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In vitro efficacy of formalin, hydrogen peroxide and copper sulfate on the scuticocilliate *Uronema marinum* at low salinity

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The scuticocilliate, *Uronema marinum* is a histophagous ciliate and the causative agent of 'scuticociliatosis' in cultured olive flounder *Paralichthys olivaceus*. In the present study, *in vitro* efficacy of hydrogen peroxide, formalin and copper sulfate on the scuticocilliate at low salinity was investigated. Each chemical showed synergistic parasiticidal effects with low salinity (salinity in 5 ppt) compared to each chemical alone (salinity in 33 ppt). At low salinity (5ppt), ciliates were killed completely within 1.5 h by exposure to 50 ppm formalin (37% formaldehyde), at 100 ppm hydrogen peroxide (30% solution) and at 100 ppm copper sulfate (20% solution). The formalin was the most effective chemical against the parasites at low salinity.

Key words : *Uronema marinum*, Olive flounder, Chemical, Synergistic effects, Low salinity

Introduction

Scuticociliate has been recognized as a serious pathogen in worldwide mariculture. Mass mortalities by the ciliates have been reported in juvenile flounder, *Paralichthys olivaceus*, juvenile turbot, *Scopthalmus maximus*, adult sea bass, *Dicentrarchus labrax*, and subadult southern bluefin tuna, *Thunnus maccoyii* (Yoshinaga and Nakazoe, 1993; Dykova and Figueras, 1994; Dragesco *et al.*, 1995; Munday *et al.*, 1997; Jee *et al.*, 2001). In Korea, mass mortalities of fry and high cumulative moralities of juveniles by scuticociliate infection frequently occur in many flounder farms. Recently, the causative agent of 'scuticociliatosis' in olive flounder was identified *Uronema marinum* by Jee *et al.* (2001).

In vitro efficacy of some chemicals against scuticociliates has been demonstrated (Novotny *et al.*, 1996; Crosbie and Munday, 1999). Currently, formalin is used as a therapeutant for scuticociliatosis in cultured flounder. However, the chemical had a little treatment effects against the scuticociliate infecting flounders. Thus, other effective therapeutants are neededto control the scuticociliate in flounder. As the ciliate is vulnerable below 5 ppt (Bassler, 1983), chemicals used for treatment of scuticocilliate infection would be more effective at low salinity. The objective of this study was to evaluate therapeutic potential of chemicals at low salinity against the scuticociliate infection in flounder.

Materials and Methods

Isolation and cloning of ciliates

A scuticocilate was isolated from the brain of moribund flounder. Fish were killed by a sharp blow to the head. The brain was excised,washed serially in sterile artificial seawater (Sigma) with capiliary pipettes and placed into 10 mL of Dulbecco's Modified Eagles Medium (DMEM)containing 0.1% antibiotic (penicillin-streptomycin, Sigma) in

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25 cm^2 flasks (Corning). After 12 h of incubation at 17℃, ciliates containing medium were transferred in 24-well polystyrene plates (Corning) and cloned by serial dilution method.

Fish cell lines and ciliates culture

The cell line, CHSE-214 (Chinook salmon embryo, ATCC CRL 1691), was obtained from the virus laboratory within the Fish Pathology Division, National Fisheries Research & Development Institute. CHSE-214 cells were cultured in 10 mL DMEM containing FBS (10%, v/v) and 50 μm/mL streptomycin and 50 I.U./mL penicillin G at 20℃, and subcultured when to be monolayer, approximately 10^5-10^6 cell densities in the 25cm^2 flasks.

The cloned ciliate was inoculated into the above CHSE-214 cells in 25cm^2 flasks and cultured for 7 days at 17℃. The CHSE-214 cells subcultured every 1 or 2 weeks were used to evaluate the efficacy as chemicals. Absence of contaminated bacteria in the subculture was confirmed on marine broth 2216 (Difco).

Determination of the efficacy of chemicals at low salinity (CS)

The chemicals tested for efficacy in killing the ciliates were37% formaldehyde (0, 25, 50, 100 ppm), 30% hydrogen peroxide solution (0, 50, 100, 200 ppm), 20% copper sulfate solution (0, 100, 200, 300 ppm). The chemicals were prepared with diluted 2 μm filtered freshwater at 5 ppt.

0.1 mL of culture suspension aliquot was transferred into each well of cell culture cluster plate (24 wells plate, Corning) and then 0.9 mL of suitably diluted chemicals or CS was added to give the desired final concentration. Ciliates density was initially 6.2 × 10^5/mL for each well. Incubation of ciliates + chemicals at low salinity was performed at 17 ℃. Each trial was performed in triplicate wells for each chemical at low salinity and appropriate controls.

