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Research Article

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Tetrodotoxin detoxification capability of *Tachypleus tridentatus*

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ABSTRACT

Although tetrodotoxin (TTX) is an extremely neuro-toxic compound, its activities have potential applications as medicines. There must be an efficient antidote for this toxin to minimize risks in clinical trial as well as in poisoning from TTX consumption. Therefore, screening for compounds which can neutralize, detoxify the toxicity of TTX or reduce its negative effects is necessary. Previous studies showed that hemolymph from Carcinoscorpius rotundicauda containing TTX binding protein could detoxify TTX in mice. This study investigated for binding protein as well as TTX detoxification capability of Tachypleus tridentatus hemolymph to hypothesize the main role in TTX detoxification. The result of this study illustrated that hemolymph Tachypleus tridentatus with the dose less than 20mg protein per mouse unit showed no capability in TTX detoxification even though there was survival prolong insignificantly. The study also checked hemolymph Carcinoscorpius rotundicauda, showing there was no capability in TTX detoxification. A major protein of hemolymph, purified hemocyanin also had no activity in TTX detoxification. In summary, TTX detoxification in capability in TTX detoxification was not due to hemolymph or hemocyanin.

Key words: tetrodotoxin, *Carcinoscorpius rotundicauda*, *Tachypleus tridentatus*, hemocyanin, hemolymph, detoxification

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INTRODUCTION

Tetrodotoxin (TTX), a venom composition of buffer fish was first isolated in 1909 and the structure illustrated in 1964 [1]. Many researches proved that TTX was produced by symbiotic bacteria, then accumulated in different organs of buffer fish [2]. Over ten TTX producing bacteria strains were found in different organs of buffer fish including *Pseudomonas* spp., *Serratia*, *Bacillus* spp., *Aeromonas*, *Actinomyces*, *Microbacterium*, *Providentia*, *Enterococcus* [3,4]. On the other hand, TTX was found commonly in various types of animal including the *California newt Tarichi torosa*, the *goby Gobius criniger*, the gastropod mollusks *Charonia sauliae*, the xanthid crab *Atergatis floridus*, and the blue-ringed octopus *Octopus maculosus* [5]. Because of its unique structure containing hydroxyl groups, an ortho ester, and a cyclic guanidine with hemiaminal showing strong affinity to preferentially neuronal, and muscle sodium channels, it is a potent neurotoxin of low molecular weight. The symptoms can appear within hours after consumption including oral numbness, muscular weakness, nausea, vomiting, and sensory deficit. In serious case, it will be followed by flaccid paralysis and caused death because of the respiratory failure [6]. Although TTX is an extremely toxic compound, it has been researching for clinical uses in relieving chronic pain and cancer.

Horseshoe crabs belong to the phylum, *Arthropoda*, along with crabs, insects, and other invertebrates with jointed legs, but their closest living relatives are spiders and scorpions. There are only four species of horseshoe crabs in existence in the world today. These are *Limulus polyphemus* found along the American Atlantic coast and in the Gulf of Mexico while *Tachypleus gigas*, *Tachypleus tridentatus* found in the Southeast and East Asia,

Carcinoscorpius rotundicauda found in the Southeast Asia [7]. These creatures are sometimes called "living fossils" because they have changed little from their fossilized relatives; the earliest species identified is approximately 450 million years old. Horseshoe crabs are a valuable resource, commercially as a fertilizer and as a source of calcium for enriching fowl grains and medicinally in identifying endotoxins. The large lateral compound eyes are used to study cellular mechanisms of retinal function in humans. Horseshoe crab blood and blood products are used in cancer research, to test sterility of drugs and antibiotics and to signal the presence of chemical poisons, endotoxins, produced by certain bacteria. Chitin, a substance found in the horseshoe crab's shell, is non-toxic, biodegradable and used in contact lenses, skin creams and hair sprays. Litmulus amebocyte lysate (LAL) derived from horseshoe crab have been commonly used in testing the purity of drug, medical equipment. This product can detect even one per trillion of endotoxin in drug, medical equipment quickly and simply. Many researches revealed that blood from horseshoe crab can detoxify tetrodotoxin (TTX). Researched results showed that the TTX dose of 10 x LD_{50} of mice intracardially injected into *Carcinoscorpius rotundicauda* caused no fatality. The resulting hemolymph extracted 24h later from this crab showed no fatality when intraperitoneally injected into mice [8] (Bow et al., 1994). Other study also revealed that hemolymph from *Carcinoscorpius rotundicauda* contains TTX binding protein, which can neutralize the toxicity of TTX [9]. To clarify the TTX detoxification of hemolymph, the hemolymph of *Tachypleus tridentatus* was used to test the TTX detoxifying ability. The detoxifying abilities of hemolymph of *Tachypleus tridentatus* was also compared with *Carcinoscorpius rotundicauda* hemolymph to clarify TTX detoxification in these horseshoe crabs.

