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Effect of Korean Mistletoe (*Viscum album* Coloratum) on the Non-Specific Immune Responses in Japanese Eel (*Anguilla japonica*)

Young-Hye Cho, Dae-Han Choi and Sang-Hoon Choi†

Department of Marine Biomedical Science, Kunsan National University, Kunsan, Chunbuk 573-400, Korea

In the present paper, the immunostimulatory effects of Korean mistletoe (*Viscum album* Coloratum) on the non-specific immune responses of Japanese eel (*Anguilla japonica*) were examined. Eel were innoculated with mistletoe, Freund's complete adjuvant (FCA), or phosphate-buffered saline (PBS) as a control into their peritoneal cavities. The number of nitrobule tetrazolium (NBT)-positive cells in the head kidney of fish was significantly increased by the second day post-injection of mistletoe. ROI products were more enhanced in mistletoe-injected fish kidney leucocytes than in FCA-injected ones. The level of lysozyme activity detected in the serum of fish 2 days after injection with mistletoe was also significantly higher than that found in the serum of the control fish. The appropriate concentration of mistletoe to induce the highest level of serum lysozyme activity was revealed to 1000 ㎍/200 g of fish. In phagocytic activity assay, mistletoe-sensitized eel kidney phagocytes captured more zymosan than did the control fish. Korean mistletoe appeared to be a good activator of the non-specific immune responses of Japanese eel.

Key Words: Mistletoe, Eel, Lysozyme, Non-specific immunity, Kidney leucocytes

Introduction

Teleost fish possess both humoral and cell-mediated immunity (Peddie *et al.*, 2002). It has been known to possess a variety of specific and non-specific defense mechanisms against invading organisms (Ellis, 1999; Sarder *et al.*, 2003). When a pathogen penetrates the physical barriers of the animal, the first lines of defense it encounters are those of the non-specific immune system. A variety of leucocyte types are involved in non-specific cellular defenses of fish, and include monocytes/macrophages, granulocytes, and non-specific cytotoxic cells (NCCs) (Iwama and Nakanishi, 1996).

Fish macrophages are the main effector cells of the natural immune response (Jewhurst *et al.* 2004) and play a crucial role as accessory cells in both the initiation and regulation of fish immunity (Shen *et al.*, 2002). Phylogenetically, fish are the oldest animals showing adaptive (specific) immune responses characterized by the presence of lymphocytes able to undergo proliferation in response to specific antigens (Ono *et al*, 1993). Furthermore, fish possess innate (non-specific) immune responses based upon soluble and cellular factors of acute inflammation (Ono *et al*, 1993). As in mammals, adaptive and innate immunity in fish appear to be coordinated to a large degree by the cytokine network (Jorgensen *et al.*, 2001). In fish, protective immune responses can be primed by vaccination so that the requisite defense parameters are already in place to

†Corresponding Author : Sang-Hoon Choi, Tel : 063-469-1886, Fax : 063-463-9493, E-mail : shchoi@kunsan.ac.kr

resist infection. Fish have the aquatic environment with a large variety of diseases. While commercial vaccines are available which successfully induce protection against bacterial diseases, little is yet understood about the nature of the protective mechanisms involved. In addition, there are several other economically important diseases of fish for which no effective vaccines exist. Immunostimulants enhance the non-specific defense mechanisms, thereby preventing infectious disease. Usually, phagocytes of fish treated with immunostimulants show enhanced activity (Sakai, 2001).

