Incobotulinumtoxina (Xeomin®) Versus Onabotulinumtoxina (Botox®): Evaluation of Clinical Onset of Action with Rating Scales and Electroneurography

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Abstract

Onset of action, duration and maximum efficacy of different botulinum toxin type A (BoNT/A) preparations have been compared mainly in in vitro studies. This single-center open study compared onset of action of the two BoNT/A preparations onabotulinumtoxina (complex size 900 kDa) and incobotulinumtoxinaA (free of complexing proteins, 150 kDa) in patients with spasticity after cerebral stroke over a 15-day treatment period. Outcome measures were changes in muscle tone, increase in passive extension of the elbow, changes in limb functionality, and variation of the amplitude of the compound muscle action potential (cMAP) determined by electroneurography. A total of 108 patients (mean age 64.9 ± 11.3 years) were included in the study, 54 in each treatment arm. Muscle tone, elbow motion range, and limb function significantly improved in both groups from baseline to day 15 after BoNT/A injection (p<0.0001). Improvements were significantly greater under incobotulinumtoxinaA compared to onabotulinumtoxinaA after 7 treatment days (p<0.0001) but were comparable after 15 days. Regarding cMAP amplitude, a faster reduction in the first 7 treatment days with no further significant reductions during the next week was observed for incobotulinumtoxinaA patients, whereas onabotulinumtoxinaA patients showed a slower, progressive reduction in action potential resulting in comparable values between the two groups after 15 days. Overall, the efficacy of both BoNT/A preparations was comparable two weeks after injection.

Keywords: Spasticity; Botulinum toxin type A; Onset of action; Electroneurography; Rating scales; Presence/absence of complexing proteins

Introduction

Botulinum toxin type A (BoNT/A) acts selectively on peripheral cholinergic nerve endings inhibiting the release of acetylcholine and is recommended for the treatment of movement disorders such as cervical dystonia and blepharospasm [1], and spasticity [2]. Most preparations consist of a high molecular weight complex of the biologically active neurotoxin, non-toxic complexing hemagglutinating and non-hemagglutinating proteins, and excipients; e.g., the complex size of onabotulinumtoxina (Botox®; Allergan Inc., Irvine, CA, USA) is 900 kDa. IncobotulinumtoxinaA (Xeomin®; Merz Pharmaceuticals, Frankfurt/M, Germany) is the only BoNT/A preparation free of complexing proteins and thus differs from other conventional preparations on the market [3,4]. It is composed of pure neurotoxin with a molecular weight of 150 kDa.

The presence or absence of complexing proteins might influence the onset of action of the different BoNT/A preparations. Various studies have demonstrated that complexing proteins stabilize and protect the neurotoxin from unfavorable conditions at low pH levels such as the acidic stomach environment; on the other hand, neutral pH values favor the dissociation of neurotoxin and protein component [5]. Although this mechanism has been described several decades ago [6-8], little is known about the neurotoxin release kinetics and the stability of the complex in respect to factors such as dilution and the presence of sodium chloride and other salts. Recently, Eisele and colleagues [5] studied the 1α dissociation kinetics of the 900 kDa BoNT/A complex identifying the factors that destabilize the complex in relation to changes in environmental pH. Their data confirmed the dependence on pH and highlighted the faster dissociation at neutral pH. Thus knowledge of the pH of the sodium chloride solution used for drug reconstitution and the environment within the injected muscle fiber is of crucial importance.

Animal studies have directly compared onset of action, duration and maximum efficacy of various BoNT/A preparations on the market [9-11]; however, electrophysiological evaluations have so far only been carried out in a few healthy volunteers [12], and pathological muscle tissue investigations are scarce. Our study compared onset of action and efficacy of onabotulinumtoxina and incobotulinumtoxinaA in patients with spasticity using clinical rating scales, and electroneurography for determination of the amplitude of the compound muscle action potential (cMAP), i.e., the response obtained from supramaximal percutaneous stimulation of nerve trunks. cMAP is the sum of muscle action potentials activated synchronously and is strictly dependant on structural and functional conditions of movement axons, neuromuscular junctions and muscle fibers. It thus can be altered by myopathies, diseases in peripheral nerves and by disorders in neuromuscular transmission brought about by iatrogenic causes, as in our case the administration of botulinum toxin. This technique was chosen because it is easy to apply and can provide consistent numerical data which allow an accurate and objective comparison of the onset times in the target muscles.

