三十、Study on Biological Assay for Paralytic Shellfish Poisons: Clinical Signs of ICR Mice Induced by Paralytic Shellfish Poisons

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ABSTRACT

In the recent past, a variety of toxins had been discovered continuously, while paralytic shellfish poisons (PSP) was the first one to be identified and the most frequently to be found as well as dangerous to human beings.

In this report PSP was extracted by 0.1 N HCl from meat of purple clams, *Soletellina diphos*, which accumulated dinoflagellate sp. as biological concentration. The extracted samples were administered via routes of intraperitoneal injection and per os to weight 20±1 g ICR strain mice, which had been aroused many clinical signs. These clinical signs include hair bristly (ruffling of fur), scratch, erecting of tail, tremor, incoordination/ataxia, circling of movement, opisthotonus and convulsion, finally death. Hindleg of guinea pigs, rats, and mice were subcutaneously inoculated with the extracted sample which caused local paresis (slang leg) of the lab-animals respectively.

All examination procedures were carried out only two hours or more. The results showed that the specific clinical signs of the mice induced by PSP such as erecting of tail, circling of movement, opisthotonus and convulsion, seems to be a rapid method for screening PSP in shellfish. The doseresponse curve of PSP in mice was also discussed in this paper.

1. INTRODUCTION

In the recent past, a variety of algal toxins had been discovered continuously, however, paralytic shellfish poisons (PSP) is the first one to be discovered and lethaled to human beings. Majority of PSP were found in warm and tropic coastal area, though it had reported toxicosis omnipresent (Huang, 1986). The PSP contained purple clams had caused 30 persons toxicosis which including 2 deaths on January 1986 in the southern Taiwan (Du, 1986).

It is usually a basic principle that consider the sources of food, the clinical symptoms and latent period of the patients as an important information to

design an adequacy and acuity method for detecting any outbreak of food borne toxicity. A little over 50% of reported outbreaks were not able to detect the causative agent due to mainly lack of or insufficient quatity of food sample. Among the confirmed incidences, PSP/gonyautoxins was appeared to be minor cause as same as other natural origins. However, the largest hapless disaster of food borne poisons was caused by PSP contained purple clam (see Fig. 2) on January 1986 in the southern Taiwan. These PSP affects man because the toxins can be accumulated safely by mussels and clams, which are subsequently eaten by human beings.

PSP, saxitoxins, decrease the permeability of excitable membranes to Na^+ (but not K^+), thus axonal action potentials are not generated and paralysis results. The toxins do not cause an initial depolarization of nerves, they act non-competively, they are approximately 100,000 times more potent than cocaine or procaine, and they are frequently used in research. Clinical symptoms of human being includes numbness, spread from lips, tongue, and finger tips, and death due to respiratory paralysis may follow in 2 to 12 hours.

Although there are several alternatives for PSP analysis such as liquid chromatography, electrochemical detection, radioimmunoassay, enzyme immunoassay (in developing) and poly clonalantibody test (Ragelis, 1988), as well as fluorometric toxin analyzer (Adams & Miescier, 1980), the mouse bioassay is still officially recognized method (AOAC, 1984). This report was tried to apply clinical signs of mice to screen and identify the PSP, especially for the quantity limitation of assay sample.

2. MATERIALS AND METHODS

Preparation of sample

Thoroughly clean the external surface of the purple clam with tap water. Shuck hard-shell clams by breaking an opening on the bill of the shell and inserting a knife to cut the adductor muscle, pushing the blade between the shells and not to cut or damage the body of the clams. Use rubber glovers when handling materials which may contain PSP. Collect 100-500 g portions of meat which to be blended until homogeneity.

Extraction of sample

Taking 10.0 g homogenized meat into 50 ml flask, and add 25 ml 0.1 N hydrochloride into, and then stirred up homogeneity. Placed the flask into boiling water bath for 10 minutes, next cooling to room temperature, and then filter it. Collecting all filtered solution and quantitated to volume 50 ml, which as was applied stock solution in the test. Adjust pH value of the extract to 3.0 by adding 1 N hydrochlorde solution if necessary.

