Annals of Physical and Rehabilitation Medicine 62 (2019) 189–192

Available online at
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com

Letter to the editor

Blocking neuromuscular junctions with botulinum toxin A injection enhances neurological heterotopic ossification development after spinal cord injury in mice

ARTICLE INFO

Keywords:
Neurogenic heterotopic ossification
Spinal cord injury
Botulinum toxin type a
Osteogenesis

Dear Editor,

Neurogenic heterotopic ossifications (NHOs) are benign ectopic bones that develop within the muscle tissue surrounding extracapsular bone joints [1] after severe lesions of the central nervous system (CNS) such as spinal cord injury (SCI) or traumatic brain injury [2]. NHOs are often diagnosed late, thereby resulting in large ossifications causing joint pain and stiffness that often progress to partial or complete joint ankyloses. Complications such as nerve and blood vessel compression and skin bedsores occur further exacerbate patient morbidity. Occurrence of NHO delays recovery from injury, interrupts rehabilitation programs and lengthens the hospital stay [3]. There is no effective pharmacological treatment to reduce the burden of NHO. Surgical resection of troublesome NHO remains the only treatment and provides some benefit for mobility [3].

Several clinical studies have attempted to identify risk factors associated with NHO to stratify predisposed patients [4]. We do not know whether muscle spasticity is a risk factor [4] or a consequence of ectopic bone growth within the muscle [5]. Botulinum toxin type A (BTA) blocks the α-motor neuron signals mediated by inhibiting the release of acetylcholine (Ach) at the neuromuscular junction (NMJ) within the muscle. Thus, it is currently injected locally in the affected muscle to reduce troublesome spasticity [6]. Identifying specific risk factors such as muscle spasticity from retrospective clinical studies is challenging without additional mechanistic insights into the NHO pathogenesis.

The purpose of this study was to assess the role of NMJ signaling during NHO development by blocking NMJ with BTA in a mouse model of SCI-induced NHO [7].

For CNS trauma-induced NHO, we developed the first mouse model of SCI-induced NHO in non-genetically manipulated mice [7]. In this model, NHO formation requires the combination of both SCI and local muscular damage caused by intramuscular injection of cardiotoxin (CDTX) [7].

Experiments involved female inbred C57BL/6 mice (5–6 weeks old) obtained from the Animal Resource Centre (Perth, Australia). A 100 unit vial of onabotulinum toxin A (BOTOX®, Allergan Australia) was diluted in 25 mL of 0.9% sodium chloride to obtain a 4 unit/mL stock concentration. To determine the effect of blocking the NMJ on NHO volume, female C57BL6 mice ($n = 13$) were injected with a first dose of 20 U/kg BTA in the right hamstring muscle (corresponding to a 80 to 95 μL volume) 4 days before surgery for optimal NMJ blockade on the day of surgery [8,9] (Fig. 1A). Equal volumes of phosphate buffered saline (PBS) were injected in left hamstring muscles as an internal control (right vs. left hamstrings) for each mouse. Mice then underwent surgery for transection of the spinal cord between T11-T13 and intramuscular injection of CDTX from the *Naja pallida cobra* in hamstring muscles of both hind limbs as previously described [10]. BTA and PBS injections in right and left hamstrings, respectively, were continued weekly for the next 3 weeks after surgery [8] (Fig. 1A). The endpoint of the experiment was 25 days after the first BTA intramuscular injection. All mice were assessed for health daily and remained healthy during the whole experiment despite their paraplegia, without adverse events. Mice were housed at a maximum of 5 mice per cage. Water was available in bottles, and food pellets were placed on the bottom of the cage to let paraplegic mice easily access water and food. All experiments were performed after approval of the University of Queensland animal ethics committee following National Health and Medical Research Council recommendations.

To quantify NHO development, both hind limbs of mice underwent micro-CT (micro-CT) on day 21 after SCI + CDTX treatment (Fig. 1B and Table 1) with an Inveon Positron Emission Tomography/CT (PET-CT) multimodality system (Siemens Medical Solutions Inc.). The parameters used for all scans and NHO volume quantification methods are in Supplementary Methods. For muscle mass and thickness, inter- and intra-observer intraclass correlation coefficients were not calculated and quantifications were not blinded. The median NHO volume in muscles treated with BTA was 3.20 mm^3 (interquartile range [IQR 2.20–4.45 mm^3]), 2.9 fold larger than the median NHO volume in muscles treated with PBS (1.10 mm^3 [0.94–2.15 mm^3], $P < 0.01$) (Fig. 1B and Table 1). BTA had no effect on mineral density of NHOs (Fig. 1C and Table 1). These data demonstrate that defective ACh signaling in response to BTA injection led to larger NHOs.

To confirm whether the current regimen of BTA injections can successfully abrogate NMJ signaling and lead to loss of muscle mass [11], we injected a parallel cohort of naïve mice ($n = 5$) weekly with BTA or PBS in right and left hamstrings following the same treatment regimen but without SCI and CDTX intramuscular injection (Fig. 2A). Hamstring thickness and mass were measured 25 days after the first BTA injection. Right BTA-injected hind limbs showed major and global muscle atrophy as compared with the contralateral side in all mice. The median thickness of hamstrings

https://doi.org/10.1016/j.rehab.2019.01.005
1877-0657/© 2019 Elsevier Masson SAS. All rights reserved.

