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The Effect of Two Different Botulinum Neurotoxin A On The Cortical Neuron Cells In Terms of Apoptosis and MMP 2, MMP 7, and MMP9 Localizations

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ABSTRACT

This study aimed to associate the possible cytotoxic and apoptotic effects of Botox (Allergan) and Dysport (Ipsen) and immunolocalization of matrix metalloproteinase (MMP) proteins with HCN2 cortical neuron cell line. Accordingly, cytotoxic potentials of Botox and Dysport were determined on different concentrations. Then, the apoptosis rates of these cells were evaluated by TUNEL method. MMP2, MMP7, and MMP9 proteins were also visualized using immunofluorescence method. There was a significant difference in cytotoxicity between those treated with 3.2, 6.4, and 12.8IU compared with the control. The Dysport 12.8 IU group was statistically more toxic than Botox group at the same concentration. Therefore, the number of apoptotic cells increased from 0.1 IU in Botox and 0.01 IU in Dysport groups compared to the control. The number of apoptotic cells was significantly higher in Dysport group at 1.6, 3.2, 6.4, and 12.8 IU concentrations than in Botox group. It was determined that MMPs increased gradually at the concentrations where the number of apoptotic cells was highest compared to the control group. As a result, we consider that it may be necessary to deal with the dose adjustment in Botox and Dysport applications, together with detailed studies to be carried out in the future.

Keywords: Apoptosis, Botulinum neurotoxin A, HCN2, MMP, TUNEL.

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Introduction

Botulinal neurotoxins (BoNTs) are derived from an anaerobic bacteria of the genus *Clostridium*. They are neurotoxins that paralyze the nerves and prevent contraction of the related muscle. These toxins reduce the release of acetylcholine on the nerve terminals and inhibit neuromuscular connection. Seven similar subtypes (A, B, C, D, E, F, G) of BoNTs that act with the hyperactivity of cholinergic nerve terminals for a long time are described [1,2]. It has been shown that botulinum neurotoxin A (BoNTA) is widely preferred in clinical application. BoNTs have been used effectively in the treatment of many diseases [2]. However, some undesirable side effects might be seen, in addition to its therapeutic effects. These effects can be seen as excessive local muscle weakness or can be caused by the undesirable distribution of the toxin due to the diffusion of neurotoxin. The leakage of BoNT into the systemic circulation is known as Botulism, which can clinically stop the respiratory system and cause death [3]. However, these neurotoxins, which are used in many areas, including cosmetics, are administered to patients at clinically safe amounts at certain intervals or continuously. It is not known whether these neurotoxins, which are likely to enter the systemic bloodstream, will affect both the surrounding cells and brain cells in the long term [4].

Nowadays, three formulations of BoNTA are administered. These are Botox (Allergan, Irvine, California), Dysport (Ipsen, Slough, UK) and Xeomin (Merz Pharmaceuticals, Greensborough, North Carolina) [3]. BoNT treatment has a large safety precaution, and this is partly dependent on the ability of the toxin to remain localized at the injection site. The diffusion to nearby muscles is one of the most common side effects of this therapy. For example, in ptosis, known as eyelid drooping, sternocleidomastoid muscle transition may occur after the injection has made into the extraocular muscle. These undesirable effects are related to the local diffusion of BoNT [5]. However, it may show different migration/diffusion properties, depending on the different compositions of BoNTA. Botox and Dysport are structurally composed of a neurotoxin (150 kDa) and a non-toxic component (Dysport 300-900 kDa, Botox 900 kDa). It has been claimed that the diffusion of the neurotoxin into adjacent tissues is slower in those with high molecular weight due to its size [5]. A study was conducted to compare the diffusion of Botox and Dysport formulation in the mouse muscle tissue, and it was emphasized that two different formulations show limited diffusion to adjacent muscles in the diffusion region, but this spread is not significant [3]. Some studies have been conducted to compare the safe use of BoNTA formulations, but its effect on cortical neuron cells in different concentrations, including safe concentration ranges, is highly limited in the literature.

Evidence that the intramuscular BoNTA injection passes directly into the central nervous system is not yet available. However, several studies reported that BoNTA had an indirect effect on the central nervous system organization through the peripheral mechanism [4-7]. This toxin reported to spread through the bloodstream in *in vivo* studies. Studies on passing the blood-brain barrier at low therapeutic doses are quite limited [4]. However, it was emphasized that BoNTA affects the brain with motor afferent feedback and changes brain activity but could not pass the blood-brain barrier [8]. There is no study investigating the effect of BoNTAs on brain tissue cells comparatively, but in some culture studies, it has been stated that neurons show sensitivity to this toxin [9,10].