Blank control

Solution of 0.1 N hydrochloride was applied in this experiment as a blank control.

Laboratory Animals

ICR strain mice weighing 20±1 g, Wistar strain rats weighing 120±10 g and Hartly strain guinea pigs weighing 300±20 g were purchased from the Laboratory Animals Breeding Center, National Taiwan University Medical College, Taipei, Taiwan. Each plastic cage was housed five mice/rats with software wood bedding in environment-controlled from maintained at 20-24°C with a relative humidity of 40-60% and a 12 hr light: dark cycle with light starting at 0600 hr. The animals were given Purina Rodent Laboratory Chow #5001 (St. Louis, Missouri, U.S.A.), and tap water ad libitum.

Five of guinea pigs were housed into a stainless wire cage without software bedding but the same condition of raising as mice and rats. Purina Guinea pig LabChow #5025 was given at libitum.

Acute Intraperitoneal Toxicity Test

Several different dosages (0.10, 0.25, 0.50, 0.75, 1.00 ml) was administered to each 5 mice per groug through the intraperitoneal injection, respectively. After the extract had been administered, observe and record any clinical sign as well as lethal time of the animals immediately.

Acute Per os Toxicity Test

For each of the extract evaluated one single oral dose at 0.1, 0.25, 0.50, 0.75, and 1.00 ml serial dosage level was administered to groups of five mice per extract, respectively. Animal were observed frequently on the day of dosing and daily thereafter for the four days observation period, and recorded any clinical sign found in the test.

Dose Response Curve

Using the sigmaplo software to computerize the dose response relationship between the toxicity of PSP (lethal time, reciprocal of second) and the dosage of administration (mouse unit).

3. RESULTS

Clinical signs of ICR mice induced by PSP

a) *Administered via per os*

After being administered the extract through oral innoculation, the mice were observed a variety of clinical signs. These clinical signs include ruffling of fur, recumbency, scratching of nasal region, tremor, incoordination/ataxia, circling of movement, and then spasm and/or convulsion. The dosage of 0.1, 0.25, 0.5 ml (equal to 0.54, 1.35, 2.7 MU, respectively) extract could not cause any death, but the dosage of 0.75 and 1.0 ml extract gavaged mice were aroused death within 90 to 120 seconds.

b) *Administered via intraperitoneal injection*

The extract was administered via intraperitoneal injection to the mice which had been induced a few clinical signs such as ruffling of fur/hair bristly, scratching of nasal region and head, circling of movement, eracting of tail, ataxia, tremor, spasm and/or convulsion, opisthotonus, and finally death (see Fig. 3). Among these clinical signs eracting of tail and circling of movement were observed almost all course of test (from the scratch to the convulsion being observed), and tremor, convulsion, and opisthotonus being induced near the same time. The death time of dosage of 0.1 ml was mean 370 seconds, 0.5 ml, 190, 1.0 ml, 50. The incidences of these clinical signs showed in following Table 1.

Table 1. Incidence of several clinical signs were observed in ICR mice after being administered with lethal dose of PSP via intraperitoneal injection

Clinical sign	Incidence (%)	Clinical sign	Incidence (%)
Erecting of tail	14/20 (70)	Spasm	10/20 (50)
Scratch	16/20 (80)	Convulsion	16/20(80)
Ruffling of fur	14/20 (60)	Opisthotonus	14/20 (70)
Tremor	14/20 (60)	Circling of movement	18/20 (90)
Ataxia/Incoordination	8/20 (40)		

c) *Administered via subcutaneous injection*

The extract also had been administered to mice of ICR strain rats of Wastar strain and guinea pigs of Hartley strain via subcutaneously injection into one single hindleg (left) of these laboratory animals, which being caused local region of paresis/slang leg (left) for over 12 hours.

