Effects of Noise Stress on the Pathological Sections of the Epinephelus Coioides and the Expression of Hsp70 Protein in the Liver

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Abstract: The changes of heat shock protein Hsp70 expression in the liver of *Epinephelus coioides* were observed after noise stress, and the liver tissue was analyzed by pathological staining. The *Epinephelus coioides* with average body mass of (42.54±6.03)g was selected as the experimental object. The unmanned noise was used as the control group, and the experimental group was 130dB/1μPa, Western blot and immunohistochemistry were used respectively. The expression and localization of Hsp70 protein in liver tissues of the *Epinephelus coioides* at 0h, 6h, 12h, 24h, 48h, 72h and 120h after noise stress were analyzed. The results showed that the Hsp70 in the liver of the *Epinephelus coioides* was mainly in the cytoplasm, and its expression level showed a trend of increasing first and then decreasing, and reached the maximum after 12h of stress, and the expression level was significantly different from that before stress. However, liver tissue cells showed no obvious pathological changes such as necrosis, inflammation and fibrosis after noise stress, and the difference between the groups was not statistically significant. In summary, noise stress causes stress response in the *Epinephelus coioides*, and the change of immune index has a certain time effect, and the damage of its physiological function is reversible.

1. Introduction

Epinephelus coioides, one of the most valuable marine fish, is delicious and popular among consumers everywhere. Because it belongs to aquatic animals, it is highly vulnerable to a variety of environmental factors. Given the enormous social and economic benefits and nutritional value of the *Epinephelus coioides*, it is particularly important to protect its growth environment [1, 2]. Environmental stress factors can be divided into physical factors, chemical factors, biological factors and process factors. Since the sensitive frequency range of marine fish acoustic signals is basically within 100~1 kHz, environmental noise is an important physical environmental stress factor affecting the life activities of marine fish [3, 4].

In recent decades, with the shortage of onshore energy, humans have gradually turned their attention to the vast and innocent ocean. Marine underwater noise generated by human activities, including commercial shipping, offshore construction, sonar detection, seismic surveys and groundwater explosions, is causing more and more noise pollution to marine life [5, 6], which can lead to underwater acoustics. Environmental changes threaten the habitat and survival of living things. Many studies have shown that noise has different effects on the behavior, physiological and biochemical characteristics of marine fish [7-9]. Monica Celi et al. studied the effects of noise pollution on motorboats on *Sparus aurata*, compared with the control group, the ACTH, cortisol, glucose, lactic acid, hematocrit, Hsp70, cholesterol, triglyceride and osmotic pressure of the fish in the noise stress group were significantly increased ($p<0.05$) [10]. Holles S et al. conducted five

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different types of noise tests on the directional response of juvenile coral reefs. The results showed that ship noise destroyed the directional behavior of coral reef fish. [11] Wysocki et al. demonstrated that underwater vessel noise can cause different freshwater fish. Such as salmon, *Gobio gobio* and *Perca fluviatilis* cortisol increased significantly [12]. Fish can make a noise stress pressure on stress response, the only known molecular reaction common to organisms is stress proteins, and the most commonly studied family of stress proteins is Hsp70 in Heat Shock Proteins (HSPs). The Hsp70 family is the most conservative, and is the most abundant protein in most circadian clocks, and is the most representative of the body tissue cells' ability to withstand environmental stress [13].

Therefore, in order to assess the impact of noise pollution, it is necessary to study the generation and propagation of noise and its impact on marine fish [14]. In this study, the *Epinephelus coioides* was treated with Western blot and immunohistochemistry, and the expression and localization of Hsp70 protein in liver tissues of the *Epinephelus coioides* at 0h, 6h, 12h, 24h, 48h, 72h and 120h after noise stress were analyzed. At the same time, pathological analysis of liver tissue was carried out to explore the relationship between noise stress time and changes of grouper biochemical indicators, in order to provide scientific reference for fish acoustic protection.

