The Application of Botulinum Toxin in Oral and Maxillofacial Surgery

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Edited by

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Preface

Botulinum toxins are proteins which are highly effective, naturally occurring neurotoxin, produced as a metabolic product of the gram-positive bacteria, Clostridium botulinum. Botulinum toxin, once a food poison, was later exploited as a biological weapon, and is currently one of the most versatile pharmaceuticals for the treatment of human diseases in ophthalmology, neurology, dermatology and oral and maxillofacial surgery.

Application of this toxin drug in the field of oral and maxillofacial surgery for both aesthetic and functional indications has been extensively reported. However, a collective documentation of its varied applications is lacking. This book is a compilation of the use of botulinum toxins in head and neck surgery and medicine. The book also covers the basic aspects of the drug, including its history, pharmacology and preparation. Dermatological applications of the drug for cosmetology, as well as its various therapeutic uses in temporomandibular joint surgery, masseter muscle hypertrophy, bruxism, trismus, maxillofacial traumatology, salivary gland diseases, facial palsy, trigeminal neuralgia and gummy smiles have been described with numerous clinical and diagrammatic illustrations. The book will find relevance with head and neck surgeons, oral maxillofacial surgeons, ENT surgeons, and dermatologists in particular and also general surgeons and dentists as well.

Chapter 1

Introduction: Know the Toxin

Kumar Nilesh, Monica Patil

The history of medicine indicates unexpected coincidences leading to the development of treatment options. One of these serendipities was the discovery of the clinical application of botulinum toxin. Botulinum toxin, once a food poison, was later exploited as a biological weapon during World War II. Currently, it is one of the most versatile pharmaceutical agents used for the treatment of human diseases in ophthalmology, neurology, dermatology, dentistry, and oral and maxillofacial surgery. Botulinum toxin is a protein, which is a highly effective naturally occurring neurotoxin. It is produced as an ametabolic by-product of the gram-positive, obligate anaerobic, rod-shaped bacteria *Clostridium botulinum* (Figure 1A).

Botulinum toxin is a two-chain polypeptide. It consists of a light chain and a heavy chain, joined by a di-sulphide bond. Botulinum toxin formed from Clostridium botulinum is initially a single chain. The biologically active form of botulinum toxin is formed after the enzymatic splitting (nicking) of the progenitor toxin molecule by the process of proteolysis. This results in the formation of the active two-chain form of botulinum toxin (Figure 1B). The heavy chain provides specificity of the botulinum toxin molecule to the cholinergic receptors. It brings about the binding of the toxin molecule at the pre-synaptic cholinergic receptors, preventing the action of acetyl cholinesterase in impulse transmission across the synaptic junction.

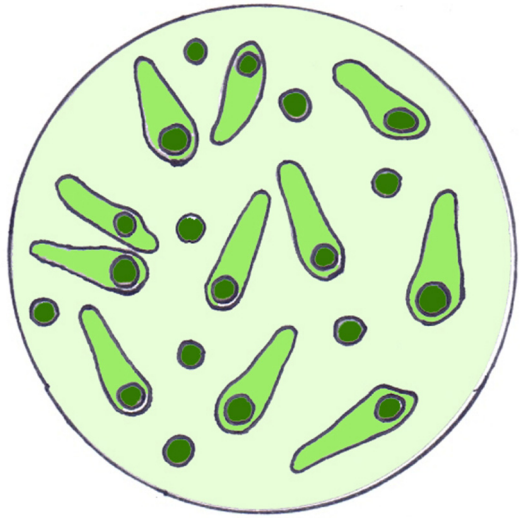

Figure 1A: Microscopic appearance of gram-positive, rod-shaped bacteria Clostridium botulinum

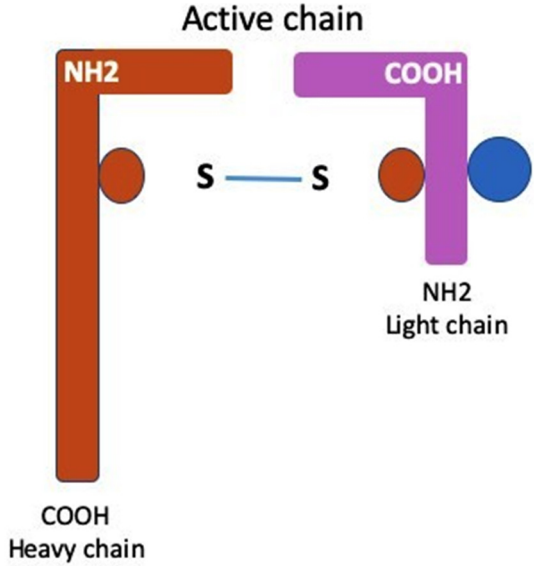

Figure 1B: Line diagram showing structure of botulinum toxin

Botulinum toxin exists in seven distinct serological forms: A, B, C (C_1, C_2), D, E, F and G. Although all the types are structurally similar, they vary serologically and antigenically. The various serotypes differ in their duration of effect and potency. Type A is the most potent neurotoxin, followed by types B and F.

