Intramuscular BoNT/A injections cause an inflammatory response in the muscle tissue of rats

Jessica Pingel1, Alexander Pacolet1, Betina Elfving2 and Litsa N Ledri1

Abstract

Objectives: The purpose of the present study was to investigate whether intramuscular BoNT/A injections cause an systemic inflammatory response and a local inflammatory response in the muscle tissue.

Methods: Thirty-two male Sprague Dawley rats treated with BoNT/A (i.m., 1IU) were divided in four groups, depending on the time of BoNT/A injection (2 days before, 1, 2, and 4 weeks before the experiment). Bio-Plex Pro Rat Cytokine 23-plex Multiplex Assay (Bio-Rad, USA).

Results: Systemic inflammation: 17 cytokines (IL-1α (p = 0.005), IL-1β (p = 0.01), IL-2 (p = 0.04), IL-4 (p = 0.03), IL-6 (p = 0.03), IL-10 (p = 0.02), IL-12(p70) (p = 0.03), IL-13 (p = 0.04), IL-17 (p = 0.03), GM-CSF (p = 0.03), INF-γ (p = 0.03), MIP-1α (p = 0.03), MIP-3α (p = 0.04), RANTES (p = 0.001), TNF-α (p = 0.04), vascular endothelial growth factor (p = 0.03), and MCP-1 (p = 0.02)) showed significantly higher expression levels 2 days after intramuscular BoNT/A injections compared to other time points (1, 2, and 4 weeks). Local inflammation: 12 cytokines (IL-1β (p = 0.02), IL-6 (p = 0.002), IL-10 (p = 0.02), IL-13 (p = 0.04), IL-17 (p = 0.02), TNF-α (p = 0.001), GM-CSF (p = 0.01), M-CSF (p = 0.04), MIP-1α (p = 0.04), MIP-3α (p = 0.002), RANTES (p = 0.02), and MCP-1 (p = 0.004)) showed higher expression levels 2 and/or 4 weeks after intramuscular BoNT/A injections compared to the other time points (2 days and 1 week).

Conclusion: Intramuscular BoNT/A injections result in a rapid systemic inflammatory response that only lasts a couple of days. At the same time, intramuscular BoNT/A injections cause an inflammatory response locally in the muscle with significantly higher cytokine levels 2 and/or 4 weeks after injections.

Keywords
BoNT/A, inflammation, cytokines, muscle, rat

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Introduction

Botulinum toxin-A (BoNT-A) is the first biological toxin that has been licensed for treatment of human disease, after the pioneering work of the ophthalmologist Alan Scott1 who used BoNT/A for the treatment of strabismus. BoNT/A is a neurotoxin that can paralyze muscles by inhibiting the release of acetylcholine which prevents muscle contraction.2 Today, the list of diseases that are treated with BoNT/A is long and includes cervical dystonia,3 blepharospasm,4 migraine,5 mandibular recontouring,6 fascial wrinkles,7 and

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numerous movement disorders.\textsuperscript{8} In cerebral palsy, BoNT/A injections are commonly used as a treatment for spasticity and in order to prevent the development of contractures.\textsuperscript{9}

However, one recent Cochrane study investigating the effects of BoNT/A injections on lower limb spasticity in children with CP showed that there was only limited evidence that BoNT/A was more effective than placebo treatment with regard to improvements of gait and range of motion.\textsuperscript{10} Furthermore, only a few studies have investigated the effect of BoNT/A injections on the muscle tissue level in children with CP. Muscle atrophy is a common side effect of BoNT/A injections on lower limb spasticity in children with CP showed that there was only limited evidence that BoNT/A was more effective than placebo treatment with regard to improvements of gait and range of motion.\textsuperscript{10} Furthermore, only a few studies have investigated the effect of BoNT/A injections on the muscle tissue level in children with CP. Muscle atrophy is a common side effect of chemical denervation.\textsuperscript{11,12} Additionally, considerable fiber atrophy has been observed after BoNT/A injections into the longissimus dorsi muscle of rabbits.\textsuperscript{13} One study observed changes in the expression of various proteins after intramuscular BoNT/A injections in rats when compared with saline injections.\textsuperscript{14} In fact, 38 proteins were associated with alterations of energy metabolism, contractile function of the muscle, transcription and translation, cell proliferation, and cellular stress response.\textsuperscript{14}

Recently, we have demonstrated significant changes of the microstructure in muscle tissue, muscle atrophy, and loss of motor control which lead to an impaired gait after intramuscular BoNT/A injections in rats.\textsuperscript{15}

