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Ichthyotoxicity of a Harmful Dinoflagellate *Cochlodinium polykrikoides*: Aspect of Hematological Responses of Fish Exposed to Algal Blooms

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To clarify the ichthyotoxic mechanisms of a harmful dinoflagellate *Cochlodinium polykrikoides*, hematological responses of the flounder *Paralichthys olivaceus* and red sea bream *Pagrus major* exposed to this algal bloom were investigated. The mortality of red sea bream was considerably larger than that of flounder, and the threshold lethal density of *C. polykrikoides* to the test fish was approximately 3,000 cells/mℓ. Blood PO_2 declined in proportion to the increasing density of algal cells. The blood PO_2 of moribund fish was about 40~60% of control test fish. Particularly, the fishes began to be killed when the blood PO_2 fell below 30~40 mmHg. However, the blood pH dropped almost 1.0 unit just before fish kill. Hemoglobin and hematocrit levels of fish exposed to *C. polykrikoides* of 5,000 cells/mℓ for 24 h and of moribund fish did not show great difference. The concentrations of plasma Na^+, K^+ and Cl^- were slightly elevated to different magnitudes except Ca^{2+}, and plasma osmolality was also increased in *Cochlodinium*-exposed fish. In the plasma cortisol level, these values of moribund flounder and red sea bream were 4~5 times higher than those of control fish. These results suggest that the drop of blood PO_2 was may be one of the principal causes of fish kill by *C. polykrikoides*, and the changes of other hematological parameters were secondary responses elicited by the decrease in blood PO_2.

Key words: blood PO_2, *Cochlodinium polykrikoides*, cortisol, fish mortality, harmful dinoflagellates, ichthyotoxicity

Introduction

Cochlodinium polykrikoides is one species of the fish killing dinoflagellates, and recent dense bloom causes serious damage to coastal fish farms in Korea. The highest alleged economic loss was US $ 95.5 million in 1995 (Kim *et al.*, 1997). Thus, harmful algal bloom of *C. polykrikoides* is still one of the most serious aquacultural problems. Nevertheless, the precise ichthyotoxic mechanisms of *Cochlodinium* are still poorly understood, and consequently there is no effective countermeasures to save fish from the blooming of *Cochlodinium*. Suffocation due to excessive covering of the fish gill surface by ruptured dinoflagellates and/or mucuslike substances secreted by them has been reported as a possible cause for fish kills (Hallegraeff, 1992). In contrast, Onoue *et al.* (1985) and Onoue and Nozawa (1989a) suggested that *Cochlodinium* species may secrete some ichthyotoxic substances such as neurotoxins, hemolysins and hemagglutinins. Moreover, they have reported that two unique paralytic shellfish poisoning (PSP) toxins were separated from the *Cochlodinium* type '78 Yatsushiro (Onoue and Nozawa, 1989b). However, the water and fat-soluble fraction of Korean type *C. polykrikoides* did not show ichthyotoxicity (Lee, 1997). In Korea, thus, fish kill by *C. polykrikoides* is generally thought to be due to suffocation, in spite of defficiency of a copious body of evidence.

Recently, we have found that *C. polykrikoides* generates reactive oxygen species (ROS) such as superoxide ion (O_2^-), hydroxyl radical ($\cdot OH$) and hydrogen peroxide (H_2O_2), and generated ROS is

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responsible for oxidative damages leading to fish kill; ROS is one of the factors inducing fish kill (Kim *et al.*, 1999). In addition, we reported that inactivation of ion transporting enzymes such as carbonic anhydrase and Na^+/K^+-ATPase and abnormal mucus secretion in gill cells were one of the principal causes of fish kill by *C. polykrikoides* (Kim *et al.*, 2000).

