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Exposure to Perchlorate Induces the Formation of Macrophage Aggregates in the Trunk Kidney of Zebrafish and Mosquitofish

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Abstract.—Environmental contamination of ground and surface waters by perchlorate, derived from ammonium perchlorate (AP) and other perchlorate salts, is of increasing concern. Exposure to perchlorate can impair the thyroid endocrine system, which is thought to modulate renal and immune function in vertebrates. This study with zebrafish *Danio rerio* and eastern mosquitofish *Gambusia holbrooki* examined the histological effects of perchlorate on the trunk kidney, which in teleosts serves excretory and hemopoietic functions and therefore may be a target of perchlorate effects. Adult zebrafish of both sexes were exposed in the laboratory to waterborne, AP-derived perchlorate at measured concentrations of 18 mg/L for 8 weeks. Adult male mosquitofish were exposed to waterborne sodium perchlorate at measured perchlorate concentrations of 1–92 mg/L for 8 weeks. Control fish were kept in untreated water. The region of the body cavity containing the trunk kidney was processed from each fish for histological analysis. Macrophage aggregates (MAs), possible markers of contaminant exposure or immunotoxic effect, were present in the hemopoietic region of the kidney in both species exposed to perchlorate. The estimated percent area of kidney sections occupied by MAs was greater in zebrafish exposed to perchlorate at 18 mg/L ($P < 0.05$) than in controls. In male mosquitofish, the incidence of renal MAs increased proportionally with sodium perchlorate concentration and was significantly different from that of controls at 92 mg/L ($P < 0.05$). These observations confirm that in fish the kidney is affected by exposure to perchlorate. The concentrations of perchlorate at which the effects were noted are relatively high but within the range reported in some contaminated habitats.

Ammonium perchlorate (AP) is a strong oxidant used primarily as a rocket propellant in military and aerospace industries and operations (Fisher et al. 2000). After the dissociation of AP into ammonium and perchlorate ions in water, the resulting perchlorate anion persists in the environment for many years (Fisher et al. 2000; Urbansky 2002). Environmental contamination of ground and surface waters by perchlorate derived from AP and other perchlorate salts is of increasing concern (Urbansky 2002).

The best known biological impact of perchlorate in animals is the impairment of thyroid gland function. Perchlorate alters thyroid function by competitively blocking iodide influx at the sodium iodide symporters that regulate the amount of iodide transported into thyroid follicles. Because iodide is necessary for thyroid hormone production, this blocking reduces the levels of thyroid hormones in blood (Wolff 1998). As a result, the pituitary gland increases its production of thyroid-stimulating hormone (TSH) in an attempt to restore thyroid hormone levels. Prolonged exposure to perchlorate causes hyperstimulation of the thyroid gland (Wolff 1998; Soldin et al. 2001). For example, histopathological studies with several vertebrate species have revealed that exposure to AP-

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derived perchlorate leads to thyroid follicle alterations such as hypertrophy, hyperplasia, angiogenesis, and colloid depletion (Fernandez Rodriguez et al. 1991; Siglin et al. 2000; York et al. 2001a; Goleman et al. 2002a; Patiño et al. 2003b). Other studies have indicated that exposure to environmentally relevant concentrations of perchlorate can affect circulating levels of thyroid hormones or TSH (Brechner et al. 2000; York et al. 2001a; Goleman et al. 2002a).

Impairments in thyroid function caused by perchlorate are likely to affect thyroid-dependent processes. Indeed, exposure to perchlorate causes developmental abnormalities in African clawed frogs *Xenopus laevis* (Goleman et al. 2002a, 2002b), deer mice *Peromyscus maniculatus* (Thuett et al. 2002), and zebrafish *Danio rerio* (Brown 1997). Conversely, perchlorate did not impair reproductive function in adult zebrafish (Patiño et al. 2003b), rats *Rattus norvegicus*, and New Zealand white rabbits *Oryctolagus cuniculus* (York et al. 2001a, 2001b). Little attention has been paid to the effects of perchlorate on other physiological functions.

