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Research Article

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Factors might increase toxicity of tetrodotoxin

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ABSTRACT

Tetrodotoxin (TTX) is a neural toxin which was commonly found in marine puffer fish genus of Lagocephalus. It could be applied in medicinal industry, which can be used in pain reducing in cancerous patient. In this study, tissues collected from Lagocephalus have been soaked in different buffers as NaCl, KH_2PO_4 and distilled water at 37°C in 24 hours. These samples were, then, injected into mice by intraperitonial injection and observed the nervous disorder symptoms. Mice that showed typical nervous symptoms and death had been recorded and analyzed. The tissue samples in different buffer were incubated and then heated at 105°C. These samples were also tested for bioassay in mice. All the symptoms and mice death were analyzed. The results pointed that toxicity in mice due to microorganism in tissues of Lagocephalus.

Keywords: *Lagocephalus*, mice bioassay, tetrodotoxin, detection

INTRODUCTION

Tetrodotoxin (TTX) is the main causative agent of thousands of intoxication cases around the world due to consuming the puffer fish. In opposite, TTX could be applied in pharmaceutical industry to create new drug for treatment of malignant cancer patient but never cause addiction, whereas, current drug such as morphine are the one that can lead to drug dependent-patients. Besides, this compound was also proven for heroin withdrawal capacity.

TTX is a potent toxin which commonly found in many marine species such as puffer fish [1], blue-ring octopus crab [2] and newt - Triturus spp [3]. TTX had been classified as a natural product which is a non-proteinous compound, its molecular weight was about 319 U and has a very low LD50 of 10 µg kg-1 in mice. TTX acts by binding to the voltage-gate sodium channels, preventing Na+ flow through the channels and consequently blocking the action potential generation. Symptoms of intoxication appear within minutes to hours and may progress from localized numbness at the mouth shortly after ingestion to respiratory failure, hypotension and even death [4].

In Vietnam, there were many studies about extraction of TTX in tetryodonniae and the serological effects of TTX on mice. To date, it still has many debates on the natural origin of TTX in the marine animal [5]. Some of them showed that TTX release by the animal itself from a specific gland under the skin in case of Japanese newt *Cynop pyrrhogaster* [6] as an example. In contrast, it was also shown that some kind of bacteria which live together with the animal were the main source of TTX production such as bacterium, Vibrionaceae, isolated some kind of marine animal [7]. The mechanism by which TTX is produced is still unknown. Due to TTX activity on sodium ion channel, in this study, we focus on effects of Na+ and K+, Phosphate to TTX which extracted from Lagocephalus by mouse bioassay and thin layer chromatography (TLC).

EXPERIMENTAL SECTION

Materials and sample preparation

Healthy mice was provided by Pharmacology Department of Drug Quality Control. Three individuals of puffer fish genus of *Lagocephalus* that were collected from Nha Trang. The internal organs such as liver, intestine and skin were isolated and soaked into NaCl, KH_2PO_4 and distilled H_2O. These samples were incubated at 37^oC for 24 hours before injection.

Buffers as NaCl, KH_2PO_4 and distilled water and extraction solution from liver, skin, intestine from three buffers above were intraperitonially injected into mice [10]. Solvents for chromatography methods were Pyridine, ethyl acetate, acetic acid, chloroform. The samples were labeled as described in table 1.

All of the centrifuged (3000rpm, 15 minutes, 4^oC) extraction solutions were divided into two groups. First group were injected directly into mice. The second group was heated in waterbath at 105^oC and let them to cool down before injection. The experiment was recorded base on the appearance of the nervous symptoms on injected mice such as convulsion, imbalance and death. Any mice survive after 24 hours were considered as alived mice and had not been counted in the result.

Thin layer chromatography (TLC)

After examining the toxicity of TTX on mice, all samples were used for TLC method. In this method, a thin layer silica gel to analyze the samples. There were two solvent system were used in this method include pyridine: ethyl acetate: acetic acid: water and chloroform: ethyl acetate: acetic acid. The optimum condition for TLC has been determined to get the best analysis. UV light system was used, at wavelength 365nm, in this method to visualize the result.

Table 1: Sample labeling role

Buffers	Livers	Intestines	Skin
Distilled water	DL	DI	DS
NaCl	NL	NI	NS
KH_2PO_4	KL	KI	KS

RESULTS AND DISCUSSION

Toxicity of the same organ extract by different buffers

The extraction solution of puffer fish organs inject into mice gave the results which was illustrate in figure 1. According to Figure 1, there was the significant difference in TTX toxicity when soaking in NaCl, KH_2PO_4 and distilled water. Figure 1A and 1C showed that liver sample, when incubate in distilled water, has lower toxicity in compare to soaking in salt buffers. While the skin sample showed the opposite result. Therefore, this difference may cause by the interaction between buffer compositions. Figure 1 also showed that the samples extracted by KH_2PO_4 have the highest toxicity due to the shortest duration of death in mice. As a result, we may suggest the way to reduce the toxicity of TTX.

