Bull. Environ. Contam. Toxicol. (1995) 54:302–308

Environmental Contamination and Toxicology

Effects of Mercuric Chloride on Chemiluminescent Response of Phagocytes and Tissue Lysozyme Activity in Tilapia, *Oreochromis aureus*

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Received: 29 January 1994/Accepted: 15 July 1994

Phagocytosis is an important defense mechanism against foreign pathogenic organisms. The cells involved are phagocytes which are comprised of peripheral blood monocytes (tissue macrophages) and polymorphonuclear (PMN) leucocytes. These cells can be activated by either particulate or soluble stimuli and thereby undergo a respiratory burst from which several reactive oxygen species (ROS) such as O_2^-, ·OH and H_2O_2 can be formed (Webb et al. 1974; Stave et al. 1984). The reactive oxygen species and some hydrolases generated within the cells are known to be the major antibacterial agents released during phagocytosis (Fridovich 1974; Gabig and Babior 1981). Allen et al. (1972) have demonstrated that chemiluminescence (CL) is emitted, *in vitro*, from phagocytizing human PMN neutrophils. A similar CL response was also encountered in fish phagocytes (Stave et al. 1984; Elsasser et al. 1986). According to Webb et al. (1974), ROS was the causative agent of the CL emitted during *in vitro* phagocytosis. Therefore, phagocytic activity can be monitored by measuring the CL response of the phagocytes.

Lysozyme is one of the potent hydrolases which are involved in the destruction of pathogens during phagocytosis. In fish, it was found predominantly in haematopoietic tissues, PMN leucocytes and monocytes (Murray and Fletcher 1976). Grinde (1989) showed that this enzyme had antibacterial activity against several pathogens in fish.

A combined oxidative and hydrolytic attack upon the engulfed pathogens allow phagocytes to kill infectious agents effectively. However, severe suppression or enhancement of these two functions caused by some exogenous factors may be detrimental to the host tissues. It has been reported that inorganic mercury could inhibit, *in vitro*, the respiratory burst and the microbicidal

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activities of human PMN leucocytes (Malamud et al. 1985; Baginski 1988). It was also reported that increased *in vitro* release of lysozyme was found in mercury-treated human PMN leucocytes (Baginski 1988). However, such work has not been reported in fish. The aim of this research was to examine whether mercury could exert similar effects on the CL response in phagocytes and tissue lysozyme activity in fish after they were exposed to different concentrations of mercuric chloride over a period of 3 wk.

MATERIALS AND METHODS

Healthy tilapia, *O. aureus,* (26-71g) were obtained from the Primary Production Department, Singapore, and were maintained in well aerated, dechlorinated water at 23-26°C. The fish were fed commercial food pellets *ad libitum*. Eighteen fish (triplicate experiments) were randomly divided into 3 groups in 50l glass tanks, and appropriate amounts of a $HgCl_2$ stock solution were added to the tank's water of the first and second groups so that final concentrations of 0.2 and 0.4 ppm Hg^{2+} of $HgCl_2$ were achieved, respectively. The third group was without mercury treatment and was used as a control. Two fish from each tank were killed at d 7, 14 and 21, and their head kidneys were removed and pooled in a petri dish containing 5 ml culture medium (L-15 supplemented with 2 % faetal bovine serum, 200 U penicillin/ml and 200 ug streptomycin/ml). Kidney cells were collected by pushing the head kidney tissues through a stainless steel mesh into a centrifuge tube. Five min later, a 1-2 ml cell suspension was carefully layered onto the top of a 34 % / 51 % Percoll gradient and then centrifuged at 1500 rpm at 4°C for 25 min. Cells lying at the 34 % / 51 % interface were collected, washed twice and finally resuspended in Hank's balanced salt solution (HBSS; Secombes 1990). The cell number was counted by a Coulter counter and the viability of these cells was over 95 % as determined by the trypan blue exclusion test. A volume of 0.93 ml cell suspension ($1x10^6$ cells/ml) and 20 ul luminol solution (0.088 mg/ml HBSS) were well mixed in a polypropylene vial and incubated at 25°C for 15 min. Immediately before measuring the CL with a luminometer (BioOrbit 1250), 50 ul opsonized zymosan (10 mg/ml HBSS) were added. The CL was recorded every 10 min for 90 min.

