Short communication

Effect of berberine hydrochloride on grass carp *Ctenopharyngodon idella* serum bactericidal activity against *Edwardsiella ictaluri*

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1. Introduction

Berberine, a kind of isoquinoline alkaloid, is the main antibacterial substance of *Coptis chinensis* Franch, and the latter has been used for the treatment of bacterial diseases of human and animals for a long history [1]. However, the *in vitro* antibacterial activity assay showed the MIC (minimal inhibitory concentration) of berberine hydrochloride was much higher than those of antibiotics. For example, MICs of berberine hydrochloride against *Aeromonas hydrophila, Vibrio vulnificus, Edwardsiella ictaluri, Escherichia coli* and *Streptococcus agalactiae* were >500 mg l⁻¹, >500 mg l⁻¹, 300 mg l⁻¹, 400 mg l⁻¹ and 100 mg l⁻¹, respectively [2]. Based only on the *in vitro* antibacterial activity, berberine hydrochloride is not supposed to have significant therapeutic effects against diseases caused by bacteria, so there should be some unknown mechanisms to support the successful treatment of bacterial diseases. *E. ictaluri* is the agent of enteric septicemia of catfish (ESC) of cultured channel catfish (*Ictalurus punctatus*). The pathogenic bacteria give great threat to farmed fish and cause considerable losses in the aquaculture industry, so it is selected to be tested in this study.

The present study is trying to investigate if berberine hydrochloride has the potential to boost fish serum bactericidal activity through activating the complement system.

2. Materials and methods

The laboratory strain of *E. ictaluri* HSN-1 was isolated from yellow catfish (*Pelteobagrus fulvidraco*) and stored at −80 °C as usual. The bacteria were grown for 48 h at 28 °C in brain heart infusion (BHI, Becton Dickinson and Company) agar slant, then it was washed with sterile phosphate buffered saline (PBS, pH7.4) and the Optical density (OD) of the suspension was adjusted to 0.1–0.5 at 600 nm in a spectrophotometer. This bacterial suspension was diluted with sterile PBS to give a concentration about 1 × 10⁵−5 × 10⁵ CFU ml⁻¹. Colony-forming units (CFU) of the bacteria were determined by plating serial dilutions on to Luria-Bertani (LB) agar plates to determine the initial CFU.

Berberine hydrochloride powder (purity, 97.1%) tested in this study was obtained from Wuhan Jinzhou Shengnong Pharmaceutical (Wuhan, Hubei province, China). It was dissolved in PBS (pH7.4) to make stock solutions. After filtration through a sterile 0.2 µm membrane (Pall Corporation, USA), stock solutions were stored at 4 °C until use. The stock solution was diluted with PBS (pH7.4) to the final concentration of 10 mg l⁻¹, 20 mg l⁻¹ or 50 mg l⁻¹.
Serum of grass carp was collected from 36 fish with an average weight of 1 kg. The blood samples were taken from the caudal vein to serum tubes without anticoagulant and allowed to clot for 2 h at room temperature. The sera were separated by centrifugation at 1500 \( \times g \) for 15 min and pooled and stored at \(-80^\circ C\). The complement activity in grass carp serum was inactivated at 52 \(^\circ C\) for 30 min [3].

The assay of serum antibacterial activity was conducted as described by Ji et al. [4]. Bacterial per cent survival (BPS) was calculated according to the following formula: 
\[
\text{BPS} = \frac{\log_{10} \text{CFU in sample}}{\log_{10} \text{CFU in control}} \times 100\%.
\]

A volume of 120 \( \mu l \) grass carp serum or heat-inactivated grass carp serum was mixed thoroughly with 20 \( \mu l \) of \textit{Edwardsiella ictaluri} HSN-1, and then 60 \( \mu l \) berberine hydrochloride (50 mg l\(^{-1}\) or 10 mg l\(^{-1}\)) was added. The final concentrations of berberine hydrochloride were 15 mg l\(^{-1}\) or 3 mg l\(^{-1}\), respectively. After being incubated at 25 \(^\circ C\) for 3 h, CFU in each sample was counted as Ji et al. [4]. For control, the same amount of PBS was used to replace berberine hydrochloride.

