Botulinum toxin immunoresistance in dystonia medical application

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Foreword

The paper hereby presented is the fourth semester project of the second year of studies at the Nat-Bas program at Roskilde University.

The theme for this project is open and could be based either in an experimental work or literature research. We consider our project *Botulinum toxin immunoresistance in dystonia medical application* a review paper; that is a combination of the first semester theme (*Application of natural science in technology and society*) and the third semester theme (*Reflection of natural science and the dissemination of knowledge in the field of natural science*).

For the first time in the Nat-Bas studies the semester project should be written as a scientific paper intended to be published in a real scientific journal.

We have decided to write a scientific paper that we would like to be published in the journal *Toxicon*. Each scientific journal has its own publication requirements and writing style. In this review paper we have tried to follow all the requirements exposed in appendix 1.

The use of *Botulinum toxin (BoNT)* in medical application is an increasing field of investigation and is directly related to: Chemistry, Biochemistry and Medical Biology. Both Chemistry and Biochemistry are subjects that we would like to continue in our studies. Another reason was that the study of this toxin in medical application is a relatively new topic. Not everything about this toxin and immunoresistance is known and further work is being done in different research centers around the world.

We have done our best in order to make this document as close as possible to a real scientific paper. The complexity of the content together with the relative newly field of investigation has made quite challenging the redaction of a review paper. However we are proud and glad to present you the outcome of our work.
Botulinum toxin immunoresistance in Dystonia medical application

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ABSTRACT

Botulinum Toxin type A is a protein produced by the bacteria Clostridium botulinum that exists in six different serotypes. The ability of this toxin is to inhibit the neurotransmitter acetylcholine at the neuromuscular junction and as a consequence of that the muscle signaling will be disrupted. The usage of BoNT-A has been considered a good alternative in minimizing the muscle disorders pain and posture but without curing the disease. The treatment with BoNT-A implicates a number of numerous injections in order to sustain symptom control and thus makes the patient vulnerable in developing neutralizing antibodies that interact against the neurotoxin ability of working. Blocking antibodies are the natural defense against the toxin and the complexing proteins present in the BoNT-A. Three different types of brands, Xeomin®, Botox® and Dysport® are used as treatments for improving the life of patience affected by dystonia. The results presented in the review conclude that Botox and Dysport contain complexing proteins, whereas Xeomin only contains the active neurotoxin and therefore reduce the immunoresistance. This review is a brief summary of the available information today, considering the immunoresistance against the BoNT-A in the patients affected by Dystonia.

Keywords:
Botulinum neurotoxin
BoNT-A
Immunoresistance
Dystonia
Complexing proteins
Blocking antibodies

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1. Introduction

Botulinum neurotoxin (BoNT) is a protein produced by the spores of *Clostridium Botulinum* bacteria. There are seven types of botulinum toxin, where all types are toxic in different degrees. BoNT is among the most potent toxins known to man. The application and usage of botulinum toxin in medical application have expanded dramatically over the last quarter of the century (Eleopra et al., 1998).

In this review article our intention is to explain the main properties of botulinum neurotoxin type A (BoNT-A). Scientists have been studying BoNT molecules to understand its structure and mechanism of action. This knowledge has been applied for the use in medical applications, mostly in movement related disorders (Truong and Jost, 2006). In this review we will briefly explain how BoNT-A can be applied in dystonia, a movement disorder associated with Parkinson’s disease. As it is today there is no cure for dystonia, the only goal is to reduce the symptoms. Patients, who are treated with BoNT-A, are able to control their movement and in some cases it also reduces pain (Jankovic, 2009). BoNT is considered as safe to use in medical applications, no long term side effect are known. Unfortunately, some patients have an immune response to BoNT after being treated with botulinum toxin (Atassi, 2004). Our intention is to explain the different types of immunoresistance as well as what are the possible reasons for the antibodies formation. Additionally, we will illustrate the ways to solve this problem.
2. BoNT-A chemistry and Dystonia application.

Chemical structure

Botulinum Toxin (BoNT) from a chemical and structural point of view is a protein. In Figure 1 we can see the protein model and the different parts of it. BoNT-A consists of a heavy amino acid chain with a molecular weight of 100 kDa and a light amino acid chain with a molecular weight of 50 kDa (LC). The heavy chain and the light chain are interconnected by a single disulfide bond (Oguma et al., 1995). The integrity of this disulfide bond is very important for the toxin’s biological activity. That is of major interest when talking about the toxic mechanism of the toxin. The disulfide bond together with the amino terminal end of the heavy chain is responsible for the translocation of the light chain into the cytosol (Sandvig, 2003).