In amphibians, perchlorate-induced thyroid disruption has been implicated in immune dysfunction (Rollins-Smith et al. 1993), and in mammals, thyroid hormones are known to influence renal excretory functions (Katz et al. 1975; Capasso et al. 1999; Prasad et al. 1999). Also, receptor activity or receptor transcripts for thyroid hormones (MacLatchy and Eales 1992) and TSH (Dutton et al. 1997; Sellitti et al. 2000; Vischer and Bogerd 2003) have been reported in the vertebrate kidney (including the teleost kidney); and perchlorate-sensitive sodium iodide symporters, similar to those found in the thyroid gland, are found in the mammalian kidney (Spitzweg et al. 2001). In most teleost fishes, the head and trunk regions of the kidney serve hemopoietic functions; the trunk kidney also produces the urine (Willett et al. 1999; Powell 2000). Thus, the possibility exists that the teleost kidney is affected by perchlorate exposure. The objective of this study was to examine the histological effects of perchlorate on the trunk kidney of zebrafish and eastern mosquitofish *Gambusia holbrooki*. The information obtained is expected to contribute to a better understanding of the ecotoxicology of perchlorate in aquatic habitats.

Methods

Fish and perchlorate exposures.—Zebrafish samples for this study were generated during a previous study of AP effects on fish reproduction and thyroid function (Patiño et al. 2003b). Briefly, adult zebrafish were exposed to AP by using a static-renewal procedure at measured perchlorate concentrations of 6 μg/L (untreated control) and 18 mg/L in 38-L glass aquaria. Four to five replicated tanks with 8 female fish each (female tanks) and two replicated tanks with 12 male fish each (male tanks) were used per concentration for a total of six or seven replicates per treatment. The exposures lasted 8 weeks. For the present study, 3–4 fish per aquarium were prepared for histological analysis of the kidney, giving a total of 22–26 fish per treatment.

Adult eastern mosquitofish samples for this study were generated in a separate study of the effects of sodium perchlorate (EM Science, Gibbstown, New Jersey) on reproduction, in the presence of measured concentrations (Anderson and Wu 2002) of 4 μg/L (untreated control) and 1, 8, and 92 mg/L, respectively, for 8 weeks with a static-renewal process in 38-L glass aquaria (J. W. Park et al., Texas Tech University, unpublished). Five replicate tanks per treatment were prepared. One or two male fish per aquarium were collected and analyzed for the present study, giving a total of six to eight fish per treatment. Four female fish were also housed in the same aquaria as the males, but they were part of a separate study of reproductive effects (J. W. Park et al., unpublished) and were not used for the present analysis.

For all experiments, aquaria were fitted with biofiltration units to maintain water quality; ammonia, temperature, pH, and dissolved oxygen were monitored regularly (Patiño et al. 2003b; J. W. Park et al., unpublished). Animal use protocols were reviewed and approved by the Animal Care and Use Committee of Texas Tech University.

Histological preparations.—After euthanasia by immersion in 1 g of MS-222 per liter, the abdominal cavity of the fish was exposed by incision; full specimens were fixed whole in Bouin's solution for 48 h at 4°C, rinsed in water for several hours, and stored in 70% ethanol. The trunk region of the fish was embedded in paraffin and serial sections of the trunk kidney (5–6 μm thick) were obtained with a microtome. Slides were stained with Harris's hematoxylin stain and counterstained with eosin (Sigma Diagnostics, St. Louis, Missouri). For zebrafish, select slides were also stained with periodic acid Shiff's (PAS) reagent and Wright's iron stain (both from Sigma Diagnostics).

Histological observations.—For each sample, the tissue section for histological observation was

chosen according to its histological integrity and quality, determined when viewing the first row of sections on the slide from left to right. A preliminary analysis of renal sections indicated that treatment with perchlorate induced a higher incidence of macrophage aggregates (MAs). No other abnormalities were apparent at the gross level in either the excretory or hemopoietic tissue compartments, but a detailed examination of excretory tissues was not performed. To quantify the incidence of MAs, a 1-mm × 1-mm ocular grid containing 121 crosshairs was positioned over the right kidney lobe, covering as much kidney tissue area as possible, at a total magnification of 100× for zebrafish and 200× for mosquitofish. Crosshairs of the grid that fell on MAs were counted. Crosshairs that fell outside the kidney or on large blood vessels were regarded as misses and subtracted from the total crosshair count. The relative (percent) area of kidney sections covered by MAs was estimated by dividing the number of crosshairs falling on MAs by the corrected total crosshair count. This quantification strategy does not discriminate between differences in area due to differences in the number of MAs or in the size of individual MAs. The same observer performed these measurements three separate times on each sample, using the same tissue section.