Apoptosis is a vital component of many events, such as normal cell cycle and embryonic development. Disorders occurring in this mechanism can cause uncontrolled processes and serious damage [11]. Matrix metalloproteinases (MMPs) are structures containing the zinc-dependent family of endopeptidases [12], which can break down the components of the extracellular matrix (ECM). MMPs normally play a role in the development of the nervous system, wound healing, and regulation of neuron or glial cell functions, or increasing their expression in cells during abnormal processes such as cancer. There are currently 24 different MMPs, and it has been reported that MMP2 and MMP9 are especially expressed from the neuron and glial cells of the brain, cerebellum, and hippocampus and their expression increases significantly in cases such as ischemia [12]. The balance of ECM components in cultured human adult neuron cells allows synapses of the neurons' extensions to occur regularly. In particular, the role of MMP2, MMP7 and MMP9 in synapse formation has been determined [12]. It is assumed that MMP1, MMP2, MMP9, and MMP13 in the nuclei of the brain, endothelial cells and cardiac myocytes regulate the activity of proteins involved in DNA repair and apoptosis [13].

Increased MMP expression in brain cells disrupts the blood-brain barrier. The disruption of the blood-brain barrier increases neuronal apoptosis [14,15]. Considering all these data, the relationship between the increased MMP2, MMP7, MMP9 localization in cells with cell apoptosis has not been fully evaluated, especially in the central nervous system pathologies in the literature.

There are many methods to detect apoptosis. TUNEL (Terminal dUTP Nick End Labeling) method is used to determine endonuclease cleavage products by enzymatically ending DNA strand breaks [16]. Terminal transferase is used to add labeled UTP to the 3 'end of DNA fragments. dUTP can then be examined under a light microscope or fluorescent microscope. Tests are available from various companies as kits. These tests also allow precise examination of fluorescence microscopy until the detection of a single cell [16]. In the present study, we aimed to evaluate the effect of Botox and Dysport application at different concentrations on HCN2 cortical neuron cells in terms of differences in apoptotic cell numbers. It was investigated whether these BoNTAs applied in different concentrations affected on the localization of MMP2, MMP7, and MMP9 in increasing apoptotic cell numbers. In this way, the safe range of these two commonly used BoNTA can be determined through the hyperpolarization-activated cyclic nucleotide-gated (HCN2) cortical neuron cells and lead to more reliable preferences in clinical practice.

Materials and Methods

Obtaining Botulinum Toxin type A

Botox (Allergan, Irvine, California) and Dysport (Ipsen, Slough, UK) were commercially obtained in 100 IU packs lyophilized. Toxins were prepared with Dulbecco's Modified Eagle's Medium (DMEM) at concentrations of 0.01, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8 IU [17].

Cell Culture

In our study, HCN2 (ATCC ® CRL-10742 ™) cortical neuron cell line was used. These cells were reproduced in DMEM by adding Penicillin-streptomycin, L-glutamine, and Fetal Bovine Serum (FBS). Passages were created in order to maintain the continuity and viability of the HCN2 cell line to be used throughout the study, and passages 6th and 11th of the HCN2 cortical neuron cell series were used in our study [18].

Cytotoxicity Test

The XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) test (Roche) method was used to determine the cytotoxic potential of toxins. In the reaction of XTT solution with mitochondria of living or early-stage apoptosis cells, the tetrazolium ring in this solution breaks down by dehydrogenase enzymes in cell mitochondria to form colored formazan crystals. The intensity of the color formed with the reaction is directly related to the mitochondrial activity. Formazan crystal does not form in the presence of dead cells. In our study, cortical neuron cells were transferred into microplate wells to 100 µl of the suspension, which is 1x105/mL. Different concentrations of Botox and Dysport (0.01, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 and 12.8 IU) were added to these cells after waiting for 4 hours for the cells to adhere and kept for 24 hours. 10 µl of XTT solution was added to the wells at the end of this period. After 2 hours of incubation, the wavelength was determined to be 570 nm and observed in microplate readers (Thermo Scientific, USA) [19].

Microscobe analysis

TUNEL assay

The TUNEL (Roche, Germany) procedure applied in our study was carried out as specified by the manufacturer. Briefly, the HCN2 cortical neuron cell line was seeded on sterile 18×18 coverslips placed in 6-well plates at 10×10^4 cells each well. Groups were then formed as outlined in the XTT cytotoxicity section, and the cells

were treated in the same way. The media on the coverslips was removed, washed twice with PBS, and air dry. Freshly prepared 4% paraformaldehyde/PBS (Sigma, Germany) was fixed at pH 7.4 at room temperature for 60 min and washed again with PBS. The cells were infused in freshly prepared Triton X-100 in 1% sodium citrate at 2–8°C for 2 min. Negative controls were treated with 50 µL label solution. The enzyme solution in the kit was mixed with the label solution and incubated. 10 min in micrococcal nuclease or DNase 1 recombinant solution (50 mM Tris–HCL, pH 7.5, 3000 U/mL-3 U/mL in 1 mg mL^{-1} BSA) to detect DNA breaks was suspended before applying the prepared mixture to the positive controls. Cells that had been passed through PBS twice were then applied to each sample with 50 µL of TUNEL blend solution (label and enzyme solution mixture) and incubated at 37°C in humid dark for 60 min. Groups were washed 3 times with PBS-Triton-X. The TUNEL was stained with 4'6'-diamidine-2-phenyl-indole dihydrochloride (DAPI) (200 nm mL^{-1}) for 5 min to observe nuclear morphology after staining. The cells were rewashed with PBS-Triton-X 100 and examined under a fluorescence microscope. The coverslip we placed under the culture plate on the slide was carefully inverted (Olympus BX51 Japan) [20].

