Orjinal Araştırma **Van Sağ. Bil. Derg.** *2024; 17(3):166-174.*
Original Research *https://doi.org/ 10.52976/vansaglik.1552892*

Immunohistochemical and Stereological Examination of the Gastrocnemius Muscle in Rats Applied with Botox

Botox Uygulanan Ratlarda Gastrocnemius Kasının İmmunohistokimyasal ve Stereolojik Olarak İncelenmesi

Mehmet Uğur Delibaş[1], Gamze Çakmak[*1]*

[1] Department of Anatomy, Faculty of Veterinary Medicine, University of Van Yuzuncu Yil, Van, Türkiye

Cited: Delibaş MU, Çakmak G. (2024). Immunohistochemical and Stereological Examination of the Gastrocnemius Muscle in Rats Applied with Botox. *Van Sağlık Bilimleri Dergisi*, 17(3), 166-174.

ABSTRACT

Objective: The aim of this study was to examine the volume density of the left gastrocnemius muscle which botox was applied on the 15th and 21st days immunohistochemically and stereologically.

Material and Method: Thirty-three-month-old Wistar Albino rats were divided into three separate groups. Botox A solution, 2.5U in 0.1ml physiological saline, was prepared and injected into the left gastrocnemius muscle of the rats in the other two groups except the control group. Rats were fixed by perfusion with 10% buffered formaldehyde. The left gastrocnemius muscle of the rats dissected one week after perfusion process. Sections were randomly selected among the first 15 sections, and each subsequent 80th section was taken by systematic random sampling method. Sections were obtained with a thickness of 5µm and 8-10 sections. The sections were stained with hematoxylin and eosin, and photographed under a microscope. Total volume values of musculus gastrocnemius sinistra were determined in accordance with the Cavalieri's Principle and using the Shtereom I package program.

Results: It was determined that the volume values between the groups showed a statistically significant difference ($p<0.05$). In addition, the effects of Botox A on the gastrocnemius muscle on the 15th and 21st days were detected immunohistochemically using Caspase 3 antibody dye.

Conclusion: The study concluded that Botox A application caused significant volumetric changes in the left gastrocnemius muscle on the 15th and 21st days, with immunohistochemical evidence of increased caspase-3 activity, indicating enhanced apoptotic processes.

Keywords: *Botox, Volume, Immunohistochemistry, Musculus gastrocnemius, Stereology*

ÖZET

Giriş: Bu çalışmanın amacı, 15. ve 21. günlerde botoks uygulanan sol gastroknemius kasının hacim yoğunluğunu immünohistokimyasal ve stereolojik olarak incelemektir.

Materyal ve Metot: Otuz üç aylık Wistar Albino sıçanlar üç ayrı gruba ayrıldı. Kontrol grubu hariç diğer iki gruptaki sıçanların sol gastroknemius kasına 0,1 ml fizyolojik tuzlu suda 2,5 U'luk botoks solüsyonu hazırlanıp enjekte edildi. Sıçanlar %10'luk tamponlu formaldehit ile perfüzyon yoluyla fiksasyona tabi tutuldu. Sıçanların sol gastroknemius kası perfüzyon işleminden bir hafta sonra diseke edildi. Kesitler ilk 15 kesit arasından rastgele seçildi ve sonraki her 80. kesit sistematik rastgele örnekleme yöntemi ile alındı. 5 µm kalınlığında ve 8-10 kesitten oluşan kesitler elde edildi. Kesitler hematoksilen ve eozin ile boyandı ve mikroskop altında fotoğraflandı. Musculus gastrocnemius sinistra'nın toplam hacim değerleri Cavalieri Prensibi'ne uygun olarak ve Shtereom I paket programı kullanılarak belirlendi.

Bulgular: ruplar arasındaki hacim değerlerinin istatistiksel olarak anlamlı bir fark gösterdiği belirlendi ($p<0,05$). Ayrıca, Botox A'nın gastrocnemius kası üzerindeki etkileri 15. ve 21. günlerde Caspase 3 antikor boyası kullanılarak immünohistokimyasal olarak tespit edildi.

Sonuç: Çalışma, Botox A uygulamasının 15. ve 21. günlerde sol gastrocnemius kasında önemli hacimsel değişikliklere neden olduğu ve artmış apoptotik süreçleri gösteren artmış kaspaz-3 aktivitesinin immünohistokimyasal kanıtı olduğu sonucuna vardı.

