Research Paper
Muscular Injection of Botox Exacerbates Seizures Through TNF-alpha and Oxidative Stress in Mice

Majid Hassanpourezatti1*, Mehdi Hosseini1

1. Department of Biology, Faculty of Basic Sciences, Shahed University, Tehran, Iran.

**Background:** Recent research has revealed the central adverse effects of Botox after intramuscular injection. The aim of this study was to examine the role of brain oxidative stress factors and circulatory cytokines as indicators of the severity of seizures following acute intramuscular (IM) injection of Botox in mice.

**Methods:** Botox (1, 5 and 30 U, IM) was injected 60 minutes before inducing maximal electroshock (MES) seizures. Nitric oxide (NO), malondialdehyde (MDA) and glutathione (GSH) levels were measured in the brain. Tumor necrosis factor-alpha (TNF-α) levels were also determined in the serum. The motor coordination was assessed after Botox administration by using the chimney test.

**Results:** Botox (5 and 10 U/kg, IM) significantly reduced the duration of hindlimb extension (HLE) and elevated levels of NO and MDA in the brain compared to the seizure group. Additionally, the administration of Botox (1 and 5 U, IM) increased the level of GSH in the brain, while 30 U decreased it. All Botox dosages demonstrated an enhancing effect on serum TNF-α levels compared to the seizure group. Botox at 5 and 30 U induced locomotor incoordination in mice.

**Conclusion:** Our results showed that IM injection of Botox can lead to the exacerbation of tonic-clonic seizures by stimulating oxidative stress in the brain and increasing circulating TNF-α levels in mice.

**ABSTRACT**

**Background:** Recent research has revealed the central adverse effects of Botox after intramuscular injection. The aim of this study was to examine the role of brain oxidative stress factors and circulatory cytokines as indicators of the severity of seizures following acute intramuscular (IM) injection of Botox in mice.

**Methods:** Botox (1, 5 and 30 U, IM) was injected 60 minutes before inducing maximal electroshock (MES) seizures. Nitric oxide (NO), malondialdehyde (MDA) and glutathione (GSH) levels were measured in the brain. Tumor necrosis factor-alpha (TNF-α) levels were also determined in the serum. The motor coordination was assessed after Botox administration by using the chimney test.

**Results:** Botox (5 and 10 U/kg, IM) significantly reduced the duration of hindlimb extension (HLE) and elevated levels of NO and MDA in the brain compared to the seizure group. Additionally, the administration of Botox (1 and 5 U, IM) increased the level of GSH in the brain, while 30 U decreased it. All Botox dosages demonstrated an enhancing effect on serum TNF-α levels compared to the seizure group. Botox at 5 and 30 U induced locomotor incoordination in mice.

**Conclusion:** Our results showed that IM injection of Botox can lead to the exacerbation of tonic-clonic seizures by stimulating oxidative stress in the brain and increasing circulating TNF-α levels in mice.

**Keywords:** Botulinum toxins, Seizures, Oxidative stress, Neuroinflammation, Brain

* Corresponding Author:
Majid Hassanpourezatti, Assistant Professor.
Address: Department of Biology, Faculty of Basic Sciences, Shahed University, Tehran, Iran.
Tel: +98 (21) 51212252
E-mail: hassanpourezatti@gmail.com, hassanpour@shahed.ac.ir

Copyright © 2024 The Author(s).
This is an open access article distributed under the terms of the Creative Commons Attribution License (CC-By-NC. https://creativecommons.org/licenses/by-nc/4.0/legalcode.en), which permits use, distribution, and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.
Introduction

Increased oxidative stress in the central nervous system (CNS) and circulatory cytokines can change the glutamate/gamma-aminobutyric acid (GABA) balance and lead to seizure discharges in brain neurons [1]. The severity of convulsions has been linked to elevated levels of oxidative stress factors and reduced antioxidants in the brain, along with changes in blood cytokine levels [2-6]. Consistent with these findings, the maximal electroshock (MES)-induced seizure model in mice has demonstrated an imbalance between oxidants and antioxidants, as well as increased blood cytokine levels correlating with seizure severity [7, 8]. Accordingly, understanding the mechanisms by which oxidative stress and inflammatory factors exacerbate seizures, and vice versa, may offer new insights for preventing seizures and epilepsy in individuals susceptible to such conditions following the use of commonly prescribed therapeutic and cosmetic agents.

