Trigeminal ganglionic neuronal death in a case positive for the botulinum neurotoxin B

Adelina Maria Jianu¹, Florinel Pop², Sorin Hostiuc³, George Cristian Curca⁴, Mugurel Constantin Rusu⁵*

Abstract: Botulinum neurotoxin B (BoNT-B) is a well known lethal agent in humans. In the last years botulinum neurotoxins have been used as therapeutic agents in pain management; their intimate effects on the peripheral nervous ganglia were however not thoroughly described. We present here a rare case of a BoNT-B serologically positive human adult in which trigeminal ganglia were found immunopositive for caspases 3 and 9. Thus the intrinsic apoptotic pathway was proposed as the cell death mechanism involving primary trigeminal neurons. Scavengers such as macrophages and resident satellite glial cells, with a CD68 immunopositivity were also identified, presumably being in the process of eliminating apoptotic remnants. Taking into account that the downregulation of metalloproteinases leads to inactivation of neuronal caspase 3, exogenous metalloproteinases, such as BoNT, may lead to apoptosis. If this causal relation, neurotoxin-to-apoptosis is valid, similar apoptotic processes can be presumed to occur in various ganglia, sensory and autonomic, involved in vital functions of the body. In conclusion, further morphological and experimental studies are needed to extensively evaluate the apoptotic effect of BoNT within the peripheral nervous ganglia in botulinum infections and BoNT treatments.

Key Words: clostridium botulinum, trigeminal ganglion, apoptosis, caspase.

Peripheral neuropathies can result from any type of neural damage, including that triggered by physical trauma, infection, inflammation, metabolic abnormalities, vascular abnormalities, neurotoxins, chemotherapeutic agents, radiation or autoimmune disease [1].

Botulinum neurotoxin (BoNT) is produced by the bacterium Clostridium botulinum under anaerobic conditions. BoNT-A through BoNT-G represent the 7 different types of BoNT [2] antigenically and serologically distinct but structurally similar. Human botulism is caused mainly by types A, B, E, and F. BoNT-B is one of the serotypes of BoNTs able to cause deadly human botulism [3].

Because of its inability to cross the blood-brain barrier, BoNT binds primarily to cholinergic nerves innervating skeletal muscles (i.e., motor nerves), parasympathetic (cholinergic) nerves, and sympathetic (cholinergic) nerves innervating sweat glands [2].

BoNTs reduce not only the release of acetylcholine, but also the release of neuropeptides involved in nociception helping therefore in cases where pain is not caused by muscular contractions, such as migraine or chronic daily headache (which includes chronic migraine). Serotypes A and B are both used therapeutically, with a clear predominance of type A [4].

1) University of Medicine and Pharmacy Timisoara, Dept. of Anatomy
2) Discipline of Pathologic Anatomy, Chair M65 – Faculty of Medicine, “Carol Davila” University of Medicine and Pharmacy, Bucharest, Romania
3) “Carol Davila” University of Medicine and Pharmacy, Dept of Legal Medicine and Bioethics, Bucharest, Romania (b) National Institute of Legal Medicine, Dept. of Forensic Pathology, Bucharest, Romania
4) (a) “Carol Davila” University of Medicine and Pharmacy, Discipline of Anatomy, Chair MD 01, Faculty of Dental Medicine, Bucharest, Romania (b) Institute of Biology – Romanian Academy, Bucharest, Romania
5) * Corresponding author: Work Address: “Carol Davila” University of Medicine and Pharmacy, 8 Eroilor Sanitari Blvd., RO-76241, Bucharest, Romania, Home Address: 1 Anastasie Panu St., bloc A2, scara 2, etaj 1, apart.32, sector 3, RO-031161 Bucharest, Romania, PHONE: +40722363705, E-mail address: anatomon@gmail.com (M.C.Rusu)
In peripheral nervous ganglia there is an incomplete blood-ganglion barrier [5, 6] that allows ions, small neurotransmitter molecules and macromolecules to cross [5]; this barrier consists of satellite ganglion cells (SGCs) that organize themselves, but not exclusively into neuronal sheaths or envelopes. Therefore, BoNT may reach the neuronal somata crossing the SGCs barrier.