Anahtar kelimeler: *Botox, Hacim, İmmunohistokimyal, Musculus gastrocnemius, Stereoloji*

* *Corresponding author: Gamze Çakmak. E-mail:* vetgamze@hotmail.com

ORCIDS: Mehmet Uğur Delibaş: 0009-0006-9634-0932, *Gamze Çakmak*: 0000-0002-3970-3040

Received: 20.09.2024, Accepted: 21.12.2024 and Pubished: 30.04.2024

INTRODUCTION

Muscles are structures consisting of special cells with contractile properties that enable living creatures to move by converting the chemical energy obtained from various nutrients into mechanical energy. These structures, which make up approximately half of the body weight in mammals, carry out metabolic activities as a result of the contractions they create within themselves and provide the creature with the ability to move externally (Hole, 1981; Vander et al., 1994; Berne et al., 2009). Muscle cells that make up muscles; It contains structures with contractile properties that act through action potentials. These cells contain chemical, mechanical and electrical stimulation properties, just like nerve cells (Weineck, 1998; Ganong, 2010). Botulinum toxin A (BTx-A) is a neurotoxin that has a weight of 150 kilodaltons (kDa) and acts by undergoing posttransitional proteolysis during biological activities. After proteolysis, the toxin is divided into two parts: 50 kDa light and 100 kDa heavy (Lacy, 1998; Tighe and Schiavo, 2013). The exotoxin of Clostridium botulinum, whose spores are extremely resistant to environmental conditions, is very sensitive to pH and temperature changes (Bulam, 2012). The toxin, which exerts its effect by rapidly and irreversibly binding to presynaptic cholinergic receptors, blocks nerve transmission at the neuromuscular junction. There are eight different serotypes of the toxin, the most common in clinical and aesthetic use is type A. Many clinical and experimental studies have been conducted on the toxin, which was first described by Justinus Kerner in 1817 (Cartee and Monheit, 2011). It has been reported that the toxin, whose use has become extremely widespread in recent years, has a therapeutic effect on more than 300 conditions such as strabismus, hyperhidrosis, hemiplegic sapaticity, Parkinson's tremor, torticollis, anal fissure and cerebral palsy (Hastad and Lacy, 1998). In rats, the gastrocnemius muscle is the strongest muscle located in the posterior part of the hind limb and causes the ankle to flex. It is the largest and most prominent muscle mass among the superficial group flexor muscles (Han Nami et al., 2013). Stereology is a branch of science that allows inferences to be made about the true purpose (area, volume, number of particles, length, etc.) of many objects, especially biological objects, with the data obtained from the images created by objects with x, y, z axes in the x and y dimensions or projections. (Baddeley, 1991; Cruz-Orive, 1999). The aim of this study was to examine the volumetric effects of Botulinum Toxin A, which has a wide range of usage today, on the gastrocnemius musculus from a stereological perspective and to determine the caspase 3 antibody activity, which is one of the oxidative stress markers.

MATERIAL and METHOD

In this study, 30 3-month-old healthy adult male Wistar Albino rats with an average weight of 250-300 g were used. Rats were kept in standard cages with 12 hours of darkness, 12 hours of light and 18-24°C. During the study, the animals were fed free diet by feeding standard pellet feed and drinking tap water. In this study, 30 male adult rats were divided into 3 groups: control, Botulinum toxin A (BTx-A) application and waiting for 15 days, and Botulinum toxin A application and waiting for 21 days. The rats were divided into groups and kept for 10 days so that they could adapt to the new environment and group elements. Ketalar was injected intraperitoneally at a dose of 50 mg/kg to the control group. Perfusion was performed without administering any experimental substance. 25U/1ml BTx-A solution was prepared by mixing 500 U of Dysport® preparation into 20 ml of 0.9% physiological saline. From the solution, 0.1 ml of 2.5U BTx-A was administered intramuscularly as a single dose injection into the m. gastrocnemius sinistra of each rat, which was detected by external palpation (Figure 1). This group was kept waiting for 15 days. Rats anesthetized with ketalar at a dose of 50 mg/kg were perfused. Rats were administered Botulinum toxin A after the injection and kept for 15 days. Perfusion was applied on the 15th day. The same procedure was applied to the third group and the rats were perfused at the end of the 21st day. For the fixation process, m. gastrocnemius sinistra of the rats, which were kept in formaldehyde for a week, and they were dissected as a whole without separating the caput laterale and caput mediale (Figure 2).

