Free Rad. Res. Comms., Vol. 17, No. 5, pp. 327–333
Reprints available directly from the publisher
Photocopying permitted by license only

EFFECT OF OZONE ON THE ACTIVITIES OF REACTIVE OXYGEN SCAVENGING ENZYMES IN RBC OF OZONE EXPOSED JAPANESE CHARR (*Salvelinus leucomaenis*)

KENJI FUKUNAGA,[1] TETSUYA SUZUKI,[1,*] AKIHIKO HARA[2] and KOZO TAKAMA[1]

[1] *Department of Food Science and Technology, Faculty of Fisheries, Hokkaido University, Hakodate 041, Japan*
[2] *Nanae Fish Culture Experimental Station, Faculty of Fisheries, Hokkaido University, Nanae, 041-11, Japan*

The effect of ozone exposure on the activities of reactive oxygen scavenging enzymes (SOD†, catalase, GSH-Px) in RBC of Japanese charr (*Salvelinus leucomaenis*) was examined. Ozone (0, 0.4 and 0.7 ppm as initial concentrations) was exposed to Japanese charr for 30 min, which definitely caused serious membrane damage to RBC of fish. Ozone exposure at 0.4 and 0.7 ppm decreased activities of both catalase and GSH-Px by 80 to 57% of the control. On the other hand, the activities of SOD remained unaffected even by 0.7 ppm ozone exposure. A hypothesis on the RBC membrane damage and participation of SOD and heme-iron was proposed.

KEY WORDS: Ozone, Japanese charr, reactive oxygen scavenge enzymes, SOD, erythrocyte, membrane damage.

INTRODUCTION

Because of its potent oxidation potential ozone has been used for bleaching, disinfection, waste treatment, aquaculture water treatment and so on. In aquaculture intensive use of ozone is widely used for disinfection of pathogen. However, improper use of ozone sometimes causes serious damage to fishes. Wedemeyer *et al.*[1] studied the acute toxicity of ozone on rainbow trout. They carried out extensive physiological experiments to elucidate the cause of death due to ozone exposure and to determine the maximum safety level on the basis of the extent of biological damage by the chronic ozone exposure. They reported that ozone acute toxicity is initiated by the imbalance of hydrominerals which leads to massive destruction of gill lamellar epithelial cells. Similar detailed studies were reported by Paller and Heidinger[2] with bluegill, by Richardson *et al.*[3] with adult white perch, and also by Lohr and Gratzck.[4]

* Please address reprint requests to Dr. Suzuki at Department of Food Science & Technology, Faculty of Fisheries, Hokkaido University, Hakodate 041, Japan.

† Abbreviations: SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; RBC, erythrocytes; GSSG, oxidized form of glutathione; LOOH, lipid hydroperoxide; Hb, hemoglobin.

They all indicated the participation of reactive oxygen species in exhibiting lethal effect on the lamellar epithelial cells of gill. However, detailed data on the damage of biomembrane lipids and proteins reactive oxygen scavenging enzymes were not reported.

In the previous studies, we described the acute toxicity of ozone from the symptomatic aspect, and from viewpoints of lipid and protein chemistry.[5,6,7,8] Short term exposure of ozone at 0.4 and 0.7 ppm to Japanese charr (*Salvelinus leucomaenis*) definitely caused lethal damage to the fish. Our earlier studies showed that the ozone exposure severely damaged lipids and proteins of erythrocytes membrane, but not so serious on gill tissue. On the other hand, it was supposed that the ozone damage must have had something to do with the reactive oxygen scavenging systems; that is, ozone or reactive oxygen species derived from ozone that are extessively formed or accumulated are supposed to affect reactive oxygen scavenging biological system of Japanese charr. We formerly found severe and selective losses of erythrocytes membranes phospholipid and protein of Japanese charr.[5,7] Following up on our previous studies, we describe in this paper how ozone exposure affects the reactive oxygen scavenging enzymes, SOD, catalase, and GSH-Px.

MATERIALS AND METHODS

Fish

Fish used in the present study were disease free one-year-old Japanese charr (*Salvelinus leucomaenis*) hatched in the fall of 1988 at the Nanae Fish Culture Experimental Station, Faculty of Fisheries, Hokkaido University. They were reared at 15°C by being fed on artificial fish food (Kyowa Hakkou Industry Ltd., Tokyo). Feeding was stopped for 24 hr before using them for the experiment. The average fish body weight was 94.7 g, and average body size was 21.0 cm. This experiment was carried out in January 1990.

