

# Investigation of the hazardous substance causing crayfish-induced rhabdomyolysis via a mouse model, a hemolysis assay, and a cytotoxicity assay

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**Abstract** The hazardous substance from *Procambarus clarkii* could cause rhabdomyolysis. Mice were administered mashed muscles and gills, intestines, and glands (GIG) obtained from cooked crayfish by gavage. Three of seventy-two mice administered with GIG (with an incidence rate of 4.17 %) appeared to have rhabdomyolysis symptoms with the serum creatine kinase (CK) content increased by more than 5 times, as well as the pathological changes in livers, spleens, kidneys, and skeletal muscles. Furthermore, the cytotoxicity and hemolysis analysis of the palytoxin revealed that the methanol/water extracts from GIG of crayfish were not inhibited by ouabain effectively, which pointed out the hazardous substance causing rhabdomyolysis was not allergen or palytoxin. These research results could be valuable in further comprehensive experiments and lay a foundation for basic rhabdomyolysis research.

**Keywords** *Procambarus clarkii* · Rhabdomyolysis · Hazardous substance · Mouse model

## Introduction

Rhabdomyolysis is a rare and serious myopathy, which is frequently triggered by freshwater fish and crustaceans. Recently, twenty-three individuals had been recognized as having Haff disease (a kind of rhabdomyolysis disease) after eating cooked *Procambarus clarkii* in the city of Nanjing. The clinical manifestations of rhabdomyolysis were mainly the increased serum creatine kinase (CK) and myoglobin (Mb), and also there appeared coffee-colored urine [1]. The release of the CK, Mb, and other toxic substances also in turn injured the kidneys and skeletal muscle via the bloodstream [2, 3].

Rhabdomyolysis is a worldwide problem. From the year 1924 to 1934, about 1,000 people who had burbot, eel, or pike as food suffered from rhabdomyolysis [2]; from the year 1934 to 1984, many cases of rhabdomyolysis appeared in Sweden and the Soviet Union; and from the year 1984 to 1997, twelve people suffered from rhabdomyolysis in America after they ate buffalo fish [1]. Besides, in the year 2001 and 2008, nine cases of rhabdomyolysis caused by crayfish and twenty-seven cases of rhabdomyolysis caused by black-finned colossoma or freshwater pompano were respectively reported in Louisiana and Brazil [4]. These cases suggested that the rhabdomyolysis might be triggered by freshwater products. In addition, mice, which were fed with hexane-soluble products extracted from the cooked buffalo fish, appeared to have muscle impairment and red-brown urine, which were consistent with the symptoms of rhabdomyolysis [1]. However, the etiology of rhabdomyolysis was not clear as yet.

In China, farming crayfish has become more and more popular with a yield of 563,281 and 486,319 t in 2011 and 2012, respectively. With the yield increasing, more and more cases of unexplained rhabdomyolysis occurred. In

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the year 2000, six cases of rhabdomyolysis caused by crayfish occurred in Beijing. During the fall of 2010, twenty-three cases of rhabdomyolysis caused by crayfish occurred in Nanjing. In China, the etiology was still unknown after more than 900 kinds of chemical substances were tested by the Centers for Disease Control and Prevention [3]. In America, the Centers for Disease Control (CDC) investigated the kinds of fish, which could lead to rhabdomyolysis, and found that the substance which could cause mice suffering from rhabdomyolysis was thermostable and soluble in hexane [2]. Crayfish-induced rhabdomyolysis might be caused by a kind of poisonous substances with the characteristics of high heat resistance and a strong toxicity [3]. Moreover, the poisonous substances might be associated with an allergen that had not yet been determined [5]. Palytoxin, which is detrimental to cells, might be one kind of hazardous substance that could cause the phenomenon of hemolysis. However, palytoxin and its analogues could be specifically inhibited by ouabain. The purpose of this article was to explain whether palytoxin and its analogues caused rhabdomyolysis and to provide fundamental data on hazardous substances that caused crayfish-induced rhabdomyolysis via a mouse model, a hemolysis assay and a cytotoxicity assay.

## Materials and methods

### Materials

The crayfish were purchased alive from the Bashi market of Xiamen, China in the fall of 2010. The crayfish were washed thoroughly, then cooled down in icy water after being cooked in water at 100 °C for 20 min. GIG (gills, intestines and glands, glands mainly including hepatopancreas and gonad) and muscles were collected, weighed, and stored at –70 °C, respectively.