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Materials and Methods

Study design and patients

This single-center open study recruited patients affected by muscular spasticity of the upper and lower limb after an ischemic or hemorrhagic stroke to evaluate onset of action and maximum efficacy of onabotulinumtoxinA and incobotulinumtoxinA in the treatment of upper limb spasticity. The study was performed at Bari Hospital, Italy in accordance with the Declaration of Helsinki and Good Clinical Practice, and was approved by the hospital’s ethics committee. All patients had signed the informed consent form. During the study patients received periodic rehabilitation consisting of stretching of injected muscles, active and passive mobilization of the upper limb, and overall daily muscle reinforcement for the first 30 days after injection and every three weeks thereafter. Patients could not participate in the study, if they were over 80 years of age, had marked muscular fibrosis in the biceps brachii muscle (evaluated by muscular ultrasound scan and ultrasonography), and presented with tendon retraction and joint blocking at the elbow (sounded by muscular-tendon ultrasonography and X-ray). Concurrent treatment with other muscle relaxants, the presence of myopathies, peripheral neuropathies, or a cardiac pacemaker, a positive anamnesis for dementia and for allergies to the study medication, and epilepsy at enrolment also led to exclusion.

Patients were divided into two groups matched by gender, age, side of spasticity, and time of onset and degree of spasticity. All received treatment with BoNT/A for upper limb spasticity. One group received a single set of intramuscular injections of incobotulinumtoxinA, the second group a single set of intramuscular injections of onabotulinumtoxinA into two sites of the muscle belly of the biceps brachii.

Outcome measures

The overall study duration was 15 days. All evaluations were carried out at baseline (during the injection session) and 7 days and 15 days after injection.

Assessment tools included a clinical outcome scale for functional evaluation of the upper limb affected by spasticity (score between 0 = not functional and 10 = full function), the Modified Ashworth Scale (MAS [13,14]) to determine muscle tone of elbow extension (from 0 = no increase in muscle tone to 4 = rigid in flexion or extension), limb goniometry for measuring the articular range of motion (ROM) of the elbow through a simple universal goniometer expressed in degrees [15], and electromyography to determine the cMAP amplitude [16,17]. The latter test was carried out using a Nicolet Viking 8 channel electromyography system with patients positioned face-up with the elbow extended or slightly bent, the forearm supine, the palm of the hand facing upwards, wrist in a neutral position, and relaxed fingers slightly bent at the interphalangeal articulations (such position was achieved with the help of a second operator). The recording electrode (concentric bipolar needle, 26 g needle diameter, recording area 0.07 mm²) was placed in the muscle belly of the biceps brachii, the earthing electrode between the recorder and the surface stimulator. Stimulation occurred at Erb’s point (supraclavicular fossa) with a frequency of 1 Hz. The current intensity was gradually increased up to maximum achievable without artifacts (high dose stimulation). cMAP amplitude was measured in millivolts (mV), from peak of negative phase to peak of positive phase (peak-peak) using the antidromic technique. The following band-pass filters were adopted: a low-pass filter that cuts the high frequencies (10-20 Hz) and a high-pass filter that cuts the low frequencies (2-5 Hz [18,19]). Outcome measures were subjective evaluation of limb function by the investigator, changes in muscle tone, and increase in passive extension of the elbow, and variation in cMAP amplitude over the treatment period; cMAP duration, area and latency were not considered.

