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Acute Exposure to *Karenia mikimotoi* Induces Oxidative Stress and Reduces Immune Parameters in the Marine Medaka *Oryzias javanicus*

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In this research, the marine medaka *Oryzias javanicus* underwent a 96 h exposure to two concentrations of the red tide dinoflagellate *Karenia mikimotoi* (1,000 and 5,000 cells mL^{-1}), and the temporal variations in biochemical responses related to antioxidant and immunity parameters were assessed in the liver tissue. The study revealed a significant increase in ichthyotoxicity with elevated cell concentrations of *K. mikimotoi*, especially evident at 96 h in marine medaka exposed to 5,000 cells mL^{-1}. At 1,000 cells mL^{-1} of *K. mikimotoi*, the opercular respiratory rate showed a significant increase, whereas exposure to 5,000 cells mL^{-1} resulted in a lowered rate. The intracellular malondialdehyde content was significantly elevated in response to both cell concentrations at 96 h. Regarding glutathione content, levels were significantly increased by exposure to both cell concentrations. Catalase and superoxide dismutase enzymatic activities experienced an increase at 1,000 cells mL^{-1} of *K. mikimotoi*, while their activities were reduced at 5,000 cells mL^{-1} at 96 h. The analysis of two immunity parameters, alternative complement pathway and lysozyme, demonstrated significantly reduced activities in the liver tissue exposed to 5,000 cells mL^{-1} of *K. mikimotoi*. These findings aim to enhance the understanding of *K. mikimotoi* toxicity in marine fish by offering insights into biochemical responses associated with harmful algal blooms.

Keywords: *Karenia mikimotoi*, Red tide, Marine medaka, Oxidative stress, Iimmunity

Harmful algal blooms (HABs) result from the excessive proliferation of various dinoflagellates, diatoms, raphidophytes, or other taxonomic groups in aquatic ecosystems, when environmental conditions favoring their blooms are established (Landsberg, 2002). These blooms, particularly associated with dinoflagellates, can inflict direct damage or release toxins that lead to mass mortalities in fish and shellfish, impacting aquaculture and fisheries (Hallegraeff, 2003). The global significance of the detrimental effects of HABs on economically vital aquaculture and fisheries has been underscored (Whyte et al., 2001; Kudela and Gobler, 2012). Dinoflagellates, diverse and abundant marine planktons, play crucial roles as food sources in aquatic trophic levels (Shumway, 1990). HABs induced by dinoflagellates, often referred to as red tides, can produce ichthyotoxic compounds even at low cell densities, leading to harmful effects through bioaccumulation in various trophic levels, including marine mammals (Landsberg, 2002; Hallegraeff, 2003; Mindy et al., 2010). Fish exposed to dinoflagellates exhibit various physical and physiological damages, such as direct physical contact with gill tissues, epithelial hyperplasia, gill clogging, anoxia, convulsions, swelling and necrosis of the lamellar epithelium, osmoregulatory disruption, and excess mucus secretion (Kim et al., 1999; Chen and Chou, 2001; Cembella et al., 2002; Landsberg, 2002; Band-Schmidt et al., 2003; Gobler et al., 2008; Shin et al., 2019; Haque et al., 2023).

Karenia mikimotoi is a prevalent species of red tide dinoflagellate known for inducing mass mortality among marine fauna in the coastal waters of the Republic of Korea (Shin et al., 2023). In this study, we aimed to analyze the acute effects of *K. mikimotoi*

on the liver tissue of the marine medaka, *Oryzias javanicus*. While potential effects of *K. mikimotoi* have been reported in marine fish, such as red seabream (Shin et al., 2023), the underlying molecular mechanisms remain poorly understood. To assess potential hazardous effects, we conducted biochemical assays targeting the antioxidant defense system and innate immunity, using concentrations of 1,000 and 5,000 cells mL^{-1} of *K. mikimotoi*. Intracellular contents of malondialdehyde (MDA) and glutathione (GSH) were monitored with analysis of enzymatic activities of catalase (CAT) and superoxide dismutase (SOD) to provide evidence of *K. mikimotoi*-mediated fluctuations in redox homeostasis. In addition, GSH-mediated antioxidant responses, along with two immune parameters-alternative complement pathway (ACH_{50}) and lysozyme -were analyzed in the liver tissue following exposure to 1,000 and 5,000 mL^{-1} of *K. mikimotoi*.

