

Evaluation of Acute Oral Toxicities from Paralytic Shellfish Toxins Based on a Three-level Response Surface Pathway Design

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ABSTRACT - Paralytic Shellfish Poisoning (PSP) occurs when humans consume shellfish contaminated with saxitoxin (STX) and its derivatives. It causes symptoms ranging from numbness and nausea to severe muscle paralysis and respiratory failure. Toxic equivalency factors (TEFs) are used to standardize the toxic effects of various PSP toxins for risk assessment. Traditional detection methods, such as mouse bioassays, have been used to set the TEFs, but ethical concerns over *in vivo* studies have shifted the focus toward analytical methods, such as high-performance liquid chromatography. However, *in vivo* data are essential for establishing TEFs, particularly for emerging marine biotoxins. This study employed a three-level response surface pathway (RSP) design, which reduced the number of animals used to evaluate the median lethal dose (LD₅₀) of STX and its derivatives. The LD₅₀ and TEF values for STX dihydrochloride, neosaxitoxin, decarbamoylsaxitoxin, gonyautoxins 1 & 4 (GTX1&4), GTX2&3, and dcGTX2&3 were 451.3 (1.00), 306.5 (1.47), 860.9 (0.52), 644.5 (0.70), 915.3 (0.49), and 2409.3 (0.19) µg/kg, respectively. These TEFs closely aligned with the WHO recommendations and prior oral LD₅₀ values, with Pearson correlation coefficients of 0.969 and 0.994, respectively. This study highlights the need for accurate TEF assignments for PSP toxins and new marine biotoxins, demonstrating that the three-level RSP design balances ethical concerns and provides reliable toxicity data.

Key words: Paralytic shellfish toxins, Toxic equivalency factor, Oral toxicity, Reduction, Three-level response surface pathway design

Paralytic shellfish poisoning (PSP) occurs when shellfish or fish accumulate PSP toxins, saxitoxin (STX) and its derivatives, from marine dinoflagellates such as *Alexandrium* and *Gymnodinium spp.*, which are then ingested by humans^{1,2}. PSP in humans typically presents with symptoms from 20 min to 5 h after consuming contaminated shellfish³. These symptoms include numbness and tingling, starting from the lips and spreading to the face and limbs, along with nausea, vomiting, and neurological effects such as dizziness and loss of coordination. In severe cases, muscle paralysis can occur, and the most severe cases may result in respiratory failure, which can be fatal⁴⁻⁶.

The toxic equivalency factor (TEF) allows comparisons of the toxicity of different chemicals that have similar harmful

effects but vary in strength⁷. This helps in assessing the overall risk when multiple chemicals are present by allowing scientists to add up their toxic effects in a standardized manner. STX and its derivatives have a common mode of action of binding to sodium channels and inhibiting neuronal conduction⁸; and thus, the TEF value is used to express the threshold levels of PSP toxins in shellfish samples, such as STX dihydrochloride (STX·2HCl) equivalents (STXeq)^{9,10}. In the United States, European Union, Canada, and South Korea, the acceptable level of PSP toxins in shellfish samples is 80 µg STXeq/100 g of shellfish meat¹¹⁻¹³. As such, assigning appropriate TEF values for different derivatives of STX is important for food safety and regulatory purposes.

Following a traditional detection method of PSP toxins, namely, the mouse bioassay (MBA, AOAC959.08), *in vivo* toxicity was measured by intraperitoneal (IP) injection of PSP toxins in mice. The results were adapted by the European Food Safety Authority (EFSA) to set TEF values for PSP toxins in 2009¹¹. While the MBA and IP injection method has the advantage of being rapid and reflecting physiological responses, it has the disadvantage of not

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reflecting ingestion through food, which is the actual route of exposure to PSP toxins in humans. To compensate for this, oral toxicity studies were conducted to reset the TEF values, and in 2016, the World Health Organization (WHO) set the revised TEF values with the updated toxicity profile of STX and its derivatives based on *in vivo* acute oral toxicity studies as well as *in vitro* assays⁹.