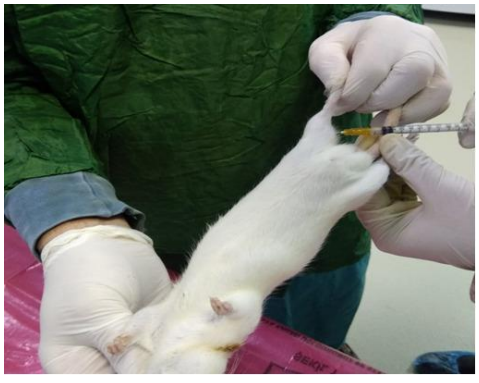

Figure 1. Botox A application to m. gastrocnemius

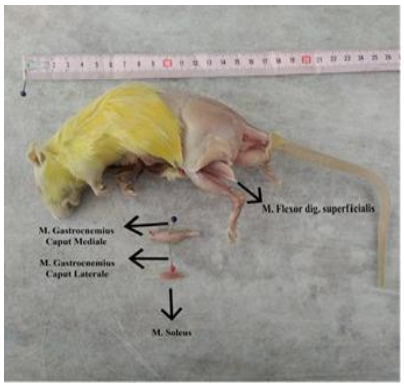

Figure 2. Dissection of sinistra. m. gastrocnemius sinistra

Since m. gastrocnemius is a small muscle, stereological stepping was not required. 5µm thick sections were obtained from the muscles. The sections were gotten by a Rotary microtome (Leica RM 2135, Leica Instruments, Nussloch, Germany). A mean of 8-10 sections were recieved. Starting by a random section among the first 15 sections, every subsequent 80th section was gotten. Sampling was carried out systematically and randomly at a rate of 1/80. The sections taken were stained with hematoxylin and eosin (Thomas and Combs, 1965; Sikandar et al., 2013). Photographs were taken at x10 magnification to calculate volume values (Figure 3, Figure 4, Figure 5). Shtereom I package program was used. Cavalieri's Principle was used for the calculation in the program (Gundersen and Jensen, 1987; Odacı et al., 2005). In the study, belonging to the control group, the observation group on the 15th day after Botox A application, and the observation group on the 21st day after Botox A application. The total volume of gastrocnemius was calculated. For volume calculations, the number of points was preferred since the numerical ratios of the calculated points can be counted instead of the volume value (Howard, 1998; Howard and Reed, 2004). Caspase-3 was detected using the streptavidin/biotin immunoperoxidase kit to obtain immunohistochemical data. Their expression was determined according to streptavidin peroxidase (ABC) (Histostain-Plus Bulk Kit; Zymed, South San Francisco, CA, USA).

Statistical Analysis

In calculating the sample size of this study, the Power of Test for each variable was determined as at least 80% and Type-1 error was 5%. Whether the continuous measurements in the study were normally distributed was by the Shapiro-Wilk (n<50) test, and since the measurements were normally distributed, parametric tests were applied. Descriptive statistics for continuous variables in the study expressed as mean and standard deviation. "ANOVA" was used for repeated measurements in "between test periods (groups)" comparisons. Following this analysis, the "Bonferroni post-hoc test" was applied to determine the tests that made the difference. The statistical significance level for the calculations was determined as p<0.05 and SPSS (IBM SPSS for Windows, ver.26) statistical program was used in the analyses.

Table 1. Application of normality test statistically.

	Group	Kolmogorov-Smirnov			Shapiro-Wilk		
		Statistic	**df**	**Sig.**	**Statistic**	**df**	**Sig.**
Total Volume (mm^3)	0.day (control group)	.168	10	.200*	.973	10	.917
	15.day	.253	10	.068	.850	10	.058
	21.day	.301	10	.011	.867	10	.093

Considering Shapiro-Wilk (n<50) test results in Table 1, parametric tests were preferred to be used in comparisons since the "values" showed a normal distribution.

Table 2. Statistical analysis of volume values of m. gastrocnemius sinistra control group and on the 15th and 21st days after Botox A application.

		N	**Mean**	**±Std. Dev.**	***p.**
Total Volume (mm^3)	0.day (control group)	10	1.741 **a**	±.057	**.001**
	15.day	10	1.409 **c**	±.096	
	21.day	10	1.567 **b**	±.100	

Significance levels according to ANOVA results in repeated measurements

a,b,c: Shows differences between test periods according to Bonferroni Post Hoc test. A statistically significant difference was observed between the test periods in terms of the volume values of m. gastrocnemius ($p<0.05$) (Table 2).