Mistletoe is a semi-parasitic woody perennial commonly found growing on oak other deciduous trees. It has been shown that extracts of European mistletoe (*Viscum album* Loranthaceae, *V. album* L.) possess a variety of biological activities such as induction of various cytokines (Mueller and Anderer, 1990; Mannel *et al.*, 1991), enhancement of natural killer (NK) cell activity (Kuttan *et al.*, 1992) and immunoadjuvant activities (Mertzer *et al.*, 1985; Hajto, 1986). Moreover, many investigators have demonstrated that the extract of *V. album* L. augmented anti-tumor effect by enhancing the cytotoxic activity of NK cells, lymphokine-activated killer (LAK) cells and macrophages. However, even if the characteristics and biological properties of European mistletoe have been studied intensively, there is only little knowledge about the biological and physiological functions of Korean mistletoe (Viscum album Coloratum, *V. album* C.), a different subspecies of *V. album* from European mistletoe. Recently, some investigators have reported that Korean mistletoe is much more effective in inducing anti-tumor and immunoadjuvant activities (Lyu *et al.*, 2000; Yoon *et al.*, 2001). Both *in vivo* and *in vitro* research have so far mainly been focused on the effect of the mistletoe on non-specific immune responses.

In this study, we investigated the effect of Korean mistletoe on a non-specific immune responses in eel. Chosen parameters, e.g., reactive oxygen intermediates (ROI), lysozyme activity and phagocytosis, were significantly augmented. The extract of Korean mistletoe would be utilized in elevating fish immune responses.

Materials and Methods

Fish

Eel (*Anguilla japonica*) weighing 200g were obtained from a commercial fish farm. The fish were kept in 70L glass aquaria with recirculated and aerated water at 18-20 ℃, and fed daily with commercial diet during the adaptation and experimental period. They were acclimated to this environment for at least 2 wks prior to use. The health status of the animals was checked daily, and they never presented clinical symptoms and none died.

Reagents

Nitroblue tetrazolium (NBT), Percoll, and Minimum essential medium (MEM) were purchased from Sigma Chemicals CO. Hanks balanced salt solution (HBSS), Fetal Bovine Calf Serum (FBS), Antibiotic-antimycotic were obtained from Gibco BRL, Grand Island, NY. Sodium Nitrite, Sulfanylamide, Phosphoric acid were purchased from ICN Biomedicals.

Extraction of mistletoe

Mistletoe growing at oak in January were harvested from Kangwondo, Korea. Used mistletoe were 1 or 2 years old, and their leaves, trunks, and fruits were cut to two joint from end of a branch followed by washing with distilled water (D.W.) and drying. The vaccum wrapped mistletoe were stored at -80 ℃ until extract. The lyophilized leaves and

trunks of mistletoe were hashed up and washed in D.W. through ion exchange resin. After washing, they were pulverized at mixer for 2 min and stirred for 16 h at 4 ℃. The mistletoe were centrifuged at 10,000 rpm for 30 min at 4 ℃ and the suspension was passed through and filtered with different pore sizes, 7.2, 0.45, and 0.22 ㎛, successively. The mistletoe extract named KM-110 was lyophilized and resuspended with D.W. in an appropriate dilution factor.

Inoculation of mistletoe

The eel were divided into 4 or 5 groups of 7 eel per group. Fish in each group were intraperitoneally (I.P) injected with 200, 500, 1000, 1500 ㎍ of mistletoe in 0.5 ml of phosphate buffered saline (PBS), respectively. The remaining group of fish was injected with an equivalent volume of sterile PBS or 1:1 emulsified Freund's complete adjuvant (FCA) as a control. At day 2 post-injection, blood and head kidney leucocytes were obtained from each fish.

Serum

Blood was collected by cutting off eel head. It was allowed to clot at 4 ℃ overnight. Serum was obtained by centrifugation at 2500 rpm for 8 min. The sera were frozen at -20 ℃ until used.

Isolation of head kidney leucocytes

The eel head kidney previously sensitized with mistletoe was dissected out by a ventral incision, cut into small fragments and transferred to 5 ml HBSS, respectively. Cell suspensions from head kidney were obtained by teasing the head kidney tissues with two slide glasses in HBSS in a Petri dish (Coring). After sedimentation of tissue debris at 4 ℃ for 1 min, the supernatants were removed. Head-kidney cell suspensions were layered over a 34-51% Percoll gradient and centrifuged at 2500 rpm for 40 min at 14 ℃. After centrifugation, the bands of leucocytes between the 34-51% interfaces were collected with a Pasteur pipette and washed twice at 1200 rpm for 8 min in HBSS. The viable cells count was determined by trypan blue exclusion.

