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In vitro Effect of Aloe on the Respiratory Burst Activity of Olive Flounder (*Paralichthys olivaceus*) Leucocytes

Ki Hong Kim†, Yoon Jung Hwang and Sung Chul Bai*

Department of Aquatic life Medicine and
**Department of Aquaculture, Pukyong National University, Pusan 608-737, Korea*

The immunostimulating effect of aloe on respiratory burst activity was investigated by measurements of the chemiluminescent responses (CL) of olive flounder (*Paralichthys olivaceus*) kidney phagocytes *in vitro*. The phagocytes incubated with 0.5 and 1 μg/ml of aloe for 24 hours showed significantly increased respiratory burst activities compared to control. The result of this study suggests that aloe can be used as an immunostimulant for olive flounder culture.

Key words : Aloe, Immunostimulant, Respiratory burst activity, Olive flounder, Chemiluminescence

An interest in the use of immunostimulants in fish farming for enhancing the activity of non-specific defence mechanisms and conferring protection against disease has increased in recent years. As antibiotics and other chemotherapeutics may induce resistant microbial strains and environmental pollution, and commercial vaccines are expensive for fish producers and are not efficacious at present against many commercially important bacterial and viral diseases (Raa *et al.*, 1992), immunostimulants are presented as an attractive alternative way of controlling fish diseases (Anderson, 1992; Secombes, 1994; Dalmo and Seljelid, 1995).

Various immunomodulators have been reported to enhance the non-specific immunity in fish, including killed bacteria and bacterial products (Anderson, 1992; Kodaina *et al.*, 1993), chitin (Sakai *et al.*, 1992; Anderson and Siwicki, 1994), levamisole (Siwicki, 1987, 1989; Siwicki *et al.*, 1990; Kajita *et al.*, 1990; Baba *et al.*, 1993; Mulero *et al.*, 1998a, b; Kim *et al.*, 1998), glucans (Yano *et al.*, 1989; Nikl *et al.*, 1993; Duncan and Klesius, 1996; Ogier de Baulny *et al.*, 1996; Santarem *et al.*, 1997), saponin (Ninomiya *et al.*, 1995), glycyrrhizin (Jang *et al.*, 1995), certain vitamins (Blazer, 1992) and hormones (Kajita *et al.*, 1992; Kitlen *et al.*, 1997).

†Corresponding author

Aloe vera leaf has been claimed to have several important therapeutic properties including acceleration of wound healing, immune stimulation, anticancer and anti-viral effects in mammals (Kahlon *et al.*, 1991; Zhang and Tizard, 1996; Stuart *et al.*, 1997). At present, however, no information is available on the effects of aloe on stimulation of fish leucocytes.

In vitro technique used to assess immunomodulation in fish is an efficacious method of carrying out a preliminary screening of different immunostimulants before performing more expensive *in vivo* experiments, and other advantages of using *in vitro* techniques are that they utilize fewer fish and less time than *in vivo* assays of this kind (Jeney and Anderson, 1993).

Therefore, this study was undertaken to investigate the *in vitro* effect of aloe on olive flounder's phagocyte activation using the chemiluminescence (CL) assay.

Materials and Methods

Fish

Olive flounders (*Paralichthys olivaceus*) with an average body weight of 460 g were purchased from a local fish market and maintained in sea water aquaria at 20 ± 1°C prior to experiment.

Isolation of Kidney Leucocytes

Kidneys were removed aseptically from flounders and passed through a 100 μm nylon mesh using minimum essential medium (MEM, Sigma) containing heparin (10 units/ml, Sigma), penicillin (100 μg/ml Sigma) and streptomycin (100 U/ml, Sigma). The resulting cell suspension was placed on a 34/51% Percoll density gradient and centrifuged at 400 ×g for 30 min at 4°C. The interphase was collected and the cells were washed twice at 400×g for 5 min in MEM containing heparin and antibiotics. The cell viability was examined with trypan blue exclusion and was evaluated to be greater than 95%. The leucocytes, including neutrophils and macrophages, were adjusted to 3.5×10^6 cells/ml MEM in experiment I, and 4×10^6 cells/ml MEM in experiment II.