Type B was probably the first botulinum toxin serotype discovered, when investigation of an outbreak of food poisoning called *botulism* in Belgium, in 1895, revealed that the causative agent was a neuroparalytic toxin produced by an anaerobic bacterium.[1] Several years later a similar incident occurred in Germany, and the bacterium was isolated. Although the bacteria that produced the two toxins were similar, as were their paralytic effects, the antisera against either toxin were not protective against the other, indicating that the two toxins were immunologically distinct. It is believed that the toxin identified in Germany was type A.[2] Type A neurotoxin is the most effective and has the longest duration of action. It is the main serotype in therapeutic use, both for aesthetic and therapeutic indications.

Botulinum toxin acts by inhibiting the exocytotic release of acetylcholine from the motor nerve terminals. Because of this property, it has been found useful in the treatment of many pathological conditions that involve excessive muscle contractions. Botulinum toxin was first developed as a therapeutic agent for the treatment of disorders that cause localized muscle hyperactivity, especially around the eyes. Ophthalmologists and neurologists were quick to appreciate the fact that botulinum toxin treatment improved the disfigurement, discomfort and disability that are associated with facial dystonias. Further investigations on botulinum toxin revealed significant benefit in pain control resulting in improvement in muscle contractions which was not associated with its neuromuscular effects. This suggested that the toxin might have an effect on pain which is independent of its neuromuscular actions.[4] Similarly, in the late 1990s, the effects of botulinum toxin were examined on the hyper-functional lines of the face.[5] Cosmetic surgeons who were using botulinum toxin for aesthetic purposes were the first to notice that in certain patients, the facial injections of the toxin also provided relief from tension and migraine headaches, making it one of the most exciting areas of botulinum toxin research.[6] In clinical practice, botulinum toxin has an excellent safety profile when used in minute quantities by experienced clinicians.

Over the past few decades physicians have constantly discovered new applications for this drug and new ways to refine its use. It has been used in various fields of both medicine and surgery. This book describes the therapeutic as well as cosmetic applications of botulinum toxin in general, with specific focus on the fields of oral and maxillofacial surgery, facial aesthetics, dermatology, and dentistry.

Learning points:
▪ Botulinum toxin are proteins which are highly effective, naturally occurring neurotoxin, produced as a metabolic product of the gram-positive, obligate anaerobic, rod-shaped bacteria, Clostridium botulinum.
▪ It is a two-chain polypeptide consisting of a light chain and a heavy chain, joined by a di-sulphide bond. Initially as single chain (precursor toxin), the final active form of the protein is a complex makeup of the two-chain neurotoxin.
▪ Botulinum toxin exists in seven distinct serological forms (A to G). These serotypes differ in their duration of effect and potency. Type A is the most potent with the longest duration of action and is most widely used for cosmetic and therapeutic purposes.
▪ Botulinum toxin, first developed as a therapeutic agent for the treatment of disorders that cause localized muscle hyperactivity, later showed significant benefit on pain and on hyper-functional lines. It finds both therapeutic and cosmetic use in the fields of oral, dental and maxillofacial surgery.

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Chapter 2

History of Botulinum Toxin: From Bane to Boon

Monica Patil, Kumar Nilesh

Since the beginning of time, humankind has been affected by several food-borne ailments. Since the time humans started preserving and storing food, it has created an optimal condition for the presence and growth of Clostridium botulinum (for example, in France ham was stored in the barrels of brine, in the Baltic poorly dried herring, in Scandinavia trout was packed in willow baskets to ferment, non-salted sturgeon roe were piled in a heap on old horsehides, lightly smoked fish in poorly heated smoking chambers, and liver sausages swung from the rafters of Austrian huts).[1] In ancient times, correlation between food consumption and subsequent death from a paralytic disease was not realized. Therefore, there are very few sources and documents available on food poisoning before the 19th century. However, some knowledge from ancient dietary laws and taboos considered the consumption of poisoned food as life-threatening. Louis Smith, in the year 1977 reported one such example of dietary taboo that was reported in his textbook on Botulism.[2] At first, this food poisoning was suspected to be atropine intoxication. However, in the old medical literature, some reported cases of intoxication with Atropa belladonna were in fact said to be cases of food-borne botulism. This was because the combination of dilated pupils and fatal muscle paralysis could not be attributed to atropine intoxication.[3]

The accurate clinical symptoms of food-borne botulism were described and published by a German physician, Justus Kerner, between the years 1817 and 1822. The idea of using botulinum toxin for therapeutic measures was also developed by him. He also described and categorized this empirical phenomena and started clinically experimenting on animals and on himself and succeeded in developing the hypotheses on the toxin's pathophysiology,

suggesting preventive measures and the treatment of botulism. Along with this, Kerner also developed the vision and ideas of future perspectives and therapeutic uses of the toxin which have been validated over the last 20 years.