For the determination of tissues lysozyme activity, 80 fish were used. These fish were randomly divided into 4 groups: the first, second and third groups were treated with 0.6, 0.4 and 0.2 ppm Hg^{2+} of $HgCl_2$ for 21 d respectively, and the fourth group was used as a control. At d 7, 14 and 21, 5 fishes from each group were sacrified. The fish were anaesthetized in 2-phenoxyethanol (0.2-0.3 ml/l) and the blood was withdrawn from their

caudal vein. The blood was kept at 4°C for 2-3 hr and then centrifuged at 3000 rpm at 4°C for 10 min. Plasma was kept at -20°C until use. Four volumes (w/v) of 0.004 M phosphate buffer (pH 6.6) were added to the kidney sample, which was then homogenized and centrifuged at 10,000 g at 4°C for 10 min. The supernatant was removed and stored at -20°C until use. Lysozyme activity was determined by a turbidimetric method (Ellis 1990). The substrate used was *Micrococcus lysodeikticus* (0.2 mg/ml 0.05 M phosphate buffer, pH 6.6 for kidney sample and pH 7.4 for plasma). Hen egg white lysozyme (Sigma Co.) was used as the standard by dissolving it in phosphate buffer saline (PBS, pH7.4). The absorbance was read at 0.5 min and 4.5 min intervals at 530 nm. The unit of lysozyme activity was defined as the amount of lysozyme that caused a decreased in absorbance of 0.001/min.

The data for both lysozyme activity and CL were analysed by ANOVA and followed by Duncan Test. A value of $p<0.05$ was considered to be significant.

RESULTS AND DISCUSSION

The results of present study (Table 1) show that lysozyme activity in plasma was increased in a dose-dependent manner. Plasma lysozyme activity in fish treated with 0.6 ppm Hg^{2+} of $HgCl_2$ was significantly higher than that of the control at d 7 and 21. Although there was no statistically significant difference of the enzyme activity at d 14, the mean values of lysozyme activity in the mercury-treated fish were higher than those of the control. Kidney lysozyme activity showed no change at d 7, but it increased in a dose-dependent manner at d 14 and 21 (Table 1). It is well-established that the kidney is a target organ of mercury intoxication and accumulates the most mercury (Meister 1981; Sin et al. 1983). In tilapia treated with 1 ppm Hg^{2+} of $HgCl_2$ for 2 hr, kidneys were able to accumulate mercury 3 times higher than the control (Allen et al. 1988). In rats, it was shown that more than 55 % of the administered inorganic mercury was found in the kidney 3 hr after treatment (Zalups 1993). Therefore, the renal tissues of the tilapia treated with $HgCl_2$ for a period of 3 wk in the present study were likely to be injured by the rapid accumulation of exogenous mercury. In fact, Bano and Hasan (1990) have pointed out that the kidney of *Heteropneutes fossilis* was damaged by mercury after being exposed to 0.2 ppm Hg^{2+} of $HgCl_2$ for 30 d, and many inflammatory cells were found in the injured kidney. Therefore, one can reasonably postulate that the increase of lysozyme activity in plasma and kidney tissues encountered in this study could be attributed to the heavy infiltration of phagocytes from peripheral blood circulation into damaged renal tissue. Since Baginski

(1988) reported that inorganic mercury, *in vitro*, enhanced the release of lysozyme from human PMN

Table 1. Changes in plasma and kidney lysozyme activities (mean ± S.D) in tilapia after a 21-day exposure to $HgCl_2$.

