한국어병학회지 제 15 권 제 2 호 (2002)
J. Fish Pathol., 15(2) : 77 ~ 82 (2002)

Effects of stress on scuticociliate killing activity of olive flounder (*Paralichthys olivaceus*) plasma in relation to humoral immunity

Se Ryun Kwon, Chun Soo Kim and Ki Hong Kim†
Department of Aquatic Life Medicine, Pukyong National University, Pusan 608-737, Korea

Effects of stress-induced suppression of humoral immunity on scuticociliate killing activity of olive flounder plasma were investigated. Changes in glucose level, alternative complement activity and lysozyme activity of plasma by handling stress were analysed in relation to *in vitro* parasiticidal activity of plasma. The plasma glucose level was about two times higher in fish after a handling stress than in control fish. Plasma lysozyme activity and natural haemolytic activity were decreased in stressed fish. The scuticociliate killing activity of plasma was significantly lower in stressed fish than in non-stressed control fish. The present results indicated that stress-induced immunodepression could be a cause of scuticociliatosis occurrence in olive flounder.

Key words: Olive flounder, Stress, Scuticociliate killing activity of plasma

Introduction

Several scuticociliate species belonging to the genera *Uronema*, *Miamiensis* and *Philasterides* are facultative histophagous parasites in marine fish (Thompson and Moewus, 1964; Cheung *et al.*, 1980; Yoshinaga and Nakazoe 1993; Dyková and Figueras, 1994; Dragesco *et al.*, 1995; Gill and Callinan, 1997; Munday *et al.*, 1997; Sterud *et al.*, 2000; Iglesias *et al.* 2001). These ciliates are characterized by their high potential for invading systemically and destroying fish tissues, leading to high mortalities in cultured fish. In Korea, scuticociliatosis is a serious problem in culturing olive flounder *Paralichthys olivaceus*, and the causative agent is identified as *Uronema marinum* by morphological characteristics (Jee *et al.* 2001).

Stress is considered one of the major problems in aquaculture, where it has been related with growth reduction, reproduction inhibition, abnormal behaviour and immunodepression, the last frequently associated with infectious disease and death (Pankhurst and Van der Kraak, 1997). Although stress-induced immunosuppression has been considered as a key factor for developing scuticociliatosis (Cheung *et al.*, 1980; Dragesco *et al.*, 1995; Munday *et al.*, 1997; Sterud *et al.*, 2000), the mechanism of the effect of stress on susceptibility to scuticociliates infections is still poorly understood.

The present experiment attempt to elucidate effects of stress-induced suppression of humoral immunity on scuticociliate killing activity of olive flounder plasma. Changes in glucose level, alternative complement activity and lysozyme activity of plasma by handling stress were analysed in relation to *in vitro* parasiticidal activity of serum.

Materials and Methods

Experimental regime

Olive flounder (*Paralichthys olivaceus*) weighing

†Corresponding Author : khkim@pknu.ac.kr

110 ± 8 g were obtained from a local fish farm and allowed to acclimatize for 2 weeks at 20℃ prior to the experiment. After acclimatization, 10 fish were divided randomly into two groups of five fish per group. The fish of the control group were kept at rest, while the fish of stress group were stressed by being held in a net in the air for 30 s at 10 min intervals for 1.5 h. After treatment, all fish were anaesthetized with 200 ㎎/ℓ tricaine methanesulfonate (MS-222, Sigma Chemical Co., USA), then blood was withdrawn by caudal vein venipuncture. Blood samples were placed on ice immediately after sampling, and the plasma were separated by centrifugation.

Isolation and culture of *Uronema marinum*

U. marinium isolated from the brain of an infected olive flounder was cultured in Eagle's minimum essential medium (MEM; Sigma) containing 200 units/㎖ of penicillin G (Sigma), 200 units/㎖ of streptomycin (Sigma) and 5% foetal bovine serum (Sigma) at 20℃.

