Toxicon 204 (2021) 5–8

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Short communication

Neosaxitoxin, a Paralytic Shellfish Poison phycotoxin, blocks pain and inflammation in equine osteoarthritis

Cecilia Montero, Gricel Riquelme, Miguel del Campo, Néstor Lagos *

Membrane Biochemistry Laboratory, Department of Physiology and Biophysics, Faculty of Medicine, University of Chile, Independencia 1027, 8380000, Santiago, Chile

ARTICLE INFO

Handling Editor: Glenn King

Keywords:
Neosaxitoxin
Osteoarthritis
Long-lasting pain blocker

ABSTRACT

The Osteoarthritis is a chronic disease characterized by a progressive deterioration of the articular cartilage producing a strong inflammatory activity and chronic pain in patients. Horses also show osteoarthritis. Since the activation and progression of the disease are similar to that of human we developed a study model in horses. In this study, we test the effect of Neosaxitoxin, a phycotoxin from Paralytic Shellfish Poison, in the remediation of osteoarthritis equine clinical symptoms such as pain (showed in lameness) and inflammation quantifying the amounts of pro-inflammatory markers like cellular infiltration, TNF-alpha and nitric oxide in the synovial fluid obtained from the horse damaged joint. The outcomes show that Neosaxitoxin blocks pain for long lasting period (average 24.7 days). Furthermore, the amounts of pro-inflammatory markers were reduced and consequently an enhanced horse's well-being was obtained. Neosaxitoxin showed to be a candidate for establishing treatment protocols for OA, being safe and effective as a pain blocker in equine osteoarthritis.

The Osteoarthritis (OA) is characterized by progressive cellular and molecular changes in the joint tissues, with a rapid progression of joint damage at level of the cartilage and subchondral bone, generated inflammation and pain. The cartilage damage develops to an established degenerative joint disease (DJD) (Bertuglia et al., 2016; Miller et al., 2014). The pathogenesis of OA is still not fully understood, making it difficult to develop effective tools for early diagnosis and therapies that improve the incidence of the disease. Most of the human tissue available for study is obtained of joint replacement, when OA lesions are in the terminal stage and little can be concluded about the factors that play a significant role in their progress. To overcome this limitation, during the last 50 years, numerous induced and spontaneous animal models have been used to study the onset and progression of OA, as well as to test new therapeutic interventions. The importance to study OA in an equine model is due to both species (human and equine) are participating in sports competitions. Therefore, the idiopathic primary OA and the post-traumatic OA related to athletic routines and races occur in both with similar progress. So, challenges exist regarding early diagnosis and the development of effective treatments that allow return to full function (Mc Coy, 2015).

Pain is the most remarkable OA clinical symptom in human and equine. Although the biological mechanism involved in pain are still unclear, it has been suggested that the local inflammation could play an important role. This hypothesis is also supported by the finding that TNF-alpha amount in synovial fluid was found to correlate with pain in Knee OA patients (Kamm et al., 2010; de Lange-Brokaar et al., 2012). TNF-alpha has been show to influence and coordinate the inflammatory response in almost all tissues. In addition to its effect on peripheral tissues, there is good evidence that can influence the excitability of nociceptors either directly or through the expression of downstream cytokines or both. TNF-alpha can produce very rapid changes in neuronal excitability by regulating voltage-gated sodium channels (VGSC) expressed by sensory nerves, producing a rapid increase in the firing rate of A and C fibers (nociceptors). These nociceptors can detect noxious signals in the innervated tissues and carry them to the dorsal horn of the spinal cord (de Lange-Brokaar et al., 2012; Miller et al., 2014).

In the past 20 years, Neosaxitoxin (NeoSTX), a phycotoxin from paralytic shellfish poison, has been widely used for its anesthetic effect in numerous studies in human and veterinary medicine (Lagos, 2014; Lobo et al., 2015; Riquelme et al., 2018; Valenzuela et al., 2019). Its main physiological effect is to inhibit the propagation of nerve impulses by reversible binding to the extracellular side of the pore of the VGSCs in excitable cells. Nevertheless, given the presence of these channels in multiple immune cells, and their implication in their activation, the immunomodulatory role of this neurotoxin has begun to be studied

* Corresponding author.
E-mail address: nlagos@med.uchile.cl (N. Lagos).

https://doi.org/10.1016/j.toxicon.2021.10.006
Received 15 July 2021; Received in revised form 4 October 2021; Accepted 12 October 2021
Available online 16 October 2021

Table 1
AEEP Lameness Scale: 0) Not present; 1) Subtle, difficult to observe; 2) Observed in a forced situation; 3) Observed when trotting; 4) Seen in any gait, but can still trot; and 5) Not support weight on damaged limb (Stashak 2004).

