Biol Res 37: 395-403, 2004

The Gonyautoxin 2/3 epimers reduces anal tone when injected in the anal sphincter of healthy adults

ROGELIO GARRIDO[1], NÉSTOR LAGOS[2], KARINNA LATTES[2], CARLOS GARCÍA[2] RODRIGO AZOLAS[1], GUNTHER BOCIC[1], ALDO CUNEO[1], HECTOR CHIONG[1], CRISTIAN JENSEN[1], ANA HENRÍQUEZ [1] and CRISTIAN FERNÁNDEZ[2]

[1] Departamento de Cirugía, Sección Proctología, Hospital Clínico Universidad de Chile.
[2] Lab. Bioquímica de Membrana, Dept. de Fisiología y Biofísica, Facultad de Medicina, Universidad de Chile, Casilla 70005, Correo 7, Santiago, Chile. Fax (56-2) 777-6916.

ABSTRACT

The primary clinical symptom of Paralytic Shellfish Poisoning is acute paralytic illness produced by paralyzing toxins. Paralytic shellfish poison is formed by a mixture of phycotoxins and their toxicity is due to its reversible binding to a receptor site on the voltage-gated sodium channel on excitable cells, thus blocking neuronal transmission. We studied the effect of the gonyautoxin 2/3 epimers by local infiltration in the anal internal sphincter of healthy voluntary adults in order to reduce anal tone. The toxin was injected after prior clinical evaluation, anoscopy and anorectal manometry. Post injection clinical examination, electromyography and anorectal manometry were performed. Resting and voluntary contraction pressures were measured and the anorectal inhibitory and anocortical reflexes were tested by manometry. Blood and urine samples were obtained from each participant, and hemogram, basic metabolic panel, and urinalysis were done both before and one week after the injection. This study shows, for the first time, that gonyautoxin 2/3 reduces the anal tone by relaxing the anal sphincters in 100 % of the participants. Manometric recordings showed a significant decrease in anal maximal voluntary contraction pressure after the toxin injection, dropping to 55.2 ± 6.2 % and 47.0 ± 6.8 % (Mean Value ± Std.Dev.) of the baseline values at 2 minutes and at 24 hours respectively after the injection. Post-injection electromyography showed that activity of the muscle was abolished. We conclude that local administration of gonyautoxin 2/3 to the anal sphincter produces immediate relaxation and a statistically significant decrease in the anal tone ($p < 0.001$).

Key terms: Gonyautoxin 2/3, Phycotoxins, PSP toxins, anal sphincter, anal fissure.

INTRODUCTION

Phycotoxins are produced by microscopic planktonic algae. In the sea these toxins are accumulated by filter feeders bivalves. When humans consume these bivalves they become intoxicated. Until now, six human illnesses associated with phycotoxins have been described: Paralytic Shellfish Poisoning (PSP), Diarrheic Shellfish Poisoning (DSP), Amnesic Shellfish Poisoning (ASP), Neurotoxic Shellfish Poisoning (NSP), Ciguatera Poisoning (CP) and Cyanobacterial Poisoning (CNP) (Hallegraeff, 1993; Yasumoto et al. 1995; Lagos, 1998). The latter is not a harmful marine issue but rather the product of certain fresh water blue-green algae that produce extremely toxic phycotoxins associated with poisoning in humans (Rodrigue et al. 1990; Long et al. 1990; Montebruno, 1993; Carmichael, 1996; Gessner et al. 1997) and animals (Lagos, 1998; Falconer, 1996; Pereira et al. 2000).

PSP –and its acute paralytic illness– poses a serious public health threat due to its high mortality rate in mammals worldwide (Lagos, 1998; Oshima, 1995; Andrinolo et al. 1999a; Lagos et al. 2000; Andrinolo et al. 2002a). Paralyzing toxins

Corresponding author: Néstor Lagos, Ph.D., Lab. Bioquímica de Membrana, Dept. de Fisiología y Biofísica, Facultad de Medicina Universidad de Chile. Casilla 70005, Correo 7, Santiago, Chile. Phone: (56-2) 678-6309, Fax: (56-2) 777-6916. E-mail: nlagos@ med.uchile.cl

Received.: January 16, 2004, In Revised Form: June 17, 2004. Accepted: June 23, 2004.

are endemic in the southern Chilean fjords due to the annual occurrence of toxic dinoflagellate blooms that produce the so-called 'red-tide' (Lagos, 1998).

