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Heavy oil exposure induces high moralities in virus carrier Japanese flounder *Paralichthys olivaceus*

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ABSTRACT

The relationship between chemical exposure and disease outbreak in fish has not been fully defined due to the limitations of experimental systems (model fish and pathogens). Therefore, we constructed a system using the Japanese flounder, *Paralichthys olivaceus*, and viral haemorrhagic septicemia virus (VHSV), and evaluated it by heavy oil (HO) exposure. The fish were exposed to HO at 0.3, 0.03, 0.003, and 0 g/L following VHSV infection at doses of $10^{2.5}$ or $10^{3.5}$ tissue culture infectious dose $(TCID)_{50}$/fish. As a result, groups given the dual stressors showed more than 90% mortality. Although VHSV infection at $10^{2.5}$ and $10^{3.5}$ $TCID_{50}$/fish without HO exposure also induced high mortality, at 68.8% and 81.3%, respectively, HO exposure induced faster and higher mortality in the virus carrier fish, indicating that chemical stressors raise the risk of disease outbreak in fish. The experimental system established in this study could be useful for chemical risk assessment.

1. Introduction

In the 20th century, many thousands of organic trace pollutants, such as polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzofurans (PCDFs), and dibenzo-*p*-dioxins (PCDDs) have been produced and, in part, released into aquatic environments (Stegeman and Hahn, 1994; van der Oost et al., 2003). The toxic effects of these chemicals on histopathological, developmental, and reproductive systems have been thoroughly investigated in various fish species (Brion et al., 2004; Hano et al., 2007; Liu et al., 2008; Viant et al., 2006; Villalobos et al., 2000; Zha et al., 2006). However, the immunotoxicity of the chemicals has not been fully investigated compared with the above toxicities.

Until now, the immunotoxicity of most chemicals has been evaluated by measuring leukocyte activities such as the phagocyte rate and the production of immunoglobulin (Arkoosh et al., 1994; Reynaud et al., 2002). However, pathogen challenge is considered to be the most important and comprehensive test for immunotoxicological screening because it is a direct test of an organism's response that has obvious biological significance at the individual and population levels (Kennedy and Farrell, 2008; Wester et al., 1994). Despite this, the relationship between toxicant exposure and the outbreak of infectious disease in aquatic organisms has not been clearly defined due to limitations of experimental systems (model fish and pathogens).

The Japanese flounder, *Paralichthys olivaceus*, is one of the most important marine fishes in Japan. From the viewpoint of a model fish, they are easily obtained from fish farms and the size is appropriate for maintenance in laboratory conditions. Moreover, the fish has high sensitivity to various pathogens including viruses, bacteria, and parasites (Isshiki et al., 2001; Kitamura et al., 2007; Rashid et al., 1994; Song et al., 2009), which allows for immunotoxic evaluation of chemicals at the individual level by experimental infection. For these reasons, we considered the Japanese flounder as a potential marine model fish. The aim of this study was to test the immunotoxicological usefulness of Japanese flounder by measuring heavy oil (HO)-induced viral disease occurrence in the fish through experimental infections.

2. Materials and methods

We tested the effect of dual stressors, viral haemorrhagic septicemia virus (VHSV) infection and HO exposure, on mortality in the Japanese flounder, *P. olivaceus*. The fish were infected with VHSV for 4 days followed by 2 days HO exposure. The detailed experimental scheme is shown in Fig. 1.

2.1. Fish

Japanese flounder (average body weight, 8.46 g) were obtained from a fish farm located in Kagawa Prefecture, Japan. The fish were placed in a 125-L holding tank supplied with re-circulating filtered seawater at 18 °C and acclimatized for 1 week before experiments. During acclimation, water temperature was gradually decreased to

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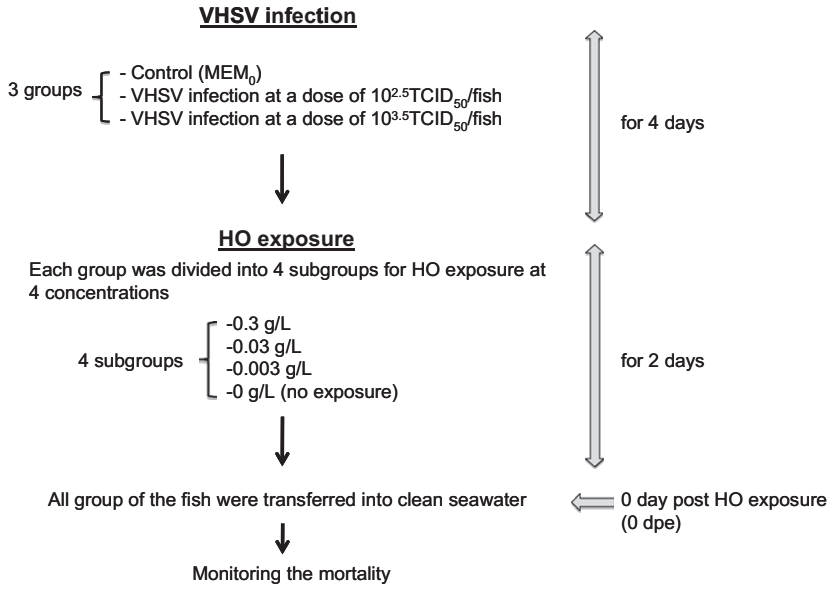

Fig. 1. Experimental scheme in the present study.