Immunofluorescence labeling

Immunofluorescent staining was performed with monoclonal mouse anti-human MMP2, MMP7 and MMP9 primary antibodies to HCN2 cortical neuron cells treated with Botox and Dysport in 3 different concentration ranges (3.2 IU, 6.4 IU, 12.8 IU), which were statistically significant with TUNEL. Cells were grown on sterile 18×18 coverslips in 6-well culture dishes. Cells were purified from the medium and washed with phosphate-buffered saline (PBS) (Sigma Aldrich, Germany). Washed cells were fixed in pH 7.4% paraformaldehyde (Merk, Germany) for 10 minutes at the room temperature. It was washed 3 times with cold PBS after fixation. Subsequently, pH 6.5 Sodium Citrate buffer heated at 95°C was treated for 10 minutes to expose tissue antigen (Sigma Aldrich, Germany). Cells were washed 2 times with PBS for 5 minutes. Cells were incubated for permeabilization in 0.1% Triton ™ X-100 (Sigma Aldrich, Germany) solution for 10 minutes. Cells were then washed with PBS for 5 minutes. The Ultra V Block (Thermo Scientific, PBQ180830, USA) was dropped and incubated for 30 minutes at room temperature to prevent non-specific binding after washing. Then, the primary antibodies MMP2, MMP7, AND MMP9 (Santa Cruz Biotechnology) were instilled onto the cells. Cells were incubated overnight at +4°C in a dark and humid environment. At the end of the period, cells were washed twice with PBS for 5 minutes. Goat Anti-Mouse IgG H&L (Alexa Fluor® 488) (ab150113) (Abcam, USA) secondary antibody was used for primary antibody. Secondary antibody was diluted to 1:300 with antibody diluent reagent (Invitrogen, USA) and applied to the cells for 1 hour in a humidity chamber. Then, nuclear staining was carried out in 4'6'-diamidine-2-phenyl-indole dihydrochloride (DAPI) (200 nm/mL) for 5 min after washing with PBS. The coverslips in the culture dishes were removed and inverted on the slide. The results were analyzed by fluorescence microscopy (Olympus BX51, Japan) using appropriate filters followed by recording [19].

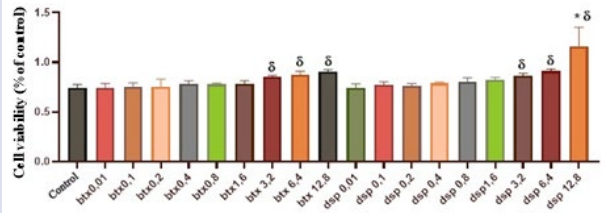
Statistical analysis

Apoptotic cells stained with the TUNEL method were counted under the microscope at 20× magnification from 5 independent regions. Apoptotic cell numbers were analyzed using the non-parametric Kruskal-Wallis test. Each group was compared with the other using the Dunn multiple comparison test.

All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, CA, USA). Each group was evaluated separately with each other using Mann-Whitney U, parametric one-way ANOVA was performed for comparing XTT results. $p < 0.05$ was considered significant.

Results

Different concentrations of Botox and Dysport 0.01, 0.1, 0.2.0.4, 0.8, 1.6, 3.2, 6.4 and 12.8 IU were applied to HCN2 cortical neurons, and the findings obtained after XTT analysis were evaluated with GraphPad Prism. Botox and Dysport 3.2, 6.4, 12.8 IU values were found to differ significantly compared to the control group. However, the Dsyport group 12.8 IU concentration value was significantly different from the Botox group in terms of cytotoxicity ($p < 0.05$) (Graph 1).



Graph 1. Graphical drawing of findings obtained from XTT analysis as a result of Botox and Dysport treatment at different concentrations to HCN2 cortical neurons with GraphPad Prism program. There is a difference in 3.2.6.4 and 12.8 IU values when the Botox and Dysport groups are compared with the control. The Dysport group differs significantly from the Botox group in terms of cytotoxicity at a concentration of 12.8 IU ($p < 0.05$).

Apoptosis cells were determined by applying in different concentrations of Botox and Dysport to HCN2 cortical neurons and marking them with TUNEL method. Apoptotic cells began to appear from a concentration of 0.1 IU in the Botox group and 0.01 IU in the Dysport group. Considering these cells labeling at similar concentrations, the number of apoptotic cells in the Dysport group is higher than in the Botox group. Apoptotic cell numbers increased in both groups as the concentration increased (Figure 1).