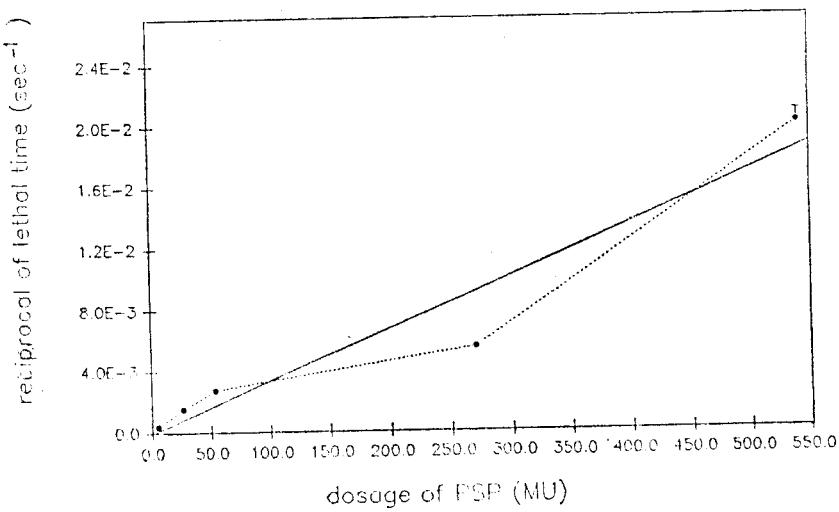

Fig. 1. Diagram of dose response relationship between toxicity (reciprocal of lethal time, sec) and dosage of PSP (Mouse Unit) in ICR mice via intraperitoneal injection. An estimate of the best linear fit was expressed as solid line.

Dose response curve of PSP via intraperitoneal administeration

The Characteristics of exposure and the spectrum of effects/toxicity come together in a correlative relationship customarily referred to as the dose-response relationship. The dose response curve (each dose $n=5$) showed that the dosage of PSP less than 0.54 MU would not induce the lethality in ICR mice. That means the sensitivity of ICR mice for detecting the toxicity of PSP was of upper this dosage.

4. DISCUSSION

Red tide/Red blooms may cause by either toxigenic marine (and/or freshwater) dinoflagellates or non-toxigenic dinoflagellates. The toxigenic marine dinoflagellates include *Gonyaulax catanella, G. tamarensis,* and the milder form of *Gymnodinium breve.* (Duffus, 1980). It is important to know that the shellfish may become poisonous even in the absences of a red tide. For instance, *G. breve* is one unarmored algae which be easily broken up by surf action and aerosolized action, and released the toxins to stimulating postganglionic cholinergic nerve fibers. In this report the pathogen was preliminarily identified as *G. tamarensis.*

Table 2. Lethal dosage of PSP in different species

Species	Dosage level (μg)	Note
Human being	456	Adams & Miescier, 1980
Mouse	30/40	Salter, *et al.*, 1989

* Dose of 124 μg PSP may cause severe symptoms of poisoning in human being.

Table 3. Detection limit of several method for analyzing PSP

Methods	Detecting limitation (μg/100 g)
Liquid chromatography	100
Mouse bioassay	30
Flourometric toxin analyzer	4

* 400 MU=80 μg/100 g

AOAC mouse bioassay has a variability of 20% and a limit of detection of 30/40 μg saxitoxin/100 g shellfish meat (a level only 2-fold less than the closure action level of 80 μg saxitoxin/100 g shellfish meat for shellfish beds.) (Adams & Miescier, 1980; Salter, *et al.*, 1989). However, the mouse bioassay method offers a higher through-put and a more rapid response time than the LC method.