Paralytic shellfish poison is formed by a mixture of phycotoxins with the structural 3,4,6-trialquil tetrahidropurine (Schantz et al. 1975; Shimizu et al. 1981; Oshima, 1995) common skeleton. Until now, 26 different naturally-occurring PSP toxins have been described (Oshima, 1995; Lagos, 1998; Pereira et al. 2000; Harada et al. 1982; Onodera et al. 1997; Lagos et al. 1999; Molica et al. 2002). PSP toxins are non-protein, low molecular weight compounds that can be classified by net charge at neutral pH into three major groups: (i) saxitoxins group (STXs) with a net charge +2; (ii) gonyautoxins group (GTXs) with net charge +1; (iii) the group of the N-sulfocarbamoyl -11-hydroxysulfate toxins (Cs) with net charge zero (Shimizu, 1993; Oshima, 1995). Of all the paralyzing toxins in natural samples, the gonyautoxin are the most abundant PSP toxins, accounting for more than 80 % of the total toxin content (Lagos, 1998; Lagos et al. 1996; Compagnon et al. 1998; Andrinolo et al. 1999b).

The high toxicity of paralyzing toxins is due to their reversible binding to a receptor site on the voltage-gated sodium channel on excitable cells, thus blocking the influx of Na^+ ions and preventing nerve and muscle cells from producing action potentials, therefore blocking neuronal transmission and causing the death of mammals by respiratory arrest and cardiovascular shock (Kao, 1966; Narahashi, 1972; Catteral et al. 1979; Strichartz, 1984; Moczydlowski et al. 1984; Guo et al. 1987; Hall et al. 1990; Strichartz et al. 1995; Andrinolo et al. 2002b; Lagos and Andrinolo, 2000; Andrinolo et al 2002a). Local application of small amounts of paralyzing toxins in striated muscles produces flaccid paralysis for periods that are dose-dependent.

This paper examines the effect of the Gonyautoxin 2/3 epimers (GTX 2/3) by local infiltration of the toxins in the anal internal sphincter muscle of healthy voluntary adults in order to reduce anal tone.

METHODS

This study was performed at the Coloproctology Section, Surgery Department, Universidad de Chile Clinical Hospital, Santiago, Chile. This was a placebo-controlled parallel group study of ten healthy, voluntary human male adults aged 24 to 48 years. An additional six volunteers comprised the parallel placebo control group and were injected with the same total dose volume, but received a toxin-free 0.9 % NaCl solution.

This study complied with the Declaration of Helsinki recommendation regarding biomedical research involving human volunteers and was approved by the institutional review board. The design and purpose of the study and the potential risks of participation were discussed with each of the volunteers before enrolment, and their written informed consent was obtained.

To be eligible for the study, volunteers were required to be healthy male adults (18 to 50 years of age) with normal sphincter tone (under 72 mmHg maximum resting pressure measured by anorectal manometry) and no anorectal pathologies such as hemorrhoids, fistula, or abscesses ever diagnosed. Under the Good Clinical Practice Guidelines, the participants were fully informed about the GTX 2/3 toxin action and molecular mechanism. Written consent was an absolute requirement of the Clinical Hospital ethics committee. This study was conducted under approval from the Universidad de Chile Clinical Hospital Ethics Committee and Instituto de Salud Pública (Reference N^o 00062) Santiago, Chile.

Before the toxin injection, each voluntary participant underwent anorectal manometry, electromyography, hemogram, basic metabolic panel and urinalysis tests. On-line fluorescent detection of toxin in urine was performed by analytical high performance liquid chromatography (HPLC); the detection limit with this method is 1 microgram in 10 microliters (Lagos, 1998). Each vial of toxin contained a sterile solution of 100 units of GTX 2/3 (Lagos, 1998; Lagos, 2002) in 1.0 ml total volume of 0.9 % of sodium chloride, without preservatives. This dose was

locally infiltrated in both sides of the anal internal sphincter using 0.5 ml in each side. An insulin syringe with a 25-gauge needle (25x5) was used for the injection. One unit of the paralyzing toxin activity corresponds to the amount of toxins enough to block neuromuscular contraction of mouse leg crural bicep for 1.5 to 2.0 hours. The gonyautoxin 2/3 was purified from shellfish highly contaminated with PSP toxins (Lagos, 1998; Lagos, 2003; Lagos, 2002). The shellfish were collected in Chilean Patagonian fjords.

A second anorectal manometry was performed two minutes after the injection. Anal pressures were measured by recording resting and maximal voluntary contraction pressures. Both the anorectal inhibitory and the anocortical reflexes of each participant were tested before and after the toxin injections. Anorectal manometries were also performed at 24 hours, 4 days, 6 days, 10 days, 12 days and 15 days after the toxin injection. Another six volunteer participants comprised the placebo control group. They were injected with the same total volume, but containing only a 0.9% sodium chloride solution, without the toxin. Anorectal manometric studies were also performed to demonstrate quantitative changes in anal pressures. The placebo control group was tested in parallel in order to make subject comparison.

Manometric recordings and an analysis of the tracing were made using a water perfusion system. The anal canal pressure was recorded by stationary pull-through technique using a water-filled micro balloon and external transducer (PVB) perfusion equipment (Medtronic Inc., Bonn, Germany). The recording and analysis of the tracing were both made by a computerized system (8 channels polygraph ID., Medtronic Polygraph with Polygram 98 version 2.2 software). Anal resting pressures were recorded in millimeters of mercury using the stationary pull-through technique and the mean pressure was identified by the computer. The maximal voluntary contraction was assessed by evaluation of the voluntary contractions of anal sphincter in each participant. Amplitude was expressed in millimeters of mercury.