Opsonization of Zymosan

Blood samples were collected with syringes from the caudal vein of the flounders before dissection. Serum was separated by centrifugation and pooled. Zymosan (Sigma) was mixed with the fresh serum and incubated at 30°C for 30 min. Zymosan was separated from the serum by centrifugation, washed three times and suspended in Hank's balanced salt solution (HBSS, Sigma).

Chemiluminescence (CL) Assay

The reactive oxygen intermediates (ROIs) produced by stimulated phagocytes was quantified using an automatic photoluminometer (Bio-Orbit 1251, Sweden). Each test cuvette (4 ml) contained 0.7 ml luminol (Sigma) made according to the method of Scott and Klesius (1981), 0.5 ml cell suspension, and 0.3 ml opsonized zymosan, which was added just prior to measurement. Blank cuvette contained luminol and cell suspension, but opsonized zymosan was replaced with MEM. The measurements were made for 100 minutes and the light emission was recorded as mV.

Priming of the Respiratory Burst Activity by Aloe

The isolated phagocytes were incubated in the presence of 0.001, 0.01, 0.1, 0.5, 1 and 10 μg/ml aloe (KIM JEONG MOON ALOE Co. Ltd.) suspended in MEM at 17°C. A control group was incubated in the absence of aloe. Respiratory burst activity was measured using the CL assay at 3, 6, 12, 24, 48 hours after aloe treatment.

Three replicates, which were originated from 3 different flounders, were used in the CL assay, and the experiment was conducted in twice (Experiment I and II) altering only cell number.

Statistics

The paired Student's *t*-test was used to determine the statistical probability, $p < 0.05$ being the lowest significance level.

Results

The effect of aloe on the respiratory burst activity of flounder kidney phagocytes is shown in Fig. 1 and 2. Aloe did not significantly increase the respiratory burst activity when assayed 3, 6 and 12 hours after incubation in all groups of cells of both experiment I and II, except a group of cells incubated

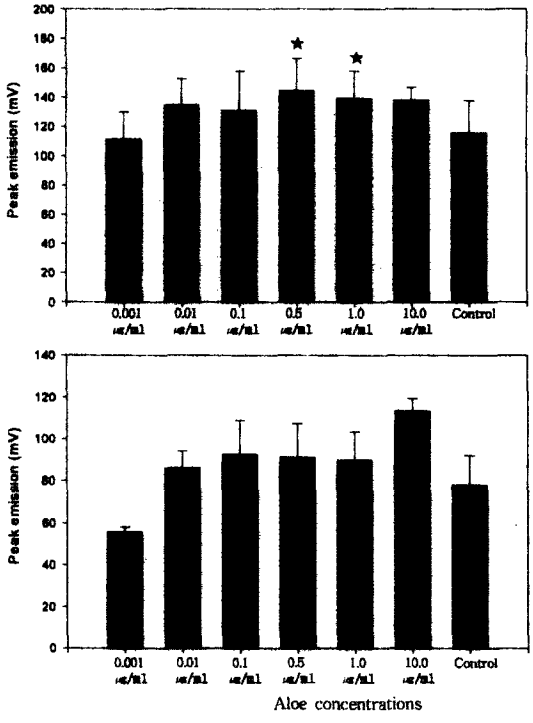

Fig. 1. The chemiluminescent response of olive flounder's kidney phagocytes which were co-incubated for 24 (A) and 48 hours (B) with the various concentrations of aloe in Experiment I. Results are means±SE. ★ Significant difference ($p < 0.05$ using a paired Student's *t*-test to compare between each aloe concentration and control).

