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Effects of traditional Chinese medicine on immune responses in abalone, *Haliotis discus hannai* Ino

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Abstract A traditional Chinese medicine (TCM) preparation was formulated from orange peel (*Pericarpium Citri Reticulatae*), hawthorn (*Crataegus pinnatifida*), astragalus (*Astragalus membranaceus* (*Fisch.*) *Bunge*), pilose asiabell root (*Radix codonopsis*), indigowoad root (*Radix isatidis*), taraxacum (*Herba taraxaci*) and malt (*Fructus Hordei Germinatus*) at a weight ratio of 1:1:1.5:1.5:1.5:1.5:2. A feeding experiment was conducted to determine the effects of TCM on innate immunity of abalone, *Haliotis discus hannai* Ino. Artificial diets containing 1%, 3%, 5% TCM preparation, 1% hawthorn or 1% astragalus, respectively, were fed to juvenile abalone (initial weight 10.38 ± 2.51 g; initial shell length 44.15 ± 4.15 mm) for 80 days. A TCM-free diet was used as a control. Each diet was fed to three replicate groups of abalone using a randomized design. The results indicated that phagocytic activity was significantly higher in abalone fed 3%, 5% TCM preparation, 1% astragalus or 1% hawthorn ($P < 0.05$). Respiratory burst activity was significantly higher in abalone fed 1%, 3%, 5% TCM preparation, 1% astragalus or 1% hawthorn ($P < 0.05$). Agglutination titre was significantly higher in abalone fed 5% TCM preparation ($P < 0.05$). Weight gain ratio (WGR), daily increment in shell length (DISL), total haemocyte count (THC), plasma protein concentration, and the activity of acid phosphatase (ACP) were not significantly affected by the TCM preparation ($P > 0.05$). These results indicate that TCM preparation can modulate the immunity of *H. discus hannai*, and it is very possible that TCM might be used as immunostimulants in abalone farming.

Introduction

Abalone are large algivorous marine gastropods, and one of the most commercially important species of gastropods in aquaculture for Asia. Over-exploitation of wild abalone has

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led to a great decrease in wild abalone numbers; thus people have begun to farm abalone for human consumption. China is the largest producer of farmed abalone in the world; however, many abalone farms have been particularly affected by epidemics of viruses and vibriosis. Therefore, the health of abalone and enhancement of their immunity are of primary concern.

Abalone lack an adaptive immune response and rely on innate immune response against microbial invasion. It is considered that agglutinin is involved in the extracellular recognition and the opsonisation of bacteria and protozoans [1,2]. Small pathogens may be agglutinated or opsonised by agglutinins, facilitating clearance by circulating haemocytes or lysed directly without their involvement. In molluscs, agranular haemocytes and granular haemocytes are considered as two distinct cell types [3]. They are involved in phagocytosis, which is generally recognized as an important way to eliminate micro-organisms or foreign materials [4]. Several kinds of reactive oxygen intermediates (ROIs) are produced during phagocytosis. These include superoxide anion (O_2^-), hydroxyl radical ($OH^\cdot$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). This process is known as the respiratory burst, and it plays an important role in anti-microbial activity [5]. Acid phosphatase is a typical lysosomal enzyme [6], which has been found in the plasma of abalone [7].

Immunostimulants can increase resistance to infectious diseases by enhancing non-specific defence mechanisms. The use of immunostimulants is an effective means of increasing the immunocompetence and disease resistance in aquaculture. Research [8–12] indicates that levamisole, vitamin C, beta-1,3-glucan, chitin, chitosan, and bovine lactoferrin can enhance innate immunity of invertebrates. However, some of the immunostimulants could not be used because of various disadvantages, such as high cost or limited effectiveness. Besides, a large number of traditional Chinese medicine (TCM) has been successfully used to cure human and domestic animal diseases since ancient times in China. Jian and Wu [13,14] find TCM can enhance the immunity of fish. However, effect of TCM on immunity of abalone is still not clear.