The 10 volunteer participants injected with the toxin were clinically evaluated at 24 hours, and then every two days between day. Vital signs, hematological parameters (hemogram), basic metabolic panel and urinalysis tests were assessed at the beginning (a day before injection) and a week after the injection. Additionally, the amount of toxin was analyzed in urine samples collected 4 hours after injecting the dose. Pulse and blood pressure, possible side effects and pain scores were recorded at each visit. The scores of injection pain and of pain two minutes after the injection were also recorded. This was evaluated by the participant subjects on a scale from 1 to 10, where 10 was the maximum value. Adverse events were monitored throughout the course of the study. Approximately 15 minutes after the injection and at each follow-up visit, the volunteers were asked a general open question, such as, "How have you been feeling since the injection/last visit?" Directed questioning and examination were then performed as appropriate.

Statistics

The Student *t*-test was used to evaluate differences in the maximum resting and voluntary contraction pressures obtained by anorectal manometry of the group injected with toxin and placebo and also before and after the toxin injection. The significance of any difference in mean was tested by the paired Student *t*-test, whereas the significance of any difference in proportions was tested by the chi-square statistic. All P values are two-tailed and shown in the Tables.

Long-term outcomes were determined after a 16-month median follow-up. This was accomplished by personal communication with the participants, most of whom worked at the same Hospital, and by clinical examination of the participants upon request.

RESULTS

No participants dropped out of the study; none were lost during the study's follow-up monitoring, nor did any suffer adverse

events or negative side effects during or after this study. Clinical laboratory tests such as the hemogram, basic metabolic panel, and urinalysis performed on each participant both before and one week after the injections did not show any significant changes. Furthermore, no toxins were detected in urine samples collected 4 hours after the injection. This clearly shows that the amount of toxin injected was under the HPLC analysis detection limit, which completely agrees with the fact that PSP toxins, once in the bloodstream, immediately move on to the extracelular fluid (Andrinolo et al. 1999a; Lagos et al. 2000; Andrinolo et al. 2002b) producing a dilution that is under the detection limits.

The participants declared that after the injection they felt anal anesthesia for an average 59.50 ± 7.12 minutes (Mean value ± Stand. Dev.), and sphincter hypotonical sensation for 40.0 ± 4.20 minutes (Mean value ± Stand. Dev.). None of the participants showed flatus incontinence or any transitory fecal incontinence (Table I).

Manometric recordings showed a significant decrease in anal maximal voluntary contraction pressure (MVCP) of the participants injected with toxins (p > 000.1). After 2 minutes, this was 55.2 ± 6.2 % (Mean value ± Stand. Dev.) of the baseline values, a decrease of 44.8 %. At 24 hours post-injection, the fall reached 53 % of the baseline. The last decrease was detected by a third anorectal manometry at 24 hours post-injection (Fig. 1, lower trace). None of the six participants injected with 0.9 % sodium chloride placebo solution showed any change in resting pressure or maximum voluntary contraction pressure – showing 68.0 ± 4.3 mm Hg and 123.0 ± 12.4 mm Hg respectively – both of which are normal average pressures according to the case experience of more than 3,000 anorectal manometries performed by the Hospital Service.

Figure 1 shows a typical manometric record where an impressive fall in anal maximal voluntary contraction pressure may be seen; in this case a decrease from 160 mm Hg (baseline, Fig. 1A) to 95 mm Hg (2 minutes post-injection, Fig. 1B) and 75 mmHg (24 hours post-injection, Fig. 1C). 15 days after the injection, all the anorectal manometry parameters were the same as those recorded before the toxin infiltration, with values in the baseline range.

Figure 2 shows an average electromyography (EMG) recorded before (upper traces) and after (lower traces) the toxin injection. This one clearly shows that the muscle activity was abolished after the toxin sphincter infiltration. The post-injection record of both sides correspond to the lower traces in Figure 1. Both the recorded amplitude and frequency decreased impressively, showing sphincter paresis (Fig. 2, lower traces).

TABLE I

Symptoms and side effects in healthy voluntary participants

Healthy voluntary adults	10
Pain during injection (Max. 10; Min. 1)	6 ± 1 £
Pain two minutes after injection (Max. 10; Min. 1)	100 % without pain
Anal anesthetic sensation (time)	59.50 ±7.12 min.
Sphincter relaxation sensation	40.0 ±4.20 minutes
Flatus incontinence	None
Fecal incontinence	None
Clinical evaluation	Immediate relaxation, 100 %
Side effects	None, 100 % of participants
Other	All asymtomatic after 24 hours

£ Mean values ±Stand. Dev.