Respiratory burst

ROI production from eel kidney cells after administration with the mistletoe was assessed by monitoring their ability to reduce nitroblue tetrazolium (Secombes, *et al.*, 1988) The leucocytes were washed one time with HBSS at 1000 rpm for 3 min at 4 ℃ and incubated in 100 ㎕ of complete media in the presence of phorbol myristate acetate (PMA, 1 ㎍/ml) and 100 ㎕/well NBT (mg/ml). After 1 hr at 25 ℃, excess amount of NBT was washed out with PBS and the leucocytes were fixed with 70% methanol. After discarding 70% methanol, the leucocytes were washed twice with PBS. The reduced formazan was solubilized with 120 ㎕ KOH and 140 ㎕ dimethyl sulphoxide (DMSO) and optical density values were read at 620 nm in an ELISA reader (ASYS HITECH, Austria).

Lysozyme Activity

Serum lysozyme activity was measured using a modified turbidimetric microtitre plate technique by Ellis (1999). Briefly, a standard suspension of 0.15 mg/ml *Micrococcus lysodeikticus* (Sigma) was prepared in 66 mM phosphate buffer (pH 6.0). Eel serum (50 ㎕) was added to 1 ml of bacterial suspension, and the decrease in absorbance was recorded at 0.5 and 4.5 min intervals at 450 nm in a spectrophotometer (SHIMADZU UV-1600PC). One unit of lysozyme activity was defined as reduction in absorbance of 0.001/min.

Phagocytic Activity

Eel head kidney leucocytes injected with mistletoe or PBS were adjusted to 1 x10^6 cells/200 $\mu\ell$ /well in 5% FBS-MEM and dispensed in 8-well slide chamber (Nunc) followed by overnight incubation at 25 ℃. Following incubation, 20 $\mu\ell$ of zymosan (mg/ml) were added to each well followed by additional incubation for 2 hr at 25 ℃. Phagocytic activity was measured by Wright's stain.

Statistical analysis

The statistical significance of differences between groups was calculated by applying Student's two-tailed t-test.

Results

In vivo effect of Korean mistletoe on the ROI production from eel kidney leucocytes

The results of the ROI production are shown in Fig.1. The level of ROI product was highly augmented in kidney cells from eel injected with 200, 500 or 1000 μg/fish of mistletoe compared with the control (Fig. 1). ROI product was significantly ($p<0.05$) enhanced in kidney cells from fish injected with 500 and 1000 μg of mistletoe and 1:1 emulsified FCA (Fig. 1).

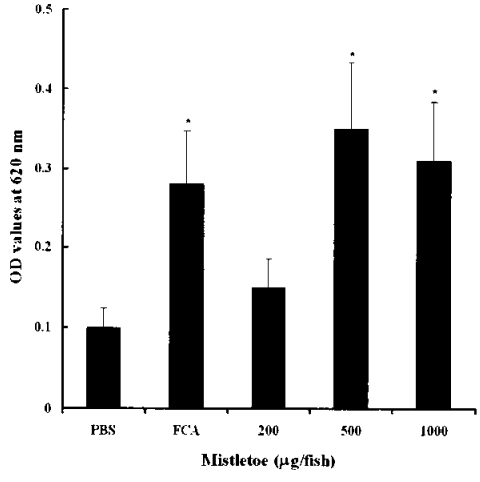

Fig. 1. NBT reduction by head kidney leucocytes from Japanese eel after injection with mistletoe. Mistletoe (200, 500 or 1000 μg/0.5 ml), 0.5 ml of PBS or 0.5 ml of 1:1 emulsified FCA were injected to 7 fish per group, respectively. Bars represent mean ± SD (n=7). Asterisks denote statistically significant differences ($P<0.05$) between control and FCA or mistletoe- injected fish.

Lysozyme activity

To study whether mistletoe has an impact on increasing lysozyme activity, sera were harvested from eel treated with 500 or 1000 μg/fish of mistletoe. Fig. 2 shows lysozyme activity in serum of eel sensitized with mistletoe. In both mistletoe treated groups, high level of lysozyme activity was observed compared to the control fish.

