

Pathological Changes and Risk Factors of Hepatopancreas Necrosis Disease of Mitten Crab, *Eriocheir Sinensis*

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Abstract

Hepatopancreas necrosis disease (HPND) is a disease and serious impacts on the industry of Chinese mitten crabs (*Eriocheir sinensis*) culture, however the actual cause of this disease is still not known. In the present study, to explore the pathogenic changes and risk factors caused by HPND, ultrathin sections of different tissues from the diseased crabs were observed with transmission electron microscope. The hepatopancreatic cells, spermatogonium, gill tissues and muscle cells of the diseased crabs showed severe structural and morphological changes. To further investigate whether HPND was caused by pathogenic microorganism, the healthy crabs were fed/injected with diseased tissues, the symptoms of HPND were not found, suggesting that HPND was not caused by virus or microsporidian infections. In addition, the toxic effect of avermectin and high pH water were also examined in this study. 40% ($p < 0.01$) crabs with HPND symptoms were found after breeding crabs in water with 9.5 pH to 10 pH for 14 days, but the crabs with no HPND symptoms were found when they were raised in water with different concentrations of avermectin. The results indicated that HPND was not caused by virus or microsporidian and might be induced by water of high pH value or other environmental factors.

Keywords: *Eriocheir sinensis*; Hepatopancreas necrosis disease; Pathological observation; Risk factors; Avermectin; pH values of water

Introduction

The Chinese mitten crab, *Eriocheir sinensis*, is one of the most economically important crustaceans in freshwater aquaculture production in China [1]. Recently, a crab disease, hepatopancreas necrosis disease (HPND), the classical symptom of HPND was hepatopancreas of diseased crab became degenerated, atrophy and whitish. Obvious hydrops could be observed in inner cavity of crab. To date, the causation of this disease is poorly elucidated.

It has been suggested that HPND of mitten crabs might be occur in the high pH of water, massive aquatic plants, high abundant of cyanobacteria, fertilizers, crab feeding and hypoxia [2]. Ding et al. [3] reported that infection of microsporidian *Hepatospora eriocheir* was associated with HPND, but animal regression test was not carried out. Meta-transcriptomes of the hepatopancreata from crabs with and without HPND were used to explore the etiology of HPND, and found that there were no striking differences in viral and microsporidian communities in the hepatopancreata of diseased and healthy crabs, however there were significant changes in the bacterial community of diseased and healthy crabs, increase of bacteria belonging to Tenericutes and Actinobacteria phyla and decrease of bacteria belonging to the Bacteroidetes phylum in crabs with HPND was suggested to associate with HPND [4]. Moreover, HPND also serious impacts on the industry of prawn culture. It was reported that *Vibrio parahaemolyticus* and *Bacillus thuringiensis* were involved in HPND of *Litopenaeus vannamei* [5], and *Vibrio parahaemolyticus* was the causative agent of acute HPND of cultured *Litopenaeus vannamei* in

Northwestern Mexico [6]. Wu et al. reported that simultaneously erupting infection of *hepatopancreas baculovirus* and vibrios was the main cause of the death of the cultured *Penaeus chinensis* with HPND [7]. Therefore, whether the HPND of crab is infected by pathogenic microorganism should be further studied.

In the present study, to further explore the etiology of HPND, the diseased crabs with HPND were collected from Yangcheng Lake Modern Agriculture Development Company, Suzhou, China, observation of the ultrathin section of tissues from the diseased crabs were observed with a transmission electron microscope and animal regression test were performed, moreover, toxic effect of avermectin and high pH water on crabs was also examined, the results indicated that HPND was not caused by virus or microsporidian and might be induced by water of high pH value and other environmental factors. This study will be helpful in diagnosis and prevention of HPND of mitten crabs.