Table 3. Analysis and statistical data of immunohistochemical results of m. gastrocnemius sinistra.

	Caspase 3 expression
Group 1	19.52±1.06[a]
Group 2	43.12±3.32[b]
Group 3	67.57±3.20[c]

a,b,c: Different letters in the same column indicate statistical difference ($p<0.05$).

RESULTS

Table 4. Volume (mm^3), coefficient of error (CE), and noise values of m. gastrocnemius sinistra of control group.

	Variables	Total Volume (mm^3)	Noise	CE
Number of Samples	R1	1.738	5562	0.0167
	R2	1.714	5482	0.0149
	R3	1.831	5862	0.0158
	R4	1.746	5588	0.0164
	R5	1.644	5263	0.0178
	R6	1.816	5812	0.0163
	R7	1.745	5584	0.0168
	R8	1.720	5504	0.0154
	R9	1.684	5389	0.0185
	R10	1.776	5652	0.0177
	Mean	**1.741**	**5569**	**0.0166**

According to Table 4 it was stated that the total average volume of gastrocnemius was 1.741 mm^3, the average number of points (Noise) was 5569, and the average coefficient of error (CE) was 0.0166.

Table 5. Volume (mm^3), noise and CE values of m. gastrocnemius sinistra after the application on the 15th day.

	Variables	Total Volume (mm^3)	Noise	CE
Number of Samples	R1	1.480	4738	0.0163
	R2	1.471	4708	0.0154
	R3	1.485	4754	0.0162
	R4	1.508	4827	0.0159
	R5	1.249	3998	0.0148
	R6	1.487	4760	0.0177
	R7	1.288	4124	0.0187
	R8	1.447	4632	0.0169
	R9	1.317	4217	0.0183
	R10	1.359	4349	0.0144
	Mean	**1.409**	**4510**	**0.0164**

According to Table 5, it is seen that average total volume is 1.409 mm^3, the average number of points is 4516, and the average coefficient of error is 0.0164.

Table 6. Volume (mm^3), noise and CE values of m. gastrocnemius sinistra after the application on day 21.

	Variables	Total Volume (mm^3)	Noise	CE
Number of Samples	R1	1.513	4842	0.0167
	R2	1.499	4798	0.0164
	R3	1.489	4765	0.0174
	R4	1.542	4936	0.0189
	R5	1.673	5354	0.0192
	R6	1.741	5572	0.0163
	R7	1.699	5438	0.0188
	R8	1.528	4892	0.0178
	R9	1.448	4635	0.0184
	R10	1.542	4937	0.0188
	Mean	**1.567**	**5016**	**0.0178**

It was observed that the results obtained in Table 6 increased compared to the results obtained from the effect of Botulinum toxin A on the 15th day. While the average total volume was 1.567 mm^3, the average number of points was calculated to be 5016 and the average coefficient of error was 0.0178. According to the results, it was observed that Botulinum Toxin A caused a volume decrease in the muscle until the 15th day, without any specific reason. It has been determined that after the 15th day, as the effect of Botulinum Toxin A on the muscle decreases, the repair, which is one of the physiological reflexes of the muscles, begins.

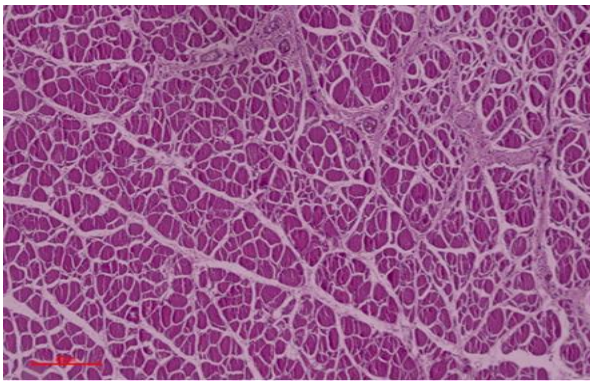

Figure 3. Control group hematoxylin eosin staining (x10) (H&E).

