FOOD CHEMICAL CONTAMINANTS

Comparison of Mouse Bioassay and Sodium Channel Cytotoxicity Assay for Detecting Paralytic Shellfish Poisoning Toxins in Shellfish Extracts

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A neuroblastoma cell culture assay was used to analyze shellfish extracts for presence of paralytic shellfish poisoning toxins (saxitoxins). Results were compared with mouse bioassays performed as part of a screening program for shellfish toxins in New Zealand. Twenty-nine samples gave negative results in both assays. Fifty-seven samples gave positive results in at least one assay. The correlation between the assays for saxitoxin equivalent levels in shellfish was 0.867. In spiking studies on shellfish extracts, the neuroblastoma assay showed a good response to added saxitoxin. Although these results support use of the neuroblastoma assay as a screening procedure for shellfish toxicity, results close to regulatory limits should be confirmed by mouse bioassay.

Several marine toxins exert their toxicity by affecting voltage-gated sodium channels (1). Saxitoxins and related compounds, which cause paralytic shellfish poisoning (PSP), inhibit sodium channel activity (2); ciguatoxins and brevetoxins, which cause neurotoxic shellfish poisoning, activate sodium channels (3). This sodium channel activity has been adapted for use in a neuroblastoma cell culture assay to detect these toxins (4, 5).

New Zealand has experienced significant outbreaks of toxic shellfish poisoning since the beginning of 1993 (6, 7), which have resulted mostly in neurotoxic shellfish poisoning and some diarrhetic shellfish poisoning in humans. Laboratory testing has also indicated the presence of domoic acid, which causes amnesiac shellfish poisoning, and saxitoxins in shellfish from New Zealand.

Presence of toxins in New Zealand shellfish was determined primarily by the mouse bioassay (8), which has a number of disadvantages (ethical objections, cost, sample size, etc). Because the size of the testing program in New Zealand is considerable, with approximately 100 key shellfish sites around the coast monitored weekly, alternative testing methods are being actively pursued. In the present study, shellfish extracts were tested for presence of PSP toxins in the neuroblastoma cell culture assay. Results were compared with those of mouse bioassays performed as part of the routine monitoring program for shellfish toxins in New Zealand.

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Experimental

Test Samples

The following types of shellfish were examined: oysters (*Tiostrea chiliensis*), tuatua (*Paphies subtriangulata*), kina (sea urchin; *Evechinus chloroticus*), pipi (*Paphies australis*), cockles (*Austrovenus stutchburyi*), scallops (*Pecten novaezelandiae*), paua (*Haliotis iris*), and green-shelled mussels (*Perna canaliculus*). Samples were removed from the shell and analyzed as whole or gut only.

Extraction and Mouse Bioassay

Aqueous extraction of shellfish and mouse bioassays were performed according to the standard method (8). Mouse bioassays were performed immediately. Aqueous extracts were stored at 4°C. Cell culture assays classified as "fresh samples" were performed within 1 month of extraction. Older samples collected up to 2 years previously and stored at 4°C were classified as "historical samples." Aqueous extracts were diluted (10×) with medium before cell culture assay. Shellfish aqueous extracts were spiked with saxitoxin standard solutions.

Standards and Reagents

Saxitoxin dihydrochloride standards for both the mouse bioassay and cell culture assay were obtained from the U.S. Food and Drug Administration (FDA). Standards were diluted (100×) with sterile water, pH was adjusted to ≤4, and solutions were stored at 4°C. The saxitoxin standard for the cell culture assay was diluted further with culture medium.

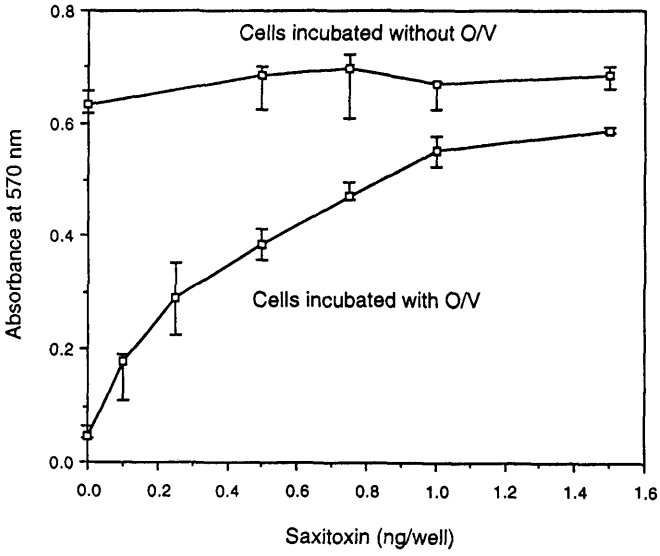

Figure 1. Saxitoxin standard curve. Median and range values for neuroblastoma cell culture assay (O = ouabain; V = veratridine).