Due to growing global ethical concerns, there is increasing pressure to reduce or ban the use of laboratory animals. As a result, the need to shift from the MBA to instrumental analytical methods, such as high-performance liquid chromatography (HPLC), for the detection of PSP toxins has become increasingly apparent¹⁴⁻¹⁶. However, in the context of biological relevance, toxicity data from mice are still crucial for establishing appropriate TEF values, not only for PSP toxins but also for newly found marine biotoxins such as cyclic imine and palytoxin. Thus, a method for *in vivo* oral toxicity test is needed that considers ethical concerns while still ensuring reproducible results in line with the 3Rs of animal research ethics—replacement, reduction, and refinement^{17,18}, particularly with the ‘Reduction’ principle.

In this study, we aimed to reevaluate the TEF of PSP toxins using a three-level response surface pathway (RSP) design, a further reduction from the four-level RSP design proposed by Dewi et al.¹⁹, and compared the results with previously reported data to validate the practicality of this method.

Materials and Methods

PSP toxins

STX·2HCl was purchased from Sigma-Aldrich (Cat. No. 93665, St. Louis, MO, USA). Other PSP toxins including neosaxitoxin (NeoSTX, CRM-00-NEO), decarbamoylsaxitoxin (dcSTX, CRM-00-dcSTX), gonyautoxins 1 & 4 (GTX1&4; CRM-00-GTX1&4), gonyautoxins 2 & 3 (GTX2&3; CRM-00-GTX2&3), and decarbamoylgonyautoxins 2 & 3 (dcGTX2&3; CRM-00-dcGTX2&3) were purchased from Cifga Laboratory (Lugo, Spain). All of these PSP toxins were obtained as solutions and used without further dilution.

Animals

Four-week-old male ICR mice were purchased from Orient Bio (Seongnam, Korea) and acclimated for 1-2 days until the mice weighed 18-22 g. Mice were maintained under a regular 12 h light/dark cycle at 22°C and 60% humidity in a specific pathogen-free facility at Gachon University (Incheon, Korea). All animal experiments were approved by the Institutional Animal Care and Use Committee of Gachon University (GU1-2022-IA0048) and performed in accordance with recommended guidelines.

Table 1. Three-level response surface pathway (RSP) design

Level 1 (n = 3)		Level 2 (n = 5)		Level 3 (n = 7)
Dose (m ₁)	No. of death	Dose (m ₂)	No. of death	Dose (m ₃)
m ₁	0	m ₁ + m ₁ /k	0	m ₂ + m ₁ /k ²
	1	m ₁ + m ₁ /k ²	1	m ₂ + m ₁ /k ³
	2	m ₁ - m ₁ /k ²	2	m ₂ + m ₁ /k ⁴
	3	m ₁ - m ₁ /k	3	m ₂ - m ₁ /k ⁴
			4	m ₂ - m ₁ /k ³
			5	m ₂ - m ₁ /k ²

m₁: starting dose (µg/kg BW), m₂: dose for level 2 (µg/kg BW), m₃: dose for level 3 (µg/kg BW), k: adjustment factor.

Three-level RSP design

The three-level RSP design, shown in Table 1, is a simplified version of the four-level RSP design proposed by Dewi et al.¹⁹. We have previously used this method to determine the acute oral median lethal dose (LD₅₀) of diarrhetic shellfish poisoning toxins, okadaic acid and dinophysistoxin-1²⁰. Briefly, three, five, and seven mice were utilized for the first, second, and third levels, respectively; at each level, mice were treated orally with each PSP toxin, followed by monitoring for 1 day to evaluate lethality. The initial dose (m₁) was set as the mean of the lowest (D_L) and highest (D_U) values of the previously reported acute oral LD₅₀, and doses for levels 2 and 3 were calculated following the equation denoted in Table 1, according to the number of dead mice in the previous level. The adjustment factor, k, was determined by using **Equation (1)**, and **Equation (2)** is presented with k clarified.