1792–1815 (Southern Germany)

During the Napoleonic wars, general economic poverty was experienced in the rural areas of central Europe because of the devastating warfare by the French, which resulted in a decline in the hygiene of everyday life, including food production. In the early 19th century, an increasing number of lethal food poisoning cases were registered by the medical administration of the Dukedom, followed by a notice that was published by the government of Stuttgart to alert the population on the harmful consumption of smoked blood sausages.

In 1811, the medical section of the Internal Affairs Department of the government of Stuttgart suspected the probable cause of sausage poisoning to be prussic acid. Later, with the involvement of the medical faculty of the University of Tü bingen, it was stated by the dean (Professor Wilhelm Gottfried von Ploucqet) that prussic acid could be a zoonic, probably organic poison. Systemic studies were then initiated on this issue by the government of Württemberg, after a medical professor (Johann Heinrich Ferdinand Authenrieth) of the University of Tü bingen suggested that further outbreaks of food poisoning should be very well recorded. He also held the local housewives responsible for inadequately boiling sausages during food preparation, resulting in them rotting from the inside.

In 1815, seven cases of intoxication were reported after liver sausage and pea ingestion. The autopsy findings of the three victims in this incident were described by a health officer (J.G. Steinbuch) in Herrenberg, Stuttgart. Another case of food poisoning was reported in the same year by a physician (Justinus Kerner), that revealed the probable cause to be various kinds of sausages. After these reports, in 1817 Professor Authenrieth decided to publish them. Subsequently, Kerner observed 76 cases, which he published in his first monograph, entitled “Neue Beobachtungen über die in Württemberg so häufig vorfallendentö dlichen Vergiftungen durch den Genuss geräucherter Würste” (“New observations on lethal poisoning occurring so frequently in Wurttemberg through the consumption of smoked sausages”). He

stated that the toxin interrupts chemical processes of nervous transmission, in the same way as rust stops conduction of electricity.[4] He then received a grant from the government of Stuttgart that helped him to escalate his research and start an animal study in 1821. He started his study by extracting the lethal substances from sausages and similar products and calling it *sausage poison*. He used this poison by mixing it with honey and then fed it to various animals, such as cats, rabbits, birds, frogs, snails, and insects such as locusts and flies. The observed clinical symptoms, particularly in cats, were very much alike those occurring in humans. As there were no observations showing disturbances in consciousness, he concluded that the poison did not affect the brain and described it as only having muscular and autonomic symptoms. The action of the toxin was observed to be in the peripheral sympathetic and parasympathetic nerves and its signal interruption, without any sensory disturbances. However, poisoning also showed lethal outcomes in autopsies, such as secondary respiratory and cardiac failure. Kerner also dauntlessly experimented on himself by ingesting a few drops of this sour poison, after which he experienced mild symptoms of sausage poisoning, such as the drying out of his tongue, palate, and pharynx in a short period of time. In 1822, he published his second monograph entitled "Das Fettgiftoder die Fettsa¨ureundihre Wirkungen auf den thierischen Organismus, ein Beytragzur Untersuchung des in verdorbenen Wu¨rstengiftigwirkenden Stoffes." (Fat poison or fatty acid and its effects on the animal organism: A contribution to the examination of poisonous substances from bad sausages), reporting the clinical evaluation of no less than 155 cases, including human autopsies, animal experiments, and all the knowledge he had gathered so far. He mentioned very interesting clinical details: "the tear fluid disappears, the gullet becomes a dead and motionless tube; in all the mucous cavities of the human machine the secretions of the normal mucus stand still, from the biggest, the stomach, towards the tear canal and the excretory ducts of the lingual glands. No saliva is secreted. No drop of wetness is felt in the mouth, no tear is secreted anymore".

The main clinical symptoms that he described were: vomiting, intestinal spasms, mydriasis, ptosis, dysphagia, and finally, respiratory failure.[5] After comparing various sausage recipes, Kerner found the common ingredients to be fat and salt. However, salt was supposed to be known as being innocent, hence, it was

assumed that the toxic changes were because of the fat, after which the denomination for the toxin; *"fat poison"* was established. This 'new poison' was concluded to be of zoonic origin, as it developed under anaerobic conditions in rotten sausages, after comparing its mode of action with the already known poisonous substances, such as atropine, scopolamine and snake venom. It was also concluded that its action was on the nervous system and that it was lethal even in small doses. Kerner repeatedly suggested storing the sausages in dry conditions to prevent further incidents of poisoning. In the final statements of his second monograph, Kerner discussed the toxin as a possible remedy for a variety of diseases, favoring a condition known as "Veitstanz" (St. Vitus dance, comparable with chorea Huntington and chorea minor) or the hyper-secretion of body fluids. More than 180 years ago, Kerner presumed that his statement on 'fatty acid' as a therapeutic agent belonged to the realm of hypothesis and may be confirmed or disproved by observations in the future.[1, 6, 7] In the 1870s, more than 50 years later, the term 'botulismus' was derived from the Latin word botulus for sausage.