CGRP is the most abundant pain mediator in primary trigeminal neurons of the trigeminal ganglion, a major sensory relay center. CGRP stimulates extravasation of inflammatory mediators that cause intracranial hypersensitivity and throbbing migraine headache. Acute migraine headaches can often be alleviated by CGRP receptor antagonists or serotonin agonists, who lower CGRP release [7]. Synaptobrevin I mediates exocytosis of CGRP from sensory neurons and its inhibition by BoNTs determines their anti-nociceptive potential [8].

It has been shown that mitochondrial functional status is controlled separately in neurites and in the neuronal soma; neuronal somatic death was shown experimentally to be macromolecular synthesis-dependent and evidence for a loss of mitochondrial cytochrome c, caspase activation, and nuclear fragmentation, has indicated that this type of cell death is entirely apoptotic [9].

The caspases are involved in apoptotic elimination of the axotomized primary trigeminal neurons [10] and the caspases cascade was shown to underlie the rat primary trigeminal apoptosis induced by neonatal capsacin administration [11].

There are no reports available on the effect of BoNT-B on the primary trigeminal neurons, in humans. We bring here such evidence, from a case positive for BoNT-B. These data may be relevant for establishing secondary sites of damage in such lethal infections or after BoNT-B use as a therapeutic agent.

Materials and methods

Autopsy was performed in an adult male patient, 62 years old, with positive serology for type B botulinum neurotoxin. The trigeminal ganglia were dissected out bilaterally during autopsy, after the brain was removed and the skull base was exposed. The ganglia were paraffin embedded and histological stains using hematoxylin-eosin (HE) were performed.

Then, immunohistochemistry for paraffin embedded specimens was performed using antibodies for CD68 and caspases, 3 and 9 (for details please observe Table 1) and the method of streptavidin-biotin complexes.

In order to get indirect proofs, we immunostained for caspases 3 and 9 five additional samples of trigeminal ganglia obtained from adult patients (aged 58-71 years, sex ratio 3:2) with no recordings of infection diseases.

Sections treated without primary antibodies served as internal negative controls. Datasheets of the primary antibodies and working metodology, (including dilution of the antibodies or incubation times, can be consulted: (1) for CD68 antibody, at: http://biocare.net/products/antibodies/c/033/; (2) for caspase 3 antibody, at: http://bsd.leica-microsystems.com/pdfs/products/cpp32-u.pdf; (3) and for caspase 9 antibody, at: http://bsd.leica-microsystems.com/pdfs/products/casp-9.pdf.

Results

The case presentation

Patient was brought to the emergency room with dysphagia, emesis, and dyspnea. Clinical examination revealed a slightly enlarged liver, longer vesicular murmur, weak bronchial rales, and a moderate salivary stasis in both pyriform sinuses. EKG revealed a sinus rhythm with pulmonary p waves in D I and D II. Next day the patients felt worse, with severe altered deglutition reflex, palpebral ptosis, marked physical asthenia, and therefore was transferred to a university hospital with a working diagnosis of myasthenia gravis, and acute respiratory insufficiency.

During transportation he entered into a profound coma (GCS=3); CT and MRI found nothing significant. Lab works revealed the following: low blood count (3.02 g/dL), high urea (135.7 mg/dL) and CK (1324 UI/L), pH=7.47. Glucose and protein levels were elevated in CSF (103 and 70 mg/dl respectively).

Clinical symptomatology suggested botulism as a possible diagnosis which was confirmed using mice intraperitoneal inoculation (positive for B type, negative for A and E type). Antibiotherapy and A + B botulinum serum were tempted but without success and the patient died in three days after the diagnostic.

An epidemiological survey was conducted but couldn’t find other cases nor the initial cause. Autopsy examination revealed cerebral stasis and edema, acute pulmonary edema, visceral anemia, miocardosclerosis, generalized I-II degree atherosclerosis, hepato- and splenomegalia, intestinal paresis, severe dystrophic hepatic lesions. Cause of death was acute respiratory insufficiency due to B type botulinum toxin intoxication.

The trigeminal ganglion

The histological examination of the slides identified cells with the size comparable to that of monocytes or small macrophages, with pleomorphic, fairly reniform nuclei, with perineuronal disposition or presenting aspects of direct phagocytosis of the neuronal somata (Figure 1).