$$D_U = m_1 \frac{(k^3 - 1)}{(k^3 - k^2)} \quad (1)$$

$$k = \frac{m_1 + \sqrt{(4D_U - 3m_1) * m_1}}{2 * (D_U - m_1)} \quad (2)$$

Statistical analysis

According to the dose-lethality relationship of each PSP toxin obtained from the acute oral toxicity study, the LD₅₀ values of PSP toxins were measured. To measure the LD₅₀ with the standard error of mean, the generalized linear regression method²¹ was utilized with R software (v.4.2.2). By dividing the mean LD₅₀ of STX by the mean LD₅₀ of the other PSP toxins, the TEF value for each toxin was determined. To assess the correlation between TEF values obtained from this study and previously reported TEF values, Pearson correlation coefficients were calculated using the ‘CORREL()’ function in Microsoft Excel.

Results

Properties of PSP toxins used in this study

The first gateway to obtain reliable and accurate toxicity data is to use validated toxicants. PSP toxins are difficult to synthesize chemically due to their complex molecular structure and the presence of multiple chiral centers²²). Given these difficulties, PSP toxins are typically extracted, isolated, and purified from toxin-producing algae, although variations in toxin concentration and composition can occur between batches. The specific toxins and their components used in this study are presented in Table 2. To minimize batch-dependent effects, PSP toxins of certified reference material (CRM) grade were used. It is worth noting that GTX1&4, GTX2&3, and dcGTX2&3 consisted of more than two types of PSP toxins in a specified ratio. The PSP toxins maintained consistent concentration and purity throughout the duration of this study.

Determination of adjustment factor (*k*) for each PSP toxin

In the three-level RSP design, setting the appropriate doses for each level is the main issue. First of all, according

to the four-level RSP design¹⁹), the starting dose (m_1) for level 1 was set as the mean between the upper limit of the previously reported acute oral LD₅₀ (D_U) and the lower limit of the previously reported acute oral LD₅₀ (D_L). In the case of STX, among previously reported acute oral LD₅₀ values in mice^{9,23-25}), 260.0 µg/kg body weight (BW) reported by the WHO⁹) and 607.0 µg/kg BW reported by Finch et al.²³) were adapted as D_L and D_U , respectively. Unlike STX, for the other toxins, only one acute oral toxicity study in mice was reported for each toxin; therefore, we used the lowest value of the 95% confidence interval range reported in the paper as D_L and the highest value as D_U . D_L values were 229.2, 809.7, 608.7, 672.0, and 2113.9 µg/kg BW for NeoSTX, dcSTX, GTX1&4, GTX2&3, and dcGTX2&3, respectively. D_U values were 310.9, 1389.0, 682.8, 919.9, and 2677.7 µg/kg BW for NeoSTX, dcSTX, GTX1&4, GTX2&3, and dcGTX2&3, respectively. Thus, the starting dose, m_1 , which is the mean of D_L and D_U for each toxin, was determined as 433.5, 270.1, 1099.3, 645.7, 796.0, and 2395.8 µg/kg BW for STX, NeoSTX, dcSTX, GTX1&4, GTX2&3, and dcGTX2&3, respectively (Table 3). Second, the doses applied at levels 2 and 3 were determined by a

Table 2. Properties of the paralytic shellfish poisoning (PSP) toxins used in this study