15 °C. After the acclimation, the fish were transferred into experimental aquaria for exposure experiments.

2.2. VHSV preparation

VHSV isolated from a diseased Japanese flounder (Kim et al., 2009) was used for the experiments. The virus was propagated in fathead minnow (FHM) cells (Gravell and Malsberger, 1965) cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) at 20 °C for 7 days. When the cytopathic effect was extensive, the supernatant was harvested and centrifuged to remove cell debris. The supernatant was used for the experiments. The virus stock was titrated in 96-well plates by the 50% end point tissue culture infectious dose ($TCID_{50}$) calculated by the Reed–Muench method (Reed and Muench, 1938).

2.3. VHSV infection

Three groups of fish (60 fish each) were prepared, and each group was placed in a 60-L tank supplied with 50 L of UV-treated and sand-filtered seawater. For virus infection, two groups of the fish were intramuscularly infected with 100 μL of VHSV at concentrations of $10^{2.5}$ or $10^{3.5}$ $TCID_{50}$/fish, respectively. The remaining group was used as a control that was intramuscularly injected with 100 μL of MEM. These tanks were maintained for 4 days at 15 °C in a temperature-holding water-bath system until HO exposure.

233. HO exposure

At 4 days post-virus infection, each group of fish was divided into four subgroups (15 fish each) transferred into 10 L tanks supplied with 8 L of UV-treated and sand-filtered seawater for HO exposure. Three subgroups were exposed to HO at concentrations of 0.3, 0.03, and 0.003 g/L for 2 days. One subgroup was used as a control without any exposure. After 2 days exposure, the fish were transferred into clean seawater and monitored for mortality for 8 days.

2.5. Statistical analysis

Statistical analyses were performed in mortality curves between the VHSV group and dual stressor group by Gehan's generalized Wilcoxon test using Graphpad Prism version 5 software program (GraphPad Prism Software Inc., San Diego, CA, USA) with Bonferroni correction. For statistical significance, a Bonferonni adjusted p value 0.0125 (0.05/4) was used.

3. Results and discussion

In this study, Japanese flounder were exposed to HO following VHSV infection for 4 days to evaluate the risk of HO exposure in virus-carrier fish. With the start of HO exposure, the mortality rapidly increased in the fish given the dual stressors. In particular, the highest HO concentration (0.3 g/L) induced 94% and 100% mortality in fish infected with VHSV at a dose of $10^{2.5}$ $TCID_{50}$/fish and $10^{3.5}$ $TCID_{50}$/fish, respectively, during 2 days HO exposure (Fig. 2a and b). Most of the other dual stressor groups also showed a high mortality of more than 80% within 2 days post-HO exposure (dpe). However, the mortalities of fish infected by virus at doses of $10^{2.5}$ or $10^{3.5}$ $TCID_{50}$/fish without HO exposure were only 37.5 and 50%, respectively, at 2 dpe. At the end of the experiment, all groups given the dual stressors showed more than 90% mortality except one group (75%) which was given 0.003 g/L of HO exposure with $10^{3.5}$ $TCID_{50}$/fish of VHSV. VHSV infection at $10^{2.5}$ and $10^{3.5}$ $TCID_{50}$/fish without HO exposure also induced high mortality, at 68.8% and 81.3%. In the HO exposure groups without VHSV infection, a 25% mortality was recorded in the fish exposed to HO at the concentration of 0.3 g/L, whereas no mortality was observed in the other groups (data not shown). There was also no mortality in the fish treated with neither HO exposure nor VHSV infection. From the results of statistical analysis, there are significant differences in mortality curves between the dual stressor groups (virus infection at the concentration of $10^{2.5}$ $TCID_{50}$/fish and HO exposure at 0.3, 0.03 or 0.003 g/L) and VHSV group without HO exposure (Fig 2a). The median survivals in the dual stressor groups exposed to 0.3, 0.03 and 0.003 g/L of HO were 5, 7 and 6 days, respectively, while it was 9 days in the VHSV group (Table 1). In the groups infected with the virus at $10^{3.5}$ $TCID_{50}$/fish, the significant difference was only observed in the group exposed to HO at 0.3 g/L (Fig 2b). These results indicate that HO exposure obviously induced fast and high mortality in the virus carrier fish.

In our previous study, we found that the bacterial number on the epidermal mucus of the fish significantly increased by HO exposure (Song et al., 2008). Also, microarray experiments have de-