1895 (Belgium)

Almost 80 years after Kerner's work, when 34 musicians of "Fanfare Les Amis Réunis" – a local brass band, relished on pickled and smoked ham at the funeral of 87-year-old Antoine Creteur in the Belgian village of Ellezelles, they all developed mydriasis and diplopia, and some of them also developed dysphagia and muscle paralysis, which led to three of them dying. The microbiologist Emile Pierre Van Ermengem, professor at the University of Ghent, formerly trained by the famous Robert Koch in Berlin, examined the ham and carried out autopsies and became the first to find the correlation of *'sausage poisoning'* with a bacterium found in the tissues of the contaminated victims and raw salted pork. In 1897, in his report on this anaerobic, spore-forming, toxin-producing bacillus, he named it Bacillus botulinus, as the term botulismus was already known at this time.[8] Subsequent investigations further discovered different serological subtypes. In the later years, van Ermengem's bacillus was renamed as Clostridium botulinum.[9]

World War II: botulinum toxin as a biological weapon

In the 1920s, a crude preparation of botulinum toxin was obtained by Herman Sommer, at the University of California by acidic preparation of a culture fluid. This was used by the US Academy of

Sciences at the beginning of World War II in a secret laboratory in Fort Detrick, Maryland. For this purpose, physicians and bacteriologists were deployed to investigate the bacteria and toxins that could be used. During this time, the United States Office of Strategic Services came up with a plan of using Chinese prostitutes as assassins for the Japanese high ranking army officers by smuggling lethal doses of botulinum toxin into their drinks or food in a pin-size gelatin capsule. These capsules were sent to Chunking, China, where they were again tested on stray donkeys before executing the program. However, the donkeys survived and the plan was abandoned. Later, it was found that donkeys could be one of the few species that were immune to botulinum toxin.[10] The first botulinum toxin for human use was produced by Edward Schantz, a scientist at Fort Detrick, in 1946. In 1972, the Biological and Toxic Weapons Convention was signed by Richard Nixon (US president) that terminated all research on biological agents used in warfare.

Medical research

After multiple unsuccessful attempts to treat strabismus, by injecting various substances into the hyperactive muscles, Alan Scott, an ophthalmologist at the Smith-Kettlewell Eye Research Institute, San Francisco, approached Edward Schantz for the botulinum toxin. In 1978, Scott received permission from the FDA to conduct a pilot study on human volunteers for the treatment of strabismus.[11] The FDA then approved the use of botulinum toxin type A, to a certain extent, in humans by 1979. Botulinum toxin effectively blocked involuntary muscle contractions, when it was injected into the larger muscles of the body. Later, in 1989, the FDA approved the use of botulinum toxin-A for hemi-facial spasms, strabismus and blepharospasms. In 1987, after injecting botulinum toxin for blepharospasm, Jean Carruthers, a Canadian ophthalmologist, observed that frown lines disappeared. She shared the same with her husband, Alastair Carruthers, who was a dermatologist. After this accidental observation, the Carruthers revolutionized aesthetic enhancement treatments by promoting botulinum toxin in cosmetic procedures and published their first report on botulinum toxin application for cosmetic purposes in 1996.[12] Since then, numerous new indications have been found after various experimental and clinical studies.

An Orphan Drug

In 1991, several batches of the botulinum toxin-A and all research findings concerning this "orphan drug" were bought by Allergan Inc., Irvine, who, as new parents, gave the substance the name Botox®.[13] Two years later, Porton Products Ltd, a British company, launched a slightly different formulation, and named it DYSPORT® (Ipsen Inc., Rockford, IL 61125-1266.), derived from the word dystonia.

In the recent past, various subtypes have been found, such as type A, B, C, D, E, F, and G, and a variety of different products containing botulinum toxin-A strains are on the market with numerous indications for the medical use of this drug. Derived from food poisoning 200 or more years ago, the toxin has made its way and, for many reasons, it is probably the most amazing substance that has been developed as a revolutionary pharmaceutical in the past decades.

Learning points:
▪ In 1821, after receiving a grant from the government of Stuttgart, Kerner started his study on sausage poisoning and observed the action of the toxin to be on the peripheral sympathetic and parasympathetic nerves and its signal interruption, without any sensory disturbances.
▪ In 1897, different serological subtypes were discovered after subsequent investigations.
▪ In the 1920s, botulinum toxin was used as a biological weapon by the US Academy of Sciences at the beginning of World War II.
▪ The first botulinum toxin for human use was produced in 1946 by Edward Schantz, a scientist at Fort Detrick.
▪ In 1978, Scott received permission from the FDA to conduct a pilot study on human volunteers for the treatment of strabismus
▪ Later on, in 1989, the FDA approved the use of botulinum toxin-A for hemi-facial spasms, strabismus and blepharospasms.
▪ The first report on botulinum toxin in cosmetic procedures was published in 1996 by Carruthers.