Horses with OA	AAEP score before NeoSTX administration	Start effect after NeoSTX administration (min)	AAEP score 4 h after NeoSTX administration	End effect (days)	AAEP score at the end of the effect
1	4	30	0	21	3
2	3	20	0	23	2
3	3	20	1	24	2
4	3	30	0	20	3
5	3	30	0	25	2
6	4	15	0	25	3
7	4	60	1	25	2
8	3	30	0	30	3
9	3	30	1	30	2
10	3	25	0	24	3
11	4	30	1	25	3
Average	-	27.7	–	24.7	–

Control Horses with OA	AAEP score before vehicle administration	AAEP score 4 h after vehicle administration
1	3	3
2	3	3
3	4	4
4	3	3

(Black and Waxman, 2013; Montero et al., 2020). NeoSTX infiltrated locally generates a prolonged anesthetic effect by direct action on neuronal activity, and also it makes a decrease in the inflammatory response by reducing the pro-inflammatory phenotype in macrophages (Lagos, 2014; Montero et al., 2020).

The aim of this study was to determine the capacity of NeoSTX to improve clinical parameters of equine osteoarthritis, considering both: a) pain manifested by lameness in the horse and b) measure pro-inflammatory markers (cellular infiltration, TNF-alpha and nitric oxide amounts) in the synovial fluid from the damaged joint. Since, NeoSTX generates long-lasting anesthetic effects (Rodriguez-Navarro et al., 2009), and due to the satisfactory results showed in chronic inflammatory pathologies (Lagos, 2014; Manríquez et al., 2015; Riquelme et al., 2018) this phycotoxin revealed as a potential candidate for establishing treatment protocols for OA.

In this investigative clinical trial, 15 horses diagnosed with OA (with a score of 3 or 4 on the AEEP lameness scale and radiological confirmation) (Table 1); from the Club Hípico de Santiago, Chile (11 horses); and from the private Haras of Santiago (4 horses with OA and 6 healthy) were tested. All horses were thoroughbred, without distinction of sex, between 3 and 5 years old, and throughout the study they remained at rest within their stables. The principles of the Chilean National Ethical Guidelines for Biomedical Research Involving Veterinarian Subjects (Comite Institucional de Cuidado y Uso de Animales de Experimentacion (CICUAL, CD:812/03)) were strictly followed in the design of this study, which was also approved by the Ethical Committee of the Medicine Faculty, University of Chile (FM 0551).

11 horses with OA were infiltrated with pure NeoSTX (300 μg) diluted in 5 ml of 0.9 % NaCl at pH 6.2. The dose was applied subcutaneously in two infiltration points (2.5ml each), located on both sides of the affected area. Following the same procedure other 4 OA horses were infiltrated with vehicle (NaCl 0.9 %). After infiltration, all horses were tested according to the American Association of Equine Practitioners' lameness scale (AAEP) (Hinchcliff et al., 2013), and they were closely observed up to 4 h. All the NeoSTX infiltrated horses showed an average of 27.7 min at which the AAEP score was lowered, launching a positive effect with score of 0 or 1 in the AAEP scale. None OA horses infiltrated with vehicle improved in the AAEP score (Table 1).

As an example of how local infiltration of NeoSTX can control chronic pain and abolish lameness in a deep injured horse a Video 1 is showing. This one summarized a follow-up for 4 h after NeoSTX infiltration to a horse with Ring Bone impairment, a pathology that generates similar symptoms to OA in horses. The evaluation outcome after NeoSTX infiltration by the lameness test showed in this video was the same performed for OA horses. The 11 horses with OA evaluated with this test, showed a long lasting effect with a mean duration of 24.7 days.

