Phycotoxins of the Paralytic Shellfish Poison: Clinical Applications

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Introduction

A drug is defined as an agent intended for use in the diagnosis, mitigation, treatment, cure, or prevention of disease in humans or in other animals. Certainly, the vast array of effective medical agents available today represents one of our greatest scientific accomplishments. It is difficulty to conceive our civilization devoid of these remarkable and beneficial agents. Drug, in the form of vegetation and minerals, have existed longer than humans. Human disease and the instinct to survive have, through the ages, led to their discovery. Throughout history, the knowledge of drug and their application to disease has always meant power.

Searching for potential clinical applications, poisonous substances from plants, animals and microorganisms have been tested in studies on animal physiology. The most well-known example is the clinical use of botulin toxin type A, which has been shown to be useful in several therapeutic approaches [1].

The first definition of a "Red Tides” was done by Okamura [2] in Japan since this phenomenon is frequently occurrence there, he defined as follows: "Akashio (red tide in Japanese) refers to the water colour change due to outbreaks of microscopic plankton which can sometimes cause the death of fish and others animals, irrespective of the colour [2]. The expression “red tide” become common in Japan during the latest half of the 1960s. The description “red tide” is visual and not scientific, and the phenomenon describes by Okamura is worldwide known as “water blooms” and more accurate Harmful Algae Blooms (HABs), since they produce serious negative impacts in marine environments. These occurrences of toxic or harmful microalgae represent a significant and seemingly expanding threat to human health, fishery resources, and marine ecosystems throughout the world [3].

A number of marine microalgae species produces phycotoxins that are responsible for massive fish kill and seafood-related poisoning in humans [4, 5]. The symptoms are often neurological and/or gastrointestinal and linked to an altered cellular excitability. Microalgae primary producers that make up the base of both marine and fresh water food webs. Some of them also produce unusual compounds that exhibit potent biological activities. They are secondary metabolites, which are not vital to the plain metabolism and growth of the organism, but they are present in constrained taxonomic groups. Among the many algal secondary metabolites that have been identified, an important number are potent biotoxins responsible for a wide array of human illnesses.

Four major illnesses associated with microalgae biotoxins and with shellfish poisoning have been described: Paralytic shellfish poisoning, Diarrheic shellfish poisoning, Amnesic shellfish poisoning and Neurotoxic shellfish poisoning [6, 7]. Paralytic shellfish poisoning, which shown as primary clinical symptom acute paralytic illness, poses the most serious threat to public health due to its high mortality rate in mammals [4].

Paralytic shellfish poisoning (PSP), is the most widespread algae derived shellfish poisoning worldwide [4]. The PSP toxins are a group of over 28 structurally related imidazoline guanidinium derivatives, non-protein phycotoxins with low molecular weight ranging from 280 to 450 Daltons. They have a common chemical skeleton (3, 4, 6-trialquil tetrahidropurine) that makes them hydrophilic, so totally soluble in water [8]. According to the net charge that these toxins show at pH 7.0, they can classified in three major groups: (1) saxitoxins (STXs) with a net charge of +2; (2) gonyaulatoxins (GTXs) group with net charge of +1, and (3) N-sulfolocarbamoyl-11-hydroxysulfate toxins (Cs) with net charge 0. The Saxitoxin was the first PSP toxin described and its structure established by x-ray analysis [9, 10] it is the most well know and studied. Also, it is the most frequently found commercially available. Nevertheless, Gonyautoxins (GTXs) and specially GTX2/3 and GTX1/4 epimers are the most abundant in molluscs extract samples and they account for the shellfish high toxicity in Chile and worldwide [4, 5, 11, 12].

The organisms that are recognized as primary sources of PSP toxins include three morphologically distinct genera of dinoflagellates, Alexandrium sp., Pyrodinium sp., and Gymnodinium sp [11] and six species of blue-green alga, Aphanizomenon flos-aquae Ralfs ex Bornet & Flahault [15], Lyngbyawollei Farlow ex Gormont [14] from North America; Anabaena circinalis Rabenhorst ex Bornet and Flahault [15] from Australia; Cylindrospermopsis raciborskii (Wolsynska) Seenayya and Subba Raja [16] and Microcystis aeruginosa Kutzing [17] from Brazil; Planktothrix Anagnostidis and Komáreck sp. [18] from Italy, and Aphanizomenonflos-aquaeRalfs ex Bornet & Flahault [19] from Portugal. The last two were found in European continental freshwaters and the two species reported from Brazil are the only two described in South American freshwater.

In Chilean littoral, these PSP toxins are produced by the dinoflagellate Alexandrium catenella, which is filtrated by bivalve
molluscs, concentrating the toxins in high amount. The PSP toxins can be purified starting from high-contaminated shellfish collected in the austral southern Chilean fjords. Actually, in these Patagonian fjords the shellfish highest toxicity ever reported [20] has been described.

Until now, all the clinical applications using PSP toxins have been performed merely in the University of Chile Clinical Hospital in Santiago, Chile. Here, due to a pioneer basic science - clinical collaboration the therapeutic usage of PSP toxins had been established. The therapeutics properties of these biotoxins after local infiltration showed to be safe and effective. This report describes the therapies, involving local infiltration of PSP toxins, characterized by immediate pain relief and muscle relaxation, both effects displayed in minutes after biotoxins infiltration with amazing successful clinical results. The data published in those clinical trials will be analysed and discussed in this chapter.

The molecular mechanism underlying the PSP Toxins clinical effects

The high toxicity of the PSP toxins is due to the reversible binding to a site receptor on the voltage-gated sodium channel on excitable cells [21, 22], blocking neuronal transmission and causing mammals death by respiratory arrest and cardiovascular shock [23-28]. PSP toxins bind with high affinity (Saxitoxin Kd lower than 2 nM) to site 1 on the voltage-dependent sodium channel, inhibiting channel opening. The voltage-dependent sodium channels play a key role in neurotransmission at both neuronal synapses and neuromuscular junctions.

Since all the PSP toxins share two guanidine groups, in carbon 2 and 8; they show positive net charge with a protic dissociation at pKa 8.22 and 11.28 [28]. The ammonium quaternary groups in their chemical structure provide them high polarity, so they cannot go across the blood-brain barrier [28]. Consequently, their main physiological effect is linked to the blocking action at axonal level impeding both, nerve impulse propagation and neuronal transmission over the neuromuscular junction. Therefore, when they are applying locally, two clinical activities are manifested simultaneous: (i) the control of pain (anesthesic activity) and (ii) the control of muscle hyperactivity (relaxant effect). In this review, we will discuss the local application of PSP toxins, which produces muscular flaccid paralysis and anesthesia for periods that are dose dependent.