2. Materials and Methods

2.1 Experimental materials

The experimental *Epinephelus coioides* was provided by the aquaculture area of Yangjiang City, Guangdong Province. The specifications are shown in Table 1.

Table 1 Body Index of *Epinephelus coioides*

Animal	weight (g) M±SD	Length(cm) M±SD
Epinephelus coioides	42.54±6.03	13.62±0.78

The experimental instrument signal transmitter is manufactured by SUin Corporation, model TFG6960A. The power amplifier and boost converter are produced by Nanjing Fo Neng Technology Industrial Co., Ltd., model HE-1500G, and the underwater sound source is produced by the Institute of Acoustics of the Chinese Academy of Sciences, model FM-70001#.

2.2 Experimental methods

The grouper was randomly divided into 2 groups and placed in a round 600 LPVC bucket, and tested after 7 days of stabilization. The salinity was 36.4 ± 0.81 ppt and the temperature was 25.61 ± 0.39°C throughout the study. The seawater used is filtered through sand, and the water is changed once a day, and the water exchange rate is 1/2. According to the preliminary test results, the sensitive frequency of the *Epinephelus coioides* is around 647 Hz. Therefore, the addition of 647 Hz, 130 dB/1μPa underwater noise is the experimental group, and no added noise is the control group. Samples were taken at 0h, 2h, 6h, 12h, 24h, 48h, 72h, and 120h after noise stress, and each group took 3 tails.

2.3 Western Blot method for detection of Hsp70 protein expression in fish liver tissues

The *Epinephelus coioides* was quickly anesthetized with 100 mg/L MS-222, and the liver tissue was dissected, washed with pre-cooled PBS for 2-3 times to remove blood stains, and stored in an ultra-low temperature freezer placed at -80°C for use. Total protein extraction: The liver tissue was cut into small pieces and placed in a homogenate tube. Add 1~2 small magnetic beads of 2mm, 10 times tissue volume PBS (add protease inhibitor), homogenate for 1min, remove the ice bath for 30min, centrifuge at 12000rpm for 10min, take the supernatant, determine the protein concentration by BCA method, the protein solution Add 5 times protein loading buffer according to the ratio of 4:1, denatured in a boiling water bath for 15 min, and stored in a refrigerator at -20°C for use. The PVDF membrane was decolorized at room temperature for 1h after 300mA constant-flow wet

transfer, and 5% skim milk (0.5% TBST) was blocked for 1h. Membranes were incubated with mouse monoclonal antibody Hsp70 (Sihma, 1:3000 dilution) overnight at 4°C, destained 3 times (5min/time) in TBST, and bound to goat anti-mouse IgG (BBI Life Sciences, 1:1000) .Incubate for 30min at room temperature, decolorize 3 times in TBST, place on a chemiluminescence system, and analyze the optical density of the target band by Alpha software.

2.4 Immunohistochemical analysis of Hsp70 localization in hepatocytes

Paraffin sections were routinely dewaxed and hydrated. After treatment with 3% hydrogen peroxide for 25 min at room temperature to remove endogenous peroxidase, slides were detached in PBS (pH 7.4) for 3 times (5 min each). The tissue was evenly covered with 3% BSA in the circle, sealed at room temperature for 30 min, washed 3 times with PBS (pH 7.4), added Hsp70 primary antibody (Sigma, 1:100) at 4°C overnight, and added secondary antibody HRP-conjugated Goat Anti-Mouse IgG (BBI Life Sciences, 1:200). DAB color development, hematoxylin counterstained nucleus. Zhonglu gum seals. Hsp70 expression was observed under a light microscope. The positive cells with brownish yellow particles in the tissue were positive for Hsp70 expression.

2.5 Histopathological examination of liver slices

The liver was fixed in 4% paraformaldehyde, repairs the block and re-fixed, 70% (30 minutes), 80% (30 minutes), 85% (30 minutes), 90% (30 minutes), 95% (30 minutes), 100% (15 minutes) and 100% (15 minutes) gradient ethanol dehydration. Paraffin embedded, production (4 micron m thick), HE staining, observed under optical microscope photographs analysis results.

