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Research Article

The Immunomodulatory Effect of Intraperitoneal Injection of Licorice Extract on Giant Salamander

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Abstract

The effect of licorice extract on non-specific immune function of giant salamanders was studied by intraperitoneal injection. The results showed that the lysozyme activity in the drug group increased, and the lysozyme activity increased with the increase of dose. From the 7th day onwards, each sampling of the drug group showed significant differences compared to the control group ($P<0.05$). The phagocytic activity of macrophages in the drug group showed some fluctuations, but there was no significant difference compared to the control group. The white blood cell volume value of the high-dose group gradually increased in the first three samplings, and showed a significant difference compared to the control group at 14 days ($P<0.05$). It decreased slightly in the last two samplings, but was still higher than the control group at the same time. The spleen organ coefficient of the low-dose group was (0.7 ±0.01) % at 28 days, higher than that of the control group (0.4 ±0.05) %, and the high-dose group was (0.7 ±0.07) % at 14 days, higher than that of the control group (0.4 ±0.02) %. Both differences were significant ($P<0.05$). After the last sampling (28 days), artificial infection with *Aeromonas hydrophila* bacteria resulted in a mortality rate of 80% in the control group, 50% in the low-dose group, and 30% in the high-dose group, all lower than the control group. The drug group also had higher immune protection rates than the control group. The results indicate that intraperitoneal injection of licorice extract can improve the immune function and disease resistance of giant salamanders to a certain extent.

Keywords

Intraperitoneal Injection, Licorice, Giant Salamander, Immune

1. Introduction

Chinese herbal medicines are widely distributed, diverse in types, have minimal toxic side effects, and are not easily polluting the environment. They also contain various immune regulating components, such as polysaccharides, glycosides, volatile oils, organic acids, alkaloids, etc. There are over 200 known Chinese herbal medicines with immune activity. Lic-

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orice is one of them. Licorice is known as the "King of Herbs" in traditional Chinese medicine and is one of the globally protected species by the World Wildlife Fund. There are about 30 species of licorice, mainly distributed in Northeast, North, and Northwest China in China. Previous studies have shown that licorice has multiple effects, such as anti-inflammatory, antiviral, anticancer, anti-allergic, hepatoprotective, and estrogenic effects, and can regulate the body's immune function [1]. Licorice is also receiving increasing attention in aquaculture [2].

The Chinese giant salamander (*Andrias davidianus*), the American giant salamander (*Cryptobranchus alleganiensis*), and the Japanese giant salamander (*A. japonicum*) are three precious aquatic protected animals that currently exist in the world. The Chinese giant salamander (*A. davidianus*) belongs to the Amphibia in classification, with the Caudata order, Cryptobranchidae family, and *Andrias* genus. Due to its cry resembling that of a child, it is commonly known as the baby fish and is a second level protected species in China, mostly found in the Qinling Mountains of Shaanxi Province. In China, artificially bred giant salamanders are allowed to be traded and have formed a large scale of breeding [3]. However, diseases often occur during the breeding process of giant salamanders, causing serious economic losses to breeders. At present, the commonly used drugs for giant salamanders are chemical drugs and antibiotics, which can easily cause pathogen resistance and drug residue, which is very harmful to the environment and human health. Therefore, this study attempts to use licorice extract as an alternative drug to investigate its immunomodulatory effects on giant salamanders, which is in line with China's disease prevention and control guidelines for developing pollution-free aquaculture and producing green aquatic products.

2. Materials and Methods

2.1. Experimental Materials

2.1.1. Glycyrrhiza Uralensis Fiseh

The seedlings were purchased from Wuwei City, Gansu Province, China and cultivated in Luonan County, Shaanxi Province. The effective ingredients were extracted by slicing the roots.

2.1.2. Andrias Davidianus

Purchased from a giant salamander artificial breeding farm in Luonan County, Shaanxi Province, with an average weight of 1048.05 ± 3.07g. Prior to the experiment, the animal was trained in a continuously flowing mountain spring water aquaculture pond for one month. During the experiment, it was kept quiet and away from light, and mixed fish and compound feed were fed.

