

## Original Paper

# Botulinum Toxin Type A - Possible Anti-Nociceptive Effect on Mice

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## REZUMAT

### *Toxina botulinică tip A - posibile efecte antinociceptive pe șoareci*

Toxina botulinică, folosită inițial în tratarea afecțiunilor neuromusculare spastice, a primit în ultimele decenii o atenție crescută și o cercetare din ce în ce mai aprofundată, mecanismele de acțiune fiind în continuare atent studiate, în vederea stabilirii altor posibile proprietăți terapeutice.

**Material și metodă:** Este un studiu experimental realizat pe 60 de șoareci CD1, împărțiți în 4 loturi de câte 15, injectați cu ser fiziologic și respectiv 3 doze diferite de toxină botulinică tip A.

**Obiective:** Studiul nostru își propune să stabilească existența unei proprietăți antinociceptive a toxinei botulinice, dependentă de doză, la distanță de locul injectării.

**Rezultate:** După analiza statistică a datelor, am putut observa posibile efecte antinociceptive ale toxinei botulinice la doze de 20 U/kg.

**Concluzii:** Putem formula o concluzie privind existența unui efect antinociceptiv al toxinei botulinice tip A, la distanță de locul injectării, fie prin mecanisme retrograde axonal, fie prin interacțiunea cu receptori  $\mu$ -opioizi centrali. Studiul reprezintă un punct de plecare pentru experimente ulterioare, privind posibilele interacțiuni ale toxinei cu alte substanțe cu efecte antialgice sau antiinflamatorii.

**Cuvinte cheie:** toxina botulinică, șoareci, durere

## ABSTRACT

Botulinum toxin, originally used in the treatment of spastic neuromuscular diseases, received increased attention in recent decades and a deeper research, further mechanisms of action being carefully studied in order to determine other possible therapeutic properties.

**Method and material:** This is an experimental study conducted on 60 CD1 mice, divided into 4 groups of 15 each, injected with saline solution and 3 different doses of type A botulinum toxin.

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**Objectives:** Our study aims to establish an anti-nociceptive properties of botulinum toxin, dose-dependent, remote from the injection site.

**Results:** We could observe possible anti-nociceptive effects of botulinum toxin at doses of 20 U / kg after the statistical data analysis.

**Conclusions:** We can draw a conclusion on the existence of an anti-nociceptive effect of type A botulinum toxin, remote from the injection site, through retrograde axonal mechanisms or interaction with central  $\mu$ -opioid receptors. The study represents a starting point for subsequent experiments for possible interactions with other substances with analgesic or anti-inflammatory effects.

**Key words:** botulinum toxin type A, mouse

## INTRODUCTION

Botulinum neurotoxin has been used in the treatment of various groups of cronic neuromuscular diseases, which include neuromuscular junction signaling, non-neuromuscular transmission, cholinergic, or non-cholinergic.

Having specificity on releasing neurotransmitter vesicles with acetylcholine in the synaptic gap, the main usage of botulinum toxin was as a muscle relaxant drug in painful or painless spastic syndromes.

We can draw a conclusion on the existence of an anti-nociceptive effect of type A botulinum toxin, remote from the injection site, through retrograde axonal mechanisms or interaction with central  $\mu$ -opioid receptors. The study represents a starting point for subsequent experiments for possible interactions with other substances with analgesic or anti-inflammatory effects. [1]. After crossing the neural membrane, into the cytosol [2], the toxin's light chain acts like a zinc-protease with proteolytic activity from its N-terminal fragment, cleaving a 9 amino acid segment in the Carboxylate end of SNAP-25 (synaptosome-associated protein 25, involved in the release of acetylcholine vesicles in the synaptic gap) [3].

In vivo studies showed that most nerve endings do not have botulinum toxin receptors, thus the protein is less effective, the translocation into the neural cytosol being performed by pinocytosis, thereby blocking cellular carrier SNARE complex [4]. However, some exceptions have been observed in vitro, showing that botulinum toxin inhibits the release of substance P from the cultures of embryonic dorsal root ganglia neurons [5] and reduces stimulated release of calcitonin gene-associated peptide (CGRP) [6]. Based on these

results botulinum toxin can inhibit in vivo the release of other substances involved in the chemical processes of pain and inflammation. This hypothesis is strengthened by the results of other studies on human subjects, in which injecting botulinum toxin in the muscles has reduced local pain, i.e. neurogenic bladder pain model [7].