Clinical trials published from 2004 to 2017

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<td>Lagos, N, García, C., Lattes, K, Lagos, M., et al.[29]</td>
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<td>Paralytic Shellfish Poison: toxins that can kill and heal</td>
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### Treatment of chronic anal fissures

Anal fissure is a cut or crack in the anal canal that may extend from the mucocutaneous junction to the dentate line, which most commonly occurs in the posterior midline. Once the tear occurs, it begins a cycle leading to repeated injury. The exposed internal sphincter muscle beneath the tear goes into spasm, which pulls the edges of the fissures apart impeding the healing of the wound. This cycle leads to the development of a chronic anal fissure. This is a common problem that causes substantial morbidity with roughly equal incidence in both genders and shows great reluctance to heal without intervention [44, 45].

The major symptoms of chronic anal fissure are permanent pain, intense pain during defecation, lasting at least four hours, bright blood on the toilet paper and on the surface of stools, as well as sphincter cramps [44]. The elevation of anal pressure in patient with chronic anal fissure may result from increased tone of the internal anal sphincter, which is clearly observed manometrically, the resting anal pressure being elevated in fissure patients [46]. Therefore, most of the therapies are focused to diminish the anal tone.

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(*) This manuscript was recommended by F1000PRIME 2015 as being of special significance in the field.
For this pathology, we proposed the use of PSP toxins to produce the internal sphincter muscle relaxation, in order to end with the ischemia allowing the capillary to start epithelized and at the same time to block pain, since PSP toxins are potent local anesthetic. Since, both effect occurs simultaneous, we expect subsequently to improve the quality of patient life from the same instant that we infiltrate with PSP toxins [30-32, 47].

As a result, the epimers Gonyautoxin 2/3 (GTX 2/3), the most abundant PSP toxin found in southern Chilean shellfish (Mytilus chilensis) was chosen for the first clinical trial. The phycotoxins were purified from highly PSP toxin contaminated shellfishes collected in Chilean Patagonia fjords [4].

The anal fissure studies were performed at the Coloprectology Section, Surgery Department, Clinical Hospital-University of Chile, Santiago, Chile. All the trials complied with the Declaration of Helsinki regarding medical research involving human volunteers, approved by an-institutional reviewing board at the Clinical Hospital Ethics Committee and the Public Health Institute, Santiago, Chile. The Public Health Institute is the equivalent to the USA, Food and Drugs Administration (FDA) in Chile. The design and purpose of each study, as well as the potential risks for the participants were discussed before enrollment. Their written informed consent was always obtained.

In general, each toxin doses consisted of a sterile solution of 100 units of GTX 2/3 epimers in 1.0 ml total volume of 0.9% NaCl, without preservatives. An insulin syringe with a 25-gauge needle was used for the injection. One unit of the paralyzing toxin activity corresponds to the amount of toxin enough to block neuromuscular contraction of a mouse leg crural biceps for 1.5 to 2.0 hours.

In the first clinical trial, the effect of GTX 2/3 was studied over the anal tone of healthy adults, showing a relaxing effect in all participants. Moreover, manometric recording showed a significant decrease in anal maximal voluntary contraction pressure after the toxin infiltration. The post-injection electromyography showed that the muscle activity was abolished practically immediately after the injection [30]. No participant suffered any adverse events or negative side effects during or after the infiltration. The clinical laboratory tests performed before and after the infiltration did not show any significant changes. An important finding was that neither flatus nor faecal incontinence were observed. Furthermore, the ano-rectal inhibitory and ano-cortical reflexes remained functional, indicating that the infiltration blocked muscle hyperactivity, but leaving enough strength for physiological functioning [30].

That study concluded that local intramuscular injection of GTX 2/3 epimers into the anal internal sphincter produced immediate sphincter relaxation, decreasing the pressure produced by voluntary contraction. Hence, the effectiveness and safety of PSP toxin injection in humans is shown for the first time. Thereafter, we were ready to test the drug for anal fissure in patients.

The theoretical background of the study refers to a temporary pharmacological immobilization of the anal sphincter for eliminating sphincter spasm, leading to the healing of the wound. This is the critical step for treating and healing anal fissure, interrupting the vicious loop of chronic anal fissure: the crack in the anal canal (injury), inflammation, pain and internal sphincter spasm.

In order to test the effect of these biotoxins, blocking pain and relax the internal sphincter, two clinical trials were performed. The first trial complained that the drug effect did not cover the full period of treatment. The protocol used for the second trial was practically in one week. The infiltration was repeated after 7 days and in the second trial, infiltration was repeated after 4 days.

In both trials, adults, between 18 and 70 years old, diagnosed with symptomatic anal fissures were enrolled. The recruited patients received clinical examination, including digital evaluation, proctoscopy and questionnaires to evaluate the symptoms. Anorectal manometries were performed before and 4 minutes after the toxin infiltration. The dose was infiltrated in a total volume of 1 millilitre into both sides of the anal fissure, within the internal anal sphincter (0.5 ml in each side). The patients were clinically evaluated 4, 7, 14, 21 and 28 days after the beginning of the treatment. The infiltration pain scores and that two minutes after the injection were evaluated by a VAS scale from 1 to 10, being 10 the maximum pain value. Long-term outcomes were determined at a14 months follow-up. In both studies the exclusion criteria were (i) evidence of anterior, posterior or both circumscribed ulcers; (ii) induration of the edges, and (iii) exposure of horizontal fibres of internal anal sphincter, with symptoms of post defecation or permanent pain, bleeding, or both. Patients with fissure history of more than two months were considered as chronic anal fissure patients. The exclusion criteria were: (i) patients younger than 18 or older than 70 years; (ii) pregnancy, and (iii) fissure associated with other pathological condition, such as haemorrhoids, fistula or anal abscesses. Both clinical trials considered pain relief and total fissure epithelisation as primary endpoints.