TABLE II

Anorectal Manometry Recordings

	Placebo injection	GTX2/3 injection
Age (years)	34.0 ± 6.9	37.4 ± 8.0
Maximum resting pressures (MRP) (mm Hg)		
Pre-injection	68.5 ± 2.5	66.2 ± 19.5
2 min post-injection	68.0 ± 4.3	62.1 ± 15.1
Maximum voluntary contraction pressures (MVCP) (mm Hg)		
Pre-injection	122.2 ± 9.9	126.0 ± 11.5
2 min post-injection	123.0 ± 12.4	69.5 ± 5.8 £
Reflexes		
Ano-rectal inhibitory reflex	100% maintained	100% maintained
Ano-cortical reflex	100% maintained	100% maintained

Values are Mean ± Stand. Dev.
£ $p < 0.05$

Figure 1-A

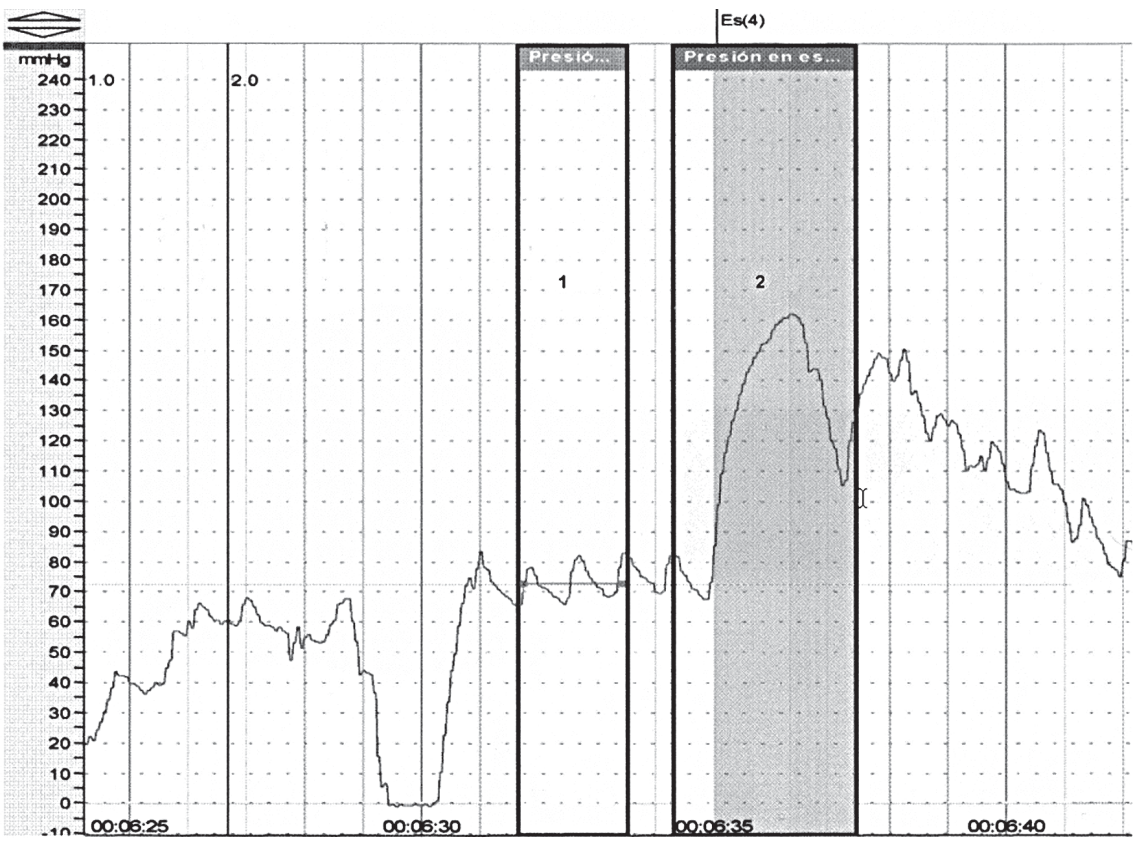


Figure 1. Manometry record of resting and maximal voluntary contraction pressures. A. Before (pre-injection). B. 2 minutes post-injection. C. 24 hours after the toxin injection in the anal internal sphincter. **1**, Maximum resting pressure and **2**, Maximum voluntary contraction pressure