2.6 Experimental data processing

Data were expressed as mean±standard deviation (Mean ± SD), statistical analysis was performed using SPSS 23 software, and multiple comparisons were performed by one-way ANOVA and Tukey method. Significant difference $P< 0.05$ is significant.

3. Experimental results

3.1 Western Blot detection of differences in Hsp70 protein expression in fish liver tissues

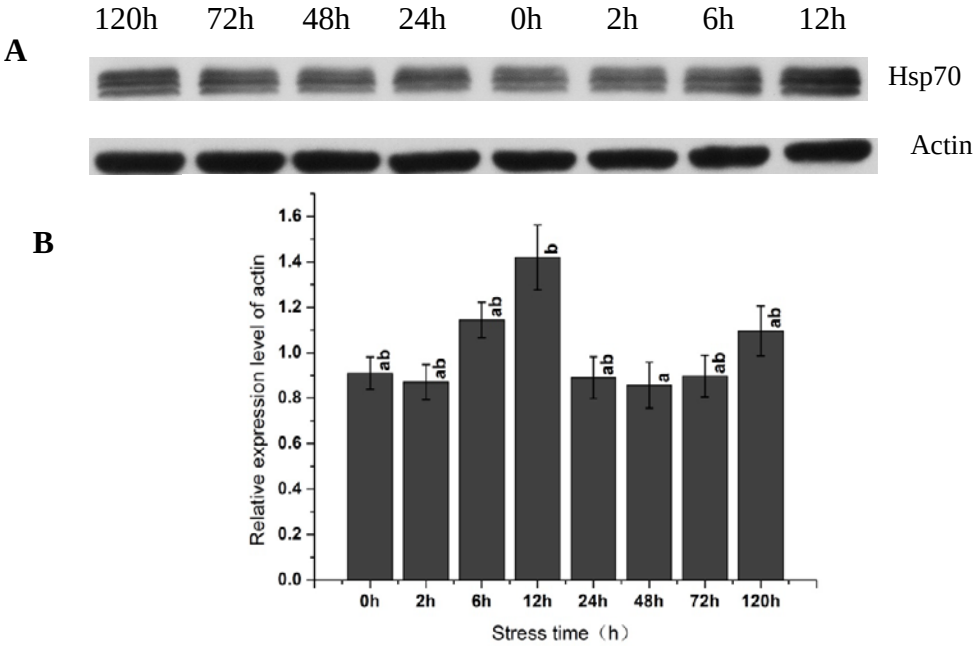

Fig.1 Effect of noise stress on Hsp70 expression in liver of *Epinephelus coioides*, The proteins were separated by SDS-PAGE, identified by immuno-blotting(A) , and quantified with analysis densitometry(B). The results shown are the mean ± S.E.M. (N = 5 control and N = 35 test specimens).

It can be seen from Fig. 1 that the expression level of Hsp70 protein in the liver of the *Epinephelus coioides* showed that the expression level increased continuously at 2h~12h, and reached the highest value at 12h, which was not significant (P>0.05). At the late stage of stress, the expression level of Hsp70 tends to be flat, but higher than that of the control group.