Table 1. Scoring system for assessing chemicals for *in vitro* efficacy against scuticociliates recovered from cultured olive flounder *Paralichthyus olivaceus* (Modified from Novotny *et al.*, 1996)

Score	Interpretation
3	No effect; highly motile and cells elliptical
2	Low effect; slowly motile and cells round
1	Moderate effect; cells stationary and irregular
0	High effect; cells lysis

Ciliates were assessed for motility and morphology after addition of the tested chemicals at low salinity using an inverted microscope (Nikon Invertoscope ID 02) and assigning acores, interpretations of which are outlined in Table 1. At appropriate time intervals (approximately every 24 h over 4 d) all ciliate were sampled to enumerate. For counting, 5 drops of 5 μL of each sample was placed on a slide glass, fixed with 5 drops of 5 μL of 5% formalin, and cell number was directly counted under a light-microscope. Five countings were made for each sample and averaged.

Results

The efficacy of chemicals alone

The effects of chemicals alone on ciliate motility and morphology are shown in Fig. 1 and 2. Ciliates were completely destroyed at 200 ppm of hydrogen peroxide, at 100 ppm of formalin, at 3 ppt of freshwater and at 200 ppm of copper sulfate. All ciliates treated with the chemicals were killed within 4 h (Fig. 1). Although ciliates showed irregular cell shape and lower motility during 4 h at 100 ppm of hydrogen peroxide, at 50 ppm of formalin, at 6 ppt of freshwater and at 100 ppm of copper sulfate, the ciliates were not completely destroyed (Fig. 2).

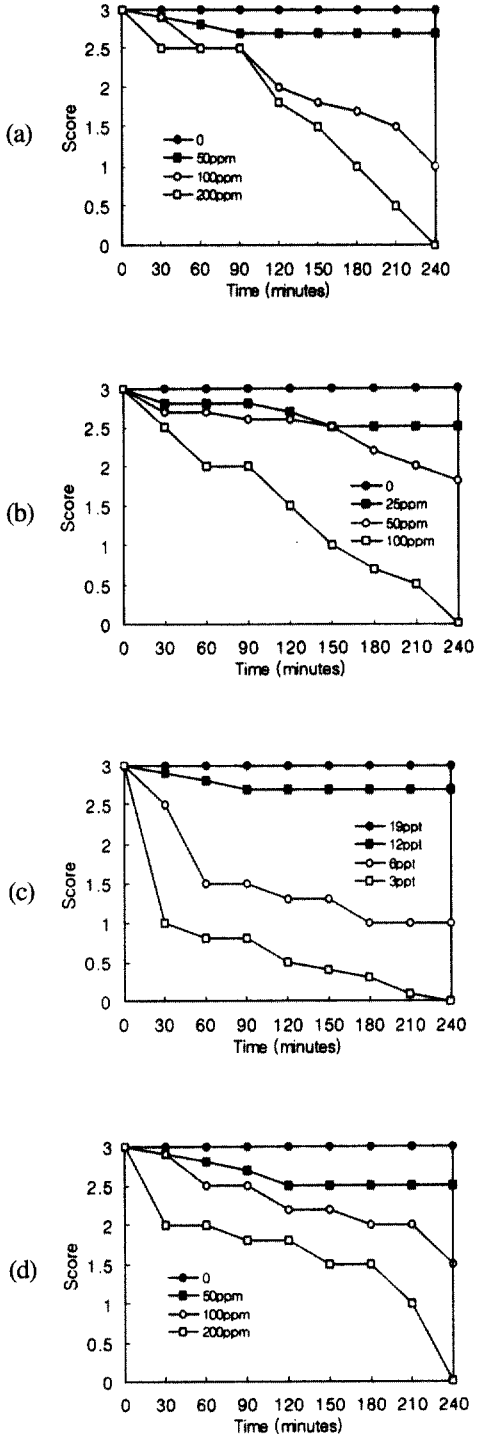

Fig. 1. Effects hydrogen peroxide (a), formalin (b), freshwater (c) and copper sulfate (d) on morphology and motility of scuticociliate, *Uronema marinum*. Each point represents the mean of triplicate experiments.

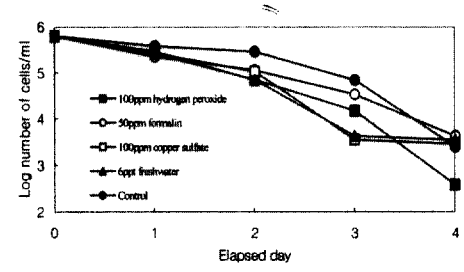

Fig. 2. Effect of hydrogen peroxide, formalin, and copper sulfate on survival of scuticociliate, *Uronema marinum*. Each point represents the mean of triplicate experiments.