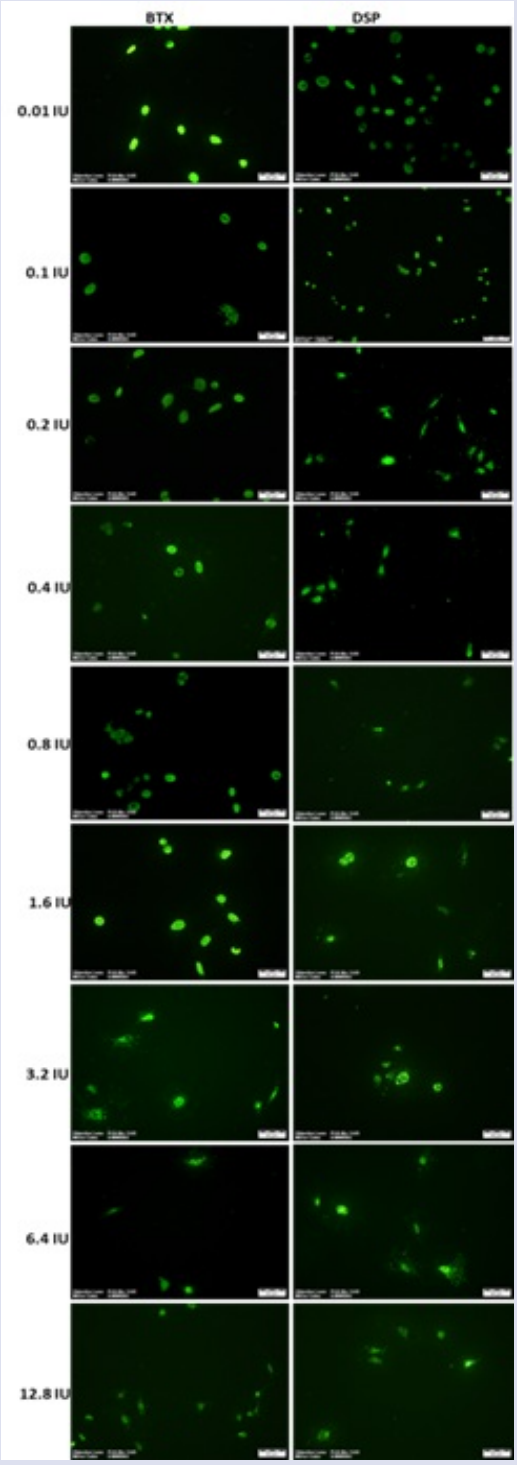
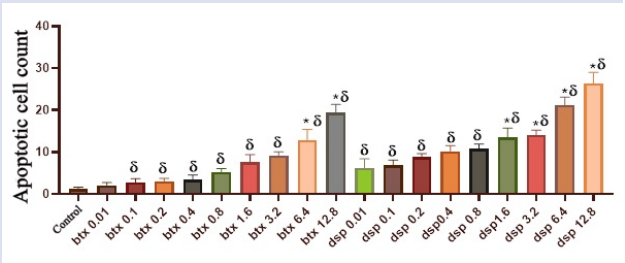


Figure 1. Fluorescence microscope image showing apoptosis cells by marking with TUNEL method as a result of Botox and Dysport treatment to the HCN2 cortical neurons at different concentrations. Apoptotic cells began to appear in the BTX group from 0.1 IU and in the DSP group from 0.01 IU concentration. As the amount of concentration increased, the number of apoptotic cells also increased (20x magnification).

The numbers obtained by applying different concentrations of Botox and Dysport to HCN2 cortical neurons and semi-quantitative scoring of apoptotic cells were evaluated in the GraphPad Prism program. The number of apoptotic cells began to increase from the concentration of Botox 0.1 IU and Dysport 0.01IU compared to the control group. In addition, Botox 6.4, 12.8 IU concentrations and Dysport 1.6, 3.2, 6.4, and 12.8 IU concentrations differ significantly in terms of cells leading to apoptosis ($p < 0.05$) (Graph 2).



Graph 2. Graphical drawing of findings obtained by semi-quantitative scoring of apoptosis cells at different concentrations of Botox (btx) and Dysport (dsp) treatments to HCN2 cortical neurons with GraphPad Prism program.

In our study, the importance of MMP2, MMP7, and MMP9 was remarkable on the number of apoptotic cells in cortical neurons that increased with increasing concentration. Therefore, MMP antibodies were labeled with the immunofluorescence method in the concentration ranges where apoptotic cells increased significantly. As the concentrations of MMP2, MMP7, and MMP9 increased in Botox and Dysport groups, the nucleus and cytoplasmic MMP localization also increased, and this ratio was found to be higher in the Dysport group than the Botox group when semi-quantitative findings were evaluated (Figures 2-7).