Table 2: T-Test Analysis

Sample treatment	Sample pairs	Significant difference	Non-significant difference
Non-heat samples	NL & KL	√	
	DL & NL	√	
	DL & KL	√	
	DS & NS		√
	DS & KS		√
	NS & KS	√	
Heated samples	DL’ & NL’		√
	DL’ & KL’	√	
	NL’ & KL’	√	
	DS’ & NS’		√
	DS’ & KS’		√
	NS’ & KS’		√

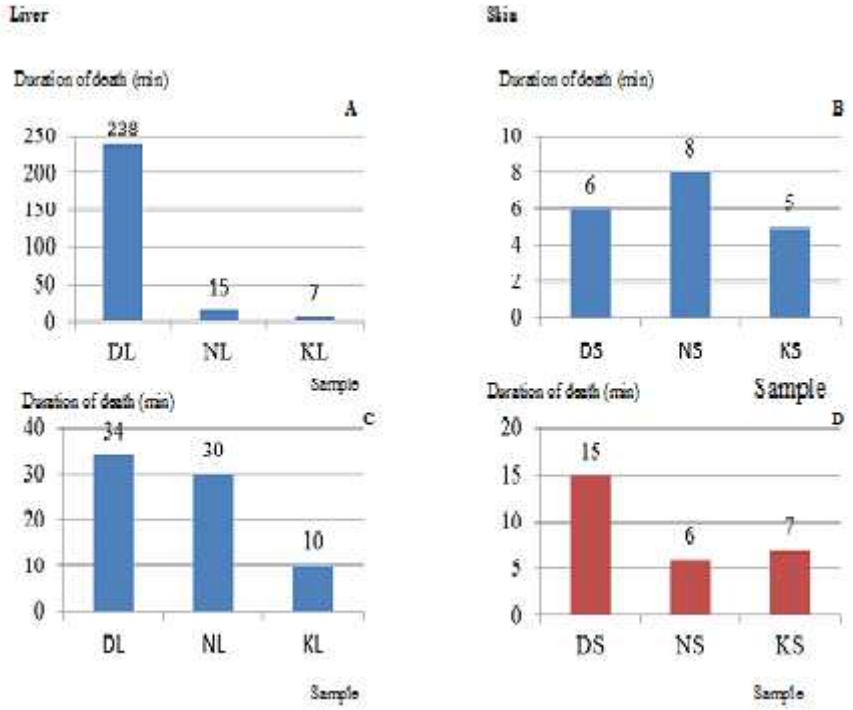

Figure 1: Toxicity of different organs in different buffers. (A) Liver sample; (B) Skin sample; (C) heated liver sample; (D) heated skin sample

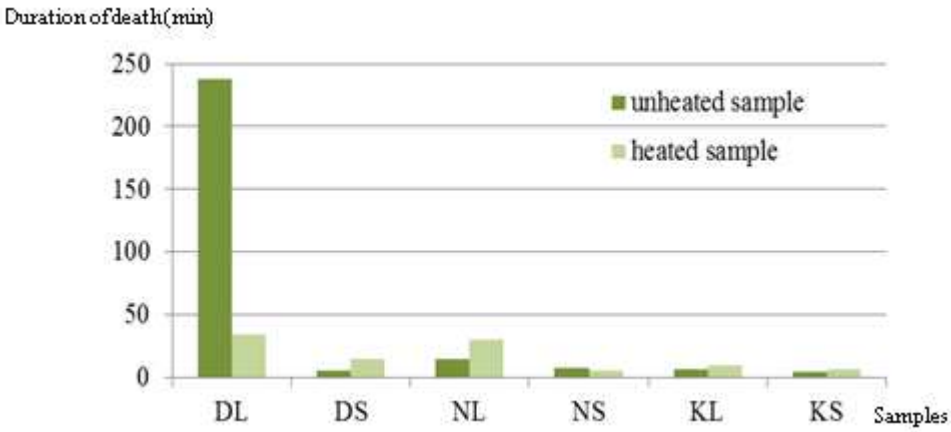

Figure 2: Effects of heat on sample toxicity

Toxicity of different organs

The results were shown in figure 2. The differences between samples were analyzed by statistical test shown in table 2. According to table 2, heated and unheated liver samples which were incubate in KH_2PO_4, NaCl and distilled water were significantly different, correspondently. It was clear that KH_2PO_4 extraction samples were highest toxicity compare with others (Figure 2). Similar results have also been obtained in other puffer fishes. This difference between samples before and after heating may suggest a way to eliminate the toxin.