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Chapter 3

Pharmacology: Understanding the Drug

Monica Patil, Kumar Nilesh

Neuromuscular transmission

To know the mechanism of action of botulinum toxin, it is important to understand the steps involved in cholinergic neurotransmission, at the neuromuscular junction. Cholinergic neurotransmission involves six basic steps: the synthesis, storage, release, binding, degradation, and recycling of acetylcholine[1] (Chart 3A).

At the first step, choline is transported from the extracellular fluid into the presynaptic neuron's cytoplasm. This is brought by a carrier system that also co-transports sodium. In the cytoplasm of the neuron, choline reacts enzymatically with acetyl coenzyme A to form acetylcholine. The acetylcholine is aggregated into the synaptic vesicles, where it is stored in granules. When an impulse is initiated and transmitted across the neuron, the action potential ultimately arrives at the nerve ending (at the neuromuscular junction). The voltage-sensitive calcium channels in the presynaptic membrane open up, causing an increase in the intracellular calcium concentration. Elevated calcium levels promote the docking and subsequent fusion of the synaptic vesicles containing acetylcholine with the cell membrane. This is conducted via a complex mechanism involving protein isoforms, ultimately resulting in the release of acetylcholine. Acetylcholine subsequently diffuses across the synaptic junction and binds at the postsynaptic nicotinic receptors on the muscle fiber. This binding activates a second messenger system that results in the contraction of the muscle. Following this, acetylcholine is rapidly broken down into choline and acetate by the enzyme, acetylcholinesterase. Choline can be recycled by the high-affinity transport system that pulls the molecule back into the neuron.[2]

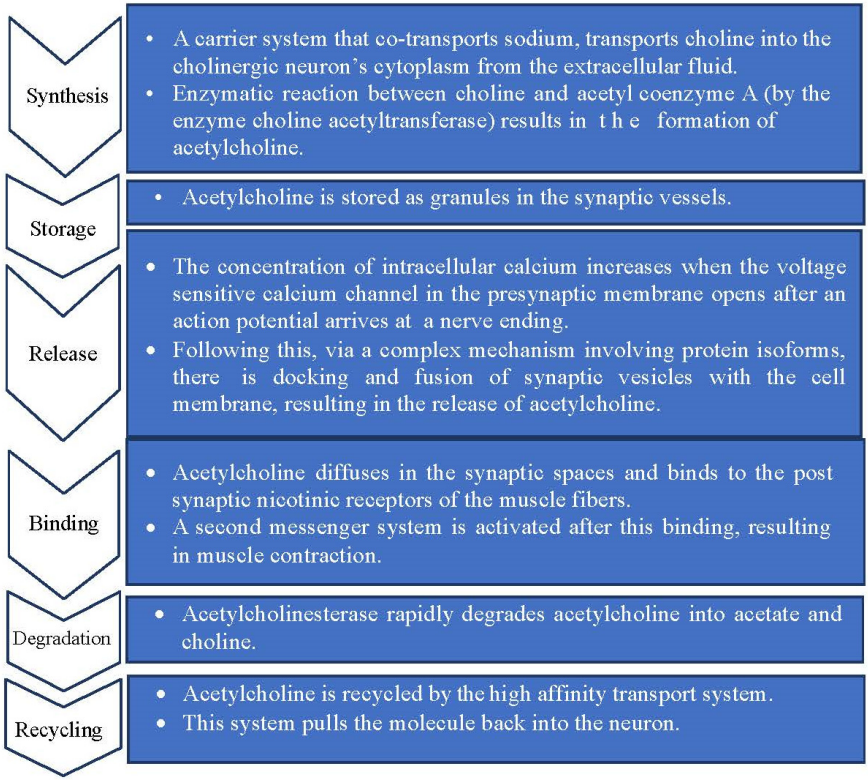

Chart 3A: Flowchart of neuromuscular transmission

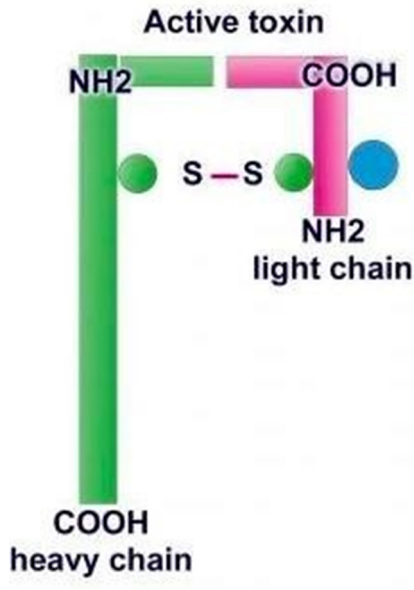

Figure 3A: Line-diagram showing structure of botulinum toxin.