Statistical analysis.—The average of the three measurements per fish was designated as the fish value for MA incidence. All fish values within each aquarium were then averaged to estimate the respective tank value. Tank values were subjected to square root transformation, as suggested for the analysis of low-value percentage data (Steel and Torrie 1980). Analyses were conducted with the STATISTICA for Windows 1998 software package (StatSoft, Tulsa, Oklahoma). The data for zebrafish were analyzed with a two-tailed Student's *t*-test, whereas the data for mosquitofish were analyzed with one-way analysis of variance (ANOVA), followed by Duncan's multiple range tests. Homogeneity of variances was assessed using the Cochran *C* statistic and the Bartlett chi-square test. Statistical differences were considered significant at overall α of 0.05.

Results

There were six and seven aquarium replicates for the zebrafish control and 18-mg/L treatments, respectively (Patiño et al. 2003b). Preliminary histological analysis showed that fish from one control male tank and one AP-treated male tank had signs of kidney mycobacterial infection, based on the presence of granulomas with acid-fast bacteria (not shown). These two aquaria were not used in the subsequent analysis, leading to a final number of replicates of five and six for the control and 18-mg/L treatments, respectively (the total number of fish analyzed per treatment was consequently also reduced to 18 and 21 fish for the control and 18-mg/L treatment, respectively). The omission of these two male tanks left only one male tank each for the control and 18-mg/L groups. Given the low sample size of male tanks in this study, a statistical analysis based on sex was not feasible.

In sections of zebrafish kidney stained with hematoxylin and eosin, MAs were recognized as clear or light-brown macrophage clusters of various sizes (Figure 1A). Some of the cells in the aggregates had inclusions that reacted with Wright's iron stain (Figure 1B), and most of the cells were positive for PAS (Figure 1C), indicating the presence of iron and polysaccharides, respectively. Sections of mosquitofish MAs stained with hematoxylin-eosin had a considerable amount of melanin and showed a dark-brown appearance (Figure 1D). In both species, renal MAs were found within the hemopoietic tissue compartment.

Zebrafish from tanks containing 18 mg/L AP had significantly greater amounts of renal MAs than the fish in the control tanks (Figure 2; $P = 0.015639$). The same results were obtained when the male tanks were excluded from the analysis (data not shown). In male mosquitofish, the increase in the incidence of renal MAs was proportional to the concentration of sodium perchlorate (Figure 3). The incidence of renal MAs in perchlorate-exposed (92 mg/L) mosquitofish was significantly different from that of the control fish (one-way ANOVA, $P = 0.0133$; Duncan's multiple range tests, $P < 0.05$).

Discussion

The results of the present study indicate that exposure to perchlorate increases the incidence of MAs in the hemopoietic tissue compartment of the trunk kidney of zebrafish and eastern mosquitofish. Ammonia (un-ionized) is toxic to fishes at relatively high concentrations (Wedemeyer 1996); accordingly, a role for ammonia in the process of renal MA formation cannot be clearly ruled out for zebrafish because ammonium perchlorate was used as source of perchlorate in these experiments. However, based on water pH and temperature (Patiño et al. 2003b) and measured total ammonia levels, the estimated value for un-ionized ammonia in the AP-treated aquaria (approximately 0.03 mg/