Entire animal handling and experimental procedures adhered to the ethical standards and were approved by the Animal Experimental Ethics Committee of Incheon National University (Incheon, South Korea). The marine medaka, *Oryzias javanicus*, utilized in this study were housed in artificial seawater (TetraMarine Salt Pro, Cincinnati, OH, USA) with a practical salinity of 31 ± 0.7 units and dissolved oxygen levels of 5.98 ± 0.69 mg O_2 L^{-1}, maintained at a temperature of 20 ± 1℃ under a 14/10 h light/darkness cycle. The fish were provided with frozen mosquito larvae and an artificial diet twice daily until reaching satiation.

A laboratory culture of *K. mikimotoi* was conducted following the methods outlined in a previous study (Shin et al., 2023). The dinoflagellate was cultivated at 20℃ in 0.001 μM f/2 medium, dissolved in filtered seawater, under continuous light conditions at 100 μmol photons/m^2/s. The cell count of *K. mikimotoi* was determined using a Leica DMLA microscope (Leica Microsystems, Wetzlar, Germany) equipped with UV epifluorescence and an AxioCam HRc camera (Zeiss, Göttingen, Germany) employing a Sedgwick-Rafter counting chamber (VWR, Langenfeld, Germany). For exposure experiments, *K. mikimotoi* was harvested during the exponential and early stationary phases to achieve final concentrations equivalent to approximately 10,000 *K. mikimotoi* cells mL^{-1} before treatment.

To assess sublethal effects, individual exposure experiments were conducted using 30 marine medaka. The fish were subjected to varying concentrations, including a control group and concentrations of 1,000, 2,000, 3,000, 4,000, and 5,000 cells mL^{-1} of *K. mikimotoi* for 96 h. Throughout the exposure period, the fish were not provided with any food. No mortality was observed in the control group during the 96 h experimental period. Survival rates were recorded at intervals of 0, 3, 6, 12, 24, 48, and 96 h for both the control and the exposed marine medaka groups. Opercular respiratory rates were measured in marine medaka exposed to concentrations of 1,000 and 5,000 cells mL^{-1}. This measurement was conducted in a transparent chamber where ten marine medaka from each concentration group were placed. The respiratory beats of each marine medaka were recorded over a 5-min period.

For the assessment of biochemical responses, sixty-three marine medaka were allocated to three groups corresponding to each concentration of *K. mikimotoi*. At intervals of 0, 3, 6, 12, 24, 48, and 96 h, three marine medaka from each concentration were collected. To obtain liver tissue samples, the individuals were anesthetized through immersion in a solution of tricaine methanesulfonate (Sigma-Aldrich, Inc., St. Louis, MO, USA). Subsequently, each marine medaka was dissected to extract liver tissues. For the analysis, three liver samples from each subgroup were combined, and these pooled specimens were subjected to analysis in triplicate.