Muscle atrophy is often associated with an inflammatory response inside the muscle.\textsuperscript{16,17} Various pro-inflammatory cytokines including C-reactive protein (CRP), interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-\(\alpha\)) have been linked to muscle atrophy and altered protein homeostasis in the muscle.\textsuperscript{18-20} In addition, both IL-6 and IL-1 administration in rats have shown to increase the breakdown of myofibrillar proteins in skeletal muscle tissue.\textsuperscript{21,22} Conditions that can provoke an inflammatory response in muscle tissue includes heart conditions,\textsuperscript{17} exercise,\textsuperscript{23} muscle injury,\textsuperscript{24} and immobilization.\textsuperscript{25}

One study showed that 4 weeks of immobilization caused an increase of TNF-\(\alpha\), interleukin-1-beta (IL-1\(\beta\)), IL-6, and nerve growth factor (NGF) in rats.\textsuperscript{26} Furthermore, high circulating levels of the pro-inflammatory cytokine IL-6 has shown to act as mediator of skeletal muscle proteolysis in patients with cancer cachexia.\textsuperscript{27} The same research group showed that an overexpression of IL-6 in transgenic mice caused muscle protein degradation and increased levels of cathepsin in skeletal muscle, indicating that IL-6 is directly involved in the regulation of muscular atrophy.\textsuperscript{28} Furthermore, whether intramuscular BoNT/A injections causes intramuscular inflammation as seen after immobilization is also still unclear.\textsuperscript{29}

The aim of the present study is to investigate whether intramuscular BoNT/A injections cause a systemic inflammatory response, and further whether the chemical denervation of the muscle following BoNT/A injections causes local inflammatory responses in the muscle tissue.

**Methods**

**Animals**

All experiments were conducted in accordance with the guidelines of the European Union (EU) Directive 2010/63/EU and were approved by the Danish Animal Experiments Inspectorate (License number: 2015—15—0201—00689). Thirty-two male Sprague Dawley rats (©2021 Taconic Biosciences, Albany NY, USA) treated with BoNT/A (i.m., 1IU) were divided in four groups, depending on the time of BoNT/A injection (2 days before, 1, 2, and 4 weeks before the experiment). Two rats were housed in each cage and were kept in a 12/12 light dark cycle with access to water and food ad libitum.

**Injections**

All animals were anesthetized with 2% isoflurane. Both hindlimbs were shaved, and the skin was disinfected. Then, a total of 1UI BoNT/A (botulinum toxin type A (Allergan\textsuperscript{30}Inc. Dubline, Ireland)) was injected in 10 µl saline into the muscles of the triceps surae (medial gastrocnemius, the lateral gastrocnemius, and soleus) muscle using a 0.5 mL syringe (Omnican\textsuperscript{31}® 20 BRAUN, Germany), and the same injection volume of saline was injected in the contralateral control leg. This dose was chosen in order to avoid any distress in the animals during the experiment. Until termination of the experiment, the welfare of the rats was routinely checked for signs of dehydration or distress etc. The rats were weighed every day following the injection in order to monitor weight loss. No weight loss or any signs of distress occurred in the rats during the experiment.

**Blood sampling**

While the animals were anesthetized, blood samples were withdrawn from a tail vein using the vacuum blood collection method\textsuperscript{32} and 2 mL collection tubes (VACUETTE® Greiner Bio-One International GmbH, Austria). The volume of the taken blood samples ranged from 600 µl to 1 mL blood. The blood samples were then spun down and the plasma was separated and stored at \(-80^\circ\text{C}\) for further multiplex analysis.

**Tissue preparation**

The rats were anesthetized by 2% isoflurane. The medial gastrocnemius was removed and dissected into smaller pieces with a scalpel (Swann-Morton, Mediq Denmark A/S). The wet weight of the triceps surae was measured immediately after removal. One piece of each muscle head was snap frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) for further multiplex analysis. After the harvest,
the animals were euthanized using pentobarbital injections into the heart while the animals were still under anesthesia (2% isoflurane). Sample preparations for the multiplex analyses were as follows: The tissue samples were dissolved in lysate using the Bio-Plex tissue sample diluent and plasma sample were diluted in the Bio-Plex plasma diluent according to the manufacturer's protocol (Bio-Rad, USA). The prepared samples were loaded on the plates and the analyses were carried out by carefully following the manufacturer’s protocol (Bio-Rad, USA).

