart et al. 1997). For this reason, Table 1 reports only the percent composition results as the
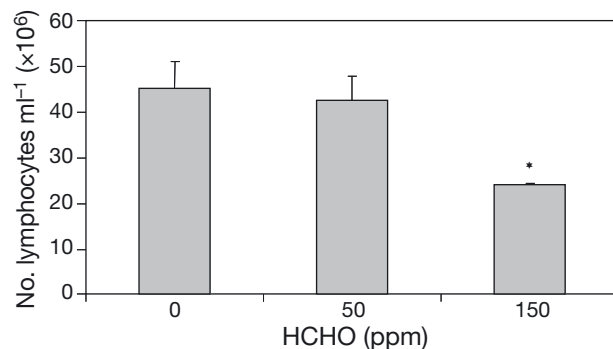

Fig. 2. *Oreochromis niloticus*. Peripheral blood lymphocyte cellularity (cells ml^{-1}) in fish exposed to 0, 50, or 150 ppm formaldehyde in water for 30 min, every other day for 2 treatments. *Different from control, p < 0.05
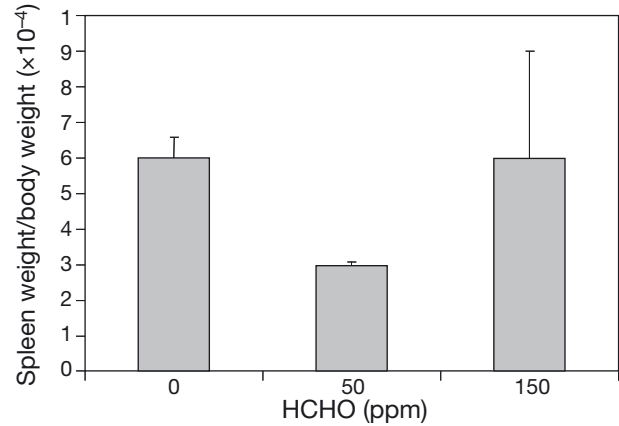

Fig. 3. *Oreochromis niloticus*. Spleen/body weight ratio in fish exposed to 0, 50, or 150 ppm formaldehyde in water for 30 min, every other day for 2 treatments

probably more reliable data. In contrast to reduced percentages of progenitors, lymphocytic cells in the pronephros showed increased percentages at both levels of enriched HCHO exposure.

Functional assays

The mitogen stimulation assay showed no significant difference among treatment groups at either level of enriched dosage (Table 2). Similarly, the ^{51}Cr release assay demonstrated no treatment-related differences (data not shown).
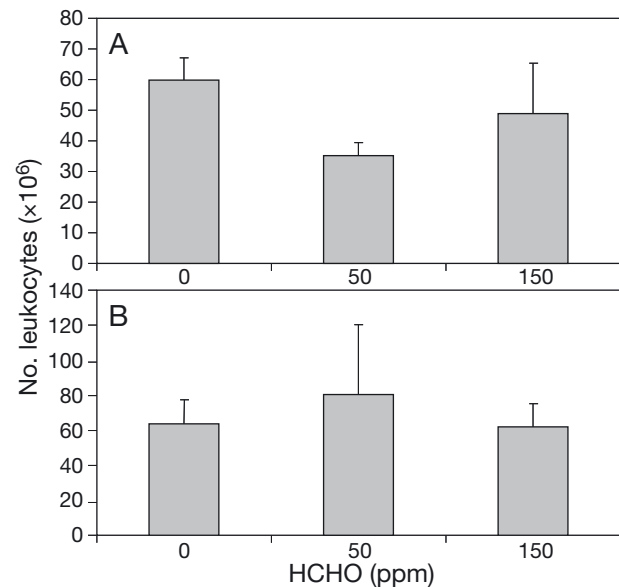

Fig. 4. *Oreochromis niloticus*. Leukocyte cellularity in the (A) spleen and (B) pronephros in fish exposed to 0, 50, or 150 ppm formaldehyde in water for 30 min, every other day for 2 treatments

Table 1. *Oreochromis niloticus.* Leukocyte percentage in the pronephros. *p < 0.05 (n = 4 fish treatment^{-1} experiment^{-1})

Conc. (ppm)	Lymphocyte	Macrophage	Heterophil	Progenitors
0	18.5 ± 2.6	10.6 ± 1.9	>1	64.3 ± 4.3
50	36.5 ± 4.1*	13.8 ± 2.7	>1	47.3 ± 1.7*
150	30.5 ± 6.5*	9.3 ± 1.8	>1	47.0 ± 2.9*

Table 2. *Oreochromis niloticus.* Mitogen proliferation of cultured fish splenocytes. Values for phytohemagglutinin (PHA) 1.0 µg ml^{-1} are reported as Δ specific absorbance (n = 4 fish treatment^{-1} experiment^{-1}). PMA: phorbol 12-myristate 12-acetate. Ion: ionomycin. PMA/Ion: 10 ng ml^{-1} PMA + 200 pg ml^{-1} Ion