Biochemical responses were assessed following the methodology outlined in our prior study (Do et al., 2022). In brief, liver tissues, combined and homogenized from each group, underwent homogenization in a cold buffer (20 mM Tris, 150 mM NaCl, 10 mM β-mercaptoethanol, 20 μM leupeptin, 2 μM aprotinin, and 100 μM benzamidine) and were then centrifuged for 30 minutes at 30,000 × g at 4℃. Following a 15 min heat denaturation of the supernatants at 75℃, thiobarbituric acid reactives (TBARs) were quantified at 535 nm using a Thermo Varioskan Flash spectrophotometer (Thermo Fisher Scientific, Tewksbury, MA, USA), with malonaldehyde bis (MDA; tetrametoxypropan, Sigma-Aldrich, Inc.) serving as a standard. The overall content of lipid peroxidation compounds was determined as nM of MDA per gram of liver tissue. Total soluble protein was gauged using the Bradford method. The intracellular GSH content in the combined liver tissues was measured employing a Glutathione Assay Kit (Catalog No. CS0260; Sigma-Aldrich, Inc.). Enzymatic activities of CAT and SOD were assessed through enzymatic methods using the SOD Assay Kit (Catalog No. 19160; Sigma-Aldrich Chemie, Switzerland) and the Catalase Assay Kit (Catalog No. CAT100; Sigma-Aldrich, Inc.), respectively. The ACH_{50} activity of *K. mikimotoi*-exposed marine medaka was analyzed using sheep red blood cells (SRBCs; 1.5×10^6 cells, National Institute of Toxicological Research, South Korea) following a previously established method (Nam et al., 2020). The lysozyme activity of *K. mikimotoi*-exposed marine medaka was measured using a turbidimetric assay in a 96-well plate (Ellis,

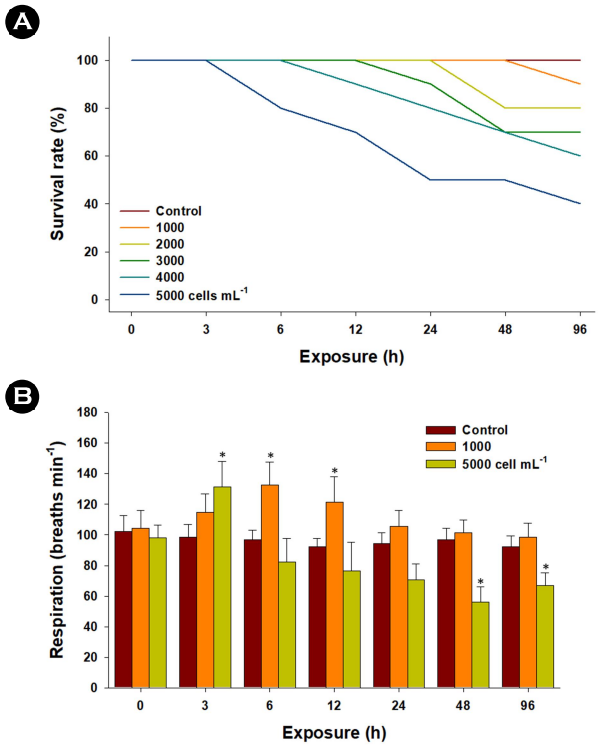

Fig. 1. Effects of different concentrations of *K. mikimotoi* on (A) survival rate (%) and (B) 5-min opercular respiration rate (breaths min^{-1}) of marine medaka for 96 h.