PSP toxin	Concentration ^{a)} (µM) and Content (%)					
	STX·2HCl	NeoSTX	dcSTX	GTX1&4	GTX2&3	dcGTX2&3
STX	54.5±3.8 (>99) ^{b)}	-	-	-	Traces	0.91±0.05 (1.0)
NeoSTX	-	51.1±3.0 (>99)	-	-	-	0.72±0.12 (0.8)
dcSTX	-	-	56.8±4.8 (>99)	-	-	0.16±0.05 (0.2)
GTX-1	-	-	-	82.0±4.7 (>80.1)	Traces	-
GTX-2	-	-	-	-	62.7±4.2 (>75)	0.26±0.04 (0.3)
GTX-3	-	-	-	-	20.1±1.8 (>24)	0.11±0.02 (0.1)
GTX-4	-	-	-	19.4±1.4 (>18.9)	Traces	-
GTX-5	-	-	-	-	-	0.35±0.05 (0.4)
GTX-6	-	-	-	-	-	0.08±0.01 (0.1)
dcGTX-2	-	-	-	-	-	62.0±4.0 (70.8)
dcGTX-3	-	-	-	-	-	23.0±2.4 (26.3)

^{a)}Concentration is presented with 95% confidence interval.

^{b)}Percentage of each toxin within PSP toxins.

specific formula, shown in Table 3, where m_1 and an adjustment factor (k) were used as variables. To obtain the k value for each toxin, the D_U and m_1 values for each toxin were substituted into Equation (2). The k values for each toxin were calculated as follows: STX·2HCl, 3.27; NeoSTX, 7.47; dcSTX, 4.61; GTX1&4, 18.40; GTX2&3, 7.30; and dcGTX2&3, 9.40 (Table 3).

Table 3. Determination of adjustment factor (k) for each PSP toxin

PSP toxin	D_L ^{a)}	D_U ^{b)}	m_1 ^{c)}	k ^{d)}	Ref.
STX·2HCl	260.0	607.0	433.5	3.27	9,23-25)
NeoSTX	229.2	310.9	270.1	7.47	24)
dcSTX	809.7	1389.0	1099.3	4.61	
GTX1&4	608.7	682.8	645.7	18.40	
GTX2&3	672.0	919.9	796.0	7.30	26)
dcGTX2&3	2113.9	2677.7	2395.8	9.40	

^{a)} D_L ; lower limit of previously reported oral LD_{50} ($\mu\text{g}/\text{kg}$ BW).

^{b)} D_U ; upper limit of previously reported oral LD_{50} ($\mu\text{g}/\text{kg}$ BW).

^{c)} m_1 ; starting dose for the three-level RSP design ($\mu\text{g}/\text{kg}$ BW).

^{d)} k ; adjustment factor.

Acute oral LD_{50} of PSP toxins obtained from the acute oral toxicity study

Detailed information, including the number of mice used in each level, dose, BW of the mice, and lethality of each level for each toxin, is listed in the Table 4. In level 1, each toxin was orally administered to three mice at the m_1 dose. Based on the number of mice that died in level 1, the dose for level 2 was determined and orally administered to five mice. Based on the number of mice that died in level 2, the dose for level 3 was determined and orally administered to seven mice. At each level, lethality was determined by counting the number of mice that died over a 24-h period. Based on the dose-lethality relationship, the acute oral LD_{50} of each toxin was calculated, and the values were as follows: STX·2HCl, 451.3 ± 13.5 ; NeoSTX, 306.5 ± 2.9 ; dcSTX, 860.9 ± 23.5 ; GTX1&4, 644.5 ± 1.0 ; GTX2&3, 915.3 ± 47.0 ; and dcGTX2&3, 2409.3 ± 13.8 ($\mu\text{g}/\text{kg}$, mean \pm standard error of the mean) (Table 4).