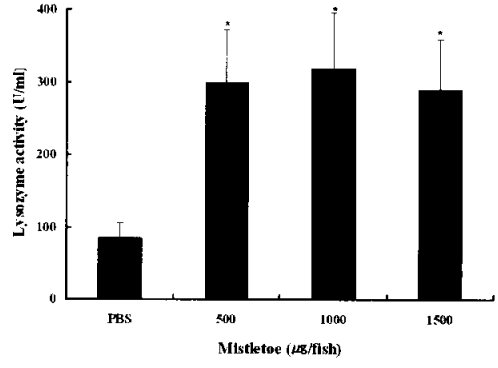

Fig. 2. Serum lysozyme activity of Japanese eel injected with mistletoe. Results are expressed as the units/ml of sera from 7 fish per group. Bars represent mean ± SD (n=7). Asterisks denote statistically significant differences ($P<0.05$) between control and mistletoe-injected fish. One unit of lysozyme activity was defined as reduction in absorbance of 0.001/min.

Phagocytic activity

To test whether mistletoe can have an influence on inducing phagocytic activity, eel kidney leucocytes were incubated for overnight and then challenged with zymosans (500 and 1000 μg/fish). As shown in Fig. 3, phagocytes from mistletoe-injected eel were observed to engulf more zymosans than

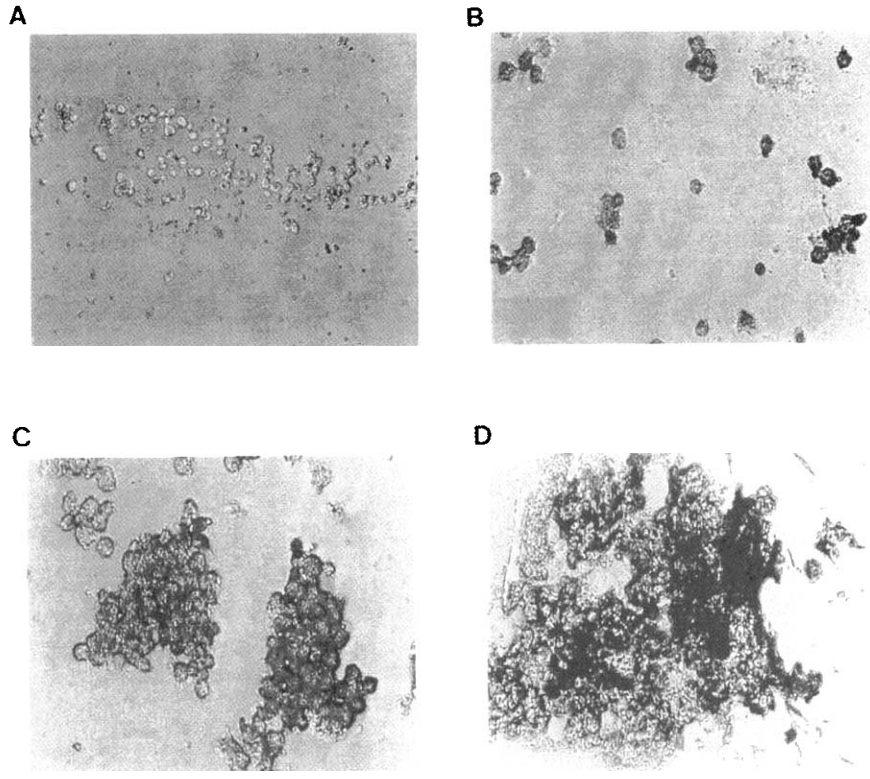

Fig. 3. Phagocytosis of mistletoe-injected eel kidney leucocytes against zymosan. A, zymosan; B, PBS- ; C, 500 μg of mistletoe- ; and D, 1000 μg of mistletoe-injected eel kidney leucocytes against zymosan (Phase contrast microscopy x 40). Cells were stained by Wright's stain

those from the control fish. Furthermore, the activated phagocytes are likely to aggregate together in a dose dependent manner. The exact amount of zymosans ingested in phagocytes was uncountable due to the aggregation of cells. However, the more aggregated cells, the more ingested zymosans.