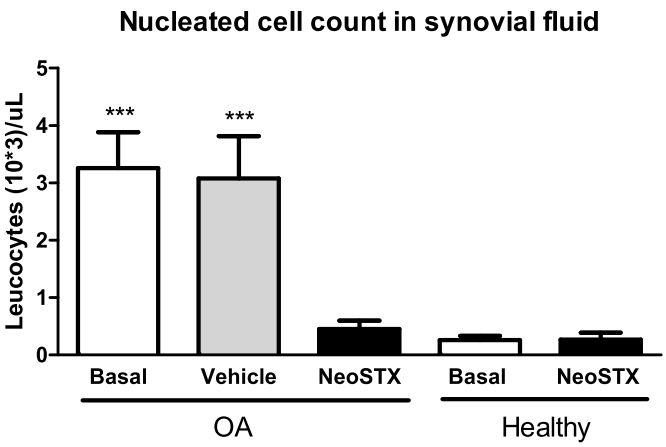

Fig. 1. Nucleated cell count in synovial fluid from NeoSTX-treated AO horses: Before (Basal) and 4 days after NeoSTX or vehicle administration. 5 ml sample of synovial fluid was taken from the injured joint. NeoSTX decreased cellular infiltration at the level of healthy horses, but not the vehicle used as control. NeoSTX in healthy horses does not vary the cell count. The cell count was carried out using a with the TC10 ™ Automated Cell Counter, according to the manufacturer's instructions for analysis of synovial fluid samples. Significant differences were observed between the OA horses basal level and those treated with vehicle, with the NeoSTX treated OA horses and the healthy horses (***$p < 0.005$).

Supplementary data related to this article can be found at https://doi.org/10.1016/j.toxicon.2021.10.006.

Synovial fluid samples were taken from all horses prior and 4 days after infiltration with NeoSTX or vehicle. In each sample the inspection of cell count, TNF-alpha and nitric oxide (NO) amounts was done. In turn, 3 healthy horses were administered NeoSTX and 3 healthy horses were administered vehicle, and the same analyzes were performed 4 days later. The results of the nucleated cell count are shown in Fig. 1; these were achieved using a TC10 ™ Automated Cell Counter (Bio Rad, USA).

At day 0, horses with OA have a high number of cells when compared with healthy horses, which shows a great cellular infiltration in the joint as a result of the pathology. After 4 days, the horses with NeoSTX decreased the number of cells in the damaged joint to similar level of the

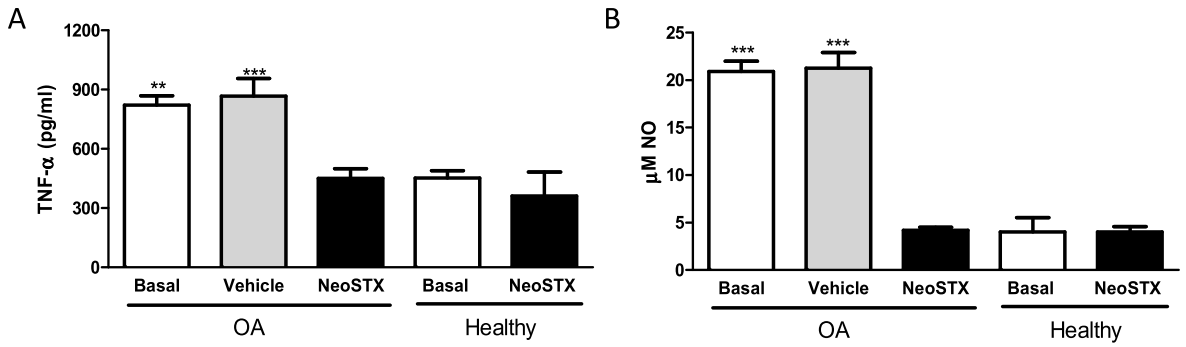

Fig. 2. TNF-alpha (A) y NO (B) concentrations in synovial fluid from NeoSTX-treated AO horses. TNF-alpha was measured by Equine TNF-alpha DuoSet ELISA (R&D systems, USA) and NO by Griess assay, 4 days after OA horses NeoSTX infiltration. The NeoSTX infiltrations in OA horses diminish the production of both pro-inflammatory markers. Not change was observed in horses infiltrated with vehicle used as control. NeoSTX infiltrated in healthy horse's make none change in both pro-inflammatory markers. Significant differences were observed between the basal state of the OA horses and those treated with vehicle, with the NeoSTX treated OA horses and the healthy horses (***$p < 0.005$; **$p < 0.01$).

healthy ones. None of the horses infiltrated with vehicle showed any improvement. As a control, NeoSTX was infiltrate in healthy horses, they did not show any change in these parameters.