We prepared a TCM formulation according to the theory of traditional Chinese medicine to study the effect of TCM on innate immunity of abalone. The main compounds of TCM that have the effect of immunoregulation are astragalus (*Astragalus membranaceus* (*Fisch*.) *Bunge*), pilose asiabell root (*Radix codonopsis*), indigowoad root (*Radix isatidis*) and taraxacum (*Herba taraxaci*). Cho and Leung [15] report that astragalus membranaceus have the effects of immunoregulation and immunorestoration both in vitro and in vivo. Pilose asiabell root can activate the proliferation of phagocytes, and suppress eclampsia. Wang et al. [16] point out that indigowoad root has the effect of anti-endotoxin. Chen et al. [17] further suggest a possible mechanism of anti-influenza activity of indigowoad root extracts. Taraxacum has been known since ancient times for its curative properties. Though quantification of individual Taraxacum constituents is not clear yet, particular attention has been given to diuretic, choleretic, anti-inflammatory, anti-oxidative, anti-carcinogenic, analgesic, anti-hyperglycemic, anti-coagulatory and prebiotic effects [18].

This study was aimed at determining the effect of TCM on growth and immune parameters of abalone, and trying a possible way to protect abalone from disease infection by applying TCM to aquaculture. For the former purpose, total haemocyte count, phagocytic activity, respiratory burst activity, agglutination titre and acid phosphatase activity were examined.

Materials and methods

Preparation of diets

Experimental diets were formulated with fishmeal, soybean powder and kelp powder to provide 30.5% crude protein, which is considered to be sufficient to maintain the optimal growth of *H. discus hannai* [19]. The dietary lipid level was 4%, which is sufficient to maintain the optimal growth of abalone [20]. The compositions of vitamins were similar to those used by Chen et al. [7], and minerals were similar to those used by Uki et al. [21].

Orange peel (*Pericarpium Citri Reticulatae*), hawthorn (*Crataegus pinnatifida*), astragalus (*Astragalus membranaceus (Fisch*.) *Bunge*), pilose asiabell root (*Radix codonopsis*), indigowoad root (*Radix isatidis*), taraxacum (*Herba taraxaci*) and malt (*Fructus Hordei Germinatus*) were ground to a 300 mesh size powder, respectively. They were then mixed at a weight ratio of 1:1:1.5:1.5:1.5:1.5:2 to compose a formulation, which was then incorporated into feed at a ratio of 1%, 3%, 5% (w/w) to prepare diet 1, diet 2 and diet 3, respectively. Hawthorn or astragalus was also incorporated into the feed at a ratio of 1% (w/w) to prepare diet 4 and diet 5, TCM-free diet was used as a control. The dietary flakes were sealed in sample bags and stored at $-20\,^\circ C$ until use.

Animal culturing

Abalone (initial weight 10.38 ± 2.51 g; initial shell length 44.15 ± 4.15 mm) used in this experiment were supplied by Pacific Fisheries Co., Dalian, China. One thousand eight hundred specimens were randomly assigned to six groups and carried out in triplicate. Tests were carried out in triplicate test groups consisting of 100 abalone each in 1400 l PVC tanks containing 1000 l of aerated seawater. Prior to the initiation of the experiment, abalone were fed the TCM-free diet for 2 weeks. When the experiment started, the experimental diets were directly given in water, and the abalone were fed ad libitum once daily at 17:00 pm. Every morning, faeces and excess diet pellets were removed to maintain water quality. During the 80-day experiment, water temperature varied between 15 °C and 22 °C, salinity between 32‰ and 33‰, pH between 7.2 and 7.8, and dissolved oxygen was no less than 7.1 mg l^{-1}.

Sample collection and analysis

At the end of the feeding trial, the abalone were not fed for 3 days. Thirty abalone from each replicate were weighed and measured. Haemolymph was collected by cutting the brood sinus in the adductor muscle with a scalpel. Haemolymph from 15 abalone in each replicate was pooled to reduce inter-individual variation, and was immediately placed on ice to retard cell clumping. Four milliliters of