In preclinical studies conducted on lab animals either after injection of carrageenan, or produced by injecting formalin, usually in the paw (intraplantar), botulinum toxin has proven effective in reducing inflammatory pain [8] and neuropathic pain in the case of chronic constriction nerve [9] or neuropathic pain induced by paclitaxel administration [10]. All these studies highlighted analgesic and anti-inflammatory properties of local type A botulinum toxin, explained by different local mechanisms, but also a central action, as evidenced by behavioral studies in laboratory animals [11], or immune-histochemical studies [12], establishing that central activity of the toxin can be achieved through a retrograde axonal transport, from the periphery to the CNS (central nervous system) [13, 14].

## METHOD AND MATERIALS

### Animals

The study included a total of 60 male CD1 mice, from the Bio-Base of the Bucharest "Carol Davila" Faculty of Medicine and Pharmacy, weighing between 35 and 50 grams, 38.5 grams on average. Animals were housed in standard transparent Plexiglas cages, in 4 groups of 15 mice each, with clean shavings, with food and water available ad libitum, with standard 12-hour lighting cycles (12 hours light / 12 hours darkness) and constant temperature of  $21^{\circ}\text{C} \pm 1^{\circ}\text{C}$ .

The study was conducted after 3 days of acclima-

tization of the mice to the new environment.

Testing was conducted in accordance to the Romanian laws on using laboratory animals for scientific purposes, regarding the ethical principles of research, stipulated in Law 43 / 11.04.2014, Law 305/2006 and the European Convention for the protection of vertebrate animals used for scientific and research purposes ETS 123/1986, amended in 2006.

#### Used substances

We used type A botulinum toxin, produced and sold by Allergan Inc. Romania, under the name of Vistabel, presented under the form of a vial of 50 U of purified white powdered toxin to be recombined in isotonic solution or sterile water: 4U/0.1 ml.

We chose the doses of botulinum toxin based on studies of neurotoxicity found in the literature (LD50 = 0.5-1.0 x 10<sup>-6</sup> mg / kg) [15] and behavioral studies [16], in order not to produce toxic effects such as paralysis or behavioral changes.

We set three doses of botulinum toxin to be administered to three groups of 15 mice each:

- Goup B – 5 U/kg /mouse,
- Group C – 10 U/kg /mouse,
- Group D – 20 U/kg /mouse,
- Group A was the reference group, and received saline solution 0,9%.

#### Stages of the study

After acclimatization of the lab mice, we injected increasing doses of botulinum toxin diluted in isotonic solution in the forehead area, subcutaneously, intramuscularly, as follows: 5U / kg/mouse to group B with 15 mice, with an average weight of 38.6 grams, 10 U / kg/mouse to group C with 15 mice, with an average weight of 38.7 grams and 20 U / kg/mouse to group D with 15 mice, with an average weight of 38.8 grams, 5 days before the actual test. On the day of testing, the control group with 15 mice with average weight 38.6 grams, was injected with saline in the forehead, subcutaneously, 20 minutes before the test.

We evaluated all four groups of mice using Hot Plate Test, using a hot plate Ugo Basile apparatus, which consists of a glass cylinder with transparent walls and with the base made of a hot plate with a constant temperature of 55°C. The test was performed for maximum 90 seconds for every mouse, in order to avoid further irreversible burn lesions to the plantar side of the paws or to the tail.

We calculated the time elapsed from the moment the mouse set foot on the hot plate to the moment he started licking his paw (opioid specific), and then the moment he jumped.

The main purpose of this test was to establish if indeed the botulinum neurotoxin has indeed any effect on pain dose-related or not, in comparison with the pain threshold observed in the mice from the control group A.

The periods of time measured were gathered into a spreadsheet in Excel and statistically analyzed with SPSS program.

The results are presented as means, standard deviations and comparative tests (T-Test), the statistically significant results having a p number <0.05.