It is usually to apply 3 dilutions fall within median death time of 5-7 minutes and sommer's table of mouse death time to evaluate the toxicity of PSP in biological method. However, in this report the author tried to use the clinical signs of ICR mice induced by PSP to access and identify the toxicity. There have found twelve kinds of derivatives upon PSP (Wu, 1986; Salter, *et al.*, 1989)

but in this report the extract which major involved gonyautoxin-II of 50%, gonyautoxin-III, 30%, gonyautoxin-I, 10%. These PSP were analysed by using reverse phase HPLC at Food and Drug Administration, U. S. A.

It was observed the clinical signs in mice, after being administered 0.1 N hydrochloride extract of PSP containing purple clams, that included ruffling of fur, scratch, erecting of tail, tremor, incoordination/ataxia, circling of movement, opisthotonus and convulsion. Among these clinical signs have some special responses especially for erecting of tail, circling of movement, tremor, and and opisthotonus, may become a criterion for evaluating and screening PSP in shellfish.

McCulloch, A. W., *et al.*, 1989, in Canada reported that Zinc from oyster tissue without PSP (900 μg/g of zinc) via intraperitoneal injection could induce some clinical signs of the test mice. The observed clinical signs include body temperature drop, extreme weakness, and cyanosis, with some deaths occurring over a period of several hours. These results may be interpreted on the basis of either zinc bioavailability or a synergistic effect of some other component present in the extract (McCulloch, *et al.*, 1989). These clinical signs were different from those characteristic of either PSP or domoic acid toxicity.

In 1989, Quilliam M. A. & Wright J. L. L. reported one mystery poisons, Amnesic Shellfish Poisons, with some unusual neurotoxic symptoms included scratching, trembling, and death by asphyxiation in mice. Lately Iverson, F. and his colleagues evidenced that domoic acid of the causitive could induce the loss of neurons in hippocampus in the laboratory animal, and change bebavior majorly of scratching. The clinical signs in human patients included vomiting and diarrhea, followed in some cases by confusion, menory loss, disorientation, and coma. Therefore clinical signs both in mice and in human beings were different from the PSP-induced.

The clinical signs of mice caused by Histamine-like poisons/Scombroid poisoning include scratch, excitation/saltatory spasm, and torsion spasm but seldom death. In the case of my laboratoy detecting of food poisons there was 318.2 mg/g histamine of assay sample which could not cause the mice dead. These do not correlate to the clinical signs induced by PSP.

Table 4. Mouse lethal time of several poisons contained in marine food

Poisons	Lethal time in mouse (min)
Tetrodotoxin(s?)	5-25
Ciguatoxins	110-360
Zinc from Oyster Tissue	120-300/several hours
Domoic acid/Amnesic Shellfish Poison	180
Paralytic Shellfish Poisons	0.7-60

The dose response relationship showed that the test limitation was 0.54 MU, which did similar to the limitation of biological method of AOAC. In the mouse