Statistical analysis

Age and value of cMAP can be considered distributed according to Gauss, therefore data are summarized as mean and standard deviation; the analysis of the effectiveness of treatment and the comparison between the different points of follow-up was made by applying a model of analysis of variance for repeated measures. Evaluating the effect of some covariates such as sex, age (divided into classes <65, ≥ 65) and location of the lesion, they were included as random effects in the model, but excluded from the final model because they were not statistically significant. Other quantitative variables (MAS, clinical outcome and goniometric measurement) are not distributed according to Gauss, therefore data were summarized as median and range; comparison between therapies and between different times of follow-up were analyzed with non-parametric methods (Kruskal-Wallis and Friedman test). Post-hoc comparisons were performed by Bonferroni correction. Qualitative variables were summarized as counts and percentages; comparison between independent samples was performed using the chi-square test. The assessment of correctness of statements and comparability of the two groups was performed using the t-test student and the Wilcoxon test (for quantitative variables) and the chi-square test (for qualitative variables). Differences between groups were considered statistically significant with a p<0.05. Comparison within a treatment group was analyzed by the Bonferroni correction; in relation to the number of comparisons of interest, a p<0.0045 was considered as statistically significant. We used SAS 9.3 software for PC; Friedman’s test was conducted using the statistical software R version 12.

Results

The study included 108 patients (mean age 64.8 ± 11.3 years) already afflicted by spasticity since 18.6 ± 2.3 months. Fifty-four patients were treated with incobotulinumtoxinA, and 54 patients received onabotulinumtoxinA. Groups did not differ significantly in baseline characteristics (all p>0.05; Table 1). Proportions of patients receiving <150 U or >150 U of their respective treatment were also comparable between the groups (Table 1). Mean neurotoxin doses were 120 ± 15.9 <150 U or >150 U of their respective treatment were also comparable. No difference between groups (Table 1).

Treatment efficacy

Table 2 summarizes the results obtained at the three time points assessed in this study for muscle tone measurement, cMAP amplitude, and evaluation of clinical outcome. cMAP results are shown in Figure 1. At

| Neurontoxin dose  | Gender | Age (years) | IncobotulinumtoxinA | p value |
|-------------------|--------|-------------|---------------------|---------|
| <150 U or >150 U | Male   | <65 years   | 67.3 ± 5.6          | 6.65 ± 5.5 | 0.487 |
|                   | Female | ≥ 65 years  | 37 (66.1%)          | 30 (57.7%) | 0.41  |
| Suggestability    | Right body side | 21 (37.5%) | 35 (62.5%)          | 22 (42.3%) | 0.912 |
|                   | Left body side  | 27 (50%)   | 33 (63.5%)          | 27 (50%)  |
| Neurotoxin dose   | <150 U or >150 U | 39 (75%)  | 13 (25%)            | 40 (76.9%) | 0.819 |

Table 1: Baseline characteristics and received treatment doses of the two study groups Data are mean ± SD or number of patients (%).
baseline, the two treatment groups were comparable for all four outcome measures.

Muscle tone assessment showed a significant reduction in Ashworth score from baseline in both treatment groups 7 days and 15 days after injection ($p<0.0001$). Comparison of the two treatments revealed significant differences after 7 days ($p<0.0001$) but not after 15 days ($p=0.969$). This was confirmed by a comparison of score reductions between incobotulinumtoxinA and onabotulinumtoxinA patients (baseline – 7 days, $p=0.0002$; baseline – 15 days, $p=0.893$).

Elbow motion range increased significantly for both treatment groups at baseline, and 7 and 15 days after botulinumtoxin A injection. Data are median (range). A comparison of score reductions between the treatment groups, baseline minus 7 days and baseline minus 15 days.

Table 2: Outcome measures at baseline, and 7 and 15 days after botulinumtoxin A injection. Data are median (range). A comparison of score reductions between the treatment groups, baseline minus 7 days and baseline minus 15 days.