Figure 1-B

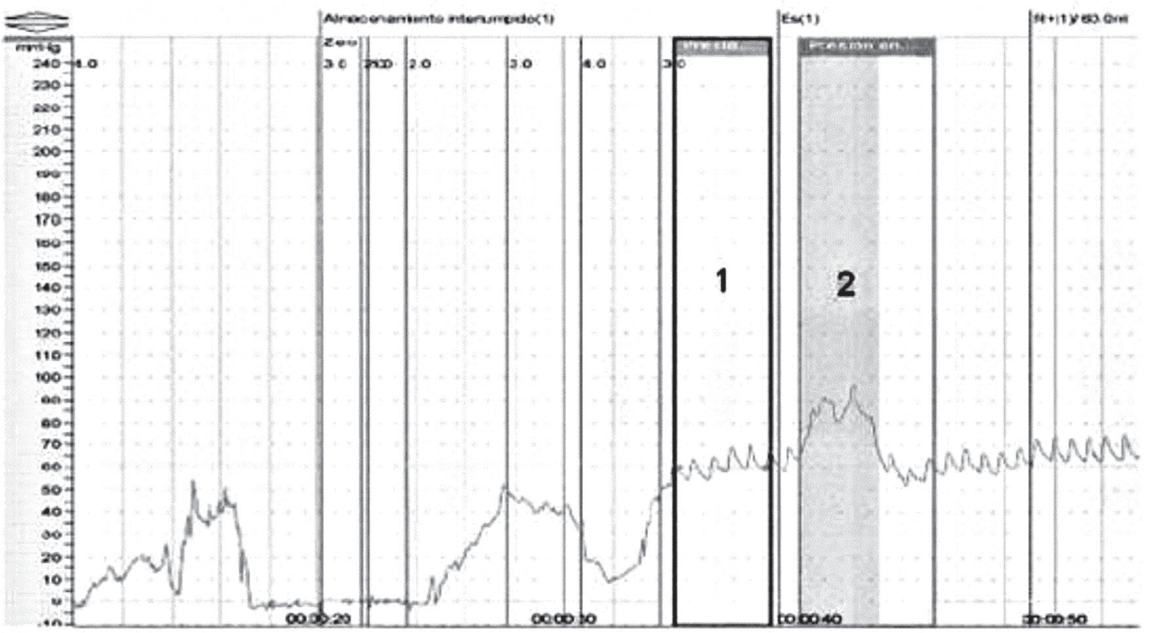
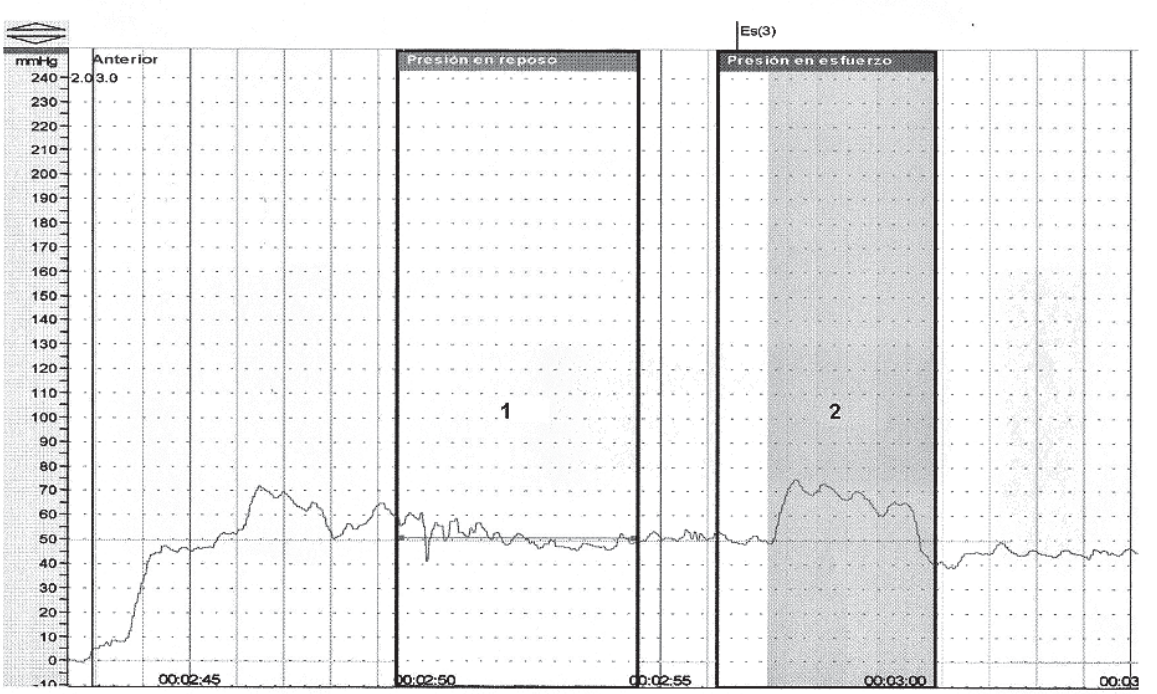