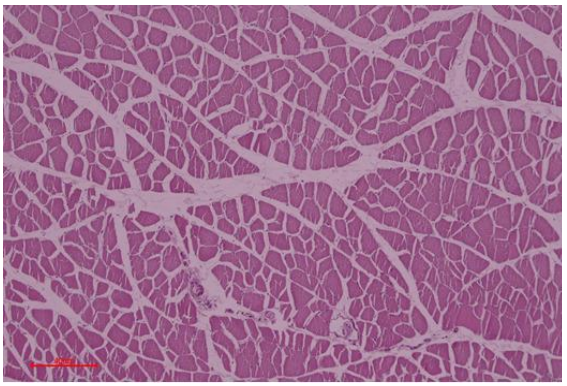

Figure 4. Image of the muscle to which Botox A was applied on the 15th day (x10) (H&E).

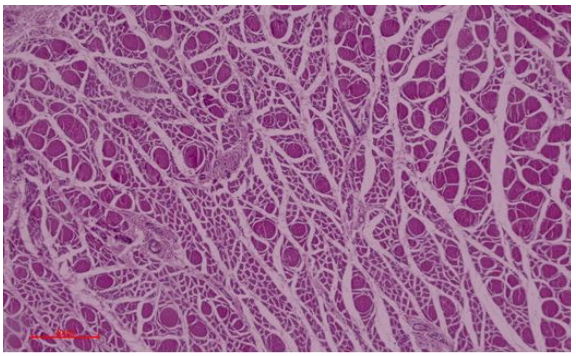

Figure 5. Image of the muscle to which Botox A was applied on day 21 (x10) (H&E).

It was revealed that the average volume values of the m. gastrocnemius sinistra were 1.741 mm^3 in the control group. It was determined that the mean volume value of the gastrocnemius was 1.409 mm^3 15th day, while the average volume value of the rats on the 21st day to which Botulinum Toxin A was applied was 1.567 mm^3.

It has been determined that it causes volume loss in the gastrocnemius for a period of time and then causes an increase in volume due to the muscle's recovery reflex in line with the decreasing effect of Botulinum toxin A.

Histopathological Results

Group 1: Control group, Caspase 3 expression of m. gastrocnemius sinistra was evaluated as negative (Figure 6).

Group 2: In the immunohistochemical examination of m. gastrocnemius on the 15th day after Botox A application, cytoplasmic Caspase 3 expression was detected in myocytes (Figure 7). A statistically significant difference ($p<0.05$) was observed when compared to the control group.

Group 3: In the immunohistochemical examination of m. gastrocnemius sinistra on the 21st day after Botox A application, severe cytoplasmic Caspase 3 expression was detected in myocytes (Figure 8). A statistically significant difference ($p<0.05$) was detected when compared to the control group.

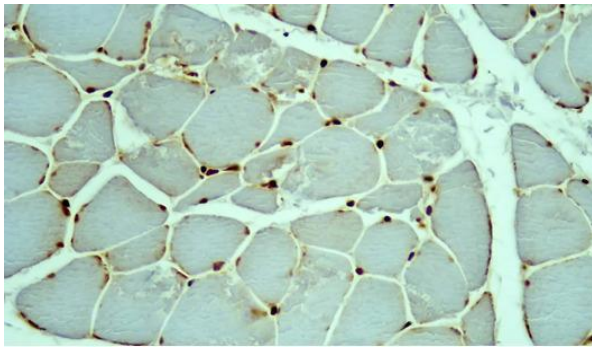

Figure 6. Control group, negative Caspase 3 expression, IHC-P

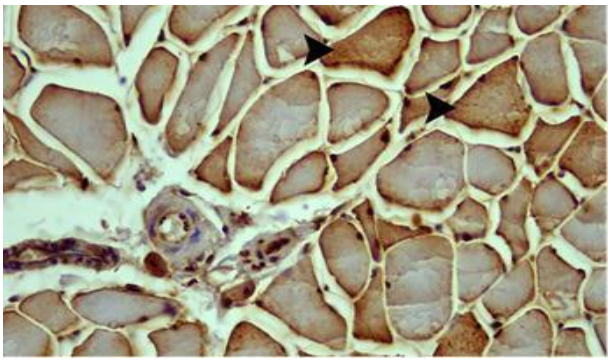

Figure 7. Moderate cytoplasmic Caspase 3 expression in m. gastrocnemius sinistra on day 15, IHC-P.