**Multiplex assays**

The levels of 23 cytokine and neurotrophin proteins were measured using a Multiplexing method (Bio-Plex Pro Rat Cytokine 23-plex Assay (Bio-Rad, USA, #12005641) which included the following targets: interleukin (IL)-1-alpha (α), interleukin (IL)-1-beta (β), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-10 (IL-10), interleukin-12 (IL-12(p70)), interleukin-13 (IL-13), interleukin-17 (IL-17), interleukin-18 (IL-18), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), C-X-C motif chemokine ligand 1 (GRO-KC), interferon (INF)-gamma(γ), monocyte chemoattractant protein 1 (MCP-1), macrophage stimulating factor (M-CSF), macrophage inflammatory protein 1-alpha (MIP1-α), macrophage inflammatory protein 3-alpha (MIP3-α), chemokine (C-C motif) ligand 5 (RANTES), tumor necrosis factor-alpha (TNF-α), and vascular endothelial growth factor (VEGF).

The sensitivity ranges of all targets are shown in Table 1. The assays were run on a Bio-Plex 200 System (Luminex xMAP Technology, BIO-RAD; #171000201) following the manufacturer’s instructions. A Bio-Plex Pro Wash Station was used for washing steps (Bio-Rad; #30034376). Muscle samples were diluted in the ratio of 1:2 and plasma samples were diluted in the ratio of 1:4 in sample diluent included in the kit. All procedures were followed as suggested by the manufacturer.

**Statistics**

All data are presented as mean ± SEM. Plasma analysis: Changes in cytokine expression over time were investigated using a one-way ANOVA with time as independent factor and observed concentration as outcome variable using SigmaPlot version 13 (Systat Software, Inc. SigmaPlot for Windows, San Jose, California, USA). For plasma samples, a Holm–Sidak post hoc test was applied in order to analyze differences between the different time points. Muscle analysis: A two-way ANOVA was performed with time and group as independent factors and observed concentration as outcome variable in order to analyze the cytokine expression over time and between the injected and the control leg using SigmaPlot version 13 (Systat Software, Inc. SigmaPlot for Windows, San Jose, California, USA). The comparison between the BoNT/A-injected leg and the control leg was performed by a paired Student’s t-test. In case of a significant main effect, a post hoc test was applied in order to analyze differences between the different time points. A p-value of <0.05 was considered to be significant. All figures were made in GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA).

**Results**

**General observations**

The results of the present study show that intramuscular injections cause a rapid systemic inflammatory response. Two days after BoNT/A injections, there are high levels of cytokines in the plasma that abate fast and are already significantly lower one week after injection. The cytokine levels in the muscle, however, show steady increasing cytokine levels throughout the 4-week protocol.

**Systemic plasma cytokine levels**

The plasma levels of all targets that showed significant changes locally in the muscle are shown in Figures 1 and 2. The plasma levels of the following targets showed
significant changes over time and the 2-day time point showed significantly higher expression levels than the other time points: IL-1α ($p = 0.005$), IL-1β ($p = 0.01$), IL-2 ($p = 0.04$), IL-4 ($p = 0.03$), IL-6 ($p = 0.03$), IL-10 ($p = 0.02$), IL-12p70 ($p = 0.03$), IL-13 ($p = 0.04$), IL-17 ($p = 0.03$), GM-CSF ($p = 0.03$), INF-γ ($p = 0.03$), MIP-1α ($p = 0.03$), MIP-3α ($p = 0.04$), RANTES ($p = 0.001$), TNF-α ($p = 0.04$), VEGF ($p = 0.03$), and MCP-1 ($p = 0.02$).

The following targets did not show any significant changes in plasma cytokine levels: IL-5 ($p = 0.665$), IL-7 ($p = 0.36$), IL-18 ($p = 0.31$), G-CSF ($p = 0.81$), and M-CSF ($p = 0.72$).

All the targets that revealed significant changes over time showed that the plasma levels of each target was significantly increased 2 days after BoNT/A injection compared to the other time points (1 week, 2 weeks, and 4 weeks).

**Muscle tissue cytokine levels**

The cytokine levels in the muscle tissue after intramuscular BoNT/A injections changes significantly over time in the following cytokines: IL-1β ($p = 0.02$), IL-6 ($p = 0.002$), IL-10 ($p = 0.02$), IL-13 ($p = 0.04$), IL-17 ($p = 0.02$), TNF-α ($p = 0.001$) (Figure 3) and further GM-CSF ($p = 0.01$), M-CSF ($p = 0.04$), MIP-1α ($p = 0.04$), MIP-3α ($p = 0.002$), RANTES ($p = 0.02$), and MCP-1 ($p = 0.004$) (Figure 4).