2.1.3. Micrococcus Lysodeikticus

Freeze dried powder was purchased from Sigma company.

2.1.4. Aeromonas Hydrophila

Purchased from Institute of Microbiology, Chinese Academy of Sciences.

2.2. Preparation of Pharmaceuticals

The crude extraction of licorice polysaccharides and glycosides was carried out using the water extraction and alcohol precipitation method according to the extraction process described in reference. In short, licorice slices are placed in 10 times the amount of 95% alcohol, refluxed and washed with alcohol at 60 ℃ to remove impurities. Alcohol waste liquid is rotary evaporated at 60 ℃ and recovered. The medicine residue is extracted with distilled water, filtered, and discarded. The water extract was centrifuged with ethanol at 4000rpm for 20 minutes, and the supernatant was discarded. The centrifuged extract was evaporated to dryness, ground into powder, and stored at 4 ℃. Mix the crude extract powder of licorice with sterile physiological saline at concentrations of 0.5% and 2% for injection.

2.3. Grouping and Sampling

The same origin and batch of giant salamanders were randomly assigned to 15 cement tanks (each with a volume of about 300L), with 5 cement tanks in the control group, 5 in the low-dose group, and 5 in the high-dose group, and 10 giant salamanders in each tank. The control group received intraperitoneal injection of sterile physiological saline, the low-dose group received intraperitoneal injection of 0.5% licorice injection, and the high-dose group received intraperitoneal injection of 2% licorice injection, with 5mL injected into each tail.

Samples were taken on the 3rd, 7th, 14th, 21st, and 28th day after abdominal injection, with 10 samples taken from each pool each time. The control group was sampled before the experiment (0d). When sampling, first weigh the body, then use a disposable medical syringe to draw blood from the tail vein, and finally weigh the spleen.

2.4. Immunological Testing

2.4.1. Serum Lysozyme Activity

Follow the method described by Parry [4]. Add 5 μL of fresh serum to 3mL of bacterial solution and measure at a wavelength of 540nm. An activity unit (U) is defined as a decrease of 0.001 in absorbance value within 1 minute.

2.4.2. Phagocytosis Activity of Renal Macrophages

Renal macrophage suspension was prepared using the Secombes method [5]. The bacterial suspension was prepared

using Thompson's method [6], except that the strain was *A hydrophila*, not *A salmonicida*. Under a microscope, observe and count 200 macrophages engulfing A Calculate the phagocytic percentage of hydrophila cells using the following formula:

Phagocytic Percent= (number of macrophages engulfing bacteria/200) × 100%

2.4.3. White Blood Cell Volume

The collected blood sample is placed in a heparinized medical white blood cell hematocrit tube, centrifuged at 22 ℃ 2000r/m for 30 minutes, carefully removed from the tube, and measured with a vernier caliper to determine the percentage of white blood cell hematocrit height to total length, which is the white blood cell volume (Leucocrit).

2.4.4. Spleen Organ Coefficient

First, anesthetize the giant salamander, then weigh it, remove its spleen, rinse it slightly with physiological saline, absorb the surface moisture with absorbent paper, and immediately weigh it on an electronic scale.

The ratio of spleen weight to body weight is called the Spleen Weight Index.

2.4.5. Artificial Infection Experiment

Wash the bacterial moss of *Aeromonas hydrophila*, which has been activated twice, with 0.5% physiological saline, and accurately prepare it using a spectrophotometer to achieve a final concentration of 2.3 × 106 CFU/mL. After the last sampling, inject 5mL intraperitoneally into each tail and observe for one week to calculate the mortality rate and immune protection rate.

Mortality Rate= (number of deaths/number of subjects) x 100%

Relative Percent Survival= (1-mortality rate of the immunization group/mortality rate of the control group) x 100%.

