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Short communication

Combination treatment against scuticociliatosis by reducing the inhibitor effect of mucus in olive flounder, *Paralichthys olivaceus*

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ABSTRACT

The olive flounder, *Paralichthys olivaceus*, is an economically important food fish in Japan and Korea. Scuticociliatosis is a major parasitic disease, and fatal infection with scuticociliates, or mixed infections with scuticociliates and other pathogenic agents (e.g., *Vibrio* spp.) cause severe mortalities in farmed olive flounders. To date, however, effective chemotherapeutic treatment of scuticociliatosis has only been reported at the *in vitro* level. In this study, we employed combination treatment, using benzalkonium chloride (to remove excess mucus from the body surface) and bronopol (to kill the parasites), to overcome the protective effect of mucus by some medicine to the scuticociliates. In the presence of the mucus mixture, the higher dose of bronopol (156 ppm) yielded morphologies and motilities similar to those of ciliates treated with the lower dose of bronopol (80 ppm) in the absence of mucus. We also investigated the *in vivo* effects of this treatment in field trials involving a total of 15,025 naturally infected flounders. We observed that short-term bath treatments with benzalkonium chloride (50 ppm) followed by bronopol (500 ppm) were effective, assessed by the relative percentage mortality (RPS) value. Thus, this study provides a notable therapeutic strategy by removing the mucus to treat scuticociliatosis in olive flounders at the aquaculture field level.

1. Introduction

The olive flounder, *Paralichthys olivaceus*, is an economically important mariculture flat fish species in Japan and Korea. The ciliates *Philasterides dicentrarachi*, *Uronema marinum*, *Pseudocohnilembus persalinus*, and *Miamiensis avidus*, which cause scuticociliatosis, have been recognized as the major parasites in the marine olive flounder aquaculture industry [1]. Their primary infections occur mainly via the gills and skin, where the ciliates feed on cells and tissue; from there, they spread into the internal organs via the blood stream [2,3]. Affected fish typically secrete excessive body mucus and display extensive dermal damage, including distinguishable gross lesions such as bleached areas and necrotic tissues [1–3].

The mucosal surfaces of fish play a critical role in their defenses by acting as a biological barrier [4]. However, traumatic skin damage or erosion of the mucosal surfaces may impair this first-line defense, triggering an abnormal mucosal shedding that often facilitates proliferation of bacteria on the body surface followed eventually by an infective process [5]. Case in point, a recent study revealed the presence of mixed infection by scuticociliates and other bacteria (e.g., *Vibrio* spp.) in infected olive flounders [6]. Although how this co-infection came about is not yet fully understood, this may suggest that abnormal mucus secretion in flounders due to persistent infection by this parasite may facilitate secondary infections (e.g. bacteria). Moreover, an overabundance of mucin, the most abundant component of the mucus layer [7], limits the interaction of the target cell surface with immune substrates and/or chemotherapeutic agents [8]. Hence, it can be deduced that treatment methods for scuticociliatosis may be hampered by excessive mucus in the body surface.

The objective of this study therefore, was to evaluate the effect of combination treatment using benzalkonium chloride (N-alkyl-N-benzyl-N,N-dimethylammonium chloride) and bronopol (bromo-2-nitropropane-1, 3-diol) against scuticociliatosis in field trials using naturally infected flounder; the former to remove the mucus and the latter to treat scuticociliatosis. The results from this study suggest that this is a promising and practical treatment strategy against scuticociliatosis.

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2. Materials and methods

2.1. Isolation and subculture of ciliates

Ciliates were isolated from the gills and ulcerated skin of infected *P. olivaceus* (mean weight 17.5 ± 2.5 g; mean length 12.5 ± 2.5 cm; $n = 50$) that had been cultured in a land-based flounder farm located in Namhae, South Korea. Ciliates were subcultured and harvested as described previously [6].

2.2. In vitro effect of mucus on the efficacy of bronopol against ciliates

Skin mucus samples of olive flounder without any gross lesions on the body surface (mean weight 12.5 ± 2.5 g; mean length 10 ± 2 cm) were collected by gentle scraping using a soft rubber spatula. The mucus was homogenized with 1 volume of 0.22-μm-filtered/autoclaved sea water and mixed with ciliates to a final cell density of 1×10^4/ml. Thereafter, this mixture (500 μl) was seeded to a sterile 24-well plate (SPL Life Science, Pochen, Korea). Control groups were prepared at the same cell density without the mucus mixture. The effects of bronopol (BP, 50%, w/v; KBNP Inc., Anyang, South Korea) on samples with and without mucus were observed under an inverted fluorescence microscope (Eclipse Ti-s; Nikon Instruments Inc., Tokyo, Japan) at 0, 5, 10, 20, and 30 min post-inoculation. Due to their high motility, the ciliates were fixed with a 2% paraformaldehyde solution prepared with 0.22-μm-filtered/autoclaved sea water (to minimize the osmosis-driven morphological changes) [9].