Figure 1-C



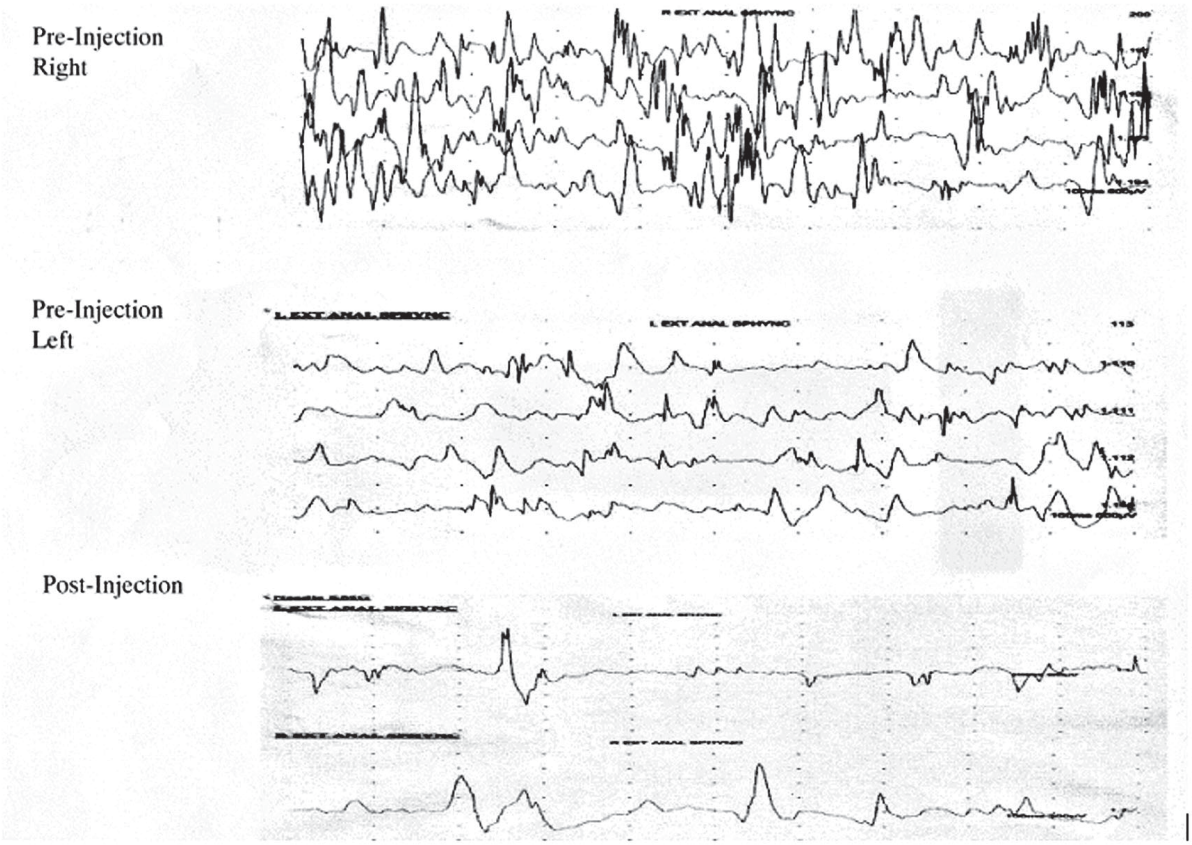


Figure 2. Electromyography record, showing the internal anal sphincter muscle activities pre (upper traces) and post toxin injection (lower traces).

After 16 months of follow-up, none of the participants experienced adverse events or negative side effects. None of the participants showed any systemic side effects or anorectal problems. All of the participants in this study are currently asymptomatic and perfectly healthy.

DISCUSSION

The fact that all the participants relaxed their anal sphincter 2 minutes after the GTX 2/3 infiltration shows that these toxins, when locally administered, produce paresis of the sphincter. Although this study does not include a large number of participants, all of them showed a decrease in anal tone. On the other hand, the six participants injected with a placebo solution did not show any change in anal tone or decrease in anal maximal voluntary contraction pressure, as occurred in those injected with toxin.

Mean scores of maximum resting pressure and maximum voluntary contraction pressure were calculated for both the placebo and toxin-injected groups. The placebo group maintained both resting and voluntary contraction pressures without any statistically significant change from those recorded at the baseline ($p > 0.05$). In contrast, the MVCP toxin recorded after the injection showed a mean score 67 ± 10.57 mmHg less than that recorded before the injection. This fall was statistically significant ($p < 0.001$), indicating that the toxin injection resulted in a diminishing of the anal tone.

An important finding was that neither flatus nor fecal incontinence were observed and that in all the participants the anorectal inhibitory and anocortical reflexes remained functional. No differences were

observed before or after the injections. Due to the low amounts of toxin used in this study the danger of flatus and fecal incontinence was eliminated. In this case, the injection blocks excessive muscle contraction but leaves enough strength for normal performance. Local peripheral application of PSP toxins interferes with neuromuscular transmission and alters the action that produces sequential sphincter paralysis (Kao, 1966; Narahashi, 1972). The toxin thus paralyzes the injected muscle but does not affect the other muscles. The paretic effect exhibited by the injected sphincter lasts for 12 days (toxin dose = 100 units). Furthermore, no side effects were observed in the participants at any time over the long-term (16-months) follow up. To our knowledge, this is the first report on testing this toxin for anal sphincter relaxation.