3. Experimental Results

3.1. Determination Results of Serum Lysozyme Activity

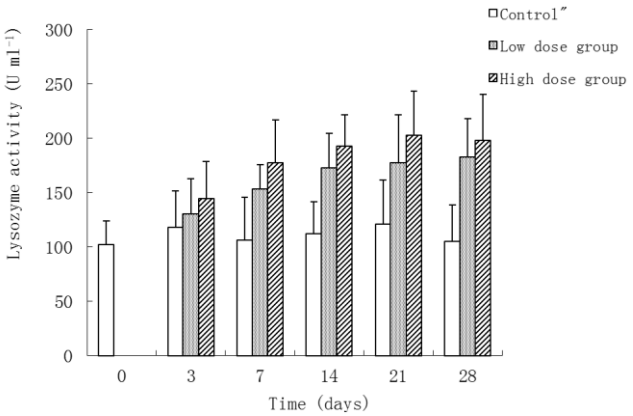

Figure 1. *Effect of intraperitoneal injection of G. uralensis Fiseh extract on lysozyme activity in A. davidianus serum.*

The effect of intraperitoneal injection of licorice extract on serum lysozyme activity in giant salamanders is shown in Figure 1. Both the low-dose group and high-dose group showed an upward trend, and lysozyme activity increased with increasing dose. Starting from the 7th day, significant differences ($P<0.05$) were observed between the drug group and the control group in each sampling.

3.2. Results of Phagocytosis Experiment

Table 1. *Effect of intraperitoneal injection of G. uralensis Fiseh extract on phagocytic activity of macrophages in A. davidianus kidney.*

Groups	Phagocytic percentage (%)					
	0d	3d	7d	14d	21d	28d
Pre experiment control	35.6±5.3	——	——	——	——	——
Control	——	43.2±5.2	48.3±4.7	38.2±5.9	49.2±7.8	36.3±6.1
Low dose	——	47.4±6.6	49.9±5.3	43.8±6.8	50.7±7.5	44.4±6.3
High dose	——	42.3±8.3	41.2±6.6	38.4±6.9	43.6±8.2	46.2±7.3

The phagocytic percentage of renal macrophages is shown in Table 1. There was no significant difference between the drug

group and the control group, and each group showed fluctuations at different sampling periods with no regularity.

3.3. Compression Test Results

Table 2. Effect of intraperitoneal injection of G. uralensis Fiseh extract on leucocrit value of A. davidianus.

Groups	White blood cell volume (%WBC)					
	0d	3d	7d	14d	21d	28d
Pre experiment control	3.4±0.5	——	——	——	——	——
Control	——	4.2±1.2	3.5±0.6	3.2±0.9	4.7±1.4	3.5±1.7
Low dose	——	3.7±0.6	3.6±1.3	4.4±0.8	4.5±0.5	3.7±1.3
High dose	——	4.3±1.3	5.5±1.3	6.8±0.9*	5.4±1.2	5.5±1.3

Note: * indicates a significant difference compared to the same period control

The low-dose group showed no difference compared to the control group during each sampling period. The high-dose group gradually increased in the first three samplings and showed a significant difference compared to the control group at 14 days ($P<0.05$). There was a slight decrease in the second two samplings, but both were higher than the control group at the same time.

3.4. Determination Results of Spleen Organ Coefficient

Table 3. Effect of intraperitoneal injection of G. uralensis Fiseh extract on spleen weight index of A. davidianus.

Groups Spleen organ coefficient (%)						
	0d	3d	7d	14d	21d	28d
Pre experiment control	0.5±0.03	——	——	——	——	——
Control	——	0.6±0.07	0.4±0.08	0.4±0.02	0.5±0.04	0.4±0.05
Low dose	——	0.5±0.02	0.5±0.03	0.6±0.05	0.4±0.09	0.7±0.01*
High dose	——	0.6±0.12	0.5±0.05	0.7±0.07*	0.4±0.13	0.4±0.07

Note: * indicates a significant difference compared to the same period control

The low-dose group had a result of (0.7 ±0.01) % at 28 days, which was higher than the control group's (0.4 ±0.05) %. The high-dose group had a result of (0.7 ±0.07) % at 14 days, which was higher than the control group's (0.4 ±0.02) %. Both differences were significant ($P<0.05$).