In the first trial, total remission of acute and chronic anal fissures was achieved in most of the cases, 98% of the patients healed at a mean time of 17.6 ± 9 days. In the second trial, total remission was achieved earlier, with a mean time of healing of 8.2 ± 2.4 days, providing a more efficient treatment, since the patients healed practically in one week. The protocol used for the second trial was then proposed to patients. Indeed, the patients included in the first trial complained that the drug effect did not cover the full period of seventh days between the injections.

The first clinical trial was initially proposed as a randomized, double-blind trial, for which the patients were consecutively recruited upon arrival to the Coloprectology Section, diagnosed and infiltrated with toxin or placebo solution (toxin-free 0.9 % NaCl solution). The double-blind trial was opened for humanitarian and ethical reasons.
since after three weeks of treatment the patients injected with toxin were healthy and the ones injected with placebo showed no improvement or worsening of their clinical condition. Moreover, 100 percentages of the patients injected with the phycotoxin showed immediate post injection anal sphincter relaxation, which was detected by digital examination and anorectal manometry. This effect was not observed following placebo injection, making unnecessary to keep the patients suffering for three or four weeks, to just keep the double-blind protocol. The double-blind protocol was opened with the authorization of the Chilean Public Health Institute and the Hospital Ethic Committee.

All patients declared to feel anaesthesia after the infiltration, and relieve from the intense anal pain. Thus, the life quality of the patients was immediately and significantly improved. During the digital examination, colorectalologists detected an immediate anal tone reduction, which persisted for at least five days. None flatus or faecal incontinences were observed, explained by the full preservation of anal-rectal inhibitory and anal-cortical reflexes. Bleeding stopped in all patients within 48 hours after infiltration and at the first clinical control (4 days after), the patients showed epithelisation of the lesion, with only modest pain when defecating. At the second clinical examination the lesion was totally epithelized with a healthy scar.

As a result, Gonyautoxin local infiltration into the internal anal sphincter produced a temporary pharmacologic immobilization of the anal sphincter muscle that eliminated sphincter spasm, the critical step, breaking the vicious circle of damage, inflammatory, pain and sphincter spasm, consequently healing the anal fissure. PSP toxins infiltrations showed an outstanding efficacy and safety in anal fissure treatment representing a new therapeutic approach. Due to its efficacy and low cost it should be preferred to any other pharmacologic treatment or surgery. Surgery implies permanent flatus and/or faecal incontinence risk, and an average of seven weeks for healing time, irreversible sphincter damage and costly hospitalization. The present 4 days protocol showed to be very efficient, shortening the healing time and the patient perception of healing. Actually, the authors recommend a safe three injections treatment: one as a starting infiltration (zero time), then twice every 4 days, in total 8 days of treatment.

About the potential intoxication that can occur with the dose implemented (20 micrograms per patients), this is far below the safe limits of international official regulations who allow consumption of shellfish with a safe limit of 80 micrograms of STX equivalent to 100 grams of shellfish meat (Fish and Fishery Products Hazards and Controls Guidance 2006).

This pathology has a prevalence of 1.2 to 1.5% of the total population between 16 to 80 years old of both sexes in developed countries (USA, Japan and Europe). In case of Chile, we have a similar data, since our population is around 17 million of people we have to deal with around 250,000 patients with this disease. In any underdeveloped country it should be similar, for that reason, this pathology is also a major social problem because the gold standard in this pathology is a sphincterotomy surgery and normally in those countries the surgeons that practice this surgery are scarce. All the patients that participate in our clinical trials, associated to anal fissure, belonged to the last economic quintile and they did not have health security. They have a poor quality of life.

These studies showed an imperative clinical use of these PSP toxins, the therapeutics properties of these biotoxins during a local infiltration in chronic anal fissure were both effective and safe, but also they showed faster recovery and unexpensive massive ambulatory treatment, that could be the solution for those countries where there is not enough surgeons available.

**Treatment of chronic tension-type headache**

According to the International Headache Society, tension-type headache (TTH) is essentially defined as bilateral headache of a pressing or tightening quality that lacks a known medical cause. TTH is classified as episodic if it occurs on less than 15 days a month, and as chronic if it occurs with larger frequency, meeting the International Headache Society diagnostic criteria (International Headache Society ICH-10 guide for headaches, 1997).

This clinical trial was designed to evaluate the therapeutic properties of Gonyautoxin 2/3 epimers local infiltration in patients with chronic TTH, to propose a safe and effective alternative to current treatment [39]. A study was initially proposed as a randomized, double-blind trial in which the patients were consecutively recruited upon arrival to the Hospital Neurology Clinic, where the patients were diagnosed and randomly injected with either toxin or a placebo solution. A randomized table generated by computer was used to designate placebo or toxin infiltration for each patient. Due to the immediate clinical effect produced by the gonyautoxin treatment, immediately instantly detected by electromyography (EMG) after the infiltration, the study was modified and continued as an open label study. Two major effects could not be maintained in the blinding as initial proposed by the study: (i) a muscle relaxant effect detected immediately by the physicians injecting the toxin, and (ii) a dropping in the pain scores, referred by the patients 5 minutes post injection, which was not observed in the placebo group. Both clinical effects were only observed after the toxin infiltration and never following placebo. Thus, 27 patients with chronic TTH were locally infiltrated with a gonyautoxin dose of 50 micrograms in 2 ml. The infiltration was applied intramuscularly in 10 sites considered as pain trigger points according to a fixed infiltration protocol (200 micro liters per injection). EMG recording was performed before and immediately after infiltration.

The TTH diagnosis was performed by neurologists according to the International Headache Society criteria and the inclusion criteria were: (i) patients with headache of a pressing or tightening quality with episodic frequency above 15 days. (ii) patients refractory to any other conventional treatment, such as orally administered analgesics, systemic muscular relaxant, corticoids and antidepressants like amitryptiline, the gold standard treatment [48, 49]. All patients had been previously treated and controlled at the University Hospital Neurology Clinic over 2 years, with symptom lasting over 3 years.

The exclusion criteria were: (i) pregnancy; (ii) use of prophylactic headache treatment a month prior the infiltration; (iii) myasthenic syndromes; (iii) muscular dystrophies; (iv) inflammatory myopathies; (v) acute and chronic polyneuropathies, and (vi) use of anticoagulant treatment, terminal illnesses (AIDS, cancer), drugs or alcohol abuse, or psychotropic substances 24-hour before infiltration. The end-points were: (a) reporting a control of the acute episode of pain at the moment of infiltration, defined as a 30% drop from the original pain score, and (b) prophylactic effect, escalation in the day number without headache, with a minimum of 14 days for being considered as successfully fulfilled end-point.