In horses diagnosed with OA, the infiltration with NeoSTX reduced the amount of TNF-alpha and NO in the synovial fluid 4 days after the administration, showing lower quantities compared to its basal concentration and those treated with vehicle as control (Fig. 2). TNF-alpha was analyzed by an Equine TNF-alpha DuoSet ELISA (R&D systems, USA) and the Griess assay was used to quantify NO. These result are consistent with the lower cellular infiltration detected in the joint.

This study shows that NeoSTX is effective in controlling chronic pain in horses diagnosed with OA. Moreover, the cellular infiltration was diminished and the amount of pro-inflammatory markers was reduced in the damaged joint.

For a long time, OA have been considered a non-inflammatory pathological condition. However, increasing evidence that inflammation is present in OA patients has brought to light the possibility that synovitis and the immune system could be active players in development and progression of OA (de Lange-Brokaar et al., 2012).

There are three main types of synoviocytes: macrophages (type A), fibroblasts (type B) and dendritic cells (type C) (Burmester et al., 1983). It is now thought that much of the cytokine expression is originally by the synovium, and predominantly from the synovial macrophages which drive the inflammatory and destructive responses in OA (Bondeson et al., 2006). Histological studies have demonstrated that OA synovial macrophages exhibit an activated phenotype and that they produce both pro-inflammatory cytokines and vascular endothelial growth factor. These cells are important for the structural damage like osteophytes form, because macrophages perpetuate inflammatory and destructive responses from the synovial fibroblasts through a combination of TNF-alpha, IL-1B and NO (Bondeson et al., 2006).

The inhibitory effect of NeoSTX on the depolarization of sensory neurons by acting on the VGSCs has been well demonstrated (Llewellyn, 2006; Lagos 2014; Alonso et al., 2016). Therefore, the effect that this phycotoxin would have on the activation of macrophages has become an important issue, since these channels play an important role in its activation (Black and Waxman, 2013; Montero et al., 2020). Consequential, therapies aimed to block pain in OA and in turn reducing inflammation, not only improve the patient's quality of life, but also reduce the progressive negative effects of this illness. New studies with therapies sustained over time are required to establish NeoSTX as an optimal candidate for OA treatment in humans and horses.

Credit author statement

Cecilia Montero participated in the design and implementation of the clinical trial, sample analysis and writing of this manuscript. Gricel Riquelme participated in the implementation of the clinical trial. Miguel del Campo, participated in the design of the clinical trial, analysis of samples and writing of this manuscript. Nestor Lagos participated in the clinical design and writing of this manuscript.

Ethical statement

The principles of the Chilean National Ethical Guidelines for Biomedical Research Involving Veterinarian Subjects (Comite Institucional de Cuidado y Uso de Animales de Experimentacion (CICUAL, CD:812/03)) were strictly followed in the design of this study, which was also approved by the Ethical Committee of the Medicine Faculty, University of Chile (FM 0551).

Funding

This study was supported by grants from the Fondo Nacional de Ciencias FONDECYT #1130037, Chile.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Alonso, E., Alfonso, A., Vieytes, M.R., Botana, L.M., 2016. Evaluation of toxicity equivalent factors of paralytic shellfish poisoning toxins in seven human sodium channels types by an automated high throughput electrophysiology system. Arch. Toxicol. 90, 479–488.

Bertuglia, A., Pagliara, E., Grego, E., Ricci, A., Brkljaca-Bottegaro, N., 2016. Pro-inflammatory cytokines and structural biomarkers are effective to categorize osteoarthritis phenotype and progression in Standardbred racehorses over five years of racing career. BMC Vet. Res. 12, 246.

Black, J.A., Waxman, S.G., 2013. Noncanonicals rol of voltage-gate sodium channel. Neuron 19, 532–542.