EXPERIMENTAL SECTION

Collection of hemolymph from *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda*

Tachypleus tridentatus and *Carcinoscorpius rotundicauda* were caught at shoreline in Nha Trang province in March, 2015, and provided by Research Institute for Aquaculture III (Vietnam). Hemolymph was collected by cardiac puncture of *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda* followed by centrifuge at 150g, 4^{o}C for 10 min. The plasma was further clarified by centrifugation at 9000g, 4^{o}C for 10 min, then the supernatant was quickly frozen in liquid nitrogen and stored at -80^{o}C for bioassay. The protein content of partially purified hemolymph was determined by Bradford assay for precise monitor in mixing with TTX. TTX was purchased from Tocris (UK).

Test for TTX detoxification in mouse

The different dosages (0 to 20mg) of hemolymph of *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda* was mixed with the LD_{50} of TTX, incubated for 60 min at 37^{o}C, then intraperitoneally injected into mice and recorded the symptoms. Mice selected for this experiment were 7-8 weeks old, weight 20±1g, and all mice were male, provided by Pasteur Institute (Vietnam). The LD_{50} of mice was determined to be 0.22µg per mouse unit. Survival time was recorded and analyzed.

SDS-PAGE

To compare the components of hemolymph of *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda*, hemolymph from these strains were detected on SDS-PAGE. Hemocyanin products from *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda* were purified by immobilized metal affinity chromatography based on the modified method of Ciria [10]. In this study, mini-IDA column (capacity 1.5ml packed volume; 0.7cm x 4cm) was washed with distill water, then charged with 10ml of CuSO4. 5H2O (6mg/ml) for 2h. The column was washed with 1M Tris-HCl, pH 7. 1ml of horseshoe crab hemolymph was applied, PBS buffer was added to wash unbound protein. The concentration of 100mM, 200mM, and 300mM imidazole was alternatively used to elute the target protein. The fractions were dialyzed with 10kDa cut-off membrane, and checked on SDS-PAGE.

RESULTS AND DISCUSSION

Survival analysis for TTX detoxification of hemolymph in mice

The incubated TTX-hemolymph mixtures were intraperitoneally injected into mice. Unfortunately, the hemolymph of both *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda* showed no TTX detoxification capability from dosage of 0-20mg per mouse unit. In the figure 1, the hemolymph dose of 0-20mg protein was combined with LD_{50} of TTX in each mouse unit showed that 0.5mg of hemolymph protein from *Carcinoscorpius rotundicauda* can elongate the death time to 40-45 minutes while the control mouse died at about 20-30 minutes after TTX only injection. When the hemolymph protein was increased to 20mg made mouse quick breath, convulsions and died at 10-20 minutes. It may be due to vigorous immune response in the presence of strange proteins or antigens. In figure 2, the *Tachypleus tridentatus* hemolymph can elongate the death time of mouse to 80-90 minutes at the dose of 0.5mg and cause quick breath, convulsions and died at 10-20 minutes in the presence of 20mg hemolymph protein.

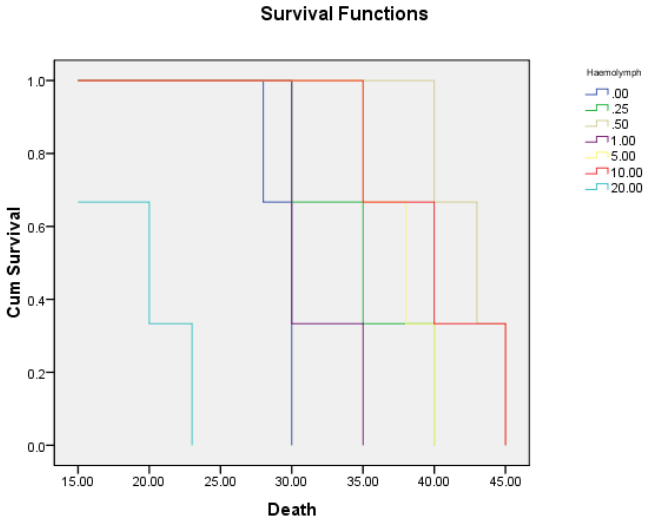

Figure 1: The death time in minutes of mice after intraperitoneal injection of incubated TTX-*Carcinoscorpius rotundicauda* hemolymph mixture

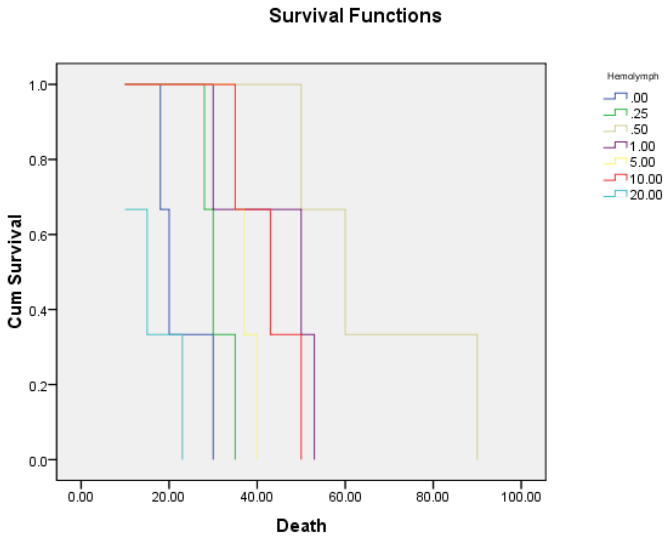

Figure 2: The death time in minutes of mice after intraperitoneal injection of incubated TTX- *Tachypleus tridentatus* hemolymph mixture

The results in figure 1 and figure 2 illustrated that hemolymph from both *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda* couldn't affect on TTX detoxification when infected with high dose TTX because hemolymph might be due to immune response to strange antigen. Actually, there were 90% hemocyanin in hemolymph.