Table 1: Comparison of cMAP amplitudes (mV) in the biceps brachii (mean ± SD) between the two treatment groups at baseline, and 7 and 15 days after botulinumtoxin A injection. Data are median (range). A comparison of score reductions between the treatment groups, baseline minus 7 days and baseline minus 15 days.

![Figure 1: Comparison of cMAP amplitudes (mV) in the biceps brachii (mean ± SD) between the two treatment groups at baseline, and 7 and 15 days after botulinumtoxin A injection.](image)

Adverse events such as asthenia of the injected muscle, weakness/paralysis of adjacent muscles, or dysphagia were not reported during the treatment period.

**Discussion**

The present study compared onset of action and efficacy of the two BoNT/A formulations onabotulinumtoxinA and incobotulinumtoxinA in the spastic human muscle. We evaluated electrophysiological, muscular, and clinical variations for a 15-day period after BoNT/A injection in two homogenous groups matched by age, gender, pathology, muscle balance, clinical outcome and treatment with intensive physiotherapy after injection. Overall, the efficacy of both BoNT/A preparations was comparable two weeks after injection but onset times were different. After seven treatment days, improvements in muscle tone, elbow motion range, and limb function were significantly greater and the reduction in cMAP amplitude was faster under incobotulinumtoxinA compared to onabotulinumtoxinA.

A reason for the difference in latent time from injection to onset of effect might be the presence or absence of complexing proteins in the BoNT/A preparations. In contrast to incobotulinumtoxinA, the active neurotoxin of onabotulinumtoxinA is encapsulated in a protein shell. The stability of such a complex seems to be controlled by the pH: the neurotoxin is protected at low pH ($20.21$) and released at neutral pH. According to Eisele and colleagues [5], dissociation from the protein complex is time and pH dependent with a half-life of less than one minute at pH 7.0. Our study compared the onset of response of the two BoNT/A preparations in muscle fibers affected by spastic hypertonia which presents a mainly acidic environment. The associated physiopathology created in the spastic muscle is a drop in associated physiopathology created in the spastic muscle. The BoNT/A preparations was comparable two weeks after injection but onset times were different. After seven treatment days, improvements in muscle tone, elbow motion range, and limb function were significantly greater and the reduction in cMAP amplitude was faster under incobotulinumtoxinA compared to onabotulinumtoxinA.

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power linked to the progressive loss of fast and anaerobic type II fibers involved in movements, whereas the function of the slow and aerobic type I fibers remains with subsequent development of hypertonia, loss of dexterity and fineness of movement [22]. In time, the muscle fibers become atrophic both regarding changes in the hematic flow and due to the negative protein balance (increasing the proteolysis, the number of protein is reduced). This imbalance influences the composition of myosin isoforms of the fibers which become slow and less powerful. Due to a decrease in muscle fibers and the rise in interposed collagen fibers, the muscle is reduced in thickness, and the following histopathological changes are observed:

- Proliferation of the extracellular matrix;
- Increase in the rigidity of the spastic muscle cell and, less so, of the spastic muscle tissue (switch from a more elastic isoform to a more rigid one);
- Reduction in the mechanical properties of the extracellular material in the spastic muscle in respect to a normal muscle;
- Changes in the capillary circulation of the muscle.

After denervation, intramuscular capillaries degenerate much faster than myofibers resulting in perivasal fibrosis with subsequent development of local foci of hypoxia which prevent the denervated muscle from recovering and establish an acid environment [22]. Developing acidity within the hypertonic muscular tissue could be the cause of a slowdown in the recovery of the neurotoxin from the protein complex and consequently influence latent time between injection and the onset of effect.