Intramuscular (IM) injection of Botulinum toxin (Botox) has been used as an anti-spasticity and analgesic medication [9, 10]. Recently, another research group has also demonstrated that a specific type of Botulinum neurotoxin exhibits convulsant effects after intrahippocampal injection [11]. Botox is commonly employed for its neuromuscular blocking action after intramuscular injection [12]. Data from an electrophysiological study indicated that injecting Botox into the intra-gastrocnemius muscle increased excitability in spinal motoneurons [13]. Additionally, a clinical study revealed alterations in cortical neuron activity following intramuscular Botox injection [14, 15]. Injection of Botox into some brain areas has been observed to increase neuronal excitability [16-18]. Furthermore, inflammatory reactions have been reported after high-dose intramuscular injections of Botox [19]. Botox can be retrogradely transported along axons to central motor neurons and trans-synaptically reach the cerebral cortex, triggering neuroinflammatory responses in glial cells [20]. Toll-like receptor 2 signaling has been implicated in both neuroinflammation and demyelination in response to Botox administration by macrophages [21]. On the other hand, studies have indicated anti-seizure activity for different types of Botulinum toxin [22, 23]. The present study was conducted to investigate the effect of intra-gastrocnemius injection of Botox on MES-induced seizures, brain oxidative/antioxidative biomarkers, and blood TNF-α levels in mice.

Materials and Methods

Botox (Allergan Inc., Dublin, Ireland), sodium valproate powder (Rouz Darou Pharmaceutical Co., Tehran, Iran), mice TNF-α enzyme-linked immunosorbent assay (ELISA) kit (Karmania Pars Gene Company, Rafsanjan, Iran), (nitric oxide [NO] [Nampox™], malondialdehyde (MDA) (Nalondi Kit) and GSH (Nargol kit) were obtained from Navand Salamat Co., Iran. Botox was prepared at concentrations of 1, 5 and 30 U/mL in phosphate-buffered saline (PBS) and injected intramuscularly (IM) into the gastrocnemius muscle of mice (n=10 per group) 60 minutes before MES-induced seizures [24]. The doses were selected based on previously published studies [25-27]. Sodium valproate was dissolved in a saline solution and administrated intraperitoneally (IP) at a volume of 1 mL/100 g of body weight. Control animals received equal volume injections of the vehicle PBS.

Experimental animals

Male Swiss albino mice (20–25 g) were used in the experiment (n=10 in each group). They were housed in groups of five per cage in a standard temperature-controlled room (23±2°C) with 60±5% humidity and a 12 hour light/dark cycle. All experiments were conducted between 8:00 AM. and 3:00 PM. following a 30 minute acclimatization period to the laboratory conditions. The mice were randomly divided into the following groups: 1) Receiving a single intramuscular injection of normal saline (1 mL); 2) Receiving normal saline (1 mL, IM) 60 min before MES; 3) Receiving Botox at doses of 1, 5 and 30 U/kg, IM, 60 min before inducing MES; and 4) Receiving valproate sodium at 400 mg/kg IP, 60 min before inducing MES, serving the positive control group.

MES-induced seizure model

Convulsions were using electrical alternating current (50 Hz, 25 mA, 0.2 s) administered through ear-clip electrodes (stainless steel alligator clips covered with saline-moistened gauze) connected to an electroshock stimulator (Borj Sanat, Iran) [28]. Seizure severity was determined by measuring the duration (in seconds) of tonic hindlimb extension (HLE). The experiments were performed following the NIH guide for the Care and Use of Laboratory Animals. Subsequent to recording the HLE duration, blood samples were gathered from the retro-orbital venous plexus of the mice. The samples were incubated at room temperature for 30 minutes, and serum was isolated by centrifugation at 1500 g for 10 minutes. The serum samples were preserved at -80°C until TNF-α level assessment. All animals were eutha-
nized, and their brains were extracted from the skull and submerged in ice-cold PBS. The cerebral cortices of the mice were harvested and sonicated using an ultrasound homogenizer (Hielscher, UP200H).