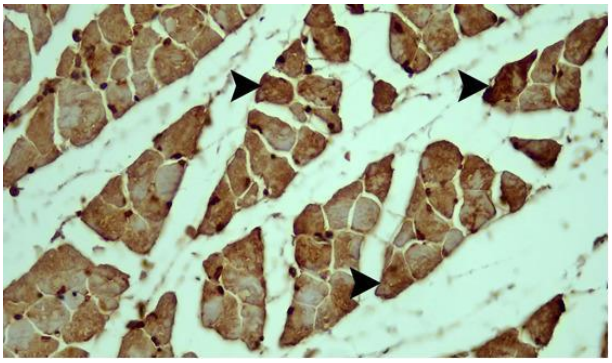

Figure 8. Severe cytoplasmic Caspase 3 expression in m. gastrocnemius sinistra on the 21st day, IHC-P.

DISCUSSION

In the study of Dressler and Saberi (2005), the mechanism of action of BTx-A on the neuromuscular junction was explained and it was stated that BTx-A blocked the release of neuroepinephrine. In a different study, it was stated that the toxin stopped sympathetic vasoconstriction in the smooth muscle cells in the porcine uterine artery and caused chemical sympathectomy (Frank and Erbguth, 2004).

Silva et al. (2021) injected 250U Dysport® preparation into both armpit areas of a female patient suffering from axillary hidradenitis and found that as a result of the treatment, the existing lesions regressed with the use of BTx-A, therefore BTx-A caused excessive eccrine sweat production in focal hyperhidrosis stated that it decreased. We believe that this regression is due to Botulinum toxin blocking the release of acetylcholine from nerve endings, and this is the case with in our study. It was concluded that it is similar to the lack of muscle function in gasrocnemius caused by BTx-A reducing acetylcholine synthesis and the atrophy resulting from this process.

Additionally, as a finding of a study, it was showed that the application of BTx-A to the vasospasm area in 11 patients with Raynaud's phenomenon caused vasodilation due to neuromuscular blocking in smooth muscles, and we have demonstrated that this situation is in full agreement with our study (Van Beek et al., 2007).

In a study conducted in 50 patients with hemiplegia, BTx-A injection was applied to the wrist and finger flexors. These applications were performed under electromyography and ultrasound guidance. While the use of

experimental animals as material and the stereology method as a method in this study differed from the aforementioned study, the results were similar to BTx-A's electromyographic, ultrasound imaging technique and stereological blocking of acetylcholine in the muscles. By observing the results of the research it was determined that the acetylcholine blockade process caused a volume decrease in m. gastrocnemius, and this situation was similar to the positive effect of BTx-A application on spasticity and joint range of motion in 50 patients with hemiplegia. It has also been stated that BTx-A application has a positive effect on daily living activities in the mentioned patients (Akkoç, 2016).

In a study conducted on New Zealand rabbits, the changes caused by BTx-A in muscles were examined electromyographically and electron microscopically, while in this study, the stereology technique and immunohistochemical examination method, which is one of the methods with the highest margin of reliability, were used. As a result of this study, it was observed that BTx-A application at doses of 0.5-2.5 ml caused a volumetric relaxation without causing structural degeneration in the muscle tissue on the 14th day after the application, and it was stated that the situation increased as the dose was increased from 0.5 ml to 2.5 ml. As a finding of this study, it was determined that there was a perfect match between the histological volume loss on the 15th day after the use of BTx-A on m. gastrocnemius sinistra and the results of the mentioned study (Işık, 2017).

Güleş (2018) injected BTx-A into m. gastrocnemius of rats, and after the injection process, starting from day 0, m. examined the volumetric change of the gastrocnemius with Magnetic Resonance Imaging (MRI). The results of the study, which attempted to explain the volumetric changes on the muscle with (MRI) imaging technique, showed that BTx-A caused a clear volume reduction on the 120th day after its use at regular and periodic intervals. While the results of the study are similar to the 2ml dose of BTx-A application in our thesis study, they differ in terms of the expected time after application and the number of groups. In addition, it has been stated that there is a decrease in volume at the cellular level in the muscle tissue within the time it takes for the volume loss to be visible in the MRI method, and the use of the stereology method in our study, which reveals this situation in the most clear and reliable way, supported the findings of the stated study.

In a study, it was stated that Botulinum toxin A, administered to DBTRG glioblastoma and SH-SY5Y neuroblastoma tumor cell lines at 5 IU for 24 hours and in addition anthranilic acid (ACA) combination at 25 µM for 30 minutes, caused activity in caspase 3 and 9 immune markers. In this study, it was aimed to examine the immunohistochemical effect of BTx-A in vitro by using experimental animals with living metabolism instead of cell culture. After BTx-A application, the inflammatory markers of the gastrocnemius sinistra on the 15th and 21st days were examined immunohistochemically, and it was shown that the results in the mentioned study were compatible with our study and BTx-A positively increased the rate of the mentioned immune markers (Akpınar et al., 2020).