In an attempt to elucidate the underlying ichthyotoxic mechanisms of *C. polykrikoides*, in the present study, we investigated the hematological responses such as pH, oxygen partial pressure (PO_2), hemoglobin, hematocrit, plasma ions and cortisol, glutamic oxalate transaminase (GOT)/glutamic pyruvate transaminase (GPT) and osmolality in fish exposed to *C. polykrikoides* blooms. We herein report that the drop of blood PO_2 is one of the immediate causes of fish kill by *C. polykrikoides*, and the changes of other hematological parameters were secondary responses triggered by the decrease in blood PO_2. In addition, the high cortisol values of fish in seawater containing harmful dinoflagellate point to the fish being stressed.

Materials and Methods

Fish and dinoflagellate

Flounder *Paralichthys olivaceus* (av. 800 g in body weight, *ca.* 35 cm in total length) and red sea bream *Pagrus major* (av. 330 g, *ca.* 25 cm) were purchased from fish farm. They were transferred into tanks with continous seawater supply, and then subjected to a 1~2 day period of adaptation before experiment. The dinoflagellate, *Cochlodinium polykrikoides*, were collected in Namhae, Korea, in summer 1999, during a bloom of this species. The density of *C. polykrikoides* was monitored by counting the number of algal cells in 0.05~0.1 mℓ subsamples using a Sedgewick-Rafter chamber.

Exposure to Cochlodinium polykrikoides

Test fishes were exposed to four different algal cell densities of 1,000, 3,000, 5,000 and 8,000 cells/mℓ for 24 h. The dinoflagellates were replenished every 12 h to the system to attain fixed densities. Five fish individuals were also kept in natural seawater as controls. During the experiments, the water temperature and dissolved oxygen in the containers were 23~24℃ and *ca.* 5 ppm, respectively.

Blood analysis

Blood samples were taken from struggling fish, exposed to *C. polykrikoides* of 5,000 cells/mℓ for 24 h, and moribund fish caused by this dinoflagellate. Blood samples were drawn by caudal puncture with a heparinized syringe. Plasma was obtained from whole blood by centrifugation at 1,200 g for 10 min and stored at −20℃ until assayed for cortisol, ions, GOT/GPT and osmolality. Blood pH and PO_2 levels were determined immediately, using a Stat Profile 5 analyzer (NOVA Biochemical Co.). Hemoglobin contents were determined on total blood by the standard method using cyanmethemoglobin at 540 nm (Van Kampen and Zijstra, 1961). Hematocrit was determined by centrifugation at 13,000 g for 5 min in microhematocrit tubes. Levels of plasma cortisol in the experimental fish were determined by radioimmunoassay (RIA) as described by Pankhurst *et al.* (1992). Plasma Na^+, K^+, Cl^- and Ca^{2+} levels were determined with ion selective electrode, respectively. Plasma osmolality was calculated as a zero-order approximation described by Tietz and Norbert (1986). GOT and GPT levels were determined from the rate of oxidation of NADH using a coupled reaction.

Results

When the test fishes were exposed to *C. polykrikoides* of 1,000 cells/mℓ, they kept silence throughout the exposure period, and not killed. However, the high density over 3,000 cells/mℓ of *C. polykrikoides* induced violent swimming. Particularly, the active pelagic red sea bream showed violent swimming at about 1 h after exposure to *C. polykrikoides*. The red sea bream in seawater containing *C. polykrikoides* of 3,000, 5,000 and 8,000 cells/mℓ exhibited mortalities of about 30, 60 and 100% within 24 h, respectively (data not shown). In contrast, the flounder exposed to *C. polykrikoides* with a density of 8,000 cells/mℓ showed mortality of 30%. This results are similar to those of our previous results (Kim *et al.*, 2000). Consequently, the extent of fish mortality was considerably larger in the active pelagic fish than in the benthic fish, and the threshold lethal density of *C. polykrikoides* to fish was approximately 3,000 cells/mℓ.