Ouabain, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Diagnostic kits of alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum creatine kinase (CK), lactate dehydrogenase (LDH), creatinine (CR), and urea nitrogen (UN) were purchased from Kehua (Shanghai, China). The hematoxylin–eosin (HE) staining kit was purchased from Beyotime (Jiangsu, China). Horseradish peroxidase-conjugated goat anti-mouse IgE (goat anti-mouse HRP-IgE) was purchased from Southern Biotech (Birmingham, AL, USA). Human hepatoma cells (SMHC-7721) were obtained from the State Key Laboratory of Cell Stress Biology (Xiamen University, China). Other reagents were all analytically pure reagents.

PBS–CB had a volume of 10 mM phosphate buffer solution (PBS, pH 7.2) with 0.9 % NaCl, 0.1 % (w/v) BSA,

1 mM CaCl<sub>2</sub>, and 1 mM sodium tetraborate, with filtration sterilization.

### Animals

Ninety Imprinting Control Region (ICR) mice (female, body weight of approximately 19.0–20.0 g, 4 weeks old, specific pathogen free, SPF), their bedding, and food were all purchased from Slac (Shanghai, China). The mice were acclimatized for 1 week before use. The animals were fed under controllable temperature (22 ± 2 °C), humidity (50–70 %), and lighting (12 h-light and 12 h-dark cycle, lights were turned on at 7:00 a. m.). Food and water were provided ad libitum [6].

### Preparation of serum and urine

Analysis of the hazardous substance for the crayfish-induced rhabdomyolysis was repeated three times (12 December 2010, 10 March 2011 and 18 June 2011) in the mouse model.

The mouse model experiment was designed according to the method of Sosa et al. [7] with some modifications. Thirty ICR mice were divided into five groups randomly and fasted overnight (12 h) before treatment. Muscles and GIG of crayfish was ground, dissolved in PBS buffer (pH 7.0, containing 0.9 % NaCl), and then administered to mice at 0.5 g and 4.0 g sample per wt/kg body weight of mouse by gavage, respectively. Control mice received PBS exclusively at the same dose. Mice were treated by gavage twice at 0 h and 12 h. After each gavage test, food was picked up for 1 h. During the observation period of 0–36 h, symptoms and mortality were recorded. After observation, the sera were collected by low-speed centrifugation at 3,000×g for 10 min at 4 °C. The tails of mice were lifted lightly in order to trigger micturition. The urine samples were collected in plastic tubes.

Measurement of serum parameters (ALT, AST, CK, and LDH) and urine parameters (CR and UN)

The serum and urine parameters were measured in the entry–exit inspection and quarantine bureau of Xiamen, China. Clinical chemistry of serum parameters and urine parameters were measured by colorimetric methods using an automatic biochemical analyzer (RILI 7600, Hitachi, Tokyo, Japan) with diagnostic kits. All the parameters were measured in triplicate.

### Measurement of IgE in serum

The enzyme-linked immunosorbent assay (ELISA) was used to measure the sera IgE levels of mice according to the

method of Pan et al. [8]. The dry muscle samples (0.15 g) and GIG samples of crayfish were ground and mixed with 25 ml PBS buffer (containing 3 % NaCl), respectively. Mice were administered with muscles or GIG through the method of gavage, respectively. The sera were collected after centrifugation with blood of mice at  $12,000\times g$  for 25 min, respectively. The concentration of IgE in the sera was measured by microplate reader (Bench mark 96, Bio-Rad, Hercules, USA). When the absorbance was more than 2.1 times compared with the control group at 450 nm, the serum sample was defined as a positive sample. All assays were performed in triplicate and the mean values were used.

#### Histological analysis

Histological analysis was designed according to the methods of Mona et al. [9] and Fiuza et al. [10] with some modifications. The liver, spleen, kidney, and skeletal muscles, which had all been embedded in paraffin after washed with PBS and fixed with 10 % (v/v) formalin, were sectioned (5  $\mu\text{m}$  thickness) by a rotary microtome (RM2235, Leica, Wetzlar, Germany). The paraffin-sectioned samples were deparaffinized, rehydrated, and stained with hematoxylin–eosin (HE) according to the instructions of the HE staining kit, and analyzed by light microscopy (50i, Nikon, Tokyo, Japan).

#### Preparation of methanol/water extract (MWE) of crayfish

The methanol/water extract (MWE) was prepared according to the method of Ledreux et al. [11] with some modifications. The dry muscles and GIG sample (1.0 g) of crayfish were ground and placed in an ultrasound chamber with 30 ml methanol/water (1:1, v/v) for 8 min. After centrifugation at  $6,000\times g$  for 20 min, the supernatant was evaporated to remove the methanol, and then dissolved in 6 ml PBS–CB with filtration sterilization after lyophilization. The filtrate was collected as MWE. MWEs of cooked *Penaeus vannamei* Boone and *Oratosquilla oratoria* were all prepared by the same method mentioned above.