3.2 Immunohistochemical analysis of Hsp70 localization in hepatocytes

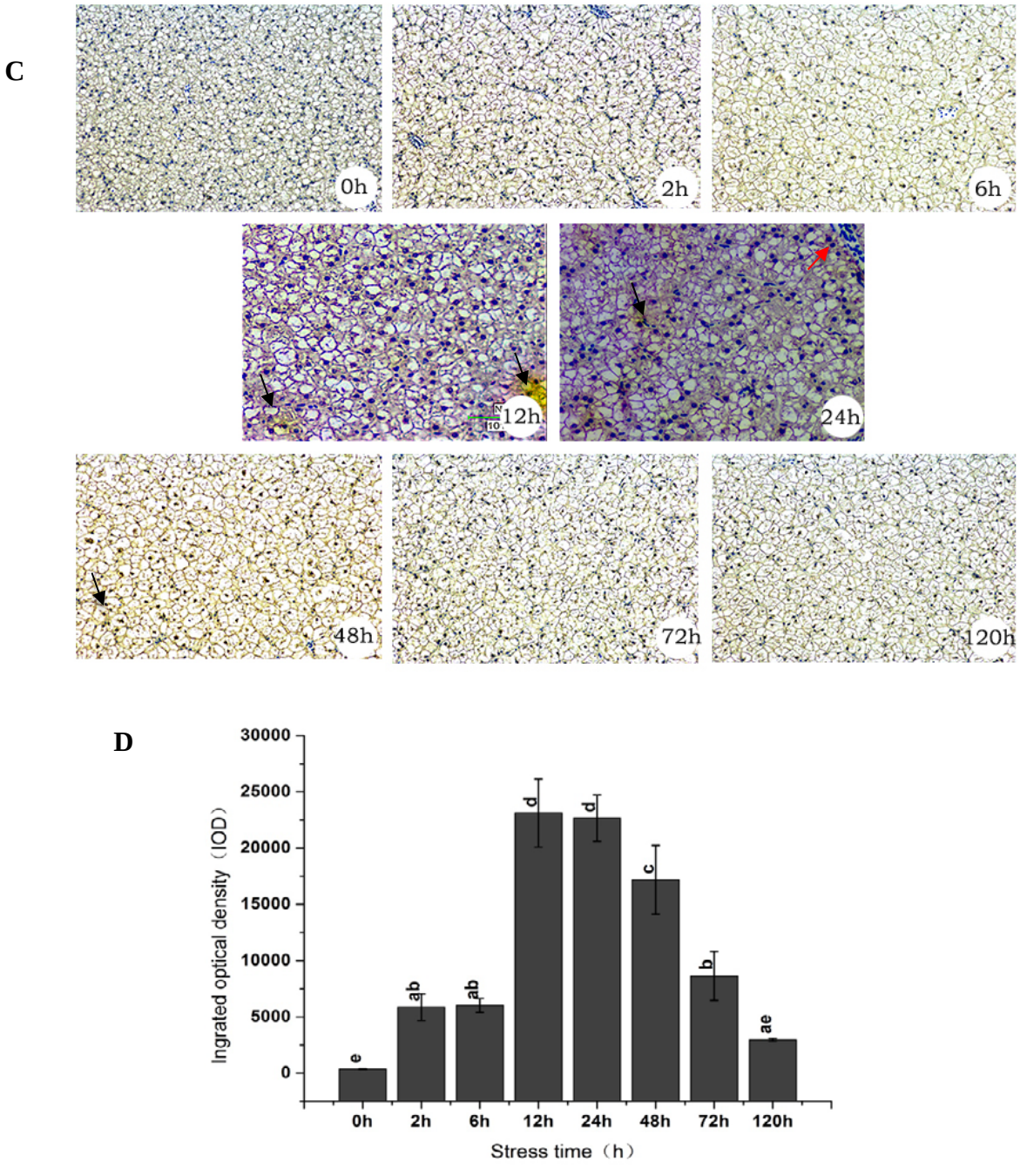

Fig.2. Histology immunohistochemistry analysis (C, 200×) and the IOD value of Hsp70 immunoexpression(D) in liver of *Epinephelus coioides* after noise stress, The results shown are the mean ± S.E.M. (N = 5 control and N = 35 test specimens).

As shown in Fig. 2, the results of immunohistochemistry showed that the nuclei stained with nuclide were blue (at the red arrow), and the positive expression of DAB was brown (shown by black arrows). The cumulative optical density (IOD) analysis of each group of samples was performed according to Image-Pro Plus 6.0 software. It was found that Hsp70 expression began to increase at 0h~12h, and increased with the increase of noise stress time, and decreased after 24h. It

is speculated that there is a certain degree of damage in the liver tissue of grouper after noise stress, which reduces the content of Hsp70.

