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Effect of Astragalus radix on proliferation and nitric oxide production of head kidney macrophages in Cyprinus carpio: an in vitro study

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Abstract: The *in vitro* effect of *Astragalus radix* water extract on the non-specific immune responses of macrophages isolated from the head kidney of *Cyprinus carpio* was evaluated. *Astragalus radix* extract stimulated the proliferation of the head kidney macrophages, and it alone had no effect on nitrogen burst activity of macrophages. But it enhanced the nitric oxide production of the mixture of macrophages and granulocytes induced by LPS. It is suggested that *Astragalus radix* could modulate the non-specific immune response of *Cyprinus carpio*.

Key words: *Cyprinus carpio*; *Astragalus radix*; macrophages; immune response

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1 Introduction

Infectious disease is one of the greatest economic threats to aquaculture, and the use of some antibiotics in aquaculture has been forbidden in China because of their negative effects on the environment, the development of antibiotic resistance and accumulation in fish and shrimp. Vaccination has been highly successful in combating many serious diseases, however, there are no effective vaccines for most pathogens and the feasibility of vaccinating against every pathogen that might affect the aquatic animals seems unlikely. Thus, the use of immunostimulants to stimulate the non-specific immune responses against multiple pathogens has attracted more and more attention in the past decades. Chinese herbs contain many immune active components such as polysaccharides, organic acids, alkaloids, glycosides and volatile oil, which can enhance immune functions[1]. *Astragalus radix* is one of common immune boosters used in human beings and animals. Its immunostimulating effects and growth promoting effects on aquatic animals have been explored through feeding animals with diets containing *Astragalus radix* by several researchers in China[2-5]. However, little has been done on its role in immune cells by *in vitro* study. The effect of *Astragalus* water extract on the immune responses of the cells isolated from the head kidney of carp was examined in this study.

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2 Material and methods

2.1 Experimental fish

Common carp (*Cyprinus carpio*) were reared at 25 ℃ in a recirculation system and fed with pellet for 8 h every day using an automatic belt feeder. Fish were about 6 month old at the start of the experiment with an average weight of about 150 g. A total of 10 fish were studied in the present experiments.

2.2 Preparation of herbal water extract

100 grams of *Astragalus radix* were soaked in 100 mL of distilled water at room temperature overnight, then boiled for 60 min. After removing the insoluble material by filtering through a filtering paper, the supernatant was further filtered through a sterilized filter (0.2μm). The extract was then diluted 10 ×, 100 ×, 1000 × and 10000 × in RPMI without phenol red.

2.3 Isolation of head kidney macrophages and granulocytes

Head kidney macrophages and granulocytes were isolated as described previously[6]. Fish were anaesthetized in 0.2 g·L^{-1} tricaine methane sulfonate (TMS), blood was removed from the caudal vene, both head kidneys were removed using sterile tools and put on the top of the nylon mesh (50μm) moistened with RPMI-hep and the tissues were teased to the pot, the cell suspension was centrifuged for 10 min. at 700g, 4 ℃ (2000 r·min^{-1} in Beckman), the upper supernatant was removed and the cell pellet was resuspended in 1 mL RPMI-hep and loaded on the top of a discontinuous percoll gradient which was prepared by adding 2 mL of each of the following densities of percoll solutions to a tube in the order of 1.08, 1.07, 1.06, 1.02 g·mL^{-1}. The gradients were centrifuged for 25 min at 700 g (2000 r·min^{-1} in Beckman), 4 ℃ and stopped without brakes. The cells at the interface of percoll between densities 1.02 and 1.06 (fraction 2, macrophage enriched); 1.06 and 1.07 (fraction 3, neutrophilic granulocyte enriched) were collected and washed twice in cRPMI-hep (10min, 700g, high break) to remove the percoll. The cells were resuspended in 1 mL cRPMI-hep or cRPMI + + and counted on a Burker counting chamber. The percentage of macrophages in fraction 2 and neutrophilic granulocytes in fraction 3 was checked to be over 90 %. The cells were then diluted to 1 ×10^7 cells·mL^{-1} and plated out in an uncoated 96-well plate (100μL·well^{-1}).

2.4 Immune assays

Proliferation of macrophages The proliferation of macrophages from head kidney was measured by Promega CellTiter 96 Aqueous non-Radioactive Cell Proliferation Assay[7].