Table 1. Antibodies used in the present study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type, species</th>
<th>Identification</th>
<th>Clonality, clone</th>
<th>Source, code</th>
<th>Positive controls used</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD68</td>
<td>IgG1 kappa, Mouse</td>
<td>macrophages, monocytes</td>
<td>monoclonal, KP1</td>
<td>Biocare, PM 033 AA</td>
<td>tonsil</td>
</tr>
<tr>
<td>CPP 32 (Caspase 3)</td>
<td>IgG1 Mouse</td>
<td>caspase 3</td>
<td>monoclonal, JHM62</td>
<td>Novocastra NCL-CPP32</td>
<td>tonsil</td>
</tr>
<tr>
<td>Caspase 9</td>
<td>IgG1 kappa, Mouse</td>
<td>caspase 9</td>
<td>monoclonal, 2C9B11</td>
<td>Novocastra, NCL-CASP-9</td>
<td>large bowel</td>
</tr>
</tbody>
</table>
Most neuronal somata had degenerative processes, cytoplasmic swelling and karyolysis and there were foci of inflammatory infiltration around some SGCs sheaths/envelopes, many of which were empty.

General histopathological aspects were characteristic for a process of trigeminal ganglioneuritis.

Immunohistochemistry for CD68 identified non-neuronal positive cells within the trigeminal ganglion; some of these were located in the interneuronal spaces and others within rarefied perineuronal sheaths/envelopes (Figure 2).

Positive immunoreactions (IRs) for caspase 3 were identified within the trigeminal neurons with two distribution patterns: (a) eccentric, intense and homogenous IRs and (b) moderate, heterogeneous, eccentric or almost complete IRs (Figure 3). All neurons on slides were immunopositive for caspase 9 but the individual appearances were variable, either intense and homogenous positive IRs or mild positive, heterogeneous IRs. Most of the SGCs displayed necrotic lesions (Figure 4).

Perineuronal edema was a constant feature evidenced within neuronal sheaths, as were the neural fibers lesion within the ganglion. These alterations emphasized the ganglionic connective stroma and the endo- and perineural sheaths (Figure 4).

Immunostaining for caspases 3 and 9 within the control trigeminal ganglia (Figure 5) revealed the absence of apoptotic neuronal and glial damage proved by un conclusive, negative immunoreaction, as for both caspases 3 and 9.

**Discussion**

Our results proved within the trigeminal ganglion apoptotic neuronal death and neurodegenerative lesions in a BoNT-B seropositive patient; apoptosis involved the intrinsic pathway, with activation of caspases 9 and 3. However, a direct causal relation is difficult to be established in a single case study, even though the neurotoxin may reach neurons due to the incomplete pattern of the blood-ganglion barrier (see Introduction).

It was shown that disruption of synaptic architecture by BoNT-C in central nervous system neurons activates distinct neurodegenerative programs in the axodendritic...
Trigeminal ganglionic neuronal death in a case positive for the botulinum neurotoxin B

Network and in the cell bodies. Neurites degenerate at an early stage by an active caspase-independent fragmentation. Later, the cell body mitochondria release cytochrome c, which is followed by caspase activation, apoptotic nuclear condensation, loss of membrane potential, and, finally, cell swelling and lysis. Recognition and scavenging of dying processes by glia also precede the removal of apoptotic cell bodies, in line with a temporal and spatial segregation of different degenerative processes [12]. According to our findings, comparable effects may occur also in the peripheral nervous system, presumably determined by BoNT-B.

A genuine analgesic effect for BoNT-A, unrelated with skeletal muscle spasmyosis has been suggested on the basis of in vitro and in vivo (animal) data. However, studies in humans designed to detect such an effect were negative, as were controlled studies of BoNT-A in patients with primary headache disorders [13].

Lesser is known regarding BoNT-B action effects associated with pain transmission and, to our knowledge, this is the first report of sensory neuronal cell death in the presence of BoNT-B. Moreover, there are no studies available to describe the histopathology of peripheral nervous ganglia in botulism, in humans.

V-SNAREs are vesicle associated proteins (e.g. synaptogamin and synaptobrevin) while T-SNAREs are plasma membrane associated proteins (e.g. SNAP proteins, syntaxin, neurexin). BoNT-B, BoNT-D, BoNT-F and BoNT-G are primarily involved in the inactivation of the V-SNARE protein synaptobrevin [2].