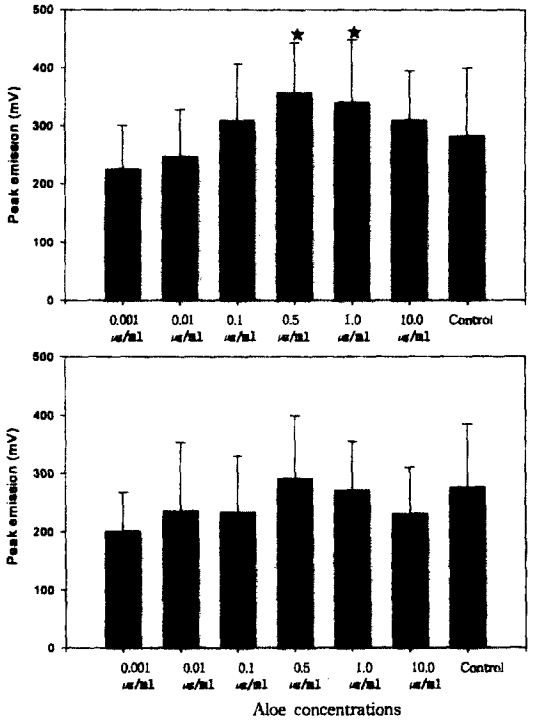

Fig. 2. The chemiluminescent response of olive flounder's kidney phagocytes which were co-incubated for 24 (A) and 48 hours (B) with the various concentrations of aloe in Experiment II. Results are means±SE. ★ Significant difference ($p < 0.05$ using a paired Student's *t*-test to compare between each aloe concentration and control).

with aloe 0.01 μg/ml for 3 hours in experiment. At 24 hours after incubation, the phagocytes showed significantly increased ($p < 0.05$) respiratory burst activity for the 0.5 and 1 μg/ml concentrations in the both trials. At 48 hours later, however, there were no significant differences in all experimental groups of cells compared to the controls.

Discussion

Fish, like other vertebrates, respond to infectious pathogens in specific and non-specific ways. However, the non-specific defences are the first a pathogen encounters, and it has been suggested that they are very important in the resistance of fish to infectious agents (Blazer, 1991). Granulocytes and macrophages possess a phagocytic activity which is the initial step in the immune response in fish, and is the major line of defence for all foreign material, including pathogenic agents (Olivier *et al.*, 1986). During phagocytosis, fish macrophages increase their oxygen consumption as well as the production of reactive oxygen intermediates (ROIs) (Chung and Secombes, 1988) such as the superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-). These ROIs play an important role in the antimicrobial activity of phagocytic cells (Allen *et al.*, 1972; Babior, 1984). Chemiluminescent response (CL) measures the respiratory burst activity of phagocytic cells in which oxygen is converted into reactive oxygen intermediates.

The result of this study suggests that aloe can be used as an immunostimulant for olive flounder culture. The phagocytes incubated with 0.5 and 1 μg/ml of aloe for 24 hours showed significantly increased respiratory burst activities. However, there were no significant differences compared to control when assayed after 48 hours incubation with the same concentrations. This result indicates that to get the most efficient immunostimulating effect of cultured flounder *in vivo*, an appropriate dose of aloe should be administered every day.

In conclusion, aloe was shown to be immunomodulating on olive flounder leucocytes *in vitro*. Phagocyte activation is important in the host non-specific defence against infectious pathogens, therefore, aloe has the potential to serve as an immunostimulant in olive flounder culture. Further experiments are required to examine whether administration of aloe to olive flounder *in vivo* can enhance non-specific immunity and resistance against harmful pathogens.

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References

Allen, R. C., Stjernholm, R. L. and Steele, R. H.: Evidence for generation of an electronic excitation state(s) in human polymorphonuclear leucocytes and its participation in bactericidal activity. Biochem. Biophy. Res. Comm., 47: 679-684, 1972.

Anderson, D. P.: Immunostimulants, adjuvants, and vaccine carriers in fish: applications to aquaculture. Ann. Rev. Fish Dis., 2: 281-307, 1992.

Anderson, D. P. and Siwicki, A. K.: Duration of protec-

tion against *Aeromonas salmonicida* in brook trout immunostimulated with glucan or chitosan by injection or immersion. Prog. Fish Cult., 56: 258-261, 1994.

Baba, T., Watase, Y. and Yoshinaga, Y.: Activation of mononuclear phagocyte function by levamisole immersion in carp. Nippon Suisan Gakkaishi, 59(2): 301-307, 1993.

Babior, B. M.: Oxidants from phagocytes: agents of defense and destruction. Blood, 64: 959-966, 1984.

Blazer, V. S.: Piscine macrophage function and nutritional influences: a review. J. Aqua. Anim. Health, 3: 77-86, 1991.