The heavy chain consists of two domains of ~50KDa each: These are the N-terminal domain (Hₙ) and the C-terminal domain (Hₖ).

![Figure 1. BoTN protein with the different subdomains: Light chain (LC) in red. Heavy chain with two sub-functional domains: in purple the C-terminal domain (Hₖ), and in blue the N-terminal translocation domain (Hₙ) (Lacy, et al., 1998).](image)

Each of these two domains has a specific function. The Hₖ domain is responsible of binding to the receptors of the pre-synaptic nerve surface. The Hₙ domain is responsible for releasing the light chain (Lc) from the endosome into the cytosol of the cell. The light chain Lc is responsible of the cleavage and the inactivation of the
SNAP-25 (essential component of the neuroexocytosis apparatus). More details about BoNT working mechanism in the Toxic action mechanism section.

**Table 1.** Summary of BoNT protein composition and chemical description.

<table>
<thead>
<tr>
<th>BoNT Protein chains</th>
<th>Chain domains</th>
<th>Chemical description</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heavy chain</td>
<td>( H_c: ) C-terminal domain</td>
<td>Termination of the protein chain with a free carboxyl group</td>
<td>Binding to the presynaptic-nerve surface.</td>
</tr>
<tr>
<td></td>
<td>( H_n: ) N-terminal domain</td>
<td>Termination of the protein chain with a free amino group</td>
<td>Translocation of the light chain inside the cytosol.</td>
</tr>
<tr>
<td>Light chain</td>
<td></td>
<td>Zn-dependent protease</td>
<td>Cleavage and inactivation of the SNAP-25</td>
</tr>
</tbody>
</table>

The explanation for the chemical interaction and folding of the tertiary structure of BoNT protein is long and complex. Here we have explained the basic chemistry of the structure. That will help to understand the working mechanism of BoTN explained in the next point.

**Toxic action mechanism of BoNT-A**

The intoxication process can be divided into four parts. The first two steps are called binding and internalization. When the protein is injected directly into the muscle it is expected that it will remain 2.5-3 centimeters from the injection area. After toxin reaches the target muscle it binds to the cell receptor with a heavy chain. It has been shown that synaptic vesicle protein 2 (SV2) is the protein receptor for BoNT-A (Dong et al., 2006). After the BoNT protein is bound to the cell surface it enters the cell by receptor-mediated endocytosis forming an endosome. (Atassi, 2004). See Figure 2 for the mechanism of action of BoNT-A.

Once the toxin is inside the cell, in the endosome, the second step starts; it is called pH dependent translocation. The pH in the endosome is lower than the pH in the cytosol (Sandvig, 2003). This pH difference makes that some hydrophobic groups of the heavy chain becomes accessible. Even though the exactly mechanism is not completely understood, it has been proved that the heavy chain, with the accessible hydrophobic groups, interacts with the endosome membrane creating some “channels” for the release of the light chain. In the endosome membrane the
disulfide bond between the heavy chain and the light chain is cleaved permitting the light chain to be released into the cell cytosol. When the light chain separated from the BoNT protein complex, the original tertiary structure of the protein is destroyed. That means that the light chain is ultimate responsible of the toxic effect of the BoNT (Simpson, 2004).

After LC separates from the toxin, the final step, called inhibition, starts. The light chain cleaves one of the proteins in the SNARE complex. The SNARE complex is widely considered to be the catalyst of the membrane fusion and essential for the release of neurotransmitter (Montal, 2010). In the motor neurons BoNT-A cleaves the SNAP-25 protein, which is the component of the SNARE complex. This complex cannot function because the vesicle and the target membrane do not bind together and therefore neurotransmitter acetylcholine cannot leave the nerve terminal. Thus, muscle contraction is disrupted. Numbers on the picture represent intoxication steps that are explained in Figure 2. Number 1 is binding, 2-internalization, 3-translocation and that last step 4- inhibition of neurotransmitter release (Turton et al., 2002).