Determination of TEFs for PSP toxins based on this study

Given that the TEF value for STX is 1.00, the TEF values

Table 4. Acute oral toxicity test based on a three-level RSP design

PSP toxin	Level 1 (n = 3)			Level 2 (n = 5)			Level 3 (n = 7)			LD_{50} ^{b)}
	Dose ($\mu\text{g}/\text{kg}$)	BW ^{a)} (g)	Lethality (%)	Dose ($\mu\text{g}/\text{kg}$)	BW ^{a)} (g)	Lethality (%)	Dose ($\mu\text{g}/\text{kg}$)	BW ^{a)} (g)	Lethality (%)	
STX·2HCl	433.5	20.8 ± 0.2	33.3	474.0	20.1 ± 0.6	80.0	461.6	19.5 ± 0.4	57.1	451.3 ± 13.5
NeoSTX	270.1	20.3 ± 0.9	0.0	306.2	19.7 ± 0.8	40.0	306.3	20.2 ± 0.6	42.9	306.5 ± 2.9
dcSTX	1099.3	19.1 ± 0.1	100.0	860.9	19.6 ± 1.0	40.0	863.3	20.3 ± 0.5	100.0	860.9 ± 23.5
GTX1&4	645.7	20.4 ± 0.2	66.7	643.8	19.7 ± 0.4	40.0	643.8	19.2 ± 0.8	42.9	644.5 ± 1.0
GTX2&3	796.0	20.6 ± 1.0	0.0	905.0	19.8 ± 1.3	40.0	905.3	19.7 ± 0.7	28.6	915.3 ± 47.0
dcGTX2&3	2395.8	19.0 ± 0.5	33.3	2422.9	19.7 ± 0.5	60.0	2422.6	19.5 ± 0.9	71.4	2409.3 ± 13.8

^{a)}BW is presented as the mean \pm standard deviation.

^{b)} LD_{50} is presented as the mean \pm standard error of mean.

Table 5. TEFs for PSP toxins used in this study

PSP toxin	TEF (EFSA, 2009 ¹¹⁾)	TEF (WHO, 2016 ⁹⁾)	TEF (Based on oral LD_{50})	TEF (This study)
STX·2HCl	1.00	1.00	1.00	1.00
NeoSTX	1.00	2.00	1.70 ^{c)}	1.47
dcSTX	1.00	0.50	0.46 ^{c)}	0.52
GTX1&4	0.94 ^{a)}	0.94 ^{b)}	0.74 ^{c)}	0.70
GTX2&3	0.45 ^{a)}	0.45 ^{b)}	0.53 ^{c)}	0.49
dcGTX2&3	0.27 ^{a)}	0.28 ^{b)}	0.17 ^{d)}	0.19

^{a)} Recalculated from TEF values of each single toxin recommended by EFSA (2009)¹¹⁾ considering the proportions.

^{b)} Recalculated from TEF values of each single toxin recommended by WHO (2016)⁹⁾ considering the proportions.

^{c)} Munday et al., 2013²⁴⁾.

^{d)} Selwood et al., 2017²⁶⁾.