Materials and Methods

Experimental animals

20 diseased crabs (*Eriocheir sinensis*) with the classical symptom of HPND and obvious hydrops in inner cavity of the diseased crab were collected on October 2015 from Yangcheng Lake Modern Agriculture Development Company, Suzhou, China. The healthy juvenile crabs, with 50% male and 50% female and each one weight about 50 g, no bacteria and microsporidian were found in the tissues by microscopy inspection that it was randomly selected from a group of crabs before being used, *E. sinensis*, were also kindly provided by Yangcheng lake Modern Agriculture Development Company.

Electron microscopy observations

Hepatopancreatic, gill, muscle, and gonadal tissues of the diseased crabs were fixed in 2.5% glutaraldehyde. The ultrathin sections were prepared according the previous report followed by observation with transmission electron microscopy (HITACHI-H7650, Tokyo, Japan) [8].

Experimental infectivity trial

Following the method reported by Ding et al. [9], approximately 1.0 g of hepatopancreas from the diseased crabs mixed with 10 mL of sterile water, the homogenate was centrifuged at $13,000 \times g$ for 20 min and the supernatant was filtered to remove bacteria via a filter having a pore size of $0.45 \mu\text{m}$. The filtered fluid was injected to healthy juvenile crabs with 10 male crabs and 10 females with each weight about 50 g (0.5 mL for each animal) followed by raising for 30 days, simultaneously, the homogenate of hepatopancreas of healthy crabs was used as a control. Moreover, the healthy crabs were only fed with the diseased or healthy tissues for 30 days to determine pathogenicity of diseased crabs and fed no other food. Each group consisted of 20 crabs and all groups were carried out in three biological repetitions. The treated animals were kept in tanks with water at 20°C during the experimental period.

Effects of elevated pH

The healthy mitten crabs with 50% male and 50% female and each weight about 50 g were cultivated at water temperature (20) according to the previous report [10] for 7 days. The crabs were divided into six groups and 20 crabs were raised in each group. The crabs in each group were raised in a 50 L tank which was used as a shelter followed by a photoperiod of 12 h of light and 12 h of dark and all groups were carried out in three biological repetitions.

The water with high pH values was set with Na_2CO_3 and NaOH. The pH meter was used to check the pH of water in a timely manner. Mitten crabs of each group were raised in water with different pH values (pH=8.0, 8.5, 9.0, 9.5 and 10.0) for two weeks. In order to maintain the stability of pH and water was replaced every 2 days.

Effects of avermectin

Avermectin, an insecticide, which is widely used in Chinese mitten crab cell cultures, to understand the toxicity effects of avermectin on crab hepatopancreata, the healthy mitten crabs (about 50g for each animal) were divided into four groups and 20 crabs with 50% male crabs and 50% female for each group were raised with different concentrations of avermectin (1 mg/mL, purchased from Wuhan Kang Shun Biological Pharmaceutical Co., Ltd. 10, 100 and 1000 times dilution according to manufacturer's instructions) for 20 days to check the toxicity effects of avermectin on healthy crabs.

Results

Characteristic symptoms and epidemiology

It had been reported that the hepatopancreas colour of HPND caused by infection of microsporidian *Hepatospora eriocheir* changed from golden yellow, light yellow to almost white from light to heavy infection. In this study, we found that the diseased crabs sampled from the crab farms had classic symptoms (Figure 1) of HPND,

hepatopancreas of the diseased crab became degenerated, atrophy and whitish. Obvious hydrops could be observed in inner cavity of diseased crab, while the healthy crab remained with golden yellow hepatopancreas (Figure 1).

The epidemiological investigation of HPND was carried out, 10% to 20% mortality rate occurred on most of farms by investigating famers in Suzhou city.