#### Hemolysis assay

The hemolysis assay was based on the method of Bignami [12] with some modifications. Blood cells were diluted 50 times with PBS–CB containing 1 M ouabain or without ouabain, respectively. Then, 100  $\mu\text{l}$  of diluted blood cells were distributed in 96-well tissue culture plates in triplicate and incubated at 37 °C for 30 min. Crayfish MWE was serially diluted (1:1, 1:5, 1:25, 1:125, 1:625 and 1:3125) with PBS–CB, meanwhile MWEs from *Penaeus vannamei* Boone and *Oratosquilla oratoria* were not diluted. Aliquots

of 100  $\mu\text{l}$  of MWE samples were severally added into the tissue culture plates mentioned above. The negative control (hemolysis rate: 0 %) was prepared by mixing the blood with PBS–CB solution (100:100  $\mu\text{l}$ ), while the positive control (hemolysis rate: 100 %) was prepared by mixing the blood solution with MilliQ ultrapure water (100:100  $\mu\text{l}$ ). After continuing incubation at 37 °C for 4 h, the tissue culture medium was centrifuged ( $4,000\times g$ , 10 min) and the value of optical density (absorbance) of the supernatants was measured at 550 nm.

#### Cytotoxicity assay

The cytotoxicity assay was based by the method of Ledreux et al. [11] with some modifications. Human hepatoma cells, SMMC-7721, were added into a 96-well tissue culture plate (10,000 cells/well), and then incubated in a CO<sub>2</sub> incubator (Thermo, Waltham, MA, USA) at 37 °C. After incubating for 24 h, the cell culture medium was removed. Then, culture medium with 500  $\mu\text{M}$  ouabain and without 500  $\mu\text{M}$  ouabain was added into corresponding wells with a volume of 200  $\mu\text{l}$  per well, respectively. After incubating for 2 h, 100  $\mu\text{l}$  different proportions of crayfish MWEs (1:1, 1:5, 1:25, 1:125, 1:625, and 1:3,125 serially diluted with medium) were added into the corresponding wells, and they continued to incubate for 20 h. At the end of the incubation period, the medium was discarded, and then MTT was added into each well. The plates were incubated for 4 h at 37 °C. Finally, the MTT was discarded and then dimethyl sulfoxide was added into each well to dissolve the formazan. The absorbance was measured at 550 nm. The control group was formed by adding the medium instead of the MWE.

#### Statistical analysis

Data were expressed as mean  $\pm$  standard deviation. Significant differences between control groups and experimental groups were calculated using the Student-Newman-Keuls test by SPSS Statistics 17.0. Data were considered significantly different when the *p* value was  $<0.05$ .

## Results

#### Analysis of the symptom induced by crayfish in the mouse model

Analysis of the hazardous substance in crayfish-induced rhabdomyolysis had been repeated three times (2010-12-12, 2011-3-10 and 2011-6-18) in the mouse model, and the three experimental results revealed uniformity. In the mouse model experiments, five GIG-treated mice (20110618-G0.5-No.2,

20110618-G0.5-No.6, 20110618-G4.0-No.3, 20110618-G4.0-No.4, and 20110618-G4.0-No.6) appeared to have muscle weakness, which is a common phenomenon in rhabdomyolysis. However, other typical allergic symptoms, such as puffiness around the eyes and mouth, diarrhea, and cyanosis, could not be observed in mice. The weights of the treated mice and the control mice were not significantly different. The results showed that maybe some hazardous substances existing in GIG caused the muscle weakness rather than GIG itself.