To investigate hematological responses of

Cochlodinium-exposed fish, we first compared the blood PO_2 and pH and the results are presented in Table 1. The blood pH values of normal red sea bream were 7.15~7.35 (av. 7.25). In contrast, the average pH in the struggling and moribund fish caused by harmful dinoflagellates were 6.96 and 6.02, respectively. Namely, the pH dropped almost 1.0 unit just before fish kill. Meanwhile, the pH in the flounder a little decreased in response to algal blooms; drop of pH values were from *ca.* 7.22 to 6.71. In the case of struggling red sea bream and moribund fish, blood PO_2 decreased to about 33 and 59% of the control fish (Table 1). Of course, PO_2 in the flounder was also decreased in struggling and moribund conditions. Likewise, the decreasing trends of PO_2 and pH were found to be out of all proportion to the level of algal cells (Fig. 1). These findings suggest that fishes began to be killed when PO_2 fell below 30~40 mmHg.

Table 1. Changes in blood pH and PO_2 of fish exposed to *C. polykrikides*

Fish		pH	PO_2 (mmHg)
Flounder	Control fish	7.22 ± 0.15	62.4 ± 10.5
	Struggling fish*	7.08 ± 0.26	48.8 ± 7.5
	Moribund fish †	6.71 ± 0.32	40.0 ± 5.4
Red sea bream	Control	7.25 ± 0.07	72.3 ± 5.3
	Struggling fish	6.96 ± 0.22	48.2 ± 4.4
	Moribund fish	6.02 ± 0.41	30.0 ± 4.7

*The fish exposed to *C. polykrikoides* of 5,000 cells /mℓ for 24 h.

† The fish were still alive but showing the symptoms including loss of equilibrium, swimming on side or upside down and grasping for breath. Data are given as mean ± SD (n=7~8).

Changes in hemoglobin (Hb) and hematocrit (Hct) of the fish exposed to *C. polykrikoides* of 5,000 cells/mℓ for 24 h and moribund fish caused by these algal cells are depicted in Fig. 2. The Hb and Hct did not greatly differ throughout the exposure time; the concentration of Hb averaged 9.8, 10.3 and 10.5 g/dℓ in control, struggling and moribund flounder, respectively. The Hct averaged 48.3% in normal fish, 51.6% in struggling and 52.3% in moribund flounder, respectively. In the red sea bream, these values were also similar to those of flounder.

The measurement of plasma cortisol levels is

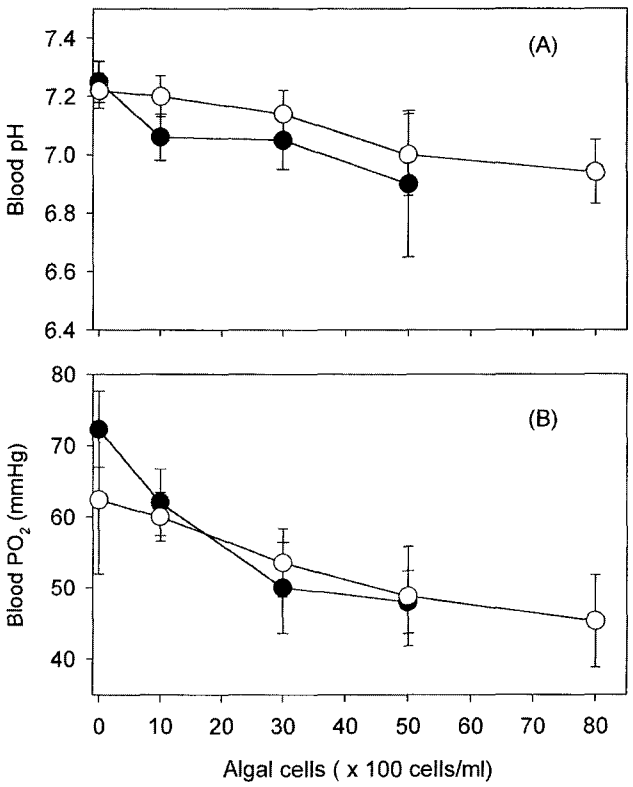

Fig. 1. Changes in blood pH (A) and PO_2 (B) of the flounder (−○−) and red sea bream (−●−) exposed to different densities of *C. polykrikoides* for 24 h. Data are means ± SD (n=3~7).

widely employed as an index of stress in fish (Barton and Iwama, 1991). Thus, the concentration of cortisol in the test fish was also measured. As expected, the concentration of plasma cortisol increased in fish to exposure to *C. polykrikoides* blooms (Fig. 3). The cortisol values of moribund flounder and red sea bream were about 8 and 21 μg/dℓ, respectively, which was 4~5 times higher than those of control fish. Consequently, the high cortisol values of fish in seawater containing *C. polykrikoides* point to the fish being stressed.