Botulinum toxin structure

Botulinum neurotoxin exist as inactive polypeptides of 150 kilodalton (kd). These are split by trypsin like bacterial protease and generate the active double-chain form of the toxin. The proportion of single to double-chain varies according to the serotype of the toxin and whether the bacterial strain expresses the appropriate protease or not.[3] The 100-kd heavy (H) chain and the 50-kd light (L) chain are linked together by heat labile di-sulfide bonds and noncovalent forces.[4] Both the H and the L chains have carboxy (COOH) and amino-terminal (NH2) terminals (Figure 3A). The H and L chains dissociate with heat and boiling, which inactivates the toxin (because neurotoxicity requires both H and L chains).[5]

Molecular actions of botulinum toxin

All the serotypes of botulinum toxin act on the peripheral nervous system. It acts at the neuromuscular junction by inhibiting the release of acetylcholine from its presynaptic terminal. In very large doses, the toxin may show autonomic effects by binding to the

nerve terminals at the autonomic cholinergic ganglia. However, at therapeutic doses, the toxin is very unlikely to be associated with any significant adverse autonomic reactions[5] (Figure 3B). The neurotoxicity of botulinum toxin involves three steps:

1. Binding
2. Internalization
3. Neuromuscular blockage

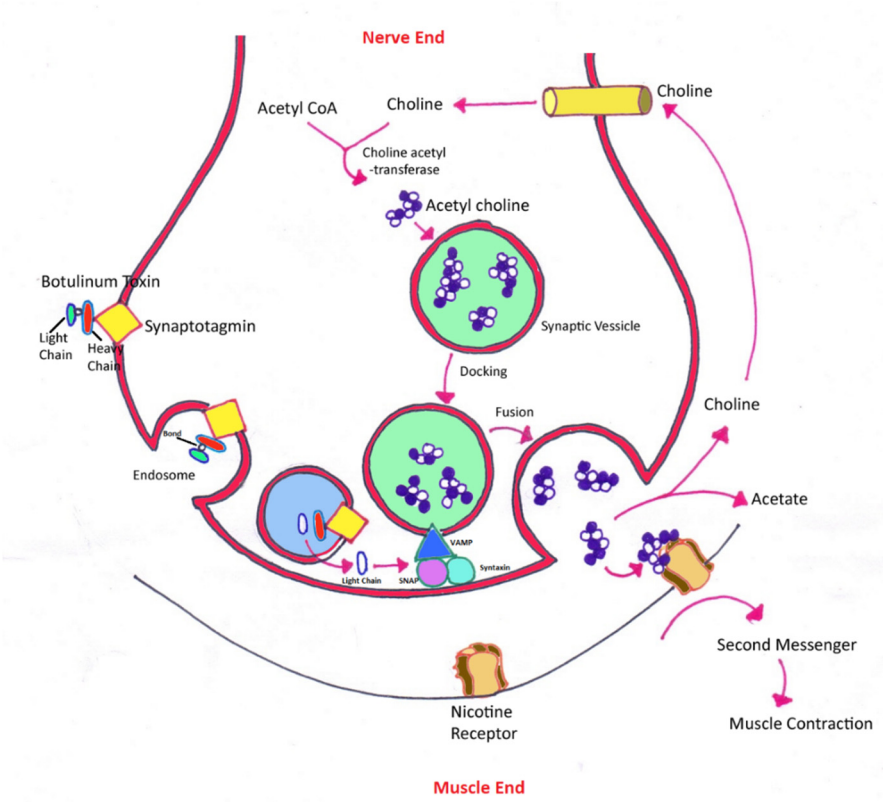

Figure 3B: Line-diagram showing acetyl choline release at the synaptic junction and mechanism of action of botulinum toxin

1. Binding

The first step involves the irreversible binding of the botulinum toxin to the presynaptic receptor. The binding happens with the help of the carboxy-terminal of botulinum toxin's heavy chain.[6-8] The exact binding site for the toxin has not been definitely identified. Previous studies suggested that distinct receptors exist for different botulinum toxin serotypes.[6] However, this view has been contradicted by the isolation of the synaptic vesicle protein, synaptotagmin. Synaptotagmin demonstrates a binding ability with botulinum toxin A, B, and E.[9]

2. Internalization

The second step involves the internalization of the botulinum neurotoxin. This occurs through receptor-mediated endocytosis. This process of internalization is partially dependent on the nerve stimulation. After internalization, the toxin molecule is present in the cytoplasmic endosomes. Subsequently, there is cleavage of the di-sulfide bond resulting in the separation of the heavy and light chains. The exact mechanism leading to this cleavage is unknown. There is a translocation of the L-chain from the endosome into the neuronal cytoplasm.[10]

3. Neuromuscular blockage

Within the nerve cytoplasm at the synaptic end, various large protein isoforms are present. These mainly include: syntaxin, vesicle associated membrane protein (VAMP) and synaptosomal associated protein (SNAP-25). These proteins form a complex platform which is necessary for the docking, fusion, and release of acetylcholine containing synaptic vesicles through the cell membrane.[11] Thus, the release of acetylcholine brings about the impulse transmission and muscle contraction.