Toxicity of the corresponding samples before and after heating

In order to understand the toxicity of TTX before and after heating, we compare the results collected from mouse bioassay and recognized that the toxicity remained steadily in the samples. They did not co-increase or co-decrease between the extractions from different samples (Figure 3). For liver sample extracted by KH_2PO_4 the duration of death was longer than that of before heating. The result was similar to that of liver extracted solution in NaCl and in distilled water. Consequently, we suggest the idea to limit TTX toxicity.

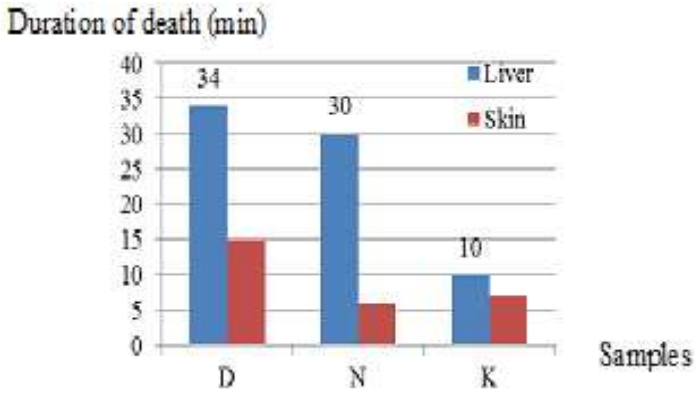

Figure 3: Comparison of the toxicity of the same organs incubated in different buffers

Thin Layer Chromatography

The toxicity of each sample was spontaneously determined by thin layer chromatography methods. Samples were analyzed in two solvent systems chloroform: ethyl acetate: acetic acid (15:5:3) and pyridine: ethyl acetate: acetic acid (15:5:3). The system of chloroform: ethyl acetate: acetic acid (15:5:3) gave the clearer result (Figure 4). Spots at the similar position in the chromatogram of non-heated and heated samples (Rf = 0.4; 0.5; 0.6; 0.9) suggested that these pots might contain TTX and its analogs based on their heat stability. The results were the base for detecting TTX in further study.

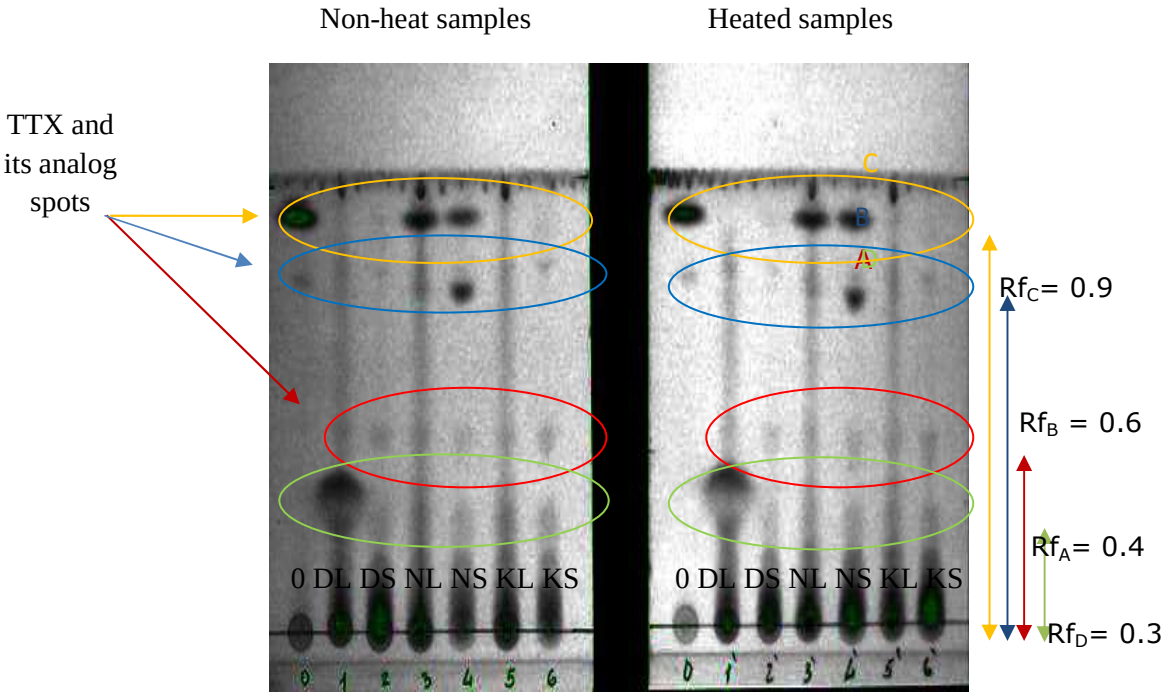

Figure 4: Thin layer chromatograph of skin and liver samples

CONCLUSION

There was the significant difference in TTX toxicity when soaking in NaCl, KH_2PO_4 and distilled water. Toxicity increased when it was in NaCl and distilled water. The significant difference of toxicity in the study might bring to a way to eliminate the toxin.

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