In the following section will focus on Dystonia, giving a general explanation about the disease and the symptoms. How BoNT is helping patients with Dystonia will also be described.
**Figure 2.** Mechanism of action of botulinum neurotoxin type A. 1-2. The BoNT protein binds to the cell receptors with a heavy chain (H$_n$(green) and H$_c$(blue). After the protein is bound to the receptors it enters the cell by endocytosis. 3. Once the protein is inside the cells pH sensitive tertiary structure gets destroyed and the light chain (LC-Yellow) is released to the cytoplasm. 4. LC cleaves the SNARE complex protein SNAP-25 to disrupt the release of neurotransmitter acetylcholine. (Turton et al., 2002)

**Dystonia**

Dystonia is a condition that is described by involuntary muscle contractions that causes slow repetitive movements or abnormal postures (Fahn et al., 1998) and can occasionally be found in rising Parkinson’s disease or in association with untreated Parkinson disease (Tolosa and Compta, 2006). The beginning of most dystonia is unknown, though the researchers believe that there may be abnormalities to the basal ganglia and cerebellum or abnormalities to the brain’s ability in processing the neurotransmitter dopamine, that help cells in the brain to communicate with each other (Neychev et al., 2008).

Classification of dystonia can be organized as generalized or focal, and can occur as a primary syndrome or secondary to another disease. There are about 50 clinical conditions reported to cause dystonia that may affect only one muscle, groups of muscles, or muscles throughout the body.
The disorder may be hereditary or caused by other features like birth associated, physical damage or adverse reaction to different medicines (e.g. tranquilizing psychiatric treatment) (Bertram and Williams, 2012).

Cervical dystonia is the most common type of dystonia. The muscles in the neck that regulates the position of the head are affected, disturbing the head to turn to one side or be dragged forward or backward (Albanese et al., 2006). Blepharospasm is the second most common focal dystonia appearing involuntary, violent affecting the contraction of the muscles controlling eye blinks. The symptoms are increasing and uncontrollably blinking while other times the spasm will force the eyes to close. Cranio-facial dystonia, oromandibular dystonia and spasmodic dysphonia are all describing dystonia that affects the muscles of the head, face and neck (Albanese et al., 2011).

The hereditary reason for dystonia is caused by mutations in the genome. The effect starts manifesting in childhood, affecting the limbs and progresses causing major disability along the maturity (Calakos et al., 2009).

An early appearance of dystonia starts at the age of 20-30 years and affects different parts of the body provoking different symptoms. Early symptoms can include a foot cramp after running some distance; in other occasion it can affect the turning of the neck when the individual is tired. The symptoms tend to remain localized with restricted progression to nearby muscles.

By regions of the body affected dystonia is organized in different localization like: a specific part of the body (e.g., writer's cramp, blepharospasm). The segmental region affects two or more adjoining parts of the body. (e.g., cranial and cervical). Multifocal region involves two or more unrelated body parts (e.g., upper and lower limb, cranial and upper limb). The last body region affected by dystonia is a generalized form that affects most or all of the body (Albanese et al., 2011). Botulinum neurotoxins type A has shown to be an effective helpful alternative for a number of movement disorders being considered the first choice treatment for patients suffering from focal dystonia although it only minimizes the disease without treating it (Colosimo et al., 2012).
The way BoNT acts in the muscle is by temporarily weakening dystonic muscles, thereby allowing for a more normal posture and function of the patient. When injected in the muscle BoNT works inside the nerve terminals to block the release of the neurotransmitter acetylcholine which serves as a neurotransmitter that acts in initiating muscle contractions (Benecke, 2012, Troung et al., 2009). The effect of BoNT begins 3 - 12 days after an injection and the muscle weakness is typically lasting between 3–4 months (Jimenez-Shahed, 2011). At the intervals of 3 months the injections effect are thought to reduce the risk of antibodies to the BoNT. The benefit of the treatment with BoNT results in the improvement of neck posture, muscle hypertrophy and pain reduction (Troung et al., 2009).

The side effects of the BoNT are hypersensitivity reactions, injection site infections, bleeding or bruising, neck pain and weakness in muscles found nearby to the injection site as a result of toxin diffusion (Troung et al., 2009). One of the major problems of using BoNT as a treatment for dystonia is the immunoresistance development against the toxin. In the next section we are going to talk about this topic.