## RESULTS AND DISCUSSIONS

Pain is a protective mechanism that signals the presence or imminence of a tissue destructions caused by painful stimuli. There are two types of pain, acute and chronic, differentiated by their duration. Acute pain is defined as a short-period type of pain or whose cause can be easily identified and treated. Chronic pain is defined as a period of at least 6 months of pain and may consist in pain with intermittent or continuous character, whose causes are difficult to identify and non-responsive to the usual analgesic therapies.

In most cases of pain treated with intramuscular injection of type A botulinum toxin in the painful area, the main theory of anti-nociceptive effect of botulinum toxin consisted in its muscle relaxant

Table 1.

Time elapsed Licking - Hot Plate (Δt - seconds)	Group - A	Group B - 5 U/kg	Group C - 10 U/kg	Group D - 20 U/kg
MEANS	5.99	7.350667	6.849333	8.255333
STDEV	1.721819	2.114603	1.532044	3.028751
TTEST		*0.025449	0.06702	*0.007651

(\*p<0.05 – statistically significant for group B; higher significance for group D)



Figure 1.



Figure 2.

Table 2.

Time elapsed Jumping – Hot Plate (Δt – seconds)	Group - A	Group B – 5 U/kg	Group C – 10 U/kg	Group D – 20 U/kg
MEANS	61.54867	51.32467	74.43067	79.90667
STDEV	19.91558	9.436035	26.8062	27.27944
TTEST		0.083166	0.146359	*0.044385

(\*p<0.05 – statistically significant for group D)

action [17], thus reducing local ischemia or reducing pain signals transmitted by afferent nerve fibers Ia from muscle spindles to the spinal cord [18].

However, the toxin’s muscle relaxant effect does not explain anti-nociceptive effects in regions other than the injection site. One possible hypothesis would be the changes in the brain or spinal cord caused by the feed-back sent from the injected muscles by intrafusal type Ia afferent nerve fibers [19] [20].

Another hypothesis, and most agreed upon at the moment, is the retrograde axonal transport [13, 14], a theory supported by studies of diabetic neuropathy induced to lab animals with a decrease of bilateral pain sensation after unilateral injection of botulinum toxin [21].

A third hypothesis is the association of botulinum toxin’s anti-nociceptive effect with central μ-opioid receptor activity, by indirect increase of endogenous opioid system’s anti-nociceptive activity or by stimulating the release / synthesis of opioid peptide or by enhancing opioid receptor function [22].

According to the studies found in the specialty literature, our study sought to demonstrate anti-nociceptive effect of botulinum toxin remote from the injection site. Thus, the data gathered and analyzed from the first part of the Hot Plate test, show

an increase in pain sensitivity threshold statistically significant for the group injected with 5 U / kg (group B) and group C injected with 20 U / kg of botulinum toxin (p <0.05). It is known that the paw-licking reaction is typical for reaching the sensitivity threshold in case of opioid substances’ effects, strengthening the third hypothesis that the toxin could interact with endogenous opioid system.

To determine whether the Hot Plate Test results in the first phase are accurate or not, (paw-licking) we continued monitoring mice on the hot plate until they started jumping, indicating the set-up of nociceptive pain. Data obtained showed a statistical significance for the group injected with 20 U / kg of botulinum toxin (group C), the other two groups not showing statistically significant outcomes compared to the reference group A.

By comparing the three different doses of botulinum toxin with the reference group, we could determine the anti-nociceptive effect dose: the third dose - 20 U/kg, but without altering overall movement function.

## CONCLUSIONS

The results we obtained demonstrate the presence of an anti-nociceptive effect of type A botu-

linum toxin, in doses of 20 U/kg/mouse, regardless of the mechanism of action agreed, effective in the treatment of acute or chronic pain, alone or in combination with other painkilling substances.

In the future we intend to combine type A botulinum toxin with other substances having painkilling or anti-inflammatory effects, central or peripheral, to determine a possible enhancing or decreasing effect on the therapeutic action of the latter. Given that the mechanisms of action of botulinum toxin are not fully understood, we aim to identify other means of action of botulinum toxin than already described in the literature, in order to discover other possible therapeutic properties.

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