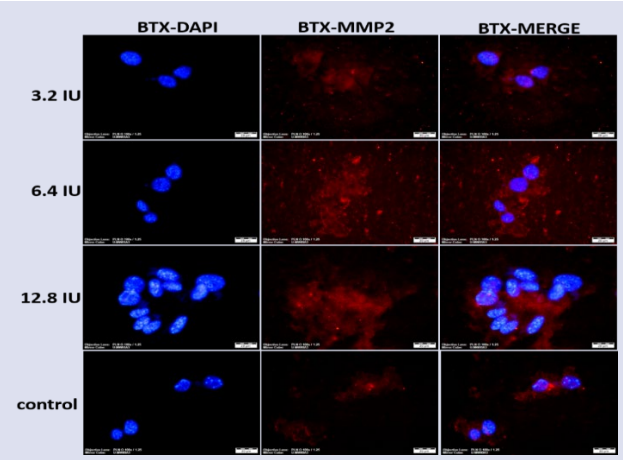


Figure 2. Immunofluorescence profile of HCN2 cortical neurons treated with Botox 3.2, 6.4, and 12.8 IU concentrations and stained with MMP2 primer antibody. The nuclei were stained by DAPI (blue). Cells were fixed in paraformaldehyde and examined under fluorescent microscope. Scale bar represents 20 µm.

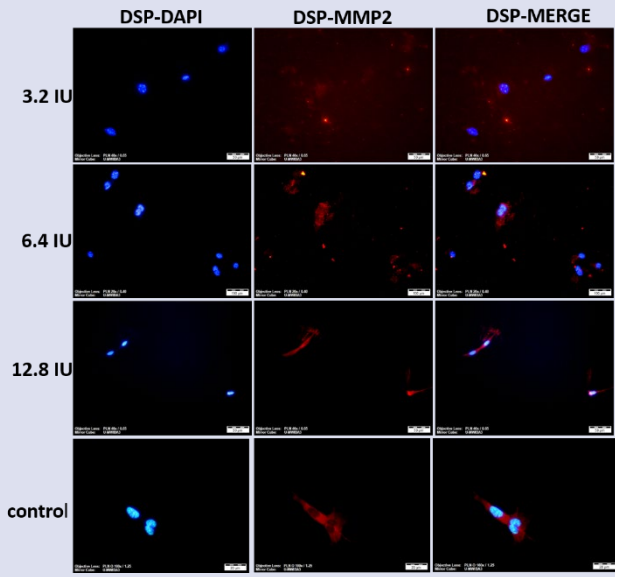


Figure 3. Immunofluorescence profile of HCN2 cortical neurons treated with Dysport 3.2, 6.4, and 12.8 IU concentrations and stained with MMP2 primer antibody. The nuclei were stained by DAPI (blue). Cells were fixed in paraformaldehyde and examined under fluorescent microscope. Scale bar represents 50 µm.

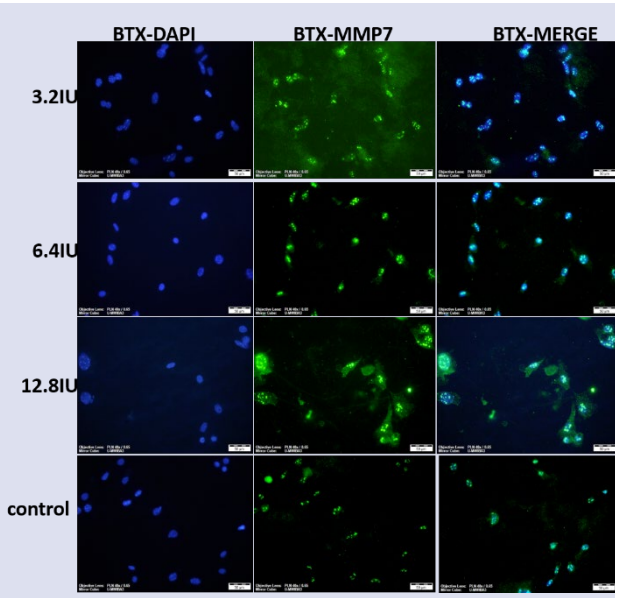


Figure 4. Immunofluorescence profile of HCN2 cortical neurons treated with Botox 3.2, 6.4, and 12.8 IU concentrations and stained with MMP7 primer antibody. The nuclei were stained by DAPI (blue). Cells were fixed in paraformaldehyde and examined under fluorescent microscope. Scale bar represents 50 µm.