Bondeson, J., Wainwright, S.D., Lauder, S., Amos, N., Hughes, C.E., 2006. The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis. Arthritis Res. Ther. 8, R187.

Burmester, G.R., Locher, P., Koch, B., Winchester, R.J., Dimitriu-Bona, A., Kalden, J.R., Mohr, W., 1983. The tissue architecture of synovial membranes in inflammatory and non-inflammatory joint diseases. I. The localization of the major synovial cell populations as detected by monoclonal reagents directed towards Ia and monocyte-macrophage antigens. Rheumatol. Int. 3, 173–181.

de Lange-Brokaar, B.J.E., Ioan-Facsinay, A., van Osch, G.J.V.M., Zuurmond, A.M., Schoones, J., Toes, R.E.M., Huizinga, T.W.J., Kloppenburg, M., 2012. Synovial inflammation, immune cells and their cytokines in osteoarthritis: a review. Osteoarthritis Cartilage 20, 1484–1499.

Hinchcliff, K.W., Kaneps, A.J., Geor, R.J., 2013. Equine Sports Medicine and Surgery: Basic and Clinical Sciences of the Equine Athlete, second ed. Saunders, W. B.

Kamm, J.L., Nixon, A.J., Witte, T.H., 2010. Cytokine and catabolic enzyme expression in synovium, synovial fluid and articular cartilage of naturally osteoarthritic equine carpi. Equine Vet. J. 42, 693–699.
Lagos, N., 2014. Clinical applications of paralytic shellfish poisoning toxins. In: Rossini, G.P. (Ed.), Toxins and Biologically Active Compounds from Microalgae, Volume 2: Biological Effects and Risk Management. CRC Press, Florida, pp. 309–329.
Llewellyn, L.E., 2006. Saxitoxin, a toxic marine natural product that targets a multitude of receptors. Nat. Prod. Rep. 23, 200–222.
Lobo, K., Donado, C., Cornelissen, L., Kim, J., Ortiz, R., Peake, R.W.A., Kellogg, M., Alexander, M.E., Zurakowski, D., Kurgansky, K.E., Peyton, J., Bilge, A., Boretsky, K., McCann, M.E., Berde, C.B., Cravero, J., 2015. A phase 1, dose-escalation, double-blind, block-randomized, controlled trial of safety and efficacy of neosaxitoxin alone and in combination with 0.2 % bupivacaine, with and without epinephrine, for cutaneous anesthesia. Anesthesiology 123, 873–885.
Manríquez, V., Caperan Castro, D., Guzmán, D., Naser, R., Iglesia, M., Lagos, N., 2015. First evidence of neosaxitoxin as a long-acting pain blocker in bladder pain syndrome. Int. Urogynecol. J. 26, 853–858.
Mc Coy, A.M., 2015. Animal models of osteoarthritis: comparisons and key considerations. Vet. Pathol. 52, 803–818.
Miller, R.E., Miller, R.J., Malfait, A.M., 2014. Osteoarthritis joint pain: the cytokine connection. Cytokine 70, 185–193.
Montero, M.C., Del Campo, M., Bono, M., Simon, M.V., Guerrero, J., Lagos, N., 2020. Neosaxitoxin inhibits the expression of inflammation markers of the M1 phenotype in macrophages. Mar. Drugs 18, 283.
Riquelme, G., Sepúlveda, J.M., Ghumgham, Z.A., Del Campo, M., Montero, C., Lagos, N., 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.
Rodriguez-Navarro, A.J., Lagos, M., Figueroa, C., Garcia, C., Recabal, P., Silva, P., Iglesias, V., Lagos, N., 2009. Potentiation of local anesthetic activity of neosaxitoxin with bupivacaine or epinephrine: development of a long-acting pain blocker. Neurotox. Res. 16, 408–415.
Stashak, T.S., 2004. Adam's Lameness in Horses, fifth ed. Lippincott Williams & Wilkins, Philadelphia USA. 2004.
Valenzuela, C., Torres, C., Muñoz, V., Simbaina, J.C., Sánchez, A., Bustamante, T., Sepúlveda, J.M., Piron, R., Del Campo, M., Lagos, N., 2019. Evaluation of Neosaxitoxin as a local anesthetic during piglet castration: a potential alternative for Lidocaine. Toxicon 164, 26–30.