Design of Acute Ozone Toxicity Experiment

Fish were taken out from the aquaculture tank at the start of the experiment. Acute ozone exposure to fish was carried out three times at 0.4 ppm as initial concentration using 2 fish per experiment. In other words, ozone exposure experiment was repeated three times; i.e., 6 fish were used in each experiment. The same was repeated for 0.7 ppm and 0 ppm (as control). Ozone was supplied continuously by silent arc discharge type ozonizer (Nihon Ozone Co. Ltd., Tokyo) from dried air. Nitrogen oxides (NO_x) formed together with ozone was removed by filtering through a column packed with KOH. The ozone concentration was measured by Indigo-Blue method[9] and the procedure of Saltzman using neutral buffered iodine.[10] Water temperature was kept at 7°C. The pH of water used ranged between 7.2 to 7.4.

After putting two fish into each ozonated water tank, their behavior was carefully observed for 30 min. Fish were also put into fresh water without ozonation, then taken out after 30 min, and used for analyses of lipid and protein profiles of RBC and gill tissue to serve as the control.

Fish exposed to ozonated water at 0.4 ppm and 0.7 ppm were taken out immediately after reaching moribund condition, and used for analyses as mentioned above. Sample collection procedure was similar to that described by Richardson *et al.*[3]

Determination of RBC Superoxide Dismutase

Erythrocytes SOD activity was determined by a modified method of Oyanagi[11,12] based on the so-called indirect assay method. Superoxide anion (O_2^-) was generated by a mixture of xanthine oxidase and its substrate xanthine, and was allowed to reduce hydroxylamine. Reagents in the reaction mixture were as follows: 0.1 mM xanthine, 1 mM hydroxylamine·HCl, 0.8 mM hydroxylamine-O-sulfonate, $6 \sim 10 \times 10^{-4}$ units/ml xanthine oxidase (Sigma Chemical Co., St. Louis, MO), 20 μM N-naphthylene diamine·HCl, 2 mM sulfanilic acid, 16.7% acetic acid in 13 mM phosphate–7 mM borate buffer (pH 8.2) as the final concentration. Assay was run for 30 min at 37°C. To the incubated reaction mixture diazo dye forming reagent (N-naphthylene diamine·HCl) was added, and the absorption of generated pink color by the reaction with the diazo-dye and nitrite during the enzyme reaction was read at 550 nm. The enzyme activity was expressed in a unit of one unit defined as the amount of enzyme that inhibited hydroxylamine reduction by 50 per cent under the assay conditions. It was expressed in NU/mg hemoglobin.

RBC Catalase Activity

RBC catalase activity was assayed by the methods of Aebi[13] and Cohen *et al.*[14] with slight modification by using hydrogen peroxide (final concentration, 10 mM) as the substrate. Enzyme units were defined as nmoles of H_2O_2 decomposed per minute per mg hemoglobin. The decomposition of H_2O_2 was directly followed by a decrease of absorbance at 240 nm using extinction coefficient of 0.00394/mmol/mm.

RBC Glutathione Peroxidase Activity

RBC glutathione peroxidase (GSH-Px) activity was assayed by methods of Paglia *et al.*[15] and Little *et al.*[16] with slight modification. This enzyme reaction is coupled to NADPH via GSSG reductase, and the rate of NADPH oxidation was measured spectrophotometrically at 340 nm. Enzyme reaction was started by addition of 0.1 ml of 5 mM H_2O_2. Incubation was carried out at 37°C. Enzyme unit was defined as μmoles of NADPH oxidized per minute per mg of Hb by using 6.22×10^{-6} as $A_{340\,nm}$.

Determination of Hemoglobin (Hb) Content

Total hemoglobin was determined after hemolysis in hypotonic buffer solution (1:10, by vol.), followed by the addition of a mixture of buffered sodium cyanide–potassium ferricyanide. The absorbance of the resulting cyanomethemoglobin was determined at 540 nm. An extinction coefficient of 11.0 was used throughout this study. The absorption spectra were measured by Hitachi spectrophotometer, type U-2000.