Survival analysis for TTX detoxification of hemocyanin in mice

To understand which components related to the survival extension, hemocyanin from *Tachypleus tridentatus* was purified (Figure 3) and then was also combined with TTX to confirm the result. In the table 1, the triplicated treatment of hemocyanin-TTX mixtures proved that hemomocyanin from *Tachypleus tridentatus* has no capability of TTX detoxification. According to the above results the TTX detoxification of isolated hemocyanin did not help mice survive in TTX, just prolong the survival time to be cure immediately by combination with the other compounds.

Mice survival was shorter when mice were injected with high dose of hemocyanin (table 1). It was also meant that hemocyanin was not good source for TTX detoxification if infected with high TTX dose. Hemocyanin may interfere the toxicity mechanism of TTX in mice temporarily, but not enough to detoxify it or help mouse survive. TTX is a

small molecule, contains many hydroxyl groups and guanidine group, easily interact and form hydrogen bonds with other polar components, but their affinity may be very weak comparing with that of sensitive sodium channels and TTX. TTX selectively binds to the sensitive sodium channels, blocks the production of action potential and block the propagation of impulse in excitable membranes. On the other hand, sodium channels involved in modulation and regulation of multiple effector functions in both excitable and non-excitable cell types including phagocytosis, motility, the release of bioactive molecules, and the regulation of Na+/K+-ATPase activities. Consequently, TTX affects widely in mice and cause death in serious cases. In this study, the hemolymph from *Tachypleus tridentatus* prolonged the death time in treated mice up to over 80 minutes while hemolymph from *Carcinoscorpius rotundicauda* could only prolong death time to 45 minutes. It could be that the hemolymph of *Carcinoscorpius rotundicauda* contains TTX, contributes the increase of TTX dose and cause mice die quickly while hemolymph from *Tachypleus tridentatus* contains no TTX. The result in table 1 reveals that prolongation of death time of *Tachypleus tridentatus* hemocyanin in mice, pointing that TTX detoxification is not due to hemocyanin, may be due to other substances.

Table 1: The death time of mice after incubated *Tachypleus tridentatus* hemocyanin-TTX injection

Mice number	TTX dose (µg/mouse unit)	Hemocyanin (µg/mouse unit)	Death time average (minutes)	Symptoms
1	0	1	Survival	No abnormal symptom
2	0	1	Survival	No abnormal symptom
3	0	1	Survival	No abnormal symptom
4	0.22	0	23	quick breath, convulsions
5	0.22	0	30	quick breath, convulsions
6	0.22	0	28	quick breath, convulsions
7	0.22	0.2	30	quick breath, convulsions
8	0.22	0.2	40	quick breath, convulsions
9	0.22	0.2	36	quick breath, convulsions
10	0.22	1	31	quick breath, convulsions
11	0.22	1	37	quick breath, convulsions
12	0.22	1	30	quick breath, convulsions
13	0.22	5	28	quick breath, convulsions
14	0.22	5	33	quick breath, convulsions
15	0.22	5	37	quick breath, convulsions
16	0.22	10	28	quick breath, convulsions
17	0.22	10	35	quick breath, convulsions
18	0.22	10	35	quick breath, convulsions
19	0.22	15	20	quick breath, convulsions
20	0.22	15	15	quick breath, convulsions
21	0.22	15	19	quick breath, convulsions

The purified hemocyanin was used to inject into mice in figure 3 emphasized that hemocyanin contained no TTX but couldn't improve mice survival. Therefore, further researches must be conducted to determine which component plays the main roles in TTX detoxification.

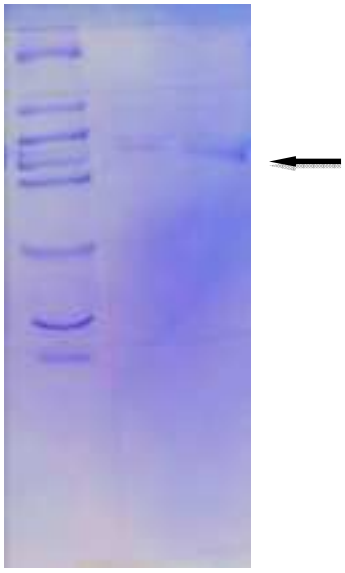

Figure 3: The SDS-PAGE result of elusion products of *Tachypleus tridentatus* hemocyanin. The arrow showed hemocyanin

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