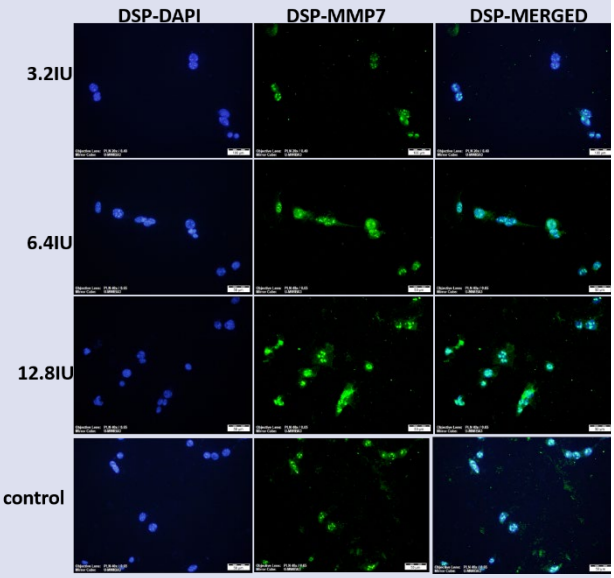


Figure 5. Immunofluorescence profile of HCN2 cortical neurons treated with Dysport 3.2, 6.4, and 12.8 IU concentrations and stained with MMP7 primer antibody. The nuclei were stained by DAPI (blue). Cells were fixed in paraformaldehyde and examined under fluorescent microscope. Scale bar represents 50 µm.

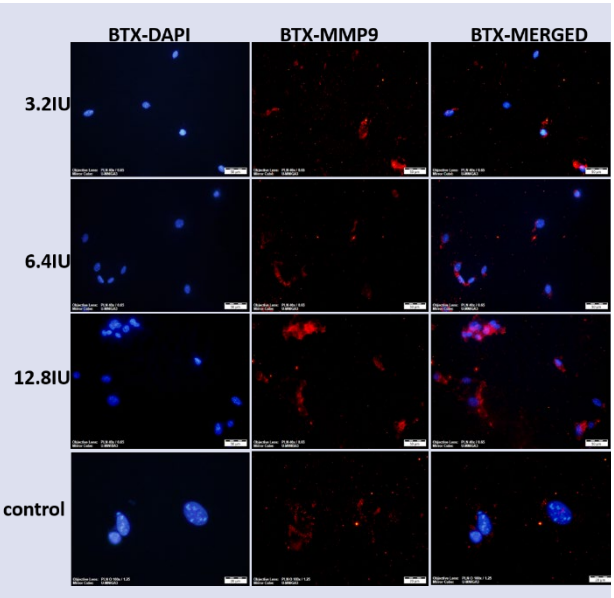


Figure 6. Immunofluorescence profile of HCN2 cortical neurons treated with Botox 3.2, 6.4, and 12.8 IU concentrations and stained with MMP9 primer antibody. The nuclei were stained by DAPI (blue). Cells were fixed in paraformaldehyde and examined under fluorescent microscope. Scale bar represents 50 µm.

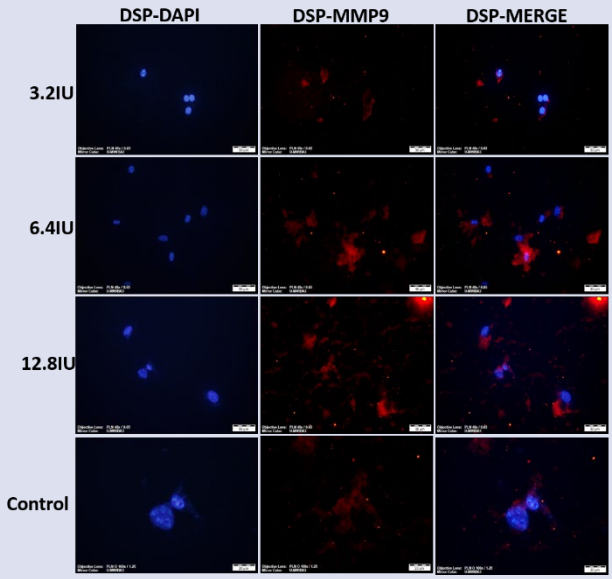


Figure 7. Immunofluorescence profile of HCN2 cortical neurons treated with Dysport 3.2, 6.4, and 12.8 IU concentrations and stained with MMP9 primer antibody. The nuclei were stained by DAPI (blue). Cells were fixed in paraformaldehyde and examined under fluorescent microscope. Scale bar represents 50 µm.

Discussion

Clostridium botulinum toxin type-A inhibits the release of acetylcholine (ACh) from stimulating pre-synaptic nerve terminals in peripheral neuromuscular junctions and the mechanism is still not fully understood [15]. BoNT consists of 7 types of neurotoxins, however, Type-A and Type-B toxins are mainly used in the clinic. BoNTA is used in medicine, especially in dermatology for various cosmetic disorders and also in the treatment of many diseases. The first type of BoNT presented is onabotulinum toxin A. It was proposed to be used as therapeutic by the Food and Drug Administration (FDA) in 2002 [21]. The second form of onabotulinum toxin A produced in France was licensed from the European Union for use for aesthetic purposes in 2006 and was approved by the FDA in 2009. BoNTA has been used for treatment in many fields, especially cosmetics [22].