3.3 Liver section histopathological examination

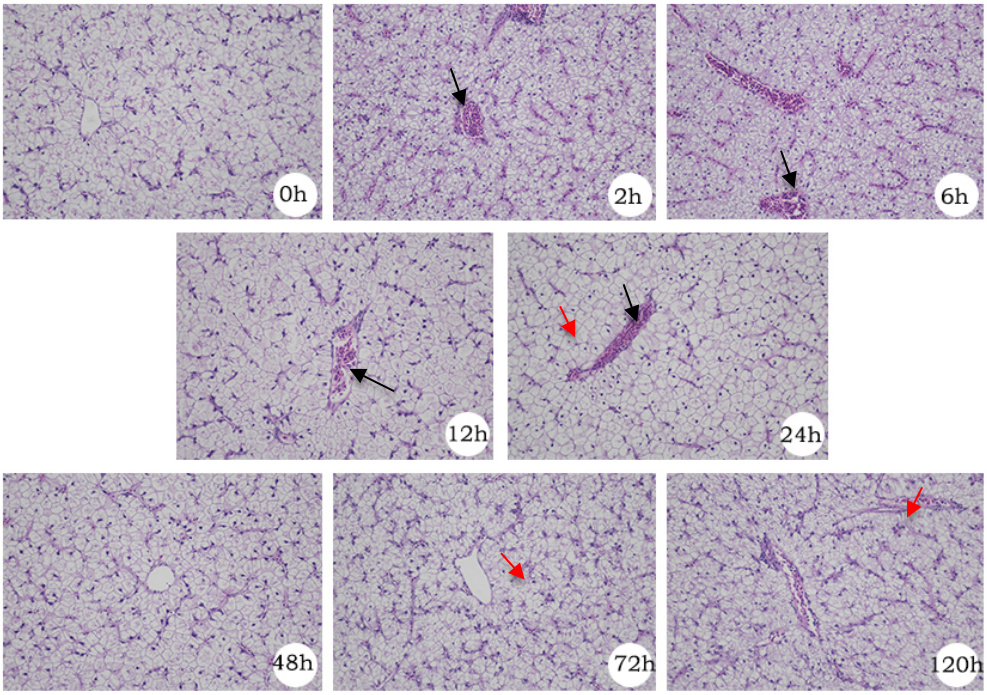

Fig.3. Pathological changes in liver of *Epinephelus coioides* after noise stress (HE staining, 200×).

Histopathological observation of liver tissue of grouper in the noise stress group (Fig. 3) showed high lipid content in the cytoplasm of hepatocytes, dissolved after tableting, and the cytoplasm of hepatocytes was sparse, mostly vacuolated (at the red arrow). Hepatocytes are arranged closely, the distribution of hepatic sinus and blood vessels is obvious, and there is red blood cell agglomeration (shown by black arrow), but no obvious pathological changes such as necrosis, inflammation and fibrosis are observed. In this experiment, the number of individual fish and the degree of lesion were compared with the blank group. After t test, the difference between the groups was not statistically significant. Therefore, this observation did not find that the noise stress has obvious toxic effects on the liver of the experimental fish.

4. Discussion

4.1 Effects of noise stress on the expression of Hsp70 in the liver of *Epinephelus coioides*

Heat Shock Protein (HSPs), also known as Stress Protein (SP), was discovered by geneticist Ritossa in 1962 when studying the pancreatic pancreas of *Drosophila* [15]. The Hsp70 family with a molecular weight of 66-78 kDa is the most conservative and important family of Hsps. According to the expression, the Hsp70 family can be further divided into inducible Hsp70 and constitutive Hsp70 (Heat stress congrate proteins 70, Hsc70). Hsc70 is an intracellular structural protein, and its expression level does not change significantly under stress conditions, while the expression level of inducible Hsp70 rapidly increases under stress conditions. As the main molecular chaperone protein in the cell, Hsp70 can prevent the unfolded nascent protein from denaturation under stress conditions to promote the refolding of aggregated protein [16, 17].