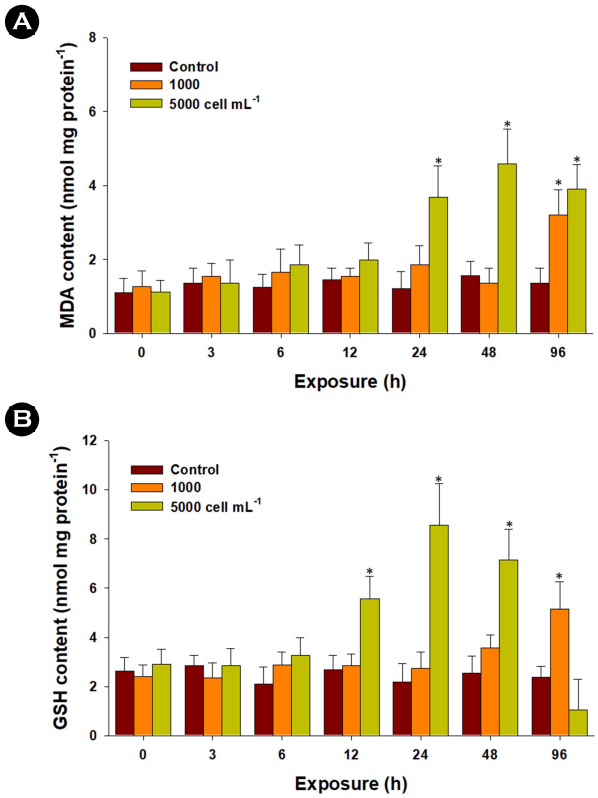

Fig. 2. Evaluation of (A) MDA and (B) GSH contents during 96 h exposure in the liver tissue of marine medaka exposed to different concentrations of *K. mikimotoi* (1,000 and 2,000 cells mL^{-1}). Data are presented as the mean ± standard deviation (S.D.). Statistically significant values are marked with an asterisk ($p < 0.05$).

1990). All data were analyzed using the statistical software package SPSS (ver. 17.0, SPSS Inc., Chicago, IL, USA) and expressed as mean ± standard deviation (S.D.).

Concentration-dependent mortality was evident with concentrations ranging from 1,000 to 5,000 cells mL^{-1} of *K. mikimotoi* (Fig. 1A). This outcome implies a threshold for both cell concentration and exposure duration of *K. mikimotoi* concerning its ichthyotoxicity in marine medaka. The opercular respiratory rates of marine medaka were significantly elevated at 3~12 h when exposed to 1,000 or 5,000 cells mL^{-1} of *K. mikimotoi* ($p < 0.05$). However, these rates were significantly reduced at 48 and 96 h in marine medaka exposed to 5,000 cells mL^{-1} ($p < 0.05$) (Fig. 1B). Consequently, the mortality observed in marine medaka exposed to 5,000 cells mL^{-1} is likely associated with significant damage to gill tissues. This damage may include alterations in gill epithelium structure, disruption of ionic homeostasis, and suffocation caused by the attachment of *K. mikimotoi*. The increased mortality is likely a result of the failure of gill function and respiration due to the direct ingestion and attachment/aggregation of *K. mikimotoi*. The detrimental effects on fish gill tissues are critical for mortality, as gills play a vital role in the absorption and transport of oxygen throughout the entire body.

Significant increases in MDA content were observed in liver tissues exposed to 1,000 cells mL^{-1} of *K. mikimotoi* at 96 h and 5,000 cells mL^{-1} at 24, 48, and 96 h ($p < 0.05$) (Fig. 2A). MDA is a byproduct of lipid peroxidation, and its measurement serves as a widely employed biomarker for assessing oxidative stress in fish (Lushchak, 2011). The elevated MDA content observed in marine medaka indicates that exposure to *K. mikimotoi* may induce the production of oxidative stressors, such as free radicals or pro-oxidant molecules, which can subsequently trigger lipid peroxidation.

Regarding GSH content, a crucial non-enzymatic antioxidant that acts as a vital protector against cellular redox-cycling fluctuations induced by exogenous oxidative stressors (Lushchak, 2011), exposure to 1,000 cells mL^{-1} of *K. mikimotoi* resulted in significantly higher levels at 96 h ($p < 0.05$). In addition, the

content significantly elevated at 12, 24, and 48 h when exposed to 5,000 cells mL^{-1} (p < 0.05) (Fig. 2B). The notable increase in GSH levels observed in marine medaka suggests the synthesis of new GSH, possibly needed to maintain the required levels for increased resistance capacity against oxidative stress as a free radical scavenger. The strongly modulated respiration rate could be considered a factor in intracellular oxidative stress, as low oxygen-triggered lipid peroxidation and subsequent oxidative stress have been well-characterized in fish (Lushchak and Bagnyukova, 2006).