**Immunoresistance to BoNT-A**

**Immunoresistance description**

As the use of BoNT in medical applications continues to increase the concern about immunoresistance to BoNT is also increasing. Immunoresistance to BoNT reduces significantly the efficiency of BoNT treatments and finally in some cases the treatment has no effect. Immunoresistance is the result of the natural occurring blocking antibodies (Zuber et al., 1993). These blocking antibodies are created by the human immunologic system in response to the presence of BoNT, their function is to neutralize and destroy the botulinum toxin from our body (Jankovic and Schwartz, 1995).

There are two types of immunoresistance:

- Primary resistance
- Secondary resistance
**Primary resistance:**

Primary resistance refers to lack of response to the very first treatment of BoNT. This is extremely rare and is mainly due to a pre-existing immunization to botulinum toxin, that is people that had suffered from botulism previously. The natural response of the body is to generate blocking antibodies to botulinum toxin. After surviving the disease; these blocking antibodies remains in the body and these persons have already developed immunoresistance (Jankovic et al., 2003).

**Secondary resistance:**

Secondary resistance is the most likely cause of treatment failure. By definition, patients who developed secondary resistance have shown prior clinical response to BoNT. That is they have developed blocking antibodies to botulinum toxin after the medical treatment has started. This secondary resistance is responsible of losing the benefit of the treatment.

The difference between primary resistance and secondary resistance is while in primary resistance the patient shows immunoresistance from the very first injection, in secondary resistance the patients have no problem with the first injections and show clinical response. As a consequence of these first injections the body starts creating the antibodies to fight BoNT-A. Consequently subsequent injections are less efficient.

The clinical use of BoNT-A consist of the botulinum neurotoxin protein in combination with other non-toxic proteins. In addition to the toxin, the non-toxic proteins are also capable to generate antibodies production, thus creating an immunoresistance problem (DasGupta, 1994). More information about these proteins will be detailed in the section: Complexing proteins.

Different brands of BoNT claims to have products generating less immunoresistance. An introduction to the three main manufactures today of BoNT and their products is presented below.
Brands of BoNT-A

In this section we would like to explain the main differences between three BoNT-A drug brands: Xeomin®, Botox® and Dysport®. They all contain the same toxin, but vary in terms of manufacturing and protein synthesis.

To begin with, they all come from the same Clostridium botulinum bacteria strains, called the “Hall” strain (Frevert and Dressler, 2010). The difference among them is the size of the protein. Botox®, the most popular and known brand of all, contains all of the complexing proteins with a molecular weight of 900kDa. Dysport® also contains complexing proteins of variable sizes between 300kDa and 900kDa (Wenzel et al., 2007). The third brand, Xeomin®, is free from complexing proteins and contains only active neurotoxin, which is 150kDa (Roggenkamper et al., 2006). In Table 2 is shown the main properties of the preparations of all three BoNT-A brands. Some studies suggested that it is complexing proteins, also called neurotoxin-associated proteins that may lead to the formation of neutralizing antibodies (Park et al., 2011). However one of the biggest problems of using Botox® and Dysport® is immunoresistance. More about complexing protein will be written in the following section.

Table 2. Properties of different botulinum toxin preparations (Juwan, et al., 2011).

<table>
<thead>
<tr>
<th>Brand name</th>
<th>Botox®</th>
<th>Dysport®</th>
<th>Xeomin®</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generic name</td>
<td>OnabotulinumtoxinA</td>
<td>AbobotulinumtoxinA</td>
<td>IncobotulinumtoxinA</td>
</tr>
<tr>
<td>Manufacturer</td>
<td>Allergan Inc (USA)</td>
<td>Ipsen Ltd (UK)</td>
<td>Merz Pharmaceuticals GmbH (Germany)</td>
</tr>
<tr>
<td>Serotype</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Complex size (kDa)</td>
<td>900</td>
<td>300-900</td>
<td>150</td>
</tr>
<tr>
<td>Complexing proteins</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Specific neurotoxin activity (U/ng)</td>
<td>137</td>
<td>154</td>
<td>227</td>
</tr>
</tbody>
</table>
Factors causing immunoresistance

Complexing proteins

The commercial product of BoNT-A is the result of a sophisticate production process. This process is based on the manufacture of the botulinum toxin from the strains of the Clostridium botulinum bacteria, and the posteriori preparation of the toxin before it can be used as a commercial product. Different excipients might be added to the toxin in order to, for example, extend the shelf life (stability) of the product. During this process possible impurities can be obtained (The Management, Design and Operation of Microbiological Containment Laboratories, 2001). Furthermore non-toxic proteins are also formed and attached to BoNT-A. These proteins are known as complexing proteins and their function is not completely understood. Figure 3 shows all the preparation processes.