The effect of GIG on the increase of the UN and CR in urine

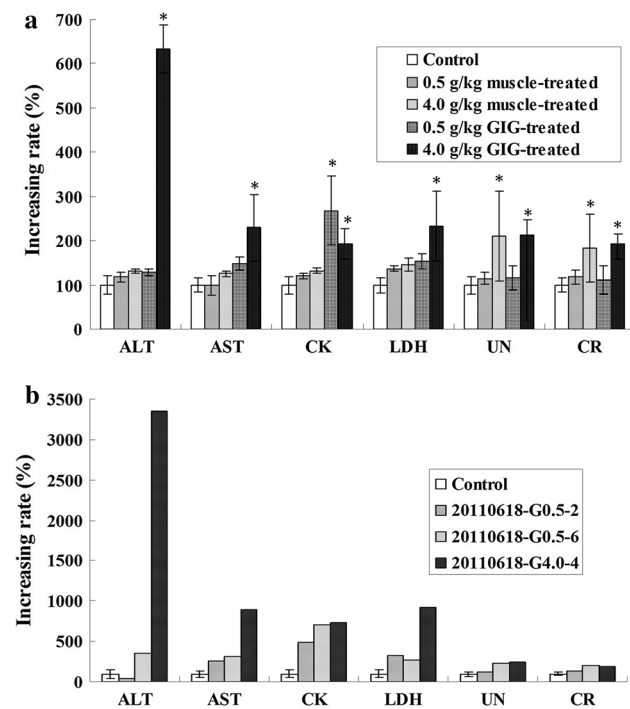
The urine parameters are shown in Fig. 1a. In the urine test, mice which were administered the mashed GIG of crayfish at the dose of 4.0 g/kg body weight of the mice showed the higher average values of 102 % of the UN and 93 % of the CR ( $P < 0.05$ ) after comparison with the control group. However, mice, which were administered with mashed muscles of crayfish at 4.0 g/kg body weight showed the

higher average values of 120 % of the UN ( $P < 0.05$ ) and 84 % of the CR ( $P < 0.05$ ) after comparison with the control group. The coffee-colored urine could not be observed in all treated mice; however, and the color of urine was darker in the GIG-treated mice (especially 20110618-G0.5-No.2, 20110618-G0.5-No.6, and 20110618-G4.0-No.4) compared to that of the control (as shown in Fig. 1b).

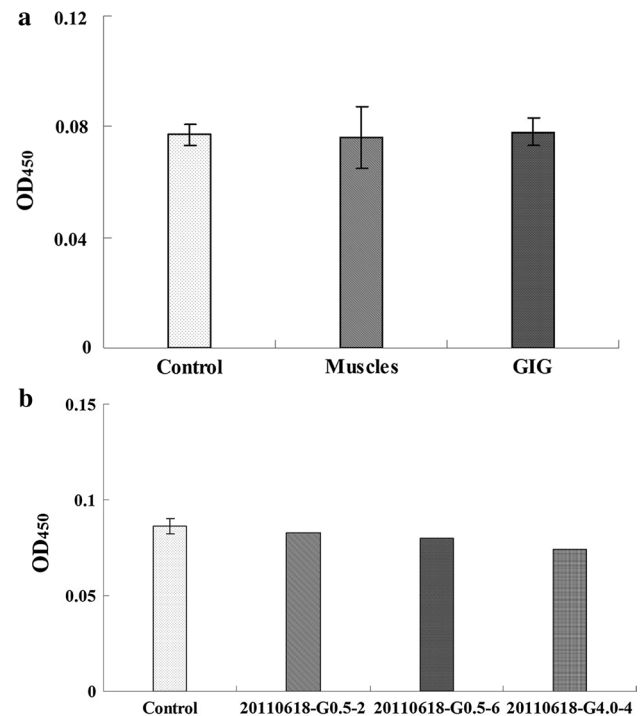
The effect of GIG on the serum parameters

As shown in Fig. 1a, no significant differences were found between control mice and muscle-treated mice. However, there were still some considerable differences between the control mice and the experimental mice treated with GIG at a dose of 4.0 g/kg body weight in the average value of serum parameters. The levels of serum parameters of ALT (534 %,  $P < 0.05$ ), AST (129 %,  $P < 0.05$ ), CK (94 %,  $P < 0.05$ ), and LDH (133 %,  $P < 0.05$ ) in GIG-treated mice were much higher than the levels in control mice, which suggested that GIG could lead to the increase of the serum parameters. Three mice with very outstanding serum parameters are shown in Fig. 1b.

The effect of allergy to rhabdomyolysis in the mice was measured by ELISA of IgE. The test results of the



**Fig. 1** Analysis of the serum and urine parameters. **a** The increasing rates of parameters in serum and urine of the treated mice (muscle-treated mice and GIG-treated mice) with different doses after comparison with control mice. The ALT, AST, CK, LDH, UN, and CR are the abbreviations of alanine aminotransferase, aspartate aminotransferase, creatine kinase, lactate dehydrogenase, urea nitrogen, and creatinine, respectively. The error bars represent standard deviations. \* $p < 0.05$  of the Student-Newman-Keuls test by SPSS Statistics 17.0 with respect to the control. **b** The increasing rates of different parameters (ALT, AST, CK, LDH, UN, and CR) in serum and urine of the three GIG-treated mice after comparison with the control mice