The immediate effect of the toxin, as well as to control a placebo
effect, EMG recording was performed, 2 minutes after toxin or placebo injections. The injection pain scores and pain duration in minutes was evaluated by asking the patients to rate their pain in a visual analog scale (VAS scale) from 1 to 10, being 10 the maximum pain value. Additionally, they were asked to rate the pain felt during the infiltration, as well as amount of paresthesia in the injected muscles, anesthesia or any other discomfort or pain after the infiltration.

Apart of oral paraesthesia reported by 12 of the 27 patients included in the trial, no other negative symptoms were described or detected during the follow up period. When a two-week period was considered as a base line, 19 of 27 patients (70%) were successfully responders to the infiltration. The patients showed a remarkable effect immediately after the infiltration, confirmed by trapezium EMG recording. All patients reported a decrease in pain score at 5 minutes post-injection. The responder above the baseline (two weeks), showed an average above 8 weeks of headache pain free, all of them without a second infiltration or the usage of any additional analgesic medication.

The pain information recorded was: average headache pain during the week before infiltration, value of pain scores just before the infiltration and 5 minutes after the infiltration.

Follow-up was performed by telephone 48 hours after the treatment for each patient. A visiting follow-up was performed 1 and 2 weeks after treatment. The patients were asked to score the overall effect of the treatment and to report any side effects. Both intensity and frequency were scored. All patients were contacted by telephone every two weeks for a long-term follow up for 50 weeks. The EMG recordings showed a dramatic abolition of the trapezius muscle activity after toxin injection, confirming its relaxant effect. This effect was never observed with placebo.

The gonyautoxin infiltration showed an abrupt and effective decrease in acute pain scores in minutes, been the immediate clinical effect of the infiltration. The patient sensation it was undoubtedly demonstrated by the EMG recording of the muscle activity post infiltration. The trapezius activity was practically abolished two minutes after infiltration. EMG recording was in agreement with the immediate pain relief reported by the patients after the infiltration, and the very low pain reported after the toxin injection [Figure 2] [39].

Considering the amazing immediate relaxant effect observed in this study, but also reported for healing of anal fissures [30, 31], the primary outcome and major improvement aimed by the authors in this study was to produce a significant drop in patient’s acute pain score. This outcome was achieved for the 83 percentage of responder patients, considering a 30 percentage drop off of the baseline headache pain. The following outcome measure was the number of days without headache episodes. This was remarkably achieved since the average duration of the prophylactic effect provided by the Gonyautoxin 2/3 epimers was above 8 weeks, meaning an average of 2 months without headache. Compared with the available treatments for chronic tension-type headache [50-52], this is an excellent prophylactic long lasting effect The effect is superior to that provided by the gold standard based on tricyclic anti depressive reporting a 30 % efficacy [48].

The gonyautoxin infiltration was well tolerated and immediately effective. The beneficial effects lasted for an average over 2 months and in some patients for several months. This long lasting anesthetic effect with some patients showing 50 weeks without headache pain was above of the one expected in the study, being this study the first trial testing gonyautoxin in treatment of chronic tension-type headache, no data is available to compare such as long lasting pain relief.

The physiological effects of this potent voltage sodium channel blocker may include a wide range of neurophysiological and neurochemical responses such as release of neurotransmitters or activation of segmental and heterosegmental antinociceptive system. On the other hand, since chronic pain is considered as an expression of neuronal plasticity and it is generated partly in the periphery and partly in the CNS, maybe this treatment with gonyautoxin could influence changes of the central sensitization generated by prolonged nociceptive input from the pericranial myofascial tissues. This central sensitization change has been postulated as a physiopathological change in tension-type headache [53].

**Achalasia: Intrasphincteric Neosaxitoxin infiltration. Evidence for lower esophageal sphincter relaxation**

Following the successful experience with PSP toxins treatment as smooth muscle relaxant for chronic anal fissure [30, 31, 37], the author tested Neosaxitoxin to yield lower esophageal sphincter relaxation in two severe Achalasia cases [34].

Achalasia is a gastrointestinal motility disorder characterized by aperistalsis of the thoracic esophagus and failure of the lower esophageal sphincter to relax during swallowing [54]. Clinically is expressed by dysphagia for solids and liquids, regurgitation of undigested food, chest pain and weight loss [55, 56]. The diagnosis is confirmed by radiological evaluation and manometric outcomes. The current methods of achalasia treatment are focused on reducing the lower esophageal sphincter (LES) pressure, thus improving the esophageal emptying. These methods include pharmacological therapy [57-62], mechanical disruption of the LES fibers (either by pneumatic dilatation or by surgical myotomy) and the use of botulin toxin [63-69].

Two patients with long standing history of achalasia were submitted to local intrasphincteric endoscopically infiltrations with Neosaxitoxin, both were diagnosed with upper endoscopies. They showed alimentary remnants in a dilated and atonic esophagus, a tight closed LES, but with no other pathology. The Barium esophagram revealed a typical “bird’s beak” appearance and a dilated and tortuous esophagus. The intraesophageal pressure recording showed an absent esophageal peristalsis, impaired LES relaxation and, in both cases, resting LES pressure was within normal range.

Figure 2: Cystoscopy infiltration protocol model. Each white dot represents a site of injection.
In order to perform the infiltration, a flexible upper gastrointestinal endoscopy was performed while the patients were under conscious sedation. The LES was visualized endoscopically by the identification of the “sphinteric rosette” at the level of the squamous-columnar junction. Through a 6-mm sclerotherapy needle, one milliliter of sterile saline solution with s dose of 5 8micrograms of Neosaxitoxin was infiltrated into each of the four sphincter quadrants. The procedure was performed in an outpatient basis.

The symptomatic response was evaluated on the basis of a symptom achalasia score according to Echards, which was the sum of the individual score for three achalasia symptoms: dysphagia, regurgitation and chest pain [64]. Patient 1 started with an achalasia score of 9 and patient 2 with a score 8. Esophageal manometries were performed before and 16 hours after the infiltration, both were treated ambulatory and evaluated every day. Clinical follow up was made using a standardized symptoms scale. Patients were contacted daily by phone for the next 10 days.