There are several suggestions about what could be the possible roles of the complexing proteins (Park et al., 2011). At the very first it was proposed that they protect the botulinum toxin in the gastrointestinal tract from gastric pH extremes (Ohishi, 1977). However, in the medical applications, when toxin is injected in vitro, complexing proteins do not have any function and therefore are not necessary. Additionally, it was also hypothesized that they might play a role in limiting the diffusion of the toxin away from the injection area or they might enhance the stability of botulinum toxin (Aoki et al., 2006). Neither of this was confirmed. Several experiments were performed and no significant difference in diffusion or in stability of the toxin were seen between only neurotoxin (150kDa) and toxin complex (900kDa) (Frevert and Dressler, 2010).

Although it seems that complexing proteins do not have a function in limiting diffusion or the stability of BoNT-A, it is conceivable that they are involved in the production of blocking antibodies. It was noticed that after the original Botox® formulation was improved and contained fewer of complexing proteins, it reduced the risk of antibody formation by 6 times (Park et al., 2011). Complexing proteins contain hemagglutinin (HA), which are lectins. It is known that lectin plays an important role in the immune system (Park et al., 2011). In Figure 3 you can see the content of BoNT preparations. The procedure can be divided in three parts. In the
first one you separate botulinum toxin from the excipients (inactive substances of the medication), whereas at the next step you can separate BoNT from the complexing properties, that contains HA, known for its immunogenicity properties (Park et al., 2011).

Figure 3. Composition of botulinum toxin preparation. Note: HA: Hemagglutinin; NHA, Non-Hemagglutinin (Park et al. 2011).

In a clinical trial program in US, 12 of 1080 patients treated with botulin toxin without complexing proteins (brand known as Xeomin®) developed blocking antibodies against the toxin. However, it is important to note, that all of these patients was treated earlier with botulin toxin with complexing proteins. It is highly possible that they have been already primed from previous treatment (Frevert and Dressler, 2010). In table 4 you can see the percentage of patients showing immunoresistance for each brand of BoNT-A in cervical dystonia.

The immunoresistance for Xeomin is not stated, because it has been approved to treat cervical dystonia not a long time ago (2005 in Europa and 2010 in USA) and there are not any appropriate statistical studies available. However, immunogenicity of Xeomin was compared with Botox and Dysport in New Zealand white rabbits. Interestingly, the results showed no antibodies formation with Xeomin, contrary to Botox and Dysport that induced the formation of neutralizing antibodies. (Blümel et al., 2006)
Table 4. The percentage of people who get immunoresistance after treatment for cervical dystonia.

<table>
<thead>
<tr>
<th>Brand name</th>
<th>Botox®</th>
<th>Botox® old</th>
<th>Dysport®</th>
<th>Xeomin®</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoresistance</td>
<td>1.2 %</td>
<td>9.5%</td>
<td>3%</td>
<td>Not stated</td>
</tr>
</tbody>
</table>

**BoNT-A biological activity.**

An ELISA sandwich (see Elisa appendix) procedure was performed using the extracted antibodies from rabbit and guinea pigs treated with *C. botulinum* type A. The purpose of this procedure was to conclude the concentration of the 150kD neurotoxin in Botox, Dysport and Xeomin (Frevert, 2010) and to determine the highest specific neurotoxin activity, which serves as a parameter for the immunological quality of a BoNT drug (Troung et al., 2009). In Xeomin the highest specific activity was 227 units/ng, Dysport had 154 units/ng and Botox with the lowest biologic activity of 137 units/ng. (See table 2)

The outcome of the experiment had shown that 100 units of Botox contain a concentration of 0.73 ng, Dysport - 0.65 ng and Xeomin contain 0.44 ng of BoNT type A (Pagan and Harrison, 2012) (See Table 5).

Table 5. The concentration of 100 U of toxin after the ELISA sandwich experiment in the Botox, Dysport and Xeomin

<table>
<thead>
<tr>
<th>Brand name</th>
<th>Botox®</th>
<th>Dysport®</th>
<th>Xeomin®</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>0.73 ng</td>
<td>0.65 ng</td>
<td>0.44 ng</td>
</tr>
</tbody>
</table>

In Frevert's paper (Frevert, 2010) the authors proposal was that 0.44 ng neurotoxin in 100 units Xeomin is as potent as the 0.73 ng neurotoxin in 100 units Botox. The result suggests that Xeomin contains only active neurotoxin, whereas Botox is likely to contain denatured neurotoxin (Frevert, 2010).