Tissue	Time (d)	Lysozyme activity**			
		$HgCl_2$ Concentration			
		Control	0.2 ppm	0.4 ppm	0.6 ppm
Plasma	7	315 ± 85	334 ± 68	376 ± 72	435 ± 90*
	14	342 ± 82	443 ± 65	419 ± 155	447 ± 67
	21	423 ± 82	422 ± 140	480 ± 78	608 ± 71*
Kidney	7	5345 ± 441	4776 ± 720	5298 ± 558	4953 ± 264*
	14	3700 ± 603	3891 ± 465	4202 ± 432*	4754 ± 351*
	21	3401 ± 590	3687 ± 235	4240 ± 430*	5142 ± 328*

** Lysozyme activity: units/ml plasma and units/g kidney
* $p<0.05$; (n = 5)

leucocytes, one cannot rule out the possibility that the increase of lysozyme activities in both plasma and kidney from d 14 until the end of the experiment might also be due to the enhanced release of lysozyme from the phagocytes being activated within renal tissue which was damaged by mercury deposition.

Fig 1. shows the CL response of head kidney cells after 7, 14 and 21 d of $HgCl_2$ exposure. The CL response of renal phagocytes in fish treated with mercury was increased throughtout the experiment. At d 7, CL response in fish treated with 0.2 ppm Hg^{2+} was higher than control and 0.4 ppm Hg^{2+} groups, but at d 14, both mercury-treated fish showed a significantly higher CL than control. The CL emission was also higher in mercury-treated fish at d 21 in terms of the total count of CL in 60 min, implied that the kidney phagocytes obtained from the mercury-treated fish were more active in phagocytosis than those of the controls. This seems to be contradictory to the findings of Malamud et al. (1985) and Baginski (1988) who showed that mercuric chloride, *in vitro*, exerted only an inhibitory effect on the CL emission of normal human PMN leucocytes within 90 min. The discrepancy could be due to phagocytes at different physiological states being used for the CL determination. In our study, the phagocytes exposed to mercury within the fish body for as long as

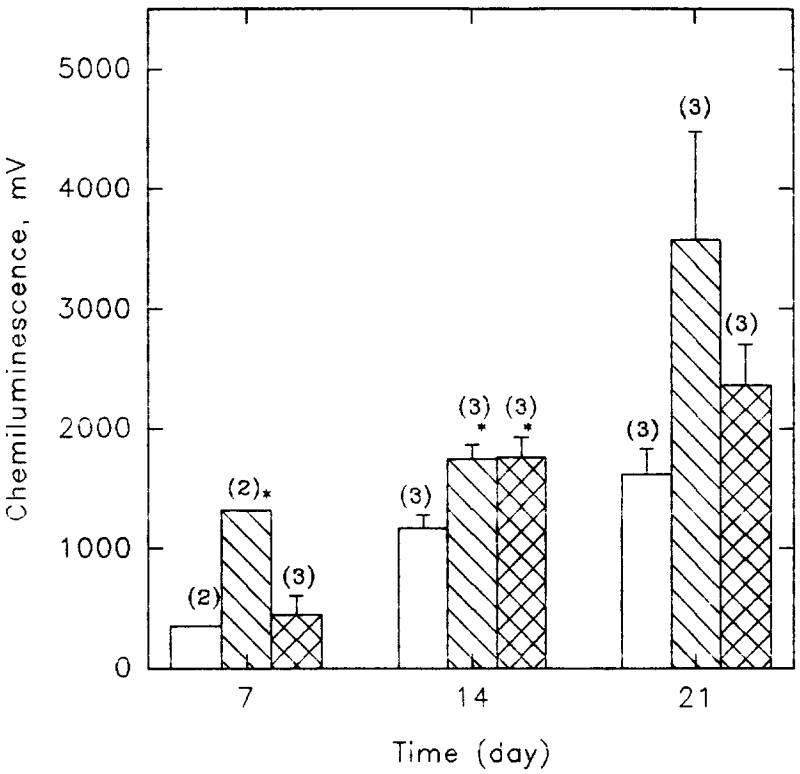

Fig 1. Effect of mercuric chloride on total count of CL in 60 min (mean ± s.e.). Number in parenthesis represents sample size. (*, $p<0.05$)
□ control; ▨ 0.2ppm Hg^{2+}; ▩ 0.4ppm Hg^{2+}

21 d might be actively involved in scavenging the damaged renal tissues.