In this context, one also has to consider the mechanism of action of BoNT/A which consists of the four fundamental processes receptor bond, internalization, translocation into cytoplasm, and enzymatic change of the target [23,24]. To cleave the neurotoxin’s target, the proteins of the SNARE complex, BoNT/A must pass from the vesicular lumen to the cellular cytoplasm. The low pH of the vesicular lumen is crucial for toxin action, because it allows translocation into the cytosol [25]. The acidic pH induces a conformational change in the translocation domain of the neurotoxin heavy chain (from a “neutral-hydrophilic” to an “acid-hydrophobic” conformation) which acts as a channel for the neurotoxin light chain to pass from the lumen into the cytosol [26]. Once exposed in the cytosol (pH neutral), the protein would bend and through the reduction of the intercatenary sulphur bridge [27], it would be released into the cytoplasm in the active form.

Protein complexes have been attributed with higher activity [28], stabilization and protection of the neurotoxin [29], and inhibition of diffusion to adjacent sites [30]. The results by Carli and colleagues [10] and Eisele and colleagues [5], however, have put the role and importance of protein complexes in therapeutic efficacy of BoNT/A preparations into question; in particular, since therapeutic equipotency of onabotulinumtoxinA and incobotulinumtoxinA has been observed in healthy volunteers [31,32], patients with cervical dystonia [33], and patients with blepharospasm [34]. Furthermore, as complexing proteins increase the bacterial protein load, their presence might increase the immunogenic risk of neutralizing antibody formation against the neurotoxin [35]. Recent study results indicated low antigenicity of long-term incobotulinumtoxinA treatment of cervical dystonia for secondary non-responders to onabotulinumtoxinA or abobotulinumtoxinA [36].

Our comparison of time of onset of the two botulinum toxins in muscle tissue affected by spastic hypertonia took into account that muscular atrophy and fibrosis in human muscle denervated for a long time are associated with clear changes in constricting vessels and microcirculation. In literature, very little attention has been paid to the structural and functional changes in skeletal muscles present after spasticity. Although muscle and neural changes are usually correlated, recent data have demonstrated that the muscular changes in spasticity cannot be explained by classic interpretations of the effects of neural changes alone. First debates regarding changes in skeletal muscles secondary to spasticity used the context of the chronic electrical stimulation model, but this has proven inaccurate [Lotta et al.]. In addition, no animal model has so far been developed that accurately reconstructs the transformation of human muscles in spasticity. It is thus important to improve our understanding of changes in the pathological human muscle. In response to a low functional demand, morpho-functional changes occur: the microvascular bed undergoes degeneration of the vascular wall and loss of capillary vessels, perfusion at rest is reduced, as is arteriolar response to vasoconstrictor and vasodilator stimuli. With spasticity consolidating, blood vessels are less present and with wall alterations (thickening associated with changes in the basal membrane). Pathogenesis of these vascular alterations is surely multifactorial and related to events such as functional disuse with subsequent reduction in muscle volume and predominance of type II fibers which are known to require less vascularization [Lotta et al]. The administration of botulinum toxin in such pathological conditions could thus expose it when in contact with an environment featuring a mainly acidic pH that could influence the scission of the remaining neurotoxin still tied to the protein complex. In view of this assumption it seems possible that the therapeutic effects of incobotulinumtoxinA and onabotulinumtoxinA may occur more quickly compared to onabotulinumtoxinA.

The results of our study thus place the doubt that protein dissociation may be influenced the condition of spasticity, since Onabotulinumtoxin A within the muscle tissue with the muscular tissue may still be partially limited by complexing proteins, notwithstanding reconstitution is done in solutions only nominally neutral; here comes the need to deepen thus determining the pH within the spastic muscle with microdialysis techniques.

Conclusion

Although efficacy of the two Botulinum toxin A preparations in the treatment of spasticity was comparable two weeks after injection, onset of action occurred earlier for incobotulinumtoxinA than for onabotulinumtoxinA. The rapid impact of incobotulinumtoxinA on functional recovery and movement might permit the implementation of an intensive rehabilitation program from the first days following injection.

A reason for the earlier onset of efficacy might be the absence of complexing proteins in the incobotulinumtoxinA preparation; however, further studies are required.

