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In Vitro Effect of Mercuric Chloride and Sodium Selenite on Chemiluminescent Response of Pronephros Cells Isolated from Tilapia, *Oreochromis aureus*

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Phagocytosis is a basic immunological function of mononuclear phagocytes and polymorphonuclear leukocytes (Olivier et al. 1986; Finco-Kent and Thune 1987). This process is a major defence mechanism in fish which involves recognition and killing of pathogenic microorganisms. It has been reported that phagocytic cells consume more oxygen and release several reactive oxygen species (ROS) during phagocytosis. This 'respiratory burst' was first quantified by measuring the chemiluminescence (CL) emitted from human polymorphonuclear leukocytes (PMNs; Allen et al. 1972) and later in fish phagocytes (Stave et al. 1984). The oxygen intermediates responsible for this CL reaction include O_2^-, $\cdot OH$ and H_2O_2 (Webb et al. 1974) which are also the major bactericidal agents in phagocytes' oxygen-dependent killing process (Fridovich 1974; Sharp and Secombes 1993). Therefore, CL response can be used as an indicator of phagocytosis (Tomita et al. 1981; Stave et al. 1984; Fletcher 1986; Moritomo et al. 1988).

Effects of some environmental pollutants on fish CL response have been evaluated. These include cadmium, copper and aluminium (Elsasser et al. 1986); tributyltin (Rice and Weeks 1989); pentachlorophenol (Anderson and Brubacher 1992), but the effect of mercuric chloride has not been well established. However, a reduction of CL response from mercury-treated human PMNs has been reported (Malamud et al. 1985; Baginski 1988). On the other hand, the interaction between mercury and its antagonist, selenium has been widely studied (Jorgensen and Heisinger 1987; Patel et al. 1988; Baatrup et al. 1989). Selenium is known as an essential element which plays an important role in the immune system (Spallholz 1990), where its main function is to eliminate hydroperoxides from cells as well as tissues. Nevertheless, an excess of selenium was reported to have suppressive effect on the functions of the natural and lymphokine-activated killer cells (Nair and Schwartz 1990). This study is designed to examine the individual effects of mercury and selenium and also

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their possible interaction on CL response of fish pronephros phagocytes, because a defect in phagocytosis may predipose fish to diseases.

MATERIALS AND METHODS

Fish were provided by the Primary Production Department, Singapore. They were acclimatized for at least 1 wk before use, and fed once a day with commercial food pellets *ad libitum*. Healthy fish ranging from 26-65g were used in each experiment, and a sample of cell suspension represents a pool of pronephros from 2 fish for each group. In brief, isolated pronephros were put in a petri dish containing 5 ml of L-15 medium (supplemented with 2 % faetal bovine serum, 100 U penicillin/ml, 100 ug streptomycin/ml and 20-30 U heparin/ml). They were then cut into small pieces and pushed through a stainless steel mesh with a glass pestle. The cells were collected and placed into cold centrifuge tubes on ice. One to two ml of cell suspension was slowly layered onto the top of a 34% / 51 % Percoll gradient, and was centrifuged at 470 g at 4 °C for 30 min. The enriched phagocytes lying in between the two density gradients were removed and washed once with L-15 medium. The resulting cell pellet was then washed once and resuspended in Hank's balanced salt solution without phenol red (HBSS; Secombes 1990). To measure the CL response, an appropriate volume of cell suspension ($1X10^6$ cells/ml), Hg^{2+} or SeO_3^{2-}, 20 ul luminol (0.088 mg/ml) and 50 ul zymosan (10 mg/ml) were added to a polystyrene cuvette to make the final volume to 1 ml. All the cuvettes were then incubated at 25 °C for 15 min. The luminol was added at 10th min while the zymosan was added immediately before measuring the CL with a luminometer at the end of the 15 min incubation (BioOrbit 1250).

Mercuric ions and selenite were added to cell suspensions respectively so that their individual effect could be investigated. The treated cell suspensions were then incubated for 15 min at 25 °C before the CL reading was recorded. Interaction of mercury and selenium was estimated in 3 ways; Hg^{2+} and SeO_3^{2-} were added simultaneously, or the cell suspension preincubated with SeO_3^{2-} for 15 min before Hg^{2+} was added and vice versa. In another experiment, the cell suspensions were incubated with Hg^{2+} or SeO_3^{2-} for 1 hr at room temperature in HBSS without phenol red (supplemented with 100 U penicillin/ml and 100 ug streptomycin/ml). The CL response was measured after the chemical-treated cells were washed twice and resuspended in fresh HBSS, without antibiotics and phenol red.

The data are expressed as mean ± S.D. and analysed by one-way ANOVA followed by Duncan Test. A value of $p<0.05$ was

considered to be significant.

RESULTS AND DISCUSSION

Phagocytosis is an important defence mechanism against various foreign pathogenic organisms. Therefore, the first contact between phagocytes and pathogens would lead to the destruction and removal of the latter from the animal body. The CL response is considered to be a sensitive method of demonstrating such early events in phagocytic recognition (Tomita et al. 1981; Stave et al. 1984; Fletcher 1986). Therefore, the CL assay has been used to assess the effects of some environmental pollutants on immunomodulatory activities (Elsasser et al. 1986; Rice and Weeks 1989; Anderson and Brubacher 1992). Welch (1980) pointed out that the CL emitted from the stimulated phagocytes depended largely on the nature of the bacteria used. In view of this, opsonized zymosan is chosen and used for phagocyte activation.