The presence of remaining denatured/inactive neurotoxin in Botox or Dysport can be associated with the manifestation of neurotoxin neutralizing antibodies in some patients. Another condition of supporting the previous statement is by looking at the amount of neurotoxin in Botox, which is estimated from the proportion of neurotoxin in the complex: the 150 kD neurotoxin found in Xeomin should be one-sixth of the 900 kD complex. Thus 5ng (reported by Botox in the medical
prescription) would be $5ng/6 = 0.83 ng$, which is closely to the 0.73 ng proved in Dr. Freverts analysis.

3. Discussion

Nowadays BoNT-A is used more and more in medical applications related to the nervous system and movement disorders. BoNT-A is not the cure of the disease but is helping in minimizing the symptoms. By this the life of the patient is better and the quality of life improved. Unfortunately BoNT-A has a limited time effect in the body; therefore the treatment should be repeated periodically (Jimenez-Shahed, 2011). Human immune system plays a role here because it considers BoNT-A as a pathogen and therefore starts the natural occurring defense reaction through blocking agents and antibodies. The function of the last ones is to neutralize and eliminate the toxin presence in our body and this is indeed a challenge in the medical field.

Body immunoresistance against BoNT-A product is the one of the main reasons of treatment failure. When a patient develops blocking antibodies the effect of BoNT-A is minimized, and finally eliminated. In order to minimize the immunoresistance effect manufacturers work with the BoNT-A protein composition and protein synthesis. The higher the purity of BoNT-A and the lower the amount of complexing proteins the lower the immunoresistance risk. It has been suggested that complexing proteins also known as non-toxic proteins that are attached to the toxic BoNT-A protein, are responsible for the immunoresistance response (Park et al., 2011).

At the same time that BoNT-A medical use is increasing the concern about immunoresistance is also increasing. Different commercial products claim different degrees of tolerance by the immune system. The new brands work in the synthesis of pure active toxin with no complexing proteins. That is the case of Merz Pharmatheutical, with Xeomin product. Merz claims (Frevert, 2010) that due to the purity of its product the immunoresistance response to the treatment is minimized.

What seems to be clear is that the purity of the BoNT-A proteins plays an essential role in the immunoresistance response. Other commercial brand, Botox have
reformulated their product in order to make it more tolerant. That is by modifying the composition of the protein by removing the complexing proteins in order to minimize the risk of immunoresistance.

The different producers of BoNT-A give no standardized information about biological activity. That means there is no standard and common method for the measurement of the biological activity of the different products. This makes it difficult to find a possible and objective scientific study since a lot of the available information is directly coming from the private industry or from public scientific studies funded by them. Results published by the industry must be taken with cautiousness even though there is some scientific reasoning behind them. It is clear that the private industry is aware that the present formulations must be improved in order to minimize the immunoresistance response. Old brands like Botox and Dysport change their product formulations to make them more efficient and less immunoresistant. A new BoNT-A brand, Xeomin, claims that their formula do not have any immunogenicity. Unfortunately, it has been approved for the treatment of cervical dystonia only a few years ago in USA and therefore there are no strong statistical studies that clearly support this statement. For some patients it takes several years to develop immune response.

4. Conclusions

It has been demonstrated that BoNT-A application in medical treatment for cervical dystonia improves the symptoms and increase the patient’s quality of life. This treatment has a strong drawback: toxin immunoresistance response in the human body. From our study we can conclude that the immunoresistance is generally related to the purity of the toxin protein and with the complexing proteins present in the product. Although the highly pure BoNT-A reduces immunoresistance, it has not been proved that immunoresistance can be completely avoided. Further work must be done in the direction of minimizing immunoresponse in the medical use of BoNT-A.
5.1 Acknowledgment

We are grateful for all the effort and continuous support from our supervisor Jette Rank. Without her comments and suggestions this work would not be as enjoyable as it has been.
5. References


Dong, M., Yeh, F., Tepp, W., H., Dean, C., Johnson E., A., Janz, R., Chapman, E., R., 2006. SV2 Is the Protein Receptor for Botulinum Neurotoxin A. Science 312, 5773, 592-596


7. Appendix

Appendix 1. Toxicon publication: Instructions for authors.