After gaining access into the neuronal cytosol, the BoNTs metalloprotease activity selectively proteolyse and disable SNARE proteins which mediate vesicular transmitter release. BoNT-B acts on vesicular protein isoforms I, II and III of synaptobrevin [referred to as Sbr, but also known as vesicle-associated membrane protein (VAMP)] – Sbr I, Sbr II and Sbr III [8]. BoNT-B cleaves Sbr I and blocks exocytosis of CGRP from mouse TGNs; accordingly, preliminary observations from initial experiments on knock-down of Sbr I indicated a substantial reduction in CGRP release. Thus, it is reasonable to consider that Sbr I isoform can mediate the release of CGRP from LDCVs – at least in sensory neurons [8]. As such, the antinociceptive effect of the BoNT-B may be CGRP-release related, by suppressing the neurogenic inflammation determined by that peptide.

BoNT could alleviate pain in neuropathies and various types of headache where neurogenic inflammation plays a role [14]. BoNT-A inhibits calcium-dependent release of substance P, a nociceptive peptide, in dorsal root ganglion neurons cultures [15] and subsequently has an antinociceptive effect. The nociceptive effect of substance P release requires, for one of its two mechanisms, extracellular calcium and an intact SNAP-25 while the other mechanism is independent of extracellular calcium and does not involve SNAP-25 [15].

The effects of BoNT-A involve attenuation of the release not only of the substance P, but also of calcitonin gene–related peptide (CGRP) and glutamate, and inhibition of vanilloid receptor activity [16]. However, there is only

Figure 4. Immunohistochemistry for caspase 9 displays various degrees of positive immunoreactions (IRs) at the level of the trigeminal neurons, intense (n1) or mild (n2). Other neuronal somata exhibit an advanced stage of disintegration (n3). Satellite glial cells appear extensively damaged by necrotic lesions and few can be accurately identified in the constitution of the neuronal sheaths/envelopes (bullets). The perineuronal edema (*) emphasizes the connective coat of the perineuronal sheaths/envelopes which is stained blue with hematoxylin (arrowheads). Also, the connective sheaths of the neural cords within the ganglion are well stained with hematoxylin (arrows) but there is an advanced degree of neural content dystrophy and disintegration of these intraganglionic cords.

Figure 5. Immunohistochemistry for caspases 3 (A) and (9) in control shows integrity of the neurons, the neuronal capsules consisting of glial cells, and of the intraganglionic neural fascicles. The immunoreactions for caspases are negative.

neurototoxic effects in a case positive for the botulinum neurotoxin B
circumstantial evidence for noncholinergic mechanisms as an explanation for the antinociceptive effect following BoNT shots [17].

Active suicidal death programs are intrinsic to cells in unicellular and multicellular organisms. Activation of these programs not only kills cells but additionally promotes mechanisms for elimination of the dying/dead cells. In multicellular animals, cells undergoing apoptotic death can be eliminated by two processes with contrasting physiopathological consequences. One is the safe, physiological clearance by phagocytosis of the dying/dead cell or of apoptotic bodies through the timely assistance of a partner cell (the scavenger); this intervention of the scavenger removes the apoptosing cells or the apoptotic bodies while they still have a near-to-intact cytoplasmic membrane thus preventing leakage of dangerous molecules.

When the scavenging mechanism fails in vivo, cell elimination is carried out by transition of full-blown apoptosis to secondary necrosis that leads to cytoplasmic membrane damage and cell disintegration [18]. Both mechanisms were evident in our case at the level of the trigeminal ganglion, involving the import of scavengers at the level of the foci of inflammatory infiltration and, respectively, extensive processes of secondary necrosis (late-stage apoptosis).

The scavenging mechanisms within the trigeminal ganglion, in our case, were conducted not only by imported macrophages from a well-represented local microcirculation but also by recruited resident SGCs switched to a macrophagic phenotype.

It was experimentally proved that after chronic constriction injury of the rat infraorbital nerve an early and transient reaction of microglia in the rostral ventromedial medulla is present [19]. The SGCs of the human trigeminal ganglion were proven to have a unique leukocyte phenotype, with features of both macrophages and immature myeloid dendritic cells. Microglia also resemble macrophages and immature myeloid dendritic cells involved in neurodegenerative disorders, and both microglia of the CNS and the SGCs of the trigeminal ganglion express common phenotypes, including the CD68 phenotype [6]. Therefore SGCs can be considered as a peripheral equivalent of the CNS microglia.