Discussion

The present results indicate that non-specific immune response of eel kidney leucocytes was affected by the injection of mistletoe. Mistletoe (*V. album*) is native to both Europe and Asia. *V. album* grows in Europe, northwest Africa, and southwest and central Asia and Japan: the Asian plant is a special variety. It is propagated by birds that eat the berriers and then excrete by wiping the sticky pulp off their beaks. Mistletoe contain lectins, protein toxins, and polysaccharides. Also, mistletoe's lectins are cytotoxic glycoproteins of approximately 10,000 molecular weight and activates macrophages and lymphocytes, leading to secretion of various kinds of cytokines. Lectins are believed to mediate pathogen recognition, which can lead to neutralization of the invading organism (Vasta *et al.*, 1994; Weis *et al.*, 1998) during the early stages of an infection (Holmskov *et al.*, 1994; Ni and Tizard, 1996; Lu, 1997). Mistletoe, therefore, has been widely used for therapeutic purpose in Europe and in Korea as well. Furthermore, mistletoe preparations had increase in natural killer cells, T-helper cells, cytokine release, and peripheral blood

mononuclear cells and lymphocytes. In mammalians, Korean mistletoe has shown 10 to 1000 times higher anti-tumor effect than Europe mistletoe. Administration with mistletoe showed an increased non-specific immune response. ROI production from eel kidney leucocytes was readily elicited by the mistletoe administration, suggesting that ROI might be good activation indicator in eel kidney leucocytes. In fact, ROIs such as the superoxide (O_2), hydrogen peroxide (H_2O_2), hydroxyl radical (OH), and singlet oxygen, play an important role in the antimicrobial activity of phagocytic cells. In *in vivo* experiments, the level of ROI production was highly augmented in kidney leucocytes from eel injected with 500 or 1000 ㎍/fish of mistletoe. At higher concentration of mistletoe, ROI was much more produced. However, mistletoe with more than concentration of 1000 ㎍/fish (200 g) failed to up-regulate the further induction of ROI production. Usually, immunostimulants do not show a linear reaction between doses and effect but a maximum effect at intermediate doses and no effect and even toxicity at high doses (Bliznakov and Adler, 1972; Gialdroni-Grassi and Grassi, 1985). The fact has been established in fish through *in vivo* (Kenyon *et al.*, 1985; Anderson and Jeney, 1992) and *in vitro* studies (Siwicki *et al.*, 1990). In *in vitro* experiment, 10 ㎍/㎖ of mistletoe was revealed to an optimal concentration to induce ROI production without any cellular cytotoxicity. In rechallenging test, mistletoe failed to reactivate eel leucocytes with an elevated ROI production (data not shown), indicating that non-specific fish immune response is similar to a mammalian system regarding to a cellular memory process.

In the assay of lysozyme activity in serum from the mistletoe-injected fish, it was found that mistletoe plays a critical role in evoking lysozyme activity from eel kidney phagocytes. In the injection of 1500 ㎍/fish of mistletoe, lysozyme activiy was down-regulated relatively to a low concentration of mistletoe.

To support the possibility of the phagocytic activity enhanced by mistletoe, zymosans were treated to either phagocytes from mistletoe-injected eel or those from mistletoe non-treated eel. Expectedly, phagocytes from mistletoe-treated eel were aggregated together and bunch of zymosans were engulfed by the phagocytes, indicating that mistletoe play an excellent role in activating the non-specific immune response in eel.

Taken together, Korean mistletoe could be a promising immunoadjuvant inducing the non-specific immune response in eel, considering that most of all indicators for non-specific immune mechanism were revealed with positive results. Based on our preliminary results, the specific immune response by mistletoe needs to be further studied and further fish immunoadjuvant for a diet should be developed in future.

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