**Fig. 2** Analysis of the IgE level in serum. **a** The optical density of IgE at 450 nm in the control mice and the treated mice (muscle-treated mice and GIG-treated mice). The error bars represent standard deviations. **b** The optical density of IgE at 450 nm in the control mice and the three GIG-treated mice. The error bars represent standard deviations

experimental groups were determined to be positive if the numerical value of sera IgE exceeded 2.1 times the values obtained in the control groups [6]. As shown in Fig. 2a, the analysis of sera IgE levels between the GIG-treated mice (sera IgE level:  $0.077 \pm 0.004$ ) and the muscle-treated mice (sera IgE level:  $0.076 \pm 0.011$ ) revealed that the results were negative after compared with the control groups (sera IgE level:  $0.078 \pm 0.005$ ). It was the same with the IgE levels of the three GIG-treated mice (20110618-G0.5-No.2, 20110618-G0.5-No.6, and 20110618-G4.0-No.4) (shown in Fig. 2b). These results suggested that the rhabdomyolysis might not be caused by allergic reactions to crayfish.

### Histological analysis

Histological analysis showed that some tissues (such as tissues of the liver, spleen, kidney, and skeleton) changed in GIG-treated mice to different degrees, especially in mice treated with GIG at a dose of 4.0 g/kg body weight. However, changes in the kidneys were only found in three GIG-treated mice (20110618-G0.5-No.2, 20110618-G0.5-No.6, and 20110618-G4.0-No.4). Moreover, in contrast to the GIG-treated groups, the phenomenon of muscle-treated mice was not obvious.

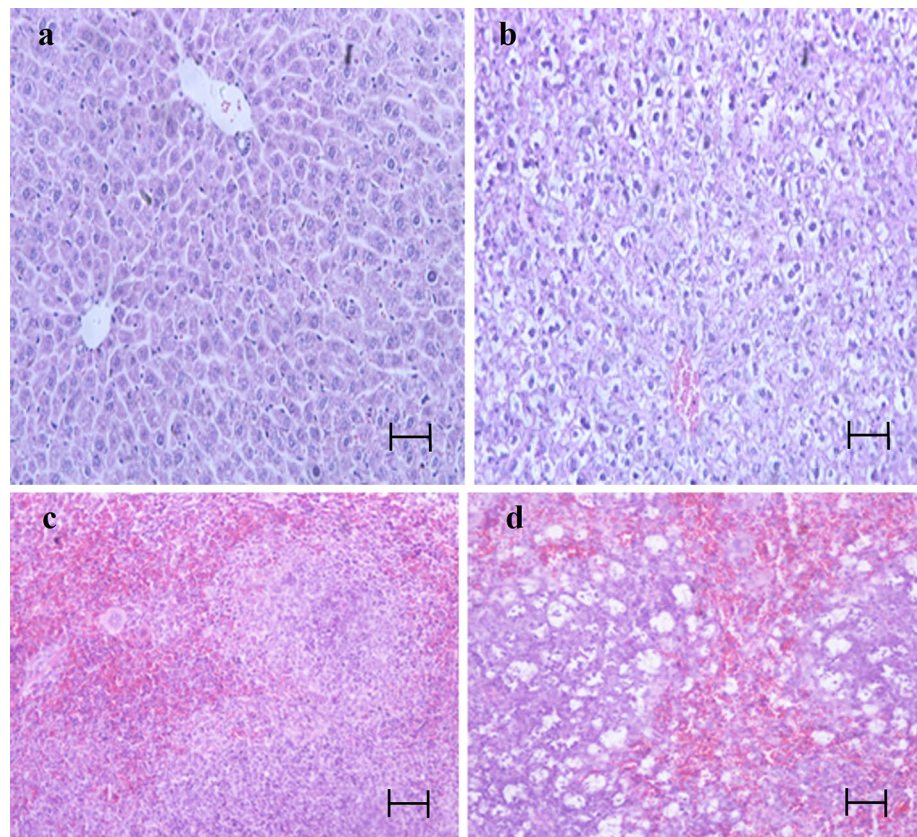
The typical changes of tissues in mice that were administered mashed crayfish GIG by the method of gavage were

observed. As shown in Fig. 3, compared with the control mice (Fig. 3a), hepatic cord disorder, hepatic sinus dilation, and hydropic degeneration were observed in the liver of the GIG-treated mice (Fig. 3b). Moreover, severe vacuolization was observed in the white pulp of the spleen of the GIG-treated mice (Fig. 3d) after comparison with that of the control mice (Fig. 3c). In the kidneys of the control mice (Fig. 4a), the cortex sections showed that the renal corpuscles and glomeruli were normal with distal and proximal convoluted tubules distributed around them. However, congestion and atrophic glomeruli were observed in the kidneys of the GIG-treated mice (Fig. 4b). Besides, after comparison with that of the control mice (Fig. 4c), the muscle fiber was tapered with a disappearance of the horizontal stripe in skeletal muscles of the GIG-treated mice (Fig. 4d). Therefore, the experimental phenomena stated clearly that some hazardous substance existing in GIG caused tissue lesions in different degrees that were believable and authentic.