Botox has a molecular mass of 900kDa and Dysport has a molecular mass of 300-900kDA. Both toxins contain the nuclear neurotoxin weighing 150 kDa and varying amounts and types of non-toxin accessory proteins. This situation changes the molecular weights and mechanisms of action of Botox and Dysport. This is expressed as bioavailability during clinical practice and therefore the neurotoxin load per hundred units varies. On the other hand, injection of a given dose of BoNT-A in a larger volume increases the area to which it initially diffuses, thus increasing the risk of excessive diffusion [23]. Possible negative effects that may occur due to the varying amount of toxin per injection and toxin load require looking at these frequently used toxins from a different place.

Local BoNT administration results in muscle paralysis or immobility of cholinergic glands. There is evidence that botulinum toxin is carried retrograde transport in the spinal cord through the motor and sensory nerves. However, there are no pathological signs or symptoms caused by the retrograde transport of botulinum toxin to the central nervous system. It is still known that the dose of Botox varies according to the type of BoNTA used and the area injected. There is no clear guideline for proper dosing. Botox must be diluted with sterile 0.9% saline before injection. In general, if the muscle is small in size, the injected solution should be more diluted. For example, several 0.1ml applications were proposed to different areas of the face and forehead for migraine treatment. In cervical dystonia patients, it varies up to 30-100 units and treatment is recommended several times, while in the clinic, this rate is 5-100 units [24]. The appropriate dose of Botox in the cosmetic field is between 20-40 units. In our literature research, we determined the dose range of Botox and Dysport applied to HCN2 cortical neurons as wide as 0.01, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 and 12.8 IU concentrations. The effect of Botox and Dysport, which are frequently used in many areas in the clinic, on cytotoxic cortical neuron cells is not previously studied. In the study, a significant difference was found between the groups treated with 3.2, 6.4 and 12.8 IU in Botox and Dysport groups compared to the control in terms of cytotoxicity. However, Dysport 12.8 IU concentration is more significant than all other groups ($p < 0.05$).

Every cell in our body lives for a certain time and dies on time. There is a controlled balance between cell death and cell proliferation. Apoptosis is an event that is regulated by many complex internal and external mechanisms in which cells are self-destroying, regulated by genes, programmed, requiring RNA, protein synthesis and energy, and maintaining homeostasis in the organism. Different methods are used to determine and display apoptosis, and one of them is TUNEL method. Terminal deoxynucleotidyl transferase (TdT) mediated dNTP nick-end labeling (TUNEL) is often used to detect *in situ* cell death. DNA strand breaks in apoptotic cells can be identified by labeling the free 3p-OH terminus with altered nucleotides (fluorescein-labeled dNTP) in a TdT enzymatic reaction. It is one of the methods frequently used in the determination of cellular apoptosis especially in neurotoxicity studies [25]. In our study, after the application of Botox and Dsyport to HCN2 cortical neurons in different concentrations, apoptotic cells were stained by TUNEL method, and the number of cells obtained was compared with the control and each other. As a result of this comparison, an increase in apoptotic cells was observed from the concentration of 0.1 IU in the Botox group and the concentration of 0.01 IU in the Dysport group. In addition, apoptotic cells are significantly higher in the Dysport group at concentrations of 1.6, 3.2, 6.4 and 12.8 IU than the Botox group.