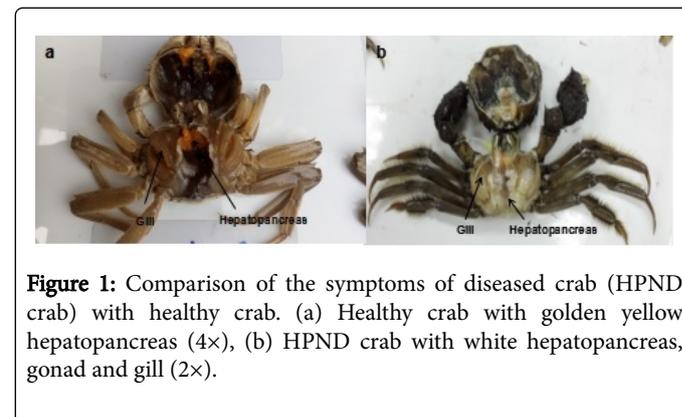


Figure 1: Comparison of the symptoms of diseased crab (HPND crab) with healthy crab. (a) Healthy crab with golden yellow hepatopancreas (4 \times), (b) HPND crab with white hepatopancreas, gonad and gill (2 \times).

Electron microscope observations

In order to determine whether the diseased crab with hepatopancreas necrosis was caused by infection of pathogenic microorganisms, the diseased crabs were inspected by a microscope, no microsporidian, parasites and fungi were found in the hepatopancreas, gill and muscle although bacteria could be observed in the gill. To further understand the pathogenic changes of the diseased crab, the ultrathin sections of tissues of the diseased crab sampled from the farms were examined with transmission electron microscopy.

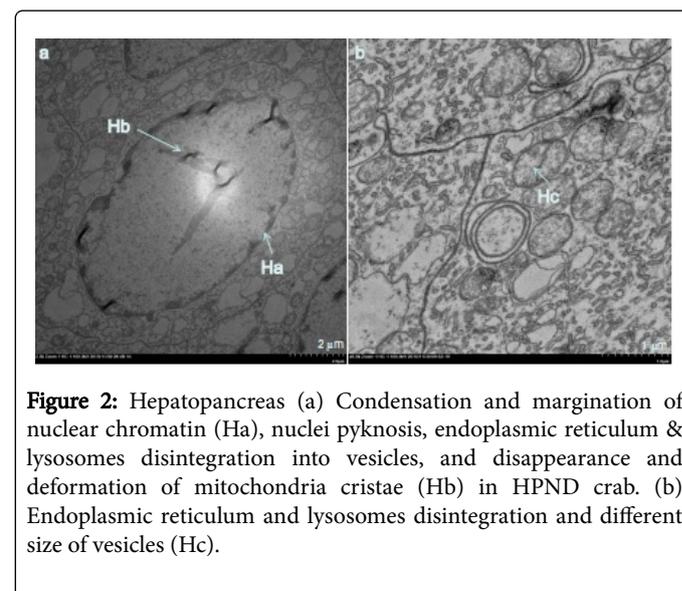


Figure 2: Hepatopancreas (a) Condensation and margination of nuclear chromatin (Ha), nuclei pyknosis, endoplasmic reticulum & lysosomes disintegration into vesicles, and disappearance and deformation of mitochondria cristae (Hb) in HPND crab. (b) Endoplasmic reticulum and lysosomes disintegration and different size of vesicles (Hc).

In hepatopancreatic cells of the diseased crab (Figure 2), condensation and margination of nuclear chromatin, disappearance and deformation of mitochondria cristae, and endoplasmic reticulum and lysosomes disintegration, and different size of vesicles could be observed Nuclear condensation, cell atrophy and intercellular space

and a lot of spermatogonium with distinct nuclear pyknosis and vesicles could be observed in gonad of the diseased crabs the membrane of spermatogonium was thickened and rucked up (Figure 3).

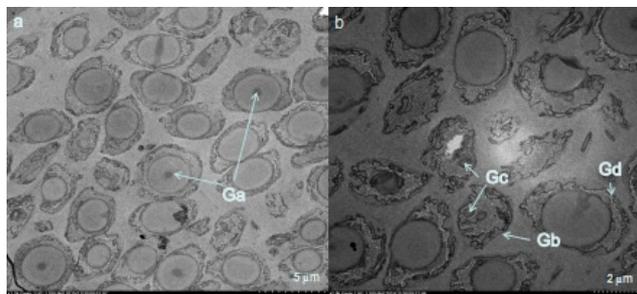


Figure 3: Gonad, (a) Nuclear condensation (Ga) in HPND crab, (b) Cell atrophy and intercellular space (Gb), scale=5000 nm. A lot of spermatogonium with distinct nuclear pyknosis and vesicles (Gc) observed in gonad of the diseased crab, the membrane of spermatogonium was thickened and rucked up (Gd).