Glucose level

Plasma glucose as an indicator of stress response was measured using a glucose oxidase/peroxidase enzymatic assay kit (Sigma). Plasma was mixed with potassium phosphate buffer containing glucose oxidase. Glucose solution (2 mg/㎖) was also mixed with buffer containing glucose oxidase as a standard. After incubation at 20℃ for 10 min, absorbance was detected at 500 nm against blank (buffer only).

Lysozyme activity

The plasma lysozyme activity was determined by a turbidimetric method (Ellis, 1990). The substrate used was *Micrococcus lysodeikticus* (0.2 mg/㎖ 0.05 M phosphate buffer, pH 7.4). The absorbancy was read at 0.5 min and 4.5 min intervals at 530 nm. The unit of lysozyme activity was defined as the amount of lysozyme that caused a decrease in absorbancy of 0.001/min.

Natural haemolytic complement activity

Natural complement activity was measured by a colorimetric haemolytic assay using isolated rabbit erythrocytes (RRBC). Briefly, rabbit blood was withdrawn from the ear vein into anticoagluant buffer (ACB; 3.15% sodium citrate and 2.45% glucose, pH 7.4) treated syringes. Blood was centrifuged at 500 g for 10 min and erythrocytes were isolated, washed with ACB and finally washed twice with gelatine veronal buffer solution (GVBS) supplemented with EGTA (GVBS-EGTA, pH 7.4). The cells were resuspended in GVBS-EGTA to concentration of 5×10^8 cells/㎖. The assay was performed with 30 ㎕ of the RRBC suspension which were incubated with 100 ㎕ of plasma dilution in GVBS-EGTA (1:4, 1:8, 1:16, 1:50, 1:64, 1:80, 1:100, 1:128, 1:512). Samples were incubated for 45 min at 20℃. At the end of incubation time, 30 ㎕ of 0.2 M EDTA were added to stop the reaction and samples were centrifuged for 3 min at 1,600 g. An aliquot of 100 ㎕ of the supernatant was taken and the absorbance of free haemoglobin was measured at 405 nm. Negative controls were done by adding 30 ㎕ of 0.2 M EDTA before the erythrocyte suspension. Spontaneous lysis (0%) and total lysis (100%) were also done by incubating 30 ㎕ RRBC with 130 ㎕ of GVBS-EGTA or 130㎕ distilled water, respectively. Lysis percentage (Y) was calculated for each dilution of plasma as:

$$Y = \{(\text{OD sample} - \text{OD negative control})/(\text{OD 100\% lysis} - \text{OD 0\% lysis})\} \times 100$$

For the calculation of complement concentration

giving rise to 50% of lysis via the alternative pathway (1 ACH50 unit), results were modified by Klerx *et al.* (1983) as a plot of:

$$\log_{10}(Y/100\text{-}Y) \text{ vs. } \log_{10}(\text{plasma dilution})$$

Complement activity (ACH50) is given in U ml^{-1} and calculated as follows:

$$ACH50 = 10^{1+P}$$

Where P is graphically determined as the point of intersection with the x axis.

Parasiticidal activity

The parasite killing activity of serum was assessed in the following manner. Plasma was diluted in HBSS (pH 7.4) as follows; 1:16, 1:32, 1:40, 1:64, 1:80, 1:128, 1:160, 1:256, 1:512. A part of plasma was heated at 45℃ for 2 h, and was used as inactivated plasma. The diluted serum was mixed with live parasite suspension (10^4 cell/$m\ell$) at 10:1 mixing ratio. The mixture was incubated at room temperature for 24 h. Lysis of the ciliates was detected by an inverted microscope, and plasma parasiticidal activity was represented the inverse of maximum dilution ratio showing 100% lysis of the ciliates.

Statistical analysis

In overall experiments, Student 's *t*-test was employed to evaluate the level of significance and the difference was considered significant when $P<0.05$.