Similarly, to anal fissure the immediate sphincter relaxation was observed and recorded at 5 minutes after infiltration. The resting lower esophageal sphincter pressure fall down in an average of 40%. After two hours the symptoms improved significantly, achieving a score of zero at four hours after infiltration. The clinical relaxation effect lasted for eight days. Again, the long lasting effect was observed.

Dysphagia was the first symptom to revert, followed by regurgitation and then chest pain. At the day fourteen after injection, the surgical myotomy was performed without difficulty. No evidence of periesophageal adhesions was found at the surgical time. Muscle samples were taken at the myotomy site in order to perform a histological evaluation of inflammation. Biopsy revealed depletion of ganglion cells in the myenteric plexus, with no inflammatory infiltration at the muscular level. No side effects were observed during and after the infiltration. Meaning that the intrasphincteric Neosaxitoxin infiltration was effective and safe in treatment of Achalasia. This therapeutic approach does not contribute to any additional risk as was showed during this protocol.

Neosaxitoxin as a local anaesthetic

The voltage-gated sodium channels are responsible for the rising phase of the action potential in the membranes of neurons [70]. At least nine distinct voltage-gated sodium channels have been cloned from mammals, being broadly divided by its affinity to Tetrodotoxin (TTX). The NaV1.1, NaV1.2, NaV1.3 and NaV1.7 are highly TTX sensitive, whereas NaV1.5, NaV1.8 and NaV1.9 are TTX resistant to varying degrees [71]. Many of these channels have specific tissue distributions, determining distinct excitation properties [72].

Local anaesthetics are compounds that reversibly block the neural conduction by occupying enough sodium channels within an axon to interrupt its activity, stopping the depolarization, thus preventing the propagation of action potential and neuronal communication [73]. The clinical use of local anaesthetics such as aminoamides (e.g. lidocaine) and amino esters (e.g. procaine), inhibit sodium channel activity by binding in the channel inner pore, toward the inside from the intracellular side of the cell [70].

In the last 25 years, there has been an interest in the local anaesthetic activity of biotoxins. They bind to the outer opening of sodium channels [74]. These agents, including TTX and saxitoxin (STX) analogues, reversibly bind to sodium channels with high affinity [75-81]. Since Neosaxitoxin is a phycotoxin that reversibly blocks the voltage-gated sodium channels at the neuronal level and being the most potent of these toxins, it should be a strong pain killer. Indeed, Neosaxitoxin has shown greater potency than other STX analogous and it is also more potent than TTX in vitro and in vivo animal studies [74, 82]. The relative potencies of these toxic compounds in vitro and in vivo experiments have been described: Neosaxitoxin > STX> TTX [74, 79].

The first pioneer study was conducted as randomized, double-blind, placebo-controlled trial, with 10 healthy volunteers [35]. Subcutaneous injections were made in the middle posterior skin of the calf, one leg receiving 50 micrograms of neosaxitoxin and the contra-lateral receiving the placebo. The anesthetic effect was evaluated using a standardized sensory and pain model, TSA II Neurosensory Analyser (Medoc Ltd, Minneapolis, MN) and also the Von Frey Technique were used to evaluate five parameters: sensory threshold for warm and cold, pain thresholds for heat and cold and mechanical touch perception threshold. Measurements were made at 0, 1, 3, 6, 9, 12, 16, 24 and 48 hours after the injections. All thermal thresholds were determined as the average of three assessments performed at 10 second intervals, from a baseline temperature of 32ºC, and with a rate of change of 1ºC/second. The upper cut-off limit was 50ºC and the lower 0ºC. Cold and warm detection thresholds were defined as the smallest change from baseline that the volunteer could perceive, and the volunteer pressed a button as soon as the specific sensation was perceived. The heat and cold pain detection threshold was the temperature perceived as painful, and the volunteers were instructed to react to the first sensation of pain.

The touch detection thresholds were determined by mechanical stimuli with a series of monofilaments of different strength. Ten hairs were used that covered the range from 0.1 to 100 force grams on a logarithmic scale. (Touch Test Sensory Evaluators, Stoelting Co, Wood Dale, IL) The touch detection threshold was defined as the least force of mechanical stimulation that produced a sensation of touch or pressure.

Localized reactions at the injection site (erythema, discoloration, hematoma, induration, swelling and blisters) and Neosaxitoxin intoxication symptoms like nausea, headache, ataxia, perioral and distal limbs paraesthesia were recorded. No adverse reactions to Neosaxitoxin were detected. Four volunteers presented small haematomas in the infiltration zone, two in the toxin group and two in the placebo group at the 24 evaluation hours, both disappeared after two weeks. No other local reactions were observed. None of the volunteers noted or showed any motor disability or discomfort during the follow up period. Two weeks after the injections the volunteers returned to the Clinical Hospital where physicians evaluated the injection sites for persistent and delayed reactions and the volunteers were questioned regarding any abnormality. Two months after the injections, the volunteers were contacted by telephone and asked about any trouble or incidents that they felt might be related to the study applications.

The data showed an effective blocking outcome of the evaluated parameters in all patients. Gradually the blocking began to revert, the heat pain being the first to return to normal values after 3 hours. Cold pain was the longest sensation abolished, achieving 24 hours of blockade. The touch detection thresholds presented a significant reduction that last until 9 hours post injection. This one also showed a progressive return to normal values.

Since Neosaxitoxin is a phycotoxin that reversibly blocks the voltage-gated sodium channels at the neuronal level and being the most potent of these toxins, it should be a strong pain killer. Indeed, Neosaxitoxin has shown greater potency than other STX analogous and it is also more potent than TTX in vitro and in vivo animal studies [74, 82]. The relative potencies of these toxic compounds in vitro and in vivo experiments have been described: Neosaxitoxin > STX> TTX [74, 79].

The first pioneer study was conducted as randomized, double-blind, placebo-controlled trial, with 10 healthy volunteers [35]. Subcutaneous injections were made in the middle posterior skin of the calf, one leg receiving 50 micrograms of neosaxitoxin and the contra-lateral receiving the placebo. The anesthetic effect was evaluated using a standardized sensory and pain model, TSA II Neurosensory Analyser (Medoc Ltd, Minneapolis, MN) and also the Von Frey Technique were used to evaluate five parameters: sensory threshold for warm and cold, pain thresholds for heat and cold and mechanical touch perception threshold. Measurements were made at 0, 1, 3, 6, 9, 12, 16, 24 and 48 hours after the injections. All thermal thresholds were determined as the average of three assessments performed at 10 second intervals, from a baseline temperature of 32ºC, and with a rate of change of 1ºC/second. The upper cut-off limit was 50ºC and the lower 0ºC. Cold and warm detection thresholds were defined as the smallest change from baseline that the volunteer could perceive, and the volunteer pressed a button as soon as the specific sensation was perceived. The heat and cold pain detection threshold was the temperature perceived as painful, and the volunteers were instructed to react to the first sensation of pain.