The obvious pathological changes could be also found in the diseased gill, the cellular membrane degraded and mitochondrial cristae was disordered and disappeared (Figure 4). Muscle tissue of leg of the diseased crabs were loose and slack.



Figure 4: Gill, The cellular membrane degraded, and mitochondrial cristae was deformed and disappeared (Ca) in HPND crab.

In muscle cells of the diseased crabs, condensation and margination of nuclear chromatin (Figure 5) injury of nuclear membranes, and mitochondria disintegration could be observed, moreover, myofibril arrangement was loose and there were many vesicles between myofibril. Obvious pathological changes could be found in hepatopancreas, muscle, gill and gonad, but microorganisms including bacteria, fungi, microsporidian and viruses were not detected, suggesting that the diseased crabs sampled from the farms were not infected by pathogenic organisms.

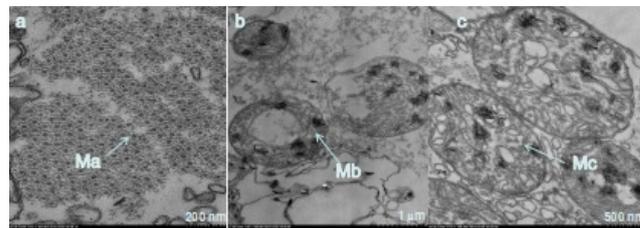


Figure 5: Muscle, (a) The myofibril of tissue from HPND myofibril arrangement was loose and there were many vesicles between myofibril (Ma), (b) Condensation and margination of nuclear chromatin (Mb), (c) Injury of nuclear membranes, mitochondria disintegration (Mc).

Experimental infectivity trial

Healthy crabs were fed with the sampled diseased crabs for 30 days, or injected with the filtered fluid prepared from hepatopancreas homogenate of the diseased crabs followed by raising for 30 days, there was no sign or symptom of HPND in the treated crabs, suggesting that the diseased crabs sampled from the farms were not infected by virus or microsporidian.

Effects of elevated pH

The results of raising crabs with different pH values of water showed that no death and no symptoms of HPND in crabs were found in the crabs raised in the water of pH 8.0- pH 9.0, the mortality rate of crabs raised in the water of pH 9.5 reached to 40% ($P < 0.01$) while at pH 10 all the crabs were died (Figure 6). The symptoms of the diseased crabs were similar with that of HPND, hepatopancreas color of the treated crab was gradually changed from yellow to whitish and finally the hepatopancreas became degenerated and atrophy.



Figure 6: Gross signs of HPND induced by water with high pH values in crabs. When healthy crabs were bred with water of high pH values, hepatopancreas nearly disappear in the intestinal tract. Four major developmental stages (a-c) of the disease were characterized, from light to heavy, with the hepatopancreas color turning from golden yellow, light yellow to almost white.

Effect of avermectin

No death and no symptoms of HPND in crabs were found after 20 days by raising in water with different concentrations of avermectin (diluted 10, 100 and 1000 times of working concentration according to manufacturer's instructions), however, a little toxic and side effects of avermectin were appeared on the crabs.