Results

Plasma glucose level was about two times higher in fish after a handling stress than in control fish (Fig. 1). Plasma lysozyme activity was decreased considerably in stressed fish (Fig. 2). Plasma complement activity, as measured by the alternative pathway, was depressed by handling stress (Fig. 3). Scuticociliate killing activity of plasma was significantly lower ($P<0.05$) in stressed fish than in non-stressed control fish (Fig. 4). Parasiticidal activity of plasma was disappeared after heat inactivation.

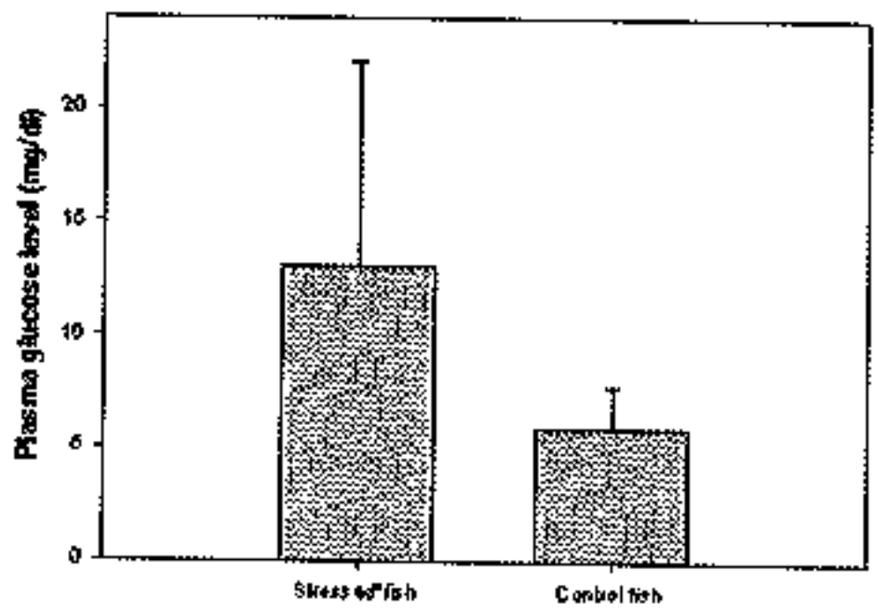

Fig. 1. Effects of handling stress on plasma glucose level of olive flounder, *Paralichthys olivaceus*. Each bar is mean ± standard error from 5 fish.

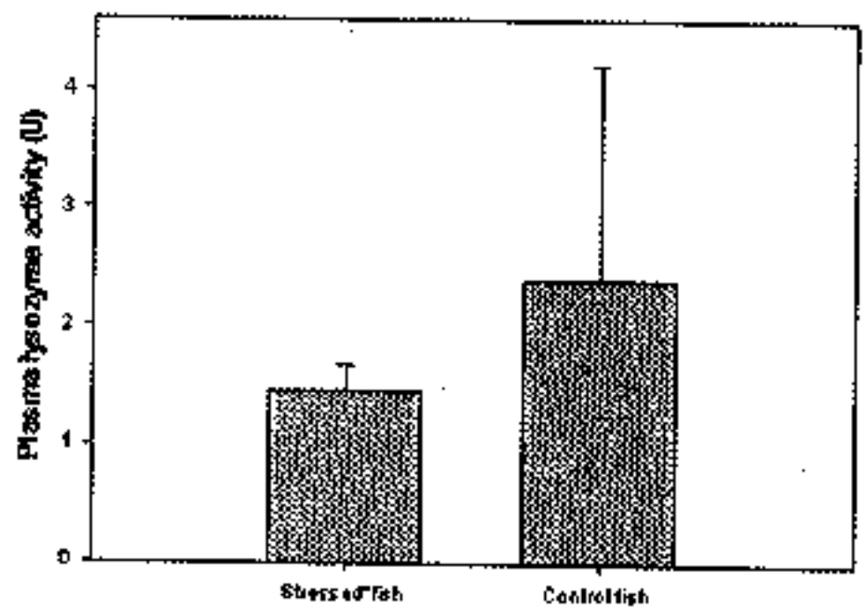

Fig. 2. Effects of handling stress on plasma lysozyme activity of olive flounder, *Paralichthys olivaceus*. Each bar is mean ± standard error from 5 fish.