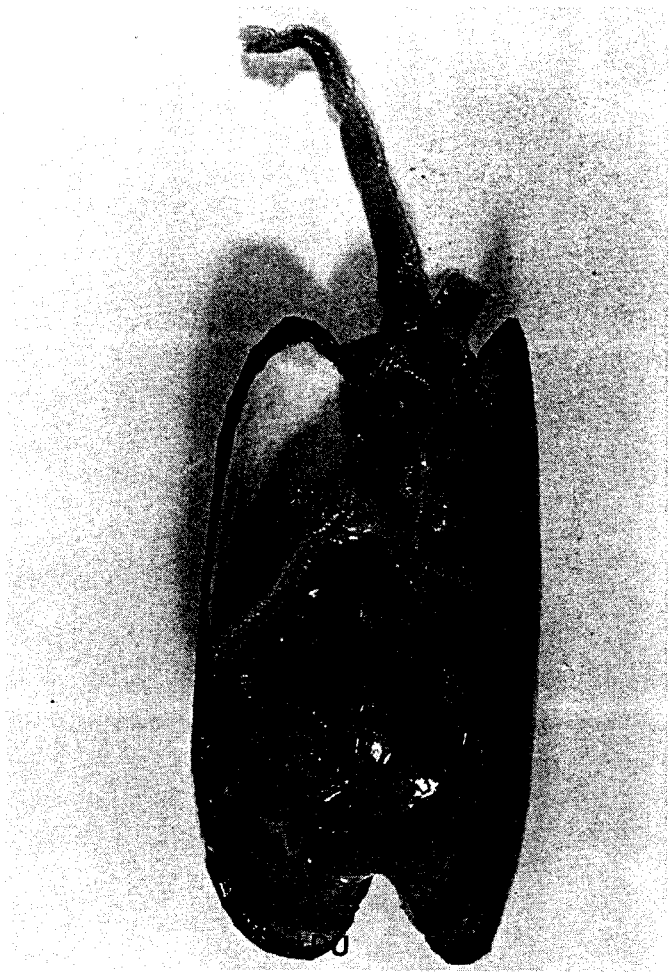

Fig. 2. Purple clam, *Soletellina diphos*, note two long siphons.

bioassay of the extract obtained from PSP containing purple clams confirmed the relation between lethality and dosage of PSP. It is an unquivocal evidence showed that the clinical signs, sources of food, and mice lethal time were useful criterion for differentiation PSP from other poisons, although the mouse bioassay method can not measure individual toxin of PSP.

Clem, J. D., 1979 reported that levels of 80 μg of PSP per 100 g of raw edible shellfish meat (equal to 400 MU), and 20 mouse units (MU) or any detected level of *G. breve* toxins per 100 g were considered potentially unsafe for humen consumption (Adams & Miescier, 1980). Thereafter the A. O. A. C. recommended that one should consider any value more than 80 μg/100 g of PSP as hazardous and unsafe for human consumption.

It is recommended that further collaborative studies be undertaken to evaluate the clinical signs induced by PSP, and hoped to improve and simplify the course of determination. For the public food safety, it is hoped to carried out the PSP examination routinely in shellfish during the period of red tide.