The third and final step in the action of botulinum toxin involves neuromuscular blockage. The L-chain of botulinum toxin contains a highly specific zinc-endopeptidase at its amino-terminal which cleaves the protein complex with its proteolytic activity. Disintegration of the protein complex prevents the fusion and release of acetylcholine from the synaptic vesicles.[5] This results in a blockage of the impulse transmission and, subsequently, the muscle contraction is prevented.

Drug and disease interaction with botulinum toxin

Various drugs act on the neuromuscular junction and interfere with the action of botulinum toxin.

- **Aminoglycoside antibiotics** enhance the action of botulinum toxin. Aminoglycosides, such as kanamycin, gentamycin, and streptomycin in large doses can avert the release of acetylcholine from the nerve endings, producing a clinical syndrome similar to botulism. This effect is believed to be related to calcium channel blockage. Symptoms rapidly subside with the elimination of the offending drug from the body.

- **Aminoquinolines** act as antagonists to the effects of botulinum toxin by acting intracellularly, inhibiting the toxins lysosomal processing or acting on the cell membrane by inhibiting the toxin binding.

- **Cyclosporine** has also reportedly been shown to bring about neuromuscular blockage due to some unknown mechanism that is characterized by muscle weakness and ventilatory failure. However, the results may be due to the anti-inflammatory or immunosuppressive effects of cyclosporine on muscle or blockage of the presynaptic calcium channels.

- **D-Penicillamine**, in immunologically predisposed individuals, may trigger the formation of acetylcholine receptor antibodies, resulting in symptoms of myasthenia gravis. This can be seen in a small percentage of patients with rheumatoid arthritis. The symptoms and the antibodies diminish within a few months after drug cessation.

- In patients with **neuromuscular transmission disorders**, the use of botulinum toxin is contraindicated. In Lambert-Eaton myasthenic syndrome (LEMS), antibodies that are directed against the tumor antigens cross-react with the voltage-gated calcium channels that are involved in acetylcholine release. This leads to a disturbance of neuromuscular transmission. The antibody-induced internalization and acetylcholine receptor's degradation causes weakness in myasthenia gravis.

Assays and pharmacological actions

Muscle assay

There are several assays that explain the pharmacological action of botulinum toxin. The multistep hypothesis of botulinum toxin has been established by the mouse phrenic nerve diaphragm model.[12] Histologic analysis and toxin radio-labelling coupled together described the relationship among the toxins, motor end plates, and receptor sites. It demonstrated that the toxin binds more rapidly to the nerves of actively contracting muscles.[13]

The mouse hypoglossal nerve assay revealed that, unlike the toxicity caused to the neuron cell body by retrograde transport due to the doxorubicin, botulinum toxin does not induce motor-neuron death but causes a chemical denervation of the neuromuscular junction.[14, 15]

The denervation is reversible and was demonstrated by studies on muscle fibers from human blepharospasms. Acetylcholine esterase stain was used to assess the denervation of striated muscles after injecting botulinum toxin and it was observed that denervation is accompanied by spreading of the acetylcholine esterase activity to cover most of the exposed sarcolemma. After 4-5 months, the distribution of acetylcholine esterase activity returned back to its normal pattern.[16] Neurogenesis, with the formation of axonal sprouts within ten days and new motor end plates aids in recovery from denervation and the nerve terminals and muscle motor end plates are therefore reconnected.[17]

The muscle atrophy is also reversible and was demonstrated by the rabbit longissimus dorsi muscle assay. Within two weeks of injection with the toxin, the muscle atrophy was seen which continued for about four weeks before reversing.[18] Similarly, patients with orbicularis oculi muscles of blepharospasm treated with botulinum toxin showed reversible denervation atrophy.[16] About 3-6 months were required for clinical functional recovery of the muscles by which time the muscle returned to about 70-80% of its original bulk.[5] However, sprouting and remodeling may continue for up to three years.[19]

Bioassay

The mouse biologic assay is currently the only accepted quantitative method for the detection of Clostridium toxins in culture, serum, and food samples and for antitoxin standardization.[20, 21] It is also the most specific and sensitive measurement of botulinum toxin activity.