The touch detection thresholds were determined by mechanical stimuli with a series of monofilaments of different strength. Ten hairs were used that covered the range from 0.1 to 100 force grams on a logarithmic scale. (Touch Test Sensory Evaluators, Stoelting Co, Wood Dale, IL) The touch detection threshold was defined as the least force of mechanical stimulation that produced a sensation of touch or pressure.

Localized reactions at the injection site (erythema, discoloration, hematoma, induration, swelling and blisters) and Neosaxitoxin intoxication symptoms like nausea, headache, ataxia, perioral and distal limbs paraesthesia were recorded. No adverse reactions to Neosaxitoxin were detected. Four volunteers presented small haematomas in the infiltration zone, two in the toxin group and two in the placebo group at the 24 evaluation hours, both disappeared after two weeks. No other local reactions were observed. None of the volunteers noted or showed any motor disability or discomfort during the follow up period. Two weeks after the injections the volunteers returned to the Clinical Hospital where physicians evaluated the injection sites for persistent and delayed reactions and the volunteers were questioned regarding any abnormality. Two months after the injections, the volunteers were contacted by telephone and asked about any trouble or incidents that they felt might be related to the study applications.

The data showed an effective blocking outcome of the evaluated parameters in all patients. Gradually the blocking began to revert, the heat pain being the first to return to normal values after 3 hours. Cold pain was the longest sensation abolished, achieving 24 hours of blockade. The touch detection thresholds presented a significant reduction that last until 9 hours post injection. This one also showed a progressive return to normal values.
Neosaxitoxin was undetected, by High Performance Liquid Chromatography (HPLC) with on line fluorescence detection method [26], in blood and urine samples collected at 1 and 4 hours after infiltration. Nevertheless, Neosaxitoxin showed to be a potent agent capable of producing the anaesthetic effect of outer sodium channel blocking compounds like TTX and STX, whose effects have long been known, since they were previously tested only in animal models [79, 83-87].

The clinical use of Neosaxitoxin showed to be effective and safe as local anaesthetic when injected in the subcutaneous plane. Moreover, at microgram dosage level a clinically sensitive blockage, in a completely reversible manner was observed. The data displayed in this report opened a whole new line of research in acute and chronic pain management with this biotoxin.

Development of a new local anesthetic

Essentially, local anesthetics effectively block and relieve pain, but with a relatively short duration of action, limiting its anesthetic effectiveness not more than 4 to 6 hours [88]. Therefore, to develop a long-acting pain blocker should improve the management of pain, but no such agent is yet available for clinical usage.

Pain relief is a public health issue of such critical relevance as to constitute an international imperative and fundamental human right [89]. It has been estimated that nearly 80% of patients that undergo a surgical procedure suffer post-operative pain and approximately 20 percentage experience severe pain [90]. Inadequate pain treatment results in unnecessary patient suffering, physiological disarrangement, reduced mobility, delayed ambulation, increased risk to present a medical complication and psychological sequels, such as depression, anxiety and stress [91]. All of the above disarrangement will extend the hospitalization length and also augment of overall health costs.

Local anesthetic relieve pain avoiding the systemic side effects produced by other analgesics, such as the opioids [89, 93]. Therefore, these types of drugs have been increasingly considered in modern surgical pain management protocols [88, 94-101]. As a result, it has been an increased interest for developing a long lasting local anesthetic that can be established by a single injection [88, 94, 95, 97, 98].

Until now, Tetrodotoxin, Saxitoxin and Neosaxitoxin have been found to yield local anesthetic properties in animal models [79, 83]. Moreover, they also showed that the effect of the outer pore-sodium channel blockers is potentiated when are co-injected with conventional local anesthetic or vasoconstrictors [74, 85]. The co-injection yielded a blockage greater than the sum of the effect of the individual drugs [74].

Since, Neosaxitoxin effect persisted over 24 hours [35] and following the idea of developing a long-acting pain blocker, the logic way to go was to evaluate the potentiation of the Neosaxitoxin local anesthetic effect in combination with bupivacaine or epinephrine in a randomized double blind clinical trial in humans.

So, ten healthy male volunteers aged 18 to 34 were enrolled. The inclusion criterion was healthy males with the ability to understand and perform the tests. The following exclusion criteria were considered: (i) consumption of any oral analgesics at least ten days before the study; (ii) drug abuse history; (iii) any sign of psychiatric disorders at the clinical assessment for recruiting the volunteers. The volunteers received all 4 formulations, with one month elapsing between the two rounds of experiments. The validated sensory and pain paradigm performed by Rodriguez [35] was again used for evaluating the effect of the treatments from 0 to 72 hours after the injections, measuring sensory pain and mechanical touch perception threshold. Two rounds of experiments were performed, every participant receiving 2 of the 4 combinations in each round, one on each forearm. Therefore, each participant received the 4 preparations during the trial. The rounds were performed with one month interval. The combination and the forearm to be injected were defined according to a computer-generated randomization table. The volunteer and the physician administrating the solutions were blind to the content of the treatment.

Two weeks after the injections the volunteers returned to the Clinical Hospital, where physicians evaluated the injection sites for persistent and delayed reactions. The volunteers were further questioned for any abnormality. Two months after the injections, the volunteers were contacted by telephone and asked about any trouble or incidents that they could associate to the study.

The data showed undoubtedly that the duration of the effect produced by combined treatments was longer than single drugs. In this study, the authors showed the potentiation of the local anesthetic effect of Neosaxitoxin in combination with bupivacaine or epinephrine in a randomized double blind clinical trial. The synergistic effect produced by these drugs was observed in all sensory and pain tested. Bupivacaine alone was the shorter blocking agent, followed by Neosaxitoxin alone. The Neo-Bupi combination showed longer blocking effect than that produced by Neo-Epi combination, mainly in the warm sensation threshold test, suggesting greater efficacy for the Neo-Bupi combination that last over 24 hours in practically all the parameters measured. These results agree with data obtained with animal models [85, 87].