It has been reported in the literature that the use of BoNTAs is safe (lethal dose in humans can reach 50% (LD50) up to 40 U/kg BW). Therefore, it is stated that its use in cosmetics is particularly safer [26]. It has been reported that BoNTA does not cause permanent changes in nerve terminals and targeted muscles, therefore it cannot cause long-term adverse or side effects in the field of dermatology [26]. However, complications are rarely reported after the application of cosmetic Botox. The most common complications are ecchymosis and purpura at the injection sites [27]. These side effects can be prevented by simple clinical precautions [28]. On the other hand, in a case report, it was emphasized that sudden death developed in a patient who was given Botox, and this situation occurred after Botox treatment for cosmetic purposes and revealed that it was positive in terms of hypersensitivity tests in forensic medicine findings. In September 2005, the FDA reported that the use of Botox caused 28 deaths between 1989 and 2003, but none of them were directly linked to cosmetic use [29]. The most common local side effect is swelling or bruising at the injection site. Serious local reactions such as blurred vision, swelling, itching, and dry mouth have also been reported. It also causes headaches or flu-like symptoms as a systemic reaction. Moreover, severe findings such as urinary retention, difficulty breathing, difficulty swallowing, and dysphonia can be seen after Botox injection [30]. In addition to all these clinical findings, in a study by Wiegand et al. [6], it is stated that BoNTA is delivered to the spinal cord system by retrograde transport in an intramuscular injection. They stated that BoNTA, which was given by marking the cat gastrocnemius muscle, was moved first in the sciatic nerve, then in the ipsilateral spinal ventral roots, then in the spinal cord segments and some dorsal roots. This information suggests that BoNTA is moved over motor and intrafusal afferent axons. Neurophysiological techniques used in cats and other animals have been reported to occur with the movement of toxins into the alpha motor neuron soma membrane [6]. In addition, BoNTA was administered directly into the spinal cord in cats under anesthesia. In the study, it was stated that BoNTA reduced cholinergic transition to Renshaw cells and the function of inhibitory interneurons [7]. In studies conducted in the rat brain, it was found that BoNTAs are primarily bound to synaptosomes and the regions of the rat brain rich in synapses such as the hippocampus and cerebellum [31]. It has been reported that toxin is spread through the bloodstream in *in vivo* studies. Studies, in which it passes the blood-brain barrier at low therapeutic doses, are quite limited [4]. However, it was reported that BoNTA affects brain tissue with motor afferent feedback and changes brain activity but does not cross the blood-brain barrier [8]. In a study investigating the effect of botulinum neurotoxins on cells obtained from the embryonic rat dorsal root ganglia by primary culture, it has been reported that neurons show high sensitivity to toxins, and especially BoNTA significantly paralyzes synaptic transmission [9]. Similarly, there are findings in fetal mouse spinal cord neurons that especially BoNTA blocks neural transmission [32]. In another study, it was thought that BoNTC causes widespread synaptic damage in the central nervous system neurons, with the structural disruption of the neuron extensions, the disruption of the communication of the cells with each other, the loss of interneuron connections and eventually disappearing these cells by undergoing apoptosis process [10]. Similarly, in a study conducted by Zhao et al. [33] in *in vitro* and *in vivo*, mouse neurons exposed to BoNTC have degenerated. They stated that BoNTs can block synaptic vesicle exocytosis and cause damage to synapses and neurons. However, they did not report that BoNTAs caused a significant neuronal disorder [33]. In our study, it was determined that two different BoNTAs applied to HCN2 cortical neurons at different concentrations increased cytotoxicity and apoptotic cell number. We consider that Dysport undergoes more cells to apoptosis than Botox due to the molecular weight difference [23] especially at high concentrations.

Based on the potential peripheral leakage and the ability of BoNTs to reach neurons, we consider that the role of MMPs, known to regulate the brain matrix of neurons and glial cells, has gained more importance with the increase in apoptotic cell number of Botox and Dysport applied in different concentrations. It is known that MMP2, MMP7, MMP9 are especially related to the regulation of the brain extracellular matrix, DNA repair and apoptosis in the brain [21,22]. The MMP protein family primarily impairs the blood-brain barrier integrity [34]. In addition to neurons in the central nervous system, they are also expressed in endothelial cells in mammals and contribute to the blood-brain barrier integrity. Kanda et al. [14] stated that with the application of BoNTAs on Drosophila, it disrupts the blood-brain barrier with the increase of MMP2 expression.

Studies have shown that MMPs can degrade the nuclear matrix and poly (ADP-ribose) polymerase (PARP), which is one of the components of the nuclear matrix [35]. PARP is activated by single-stranded DNA breaks induced by peroxynitrite and is responsible for repairing single-stranded DNA breaks. Thus, PARP can be inactivated by MMPs via proteolytic cleavage. Inhibition of DNA repair can cause apoptosis, and excessive amounts of PARP also cause energy depletion of cells, resulting in apoptosis. It has been stated that apoptosis is induced in various tissues and cells by increasing the expression of MMP2, MMP7 and MMP9 [36]. Studies on the damage caused by Botox and Dysport on neurons are very few and controversial. In some histological and electron microscopic study findings, it is stated that Botox causes muscle atrophy by disrupting the thin structure of the muscle [37]. It has been stated that Botox treatment induces apoptosis in glioblastoma and neuroblastoma cells due to mitochondrial damage together with excessive ROS production [38]. Recently, it has been reported that the positive effects of Botox treatment especially on peripheral nerve damage and damage after

spinal cord injuries [39]. However, due to the long-term and continuous nature of these treatments, their possible effects on the neurons of the central nervous system cannot be ignored. In a study, it was emphasized that botulinum toxins cause intense degeneration in human primary neuron culture cell lines, not only in the perisynaptic areas of neurons, but also in their perikaryons [40]. The receptors are exposed to the toxin in cultured neurons because the neurons lack both the myelin sheath and the perineurium that normally surrounds peripheral nerves from the outside at their ends (the presynaptic terminals of the endplate). Thus, the toxin can cause more significant damage. It is still insufficient to explain the cause of death of healthy neurons by apoptosis at clinical concentrations recommended for therapy. However, the cells undergo apoptosis as a result of Botox and Dysport applied to healthy cortical neuron cells at various concentrations. Cells undergoing apoptosis were remarkably significant at 3.2, 6.4 and 12.8 IU Botox and Dysport concentrations. The toxin applied on healthy cells may have caused mitochondrial damage with the increase in the amount of intracellular ROS, as well as an increase in MMP2, MMP7, and MMP9 immunolocalizations. Studies can be developed and expanded, which may lead to dose limitations in the use of these toxins, which have a wide area of use.

Conflicts of interest

There are no conflicts of interest in this work.

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