

Determination of neosaxitoxin in biota of Lake Baikal: preliminary observations

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ABSTRACT. Benthic biofilms, macrocolonies and net plankton samples collected in summer from well warmed-up bays and shallow waters of Lake Baikal during the mass development of cyanobacteria were studied. The contents of saxitoxin and neosaxitoxin were determined by means of liquid chromatography mass spectrometry in the form of 2,4-dinitrophenylhydrazine derivatives. The production of paralytic shellfish toxins and the saxitoxin/neosaxitoxin ratio depend on the species of cyanobacteria were also determined. Nowadays, the planktonic and benthic cyanobacteria of Lake Baikal can be considered as a natural source of neosaxitoxin only for research purposes. However, with obtaining cultured strains among the studied species, they can be used soon as producers.

Keywords: Neosaxitoxin, Lake Baikal, high performance liquid chromatography, mass spectrometry, derivatization, 2,4-dinitrophenylhydrazine

1. Introduction

Neosaxitoxin (NEO) is a compound of the group consisting of ~60 structurally related natural chemicals called paralytic shellfish toxins (PSTs). Some representatives of dinophytes, including species of the genus *Alexandrium* (*A. andersoni*, *A. catenella*, *A. excavatum*, *A. fundyense*, *A. minutum*, *A. ostenfeldii*, *A. tamarense*, and *A. tamiyavanichi*), as well as species *Gymnodinium catenatum* and *Pyrodinium bahamense*, are saxitoxin producers in marine resources (Llewellyn, 2006). At the same time, cyanobacteria of genera *Anabaena*, *Cylindrospermopsis*, *Aphanizomenon*, *Planktothrix*, *Lyngbya*, etc. are the PST producers in freshwater bodies (Wiese et al., 2010). In addition to the direct production by cyanobacteria, NEO, and some other PST variants, can be a product of enzymatic oxidation of gonyautoxins 1 and 4 (GTX1/4) (Sugawara et al., 1997).

Filter feeding shellfish that consume dinophytes is capable of bioaccumulating PSTs in high concentrations, which is hazardous to humans who use them as a food (Negri et al., 1995). In freshwater bodies, PSTs are mainly orally transmitted to humans and animals, for example, via drinking water.

Neosaxitoxin and its analogues target integral membrane protein (voltage-gated sodium channel (VGSCs or Nav channel) involved in the transmission of electrical signals in cell and the distribution of the action potential, basic physiological processes of the nervous system (Cestèle and Catterall, 2000; Yu and Catterall,

2003). The toxic effect of PSTs on living organisms arise from sodium channel blocking of electrically excitable membranes in nerve and muscle cells by the guanidine fragment in the structure of the toxin. The toxic effect of PSTs on living organisms occurs when the sodium channel of electrically excited membranes in nerve and muscle cells is blocked by a fragment of guanidine in the structure of the toxin. This leads to a decreased production of nerve impulses in tissues and organs. An acute toxicity arises even at minimal doses of saxitoxins (STXs) and can result in rapid death from paralysis of respiratory muscles, whose membranes are rich in sodium channels.

Blocking of sodium channels by saxitoxins in nerve cells is similar to tetrodotoxin (TTX) action mechanism. Such a unique feature of STXs made it possible to study neosaxitoxin as an analgesic for local anaesthesia in clinical practice (Rodriguez-Navarro et al., 2007; 2011) and veterinary medicine (Riquelme et al., 2018; Varela et al., 2019).

After a single injection, neosaxitoxin (individually or combined) has provided prolonged analgesia from one to three days. NEO is more effective and less toxic than STX (Manríquez et al., 2015; Rathmell et al., 2015), and currently it is in clinical trial phase no. 1 (Rodriguez-Navarro et al., 2007; 2011).