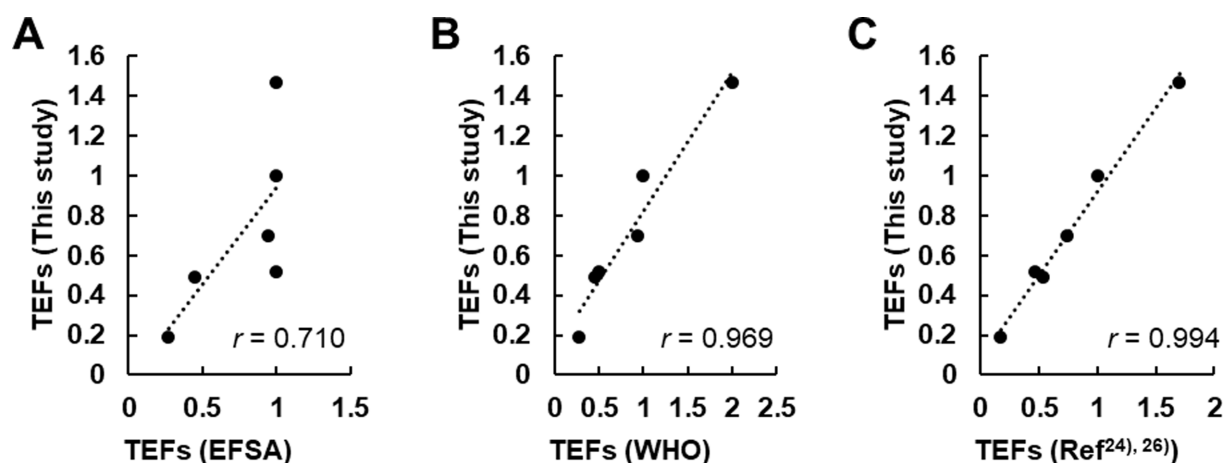


Figure 1. Correlation of TEFs obtained from this study with TEFs from the EFSA, WHO, or previous reports. TEFs obtained from this study were moderately correlated with the TEFs recommended by the EFSA (A) with a correlation coefficient of 0.710, whereas they were highly correlated with the TEFs recommended by the WHO (B) and the TEFs previously reported (C) with a correlation coefficient (r) of 0.969 and 0.994, respectively.

were 1.47, 0.52, 0.70, 0.49, and 0.19 for NeoSTX, dcSTX, GTX1&4, GTX2&3, and dcGTX2&3, respectively (Table 5). In Table 5, the TEF values previously recommended by the EFSA or WHO are listed along with the TEF values defined by acute oral LD₅₀ from references^{24,26}. TEF values proposed by the EFSA or WHO are for each single toxin. Therefore, in Table 5, the numbers labeled as the TEF values of GTX1&4 proposed by the EFSA or WHO are the author's recalculations of the TEF values of GTX1 and GTX4, based on the proportions of GTX1 and GTX4 in the GTX1&4 solution used in this study. Similarly, the numbers labeled as the TEF values of GTX2&3 and dcGTX2&3 proposed by the EFSA or WHO are the author's recalculations based on the proportion of corresponding toxins. The TEF determined in this study for NeoSTX, was 1.47, which is lower than the 2.00 recommended by WHO⁹ and 1.70 reported by Munday et al.²⁴. Although, the TEFs were based on acute oral toxicity data from the WHO, the TEFs calculated using the oral acute LD₅₀ values obtained from this study showed significant correlations, with correlation coefficients of 0.969 and 0.994, respectively (Fig. 1).

Discussion

PSP is caused by consuming shellfish or fish contaminated with PSP toxins, primarily STX and its derivatives⁸. These derivatives include Neo-STX, dcSTX, GTX1-4, and dcGTX1-4⁴. Symptoms of PSP in humans typically appear 20 min to 5 h after ingestion and include numbness, tingling, nausea, neurological issues such as dizziness and muscle paralysis, and even death due to respiratory failure^{4,5}. Traditionally, PSP toxin detection has

been conducted using MBAs following the method described in the AOAC Official Method 959.08. In the MBA, PSP toxins are extracted using a 3 mM hydrochloride solution from the edible part of shellfish, and 1 mL of the extract is administered to mice by IP injection. Based on the BW of the mice and time to death, the mouse unit (MU) of the extract is determined²⁷. However, these tests do not reflect human consumption routes, and thus, more recently, oral toxicity studies have led to revised TEFs set by the WHO in 2016⁹.

Since 1959, when the 3Rs principles for humane animal experiments were proposed, considerations of animal welfare and ethics in *in vivo* experiments have expanded^{17,18,28}. Specifically, the "replacement" aspect of the 3Rs has been emphasized, and instrumental methods such as HPLC and liquid chromatography with tandem mass spectroscopy, which have the advantage of accurately measuring the chemical concentrations of toxins, are gaining traction to replace MBA in detecting PSP toxins^{29,30}. However, as these analytical methods have the limitation of not reflecting the physiological effects of toxins in the body, the biological relevance of *in vivo* experiments is still highly valued and considered crucial for establishing TEF values for PSP toxins and newly discovered marine biotoxins⁷.