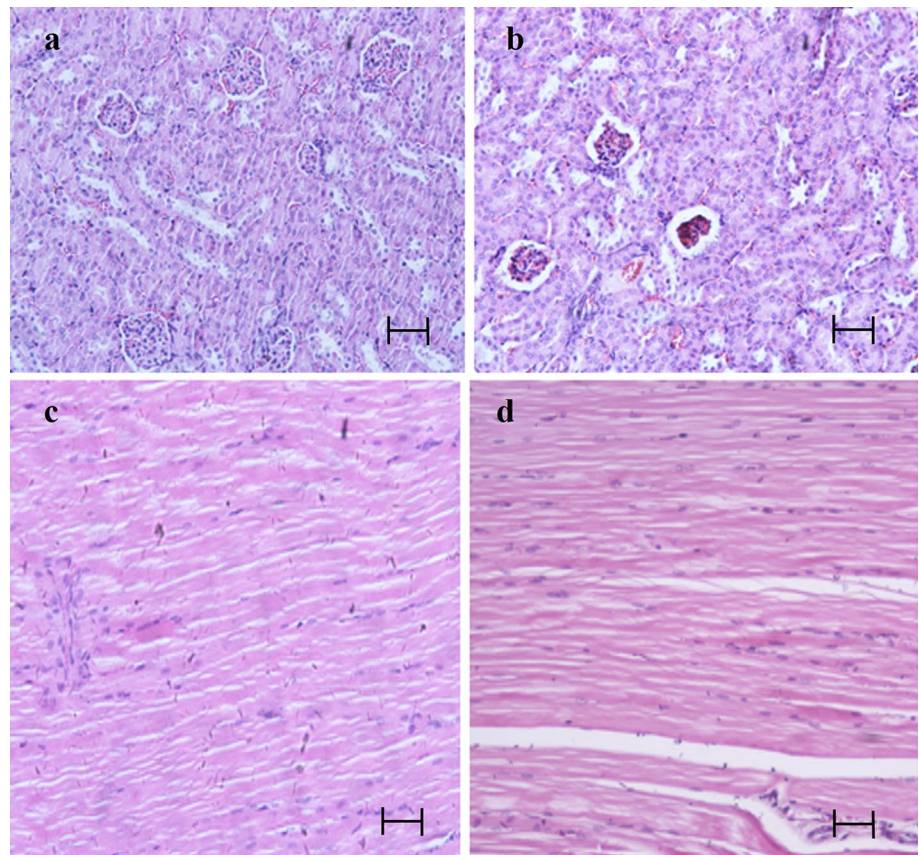
### Hemolysis and cytotoxicity assay

The assay of hemolysis could be detected and analyzed by measuring the absorbance of the hemoglobin, which was released from incubating red cells. The hemolysis results are shown in Fig. 5a. From the experimental data,

**Fig. 3** The histological examination of the liver and the spleen of the control mice and the GIG-treated mice. The liver (**a** the control mouse; **b** the mouse treated with GIG at 0.5 g/kg). The spleen (**c** the control mouse; **d** the mouse treated with GIG at 0.5 g/kg). Hematoxylin–eosin stain (bars = 250  $\mu$ m, eyepiece magnification:  $\times 20$ )



**Fig. 4** The histological examination of kidneys and skeletal muscles from the control mice and the GIG-treated mice. The kidneys (**a** the control mouse; **b** the mouse treated with GIG at 4.0 g/kg). The skeletal muscles (**c** the control mouse; **d** the mouse treated with GIG at 4.0 g/kg). Hematoxylin–eosin stain (bars = 250  $\mu$ m, eyepiece magnification:  $\times 20$ )



hemolysis could be caused by MWE of GIG from crayfish evidently; however, it could not be caused by MWE of GIG from *Penaeus vannamei* Boone and *Oratosquilla oratoria*. Muscles from crayfish, *Penaeus vannamei* Boone and *Oratosquilla oratoria*, also could not cause hemolysis. Furthermore, the hemolysis could not be inhibited by ouabain.