In conclusion, the findings of the present study showed that mercury enhanced both renal tissue lysozyme and phagocyte CL activities in tilapia after 14 and 21 d of mercuric chloride exposure. This increase may suggest that the renal tissues were damaged and inflamed by the prolonged accumulation of mercury in the kidney. Although lysozyme and ROS are known to be the important antimicrobial agents for the host defence mechanisms against infection, excess production of both caused by exogenous factors such as mercury may also lead to the injury of adjacent normal tissues. This results in the induction of an inflammatory reaction which may further weaken the resistance of the fish towards pathogens. Work on determining the susceptibility of fish to infectious agents after mercury exposure is now being undertaken.

Acknowledgments. This work was supported by a research

grant from National University of Singapore.

REFERENCES

Allen RC, Stjernholm RL, Steele RH (1972) Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. Biochem Biophys Res Commun 47(4):679-684.

Allen P, Sin YM, Wong MK (1988) Acute effects of mercuric chloride on intracellular GSH levels and mercury distribution in the fish (*Oreochromis aureus*). Bull Environ Contam Toxicol 40:178-184.

Baginski B (1988) Effect of mercury chloride on microbicidal activities of human polymorphonuclear leukocytes. Toxicology 50:247-256.

Bano Y, Hasan M (1990) Histophatological lesions in the body organs of catfish (*Heteropneustes fossilis*) following mercury intoxication. J Environ Sci Health B25(1):67-85.

Ellis AE (1990) Lysozyme assays. In: Stolen JS, Fletcher TC, Anderson DP, van Muiswinkel WB (eds.) Techniques in fish immunology, SOS Publication, Fair Haven, NJ, p197.

Elsasser MS, Roberson BS, Hetrick FM (1986) Effects of metals on the chemiluminescent response of rainbow trout (*Salmo gairdneri*) phagocytes. Vet Immunol Immunopathol 12:243-250.

Fridovich I (1974) Superoxide radical and the bactericidal action of phagocytes. N Engl J Med 290:624-625.

Gabig TG, Babior BM (1981) The killing of pathogens by phagocytes. Ann Rev Med 32:313-326.

Grinde B (1989) Lysozyme from rainbow trout (*Salmo gairdneri* Richardson) as an antibacterial agent against fish pathogens. J Fish Dis 12:95-104.

Malamud D, Dietrich SA, Shapiro IM (1985) Low levels of mercury inhibit the respiratory burst in human polymorphonuclear leukocytes. Biochem Biophys Res Commun 128(3):1145-1151.

Meister A (1981) On the cycles of glutathione metabolism and transport. Curr Top Cell Reg 18:21-58.

Murray CK, Fletcher TC (1976) The immunohistochemical localization of lysozyme in plaice (*Pleuronectes platessa* L.) tissues. J Fish Biol 9:329-334.

Secombes CJ (1990) Isolation of salmonid macrophages and analysis of their killing activity. In: Stolen JS, Fletcher TC, Anderson DP, van Muiswinkel WB (eds.) Techniques in fish immunology, SOS Publication, Fair Haven, NJ, p197.

Sin YM, Lim YF, Wong MK (1983) Uptake and distribution of of mercury in mice from ingesting soluble and insoluble mercury compounds. Bull Environ Contam Toxicol 31:605-612.

Stave JW, Roberson BS, Hetrick FM (1984) Factors affecting the chemiluminescent response of fish phagocytes. J Fish Biol 25:197-206.
Webb LS, Keele BB Jr, Johnston RB Jr (1974) Inhibition of phagocytosis-associated chemiluminescence by superoxide dismutase. Infect Immun 9(6):1051-1056
Zalups RK (1993) Early aspects of the intrarenal distribution of mercury after the intravenous administration of mercury chloride. Toxicology 79:215-228.