Quadruplicated monolayers of 10^6 cells per well (in cRPMI + +) in a flat-bottomed 96-well microtiter plate were incubated with different stimuli for 96 h at 27 ℃, under 5 % CO_2. To measure proliferation of macrophages in head kidney, 20μL cRPMI + + (control) or lipopolysaccharide (LPS, 500μg·mL^{-1}) or different dilution of herbs were added.

After incubation, 10μL Cell Titer was added to each well. After 3 h incubation at 27 ℃, under 5 % CO_2, the optical density was determined in an ELISA reader at 492 nm with reference at 690 nm.

Measurement of nitric oxide production The measurement of nitric oxide production by macrophage and the mixture of macrophages and neutrophilic granulocytes were done by quantifying nitrite content from the supernatants of the two fractions using the Griess reaction.

Quadruplicated monolayers of 10^6 cells per well (in cRPMI + +) were allowed to adhere in a flat-bottomed 96- well microtiter plate. After incubation of 1.5 h at 27 ℃, the non-adherent cells were removed by rinsing with cRPMI without phenol red. The fresh 100μL cRPMI + + was added to each well, the monolayers were incubated with 20μL cRPMI + + or LPS (500μg·mL^{-1}) or different dilution of herbs for 96 h at 27 ℃, under 5 % CO_2.

After incubation, 75μL of the supernatants was removed from the individual wells and placed in a separate 96-well microtitre plate. 100μL of 1 % sulphanilamide in 2.5 % phosphoric acid was added to each well, followed by adding 100μL of 0.1 % N-naphthyl-ethylenediamine in 2.5 % phosphoric acid.

After mixing, the optical density was determined in an ELISA reader at 540 nm with 690 nm as a reference.

2.5 Statistics

The data were compared using student's t-test and a one-way analysis of variance (anova). The results are expressed as mean optical density (OD) + SD and differences were considered statistically significant at $P < 0.05$.

3 Results

3.1 Proliferation of macrophages in head kidney

To evaluate the effect of *Astragalus radix* on proliferation of immune cells, macrophage-enriched fraction 2 cells (quadruplication) were stimulated with RPMI + + (control), LPS (positive control) and different dilutions (10 ×, 100 ×, 1000 ×, 10000 ×) of *Astragalus* water extracts. The optical density was measured after 4 days incubation. As shown in figure 1, proliferation of macrophage stimulated by LPS was significantly higher than that of the control, indicating that macrophages were in normal condition. The dose response effect of *Astragalus* water extracts on the proliferation of macrophages was observed. The proliferation of macrophages was shown to be stimulated by 10 ×, 100 ×, 1000 × and 10000 × dilution of *Astragalus* water extract.

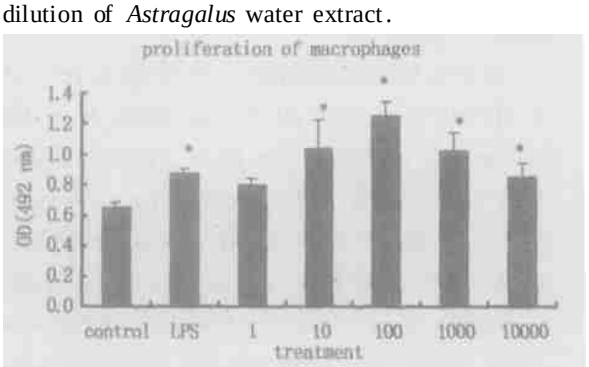

Fig. 1 Proliferation of macrophages in head kidney after 4 days incubation
This is one representative out of 4 independent experiments

3.2 Nitric oxide production

To investigate the role of *Astragalus radix* on the immune function of macrophages, nitrogen burst activity of macrophages in head kidney was assessed. macrophage-enriched fraction 2 cells (quadruplication) were stimulated with RPMI + + (control), LPS (positive control) and different dilutions (10 ×, 100 ×, 1000 ×, 10000 ×) of *Astragalus* water extract. The optical density was measured after 4 days incubation. Results indicated that different dilutions of *Astragalus* water extracts had no effect on nitrogen burst activity of macrophages (Figure 2).