2.3. The in vivo effects of benzalkonium chloride and bronopol

The toxicities of benzalkonium chloride (CleanpiaTM, 2.5% w/v; KBNP Inc., Anyang, South Korea) and bronopol against olive flounders (mean weight 12.5 ± 2.5 g; mean length 10 ± 2 cm) were evaluated at 30, 50, and 100 ppm ($n = 10$ flounders for each concentration) and 300, 600, and 1000 ppm ($n = 9$ for each concentration), respectively. Mortality was monitored for 72 h, and the median lethal concentration time (LCt_{50}) was determined for each compound [10]. To examine the ability of benzalkonium chloride to remove the mucus layers of fish, 40 olive flounders without any gross lesions on their body surfaces (mean weight 12.5 ± 2.5 g; mean length 10.0 ± 2.0 cm) were separated into four groups, placed in 100-L fiberglass plastic aquaria containing 30 L of 0.45 μm-filtered sea water per tank, and exposed to benzalkonium chloride (20, 30, 50, or 100 ppm) at 20 ± 2 °C. After 2 h, mucus samples were collected from the upper phase (2 L) of sea water in each tank and homogenized with 1 volume of Tris-buffered saline (TBS, 50 mM Tris–HCl, pH8.0, and 150 mM NaCl). The secretion of mucus before and after treatment in each group was determined by measuring the protein concentration in clarified mucus extract (CME) as described previously [11].

As a lab-scale experiment, a total of 54 flounders (mean weight 12.5 ± 2.5 g; mean length 10.0 ± 2.0 cm) that had been naturally infected with scuticociliates and showed dermal lesions of various sizes were randomly placed in six 100-L aquaria and exposed to co-treatments using benzalkonium chloride (50 ppm) for an hour followed by bronopol (300, 500, or 600 ppm for an hour; $n = 9$ flounders for each concentration), or bronopol alone (300, 500, or 600 ppm for an hour; $n = 9$ for each concentration). As controls, nine fish were placed in an aquarium without any chemotherapeutic agent. Prior to the experiments, the water flow was stopped and aeration was provided in each tank. Thereafter, water flow was continuously supplied using a re-circulating system. The experiments were carried out in 0.45-μm-filtered sea water held at 20 ± 2 °C. The chemical compounds were reapplied daily for 8 days. Mortality was recorded.

2.4. Field trials

The efficacy of sequential chemotherapy using benzalkonium chloride followed by bronopol was assessed in field trials at a land-based flounder farm with a flow-through system (Jeju Island, South Korea). The water turnover rates in the 13 × 32 × 2.5-m rectangular tanks generally ranged from 12 to 20 times per day. The average weight of the fish was about 200 g and the stocking density was 20–40 kg/m^2 of bottom area. Seven days before the experiment, a total of 15,025 flounders showing typical dermal damage due to scuticociliatosis were selected. Of them, 9162 fish were randomly placed in three tanks (treatment groups) and the remaining 5863 fish were distributed to two tanks (untreated controls). From the treatment groups, about 1000 infected flounders were randomly selected and scuticociliate infection was confirmed by microscopic observation. Flounders from the treatment groups were exposed to a short-term (1-h) bath in 50 ppm benzalkonium chloride followed by a short-term (1-h) bath in500 ppm bronopol, and then transferred to tanks. During the 6-day exposure period (August 7 to 12, 2012), this sequential treatment was performed once a day. During the 17-day experimental period (August 7 to 23, 2012), fish were not fed, and mortality was monitored six times per day. The average temperature was 26.7 °C.

2.5. Statistical analysis

Kaplan–Meier analysis was used to generate survival curves, and the log-rank test was used to analyze significant ($P < 0.05$) differences between control and treated olive flounders by using SPSS software (version 17.0). Relative percent survival (RPS) was employed to assess significant differences in mortalities and calculated as: RPS = 100% × (1 − % mortality of treated fish/% mortality of control fish).

3. Results and discussion

In this study, we did not induce experimental infections by immersing fish with identified species of scuticociliates [6,12] because our final goal was to investigate the effect of our therapeutic strategy by removing the mucus in naturally infected olive flounders, where scuticociliatosis may be caused by mono- or co-infection(s) of *P. dicentrarachi*, *U. marinum*, *P. persalinus*, and *M. avidus* [1].

As mucus may impede the action of chemotherapeutic agents, we first evaluated the *in vitro* effect of bronopol on ciliates in the presence or absence of mucus. Bronopol is considered to be a suitable alternative to the carcinogen, formaldehyde, in food production, water disinfection, healthcare products, and the aquaculture industry [13]. The responses of ciliates to bronopol were characterized based on changes in their morphology and motility, which were scored as previously described [6]. Interestingly, we found that in the presence of the mucus mixture, the higher dose of bronopol (156 ppm) yielded morphologies and motilities similar to those of ciliates treated with the lower dose of bronopol (80 ppm) in the absence of mucus (Fig. 1(a)). In the latter case, the motility/morphology scores at 5, 10, 20, and 30 min after exposure were 3/4 (decreased motility/no morphological change), 2/2 (about 50% of ciliates stationary but still beating/about 50% of ciliates with round or irregular shapes), 0/1 (no motility/more than 50% of ciliates with round or irregular shapes), and 0/0 (no motility/extensive lysis), respectively (Fig. 1(b)). This observation suggests that bronopol is efficacious against scuticociliates, but its action may be impeded by