One mouse unit (MU) is defined as the median intraperitoneal dose required to kill 50% of a batch of 18 to 20 g of female Swiss-Webster mice (LD50) in over three to four days.[22-24] The original assay which was measured in monkeys, was found to be less narrow compared to the lethal dose effects in mice.[25] The LD50 of botulinum toxin-A for humans, extrapolated from the studies in monkeys, is estimated to be about 40 MU/kg.[26] For a normal human of 70 kg weight, the LD50 is found to be in the range of 2500–3000 MU. The presence of possible species variability in the botulinum toxin sensitivity prevents an accurate calculation of the toxic dose for humans.[5] Nevertheless, the safety margin for a therapeutic dose is quite wide. For example, in the treatment of glabellar frown lines, the required average number of units is about 30 units. However, the most significant drawback of the mouse bioassay is that it does not provide an accurate characterization of botulinum toxin potency in human subjects.[27] Clinical potency of the toxin in humans is also dependent on the targeted muscle, the toxin dose, and the volume.

Immunoassays

Compared to the mouse bioassay, assays that use monoclonal antibodies, like immunodiffusion and the hemagglutination assay are still not sensitive enough. Radio-immunoassay is also inapplicable for the sample's routine testing because of the requirement to radiolabel the toxin and it also requires a suitable radiologic facility.

However, the enzyme-linked immunoassay (ELISA) shows the greatest potential as a replacement for the bioassay, as the standard technique using polyclonal antibodies is reasonably rapid, highly specific, and can be applied to the testing of a large number of specimens.[20] The redox cycle amplification system, or an enzyme-linked coagulation amplification system, can further improve the sensitivity of the ELISA.[28] Nevertheless, the inability of all ELISA based assays to distinguish between active and inactive

forms of toxin remains the major disadvantage. A specific assay based on botulinum toxin endopeptidase activity that directly measures the biologic activity of the toxin is being investigated.[29-30]

Yet, the mouse bioassay may not be totally replaced by these in vitro assays, as they do not provide a measurement of other parameters that contribute to the overall toxicity and therapeutic potency, making the mouse bioassay the gold standard for toxin detection and standardization.

Immunogenicity

The toxin's immunological properties can cause stimulation of antibody production, that can potentially lead to ineffective treatments. The minimum dosage and injection schedule required to induce antibody formation are unknown. However, it has been shown that immunogenicity depends on the dose per session, the cumulative dose and the frequency of administration.

Biglan et al. published a study which revealed that there was an absence of antibody formation in patients who received less than 50 IU per session.[31] Gonnering published a study in which he reported antibody response in the patients of syndromic facial spasms who received doses in the range of 150-300 IU per session, but which was not seen in patients who received doses up to 52.5 IU for a period of three years.[32] Jankovic and Schwartz tested antibody production in cervical and oromandibular patients.[33] A statistically significant difference was seen in patients who received a cumulative dose of 1709 IU with antibodies and patients who received a cumulative dose of 1066 IU without antibodies. In a study on patients with torticollis, who were treated with doses ranging from 150-300 IU, these patients demonstrated a prevalence of neutralizing antibody in 4.3% of cases.[34] It was also noted that patients who were resistant to botulinum toxin required more frequent and booster injections, two to three weeks post-treatment. However, in patients with blepharospasm or patients treated for dermatologic problems, antibody formation has not been reported to date.

The mouse neutralization assay is the most widely used test. However, this test is time consuming and expensive. More rapid immunoassays have been developed to detect the antibodies. However, their lower specificity and lack of correlation between

clinical resistance and detected antibodies remains the major drawback. The combination of these two types of tests may give the most sensitive and specific results.

Using the smallest effective dose possible, treatment intervals of at least three months and avoidance of booster injections may minimize antibody resistance. Patients with botulinum toxin-A resistance may respond to botulinum toxin-B.

Adverse effects

The uncommon minor adverse effects of botulinum toxin injection are: hematoma, bruising, itching and pain at the injection site. All of these effects range from mild to moderate in intensity and recover after some time. Improper muscle injection can also result in transient ptosis, lip drooping, ectropion, and diplopia. Severe anaphylaxis, attributable to botulinum, have been reported after almost a decade of therapeutic application.[36] Local changes in muscle fiber size and electromyographic abnormalities may be the long-term complications of botulinum toxin. However, they are mild and do not have any clinical significance. There is no central nervous system effect of botulinum toxin, as it does not cross the blood brain barrier.[37]

Learning points:
▪ Cholinergic neurotransmission involves six steps: synthesis, storage, release, binding, degradation, and recycling of acetylcholine.
▪ The mechanism of neuromuscular transmission of botulinum toxin involves three steps; binding, internalization, and neuromuscular blockage.
▪ Botulinum toxin acts at the neuromuscular junction by inhibiting the release of acetylcholine from its presynaptic terminal.
▪ Aminoglycoside antibiotics enhance the action of botulinum toxin, whereas aminoquinolines act as antagonists to the effects of botulinum toxin.
▪ There are several assays that explain the pharmacological action of botulinum toxin including: muscle assay, bioassay, and immunoassay.