The amount of toxin required for *in vivo* toxicity studies is higher than that required for HPLC, and the toxicity of the same toxin is generally weaker orally than intraperitoneally, requiring higher doses of toxin for oral administration than for IP injection^{16,23,31}. In this situation, acquiring a large quantity of CRMs necessary for obtaining accurate results is costly. Additionally, due to the nature of these toxins, chemical synthesis is not possible, and they must be

extracted from toxin-producing microalgae, leading to an unstable supply. Moreover, with the exception of STX, the CRM for the remaining PSP toxins is entirely dependent on specific manufacturers. In other words, while establishing appropriate TEF values based on oral toxicity tests, we must focus on the principle of reduction within the 3Rs, considering both ethical concerns and financial issues. In line with the principle of reduction, the OECD Guideline 425 (up-and-down procedure, UDP)³² is a method for minimizing animal use in acute toxicity testing, but it has limitations in accurately estimating toxicity levels and reflecting complex toxic mechanisms. To address these issues, Dewi et al.¹⁹ proposed the four-level RSP, which allows for more precise analysis of reactions at different dosage levels and a more accurate estimation of toxicity curves. The RSP offers greater efficiency and accuracy compared with the UDP, providing more useful results in acute toxicity testing.

In this study, we performed *in vivo* toxicity studies with the three-level RSP design^{19,20} omitting the fourth level from the four-level RSP to further reduce the number of mice from 24 to 15 (Table 1). As the three-level RSP is designed to minimize the number of mice used while still obtaining reliable results, the key factors in utilizing this method in oral toxicity studies of biotoxins are, 1) appropriate toxin materials; 2) the starting dose (m_1); and 3) the adjustment factor (k). In the present study, all toxins used were CRM purchased from Sigma-Aldrich (for STX) and Cifga Laboratory (for the others) (Table 2). The m_1 value should be determined based on all available information, aiming to be close to the expected LD_{50} . The k value should be set so that, from level 1 to level 3, the dose for each subsequent level efficiently approaches the LD_{50} based on the lethal dose of the previous stage. Based on this, the m_1 and k for each PSP toxin were determined (Table 3). Determining m_1 is critical, and this requires previously reported LD_{50} values. In this study, there was a lack of reference papers providing LD_{50} values for toxins other than STX. Consequently, the limited range of test doses was a result of relying solely on the studies by Munday et al.²⁴, and Selwood et al.²⁶ To address this limitation, conducting preliminary experiments to determine m_1 before applying the three-level RSP to unknown marine biotoxins could be considered.

In three-level RSP as well as in the UDP and four-level RSP, the LD_{50} is determined based on the relationship between dose and lethality at each level^{19,32}. Therefore, it would be recommended to have at least one dose with a lethality below 50% and one above 50%. In this experiment, this was the case for STX, dcSTX, GTX1&4, and dcGTX2&3. However, for NeoSTX and GTX2&3, all levels showed lethal rates below 50%. In such cases, the LD_{50} was extrapolated based on the dose-lethality relationship. To

determine this, a generalized linear regression method²¹) was applied to calculate the LD_{50} for all toxins (Table 4). Munday et al. reported oral LD_{50} values of STX, neoSTX, dcSTX, GTX 1&4 and GTX 2&3 with 95% confidence intervals, which were 442.9 (379.3-483.9), 271.0 (229.2-310.9), 855.8 (809.7-1389.0), 662.3 (608.8-682.8), and 881.6 (739.3-1012.1) $\mu\text{g}/\text{kg}$, respectively²⁴. Selwood et al. reported an oral LD_{50} value of dcSTX with 95% confidence intervals which was 2511.9 (2113.8-2677.5) $\mu\text{g}/\text{kg}$ ²⁶. The LD_{50} values determined in this study were found to fall within the 95% confidence intervals of the LD_{50} values reported by Munday et al.²⁴ and Selwood et al.²⁶

Based on the LD_{50} , the TEFs for examined PSP toxins against STX were determined and compared with the previously recommended TEFs by the EFSA¹¹ and WHO⁹), as well as TEFs calculated based on oral LD_{50} reported by other researchers^{24,26} (Table 5). To some extent, the TEF of NeoSTX obtained from our study was lower than that recommended by the WHO and reported by other researchers. Nevertheless, the TEF values obtained from the present study were highly correlated with those from the WHO and based on the acute oral LD_{50} reported by others (Fig. 1).