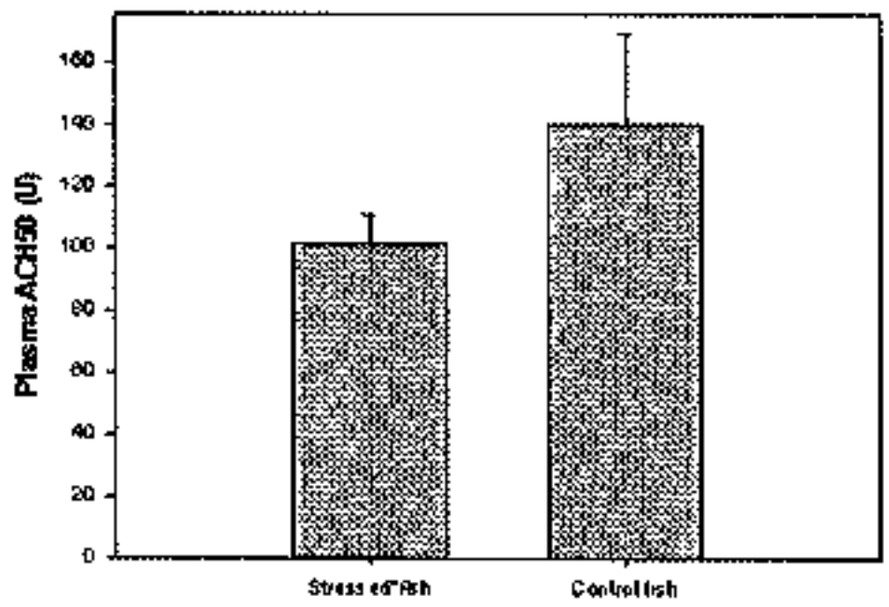

Fig. 3. Effects of handling stress on plasma activity of alternative complement pathway (ACH) of olive flounder, *Paralichthys olivaceus*. Each bar is mean ± standard error from 5 fish.

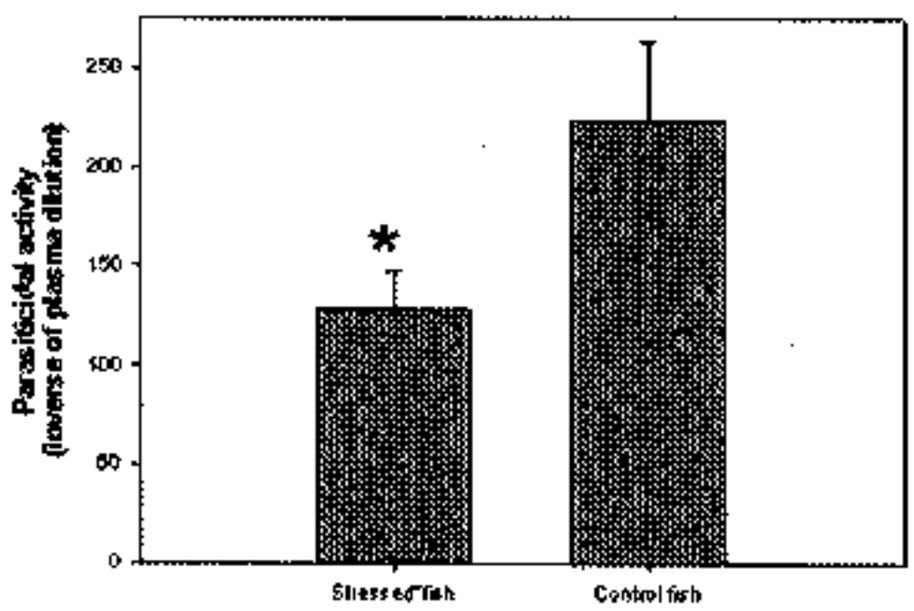

Fig. 4. Effects of handling stress on plasma parasiticidal activity of olive flounder, Paralichthys olivaceus. Each bar is mean ± standard error from 5 fish. The asterisk on the bar means statistical difference at $P<0.05$.