The two antioxidant parameters, CAT and SOD enzymes, constitute the primary line of defense against oxidative stress. In the liver tissue exposed to 1,000 cells mL^{-1} of *K. mikimotoi*, CAT activity exhibited a significant increase at 96 h (p < 0.05). The activity was also markedly elevated at 12 and 24 h but experienced a reduction with exposure to 5,000 cells mL^{-1} at 48 and 96 h (Fig. 3A). Furthermore, upon exposure to 1,000 cells mL^{-1} of *K. mikimotoi*, there was a significantly higher activity of the SOD enzyme detected from 6 to 96 h (p < 0.05) (Fig. 3B). Additionally, a significantly higher level of SOD activity was measured in the liver tissue exposed to 5,000 cells mL^{-1} of *K. mikimotoi* at 24 h (p < 0.05). However, the activity of the SOD enzyme significantly decreased with exposure to 5,000 cells mL^{-1} of *K. mikimotoi* at 96 h (p < 0.05). Specifically, SOD transforms two superoxide radicals into hydrogen peroxide, and CAT breaks down hydrogen peroxide into water and molecular oxygen, thereby mitigating the adverse effects of free radicals (Lesser, 2006; Lushchak, 2011). The observed increase in enzymatic activities of CAT and SOD in marine medaka suggests a stress response and adaptive metabolism to counteract oxidative stress. In the gill cell line RTgill-W1 exposed to the raphidophyte *Chattonella marina*, a significant increase in SOD enzyme activity was noted (Dorantes-Aranda et al., 2015). However, their concurrent reduction in CAT and SOD

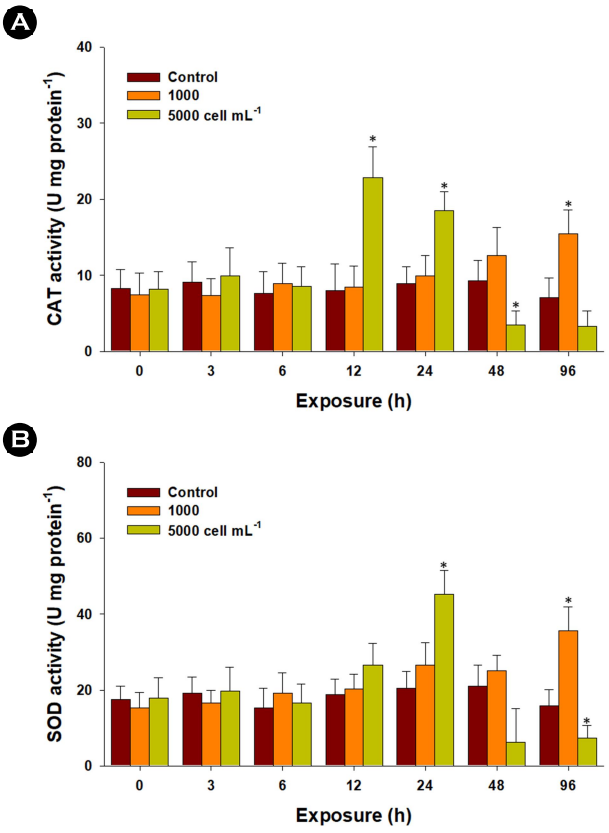

Fig. 3. Evaluation of (A) CAT and (B) SOD activities during 96 h exposure in the liver tissue of marine medaka exposed to different concentrations of *K. mikimotoi* (1,000 and 2,000 cells mL^{-1}). Data are presented as the mean ± standard deviation (S.D.). Statistically significant values are marked with an asterisk (p < 0.05).