In *Cochlodinium*-exposed fish, the concentrations of the plasma ions such as Na^+, K^+, Cl^- slightly increased. However, plasma Ca^{2+} remained nearly constant during *C. polykrikoides* exposure (Fig. 4). In addition, plasma osmolality in flounder exposed to this algal cells also increased from control levels of ca. 345 mosm/kg to 355 mosm/kg in struggling fish, and 382 mosm/kg in moribund fish (Fig. 5). The changes of plasma osmolality in red sea bream were also similar to that of flounder. On the other

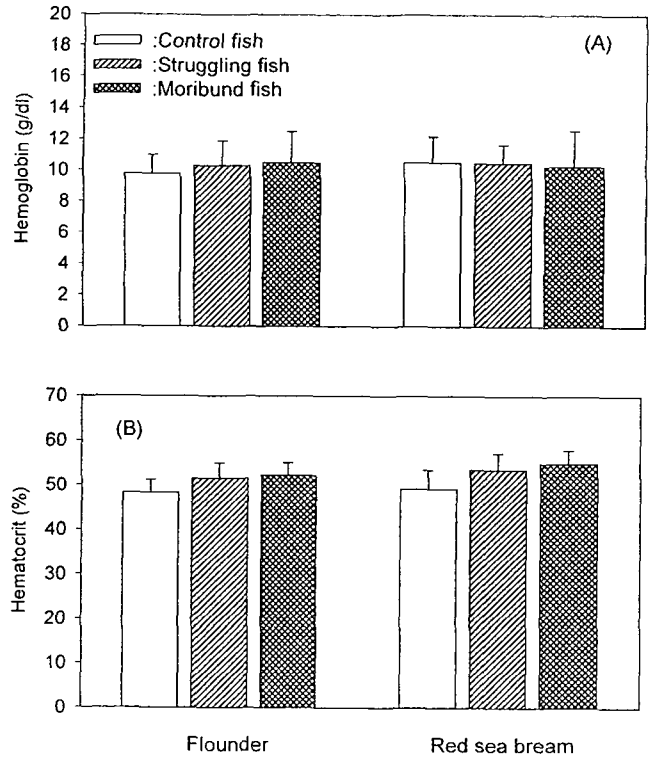

Fig. 2. Changes in hemoglobin (A) and hematocrit (B) of the flounder and red sea bream exposed to *C. polykrikoides*. *Cochlodinium*-exposure conditions were same as in Table 1. Data are means ± SD (n=3~4).

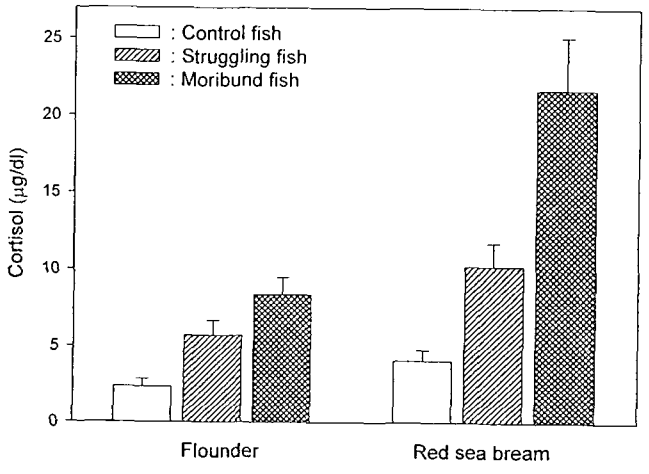

Fig. 3. Effects of the *C. polykrikoides* on the levels of plasma cortisol in the flounder and red sea bream. *Cochlodinium*-exposure conditions were same as in Table 1. Data are means ± SD (n=5~6).

hand, no changes in GOT/GPT were observed on fish subjected to harmful dinoflagellates (data not shown).