Statistic Analysis

Statistic evaluation of data were carried out by student's t-test for the 6 fish.

RESULTS AND DISCUSSION

Changes in the Activities of SOD, Catalase, and GSH-Px from RBC

In Figures 1 to 3 activities of SOD, catalase and GSH-Px in RBC from ozone exposed fish are shown in comparison with those of control fish. All the data are expressed on the basis of hemoglobin from 6 fish for each group.

It is quite obvious that activities of both catalase and GSH-Px in RBC dropped with increasing ozone concentration; i.e., by 0.4 ppm ozone exposure catalase activity decreased to 67.3% of that of the control RBC, and to 57.1% by 0.7 ppm ozone exposure. The decrease ratio of GSH-Px was not so much as that of catalase, however, its activity was decreased by ozone exposure; down to 80.4% and 66.7% by 0.4 ppm and 0.7 ppm ozone exposure, respectively. In contrast to those two enzyme activities those of SOD did not show any significant changes.

Under ideal condition reactive oxygen species scavenging enzymes work in good harmony not to give rise to O_2^-, H_2O_2, LOOH, highly reactive hydroxy radical ($\cdot OH$) and so on. However, once the harmony is disturbed by whatever some reason, harmful reactive oxygens are formed and consequently cause damages on biological functions.

The fact that SOD activity remained unchanged even under acute ozone exposure but there was a decrease in catalase and GSH-Px suggests possible accumulation of H_2O_2 or formation of reactive oxygen derived from H_2O_2 such as $\cdot OH$ by Fenton's reaction,[17] Harber and Weiss reaction[18] and/or hypochlorite ion (OCl^-) by myeloperoxidase in the presence of Cl^- ion[19] *in vivo*. Since we have not directly identified the formation of $\cdot OH$ radical and ClO^- by an appropriate technique such as electron spin resonance using spin trapping technique nor analyzed antioxidative substances, we should not give any conclusive remarks on the mechanism of ozone damage to Japanese charr that was used in the series of our study. However, it is conceivable that reactive oxygen species played a key role in causing damage to

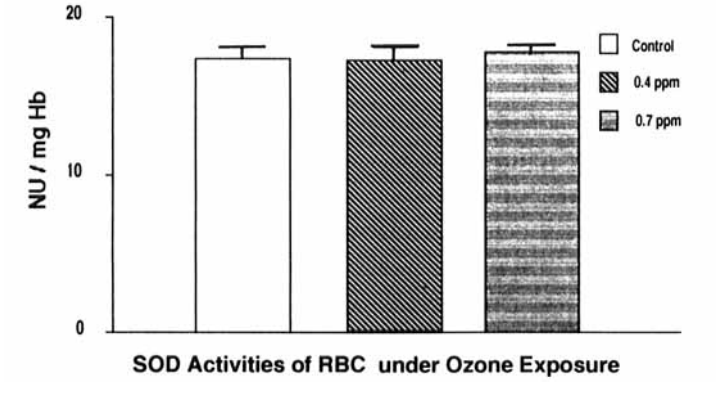

FIGURE 1 Change in SOD activities of Japanese charr RBC by ozone exposure. Japanese charrs were kept in fresh water containing 0, 0.4 and 0.7 ppm ozone as the initial concentrations for 30 min, then SOD activities were measured. No difference in SOD activity was observed among RBC with or without ozone exposure. Bars are SOD activities represented by NU/mg Hb of RBC as means ± S.E.M. for 6 fish. Experimental details are in the text.

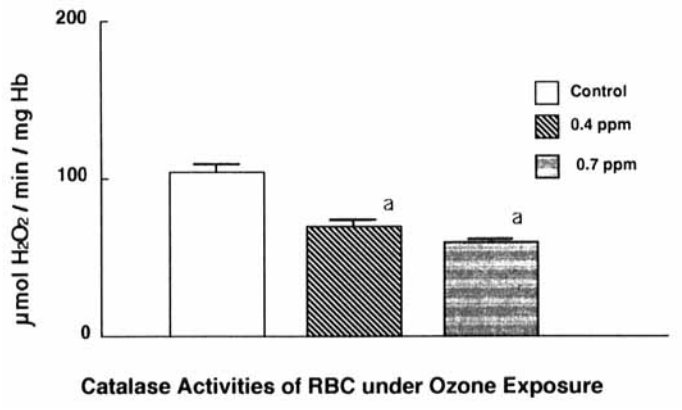

FIGURE 2 Effect of ozone exposure on catalase activities of RBC. Catalase activity drop was noticed under ozone exposure to fish for 30 min. It is obvious that decrease of catalase activity was ozone concentration dependent. Bars are catalase activities represented by μmol H_2O_2/min/mg Hb as mean $\pm$ S.E.M. for 6 fish. (a) Significant difference with $p < 0.001$. Experimental details are in the text.