The palytoxin and the analogue of palytoxin, which could be prevented by pre-treating with the ouabain, were detrimental to cells [11]. The SMMC-7721 cells were used to detect the palytoxin-like compounds in MWE of crayfish. The results of the cytotoxicity assay of MWE are shown in Fig. 5b. The MWE of muscles and GIG could influence the vitality of SMMC-7721 cells, and with the increase of the concentration of the MWE, the toxic effect was more serious. At the same time, the cells including the ouabain also could not inhibit the toxic response.

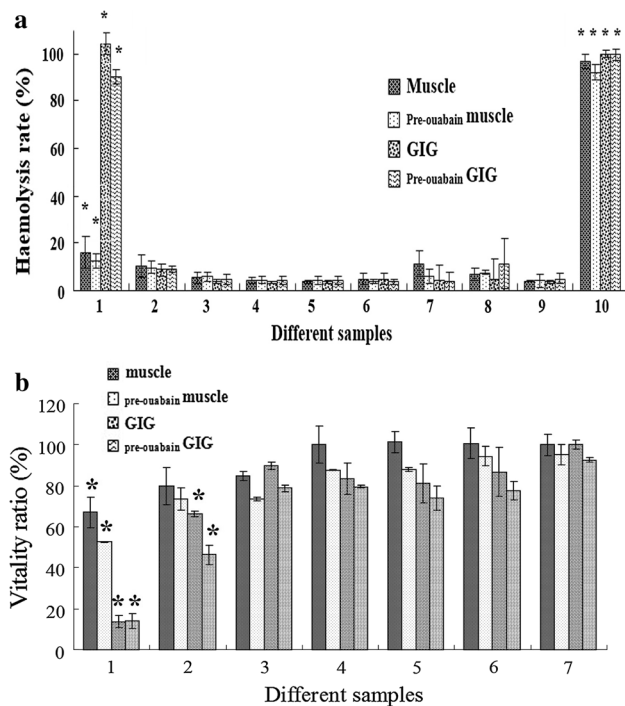
The results of hemolysis and the cytotoxicity assay suggested that the hazardous substance in MWE of crayfish was neither palytoxin nor the analogues of palytoxin.

## Discussion

Haff disease is defined as rhabdomyolysis after eating cooked seafood. In this study, the symptoms of five

GIG-treated mice were similar to the symptoms in rhabdomyolysis, especially three of them (20110618-G0.5-No.2, 20110618-G0.5-No.6, and 20110618-G4.0-No.4). Research has shown that most patients mainly have diffuse myalgia and chest pain, and those with mild symptoms recovered quickly within 2–3 days [1, 2]. This was the same phenomenon that appeared in our experiment. Furthermore, there were no typical allergic symptoms in mice according to the evaluation of allergic symptoms [6]. Thus, crayfish GIG may possibly contain one or more hazardous substances that can cause rhabdomyolysis in some mice.

The crayfish GIG contains certain kinds of enzymes in the striated muscle, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactic dehydrogenase (LDH), which are very likely to be released into the blood stream when the striated muscle get injured [13]. CK is an experimental indicator used for defining rhabdomyolysis when it is 5 times more than the normal value [1]. Numerous reports have described that ALT, AST, and LDH would increase more than fivefold when someone has suffered from rhabdomyolysis [14–16]. The phenomenon of rhabdomyolysis was found in three GIG-treated mice with high levels (5 times or higher compared with the normal value) of CK, AST, ALT, and LDH (Fig. 1b), and the level of ALT was 34 times higher than the normal



**Fig. 5** The hemolysis assay and cytotoxicity assay of the MWE of crayfish. **a** The hemolysis assay of the MWE of crayfish. 1–6 The dilution of MWE of crayfish (dilution ratios were 1:1, 1:5, 1:25, 1:125, 1:625, and 1:3,125, respectively), 7 1:1 dilutions of MWE from *Penaeus vannamei* Boone, 8 1:1 dilutions of MWE from *Oratosquilla oratoria*, 9 PBS–CB, 10 ultrapure water. **b** The cytotoxicity assay of the MWE of crayfish. 1–6 The dilution of MWE of crayfish (dilution ratios were 1:1, 1:5, 1:25, 1:125, 1:625, and 1:3,125, respectively), and 7 medium (control group). The error bars represent standard deviations. \* $p < 0.05$  of the Student–Newman–Keuls test by SPSS Statistics 17.0 with respect to the control

value. The average values of the GIG-treated mice were significantly higher than the muscle-treated and the control mice (Fig. 1a), from which we deduced that the three GIG-treated mice had suffered from rhabdomyolysis but the others had not, and the GIG had a greater probability of containing the hazardous substance that could cause the rhabdomyolysis.