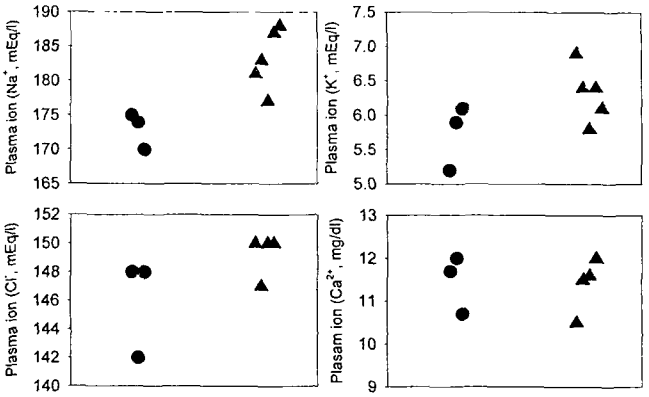

Fig. 4. The concentrations of plasma Na^+, K^+, Cl^- and Ca^{2+} of the moribund red sea bream by *C. polykrikoides* (▲), compared with those of control fish (●).

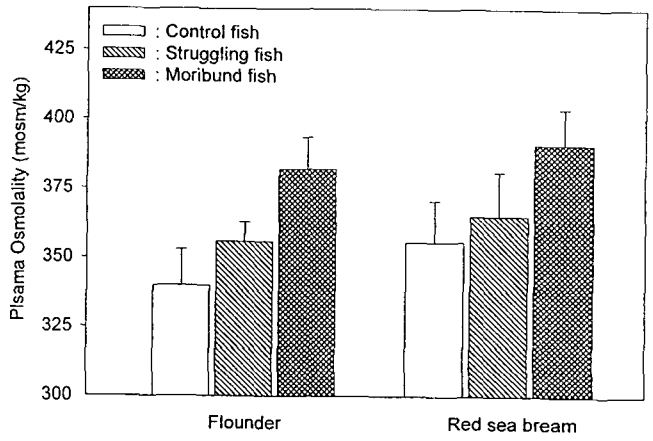

Fig. 5. Plasma osmolality of the flounder and red sea bream exposed to *C. polykrikoides*. Data are means ± SD (n=4~5).

Discussion

Although some opinions have been proposed on the cause of mass mortality of fish associated with *C. polykrikoides*, it is generally accepted that suffocation is one of the direct causes of fish kill (Hallegreff, 1992; Lee, 1997). However, the reason why the fish die from suffocation, in spite of the sufficient oxygen in water, is unanswered. Previously, thus, we investigated the physiological and/or biochemical responses related to fish exposed to *C. polykrikoides;* abnormal mucus secretion and the inactivation of ion transporting enzymes such as carbonic anhydase and Na^+/K^+-ATPase were one of important factors to the fish kill.

As shown in Table 1 and Fig. 1, the blood pH and PO_2 were reduced in *C. polykrikoides*-exposed fish. These phenomena are similar to those observed in yellowtail *Seriola quinqueradiata* exposed to *Chattonella marina* (Ishimatsu *et al.*, 1990; 1997). Although the precise mechanisms of the PO_2 drop during *Chattonella* exposure are still poorly understood, they proposed that blockade of the gill surface by mucus-like substance and reduced ventilatory conductance were involved in the hypoxemia (Hishida *et al.*, 1997). On the other hand, as the function of carbonic anhydrase in the fish gill is considered to be the transport of H^+ and CO_2 into surrounding medium, this enzyme activity is associated with the changes of blood pH and PO_2 (Dimberg *et al.*, 1981; Houston and Mearow, 1982). In this respect, several works have been reported that the inactivation of carbonic anhydase by harmful dinoflagellates resulted in physiological changes such as the reductions of blood pH and PO_2 (Endo *et al.*, 1985; Sakai *et al.*, 1986). In addition, the branchial edema interferes with oxygen transfer across the gill (Paert *et al.*, 1982).