Language

Paper should be written in good English (American or British usage is accepted, but not a mixture if these).

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).
Tables

Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

Reference style

Text: All citations in the text should refer to:

1. **Single author:** the author's name (without initials, unless there is ambiguity) and the year of publication.
2. **Two authors:** both authors' names and the year of publication.
3. **Three or more authors:** first author's name followed by 'et al.' and the year of publication.

**Example:**

Reference to the journal publication:

Appendix 2. Neurotransmitters

Neurotransmitters are molecules secreted by the neurons at synapses that diffuse a very short distance to bind to receptors on the target cells. In figure 5 this process is shown. Acetylcholine is an important neurotransmitter (see figure 4) of the nervous system functions. Muscle stimulation, memory formation and learning are processes where acetylcholine is present (Vanhoutte et al., 1986).

Acetylcholine is found in the neuromuscular junction of vertebrates. BoNT disrupt the neurotransmission of acetylcholine. Intoxication of BoNT to humans can be fatal because muscle required for breathing fail to contract when acetylcholine release is blocked.

Figure 4. Chemical structure of acetylcholine.

Figure 5. A neuron synapse with the synaptic vesicle containing acetylcholine. Since BoNT is blocking the release of the acetylcholine, cell signaling is stopped.
Appendix 3. SNARE proteins

Chemical synaptic transmission or neurotransmission is essential for cell to cell communication. In the living cells, membrane fusion is mediated via a specialized set of proteins present in opposing bilayers. These proteins are called SNARE proteins. They are responsible for the neurotransmitter acetylcholine release from the nerve terminal to the synaptic cleft.

There are two kinds of SNARE proteins: v-SNARE (vesicle-SNARE) that is located on the vesicle membrane (Sollner et al., 1993b) and t-SNARE (target-SNARE) that is located on the target membrane. SNARE complex contains three proteins: VAMP (vesicle-associated membrane protein) syntaxin 1, and SNAP-25 (synaptosomal-associated protein of 25 kDa). VAMP (Trimble et al., 1988) is located on the vesicle and therefore is v-SNARE and syntaxin (Bennett et al., 1992) with SNAP-25 (Oyler et al., 1989) are anchored on the membrane and are classified as t-SNARE. They all form the crystal structure of the neuronal SNARE core complex. It has been shown that the complex contains a coil of syntaxin, a coil of VAMP, and two coils of SNAP-25: SNAP-25 amino-terminal helix, and SNAP-25 carboxy-terminal helix (Sutton et al., 1998). In the figure 6 can be seen the structure of SNARE complex. As it was mentioned before VAMP protein is anchored to the vesicle membrane and syntaxin and SNAP-25 is attached to the plasma membrane.
Figure 6. The complex of the SNARE proteins. Complex contains four helices: blue - VAMP, red-syntxin, green-SNAP-25 amino-terminal helic and SNAP-25 carboxy - terminal helix. VAMP protein is integrated on the vesicle membrane and syntxin with SNAP-25 helices are located on the plasma membrane.

The ability of botulinum and tetanus neurotoxins to inhibit neurotransmission was used as a tool to study SNARE proteins and their mode of action. Botulinum and tetanus toxins cleave the one of the protein of the SNARE complex that leads to SNARE inactivation that prohibits the fusion of the cell membrane and vesicle. (Pellizzari et al., 1999).

The understanding of synaptic vesicle membrane fusion requires a lot of biochemical and structural knowledge about SNARE proteins. It is not our intention to explain in details how neurotransmitters are released from the nerve terminal to the synaptic cleft. Each step of the model pathway explained below involves the action of additional regulatory factors that will not be mentioned. During the fusion two SNAREs: v-SNARE and t-SNARE bind together and forms a bundle. As it can be seen in the figure 7, initially all the proteins from the SNARE complex are unbound to each other. When the vesicle arrives, the nucleation of the ternary complex starts. The change in the calcium concentration brings v-SNARE and t-SNARE together,
called zippering. In this process v-SNARE get in to the contact with SNAP-25 and syntaxin and form a coiled bundle what brings the vesicle and plasma membrane into the contact. Zipping disrupts the lipid bilayer structure that causes hemifusion. Hemifusion is followed by complete fusion that opens fusion pores. When the pores widens neurotransmitters are released from the vesicle to the synaptic cleft.  (Lin and Scheller, 2000, Nelson and Cox, 2008)