Blazer, V. S.: Nutrition and disease resistance in fish. Ann. Rev. Fish Dis., 2: 309-323, 1992.

Chung, S. and Secombes, C. J.: Analysis of events occurring within teleost macrophage during the respiratory burst. Comp. Biochem. Physiol., 89B: 539-544, 1988.

Dalmo, R. A. and Seljelid, R.: The immunomodulatory effect of LPS, laminaran and sulphated laminaran [(1,3)-D-glucan] on atlantic salmon, *Salmo salar* L., macrophages *in vitro*. J. Fish Dis., 18: 175-185, 1995.

Duncan, P. L. and Klesius, P. H.: Dietary immunostimulants enhance nonspecific immune response in channel catfish but not resistance to *Edwardsiella ictaluri*. J. Aquat. Anim. Health, 8: 241-248, 1996.

Jang, S. I., Marsden, M. J., Kim, Y. G., Choi, M. S. and Secombes, C. J.: The effect of glycyrrhizin on rainbow trout, *Oncorhynchus mykiss* (Walbaum), leucocyte responses. J. Fish Dis., 18: 307-315, 1995.

Jeney, G. and Anderson, D. P.: An in vitro technique for surveying immunostimulants in fish. Aquaculture, 112: 283-287, 1993.

Kahlon, J. B., Kemp, M. C., Carpenter, R. H., McAnalley, B. H., McDaniel, H. R. and Shannon, W. M.: Inhibition of AIDS virus replication by acemannan in vitro. Mol. Biother., 3: 127-135, 1991.

Kajita, Y., Sakai, M., Atsuta, S. and Kobayashi, M.: The immunomodulatory effects of levamisole on rainbow trout, *Oncorhynchus mykiss*. Fish Pathol., 25(2): 93-98, 1990.

Kajita, Y., Sakai, M., Kobayashi, M. and Kawauchi, H.: Enhancement of non-specific cytotoxic activity of leucocytes in rainbow trout *Oncorhynchus mykiss* injected with growth hormone. Fish Shellfish Immunol., 2: 155-157, 1992.

Kim, K. H., Hwang, Y. J. and Bai, S. C.: Enhancement of chemiluminescent response of phagocytic cells from juvenile rockfish, *Sebastes schlegeli*, by oral administration of levamisole. J. Fish. Sci. Tech., 1: (in press), 1998.

Kitlen, J. W., Hejbol, E. K., Zinck, T., Varming, K., Byatt, J. C. and McLean, E.: Growth performance and respiratory burst activity in rainbow trout treated with growth hormone and vaccine. Fish Shellfish Immunol., 7: 297-304, 1997.

Kodaina, H., Hirota, Y., Mukamoto, M., Baba, T. and Azuma, I.: Activation of rainbow trout (*Oncorhynchus mykiss*) phagocytes by muramyl dipeptide. Dev. Comp. Immunol., 17: 129-140, 1993.

Mulero, V., Esteban, M. A. and Meseguer, J.: In vitro levamisole fails to increase seabream (*Sparus aurata* L.) phagocyte functions. Fish Shellfish Immunol., 8: 315-318, 1998b.

Mulero, V., Esteban, M. A., Munoz, J. and Meseguer, J.: Dietary intake of levamisole enhances the immune response and disease resistance of the marine teleost gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol., 8: 49-62, 1998a.

Nikl, L., Evelyn, T. P. T. and Albright, L. J.: Trials with an orally and immersion-administered β-1,3-glucan as an immunoprophylactic against *Aeromonas salmonicida* in juvenile chinook salmon *Oncorhynchus tshawytscha*. Dis. Aquat. Org., 17: 191-196, 1993.

Ninomiya, M., Hatta, H., Fujiki, M., Kim, M., Yamamoto, T. and Kusuda, R.: Enhancement of chemotactic activity of yellowtail (*Seriola quinqueradiata*) leucocytes by oral administration of quillaja saponin. Fish Shellfish Immunol., 5: 325-328, 1995.

Ogier de Baulny, M., Quentel, C., Fournier, V., Lamour, F. and Le Gouvello, R.: Effect of long-term oral administration of β-glucan as an immunostimulant or an adjuvant on some non-specific parameters of the immune response of turbot *Scophthalmus maximus*. Dis. Aquat. Org., 26: 139-147, 1996.