The targets that showed significant changes over time showed significant changes at the following time points: IL-1β (2 weeks $p = 0.02$; 4 weeks $p = 0.02$), IL-6 (1 week $p = 0.003$, 4 weeks $p = 0.006$), IL-10 (2 weeks $p = 0.01$, 4 weeks $p = 0.02$), IL-13 (4 weeks $p = 0.04$), IL-17 (2 weeks $p = 0.02$), TNF-α (2 weeks $p = 0.001$, 4 weeks $p = 0.01$) (Figure 3) and further GM-CSF (1 week $p = 0.001$, 2 weeks $p = 0.003$).
The following targets did not show significant changes: IL-1α (p = 0.86), IL-2 (p = 0.29), IL-4 (p = 0.08), IL-5 (p = 0.82), IL-7 (p = 0.11), IL-12p70 (p = 0.83), IL-18 (p = 0.46), G-CSF (p = 0.33), GRO-KC (p = 0.38), IFN-γ (p = 0.61), and VEGF (p = 0.29).

**Discussion**

The systemic levels of 17 cytokines were significantly increased 2 days after injection when compared to the other time points (1 week, 2 weeks, and 4 weeks). However, these increased cytokine levels decreased very quickly and already one week after injection the cytokine levels were significantly lower and stayed significantly lower throughout the entire protocol.

Increased systemic levels of cytokines after intramuscular injections are also seen after other treatments such as vaccine injections. One study showed that the inflammatory response of different cytokines after intramuscular vaccine injections increased and peaked within 3 to 48 hours and then abated to baseline levels after 7 days. This response is somewhat similar to the acute systemic inflammatory response that has been observed in the present study where several cytokines show high systemic levels 2 days after BoNT/A injection which then abate to what can be assumed close to normalized levels 4 weeks after injection (Figures 1 and 2). Such a rapid increase of cytokines after intramuscular injections is not dangerous and is a

![Figure 2](image-url). Plasma cytokine levels after intramuscular BoNT/A injections at four different time points (2 days after BoNT/A injection, 1 week after BoNT/A injection, 2 weeks after BoNT/A injection, and 4 weeks after BoNT/A injection). * indicates an overall significant difference over time. # indicates a significant time point when compared to the other time points. Data are shown as mean ± SEM.
normal immune response that is necessary for the development of adaptive immunity.\textsuperscript{31}

In addition, when analyzing the muscle tissue for cytokine levels at the same time points, this study revealed that the cytokine levels were significantly increased in the BoNT/A-injected leg when compared to the control leg. Furthermore, the cytokine levels were significantly increased two and/or 4 weeks after intramuscular BoNT/A injections. Previous studies have determined the cytokine expressions in the skeletal muscle of rats after denervation using unilateral sciatic nerve transections.\textsuperscript{32} Here, an increase of pro-inflammatory cytokines, including IL-6, was observed in the muscle tissue that increased with time and peaked at the last time point of the protocol 4 weeks post-denervation.\textsuperscript{32}

Figure 3. Muscle tissue cytokine levels after intramuscular BoNT/A injections at four different time points: 2 days after BoNT/A injection, 1 week after BoNT/A injection, 2 weeks after BoNT/A injection, and 4 weeks after BoNT/A injection. The open circles represent the control leg and the closed black squares represent the BoNT/A-injected leg. * indicates an overall significant difference over time. # indicates a significant difference at a certain time point when compared to the other time points. Data are shown as mean ± SEM.

Also, immobilization studies have shown an increase of cytokine levels at tissue level.\textsuperscript{26} One study showed that cast immobilization did not provoke any early inflammatory response when measured one and 3 days after casting.\textsuperscript{33} However, after one and 2 weeks of immobilization, a significant increase of skeletal muscle macrophages was observed, indicating that macrophage infiltration may play an important role in mediating the development of muscle atrophy during immobilization.\textsuperscript{33} Since there is no rapid inflammatory response in the muscle tissue as observed in the plasma, these findings indicate that the intramuscular inflammatory response is a response to the chemical denervation of the muscle and not a response to the injection itself. Furthermore, it is well known that cytokine levels increase after immobilization and can act on muscle tissue.
Figure 4. Muscle tissue cytokine levels after intramuscular BoNT/A injections at four different time points: 2 days after BoNT/A injection, 1 week after BoNT/A injection, 2 weeks after BoNT/A injection, and 4 weeks after BoNT/A injection. The open circles represent the control leg and the closed black squares represent the BoNT/A injected leg. * indicates an overall significant difference over time. # indicates a significant difference at a certain time point when compared to the other time points. Data are shown as mean ± SEM.
development. However, besides the increased stiffness, individuals with cerebral palsy also have significantly smaller muscles than their typical developed peers.\textsuperscript{42} Recent studies suggest that the smaller muscles might be explained by impaired muscle growth.\textsuperscript{33,44}