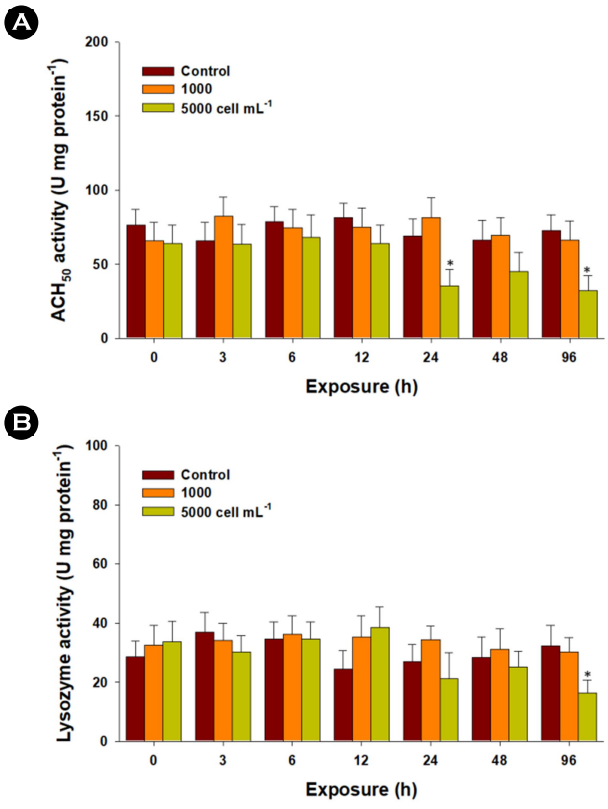

Fig. 4. Evaluation of (A) ACH$_{50}$ and (B) lysozyme activities during 96 h exposure in the liver tissue of marine medaka exposed to different concentrations of *K. mikimotoi* (1,000 and 2,000 cells mL^{-1}). Data are presented as the mean ± standard deviation (S.D.). Statistically significant values are marked with an asterisk (p < 0.05).

enzymes might be indicative of a failure in regulating oxidative homeostasis.

In general, fish experience immunological fluctuations in response to environmental stressors, although studies on HAB-triggered immune responses are limited. The immune parameters ACH_{50} and lysozyme play crucial roles in innate immunity, immunosuppression, and homeostasis in fish (Boshra and Sunyer, 2006; Saurabh and Sahoo, 2008). The complement system is pivotal in innate immunity, offering a broad spectrum of immune recognition capabilities. A significant decrease in ACH_{50} activity was observed in liver tissues exposed to 5,000 cells mL^{-1} of *K. mikimotoi* at 24 and 96 h ($p < 0.05$) (Fig. 4A). Lysozyme activity measurement is a consistent method for monitoring the potential impact of exogenous stress on innate immunity. Similarly, exposure to 5,000 cells mL^{-1} of *K. mikimotoi* resulted in significantly lower levels at 96 h ($p < 0.05$) (Fig. 4B). The noteworthy decreases in enzymatic activities of ACH_{50} and lysozyme indicate *K. mikimotoi*-triggered immune suppression. As the liver is a crucial organ synthesizing complement components (Boshra and Sunyer, 2006), the significantly decreased ACH_{50} activity implies a potential disruption in liver function. In fish, macrophages, neutrophils, and monocytes release lysozymes that directly lyse or degrade pathogen cell walls (Magnadóttir, 2006). Previously, the dinoflagellate *Alexandrium affine* (6,000 and 7,000 cells mL^{-1}) induced immunosuppression by significantly decreasing lysozyme activity and total Ig levels in the gill and liver tissues of red seabream, *Pagrus major* (Haque et al., 2021). Therefore, the reduced lysozyme activity signifies significant immune suppression, potentially increasing the vulnerability of marine medaka to diseases and exogenous pathogens, allowing unchecked inflammation.

To mitigate the detrimental effects of HABs and subsequent economic losses, it is crucial to comprehend the precise ichthyotoxicity mechanisms. In summary, all parameters of the antioxidant defense system and immunity analyzed in the liver tissue support the induction of oxidative stress and immunosuppression by *K. mikimotoi* in this species. Our results also imply that even environmental concentrations of HABs can inhibit respiration rates and may induce mass mortality in fish. Detecting early biological signals in aquatic animals in response to HABs will be useful in predicting the occurrence of HABs and establishing prevention plans in aquaculture and fisheries.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Acknowledgement

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