In previous studies, reduction of ion transported-enzymes activity in gill and the formation of edema has been confirmed as the cause of anoxia in fish exposed to *C. polykrikoides* (Kim *et al.*, 1999; 2000). Therefore, these facts led us to speculate that, at least to a certain extent, the formation of edema and inactivation enzyme activity may be deeply involved in the blood PO_2 drop. Of course, the possibility would be entirely excluded that the clogging of gas exchange surface of the gills with *C. polykrikoides* may also be involved in the PO_2 decline. In any event, these phenomena is one of important symptoms in fish kill mechanism, in spite of the general concept on the cause of fall in PO_2 and pH in planktons-exposed fish is not still established.

Since no change in blood Hb is shown in Fig. 2, the increasing trends of Hct may indicate adverse effects of *C. polykrikoides* on the cell volume, *eg.* the expansion of cell volume. Moreover, responses of Hct to the lowered blood PO_2 are thought to be mediated by the sympathetic autonomic nervous system in fish (Endo *et al.*, 1992; Ishimatsu *et al.*, 1997). Therefore, the study on the nervous system in fish subjected to *C. polykrikoides* is also required.

Blood flow and arterial oxygen content are important factors in oxygen delivery to the tissues. The former is determined by blood pressure, and the latter is affected by PO_2, hemoglobin concentration and characteristics of a blood oxygen dissociation curve. Particularly, at this point, the blood PO_2 is complexly affected by various factors such as flow rates of water and blood through the gills, venous oxygen content and the PO_2 of respiratory medium (Cameron, 1989). Thus, on the basis of this facts, we cannot also exclude the possibility of the changes of these factors involved in oxygen transfer during exposure to *C. polykrikoides.*

The concentrations of plasma ions such as Na^+, K^+, Cl^- except Ca^{2+} were all elevated to different magnitudes in moribund fish (Fig. 4). Since chloride cells are responsible for active extrusion of Cl^- and Na^+ in fish (Foskett *et al.*, 1983), it would be resonable to assume that the accumulation of these ions is a result of dysfunctions of chloride cells in *Cochlodinium*-exposure fish. Moreover, Ishimatsu *et al.* (1997) reported that the inhibition of Na^+/K^+-ATPase by severe hypoxemia was linked to the increase plasma K^+ levels. Thus, we can guess that the rise of K^+ levels are induced by *C. polykrikoides*-mediated enzyme inhibition. Likewise, the plsma osmolality increase could be due in some degree to increasing of sodium ion.

It is general acceptance that the levels of plasma catecholamine and cortisol are widely employed as an index of stress in fish (Barton and Iwama, 1991; Grampel *et al.*, 1994). The levels of cortisol in unstressed fish are low but increase up to several hundred-fold under stressful conditions (Pickering and Pottinger, 1989; Sumpter *et al.*, 1985). As can be seen in Fig. 3, the level of cortisol was also increased in *Cochlodinium*-exposed fish. This result is consistent with that of Tsuchiyama *et al.* (1992) who showed that plasma catecholamine in yellowtail following exposure to *Chattonella* rose 350-fold from 0.5 nM. Moreover, if plotted against blood PO_2, a reasonably good correlation was found in the level of plasma cortisol and the blood PO_2. Thus, it is assumed that the elevation of plasma cortisol after *Cochlodinium* exposure may be a consequence of the decreased PO_2, as in *Chattonella.* So, we consider that the hypoxemia is the vital physiological disorder which leads to secondary responses

such as elevations of plsama ions, and the eventual death.

In conclusion, the results obtained in this work strongly indicate that the drop of blood PO_2 may play a prominent role in fish kill mechanism by *C. polykrikoides,* and most other hematological changes were secondarily brought about by the PO_2 drop. What we can infer from this findings is that the gill of fish subjected to *C. polykrikoides* blooms could be damaged, *eg.* the loss of functional and structural integrity of cell membranes, by some factors containing generated reactive oxygen species.

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