Discussion

The present *in vitro* study suggests that lysozyme activity and alternative pathway of complement activation are the innate resistant factors in olive flounder to scuticociliates. Stress-induced suppression of those humoral immunity was positively correlated with decrease of plasma parasiticidal activity.

Blood glucose level increase in response to most types of stressors, and is used commonly as an indicator of stress in fish (Barton and Iwama, 1991). In the present study, plasma glucose levels in olive flounder increased by handling stress. The alteration in glucose metabolism enabled the fish to cope with the maladaptive effects of the stress (Mazeaud and Mazeaud, 1981; Vijayan *et al.*, 1991). It is widely believed that stress suppresses immune function and increases susceptibility to disease (Kort, 1994; Cohen *et al.*, 1991).

Fish can counteract against parasite infections by a number of non-specific humoral immune responses. Complement is a part of the vertebrate immune system and is composed of a series of proteins in the plasma. Activation of the complement cascade is involved in lysis of some foreign cells, opsonisation, chemotaxis of macrophages and anaphylaxis (Leid, 1988). The lytic activity of complement occurs in a cascade fashion and terminates with holes punched into the cell membrane of the activating organism (Sakai, 1992). Of the humoral immune factors, lysozyme is the most abundant and widespread enzyme, and splits the exposed peptidoglycan wall of susceptible bacteria (Roitt, 1997). Stress has been shown to reduce activities of alternative complement pathway and lysozyme in fish (Mock and Peters, 1990; Tort *et al.*, 1996). In the present study, those two humoral activities were decreased by handling stress, also.

Sigh and Buchmann (2001) reported that theronts of *Ichthyophthrius multifiliis*, an obligate parasitic ciliate of fish, were immobilized and lysed by non-immune fish serum through activation of alternative complement pathway. Forward and Woo (1996), also, demonstrated that the alternative pathway of complement activation is the mechanism of innate immunity against *Cryptobia salmositica*, a flagellate parasite of fish. In the present results, parasiticidal activity of plasma in olive flounder was significantly decreased by stress. Moreover, heat-inactivated plasma lost completely the parasiticidal activity. Thus, the present results indicated that stress-induced immunodepression could be a cause of scu-

ticociliatosis occurrence in olive flounder.

Acknowledgements

This work was supported by Pukyong National University Research Fund in 1999.

References

Barton, B. A. and Iwama, G. K. : Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annu. Rev. Fish Dis., 1: 3-26, 1991.

Cheung, P. J., Nigrelli, R. F. and Ruggieri, G. D. : Studies on the morphology of *Uronema marinum* Dujardin (Ciliatea: Uronematidae) with a description of the histopatology of the infection in marine fishes. J. Fish Dis., 3: 295-303, 1980.

Cohen, S., Tyrrell, D. A. J. and Smith, A. P. : Psychological stress and susceptibility to the common cold. New Engl. J. Med., 325: 606-612, 1991.

Dragesco, A., Dragesco, J., Coste, F., Gasc, C., Romestand, B., Raymond, J. and Bouix, G. : *Philasterides dicentrarchi*, n. sp. (Ciliophora, Scuticociliatida), a histophagous opportunistic parasite of Dicentrachus labrax (Linnaeus. 1758), a reared marine fish. Eur. J. Protistol., 31: 327-340, 1995.

Dykova, I. and Figueras, A. : Histopathological changes in turbot *Scophthalmus maximus* due to a histophagous ciliate. Dis. Aquat. Org., 18: 5-9, 1994.

Ellis, A. E. : Lysozyme assays. In: Techniques in Fish Immunology (J. S. Stolen, T. C. Fletcher, D. P. Anderson & W. D. van Muiswinkel, eds) pp.101~104. Fair Haven, NJ: SOS Publications, 1990.