This study concludes that the local intramuscular injection of GTX 2/3 epimers in the anal internal sphincter produces immediate relaxation of the sphincter with a decrease in voluntary contraction pressure and a significant decrease in anal tone. As a result of these findings, the local administration of paralyzing toxin should be an effective therapy for anal fissure. Temporary pharmacological immobilization of the anal sphincter in order to eliminate sphincter spasm should be a critical step in treating and healing anal fissure as it breaks the vicious cycle of inflammation, pain, and sphincter spasm.

Immobilization of healing tissues is a fundamental therapeutic principle, and treatment with paralyzing toxins may be found to be applicable in other pathologies such as blepharospasm, tics, tremors, bruxism, hemifacial spasm, cervical dystonia, cerebral palsy, pain brought on by muscle spasms (writer's and musician's cramps), hand tremors, and spasmodic dystonia, and others in which muscle hypertonicity results in stiff, awkward movements.

Due to the effectiveness and safety of paralyzing toxin injections as shown in this study, a trial test of these for healing anal fissure is currently underway at our Clinical Hospital, under approval from the Universidad de Chile Clinical Hospital Ethics Committee and Instituto de Salud Pública (Chile).

ACKNOWLEDGMENTS

This study was supported by grants FONDECYT 1020090 and CSMAR 02/5-2. DID, Universidad de Chile and Programa de Cooperación Científica Internacional CONICYT/GRICES. The authors would like to thank Gladys Del Peso, Medical Technologist, Departamento de Rehabilitación, Hospital Clínico Universidad de Chile, who helped recording the electromyography data. C. Garcia is currently a student of the USACH Biotechnology Ph.D. Program. We thank V. Iglesias for her statistical advice (Public Health School, University of Chile).

REFERENCES

ANDRINOLO D, MICHEA LF, LAGOS N (1999a) Toxic effects, pharmacokinetics and clearance of Saxitoxin, a component of Paralytic Shellfish Poison (PSP), in cats. Toxicon 37: 447-464

ANDRINOLO D, SANTINELLI N, OTAÑO S, SASTRE V, LAGOS N (1999b) Paralytic shellfish toxins in mussels and Alexandrium tamarense at Valdes Peninsula, Chubut, Patagonia Argentina: Kinetic of a natural depuration. J Shellfish Res 18: 203-209

ANDRINOLO D, IGLESIAS V, GARCÍA C, LAGOS N (2002a) Toxicokinetics and toxicodynamics of gonyautoxins after an oral toxin dose in cats. Toxicon 40: 699-709

ANDRINOLO D, GOMES P, FRAGA S, SOARES-DA-SILVA P, LAGOS N (2002b) Transport of the organic cations Gonyautoxin 2/3 epimers, a Paralytic Shellfish Poison Toxin, through the human and rat intestinal epitheliums. Toxicon 40: 1389-1397

CARMICHAEL WW (1996) Liver failure and human deaths at a haemodialysis center in Brazil: microcystins as a major contributing factor. Harmful Algae News. IOC-UNESCO 15: 11

CATTERALL WA, MORROW CS, HARTSHORNE RP (1979) Neurotoxin Binding to Receptor Sites Associated with Voltage-sensitive Sodium Channels in Intact, Lysed, and Detergent-solubilized Brain Membranes. J Biol Chem, 254: 11379-11387

COMPAGNON D, LEMBEYE G, MARCOS N, RUIZ-TAGLE N, LAGOS N (1998) Accumulation of PSP toxins in the bivalve *Aulacomya ater* and two carnivorous gastropods *Concholepas concholepas* and *Argobuccinum ranelliformes* during an Alexandrium catenella bloom in southern Chile. J Shellfish Res 17: 67-73

FALCONER IR (1996) Potential impact on human health of toxic cyanobacteria. Phycologia 36: 6-11

GESSNER BD, BELL P, DOUCETTE GJ, MOCZYDLOWSKI E, POLI MA, DOLAH FV, HALL S (1997) Hypertension and identification of toxin in

human urine and serum following a cluster of mussel-associated paralytic shellfish poisoning outbreaks. TOXICON 35: 711-722

GUO X, UEHARA A, RAVINDRAN A, BRYANT SH, HALL S, MOCZYDLOWSKI E (1987) Kinetic basis for insensitivity to tetrodotoxin and saxitoxin in sodium channels of canine heart and denervated rat skeletal muscle. Biochem 26: 7546-7556

HALL S, STRICHARTZ G, MOCZYDLOWSKI E, RAVINDRAN A, REICHARDT PB (1990) The saxitoxins; sources, chemistry, and pharmacology. In: HALL A, STRICHARTZ G (eds) Marine Toxins: Origin, Structure and Molecular Pharmacology. Washington, DC: American Chemical Society Symposium series 418. Am Chem Soc pp: 29-65

HALLEGRAEFF GM (1993) A review of harmful algal blooms and their apparent global increase. Phycologia 32: 79-99

HARADA T, OSHIMAY, YASUMOTO T (1982) Structure of two paralytic shellfish toxins. Gonyautoxins V and VI, isolated from a tropical dinoflagellate, *Pyrodinium bahamense* var. *compressa*. Agric Biol Chem 46: 1861-1864