The potentiation effect associated with the combination of either Neo-Bupi or Neo-Epi may be related to a vasocostriction produced by either epinephrine or low doses of bupivacaine, as shown that vasoconstrictors enhance and extend the duration of amide type-local anesthesia [102]. One logical explanation for this prolonged effect is that epinephrine reduces the drug elimination from the injection site, probably by decreasing local blood flow [103]. Vasoconstriction, as reported for conventional local anesthesia, might also reduce the Neosaxitoxin clearance from the action site. Another explanation for that synergism is that the sodium channel pore is simultaneously blocked on the external and internal sites, by Neosaxitoxin and bupivacaine, respectively, in agreement with the idea of two separates and independent sites on the sodium channel, implying that the occupation of one site does not restrict or limit the access to the other [104].

The precise magnitude of this potentiation has to be tested in future studies associated to specific pathologies where pain is the main issue. Nevertheless, the maximal lasting effect showed by either drug alone was amplified among 1.5 to 3 times in magnitude when both drugs were applied together.

The safety of Neosaxitoxin is again showed in this study; this is the second clinical trial where the anesthetic effect of Neosaxitoxin is shown in humans.
The long acting anesthesia and potentiation subjects are very relevant for current medical practice. A long acting local anesthetic combination would be an important component of a multimodal analgesic management. Multimodal analgesia, in which different types of analgesics are used in order to block distinct sites of the pain pathway. This is more and more recognized as an effective management of clinical pain [105].

The local nerve conduction blocking can be a supplement for systemic analgesics, such as non-steroidal anti-inflammatory drugs and opioids. Additionally, this strategy would allow a reduction of the respective doses, decreasing the incidence and severity of adverse reactions. The data showed in these studies supports the idea that Neosaxitoxin is a new long-acting pain blocker, with highly potential clinical usage, that will play a crucial role in the management of clinical pain in order to improve the patients’ quality of life.

Based on the promising results obtained with the clinical use of Neosaxitoxin, a randomized, double blind clinical trial comparing Neosaxitoxin versus Bupivacaine for wound infiltration in patients undergoing laparoscopic cholecystectomy was performed by Rodriguez-Navarro [106]. In this study, the authors follow the postoperative course of patients undergoing laparoscopic cholecystectomy under a standardized general anesthesia plus wound infiltration with either Neosaxitoxin or bupivacaine.

The aim was to compare both local blocking pain effects after 12 hours post-surgery. Patients received pre-incisional infiltration of laparoscope entry sites with 20 millilitres containing either Neosaxitoxin total dose of 100 micrograms or Bupivacaine 50 milligrams total dose. The primary outcome measured was the visual analog pain score at 12 hours post-surgery. The second one included patient pain scores at rest or allowed to move. Additionally, the extra analgesic usage, the functional recovery, and any possible adverse effects were also recorded. The study includes 137 patients, 69 randomized to Neosaxitoxin and 68 to Bupivacaine infiltrations.

Average pain scores at rest and with movement 12 hours post-surgery were lower in the Neosaxitoxin group compared with the bupivacaine group (PG 0.01). Additional pain measures and recovery parameters also favour Neosaxitoxin. No serious adverse events occurred, and none adverse events were more frequent in the Neosaxitoxin treated group. Neosaxitoxin shows promising as a long-acting local anesthetic. Future studies will examine dose response, formulation of combinations, and safety with dose escalation.

**A Long-acting pain blocker**

**Neosaxitoxin as a long-acting pain-blocker in Bladder Pain Syndrome**

Bladder Pain Syndrome (BPS) is defined as a “chronic pelvic pain, pressure, or discomfort of greater than 6 months duration perceived to be related to the urinary bladder, accompanied by at least one other urinary symptom, like persistent urge to void or urinary frequency. Confusables diseases as the cause of the symptoms must be excluded” [107, 108]. The Bladder Pain Syndrome Committee of the International Consultation on Incontinence has referred to Interstitial Cystitis (IC) as the BPS since 2010 [107, 108]. BPS is not a life-threatening illness, but it has recently been acknowledged as a major health issue, which seriously affects patients’ quality of life, and is often accompanied, by sleep and depressive disorders, anxiety, and recurrent urinary tract infections. Consequently, ordinary daily activities are usually avoided [109]. This syndrome affect both sexes, but women are more commonly affected and 90% of patients are Caucasian [110, 111]. The prevalence in USA is to 60-70 cases per 100,000 [108]. While BPS has a multi-factorial etiology, the most accepted theory corresponds to an injury or dysfunction of the glycosaminoglycan layer, which shields the urothelium [112]. This alteration may change the urothelium’s permeability via abnormal diffusion of toxic compounds from the urine to the submucosa, leading to sensory nerve activation, neurogenic inflammation, pain, and fibrosis, with pain being the most distinctive symptom reported by patients. This injury can be caused by bacterial cystitis, childbirth, pelvic surgery, or urological procedures [109, 112]. The report analysed now describes the therapy involving local infiltration of Neosaxitoxin in urothelium submucosa to block pain caused by BPS, thus improving patient’s quality of life of patients with this chronic, debilitating condition.

The authors designed this study to also evaluate the clinical efficacy of Neosaxitoxin as a long-lasting pain blocker in the treatment of Bladder Pain Syndrome (BPS). Patients with a diagnosis of BPS received a total dose of 80 micrograms of Neosaxitoxin in 0.9% NaCl, pH 6.5, isosmotic. The infiltration was performed via cystoscopy, under spinal anesthesia. Questionnaires were applied immediately before and 7, 30, and 90 days after the procedure to measure the patients’ reported pain severity and quality of life.

In this pilot study, again all patients successfully responded to treatment. Furthermore, the analgesic effect lasted for the entire 90 days of follow-up, without the need of a second infiltration, and no adverse reactions to Neosaxitoxin were detected. Upon infiltration, all patients who were previously refractory to conservative, as well as more aggressive treatments, showed significant clinical improvements. Nevertheless, pain the main symptom of BPS, was blocked. It is important to highlight that the analgesic effect produced by Neosaxitoxin infiltration lasted for the entire 90 days of follow-up in all patients, and this pain blocking effect only required one local intervention. Therefore, Neosaxitoxin infiltration generates a pain blockage that is locally maintained over a long period of time (above days). Furthermore, no patient experienced adverse effects such as nausea or ataxia during or after this treatment for the three months of clinical follow-up. For the first time, the effects of Neosaxitoxin on blocking the neuronal transmission of pain is shown, when is locally infiltrated into the bladder submucosa.