Various marine biotoxins, including STX, are assessed by assigning TEFs to the derivatives of each reference toxin and calculating the toxicity in the sample as an equivalent amount of the reference toxin^{12,33,34}. Therefore, assigning appropriate TEFs to these derivatives is essential not only for PSP toxins but also for proactively addressing emerging marine biotoxins, such as cyclic imines and palytoxins, which are not yet regulated, and for developing strategies to ensure food safety⁷. This process requires not only well-designed experiments to accurately determine LD_{50} but also long-term data accumulation and analysis. By securing sufficient toxicity data on various derivatives and new toxins, it will be possible to respond swiftly and accurately in the event of future toxin outbreaks. Meanwhile, there is limited data available for the chronic toxicity of marine biotoxin using *in vivo* systems, and this study also focused on how to reduce the number of mice for the acute toxicity study. In the future, further studies will be needed to ethically optimize *in vivo* chronic toxicity tests to assess toxicity that may result from continuous exposure to doses that do not exceed the regulatory threshold.

In conclusion, despite the ethical concerns and practical challenges associated with *in vivo* toxicity studies, appropriate experimental design is essential for the effective toxicity assessment of PSP toxins and other marine toxins. The three-level RSP method used in this study can enhance the efficiency of acute oral toxicity studies while providing reliable data, making it a key contributor to the development of future toxin management systems.

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국문요약

마비성 패류독소 중독증(paralytic shellfish poisoning; PSP)은 사식독신과 그 유사체로 오염된 패류를 섭취했을 때 발생하며, 저림, 구토 등의 증상에서부터 근육 마비와 심각한 경우 호흡 마비로 이어져 사망에 이를 수 있다. 독성등가계수(toxic equivalency factors; TEFs)는 다양한 마비성 패류독소의 독성을 표준화하여 위험성을 평가하는데 사용된다. 마비성 패류독소를 검출하기 위해 사용되던 마우스 생체 실험(mouse bioassay; MBA)에 대한 윤리적 문제가 제기되면서 고성능액체크로마토그래피와 같은 기기 분석법으로의 전환이 시도되고 있지만, 유사체들의 적절한 TEF를 설정하기 위해서는 여전히 동물 모델을 통한 생체 내 독성 데이터가 필수적이다. 본 연구에서는 동물 수를 줄이면서도 신뢰할 수 있는 경우투여 독성 결과를 얻기 위해 삼단계 반응표면-경로 (three-level RSP) 설계를 사용했다. 인증 표준 물질을 이용하여 각 독소의 초기 용량과 조정 계수를 결정하고 시험을 진행했으며, STX.2HCl, NeoSTX, dcSTX, GTX1&4, GTX2&3, dcGTX2&3의 반수치사량 (및 TEF) 값은 각각 451.3 (1.00), 306.5 (1.47), 860.9 (0.52), 644.5 (0.70), 915.3 (0.49), 2409.3 (0.19)로 나타났다. 도출된 TEF 값은 2016년 WHO에서 권고한 TEF 값뿐만 아니라, 이전에 보고된 경구 투여 반수치사량을 기반으로 한 TEF 값과 강한 상관관계를 보였다. 본 연구는 마비성 패류독소 뿐만 아니라 신규 미관리 해양생물독소에 대해 적절한 TEF를 설정하는 데 있어 삼단계 반응표면경로 설계를 윤리적 우려와 신뢰할 수 있는 독성 데이터의 필요성 사이에서 효과적으로 균형을 맞출 수 있는 방법으로 제안한다.

Conflict of interests

The authors declare no potential conflict of interest.

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