In addition, 3 of the 72 treated mice (including muscle-treated and GIG-treated mice) suffered from rhabdomyolysis (with an incidence rate of 4.17 %), which had a higher incidence rate than the crowd morbidity. For example, in Nanjing where the crayfish-induced rhabdomyolysis broke out, only 23 people suffered from rhabdomyolysis in the three million people who ate crayfish every month. This situation might be attributed to the fact that the dose in mice in our study was possibly higher than the human daily intake and most people ate muscles, however, very few ate GIG. Therefore, there was a small probability that crayfish could cause the rhabdomyolysis in daily intake. In the experiment, some mice treated with crayfish GIG had

suffered from rhabdomyolysis but others not, this reflected the individual differences in rhabdomyolysis.

In the process of rhabdomyolysis, the muscle cells (containing Mb) were released into the bloodstream, they reached the kidneys, easily blocked the renal tubule, and finally affected the renal function [3]. So, the change in renal function was a typical histological lesion in rhabdomyolysis. In this study, serious changes were observed in the kidneys of three GIG-treated mice (20110618-G0.5-No.2, 20110618-G0.5-No.6, and 20110618-G4.0-No.4), which were paralyzed and had high levels of serum parameters (Fig. 1b). Thus, these three GIG-treated mice suffered from rhabdomyolysis. The IgE antibody is an allergic reaction medium, under normal circumstances, the IgE content is at a low level in the serum. When an allergy occurs, the IgE levels are significantly increased. So, the elevated IgE in serum was the most powerful indicator for allergic disease. However, with the IgE levels were not increased significantly in experiments (Fig. 2), the rhabdomyolysis might not be caused by allergic reactions of crayfish.

The etiology of crayfish-induced rhabdomyolysis has not yet been determined. In Japan, it was speculated that palytoxin, which existed in marine fish (such as serranid fish, salmon and cowfish), could be enriched by food chains, and thereby caused rhabdomyolysis poisoning [17–19]. However, the hazardous substance had not yet been determined though more than 900 types of chemical substances were tested in China [3]. In the present research, the symptoms of the GIG-treated mice [paralysis, the high value of serum CK, diseased kidneys and skeletal muscles (Fig. 4)] were similar to the reports of palytoxin-treated mice [7]. However, whether the hazardous substances, which caused crayfish-induced rhabdomyolysis, were palytoxin and its analogues, has not been determined, so more data on palytoxin is needed, such as the assays of hemolysis, cytotoxicity, and immunoassays.

In the hemolysis analysis, palytoxin and its analogues, which caused the phenomenon of hemolysis, were specifically inhibited by ouabain [20]. Palytoxin was bound to the sodium pump and converted the enzyme into a channel, besides ouabain was a glycoside poison, which could bind to and inhibit the action of the  $\text{Na}^+/\text{K}^+$  pump in cell membranes. Their binding sites were not identical; however, they shared some structural determinants for binding [21]. Furthermore, the hemolysis assay (incubation at 37 °C for 4 h) was sensitive enough to detect 0.01 ng palytoxin/ml in alcohol/water extract [12], which corresponds to 0.1  $\mu\text{g}$  palytoxin/kg of shellfish [11]. This concentration was 300 times lower than the proposed regulatory threshold of 30  $\mu\text{g}/\text{kg}$  shellfish [22]. Thus, a hemolysis assay was subsequently carried out.

In our hemolysis analysis, only the MWE of GIG induced significant differences in hemolysis after compared

with PBS–CB, *Penaeus vannamei* Boone, and *Oratosquilla oratoria* (Fig. 5). Furthermore, the phenomenon of hemolysis could not be inhibited by ouabain too. Moreover, a crayfish is a kind of freshwater crustacean, so in the food chain of the freshwater crustacean, palytoxin could not be abundant. Therefore, the principal hemolytic agent in MWE of crayfish GIG could not be palytoxin and its analogues, and palytoxin and its analogues were probably not in the crayfish. Thus, the hazardous substance, which could cause the rhabdomyolysis, was not palytoxin; it might be an unknown hazardous substance.

These research results clearly demonstrate that the rhabdomyolysis caused by crayfish related to an unknown hazardous substance (neither allergen nor palytoxin) that existed in the GIG of crayfish, which could be a valuable result for further comprehensive experiments and lay a foundation for basic rhabdomyolysis research.

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