To further demonstrate the activating function of berberine hydrochloride to complement system, a complement consumption experiment was conducted [5]. The activity of complement was assayed using rabbit red blood cells (RaBRC) as targets. RaBRC in Alsever’s solution [5] were washed 3 times with PBS by centrifugation at 750 \( \times g \) for 5 min and the cell concentration is adjusted to \( 1 \times 10^8 \) cells ml\(^{-1}\). Thirty microlitres (30 \( \mu l \)) of diluted grass carp serum was thoroughly mixed with 10 mg l\(^{-1}\), 20 mg l\(^{-1}\) or 50 mg l\(^{-1}\) of berberine hydrochloride in the test tubes at 20 \(^\circ C\) for 1.5 h with agitation. For control, equal volume of PBS was used to replace berberine hydrochloride. RaBRC (60 \( \mu l \) of \( 1 \times 10^8 \) cells ml\(^{-1}\)) were added to each tube and further incubated at 20 \(^\circ C\) with occasional shaking. After 1.5 h, the sera were centrifuged at 1600 \( \times g \) for 5 min. Absorbance of the supernatants were measured at 414 nm in a spectrophotometer. Triplicates were set up for each blood sample. The values of maximum (100%) and minimum (spontaneous) haemolysis were obtained by adding 3.44 ml of distilled water or PBS to 60 \( \mu l \) of RaBRC, respectively. Complement-induced percent haemolysis of RaBRC was calculated as the follows (From Ref. [6]):

\[
\text{Percent haemolysis} = \frac{[A414 \ (\text{sample})] - [A414 \ (\text{spontaneous lysis})]}{[A414 \ (100\% \text{ haemolysis})]} \times 100\%.
\]

Statistical Product and Service Solutions (SPSS) software was used to determine whether there was significant variation among treatments at the 5\% level. The data were analysed by Least-Significant Difference (LSD) in Analysis of Variance (ANOVA). Treatment-means and standard errors were estimated.

### 3. Results and discussion

The BPSs of \textit{E. ictaluri} HSN-1 in grass carp sera treated with two concentrations of berberine hydrochloride were 65.0\% and 64.4\%, respectively, and the BPS of normal serum was 69.3\% (Fig.1), indicating that bactericidal activity of grass carp serum treated with 15 \( \mu g \) ml\(^{-1}\) or 3 \( \mu g \) ml\(^{-1}\) berberine hydrochloride increased, showing significant differences (\( P < 0.05 \)). The BPSs of \textit{E. ictaluri} HSN-1 in the heat-inactivated grass carp serum which were pre-added with two concentrations of berberine hydrochloride didn’t change significantly compared with PBS control group (Fig. 1).

In the complement consumption assay, grass carp normal serum pre-treated with 10 mg l\(^{-1}\), 20 mg l\(^{-1}\) or 50 mg l\(^{-1}\) of berberine hydrochloride showed significant lower haemolysis activity than control sera (treated with PBS) (\( P < 0.01 \)). The percent haemolysis for the three concentrations of berberine hydrochloride were 64.7\%, 61.6\% and 65.7\%, respectively, and the value for the control serum was 79.2\% (Fig.2), suggesting that complement consumption had occurred, and it was supposed to be induced by the addition of berberine hydrochloride.

Cultured fish in modern intensive aquaculture are exposed to various stresses, ranging from environment pollution to handling. Crowding stress, transportation and other acute stress can dramatically harm complement activity in fish [7,8]. It is desirable to search for substances that have the ability to stimulate complement activity in fish.
complement activities in fish. Berberine, a plant alkaloid with a long history of medicinal use in both Indian and Chinese medicine, has antimicrobial activity against bacteria, fungi, protozoans, viruses, helminths and Chlamydia at certain concentrations [9].

In the present study, the BPSs of the bacteria tested in grass carp normal serum which were pre-treated with 15 mg l\(^{-1}\) or 3 mg l\(^{-1}\) berberine hydrochloride were significantly lower than those in the normal serum control and in the heat-inactivated sera, suggesting that berberine hydrochloride could enhance the serum bactericidal activity in grass carp in a way. The complement consumption experiment showed that the haemolysis activity of serum pre-treated with 10 mg l\(^{-1}\), 20 mg l\(^{-1}\) or 50 mg l\(^{-1}\) of berberine hydrochloride were significant lower than control group. The finding suggested that the addition of berberine hydrochloride into sera can activate complement system of grass carp resulting in complement consumption. When considering the findings of both experiments, it is reasonable to infer that the enhancement to serum bactericidal activity by berberine hydrochloride treatment may involve the complement system. In other word, berberine hydrochloride can elevate the bactericidal activity of fish serum through activating its complement system. Of course, further studies will be needed to explore the specific mechanism. The results from the present study may provide partially an explanation why herb medicines contain berberine hydrochloride, such as C. chinensis Franch, can get used in aquaculture for the prevention and treatment of fish diseases caused by some kinds of bacteria.

In conclusion, a kind of Chinese traditional medicine was found to enhance the complement-mediate bacteria killing ability.

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References