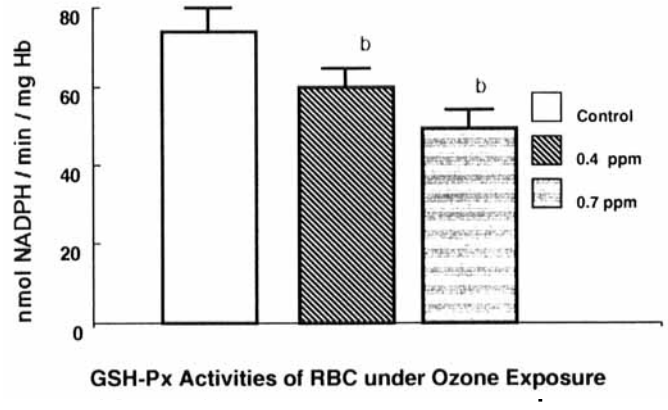

FIGURE 3 Effect of ozone exposure on GSH-Px activities of RBC. GSH-Px activity of RBC decreased by ozone exposure to Japanese charr at 0.4 and 0.7 ppm for 30 min. Bars are GSH-Px activities represented by rate of NADPH oxidation in nmol/min/mg HB as mean $\pm$ S.E.M. for 6 fish. (b) Significant difference with $p < 0.05$. Experimental details are in the text.

blood components especially to RBC membrane components. Although the detailed mechanism has not yet been made clear, both catalase and GSH-Px may have been attacked by reactive oxygen at a very early stage of ozone exposure. In such a case participation of iron involving Fenton's reaction is highly likely. The reason why SOD remained unchanged even under considerably high ozone concentration could be due to its highly resistant three dimensional protein conformation.

It is reasonable to assume that ozone damage started from the gill where gas exchange process goes on. However, the damage by ozone and/or by reactive oxygen derived from ozone would start with blood components rather than gill tissue.

Concerning the mechanism of ozone damage on Japanese charr, we would like to put forward our hypothesis as follows. Firstly, some reactive oxygen species derived from ozone reacts with hemoglobin to cause conformational change, then the degenerated hemoglobin gives rise to O_2^-.[20] The O_2^- thus generated initiates the chain reaction to give hydrogen peroxide mediated by SOD. Another possibility is that Hb-O_2 complex generates O_2^- from O_3 that penetrated gill lamellar cells. However, the pathway and mechanism of the generation of O_2^- from ozone still remains unrevealed.

With respect to the cause of death by choking, it is considered not to be due to imbalance of electrolyte as the primary cause but rather injury to blood component, i.e., hemolysis and subsequent reduction of oxygen carrier capacity, lipid peroxidation also caused by hemolysis, blood coagulation mediated by prostaglandins depleted from thrombocytes which leads to congestion of RBC and thrombocytes in blood veins and arteries especially in gill and peripheral blood vessels.

Activities of reactive oxygen scavenging enzymes in the gill under ozone exposure is now under investigation. Fluctuations of reactive oxygen scavenging substances in the gill and blood by ozone exposure are also being investigated.

Acknowledgement

The authors express their sincere thanks to Mr. Shizuo Kimura of Nanae Fish Culture Experimental Station for his continual kind help in providing experimental fish and facility service. We express deepest gratitude to Prof. K. Kator of Faculty of Sciences, the University of Tokyo, and to Drs. Kohei Yamauchi and Satoru Suzuki of our faculty for their kind suggestions and information. Special thanks are due to Messrs. M. Arita, K. Mizuno, K. Yoshida of our laboratory for their technical assistance in carrying the present study.