Han et al. (2013) examined the proteomic differences in the muscles after BTx-A injection into m. gastronemius of the rat using the western blot technique and confocal microscopy. As a result of this study, it was determined whether there was a change in the ratios of a total of 38 proteins, including the inflammatory marker with Caspase 2 protein structure. and also to examine the histological features in the muscles on the 3rd, 7th, 14th and 56th days after the injection of BTx-A application in terms of indicating a pathological condition. Contrary to the methods in the mentioned study, in our study, we examined the volume change of BTx-A on m. gastrocnemius sinistra with the stereological method, which reveals the volume and cell number most clearly, and also the histological/immunohistochemical findings on the 15th and 21st days after the application of BTx-A applied to m. gastrocnemius sinistra is intended to be revealed. It was determined that the results obtained from our study and both studies were similar. After the application of BTx-A to m. gastrocnemius sinistra, it was observed that there was cytoplasmic Caspase 3 immunohistochemical activity in myocytes. In addition, the results of our study; It was disclosed that the intensity of cytoplasmic Caspase 3 immunohistochemical activity in myocytes increased linearly with the increase in the time (0. 15. 21. days) after BTx-A was applied to the gastrocinemius muscle.

As a result, it was determined that after the application of Botulinum toxin Type A to m. gastrocnemius sinistra, it caused a stereologically significant volume change on the 15th and 21st days, and that there was an increase in caspase 3 antibody examinations immunohistochemically on the mentioned days. When the studies are evaluated, it is

inevitable that there are many research topics about muscles, which are one of the movement systems and elements that are of great importance in terms of vital activities, and therefore examining the mechanism of action on the structure and function of muscles will be of great benefit to humanity. Experimental animals were used in our study in order to provide solutions to diseases frequently encountered especially in the field of physical therapy and rehabilitation and to be able to actually be applied to humans. In addition, the use of the stereology method, which is one of the methods with the effect mechanism of BTx-A, which is widely used today and gives the results are very close to reality, and this method is supported by the immunohistochemical technique, which reveals the structural features of the tissues in the most clear way, constitutes the most important element of our study. Due to the scarcity of similar studies, such as examining any chemical substance administered by the in vivo method using the stereological method in terms of causing any volume change in living tissues, our study will be a reference source in its field and from now on; We believe that it will lead to similar studies on many chemicals, especially BTx-A.

Acknowledgment

This study was summarized from the master thesis named "Stereological and Immunohistochemical Investigation of Botox-Applied Gastrocnemius Muscle in Adult Male Rats" This research, named "Stereological and Immunohistochemical Investigation of Botox-Applied Gastrocnemius Muscle in Adult Male Rats TYL-2022-9745" was supported by the Scientific Research Projects Coordination Unit of Van Yuzuncu Yil University. We thank Van Yuzuncu Yil University Scientific Research Projects Coordination Unit for their support.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

This study was carried out by Van Yuzuncu Yil University approved dated 04/29/2021 and numbered 2021/04-21 by van Yuzuncu Yil University Animal Experiments Local Ethics Committee.

Author Contribution

Material and Method: GÇ, MUD; References: GÇ, MUD; Writing the article: GÇ, MUD

REFERENCES

Akkoç F (2016). İnme Sonrası Spastik Hemipleji Hastalarında El Bilek ve Parmak Fleksör Kaslarında Uygulanan Botulinum Toksin Enjeksiyonlarında Ultrason ve Elektramiyografi Rehberliğinin Retrospektif Olarak Karşılaştırılması. Master Thesis, Celal Bayar Üniversitesi Tıp Fakültesi Fiziksel Tıp ve Rehabilitazyon Anabilim Dalı, Manisa.

Akpınar O, Ozsimsek A, Guzel M, Nazıroglu, M. (2020). Clostridium botulinum neurotoxin A induces apoptosis and mitochondrial oxidative stress via activation of TRPM2 channel signaling pathway in neuroblastoma and glioblastoma tumor cells. *Journal of Receptors and Signal Transduction,* 40(6), 620-632.