One of the side effects that intramuscular BoNT/A injections have is muscle atrophy.\textsuperscript{45} In some situations where BoNT/A is used, muscle atrophy is a favorable effect. An example is when BoNT/A injections are used for muscle contouring in plastic surgery settings.\textsuperscript{36,47} However, when treating individuals with cerebral palsy, muscle atrophy is rather an unwanted side effect.\textsuperscript{48} Even though we did not measure muscle atrophy in the present study, the long-lasting presence of inflammatory cytokines might indicate muscle atrophy after intramuscular BoNT/A injections. Furthermore, in the present study, it was unfortunately not possible to compare the serum concentrations of all cytokines with a control group. In this study, the rats were their own controls which enabled a paired comparison between the BoNT/A-injected leg and the saline-injected leg. However, the systemic levels of the cytokines could only be compared at different time points. Nevertheless, a single saline injection should theoretically not cause any lasting systemic inflammatory response. In the present study, 32 rats were divided into four groups (2 days, 1 week, 2 weeks, and 4 weeks). The number of included animals was not based on a prior sample size calculation since the use of multiplex analyses with more than 20 target proteins (variables) makes it difficult to validly estimate the required sample size to reach 80\% power for each variable. However, in previous studies, we have successfully used \((n = 8)\) rats in each group in order to investigate the effect of intramuscular BoNT/A injections in rats.\textsuperscript{15,49} Another limitation in the present study is that only male rats were used. We cannot rule out that differences would occur when comparing male and female rats. Finally, the rats that were investigated in the present study were still under development. We cannot rule out that full-grown adult rats might have reacted differently on the BoNT/A injections. However, BoNT/A injections are a routine treatment in children with CP; therefore, the present findings of how intramuscular BoNT/A injections affects juvenile rats is, nevertheless, important.

## Conclusion

In summary, the present study observed a rapid systemic inflammatory response to intramuscular BoNT/A injections that abated quickly and was significantly decreased already one week after injection. Altogether, the systemic expression of 17 cytokines were significantly increased 2 days after injection when compared to the other time points (1 week, 2 weeks, and 4 weeks) post-injection. On the other hand, 12 inflammatory cytokines were significantly increased locally in the muscle tissue at either two and/or 4 weeks after BoNT/A injection when compared to the other time points and were also significantly elevated when compared to the control leg. These findings indicate that the intramuscular inflammatory response occurs as a response to the chemical denervation rather than to the injection itself and might be an indication of muscle atrophy.

## Perspectives

In order to further elaborate the inflammatory response in skeletal muscle after intramuscular BoNT/A injections, we suggest to perform measurements of muscle strength as well as measurements of intramuscular cytokine levels in order to see the correlation between the effect of muscle-induced paralysis and inflammation.

## Author contributions

J. P. writing of the first draft, analyzing data, conducting analysis, conducting the study, designing the project. A. P. writing of the first draft, analyzing data. B. E. writing of the first draft, analyzing data, conducting analysis. L. N. Ledri. writing of the first draft, analyzing data, conducting analysis, conducting the study, designing the project.

## Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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## Ethical approval

All experiments were conducted in accordance with the guidelines of EU Directive 2010/63/EU and were approved by the Danish Animal Experiments Inspectorate. Ethical approval for this study was obtained from * The Danish Animal Experiments Inspectorate (License number: 2015–15–0201–00689). NAME OF ETHICS COMMITTEE OR INSTITUTIONAL REVIEW BOARD (APPROVAL NUMBER/ID)*. Animal welfare: All experiments were conducted in accordance with the guidelines of the European Union (EU) Directive 2010/63/EU.

## Consent for publication

All authors have read and accepted the current manuscript.
Data availability
All generated data are presented in the manuscript and all raw data are available on request to the corresponding author.

Code availability
Not applicable - no special software or custom program computer code was necessary to analyze the present data.

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