The BPS significantly impacts the quality of life of patients by generating debilitating pain and urinary frequency and urgency that, in some cases, leads to social isolation. For this reason, many women affected by BPS have associated psychiatric disorders, such as depression and anxiety [108]. Other medical conditions associated with BPS are inflammatory and painful chronic conditions such as fibromyalgia, vulvodynia, irritable bowel syndrome, and chronic fatigue syndrome [108, 113, 114]. Authors reported, that after Neosaxitoxin infiltration, all patients had remission of their symptoms. The BPS significantly impacts the quality of life, which was evidenced by the reduction in the Interstitial Cystitis Problem Index (ICPI score).

Moreover, with this treatment, all psychiatric and pharmacological costs were eliminated.

This pilot study, showed the efficacy and safety of Neosaxitoxin as a long acting pain-blocker for BPS. It was well tolerated by patients, who experienced extended pain relief and associated beneficial effects over the 90 days of follow-up.

Until now, Neosaxitoxin has only been clinically applied in the Hospital Clinico de la Universidad de Chile in Santiago, where a
pioneering collaboration between basic science investigators and clinicians has demonstrated the therapeutic properties of these biotoxins upon a local infiltration intervention, which is both effective and safe [115].

Conclusion
Paralytic Shellfish Poison Toxins, when applied locally, have been established to be effective and innocuous in all the clinical trials. They showed a remarkable muscle relaxant and an amazing local pain killer effect. Both properties usually occur and aren’t possible to separate, also are manifested almost instantly. Considering instantly, as the outcome relaxation and/or anesthetic effect measured and recorded in minutes after the injection. Neosaxitoxin, the most potent of all PSP toxins, also showed to be a strong long-lasting local anesthetic drug when was infiltrated along and even better when it was applied in combination with epinephrine or bupivacaine. The last one was the best Neosaxitoxin drug potentiation for the long lasting effect as pain killer. Without doubt for Neosaxitoxin, the best applications as a pharmaceutical drug are to come mainly by its faster high healing rate. On the other hand, considering the immobilization of healing tissues as a fundamental therapeutic principle, treatment with PSP toxins may be found to be applicable in other pathologies in which muscle hypertonicity results in stiff, awkward movements.

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References
2. Okamura K. AkashioniTsuite. Suisan KoushuSikenjoKenkyuHokoku. 1916;12:26-41. [Crossref]
4. Lagos N. Microalgae blooms: a global issue with negative impact in Chile. Biol Rev. 1998;31:375-386. [Crossref]
5. Lagos N. Paralytic shellfish poisoning phycotoxins: Occurrence in South America. Comments on Toxicology. 2003; 9:175-193. [Crossref]
27. Moczydlowski D O, LF Michea and N Lagos. Toxic effects, pharmacokinetics and clearance of saxitoxin, a component of paralytic shellfishpoisoning, in dogs. Toxicon. 1986; 24:175-186. [Crossreference]
41. Garcia C, Barriga A, Diaz JC, M and Lagos N. Route of metabolization and detoxication of Paralytic Shellfish Toxins in humans. Toxicon. 2010; 55:135-144. [Crossref]
44. Madoff R D and JW Fleshman. AGA technical review on the diagnosis and care of patients with anal fissure. Gastroenterology. 2003; 124:235-245. [Crossref]
46. Hancock BD. The internal sphincter and anal fissure. Br J Surg. 1977; 64:92-95. [Crossref]
54. Richter JE. Oesophageal motility disorders. The Lancet. 2001; 358:823-828. [Crossref]
55. Park W and MF Vaezi. Etiology and pathogenesis of achalasia: the current understanding. Am J Gastroenterol. 2005; 100:1404-1414. [Crossref]
60. Katzen D A and D O Castell. Use of botulinum toxin as a diagnostic/therapeutic trial to help clarify an indication for definitive therapy in patients with achalasia. Am J Gastroenterol. 1999; 94:637-642. [Crossref]

94. Moiniche SA Dahal and JB Dahl. Subcutaneous infiltration with ammonium sulphate 10% does not prolong the local anaesthetic duration of lidocaine in humans. ActaAnaesthesiologicaScandinavica. 2000; 44:878-883. [Crossref]


96. Hutchison RW. Challenges in acute post-operative pain management. Am J Health Syst Pharm. 2007; 64:S2-5. [Crossref]


98. Ballantyne JC. Opioid analgesia: perspectives on right use and utility. Pain Physician. 2007; 10:479-491. [Crossref]


102. Nordin P, H Zetterström, U Gunnarsson and E Nilsson. Local, regional, or general anaesthesia in groin hernia repair: multicenter randomised trial. The Lancet. 2003; 362:853-858. [Crossref]

103. Bisgaard T. Analgesic treatment after laparoscopic cholecystectomy: a critical assessment of the evidence. Anesthesiology. 2006; 104: 835-846. [Crossref]

104. Bernards CM and D J Kopacz. Effect of epinephrine on lidocaine clearance in vivo: a microdialysis study in humans. Anesthesiology. 1999; 91:962-968. [Crossref]

105. Wagner HH and W Ulbricht. Saxitoxin and procaine act independently on separate sites of the sodium channel. Pflugers Arch. 1976; 364:65-70. [Crossref]


109. Offiah I, SB McMahon and B A O'Reilly. Interstitial cystitis/bladder pain syndrome: diagnosis and management. IntUrogynecol J. 2013; 24:1243-1256. [Crossref]


111. Rovner E. From 2014, Nov 02, 2017 Interstitialcystitis.”Retrieved Jan 24, 2018.[Crossref]

112. Parsons CL. The role of the urinary epithelium in the pathogenesis of interstitial cystitis/prostatitis/urethritis. Urology. 2007; 69:9-16. [Crossref]

113. Rossberger J, M Fall, O Jonsson and R Peeker. Long-term results of reconstructive surgery in patients with bladder pain syndrome/interstitial cystitis: subtypeing is imperative. Urol. 2007; 70:638-642. [Crossref]