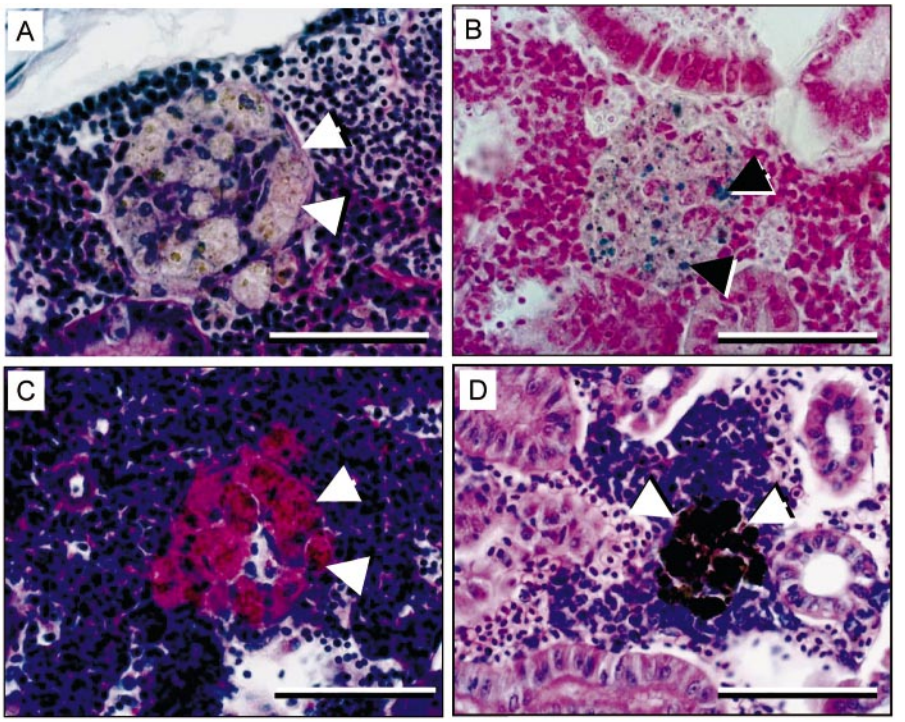

FIGURE 1.—Photomicrographs of macrophage aggregates (MAs; arrowheads) in trunk kidney of **(A–C)** zebrafish and **(D)** mosquitofish. Panel (A) shows a macrophage aggregate with light brown pigmentation (hematoxylin and eosin [H&E] stain), panel (B) an MA with hemosiderin (blue) deposits (iron stain), panel (C) an MA with polysaccharide (red) deposits (periodic acid−Schiff stain), and panel D an MA with dark melanin deposits (H&E stain). Bars are as follows: (A–C), 50 μm; (D), 100 μm.

L; R. Patiño, unpublished) only slightly exceeds the range generally recommended for fishes (Wedemeyer 1996). Further, the AP-treated fish appeared healthy and showed normal feeding and reproductive behavior as well as normal spawning success (Patiño et al. 2003b). More importantly, sodium perchlorate also increased the incidence of renal MAs in mosquitofish (present study). These observations suggest that increased renal MA formation in both species was a specific response to perchlorate exposure.

Histology of mosquitofish thyroid follicles was not performed, but histological examination of the thyroid follicles of zebrafish indicated that exposure to perchlorate caused severe angiogenesis, hypertrophy, hyperplasia, and colloid depletion (Patiño et al. 2003b). Thus, the effect of perchlorate on renal MA formation may have been indirect, acting through alterations in brain–pituitary–thyroid activity and in thyroid hormone or TSH levels. Consistent with this hypothesis, receptor activity or receptor transcripts for thyroid hormones (MacLatchy and Eales 1992) and TSH (Dutton et al. 1997; Sellitti et al. 2000; Vischer and Bogerd 2003) have been reported in the vertebrate kidney, including the teleost kidney. Also, the hemopoietic compartment of the teleost trunk kidney contains lymphocytes (Rijkers et al. 1980; Danilova and Steiner 2002), and thyroid hormones are believed to influence the process of lymphopoiesis in various vertebrate taxa (Rollins-Smith et al. 1993; Hastings et al. 1997; Dorshkind and Horseman 2000). To examine the hypothesis that the thyroid system is involved in perchlorate-dependent renal MA formation, however, researchers will need to localize thyroid hormone and TSH receptors to specific tissue compartments (excretory or hemopoietic) and cells within the trunk kidney.

Direct effects of perchlorate exposure on renal excretory function are also possible, given that perchlorate-sensitive sodium iodide symporters have been reported in mammalian kidney tubules (e.g., Spitzweg et al. 2001). At high doses that severely affected several organ systems, AP was toxic to the mammalian kidney (Sellivanova and Vorobieva 1968). However, even if sodium iodide

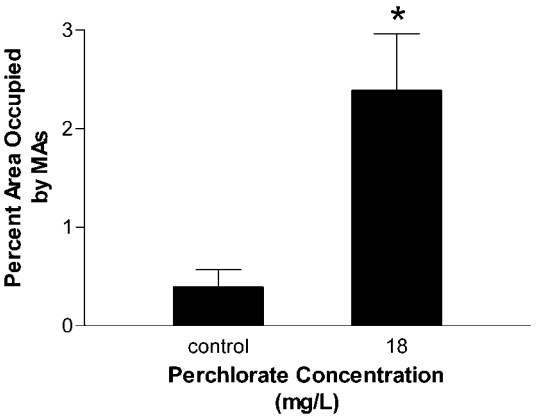

FIGURE 2.—Estimated percent area (+SE) of zebrafish kidney sections occupied by macrophage aggregates (MAs). Male and female fish were exposed to control water or water containing ammonium perchlorate for 8 weeks. The incidence of renal MAs was significantly higher in zebrafish exposed to a measured perchlorate concentration of 18 mg/L (asterisk; Student's *t*-test on square-root-transformed data, $P < 0.05$; $n = 5$–6 aquaria per treatment; the untransformed data are shown on the graph).