3.5. Results of Poison Attack Experiment

Table 4. Effect of intraperitoneal injection of G. uralensis Fiseh extract on mortality rate of A. davidianus induced by A. hydrophila infection.

Groups	Number of subjects	Number of deaths (%)	Mortality (%)	Immune protection rate
Control	10	8	80	0

Groups	Number of subjects	Number of deaths (%)	Mortality (%)	Immune protection rate
Low dose	10	5	50	37.5
High dose	10	3	30	62.5

After the last sampling (28 days), artificial infection with A The mortality rate of *hydrophila* bacteria was 80% in the control group, 50% in the low-dose group, and 30% in the high-dose group, all lower than the control group, while the immune protection rate of the drug group was also higher than that of the control group.

4. Discussion

A. hydrophila belongs to the *Vibrionaceae* family and the *Aeromonas* genus. It is a type of motile *Aeromonas* and a common opportunistic pathogen in aquatic environments [7]. It can cause sepsis in various aquatic animals, including giant salamanders [8]. *A. hydrophila* can invade the body through the skin and intestines, colonize skin and intestinal cells, and then multiply in large numbers to absorb host nutrients, leading to a decrease in the body's resistance. The pathogenicity of *A. hydrophila* is closely related to its virulence genes. Research has shown that hemolysin, outer membrane proteins, and serine proteases of *A. hydrophila* are important virulence genes [9]. Once *A. hydrophila* enters the body, it will colonize and proliferate in different tissue cells, producing exotoxins that can cause damage to the body of giant salamanders under the action of various virulence factors. Infected diseased salamanders usually exhibit increased surface mucus, enlargement of the liver, kidneys, pancreas, and lungs, varying degrees of congestion and bleeding in muscles, intestines, stomach, etc., abdominal distension with water accumulation, accompanied by hemolysis [10], seriously affecting the energy metabolism of the giant salamander, disrupting the homeostasis of the internal environment, reducing its immune system, and ultimately causing its death. Therefore, *A. hydrophila* is an important pathogenic bacterium in the breeding process of giant salamanders, which poses great harm and urgently needs to find effective prevention and control measures.

Non-specific immune response, also known as innate immune response, is the body's first line of defense against pathogen attacks and plays a crucial role in preventing infections and activating specific immune responses. When pathogens invade the body, they will face a series of immune cells and molecules that interact and initiate inflammatory responses. Among them, immune cells include monocytes, macrophages, neutrophils, non-specific cytotoxic cells, natural killer cells, mast cells, etc. Immune molecules include lysozyme, complement, transferrin, interferon, antiprotease, and C-reactive protein [11-15]. The above results are highly consistent with the experiments conducted by the author using crucian carp. Wang Wenbo et al. crude extracted licorice and then studied the immune regulatory effect of licorice crude extract on crucian carp by mixed feeding and intraperitoneal injection. Through the detection of various indicators of humoral and cellular immunity, as well as the detection of immune protection rate and cortisol level after artificial challenge, it was believed that licorice crude extract had an immune regulatory effect on crucian carp, enhancing the fish's resistance to *A. hydrophila*. The immune protection rates of the high drug group and low drug group were 21.4% and 35.7%, respectively, while the control group was 0 [2].

In summary, intraperitoneal injection of crude extract of licorice promoted serum lysozyme activity, blood leukocyte count, spleen development, and resistance to *A. hydrophila* in giant salamanders. This suggests that licorice has a certain regulatory effect on the non-specific immunity of giant salamanders and has achieved good disease prevention and control effects. It lays the foundation for the industrial deep processing of giant salamanders and has good application value. It also provides a new solution for the development of non-antibiotic, green and pollution-free industries in giant salamanders, which is of great significance for completing national and provincial fishery industry upgrading and technological innovation.

Abbreviations

WBC White Blood Cell

Author Contributions

Wenbo Wang: Conceptualization, Supervision, Writing – original draft, Writing – review & editing
Pin Liu: Investigation, Methodology
Yue Ning: Data curation, Resources
Yalong Feng: Software, Validation
Lingling Dou: Formal Analysis, Resources, Visualization

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Conflicts of Interest

The authors declare no conflicts of interest.

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