Regarding a possible correlation between BoNT-B and caspase activation we obtained circumstantial proofs as in non-botulism infectious processes a similar pattern was not found.

Survival of developing neurons is believed to depend upon proper and timely synapse formation, for which synaptic proteins (such as the SNARE proteins) are crucially important. Overinhibition of developing neurons impairs synaptic protein function and activity-induced synaptic plasticity, which could in turn result in permanent neuronal loss [20]. BoNTs are known for their metalloprotease (MP) activity [8, 21] by which they selectively proteolyse and disable SNARE proteins which are mediating vesicular transmitter release [8]. However we could not identify any link between the MP activity and caspases 3 and 9 activation we evaluated within the primary trigeminal neurons, except for a very recent study, of Scuteri et al. (2010, in press), performed on dorsal root ganglion neurons cultures, that emphasizes the importance of the matrix metalloproteinases’ (MMPs) down-regulation for neuronal survival. These authors demonstrated that the down-regulation of MMPs led to caspase 3 inactivation and brought a first proof of a direct link between MPs and neuronal apoptotic death [22]. Even though it is an in vitro study, it can be, at least partly, correlated with our evidences, gained ex vivo, of neuronal caspase-3 activation from an external source of MPs (the BoNT-B neurotoxin).

As further discusses Scuteri et al. the down-regulation of MMPs by undifferentiated mesenchymal stem cells (MSCs) may be used as a promising tool treating neurodegenerative pathologies [22]. According to these findings, the MSCs have to be further checked as a possible adjuvant component of the BoNTs used in the migraine therapy, in order to protect against the primary sensory neurons apoptosis. As it was already pointed, the BoNT use in pain therapy seems a promising treatment that must be further evaluated [23].

Even though local administration of BoNT, in experiments or as medication, may not relate to neurodegenerative lesions in sensory ganglia, this issue must be further checked. For example, local administration of BoNT was used in experiments of TMJ articular disc degeneration in rats, by injecting the toxin in the masseter muscle [24]. Also, local infiltration of BoNT is useful in cases of TMJ dislocation [25, 26]. Other conditions such as sialorrhea, muscle movement disorders, and facial nerve palsy can also be cured with this drug [27].

**SWO(T)** analysis of the case reported

**Strong points:** (1) it brings the first time evidences for the intrinsic pathway of the primary trigeminal neurons apoptosis, with activation of caspases 9 and 3, in neurodegenerative lesions; (2) it raises doubts on the BoNT use in various aligias, that was not previously evaluated experimentally for its effect on sensory ganglia; (3) the results support the APC phenotype of the satellite glial cells.

**Weak points:** (1) this is a single case study; (2) the link between the neurotoxin and the direct effect on the primary trigeminal neurons is not missing but is weakly supported by very few references; (3) as the findings are a proof of serendipity, other sensory ganglia were not evaluated.

**Opportunities:** (1) experimental models can be further evaluated leaving from the present findings; so, BoNT may be administered (after preliminary “fine tuning” of doses) in the general circulation in experimental rodents that may be left untreated with antitoxin, and peripheral ganglia may be obtained at different stages before and after their death, to get the dynamics and extent of the degenerative lesions. In such experiments, an electron microscopic
evaluation can succeed the immunohistochemical one and different experimental lots may be used to check whether or not the local administration of BoNT may lead to neurodegeneration; (2) if larger studies on human samples and the experimental studies will strongly support the results in the present case, antiapoptotic agents should be further evaluated as adjuvant therapy in BoNT use for various cephalalgias.

Acknowledgements

This paper is also supported by the Sectoral Operational Programme Human Resources Development (SOPHRD), financed from the European Social Fund and by the Romanian Government under the contract number POSDRU/89/1.5/S/64109

Abbreviations:
BoNT – botulinum neurotoxin;
CGRP – calcitonin gene-related peptide;
LDCV – large dense core vesicle;
Sbr – synaptobrevin;
SGC – satellite ganglion cell;
SNARE – soluble NSF (N-ethylmaleimide sensitive factor) attachment protein receptor;
TGN – trigeminal ganglionic neurons;
VAMP - vesicle-associated membrane protein;
SNAP - synaptosomal-associated protein.

References