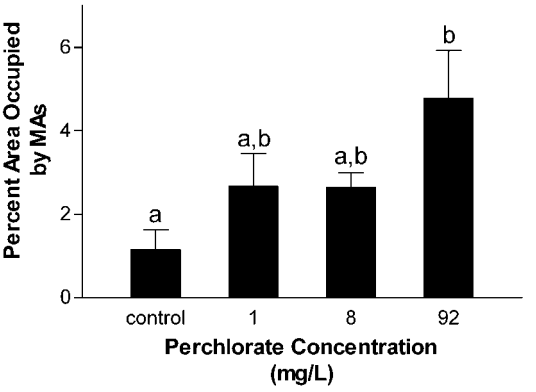

FIGURE 3.—Estimated percent area (+SE) of mosquitofish kidney sections occupied by macrophage aggregates (MAs). Male fish were exposed to control water or water containing various concentrations of sodium perchlorate for 8 weeks. The incidence of renal MAs was significantly higher in mosquitofish exposed to a measured concentration of perchlorate of 92 mg/L, whereas the incidences of renal MAs at 1 and 8 mg/L were intermediate between those of the control and 92-mg/L treatments (bars with common letters are not significantly different; one-way ANOVA and Duncan's multiple-range test on square-root-transformed data, $P < 0.05$; $n = 5$ aquaria per treatment; the untransformed data are shown on the graph).

symporters were found in fish kidney tubules, it is unclear how such occurrence could be tied to the perchlorate-dependent MA response of the hemopoietic tissue compartment observed in this study.

The role of macrophage aggregates or centers is generally considered to be the recycling and removal of cellular debris; the sequestering, destruction, and removal of cellular toxicants; antigen trapping and presentation to lymphocytes; and other functions (Tsujii and Seno 1990; Wolke 1992; Agius and Roberts 2003). In fishes, exposure to environmental contaminants is known to increase the incidence of MAs in some tissues, a response that may indicate the occurrence of tissue injury or immunotoxic response (Weeks et al. 1992; Wolke 1992; Fournie et al. 2001; Agius and Roberts 2003). Because the MAs of zebrafish and mosquitofish kidneys were associated with the hemopoietic tissue compartment, it is possible that the blood cell–forming or immune function of this tissue was affected by perchlorate exposure. Zebrafish MAs contained hemosiderin and PAS-positive components. The presence of hemosiderin, a protein-bound iron pigment, indicates the breakdown and storage of iron-containing cellular products such as hemoglobin (Wolke 1992). Therefore, an increase in the amount of hemosiderin-containing MAs supports the notion that the erythropoietic function of the trunk kidney is affected by perchlorate exposure. PAS-positive reactions indicate the presence of polysaccharide material, a common feature of fish tissue MAs (Herraez and Zapata 1986; Patiño et al. 2003a). Renal MAs of various fish species typically contain melanin (Tsujii and Seno 1990; Herraez and Zapata 1991; Weeks et al. 1992; Fournie et al. 2001; Agius and Roberts 2003). The significance of melanin in MAs is unclear, but one possible function is to protect the cells against injury from oxidizing radicals (Henninger and Beresford 1990; Rozanowska et al. 1999; Jacobson 2000). The results of the present study confirmed the presence of significant melanin deposits in renal MAs from mosquitofish but not in zebrafish. We do not know whether the difference in melanin content of renal MAs between the two species reflects species-dependent differences in their function or differences in the perchlorate exposure regimens used for each species.

In conclusion, the results of the present study suggest that exposure to perchlorate can affect physiological functions other than thyroid activity and general development in fishes. The concentrations at which perchlorate had clear effects on the incidence of renal MAs (18 mg/L in zebrafish

and 92 mg/L in mosquitofish) are at the high end of perchlorate concentrations previously reported in the environment—perchlorate concentrations as great as 33 mg/L have been reported in some contaminated aquatic habitats (Smith et al. 2001). Thus, the environmental relevance of the present findings is uncertain. However, it has been reported that the magnitude of the thyroidal effects of perchlorate are a function of not only exposure concentration but also exposure duration (Fernandez Rodriguez et al. 1991; S. Mukhi et al., Texas Tech University, unpublished). Thus, if the thyroid system were involved in the mechanism of perchlorate-induced kidney MAs, exposures to perchlorate at lower concentrations but for longer periods of time than those used in the present study might also affect the structure and function of the fish kidney.

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