Discussion

E. sinensis hepatopancreas albinism was often occurred in some farms of Suzhou city, the symptoms of the diseased crab was very similar with HPND, electron microscope observation showed that no pathogens were found in tissues or cells of diseased crabs, which indicated that *E. sinensis* hepatopancreas albinism was caused by non-living agents [11], while a microsporidian *Hepatospora eriocheir* was found in the HPND crabs, suggesting that HPND was associated with infection of *H. eriocheir*, but experimental infectivity trial was not performed with *H. eriocheir*, so we cannot conclude the possibility that crab with hepatopancreas necrosis was infected by *H. eriocheir*. Analysis of meta-transcriptome data of the hepatopancreata from crabs with and without HPND indicated microsporidia are unlikely to cause HPND [4]. In this study, no microsporidian, parasites and fungi were found in the hepatopancreas, gill and muscle of the diseased crab sampled from the farms by microscopy inspection, moreover, transmission electron microscopy observation showed that there were no virus and microsporidian in the diseased crabs with hepatopancreas necrosis, furthermore, crabs with HPND could not be found in recursive infection experiment, suggesting that the diseased crabs sampled from the farms was not caused by virus and microsporidian infection.

Similar pathologic changes such as condensation and margination of nuclear chromatin, disappearance and deformation of mitochondria cristae, endoplasmic reticulum disintegration, could be observed in the hepatopancreas, gill, muscle and gonad of the diseased crabs, these abnormal changes were very similar with cell apoptosis caused by chemical stimulation. In this study, we found that hepatopancreas color of the raised crabs in water with high pH values was gradually changed from yellow to whitish and finally the hepatopancreas became degenerated and atrophy. But crabs with symptom of hepatopancreas necrosis were not found when crabs were raised in water with different concentrate of avermectin, suggesting that HPND of crab might be induced by water of high pH value.

The pH of water has great influence on breeding of mitten crabs. It was found, however that massive aquatic plants such as *Potamogeton crispus*, *Ceratophyllum demersum* and *Vallisneria asiatica* [12,13], and algae blooms increase the pH of water, which adversely affect the breeding of mitten crabs [14]. The results of this study showed that 40% crabs were died and had symptoms of hepatopancreas necrosis when healthy crabs were raised at the water of pH 9.5, suggesting that hepatopancreas necrosis of crabs was associated with high pH value of water.

Insecticide avermectin is widely used in Chinese mitten crab cell cultures, the main indication for use of avermectins is infestations with ectoparasitic copepods, avermectin is well-documented as very effective on all developmental stages of the parasites [15]. Previous study indicated that the toxicity of avermectin on juvenile Chinese mitten crab was low [16]. In the present study, a little toxic effects of avermectin on the crabs was observed, however, no death in crabs was found after 20 days by raising in water with different concentrations of avermectin, further indicating the toxicity of avermectin on crab was lower. A simulated aquaculture pond ecosystem was used to study the environmental behavior of avermectin with Ultra-performance liquid chromatography tandem mass-spectrometry, the results indicated that avermectin was transferred to the sediment, *Elodea nuttallii*, and aquatic animals, however avermectin was only detected in the gill of Chinese mitten crab but was not detected in hemolymph, muscle, or hepatopancreas [17]. In this study, no symptoms of HPND in crabs

were found after 20 days by raising in water with different concentrations of avermectin, suggesting HPND might be not induced by avermectin. Aquaculture ecosystem usually was polluted with insecticide, moreover, some insecticides, for example pyrethroids, were used in crab culture. Carboxylesterase is the major enzyme that breaks down pyrethroid, Shen et al. reported that the expression of carboxylesterase family genes was significantly up-regulated in the crabs with HPND [4], moreover, pyrethroid was known to harm the hepatopancreas [18], therefore, whether insecticides are involved in HPND in crabs that should be further studied

Conclusion

Electron microscopic observations indicated that the hepatopancreatic cells, spermatogonium, gill tissues and muscle cells of the crabs with HPND showed severe structural and morphological changes, but microorganisms including bacteria, fungi, microsporidian and viruses were not detected; the healthy crabs were fed/injected with diseased tissues, the symptoms of HPND were not found, suggesting that the diseased crabs with HPND sampled from the farms were not infected by pathogenic organisms. In addition, examination of the toxic effect of avermectin and high pH water indicated that HPND can be induced by water of high pH value and little toxic and side effects of avermectin were appeared in the crabs.

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