Prolonged analgesic effect of NEO at extremely low therapeutic doses (50–100 µg/day) is due to its high affinity and specificity for targets of the peripheral nervous system. Owing to low concentrations together

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with the inability to overcome the blood-brain barrier and affect the central nervous system, there are no dose-dependent side effects as well as physiological and psychological dependences, in contrast to narcotic analgesics (Rodriguez-Navarro et al., 2011; Riquelme et al., 2018; Varela et al., 2019).

We previously reported the presence of saxitoxin and its analogues' producers in Lake Baikal and other water bodies of the Irkutsk Region (Belykh et al., 2020). All investigated samples, except for plankton samples collected near the Turka settlement (August 2010), were analysed only by enzyme-linked immunosorbent assay (ELISA), while PST variants were identified by the MALDI-TOF/TOF method.

The plankton samples from Barguzin Bay contained STX and NEO; those from Kurkut Bay – STX and decarbamoylated derivatives of gonyautoxins, dcGTX1/4 and dcGTX2/3 (Lake Baikal, August 2010) (Belykh et al., 2015). In benthic biofilms (settlements of Listvyanka and Bolshiye Koty, September 2015), eight variants with different toxicity (STX, NEO, dcGTX1/4, dcGTX2/3, GTX5, dcSTX, dcNEO, and doGTX2/4) were detected (Belykh et al., 2016).

A preliminary assessment of the total STX content in water, planktonic and benthic samples was carried out by ELISA method using the Abraxis Saxitoxin ELISA kit test system (Abraxis LLC, United States) with a determination limit of 0.015 µg/L. ELISA has a high sensitivity, nevertheless it cannot assess the concentration of individual toxins. Moreover, the specificity of the test system depends on the STX nature and varies from 100% for saxitoxin to 1.3% for neosaxitoxin as well as <0.2% for gonyatoxin 1 and 4.

High performance liquid chromatography-mass spectrometry (HPLC-MS) combines the ability of high sensitive mass spectrometric detection of individual saxitoxins and capable to selective separation of studied mixtures thus can be considered as one of the powerful and universal methods for determination of target compounds.

Therefore, we aimed to study phytoplankton and phytobenthos of Lake Baikal as a natural source of pharmacologically significant substance – neosaxitoxin, using HPLC-MS.

2. Materials and methods

The following equipment and materials were used in present study:

An ultrasonic homogenizer UP100H (Hielscher Ultrasonic, Germany); MiniSpin microcentrifuge (Eppendorf, Germany); Freeze dryer CoolSafe (Scanlab, Denmark); Concentrator Plus vacuum centrifuge (Eppendorf, Germany); Analytical + analytical balances (OHAUS Europe, Switzerland); Sonorex ultrasonic bath (Bandelin, Germany); household refrigerator with a freezer (Stinol, Russia);

Agilent 6210 time-of-flight mass spectrometer with electrospray ionization (ESI-MS-TOF) coupled to an Agilent 1200 liquid chromatography system (HPLC-DAD) with a Zorbax 300SB-C18 (5 µm; 2,1 × 150 mm)

column. Eluent A was 0,1% heptafluorobutyric acid (HFBA) in water, and eluent B was 0,1% HFBA solution in acetonitrile. The column temperature was 35°C. The column was preconditioned with 100% eluent B for 15 min at 0,2 ml/min, then 10% eluent B for 15 min at 0,2 ml/min. Gradient elution (10% eluent B to 100% eluent B) was performed at a flow rate of 0,15 ml/min for 20 min. The detection mode was electrospray ionization with positive ion registration (ESI+). The range of detection was 100 to 600 Da, and the ion source temperature was set at 250°C, gas flow was 3,5 L/min, nebulizer was 45 psi.

Distilled water, acetic acid (analytical grade, Reakhim, Russia), chloroform (analytical grade, Kriokhrom, Russia), acetonitrile (HPLC grade, AppliChem, Germany); trifluoroacetic acid (analytical grade, Panreac, Germany); heptafluorobutyric acid (99.5%, FluoroChem, UK); 2,4-dinitrophenylhydrazine (double recrystallized from ethanol, Reakhim, Russia), standard saxitoxin solution with a concentration of 63.3 µmol/L (National Research Council, Canada).