Assessment of oxidative/antioxidative stress biomarkers

After centrifugation, the supernatant was collected, and the concentrations of NO, MDA and GSH in brain supernatants were quantified respectively by the Natrix™ Kit, thiobarbituric acid reactive substances (TBARS) test, and NarGul™–Glutathione (GSH) Assay Kit-GSH (Navand Salamat, Urmia, Iran) based on the manufacturer’s instructions [29, 30]. The protein content in supernatants was assayed using the Bradford method by a commercial standard kit (Nadford™, Navand Salamat).

Assessment of inflammatory biomarkers

Serum TNF-α levels were determined by the ELISA method (Karmania Pars Gene Company, Rafsanjan, Iran).

Motor coordination assay

The motor dysfunction of mice was evaluated by a chimney test [31]. In this test, the animals were observed moving backward and upwards in a vertical cylinder (3 cm inner diameter, 25 cm long) 45 minutes post-Botox administration. Failure of the animals to ascend backward into the cylinder within 60 seconds was indicative of motor incoordination.

Statistical analysis

The results were analyzed by GraphPad Prism software, version 5.0 using one-way ANOVA, followed by the Tukey-Kramer test for multiple comparisons. Data are presented as Mean±SEM (n=10). The statistical program used was Fisher’s exact probability test was used to analyze qualitative variables from the chimney test. Statistical significance was considered at P<0.05.

Results

Botox enhanced MES-induced seizure severity

The effect of Botox on HLE duration is presented in Figure 1A. Injection of Botox at 1 U (IM), had no significant impact on the duration of tonic HLE in the MES model (Figure 1A). However, Botox at 5 and 30 U (IM) significantly increased the duration of HLE in mice. Conversely, sodium valproate (400 mg/kg, IP), used as a positive control, significantly reduced (P<0.001) the HLE duration in MES-induced seizures compared to the saline+MES group (Figure 1A).

Botox increased brain NO levels in mice receiving MES

MES-induced seizures had no significant effect on brain NO levels of mice compared to saline-treated control animals (Figure 1B). In mice pretreated with Botox, MES-induced seizures exhibited a tendency to elevate brain NO content in a dose-independent manner as opposed to the saline+MES group.

Botox increased brain MDA content in mice receiving MES

MES-induced seizures also significantly (P<0.01) increased brain MDA levels compared to the saline-treated control group (Figure 1C). Pretreatment with all doses of Botox intensified the enhancing effect of MES on brain MDA concentration when compared to the saline+MES group.

Botox enhanced brain GSH levels in mice receiving MES

MES-induced seizures significantly (P<0.01) increased brain GSH levels compared to the saline-treated control group (Figure 1D). The administration of Botox at 1 and 5 U significantly amplified the effect of MES-induced seizures on GSH levels in the brain, while at 30 U it decreased it compared to the saline+MES-induced seizure group.

Botox increased circulatory TNF-α levels of mice receiving MES

Seizure induction by MES significantly (P<0.01) increased serum TNF-α levels in mice. Acute administration of Botox dose-dependently elevated serum TNF-α levels in mice following MES-induced seizures (Figure 2).

Locomotor coordination

The statistical analysis revealed that the sole administration of 30 U of Botox significantly (P<0.0001) induced motor impairments in the chimney test (Table 1). This outcome suggests the onset of motor dysfunction following the administration of 30 U of Botox and seizure induction.

The mean time spent by each group of mice to climb the chimney was presented as Mean±SEM time. The
Figure 1. Determination of A) Hind leg extension (HLE) duration, B) NO, C) MDA and D) GSH levels in the brain of mice treated with Botox (1, 5 and 30 U/kg, IP) after MES-induced seizures.