Forward, G. M. and Woo, P. T. K. : An in vitro study on the mechanism of innate immunity in *Cryptobia*-resistant brook charr (*Salvelinus fontinalis*) against *Cryptobia salmositica*. Parasitol. Res., 82: 238-241, 1996.

Gill, P. A. and Calinan, R. B. : Ulcerative dermatitis associated with *Uronema* sp. Infection of farmed sand whiting *Sillago ciliata*. Aust. Vet. J., 75: 357, 1997.

Iglesias, R., Parama, A., Alvarez, M.F., Leiro, J., Fernandez, J. and Sanmartin, M.L. : *Philasterides dicentrarchi* (Ciliophora, Scuticociliatida) as the causative agent of scuticociliatosis in farmed turbot *Scophthalmus maximus* in Galicia (NW Spain). Dis. Aquat. Org., 46: 47-55, 2001.

Jee, B. Y., Kim, Y. C. and Park, M. S. : Morphology and biology of parasite responsible for scuticociliatosis of cultured olive flounder *Paralichthys olivaceus*. Dis. Aquat. Org., 47: 49-55, 2001.

Klerx, J. P. A. M., Beukelman, C. J., Van, D. H. and Willers, J. M. N. : Microassay for colorimetric estimation of complement activity in guinea pig, human and mouse serum. J. Immunol. Methods, 63: 215-220, 1983.

Kort, W. J. : The effect of chronic stress on the immune system. Adv. Neuroimmunol., 4: 1-11, 1994.

Leid, R. W. : Parasites and complement. In: Advances in Parasitology, vol. 27, (Baker, J. R. and Muller, R. eds.), Academic press, London, pp. 131-168, 1988.

Mazeaud, M. M. and Mazeaud, F. : The role of catecholamines in the stress response of fish. p. 49-75. In: Stress and fish (Pickering, A. D. eds.), Academic Press, London, 1981.

Munday, B. L., ODonoghue, P. J., Watts, M., Rough, K. and Hawkesford, T. : Fatal encephalitis due to the scuticociliate *Uronema nigricans* in sea-caged, southern bluefin tuna *Thunnus maccoyii*. Dis. Aquat. Org., 30: 17-25, 1997.

Pankhurst, N. W. and Van der Kraak, G. : Effects of stress on reproduction and growth of fish. In: Fish Stress and Health in Aquaculture (Iwama, G. K., Pickering, A. D., Sumpter, J. P. and Schreck, C. B. eds.), Society for Experimental Biology, Cambridge, pp. 73-93, 1997.

Roitt, I. : Essential immunology (9th eds.): Innate immunity. Blackwell Science, pp. 3-20, 1997.

Sakai, D. K. : Repertoire of complement in immunological defense mechanisms of fish. Annu. Rev. Fish Dis., 2: 223-247, 1992.

Sigh, J. and Buchmann, K. : Comparison of immobilization assays and enzyme linked immunosorbent assays for detection of rainbow trout antibody titres against *Ichthyophthirius multifiliis* Fouquet, 1976. J. Fish Dis., 24: 49-51, 2001.

Sterud, E., Hansen, M. K. and Mo, T. A. : Systemic infecion with *Uronema*-like ciliates in farmed turbot, *Scophtalmus maximus* (L.). J. Fish Dis., 23: 33-37, 2000.

Thompson, C. L. Jr. and Moewus, L. : *Miamiensis avidus* n.g. n.s., a marine facultative parasite in the ciliate order Hymenostomatida. J. Protozool., 11: 378-381, 1964.

Vijayan, M. M., Ballantyne, J. S. and Leatherland, J. F. : Cortisol-induced changes in some aspects of the intermediary metabolism of *Salvelinus fontinalis*. Gen. Comp. Endocrinol., 82, 476-486, 1991.

Yoshinaga, T. and Nakazoe, J. : Isolation and *in vitro* cultivation of an unidentified ciliate causing scuticociliatosis in Japanese flounder (*Paralichthys olivaceus*). Gyobyo Kenkyu, 28: 131-134, 1993.