Conc. (ppm)	PHA (1.0 µg ml^{-1})	PHA (10.0 µg ml^{-1})	PMA/Ion
0	0.068 ± 0.033	0.223 ± 0.038	0.353 ± 0.033
50	0.016 ± 0.009	0.175 ± 0.071	0.310 ± 0.034
150	0.095 ± 0.025	0.301 ± 0.044	0.327 ± 0.085

DISCUSSION

Although shown to be a carcinogen (Vasudeva & Anand 1996) and mutagen (Mackerer et al. 1996), formaldehyde has been classified as a non-immuntoxic chemical based on rodent studies (Arts et al. 1997, Casanova & Heck 1997), as well as on observed effects *in vitro* and *in vivo* (Mennear 1997). Nonetheless, its identification as a carcinogen suggests the possibility that HCHO may induce some immune effects. Various reports of HCHO's effects in several species demonstrate immunomodulatory potential. For example, in the human population, HCHO can cause increased sensitivity to multiple chemicals in individuals co-exposed to formaldehyde (Ziem & McTamney 1997, Sorg et al. 1998). Studies have shown formaldehyde to bind to human serum albumin, where it acts as a hapten (Li et al. 2007). A humoral immune response to the albumin-bound formaldehyde has been suggested as a useful biological marker for HCHO exposure. Dogs and rabbits have been shown to produce a measurable immune response to immunization with intravenous HCHO (Patterson et al. 1985). In experimental rodents, topical application of HCHO induced a dose-dependant activation of regional draining lymph nodes in rats (Arts et al. 1997) and also induced production of Th-1 inflammatory cytokine secretion in mice (Dearman et al. 1997). Lymph node weights have been increased significantly following oral exposure to HCHO in rats (Arts et al. 2008). Thus, mammals respond immunologically to HCHO exposure in a number of ways.

The present study evaluated several piscine immune parameters, including functional responses, following HCHO exposure. Doses used in the present work fall well within therapeutic ranges used commercially. Therapeutic regimens useful in the aquaculture industry range from short-term immersion in very high concentrations (≤15 min, 1000 to 2000 ppm) through long-term exposure to low concentrations (up to 4 h, 15 to 25 ppm) (Schnick 1974). Exposure to 200–300 ppm for 1 h has been considered typical in the industry (Buchmann et al. 2004). In addition, frequent long-term exposure to low concentrations of formalin (25 ppm, 4 h, 4 times per week, over several months) is also used prophylactically (Bodensteiner et al. 1993).

In general, the present studies produced no-effect immunologic data for formadehyde. Spleen/body weight ratios, total cellularity, mitogen response, and mixed lymphocyte reactions were not significantly altered. Natural killer cell activity was not significantly changed. In this regard, the response of the piscine immune system was similar to that reported in a previous study examining similar endpoints in B6C3F1 mice in which no significant immunosuppression was observed (Dean et al. 1984).

Two significant changes were observed in the present work. First, peripheral blood lymphocytes were decreased by exposure to 150 ppm formaldehyde. No change was observed in splenic lymphocyte number. There was limited evidence of increased lymphocytes in the pronephros, suggesting the possibility of sequestering of cells in this organ. This effect, if real, did not appear sufficient in magnitude to account for the decreased lymphocytes observed in peripheral circulation. Lymphocytes may also have been sequestered in some other compartment or tissue in response to HCHO exposure (e.g. thymus, mucosal, or non-lymphoid organ). The second change in HCHO-exposed fish was a significant decrease in the number of progenitor cells in the pronephros. Changes affecting the progenitor compartments of immune cells are not likely to be immediately reflected in evaluation of parameters involving the lymphoid cellularity of peripheral organs or circulating blood, nor will such changes affect the function of mature lymphoid cells already in the periphery. Such cells may well be able to mount control-level immune response for some period of time. However, decreases in progenitor cells would ultimately be expected to affect the number of cells available to colonize the periphery and come to the defense of the organism. Should depletion in the progenitor compartment be protracted, effects in the periphery and, thus, in the immune competence of the individual could eventually manifest under conditions of stress, or in instances of immune challenge. Decreases in the progenitor ranks, therefore, may potentiate immunosuppression.

It remains uncertain whether the cellular effects observed in the present study are immunotoxic effects. In rodents, a chemical-induced decrease in peripheral blood leukocyte counts has a relatively low predictive value for immunosuppression, such that actual immune suppression occurs only about half the time this effect is observed (Luster et al. 1993). Reduced numbers of hematopoietic progenitor cells appear to be a more sensitive indicator of immune suppression; however, data are lacking to calculate a predictive value (Holladay & Smialowicz 2000). Thus, immune challenge assays are the next required step to determine if the present HCHO exposures may be immunosuppressive in fish. This will be important to determine, since the dose levels used in this work were similar to those routinely used in aquaculture facilities.

In conclusion, the present data suggest that certain components of the piscine immune system, specifically the lymphoid progenitor compartment, may be negatively affected by formaldehyde exposure. Damage to this compartment is particularly noteworthy, given possible delayed effects of chemical exposure on future immune response of the individual.

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