Note: Animals were sacrificed 30 minutes after the behavioral response. Each bar represents the Mean±SEM of 10 animals per group; **P<0.01 compared with the saline+MES group (one-way ANOVA followed by Dunnett’s test).
chimney test time was statistically analyzed by Fisher’s exact probability test; "P<0.01 compared to the vehicle-treated group.

Discussion

The findings of the present study showed that IM injection of Botox in mice triggers seizures by activating oxidative stress in the brain and increasing TNF-α levels in the blood. This outcome contradicts a case study that indicated that intramuscular injection of Botulinum toxin reduces refractory focal motor convulsions [32]. These findings align with those of Montastruc et al. [33] and Caleo and Restani [34] on the adverse effects of Botox. Our results revealed a significant rise in brain NO and MDA levels, a decrease in GSH levels, and an increase in TNF-α levels in the serum of the Botox-treated group. The results are consistent with findings indicating that reported MES-induced seizures lead to an imbalance in oxidative and antioxidant levels in the brain and an increase in inflammatory factors [35]. The underlying mechanism for another neurotoxin, such as kainic acid, also involves an increase in NO and MDA levels in the brain, as well as an up-regulation in the expression of inflammatory cytokines [36].

In this study, Botox pretreatment led to the exacerbation of seizure severity after MES. This observation is supported by other studies demonstrating that cholinesterase inhibitors increase seizure severity [37]. Additionally, the current study revealed that the administration of Botox before MES-induced seizures significantly elevated NO and MDA levels while concurrently decreasing GSH levels in the brain. The presence of oxidative stress and a reduction in antioxidant reserves in brain tissue is indicative of mitochondrial dysfunction, a phenomenon observed in some individuals following Botox treatment [38]. Previous evidence has demonstrated that MDA is not only generated in the nervous system due to seizures but also contributes to the worsening of seizures [39, 40]. Ho et al. [41] showed neuroinflammation and oxidative stress following the administration of Botox. The upregulation of AMPA receptors and endocytosis of GABAergic receptors in CNS neurons are mechanisms.

Table 1. Effect of Botox administration on motor coordination of mice receiving MES-induced seizures in the chimney test

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Mean±SEM (Time (s))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle+MES seizures</td>
<td>16.3±1.7</td>
</tr>
<tr>
<td>Botox+MES seizures (1 U/kg)</td>
<td>17.3±1.1</td>
</tr>
<tr>
<td>Botox+MES seizures (5 U/kg)</td>
<td>14.8±1.9</td>
</tr>
<tr>
<td>Botox+MES seizures (30 U/kg)</td>
<td>10±2.5**</td>
</tr>
</tbody>
</table>

S: Second.
activated by TNF-α, which may mediate the effect of Botox in enhancing seizures [42]. The findings showed an increase in GSH levels in the brains of Botox-treated mice following seizure induction. According to the literature, our results showed an increase in GSH levels in the brain, which strongly supports how seizure activity can lead to the accumulation of ROS in the brain. Consequently, epileptic animals have higher GSH levels than controls to combat seizures [43, 44]. In this regard, Botox has been shown to enhance antioxidant defense mechanisms by activating antioxidant genes and inhibiting proinflammatory cytokines [45-47]. Botox treatment before MES-induced seizures also resulted in an increase in blood TNF-α levels. A direct correlation was observed between blood TNF-α levels and seizure severity [48]. In addition, Botox treatment could increase C-reactive protein and TNF-α levels in the serum [49].

Conclusion

The present study showed that inducing a redox imbalance in the brain and elevating TNF-α levels in the blood are critical risk factors for the exacerbation of generalized tonic-clonic seizures following intramuscular injection of Botox in mice.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of Shahed University (Code: IR.SHAHED.REC.1399.146).

Funding

This research did not receive any grant from funding agencies in the public, commercial, or non-profit sectors.

Authors' contributions

Conceptualization, methodology, supervision, funding administration, data analysis and writing: Majid Hassanpourezatti; Investigation and data collection: All authors.

Conflict of interest

The authors declared no conflict of interest.

Acknowledgements

The authors express their gratitude to the Vice-Chancellor of Research at Shahed University.

References