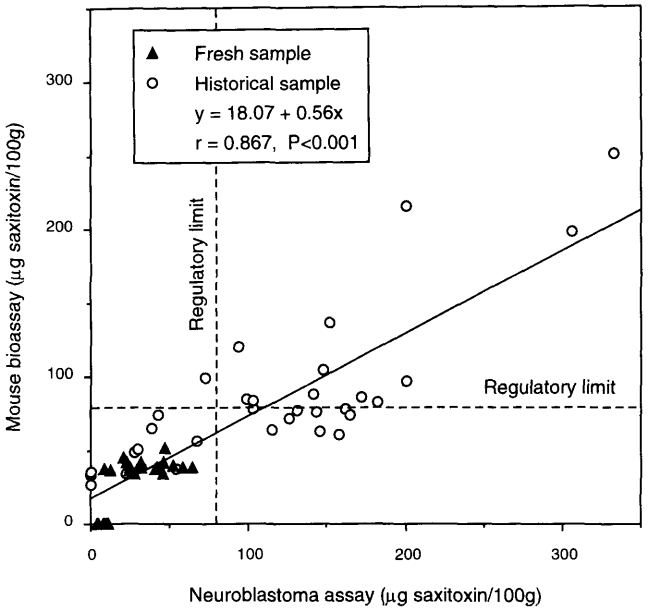

Figure 2. Comparison of mouse bioassay and neuroblastoma cell culture assay of shellfish extracts.

Stock ouabain (10 mM, aqueous; Sigma Chemical Co., St. Louis, MO) and veratridine (1 mM, 0.01M HCl; Sigma) solutions were prepared 24–72 h before use and stored at 4°C. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT; Sigma) stock solution (5 mg/mL in phosphate-buffered saline [PBS]) was prepared and stored at 4°C for up to 1 month. MTT was diluted with culture medium (1:6) before use.

Cell Culture Assay

The basic method has been described previously (4, 5). Slight modifications were made for the procedure in these experiments. Briefly, mouse neuroblastoma cells (Neuro-2a, ATCC CCL-131) were grown in RPMI 1640 complete medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, 20 mM Hepes, 25 mM bicarbonate, 1.7 mM glutamine, 1 mM sodium pyruvate, and 55 µg gentamicin sulfate/mL. Cells were incubated in closed containers at 37°C. For the assay, 96-well plates were seeded with 200 µL medium containing 1×10^5 mouse cells/mL and then incubated overnight at 37°C. The medium was then removed (by flicking the plate out and blotting it on paper towels) and replaced with fresh medium containing 0.5 mM ouabain and 0.05 mM veratridine. Control wells that were used to check for non-saxitoxin-related activity received medium diluted with 0.005M HCl (9:1) but no veratridine or ouabain. Samples or standards (10 µL) were then added to the control and experimental wells. Each plate containing samples also included standards at 2 different concentrations (1 above and 1 below the regulatory limit). Samples and standards were assayed in replicates of 3 or 4. The outer ring of wells in each plate received 200 µL PBS.

Plates were sealed with tape to minimize evaporation and incubated for 18–24 h at 37°C. Medium was again removed, and cell viability was assessed by adding 60 µL diluted MTT solution. After incubation at 37°C for 45–50 min, MTT was removed and 100 µL dimethyl sulfoxide was added. Absorbance was measured by using a plate reader (Bio-Tek EL 309) reading at 570 nm with reference at 630 nm. Median values of replicates were used for data analysis.

Results

Linearity of absorbance response was tested by using serial dilutions of cells incubated for 24 h. Absorbance response was linear to a concentration of 1×10^5 cells/mL (linear regression, r = 0.99, slope = 0.51).

Addition of ouabain and veratridine reduced cell viability by 69–92% (mean, 81%). The effect of added saxitoxin standards is shown by the standard curve in Figure 1. Saxitoxin without ouabain and veratridine had no effect.

Results of neuroblastoma assay and mouse bioassay were compared (Figure 2). Assays included both "fresh" samples (n = 24) analyzed shortly after extraction by mouse bioasssay and older "historical" samples (n = 33). The 57 results shown are those for samples that gave nonzero results in either the mouse assay or the neuroblastoma assay. Not shown are results for 29 fresh samples that were zero in both assays. The average deviation for 13 (nonzero) samples analyzed in duplicate was 9 µg saxitoxin per 100 g shellfish.

Results of spiking studies are shown in Table 1. All spiking studies were performed on fresh extracts.