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Simpson DM, Blitzer A, Brashear A, Cornella C, Dubinsky R, et al. (2008) Assessment: Botulinum neurotoxin for the treatment of movement disorders (an evidence-based review): Report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology. Neurology 70: 1699-1705.

2. Wissel J, Ward AB, Ertzgaard P, Bensmail D, Hecht MJ, et al. (2009) European consensus table on the use of botulinum toxin type A in adult spasticity. J Rehabil Med 41: 13-25.
3. Dressler D (2012) Five-year experience with incobotulinumtoxinA (Xeomin®): The first botulinum toxin drug free of complexing proteins. Eur J Neurol 19: 385-389.

4. Lorenc ZP, Kenkel JM, Fagien S, Hirmand H, Nestor MS, et al. (2013) IncobotulinumtoxinA (Xeomin): background, mechanism of action, and manufacturing. Aesthet Surg J 33: 185-225.

5. Eisele KH, Fink K, Vey M, Taylor HV (2011) Studies on the dissociation of botulinum neurotoxin type A complexes. Toxicon 57: 555-565.

6. DasGupta BR, Boroff DA (1968) Separation of toxin and hemagglutinin from crystalline toxin of Clostridium botulinum type A by anion exchange chromatography and determination of their dimensions by gel filtration. J Biol Chem 243: 1065-1072.

7. Dasgupta BR, Boroff DA, Cheong K (1968) Cation-exchange chromatography of Clostridium botulinum type A toxin on amberlite IRC-50 resin at pH 5.55. Biochim Biophys Acta 168: 522-531.

8. Sugii S, Sakaguchi G (1975) Molecular construction of Clostridium botulinum type A toxins. Infect Immun 12: 1262-1270.

9. Chung ME, Song DH, Park JH (2013) Comparative study of biological activity of four botulinum toxin type A preparations in mice. Dermatol Surg 39: 155-164.

10. Carli L, Montecucco C, Rossetto O (2009) Assay of diffusion of different botulinum neurotoxin type A formulations injected in the mouse leg. Muscle Nerve 40: 374-380.

11. Kim SH, Kim SB, Yang GH, Rhee CH (2012) Mouse compound muscle action potential assay: An alternative method to conduct the LDA...&E. J Biol Chem 243: 1065-1072.

12. Eleopra R, Tugnoli V, Quaralle R, Rossetto O, Montecucco C (2004) Different types of botulinum toxin in humans. Mov Disord 19 Suppl 8: S53-S59.

13. Aboelhasani H, Ansari NN, Naghdi S, Mansouri K, Ghotbi N, et al. (2012) “Comparing the validity of the Modified Modified Ashworth Scale (MMAS) and the Modified Tardieu Scale (MTS) in the assessment of wrist flexor spasticity in patients with stroke: protocol for a neurophysiological study” BMU Open 2: e001394.

14. Li F, Wu Y, Li X (2014) Test-retest reliability and inter-rater reliability of the Modified Tardieu Scale and the Modified Ashworth Scale in hemiplegic patients with stroke. Eur J Phys Rehabil Med 50: 9-15.

15. Ohn SH, Yoo WK, Kim DY, Ahn S, Jung B, et al. (2013) Measurement of synergy and spasticity during functional movement of the post-stroke hemiplegic upper limb. J Electromyogr Kinesiol 23: 501-507.

16. Sakamoto T, Tori Y, Takahashi M, Ishida S, Goto Y, et al. (2009) “Quantitative determination of the biological activity of botulinum toxin type A by measuring the compound muscle action potential (CMAP) in rats” Toxicology 54: 85-57.

17. Tori Y, Goto Y, Takahashi M, Ishida S, Harakawa T, et al. (2010) “Quantitative determination of biological activity of botulinum toxin utilizing compound muscle action potentials (CMAPs), and comparison of neuromuscular transmission blockage and muscle flaccidity among toxins” Toxicology 55: 407-414.