3. Results and discussion

Plankton samples were taken in mid-July-August, during the mass development of cyanobacteria, from bays and shallow waters that warm up well in summer. The sampling method was chosen depending on the habitat and species of cyanobacteria. Plankton was sampled using the Apstein net (20 µm) from a depth of 0–5 m. *Nostoc pruniforme* existing in nature as separate large spherical colonies were manually collected from the soil surface of edge water zone. *Gloeotrichia echinulate*, a species forming freely floating spherical colonies (up to 2–5 mm in diameter) and visible in water, were collected together with the surface water. The samples were frozen and delivered to the laboratory. In total, eight samples, whose sampling sites are shown in the Table, were studied.

After thawing at room temperature, large colonies were shredded into small parts (less than 5 mm); the small ones were filtered through the fabric. Unstructured samples were mixed well (more than 5 min), placed to the Petri dishes, and large inclusions (particles of sand, wood and etc.) were removed. The obtained samples were further lyophilized. After that, different parts of the dried sample from the dish (centre and several points throughout the area) were transferred to a mortar and ground for five minutes. Ground samples were transferred to test tubes and stored at –20°C until analysis.

The extraction of toxins and its derivatization with 2,4-dinitrophenylhydrazine were carried out following the procedure, which was previously proposed by us for saxitoxin (Grachev et al., 2018; Zubkov et al., 2018). The table shows the results of the study.

The present study has revealed that *Nostoc pruniforme* and *Gloeotrichia echinulate* are capable of simultaneous production of STX and NEO. The samples of benthos, where the species *Tolypothrix distorta* is a likely producer of PSTs, have indicated only STX or

Table 1. Results of saxitoxin and neosaxitoxin content in biota samples from the coastal zones of Lake Baikal

Entry	Sampling site	Sample type	Probable PST producer/Dominant species	STX, µg/kg of dry mass	NEO, µg/kg of dry mass
1	Listvennichny Bay	Benthic fouling	<i>Tolypothrix distorta</i>	900 ± 150	nd
2	Bolshoye Goloustnoye settlement	Benthic fouling	<i>Tolypothrix distorta</i>	nd	nd
3	Bolshiye Koty settlement	Benthic fouling	<i>Calothrix</i> sp.	nd	nd
4	Aya Bay (Lake Baikal)	Benthic fouling	<i>Chamaesiphon confervicolus</i>	250 ± 100	nd
5	Mukhor Bay	Macrocolonies	<i>Nostoc pruniforme</i>	3800 ± 600	1300 ± 200
6	Mukhor Bay (the Kuchelga River, estuary)	Net plankton sample	<i>Gloeotrichia echinulata</i>	430 ± 100	2200 ± 300
7	Mukhor Bay (the Kuchelga River, 1,5 km to the north of the estuary)	Net plankton sample	<i>Gloeotrichia echinulata</i>	400 ± 100	2300 ± 400
8	The Ushkany Islands	Macrocolonies	<i>Nostoc commune</i>	nd	nd

no toxins, as in the sample consisting mainly of the *Calothrix* sp. filaments.

The ratio of toxin variants in samples depends on the species of dominant cyanobacteria. For example, plankton samples, in which *Gloeotrichia echinulata* (entry 6 and 7) prevailed, contain the greatest amount of NEO (Table). The *Nostoc pruniforme* macrocolonies contain the maximum amount of STX (entry 5).

The concentration of NEO in the sample with dominant *Gloeotrichia echinulata* from Lake Baikal (Table) is comparable with other members of the order Nostocales, e.g. *Aphanizomenon* DC-1 from Dianchi Lake (China), 2279 µg/kg (Liu, 2006), and more than nine times higher than in *Aphanizomenon flos-aquae* isolated from the water of Montargil Lake in Portugal (Pereira, 2000).