The results showed that the expression level of Hsp70 protein in the liver of the *Epinephelus coioides* showed that the expression level continued to rise at 2h~12h, indicating that the noise could induce the expression of Hsp70 in the *Epinephelus coioides*, but it was not significant. It is speculated that after the fish is stimulated by noise, it will cause certain damage to the liver of the

body, and induce the synthesis of Hsp70. The combination of Hsp70 and other abnormal proteins can reduce the risk of producing insoluble aggregates, thereby enhancing the protection of cells and improving the body's ability to adapt to the environment. It is the physiological manifestation of the autonomous adaptation of the fish body. When the expression level of Hsp70 reached the highest value at 12h, the expression level decreased and reached the level of the control group, but the expression level was higher than that of the control group. It is possible that the tested fish body establishes a new physiological balance after noise stimulation. At the same time, related studies have shown that the expression of Hsp70 can only protect cells from damage to a certain extent. If the stress exceeds certain intensity or further prolongs the exposure time, it may cause abnormalities in cell membrane structure and protein composition, thereby inhibiting the expression of Hsp70 gene.

4.2 Immunohistochemical analysis of Hsp70 localization in hepatocytes

After noise stress, the cytoplasm of fish liver positive cells showed yellow and yellowish brown, the nucleus was blue, and the nucleoli region was occasionally stained positively in the nucleus. Hsp70 was mainly found in the cytoplasm. As an important molecular chaperone, Hsp70 can participate in the synthesis and localization of proteins in cells, the maturation of proteins, the degradation and regulation of misfolded proteins. The cumulative optical density (IOD) analysis of each group of samples was performed according to Image-Pro Plus 6.0 software. It was found that the IOD value showed an upward trend from 0h to 12h, indicating that the expression level of Hsp70 in the liver tissue of grouper increased after noise stress. Among them, the peak value was reached at 12h, and the difference was significant ($p<0.05$) from 2h to 72h after stress. It is speculated that the grouper is frightened and produces a stress response after noise stress. The fish body regulates the damage caused by noise by increasing the expression of Hsp70 in the cytoplasm of the liver. After 48 hours of stress, the expression level decreased, and the tested fish may establish a new physiological balance after noise stimulation. This experiment can only reflect the response of the liver tissue of the *Epinephelus coioides* to the noise stress, because the synthesis of Hsp70 is tissue-specific, and different test species, different research organizations and different stress factors, the synthesis of HSP70 has different degrees of difference.

4.3 Histopathological changes in liver tissue

The liver tissue sections of grouper showed that the lipid content in the cytoplasm of hepatocytes was high, dissolved after tableting, the liver cells were closely arranged, the cytoplasm was sparse, and it was vacuolated, in the venous cavity in the liver slices after stress. There were different degrees of red blood cell aggregation, and the distribution of hepatic sinus was obvious. No obvious pathological changes such as necrosis, inflammation and fibrosis were observed. Combined with the above immunoenzyme activity and Hsp70 expression, it is speculated that noise stress promotes the metabolism of intracellular phosphorus in the liver, causing the lipid to rise and the red blood cells in the cavity to aggregate, it is possible that red blood cells accumulate due to the vasoconstriction of certain non-auditory systems induced by noise transmitted through the sensory system of the fish to the central nervous system of the brain [18].

5. Conclusion

The results of this study showed that after noise stress, the expression level of Hsp70 in the liver tissue of the *Epinephelus coioides* was significantly increased first and then decreased, but there was no obvious effect at the cell level, and the damage in physiological function was reversible. However, this paper only studies the expression pattern of Hsp70 noise response from the protein level, and further studies on its gene expression pattern and its regulation mechanism after stress. Moreover, the expression level of HSP70 is not always parallel with the intensity of the harmful factors, and the mechanism is complicated. De Pomerai [19] indicated that different pollutants induce the expression

of different HSPs, and that only one HSPs family is not sufficient as an indicator of environmental pollution.

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