Olivier, G., Eaton, C. A. and Campbell, N.: Interaction between *Aeromonas salmonicida* and peritoneal macrophages of brook trout (*Salvelinus fontinalis*). Vet. Immunol. Immunopathol., 12: 223-234, 1986.

Raa, J., Roerstad, G., Engstad, R. and Robetsen, B.: The use of immunostimulants to increase resistance of aquatic organisms to microbial infections. Dis. Asian Aquacult., 1: 39-50, 1992.

Sakai, M., Kamiya, R., Ishii, S., Atsuta, S. and Kobayashi, M.: The immunomodulating effects of chitin in rainbow trout *Oncorhynchus mykiss*. Dis. Asian Aquacult., 1: 413-417, 1992.

Santarem, M., Novoa, B. and Figueras, A.: Effects of β-glucans on the non-specific immune responses of turbot (*Scophthalmus maximus* L.). Fish Shellfish Immunol., 7: 429-437, 1997.

Scott, A. L. and Klesius, P. H.: Chemiluminescence: A novel analysis of phagocytosis in fish. Develop. Biol. Standard, 49: 243-254, 1981.

Secombes, C. J.: Enhancement of fish phagocyte activity. Fish Shellfish Immunol., 4: 421-436, 1994.

Siwicki, A. K.: Immunomodulating activity of levamisole

in carp spawners, *Cyprinus carpio* L. J. Fish Biol., 31: 245-246, 1987.

Siwicki, A. K.: Immunostimulating influence of levamisole on nonspecific immunity in carp (*Cyprinus carpio*). Dev. Comp. Immunol., 13: 87-91, 1989.

Siwicki, A. K., Anderson, D. P. and Dixon, O. W.: *In vitro* immunostimulation of rainbow trout *Oncorhynchus mykiss* spleen cells with levamisole. Dev. Comp. Immunol., 14: 231-237, 1990.

Stuart, R. W., Lefkowitz, D. L., Lincoln, J. A. Howard, K., Gelderman, M. P. and Lefkowitz, S. S.: Upregulation of phagocytosis and candidicidal activity of macrophages exposed to the immunostimulant acemannan. Int. J. Immunopharmacol., 19: 75-82, 1997.

Yano, T., Mangidaar, R. E. P. and Matsuyama, H.: Enhancement of the resistance of carp *Cyprinus carpio*, to experimental *Edwardsiella tarda* infection, by some β-1,3-glucans. Nippon Suisan Gakkaishi, 55: 1815-1819, 1989.

Zhang, L. and Tizard, I. R.: Activation of a mouse macrophage cell line by acemannan: the major carbohydrate fraction from Aloe vera gel. Immunopharmacology, 35: 119-128, 1996.

알로에가 *in vitro*에서 넙치 백혈구의 호흡폭발에 미치는 영향

김기홍 · 황윤정 · 배승철*

부경대학교 수산생명의학과, *양식학과

알로에가 *in vitro*에서 넙치의 비특이적 면역반응에 미치는 영향을 조사하기 위하여 신장으로부터 분리된 백혈구의 호흡폭발을 chemiluminescent(CL) 반응을 이용하여 측정하였다. 알로에 분말을 0.001, 0.01, 0.1, 0.5, 1.0, 10.0 μg/ml의 농도로 MEM에 희석하여 넙치의 식세포와 함께 배양한 후 3, 6, 12, 24, 48시간째에 CL 반응을 이용하여 호흡폭발능을 측정한 결과, 알로에 0.5 μg/ml과 1.0 μg/ml의 농도에서 24시간 동안 배양한 실험구만이 알로에를 첨가하지 않은 대조구에 비해서 유의성 있게 호흡폭발능이 증가하였다. 이러한 결과는 알로에가 양식 넙치의 비특이적 면역능을 증강시킬 수 있음을 시사하며, 앞으로 *in vivo* 실험을 통한 검증이 필요할 것으로 사료된다.

Key words: Aloe, Immunostimulant, Respiratory burst activity, Olive flounder, Chemiluminescence