References

1. G.A. Wedemeyer, N.C. Nelson and W.T. Yasutake (1979) Physiological and biochemical aspects of ozone toxicity to rainbow trout (*Salmo gairdneri*). *J. Fish. Res. Board Can.*, **36**, 605–614.
2. M.H. Paller and R.C. Heidinger (1980) Mechanisms of delayed ozone toxicity to bluegill *Lopomis macrochinus*. *Environ. Pollut.* (*Ser. A Ecol. Biol*), **22**, 229–240.
3. L.B. Richardson, D.T. Burton, R.M. Block and A.M. Stavola (1983) Lethal and sublethal exposure and recovery effects of ozone produced oxidants on adult white perch *Morone americana*. *Water Res.*, **17**, 205–214.
4. A.L. Lohr and J.B. Gratzek (1986) The effects of an activated air oxidant on selected water quality parameters in a closed aquatic system. *J. Aquatic Sci.*, **4**, 85–93.
5. K. Fukunaga, T. Suzuki, S. Suzuki, K. Takama, M. Arita, A. Hara, K. Yamauchi, K. Ishizaki and N. Shinriki (1990) Acute toxicity of ozone to Japanese charr (*Salvelinus leucomaenis*). *Free Radical Biol. & Med.*, **9** (suppl. 1), 119.
6. K. Fukunaga, T. Suzuki, M. Arita, S. Suzuki, A. Hara, K. Yamauchi, N. Shinriki, K. Ishizaki and K. Takama (1992) Acute toxicity of ozone against morphology of gill and erythrocytes of Japanese charr (*Salvelinus leucomaenis*). *Comp. Biochem. Physiol.*, **101C**, 331–336.
7. K. Fukunaga, T. Suzuki and K. Takama (1991) Effect of ozone exposure on the compositions of gill and erythrocytes membrane lipids and protein of Japanese charr (*Salvelinus leucomaenis*). *Comp. Biochem. Physiol.*, **100B**, 481–487.
8. K. Fukunaga, T. Suzuki, A. Hara and K. Takama (1991) The effect of ozone exposure on gill protein and ATPase activity of Japanese charr (*Slavelinus leucomaenis*). *Nippon Suisan Gakkaishi*, **58**, 171.
9. H. Schechter (1973) Spectrophotometric method for determination of ozone in aqueous solutions. In *Water Research*, vol. 7. London: Pergamon Press, pp. 729–739.
10. B.E. Saltzman (1965) Determination of oxidants (including ozone): Neutral buffered potassium iodide method. In *Selected Methods for the Measurements of Air Pollutants*. Public Health Service Publ. 999-AP-11, p. D-X-D5.

11. Y. Oyanagi (1984) Evaluation of assay methods and establishment of kit for superoxide dismutase activity. *Anal. Biochem.*, **142**, 290–296.
12. Y. Oyanagi (1984) Establishment of nitrite-kit for SOD activity determination. *Medical Technol.*, **4**, 63–73.
13. H.E. Aebi (1981) Catalase. In *Methods of Enzymatic Analysis*, vol. II. New York, NY:Academic Press, pp. 273–285.
14. G. Cohen, P. Dembic and J. Marcus (1970) Measurement of catalase activity in tissue extract. *Anal. Biochem.*, **34**, 30–38.
15. D.E. Paglia and W.N. Valentine (1967) Studies on the quantitative and qualitative characterization of erythrocytes glutathione peroxidase. *J. Lab. Clin. Med.*, **70**, 158–167.
16. C. Little, P. Olinesce, K.G. Reid and P.J. O'Brien (1970) Properties and regulation of glutathione peroxidase. *J. Biol. Chem.*, **245**, 3632–3636.
17. I. Fridovich (1980) Biological effects of the superoxide radical. *Arch. Biochem. Biophys.*, **247**, 1–11.
18. M.S. Baker and J.M. Gebicki (1984) The effect of pH on the conversion of superoxide to hydroxyl free radicals. *Arch. Biochem. Biophys.*, **234**, 258–264.
19. M. Ducdoclon, L. Gazzolo and G.A. Quash (1984) Cellular myeloperoxidase activity in human monocytes stimulated by hyposialylated immunoglobulins and rheumatoid factors. *Immunol.*, **52**, 291–297.
20. B. Halliwell and J.M.C. Gutteridge (1989) *Free Radicals in Biology and Medicine*, 2nd. ed. Oxford: Clarendon Press, pp. 139–141.

Accepted by Professor Barry Halliwell.