**Figure 7.** The function mechanism of SNARE complex in neurotransmitter release. In the relax mode v-SNARE is unbound to t-SNARE. The nucleation brings both SNAREs into the contact. The increase of calcium induces zippering that causes tension on lipid bilayer. In the next step hemifusion starts followed by complete fusion that opens pore and neurotransmitters can leave the vesicle (Lin and Scheller, 2000, Nelson and Cox, 2008).
Appendix 4. Immune system

The immune system is a complex mechanism, which has the ability to respond to substances called antigens that are recognized as foreign attackers for our body (Guyton and Hall, 2005). The consistence of immune system is of several types of cells and proteins that differentiate each other by the activity in fighting foreign invaders.

The first part of the immune system that encounters attackers such as bacteria is a group of proteins that forms the basis for a particular system called the innate immunity (Litman et al., 2005). These proteins are found freely in the blood and can rapidly reach the site of an invasion where they can react directly with antigens. This process of developed immunity is the basis of vaccination. The innate system is found in all plants and animals (Janeway et al., 2001). If the invaders successfully escape the innate response, a second immune system called adaptive immune system is activated by the innate immunity and acts as a barrier to the pathogen (Mayer, 2006). The immune system adjusts its response during an infection to improve the detection of the pathogen. This improved response is then remembered after the pathogen has been eliminated, in the form of an immunological memory.

The immunological memory prepares the adaptive immune system to act faster and stronger each time this pathogen is present. The adaptive system is retained only by the vertebrates (Mayer, 2006). The innate immunity contains white blood cells called leukocytes. There are five different types of leukocytes (phagocytes, mast cells, eosinophils, basophils, and natural killer cells) which derivate from a multipotent cell in the bone marrow (Alberts et al. 2002) (See figure 8). The leukocytes have many things in common, but they are all distinct in form and function inside the immune system by identifying and eliminating pathogens that cause infection (Janeway et al., 2001). The immune response occurs when bacteria or any other cause, damages tissues in our body. The damaged cells release chemicals like histamine that generate the blood vessels to leak fluid into the tissues, causing swelling. The chemicals attract the white blood cells called phagocytes that "eat" microorganisms and damaged cells. (Firestein, 2007).
Mast cells live in connection tissues and mucous membranes and their role is to regulate the inflammatory response (Krishnaswamy et al., 2006).

Eosinophils and basophils are phagocytes that travel throughout the body in hunting invaders pathogens. They secrete chemical mediators that are involved in defending against parasites and play a role in allergic reactions (Kariyawasam and Robinson, 2006). Natural killers cells role is to attack and destroy tumor cells or cells that have been infected by viruses (Middleton et al., 2002).

The adaptive immune system contain special type of leukocytes cells called lymphocytes which, like all blood cells, originate from multipotent cells in the bone marrow (Alberts et al., 2002). Lymphocytes that migrate from the bone marrow to the thymus, mature into T cells while lymphocytes that mature in the bone marrow develop as B cells (Campbell and Reece, 2007), (See Fig. 6). T cells attack antigens directly and help control the immune response. They also release chemicals, known as cytokines, which coordinate the overall immune response (Goronzy and Weyand, 2007). B cells are best known for making antibodies that will bind to an antigen and marks the antigen for destruction by other immune system cells (Campbell and Reece, 2007).

**Figure 8.** The Human immune system composition in the blood and lymph
Appendix 5. ELISA

Elisa sandwich overview:

Enzyme-linked immunosorbent assay (ELISAs) is a biochemical technique used in determining and measuring the quantity of substances such as peptides, proteins, antibodies and hormones.

There are two types of the ELISA technique, depending on the purpose of the experiment:
The first technique is called Direct ELISA and the purpose is to detect the presence of a particular antigen in a sample. The second technique, called Indirect ELISA is to determine the presence of a specific antibody in the sample. The techniques mentioned in this project is for determining the concentration of the 150kD neurotoxin and is so called sandwich ELISA techniques. The principle of this procedure is to measure the amount of antigen (e.g. the neurotoxin) between two layers of antibodies.
The method involves one antibody that is bound to a 96-plate well. The antigen is added and bound to the antibody. Unbound products are then wash away and a labeled secondary antibody also called the detection antibody is added, therefore completing the “sandwich”. The assay is quantified by measuring the amount of labeled secondary antibody through the use of a colorimetric substrate (Overview of ELISA, 2012).