It is worth to be mentioned that the required single dose of NEO as a local anaesthetic for an adult is 50–100 µg (Rodriguez-Navarro et al., 2007; 2011). The low concentration of NEO in cyanobacteria from Lake Baikal and a short period of their mass development allows us to conclude that it is inappropriate to consider natural samples from the lake as a source of NEO for commercial uses.

At present time, an organic synthesis approach can be the main source of NEO for pharmaceutical purposes. Despite the method is extremely laborious, the target yield for (+)STX, as an example, is up to 70% yield (Fleming et al., 2006).

On the other hand, the colonies of *Nostoc pruniforme* and *Gloeotrichia echinulata* are convenient for collection; the concentrations of toxins in them are sufficient for scientific purposes. Therefore, we assume that with the capabilities of modern analytical equipment for extracting individual toxins one can consider Lake Baikal as a natural source of toxins for research aims. Furthermore, upon obtaining pure cultures of cyanobacteria and choosing the optimal cultivation conditions, their use in medicine is also possible.

4. Conclusions

Present study revealed the production of neosaxitoxin by cyanobacteria in various habitats of Lake Baikal. The *Nostoc pruniforme* and *Gloeotrichia echinulata* cyanobacteria are capable of simultaneous production of NEO and STX. Samples of benthos, where the species *Tolypothrix distorta* is a probable producer of PSTs, contained only STX. The production and the ratio of toxin variants in phytoplankton depend on species of cyanobacteria. Thus, plankton samples with prevailing *Gloeotrichia echinulata* showed the greatest amount of neosaxitoxin. The *Nostoc pruniforme* macrocolonies contained the maximum amount of saxitoxin. Nevertheless, Baikal cyanobacteria produce a small amount of neosaxitoxin; therefore, it is possible to consider Lake Baikal as a natural source of neosaxitoxin only for research purposes.

Acknowledgements.

Analytical equipment was provided by the Shared Research Facilities for Physical and Chemical Ultramicroanalysis LIN SB RAS. This work was carried out within the State Task (Project No. 0345-2018-0005, Project No. 0345-2018-0003) and supported by the Russian Foundation for Basic Research and the Government of the Irkutsk Region (RFBR; Grant No. 17-44-388091)

References

- Belykh O.I., Tikhonova I.V., Kuzmin A.V. et al. 2020. Toxin-producing cyanobacteria in Lake Baikal and water bodies of the Baikal region (review). *Teoreticheskaya i prikladnaya ekologiya* [Theoretical and Applied Ecology] 1: 21-27. (in Russian)
- Belykh O.I., Glagkikh A.S., Tikhonova I.V. et al. 2015. Identification of cyanobacterial producers of shellfish paralytic toxins in Lake Baikal and reservoirs of the Angara river. *Microbiology (Mikrobiologiya)* 84: 98-99. DOI: 10.1134/S0026261715010038