Discussion

The detection limit of the assay was about 0.1 ng saxitoxin per well, which is comparable with results from other laboratories (5, 9). A standard 10-fold dilution of extract with medium was used to avoid cytotoxicity unrelated to sodium channel activity; thus, 0.1 ng saxitoxin/well was equivalent to 20 µg saxitoxin/100 g shellfish. A lower dilution of extract may have been

Table 1. Results of neuroblastoma assays of aqueous extracts of shellfish spiked with saxitoxin (STX)

Species	Mouse bioassay result, μg STX/100 g	Neuroblastoma assay result, μg STX/100 g	Neuroblastoma assay result, μg STX/100 g, for sample spiked with STX at 100 μg/100 g	Increase due to spike, μg STX/100 g
Cockle	0	0	44	44
Mussel	0	0	75	75
Oyster	0	12	91	79
Cockle	0	16	101	85
Cockle	0	0	91	91
Oyster	0	18	109	91
Tuatua	0	0	106	106
Cockle	0	8	119	111
Tuatua	0	0	113	113
Pipi	0	0	118	118
Mussel	0	16	136	120
Oyster	0	0	126	126
Scallops	0	0	126	126
Scallops	0	0	127	127
Mussel	0	0	134	134

feasible, but the level chosen gave sufficient sensitivity for the screening procedure. No cytotoxicity unrelated to sodium channel activity was observed for any of the shellfish types tested. The regulatory limit of 80 μg saxitoxin per 100 g shellfish is equivalent to 0.4 ng saxitoxin/well (8).

All fresh aqueous extract samples had PSP levels below the regulatory limit for saxitoxin. Consequently, some older positive samples that had been retained from an occurrence of PSP toxins in shellfish in 1993 were examined. In addition, samples were spiked to give results above the regulatory limit.

The fresh aqueous extracts fell into 2 groups: those that were zero in the mouse bioassay and very low in the neuroblastoma assay and those that showed about 35–50 μg saxitoxin/100 g in the mouse bioassay but gave a broader range of results in the neuroblastoma assay. This was a result of the procedure used in our screening program for the mouse bioassay. Death times are recorded up to 1 h after injection, which corresponds to about 35 μg saxitoxin/100 g shellfish (8). Deaths after 1 h, which would generate mouse bioassay results between zero and 35 μg saxitoxin/100 g, are not recorded and are entered as zero. The neuroblastoma assay gives greater discriminating power at low levels of saxitoxin for a large-scale routine screening program.

Data shown in Figure 2 exclude results from the 29 fresh samples in which saxitoxin was undetected in both assays. If these results were included, the equation for the regression line would have been $y = 8.83 + 0.62x$ ($r = 0.903$, $p < 0.001$).

The neuroblastoma assay gave higher results for historical samples than did the mouse bioassay, as shown by the slope of the regression line. This indicates that the PSP activity of these samples was not lost during storage, although 4 historical samples that gave low mouse bioassay results gave zero results in the neuroblastoma assay. Both assays were calibrated by the same standards. The mouse bioassay was calibrated weekly, and the neuroblastoma assay included standards with each run. Further study is required to determine the factors that might cause higher results in the neuroblastoma assay.

Results that are below the regulatory limit in the mouse bioassay but above it in the neuroblastoma assay are of practical importance. Only one result was above the regulatory limit in the mouse bioassay and below it in the neuroblastoma assay. These results suggest that the neuroblastoma assay should be backed up by mouse testing when a result is above or close to the regulatory limit. This would improve quality assurance for economic decisions such as those regarding the closure of shellfish beds.

The spiked samples showed reasonable agreement between amount added and increased response in the neuroblastoma assay (Table 1). The median was 111 μg saxitoxin/100 g sample, with a range of 44–134 μg/100 g. The reason for the anomalous cockle result of 44 μg saxitoxin is unknown. Two other results did not exceed the regulatory limit despite addition of the equivalent of 100 μg saxitoxin/100 g shellfish. The low levels of saxitoxin detected by the neuroblastoma assay in some unspiked samples (Table 1) are not false-positive results but would be below the detection limit of the mouse bioassay.

Results from this testing indicate that the neuroblastoma cell culture assay is applicable to a variety of shellfish types and gives results comparable with those of the mouse bioassay. The assay is limited by the requirement of 18–24 h incubation after addition of samples; however, recently reported modifications (10) involving antagonism with sodium channel-enhancing toxins may reduce assay time to 4–8 h. The spectrum of saxitoxins present in the shellfish is unknown, and it is possible that those containing a different spectrum of toxins may present an altered relationship with the mouse bioassay results. However, both mouse and neuroblastoma assays are based on the effects on sodium channels, which is the primary determinant of saxitoxin toxicity.

Results that are close to the regulatory limit in the neuroblastoma assay should be confirmed by the mouse bioassay when the outcome would have potentially important economic consequences. The large number of fresh samples that were zero in

both assays indicates that the neuroblastoma assay could be used as a screening tool to eliminate such samples before mouse bioassay. Although more data are required to validate the method, the neuroblastoma assay seems to be a convenient initial screening procedure for PSP toxins.

Acknowledgments

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