18. Preston DC, Shapiro BE (2013) Electromyography and Neuromuscular Disorders. In: Clinical-Electrophysiologic Correlates , Elsevier Saunders.

19. Ubaii E (2000) Pictorial Atlas of Electroneurography. Scienza Medica.

20. Chen F, Kuziemko GM, Stevens RC (1998) Biophysical characterization of the stability of the 150-kilodalton botulinum toxin, the nontoxic component, and the 900-kilodalton botulinum toxin complex species. Infect Immun 66: 2420-2425.

21. Sharma SK, Fu FN, Singh BR (1999) Molecular properties of a hemagglutinin purified from type A Clostridium botulinum. J Protein Chem 18: 29-38.

22. Lotta S, Scoetl R, Affonsi E, Saitta A, Nicolotti D, et al. (1991) Morphometric and neurophysiological analysis of skeletal muscle in paraplegic patients with traumatic cord lesion. Paraplegia 29: 247-252.

23. Schiavo G, Rossetto O, Montecucco C (1994) Closstridial neurotoxins as tools to investigate the molecular events of neurotransmitter release. Semin Cell Biol 5: 221-229.

24. Colasante C, Rossetto O, Moriato L, Pirazzini M, Molgò J, et al. (2013) Botulinum neurotoxin type A is internalized and translocated from small synaptic vesicles at the neuromuscular junction. Mol Neurobiol 48: 120-127.

25. Pirazzini M, Rossetto O, Bolognese P, Shone CC, Montecucco C (2011) Double anchorage to the membrane and intact inter-chain disulfide bond are required for the low pH induced entry of tetanus and botulinum neurotoxins into neurons. Cell Microbiol 13: 1731-1743.

26. Koriazova LK, Montal M (2003) Translocation of botulinum neurotoxin light chain protease through the heavy chain channel. Nat Struct Biol 10: 13-18.

27. Rossetto O, Rigoni M, Montecucco C (2004) Different mechanism of blockade of neuroexocytosis by presynaptic neurotoxins. Toxicon Lett 149-91-101.

28. Sharma SK, Singh BR (2004) Enhancement of the endopeptidase activity of purified botulinum neurotoxins A and E by an isolated component of the native neurotoxin associated proteins. Biochemistry 43:4791-4798.

29. Bin MF (2009) Basic and clinical aspects of BOTOX. Toxicology 54: 676-682.

30. Sharma SK, Ramzan MA, Singh BR (2003) Separation of the components of type A botulinum neurotoxin complex by electrophoresis. Toxicology 41: 321-331.

31. Jost WH, Koli A, Brinkmann S, Comes G (2005) Efficacy and tolerability of a botulinum toxin type A free of complexing proteins (NT 201) compared with commercially available botulinum toxin type A (BOTOX) in healthy volunteers. J Neural Transm 112: 905-913.

32. Woitharth K, Müller C, Sassion I, Comes G, Grafe S (2007) Neuropathological double-blind trial of a botulinum neurotoxin type A free of complexing proteins. Clin Neurapharmacol 30: 86-94.

33. Benecke R, Jost WH, Kanovsky P, Ruzicka E, Comes G, et al. (2005) A new botulinum toxin type A free of complexing proteins for treatment of cervical dystonia. Neurology 64: 1949-1951.

34. Roggenkämper P, Jost WH, Bilhari K, Comes G, Grafe S, NT 201 Blepharospasm Study Team (2008) Efficacy and safety of a new Botulinum Toxin Type A free of complexing proteins in the treatment of blepharospasm. J Neural Transm 113: 303-312.

35. Benecke R (2012) Clinical relevance of botulinum toxin immunogenicity. BioDrugs 26: e1-9.

36. Heffter H, Hartmann C, Kahlen U, Moll M, Bigalke H (2012) Prospective analysis of neutralising antibody titres in secondary non-responders under continuous treatment with a botulinum toxin type A preparation free of complexing proteins – a single cohort 4-year follow-up study. BMJ Open 2: e000646.