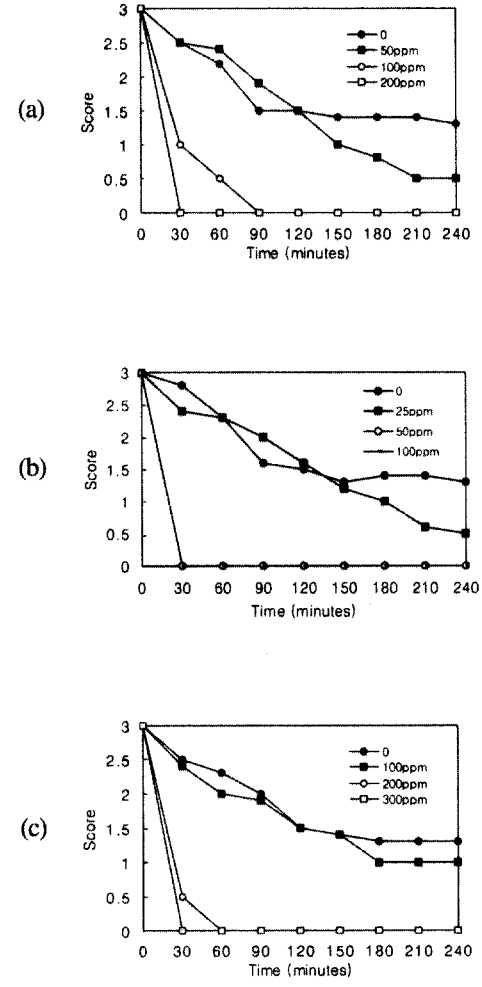

Fig. 3. Effects of hydrogen peroxide (a), formalin (b) and copper sulfate (c) at low salinityon morphology and motility of scuticociliates, *Uronema marinum*. Each point represents the mean of triplicate experiments.

The efficacy of chemicals at low salinity (CS)

The effects of CS on ciliate motility and morphology are shown in Fig. 3. Ciliates were killed at 100 ppm of hydrogen peroxide, at 50 ppm of formalin and at 100 ppm of copper sulfate. All ciliates treated with CS were killed within 1.5 h (Fig. 3). Although the ciliates showed irregular cell shape and lower motility during 4 h at lower concentration of CS, the ciliates were not completely destroyed.

Discussion

Two important possibilities of our results include: (1) The parasitic phase of *Uronema marinummay* have resistance to a low concentration of chemicals. (2) Formalin can be used effectively to control scuticociliatosis in cultured olive flounder at low salinity.

All chemicals tested here are commonly used in Korea, as parasiticides of ectoparasites. Formalin is currently approved for use in aquaculture in USA as a fungicide and parasiticide, typically at 167 to 250 ppm for 1 h bath (Sindermann, 1970; Poupard, 1978; Scott, 1993). Hydrogen peroxide has been used to treat freshwater fish for ectoparasite, typically at 1.5 g/L to 2 g/L (Bruno and Raynard, 1994). Copper sulfate solution is commonly used for the prevention and control for common parasitic fish disease (Herwig, 1979; Griffin, 1989). Freshwater bath has been applied to eliminate ectoparasite using osmoregulatory control of fishes (McVicar and Richards, 1981; Schnich, 1988; Svendsen and Haug, 1991).

In this in vitro test, all chemicals proved lethal to the ciliates. During 4 h exposure at 200 ppm of hydrogen peroxide, at 100 ppm of formalin, at 3 ppt of freshwater and at 200 ppm of copper sulfate, the ciliates killed completely, The result concurs with other in vitro trial on scuticociliates, *Anophryoides haemophila*, a pathogen of Americal lobster (Novotny et al., 1996) or *Uronema nigricans*, a pathogen of southern bluefin tuna (Crosbie and Munday, 1999).

In this *in vitro* test, chemicals revealed a synergistic effect on ciliates at low salinity. Within 1.5 h exposure to 50 ppm formalin, 100 ppm hydrogen peroxide and 100 ppm copper sulfate at 5 ppt, the parasite was completely destroyed. The synergistic effect is probable that the freshwater render ciliates to osmotic stress and then the ciliates were possibly more susceptible to the chemicals.

In the present study, formalin was most effective at low salinity against the parasite during in vitro trials. Majority of ciliates were irregular in shape with no movement 30 min after exposure to 50 ppm formalin at 6 ppt. However, at this concentration and exposure time, formalin alone (salinity, 32 ppt) did not affect on the ciliates. On the other hand, 80% of fresh water (salinity, 6 ppt) did not destroy the ciliates.

In preliminary trials, formalin was more effective in treating infected flounder at low salinity than formalin on freshwater alone (unpublished). Although the pathological effects of formalin (Cruz and Pitogo, 1989) and the detrimental effects of fresh water bath (Bassleer, 1984) on fish are well known, we could not observe any side effect of the combined formalin such as rapid gasping and loss of equilibrium. On the contrary we observed that the fish treated the combination became active in swimming and feeding.

In conclusion, the data reported here indicate that formalin can be used effectively at low salinity to control scuticociliatosis in cultured olive flounder.

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