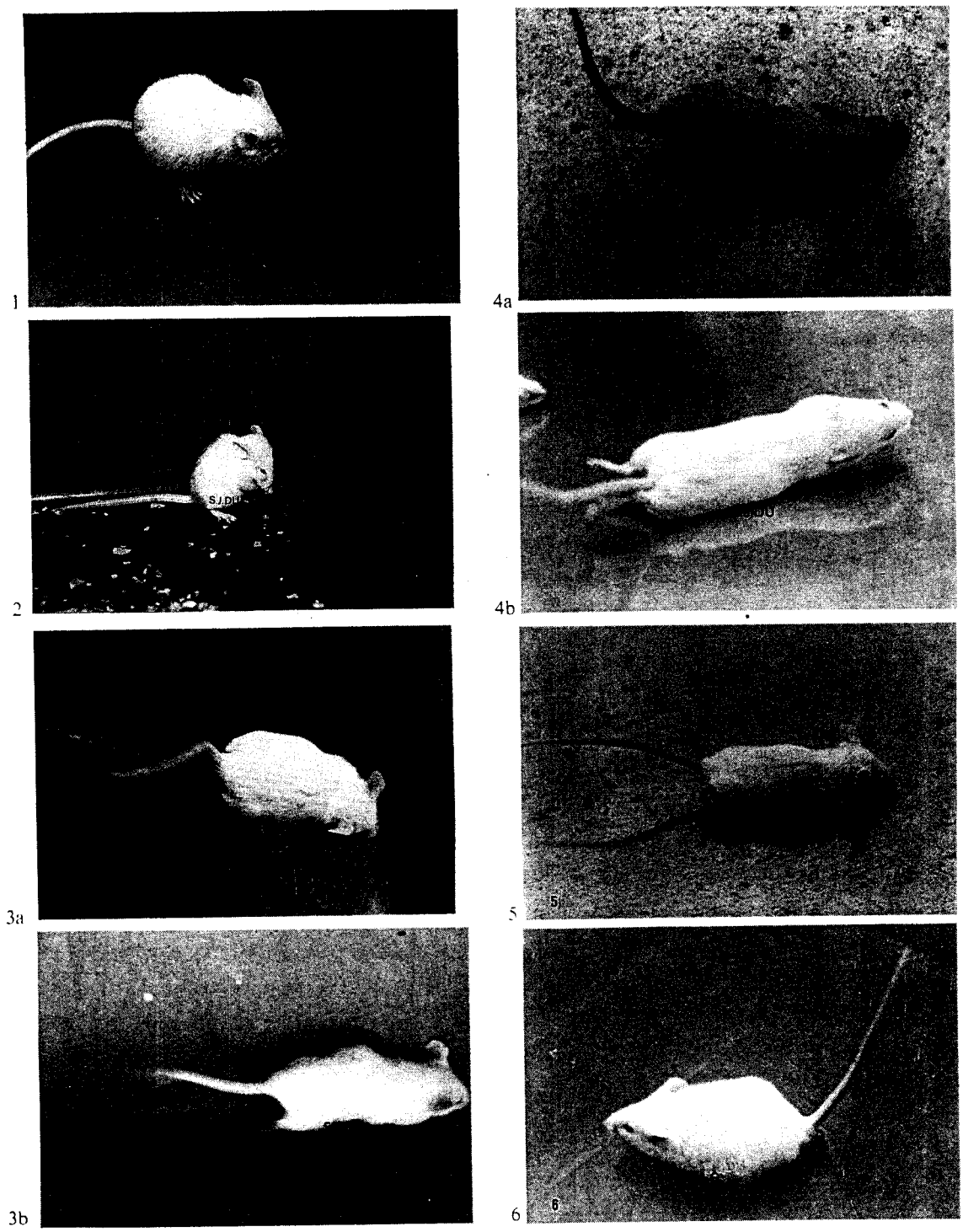

Fig. 3. Effect of treatment with PSP/the extract via intraperitoneal injection. Several clinical signs of ICR mice observed as the photographies. 1. ruffling of fur, note the posture of stand on and apathy. 2. scratch of nasal region, note also the hair bristly. 3. tremor, a) note special for the tail, b) and also note the extremities. 4. opisthotonus, a) & b) note the arch curvature of neck and vertebrae against body axon. 5. clonic spasm, note the left hind leg spasm and tail-erecting, 6. erecting oftail, note the concomitant with scrotum-protruding.

5. ACKNOWLEDGMENTS

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麻痺性貝毒誘發ICR系小鼠之臨床症狀

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摘　要

麻痺性貝毒 (Paralytic Shellfish Poisons) 是最早被發現的藻類毒素，近年來亦相繼發現其他的藻類毒素，但是仍以麻痺性貝毒的發生最爲頻繁，且對人類的健康危害甚鉅。

本報告以鹽酸抽取遭受麻痺性貝毒汚染的西施貝 (Purple Clam, *Soletellina diphos*) 其可食用部位中所含之麻痺性貝毒，經腹腔注射或口服投予 20±1 g ICR 系小鼠後，於臨床上均可觀察到不同程度之反應，計有：逆毛、搔抓、擧尾反應、震顫、共濟失調、迴旋運動、後弓反張、痙攣等，甚至死亡；其中屬於特異性反應者計有擧尾反應、迴旋運動、後弓反張。同時經後肢局部皮下注射，亦可誘發該試驗動物之後肢麻痺。

全部檢驗時程僅需二小時餘。試驗小鼠於投予檢品後所觀察到的特異性臨床症狀，似宜作爲一快速篩選麻痺性貝毒的方法。文中亦將依麻痺性貝毒對小鼠致死時間的反應，進行劑量反應曲線之探討。