- Belykh O.I., Tikhonova I.V., Kuzmin A.V. et al. 2016. First detection of benthic cyanobacteria in Lake Baikal producing paralytic shellfish toxins. *Toxicon* 121: 36-40. DOI: 10.1016/j.toxicon.2016.08.015
- Cestèle S., Catterall W.A. 2000. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. *Biochimie* 82: 883-892. DOI: 10.1016/s0300-9084(00)01174-3
- Grachev M., Zubkov I., Tikhonova I. et al. 2018. Extensive contamination of water with saxitoxin near the dam of the Irkutsk hydropower station reservoir (East Siberia, Russia). *Toxins* 10: 1-12. DOI: 10.3390/toxins10100402.
- Fleming J.J., Du Bois J. 2006. A Synthesis of (+)-saxitoxin. *Journal of the American Chemical Society* 128: 3926-3927. DOI: 10.1021/ja0608545
- Liu Y.M., Chen W., Li D.H. et al. 2006. Analysis of paralytic shellfish toxins in Aphanizomenon DC-1 from Lake Dianchi, China. *Environmental Toxicology* 21: 289-295. DOI: 10.1002/tox.20182
- Llewellyn L.E. 2006. Saxitoxin, a toxic marine natural product that targets a multitude of receptors. *Natural Product Reports* 23: 200-222.
- Manríquez V., Castro Caperan D., Guzmán R. et al. 2015. First evidence of neosaxitoxin as a long-acting pain blocker in bladder pain syndrome. *International Urogynecology Journal* 26: 853-858. DOI: 10.1007/s00192-014-2608-2
- Negri A.P., Jones G.J. 1995. Bioaccumulation of paralytic shellfish poisoning (PSP) toxins from the cyanobacterium *Anabaena circinalis* by the freshwater mussel *Alathyria condola*. *Toxicon* 33: 667-678. DOI: 10.1016/0041-0101(94)00180-g
- Pereira P., Onodera H., Andrinolo D. et al. 2000. Paralytic shellfish toxins in the freshwater cyanobacterium *Aphanizomenon flos-aquae*, isolated from Montargil reservoir, Portugal. *Toxicon* 38: 1689-1702. DOI: 10.1016/s0041-0101(00)00100-8
- Rathmell G.P., Strichartz G., Wanderer G. 2015. Neosaxitoxin versus traditional local anesthetics: mechanism of action and sites of notable effect. *Anesthesiology* 123. DOI: 10.1097/01.anes.0000470956.02665.a6
- Riquelme G., Sepúlveda J.M., Al Ghumgham Z. et al. 2018. Neosaxitoxin, a Paralytic Shellfish Poison toxin, effectively manages bucked shins pain, as a local long-acting pain blocker in an equine model. *Toxicon* 141: 15-17. DOI: 10.1016/j.toxicon.2017.11.004
- Rodríguez-Navarro A.J., Lagos N., Lagos M. et al. 2007. Neosaxitoxin as a local anesthetic: preliminary observations from a first human trial. *Anesthesiology* 106: 339-345.
- Rodríguez-Navarro A.J., Berde Ch.B., Wiedmaier G. et al. 2011. Comparison of neosaxitoxin versus bupivacaine via port infiltration for postoperative analgesia following laparoscopic cholecystectomy a randomized, double-blind trial. *Regional Anesthesia and Pain Medicine* 36: 103-109.
- Sugawara A., Imamura T., Aso S. et al. 1997. Change of paralytic shellfish poison by the marine bacteria living in the intestine of the Japanese surf clam, *Pseudocardium sybillae*, and the brown sole, *Pleuronectes herensteini*. *Scientific Reports of Hokkaido Fisheries Experimental Station* 50: 35-42.
- Varela X., Del Campo M., Piron R. et al. 2019. Effect of neosaxitoxin on epidural anesthesia in cats: a promising alternative to conventional anesthetics. *International Physiology Journal* 2: 4-10.
- Wiese M., D'Agostino P.M., Mihali T.K. et al. 2010. Neurotoxic alkaloids: saxitoxin and its analogs. *Marine Drugs* 16: 2185-2211. DOI: 10.3390/md8072185
- Yu F.H., Catterall W.A. 2003. Overview of the voltage-gated sodium channel family. *Genome Biology* 4. DOI: 10.1186/gb-2003-4-3-207
- Zubkov I.N., Kuzmin A.V., Tikhonova I.V. et al. 2018. Determination of saxitoxin by HPLC-MS with the 2,4-dinitrophenylhydrazine precolumn derivatization. *Izvestiya Vuzov. Prikladnaya Khimiya i Bitekhnologiya [Bulletin of Universities. Applied Chemistry and Biotechnology]* 8: 25-32. (in Russian)