KAO CY (1966) Tetrodotoxin, saxitoxin and their significance in the study off excitation phenomenon. Pharm Rev 18: 997-1049

LAGOS N, COMPAGNON D, SEGUEL M, OSHIMA Y (1996) Paralytic Shellfish Toxin Composition: A Quantitative Analysis In Chilean Mussels and dinoflagellate. In: YASUMOTO T, OSHIMA Y, FUKUYO Y. PARIS (eds) Harmful and Toxic Algal Blooms. Intergovernmental Oceanographic Comm of UNESCO pp: 121-124

LAGOS N (1998) Microalgal bloom: a global issue with negative impact in Chile. Biol Res 31: 375-386

LAGOS N, ONODERA H, ZAGATTO PA, ANDRINOLO D, AZEVEDO SMFQ, OSHIMA Y (1999) The first evidence of paralytic shellfish toxins in the freshwater cyanobacterium *Cylindrospermopsis raciborskii*, isolated from Brazil. Toxicon 37: 1359-1373

LAGOS N, ANDRINOLO D (2000) Paralytic Shellfish Poisoning (PSP): Toxicology and Kinetics. In: BOTANA LM (ed) Seafood and Freshwater Toxins: Mode of Action, Pharmacology and Physiology. New York: Marcel Dekker, Inc. pp: 203-215

LAGOS N (2002) Principales toxinas de origen fitoplantónico: Identificación y cuantificación mediante cromatografía líquida de alta resolución (HPLC). In: SAR EA, FERRARIO ME, REGUERA B (eds) Floraciones Algales Nocivas en el Cono Sur Americano. Madrid: Instituto Español Oceanográfico pp: 57-76

LAGOS N (2003) Paralytic shellfish poisoning phycotoxins: occurrence in South America. Comments on Toxicol 9: 1-19

LONG RR, SARGENT JC, HAMMER K (1990) Paralytic shellfish poisoning: A case report and serial electrophysiologic observations. Neurol 40: 1310-1311

MOCZYDLOWSKI E, HALL S, GARBER SS, STRICHARTZ GS, MILLER C (1984) Voltage-dependent blockade of muscle Na^+ channels by guanidinium toxins: effect of toxin charge. J Gen Physiol 84: 687-704

MOLICA R, ONODERA H, GARCÍA C, RIVAS M, ANDRINOLO D, NASCIMENTO S, LAGOS N (2002) Toxins in the freshwater cyanobacterium *Cylindrospermopsis raciborskii*, isolated from Tabocas reservoir in Caruaru, Pernambuco, Brazil. Phycologia 41: 606–611

MONTEBRUNO D (1993) Intoxicación por ingesta de mariscos contaminados con VPM (aspectos médicos y forenses). Rev Sanidad Def Nac 9: 127-132

NARAHASHI T (1972) Mechanism of action of tetrodotoxin and saxitoxin on excitable membranes. Fed Proc 31: 1124-1132

ONODERA H, SATAKE M, OSHIMA Y, YASUMOTO T, CARMICHAEL WW (1997) New saxitoxin analogues from the freshwater filamentous cyanobacterium *Lyngbya wollei*. Natural Toxins 5: 146-151

OSHIMA Y (1995) Postcolumn derivatization liquid chromatographic method for paralytic shellfish toxins. J AOAC Intl 78: 528-532

PEREIRA P, ONODERA H, ANDRINOLO D, FRANCA S, ARAUJO F, LAGOS N (2000) Paralytic shellfish toxins in the freshwater cyanobacteria *Aphanizomenon flos-aquae*, isolated from Montargil Reservoir, Portugal. Toxicon 38: 1689-1702

RODRIGUE D, ETZEL R, MAY S, PORRAS E, VELÁSQUEZ O, TAUXE R, KILBOURNE E (1990) Lethal paralytic shellfish poisoning in Guatemala. Am J Trop Hyg 42: 267–271

SCHANTZ EJ, GHAZAROSSIAN VE, SCHONES HK, STRONG FM (1975) The Structure of Saxitoxin. J Am Chem Soc 97: 1238-1239

SHIMIZU Y, HSU CH, GENENAH A (1981) Structure of saxitoxin in solutions and stereochemistry of dihydrosaxitoxins. J Am Chem Soc 103: 605-609

SHIMUZU Y (1993) Microalgal Metabolites. Chem Rev 93: 1685-1698

STRICHARTZ G (1984) Structural determinants of the affinity of saxitoxin sodium channel. J Gen Physiol 84: 281-305

STRICHARTZ GS, HALL S, MAGNANI B, HONG CY, KISHI Y, DEBIN JA (1995) The potencies of synthetic analogues of saxitoxin and the absolute stereoselectivity of decarbamoyl saxitoxin. Toxicon 33: 723-737

YASUMOTO T, FUKUI M, SASAKI K, SUGIYAMA KD (1195) Determination of Marine Toxins in Foods. J AOAC Intl 78: 574-582