Our results show that an addition of Hg^{2+} to the cell suspensions resulted in a dose-dependent suppression of CL response. Cells treated with 0.2 and 0.4 ppm Hg^{2+} showed a significant decrease of peak CL response (Table 1). On the contrary, an increase of CL was encountered in cells treated with the increasing dosages of SeO_3^{2-}(Table 1). However, the 0.2 ppm Hg^{2+}-treated cells could restore the

Table 1. Changes in peak CL response of pronephros cells exposed to individual Hg^{2+} or SeO_3^{2-}. The results are pooled from 2 separate experiments and converted to % of control (mean ± SD). Data with the same superscripts have no significant difference between them ($p>0.05$).

Chemical	Concentration (ppm)	n	Peak CL (% of control)
Hg^{2+}	control	5	100
	0.05	5	96.6 ± 10.8^a
	0.1	5	82.8 ± 12.1^a
	0.2	5	54.7 ± 13.6^b
	0.4	5	30.5 ± 2.3^c
SeO_3^{2-}	control	6	100
	5	6	103.2 ± 5.2^a
	15	6	113.1 ± 6.4^a
	25	6	130.8 ± 14.8^b

Table 2. Restoration of CL emitted from pronephros phagocytes after the chemical-treated cells were washed and resuspended in fresh HBSS. The results are pooled from 2 separate experiments. Data with the same superscripts have no significant difference between them ($p>0.05$).

Chemical	Concentration (ppm)	n	Peak CL (% of control)
Hg^{2+}	control	8	100
	0.05	8	98.1 ± 22.3^{a}
	0.2	8	97.7 ± 25.5^{a}
SeO_3^{2-}	control	8	100
	5	8	93.6 ± 17.9^{a}
	25	7	115.9 ± 20.9^{a}

CL response to their normal level if they were washed after the mercury treatment (Table 2). This suggests that the suppression of CL at this mercury concentration is not due solely to the direct cytotoxic effect of Hg^{2+}. It is plausible that the Hg^{2+} which by reacting with a membrane enzyme, possibly the NADPH oxidase, may contribute to the observed CL suppresion. Malamud et al. (1985) have suggested other target sites at the cell membrane for mercury action because preincubation with NADPH provides only partial protection to the PMNs. Seko et al. (1989) proposed that selenium could interact with the reduced glutathione (GSH) and thus enhances the release of O_2^-. If that is so, the increased mean peak CL in 25 ppm selenite-treated phagocytes after washing (Table 2) might also have been resulted from the interaction of selenium with the intracellular GSH.

Table 3 shows the effects of interaction of Hg^{2+} with SeO_3^{2-} on CL response. The results indicate that selenium at its 10X concentration to mercury intoxication has no antagonistic effect, but a 20 ppm selenite induced an increase of about 25 % and 15 % of CL response in 0.2 ppm and 0.4 ppm Hg^{2+}-treated cells respectively. In other words, the CL increase requires 50 to 100 times of SeO_3^{2-} to Hg^{2+}. Since cells treated with 25 ppm SeO_3^{2-} alone also increased 31 % of peak CL (Table 1) as compared to the control, non-treated group, the increase of CL in the cells treated with both the mercury and selenium (Table 3) is not likely due to the counter effect of selenium on mercury. On the contrary, the results seem to indicate that mercury and selenium may affect the CL response of phagocytes independently.

Table 3. Interactions of Hg^{2+} with SeO_3^{2-} on CL response. Data are expressed as % of control and pooled from 3 separate experiments. A, both chemicals were added simultaneously; B, cells were preincubated with SeO_3^{2-} for 15 min and then added with Hg^{2+} or vice versa (C). Data with the same superscripts have no significant difference between them ($p>0.05$).

Group	Hg^{2+} (ppm)	SeO_3^{2-} (ppm)	n	Peak CL (% of control)
A	0.2	2	6	53.2 ± 20.4^{abcde}
	0.2	20	6	85.1 ± 23.2^{a}
	0.4	4	6	26.4 ± 20.3^{de}
	0.4	20	6	45.9 ± 26.5^{bcde}
B	0.2	2	5	54.3 ± 22.3^{abcd}
	0.2	20	6	78.5 ± 36.5^{ab}
	0.4	4	6	31.5 ± 23.0^{cde}
	0.4	20	6	45.8 ± 35.5^{de}
C	0.2	2	6	47.7 ± 26.4^{abcde}
	0.2	20	6	81.8 ± 29.5^{abc}
	0.4	4	6	23.4 ± 15.4^{e}
	0.4	20	6	44.0 ± 43.2^{de}

The suppression of CL response by Hg^{2+} in this study is in accordance with the findings in human PMNs, but its effect can be reversible at low concentration, which is contradictory to the report of Malamud et al. (1985) in human phagocytes. It has been shown that a higher phagocyte CL response was found in tilapias which were pre-treated with water-borne $HgCl_2$ (Low and Sin 1995). This CL increase was interpreted as a result of tissue damage caused by the mercury. Spallholz (1990) showed that a higher CL response was seen in selenium-deficient animals. On the other hand,pronephros cells treated with 25 ppm SeO_3^{2-} *in vitro* also showed a significant increase in CL response (Table 1). This discrepancy raises an interesting question as to whether the action of selenium *in vitro* is similar to that of the *in vivo* system. As pointed out by Chang and Suber (1982), selenium alleviates the toxicity of mercury through a selenoprotein, glutathione peroxidase (GSH-Px). Jorgensen and Heisinger (1987) further showed that Hg-Se complexes are formed in the animal body after a simultaneous injection of mercury and selenium. However, our present *in vitro* findings show

that selenium has no effect on CL response in pronephros phagocytes treated with mercury. Therefore, more work has to be done to reveal the actual protective mechanisms of selenium against mercury poisoning *in vivo*. In conclusion, the immunotoxicological effect of environmental toxicants such as mercury on fish can be easily demonstrated, *in vitro*, by determining the phagocytic activity of their pronephros cells using the simple and sensitive method of CL assay.

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