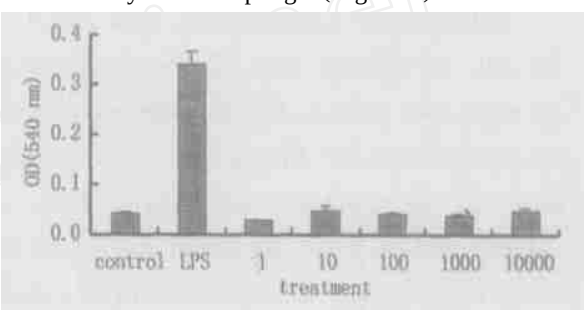

Fig. 2 Nitric oxide production of macrophages in head kidney after 4 days incubation
This is one representative experiment
out of 4 independent experiments

However, when the mixture of macrophage-enriched fraction 2 cells and granulocyte-enriched fraction 3 cells (quadruplication) was stimulated with RPMI + + (control), LPS (positive control), *Astragalus* water extract (10 ×) and combination of LPS and *Astragalus* water extract (10 ×), we found that although *Astragalus* alone had no effect on the nitrogen burst activity of the mixture of the macrophages and granulocytes, it could enhance the nitric oxide production stimulated by LPS (Figure 3).

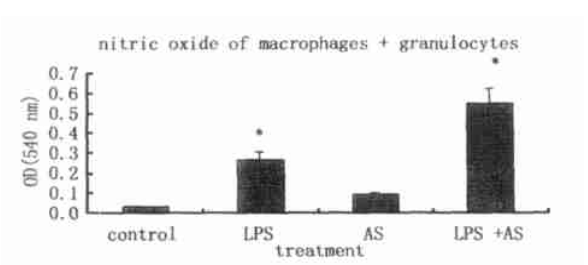

Fig. 3 Nitric oxide production of the mixture of macrophages and granulocytes in head kidney after 4 days incubation
This is one representative experiment
out of 3 independent experiments

4 Discussion

4.1 Effect of Astragalus water extract on proliferation of macrophages

Macrophages are one of the most important players in the immune system, they could not only phagocyte the pathogens, but also produce cytokines and act as an antigen presenting cell as well. The present study showed that *Astragalus* water extract could stimulate the proliferation of macrophages, meaning that once the fish are attacked by the pathogens, more macrophages are available to combat the pathogens. Therefore, *Astragalus radix* could be used as immunostimulants for fish.

4.2 Effect of Astragalus water extract on nitric oxide production

Recently nitric oxide (NO) has been established as an important and universal intercellular messenger that acutely affects important signaling pathways. NO is produced from L-arginin by the enzyme NO synthase (NOS), which occurs in various tissues in Ca^{2+}-dependent, constitutive (cNOS) and Ca^{2+}-independent, inducible (iNOS) isoforms[7]. The biological significance of NO is that it has microbicidal activity and its immunomodulatory capabilities.

Our study showed that *Astragalus* alone did not stimulate higher NO production, however, when the mixtures of macrophages and granunolytes were stimulated with *Astragalus* water extract combined with LPS, the NO production secreted by the mixture of macrophages and granulocytes was significantly higher. Therefore, we deduced that there must be some interaction between macrophages and granulocytes, either macrophages or granulocytes need something produced by the other in order to stimulate higher NO production. It has been demonstrated in gilthead seabream (*Sparus aurata*) that a macrophage-activating factor was produced by leukocytes in the head kidney. NO production could be induced with combined treatment of macrophage-activating factor and LPS, while LPS alone failed to induce NO production[8]. Whether macrophage-activating factor involved in inducing NO production in carp needs to be further studied, however, we do know that a number of cytokines could be produced by the stimulation of active components isolated from *Astragalus radix*.

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黄芪对鲤头肾中巨噬细胞的增殖和一氧化氮产量的影响:离体研究

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摘要:用密度梯度离心分离鲤头肾中的巨噬细胞和嗜中性粒细胞。用 RPMI 细胞培养液对分离的细胞进行原代培养,在细胞培养液中加入不同浓度的黄芪水提取液来研究黄芪对鲤头肾中免疫细胞非特异性免疫应答的影响。黄芪水提取液单独使用时能刺激鲤头肾中巨噬细胞的增殖,但不能刺激巨噬细胞的氮暴发活性。用黄芪和脂多糖(LPS)混合刺激巨噬细胞和中性粒细胞时,黄芪水提取液能显著提高脂多糖刺激所产生的一氧化氮的产量。研究结果表明,黄芪不仅能刺激巨噬细胞数量的增加,而且能协同脂多糖增强头肾中免疫细胞的功能,从而对机体的非特异性免疫起调节作用。

关键词:鲤;黄芪;巨噬细胞;免疫应答

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