Baddeley AJ (1991). Stereology In: Spatial Statistics and Digital Image Analysis. Washington DC, Natural Resources Company, 181-216.

Berne RM, Levy MN, Koeppen BM, Stanton BA (2009). Berne & Levy Physiology. 6th Edition, USA, Elsevier Mosby.

Bulam H (2012). The Inhibitory Effect of Platelet Rich Plasma Mesotheraphy on Botulinum Toxin Type-A Induced Skeletal Muscle Paralysis. Master Thesis, Gazi Üniversitesi, Institute of Health Sciences, Ankara.

Cartee TV, Monheit GD (2011). An overview of botulinum toxins: past, present, and future. *Clinics in Plastic Surgery,* 38(3), 409-26.

Cruz-Orive LM (1999). Precision of Cavalieri sections and slices with local errors. *Journal of Microscopy,* 193(3), 182-198.

Dressler D, Saberi FA. (2005). Botulinum toxin: Mechanisms of action. *European Neurology,* 53(1), 3-9.

Frank J, Erbguth MD (2004). Historical notes on botulism, Clostridium botulinum, botulinum toxin, and the idea of the therapeutic use of the toxin *Movement Disorders* 19(8), 2-6.

Ganong WF (2010). Medical Physiology. 23rd Edition, New York, McGraw-Hill, Companies.

Gundersen HJ, Jensen EB (1987). The efficiency of systematic sampling in stereology and its prediction. *Journal of Microscopy,* 147(3), 229-263.

Güleş ME (2018). Botulinum Toksinin Kas Hacmi Üzerine Etkisi; Deneysel Çalışma. Master Thesis, Bezmialem Vakıf Üniversitesi, Institute of Health Science, İstanbul.

Han Nami, Kim HD, Eom MJ, You JM, Han J, Kim HK, et al. (2013). Proteomic changes in rat gastrocnemius muscle after botulinum toxin an injection. *Annals of Rehabilitation Medicine,* 37(2), 157-166.

Hastad DN, Lacy AC (1998). Measurement and Evaluation in Physical Education and Exercise Science. 3rd edition, Boston, Allyn & Bacon.

Hole JWJ (1981). Human Anatomy and Physiology. 2nd Edition, USA, Wm. C. Brown Company Publishers.

Howard CV, Reed MG (2004). Unbiased Stereology: Three-Dimensional Measurement in Microscopy. 2nd Edition, London, Garland Science, 34-39.

Howard CV (1998). Unbiased Stereology: Three-Dimensional Measurement in Microscopy. 1st Edition, UK, BIOS Scientific Publishers.

Işık VM (2017). Tavşan anterior auricular kas modelinde botulinum toksin A doz değişken etkisinin; görsel elektronöromiyografik ve elektromikroskobik olarak değerlendirilmesi. Master Thesis, Ankara Eğitim ve Araştırma Hastanesi, Plastik, Rekonstrüktif ve Estetik Cerrahi Kliniği, Ankara.

Odaci E, Yıldırım S, Bahadır A, Canan S, Sahin B, Bas O et al. (2004). The possible error sources of new stereological methods and their solutions. *Turkiye Klinikleri Journal of Medical Sciences,* 24, 78-87.

Sikandar A, Cheema AH, Younus M, Zaneb H (2013). Mycobacterium avium subspecies paratuberculosis multibacillary infection (Johne's disease) in a Teddy goat. *Pakistan Veterinary Journal,* 33(2), 260-262.

Silva EG, Lima JJC, Costa NP (2021). Use of botulinum toxin in hidradenitis suppurativa. *Surgical & Cosmetic Dermatology,* 13:e20210013.

Thomas CE, Combs CM (1965). Spinal cord segments. B. Gross structure in the adult monkey. *American Journal of Anatomy,* 116(1), 205-216.

Tighe AP, Schiavo G (2013). Botulinum neurotoxins: Mechanism of action. *Toxicon,* 67, 87-93.

Van Beek AL, Lim PK, Gear AJL, Pritzkerve MR (2007). Management of vasospastic disorders with botulinum toxin A. *Plastic and Reconstructive Surgery,* 119(1), 217-226.

Vander AJ, Sherman JH, Luciano DS (1994). Human Physiology: The Mechanism of Body Function. 6th Edition, New York, McGraw-Hill.

Weineck J (1998). Sporda İşlevsel Anatomi. (Çev. AS Elmacı) Ankara, Bağırgan Yayınevi.