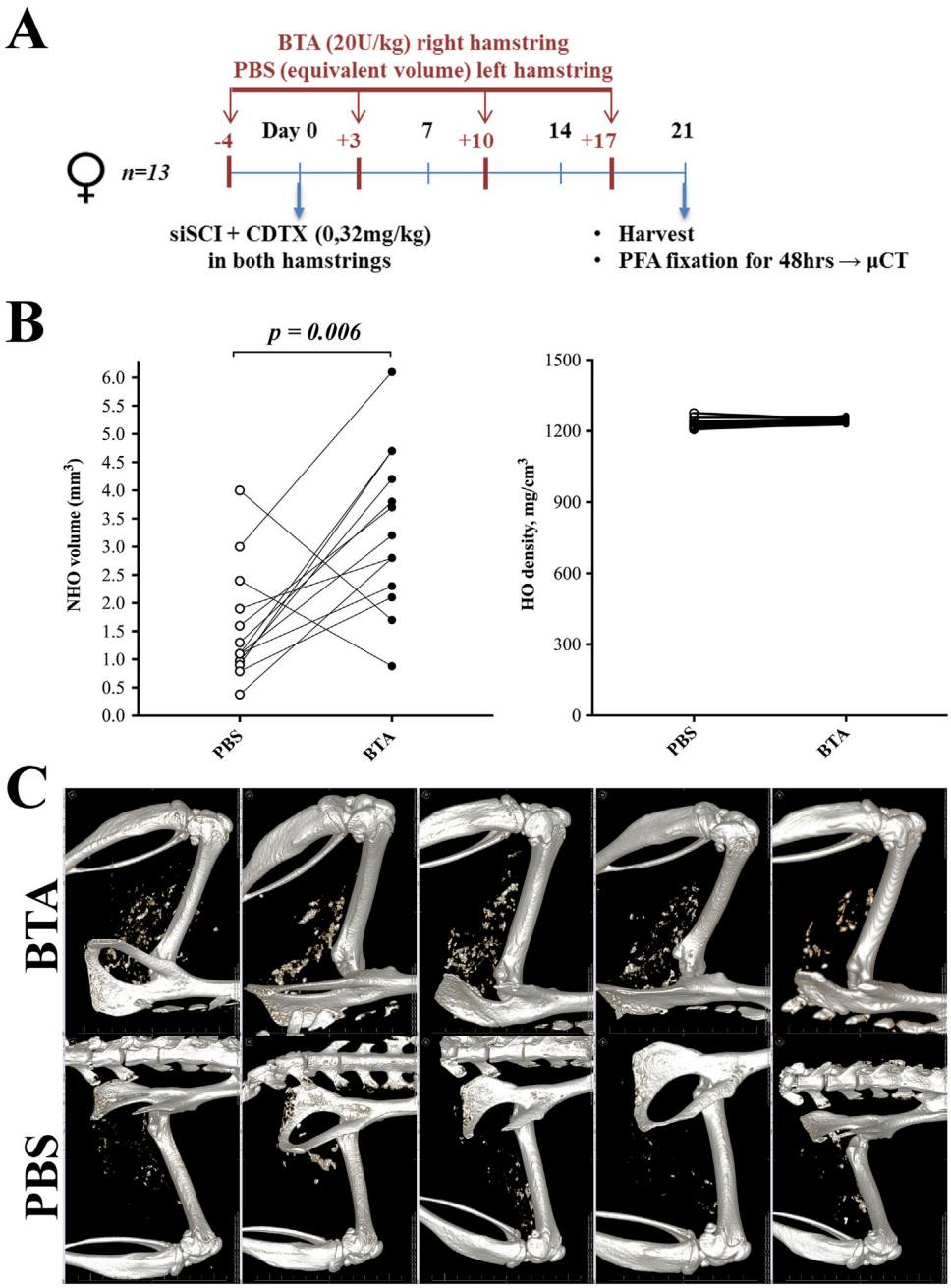

Fig. 1. Neurogenic heterotopic ossification (NHO) volume and mineral density in mouse hind limbs by micro-CT at 21 days after spinal cord injury (SCI) + cardiotoxin (CDTX). A. Injection schedule of botulinum toxin type A (BTA) in the right hamstring muscle and phosphate buffered saline (PBS) in the left hamstring muscle. B. NHO volume (mm^3) was measured by micro-CT in PBS-treated (open circles) and BTA-treated limbs (closed circles). Paired muscles for each individual mouse are joined by a line. C. Mineral density in PBS- and BTA-treated limbs for individual mice. Significance was calculated by paired *t* test (n = 13 mice). D. Representative 3-D reconstructed CT images of NHO at 21 days after surgery. BTA (right hamstring) or PBS (left hamstring) injections.

Table 1
Assessment of mice with neurological heterotopic ossification (NHO) in limbs treated or not with botulinum toxin A.

Variable	Treatment	No. of mice	Median [IQR]	*P*-value[a]
NHO volume (mm^3)	BTA	13	3.20 [2.20–4.45]	0.006
	PBS	13	1.10 [0.94–2.15]	
NHO density (mg/cm^3)	BTA	13	1242 [1238–1251]	NS
	PBS	13	1224 [1217–1243]	
Muscle thickness (mm)	BTA	5	1.34 [1.10–1.58]	<0.0001
	PBS	5	2.95 [2.79–3.13]	
Muscle mass (g)	BTA	5	0.59 [0.54–0.61]	0.0001
	PBS	5	0.85 [0.79–0.92]	

IQR, interquartile range; PBS, phosphate buffered saline; NS, not significant
[a] By paired *t* test.

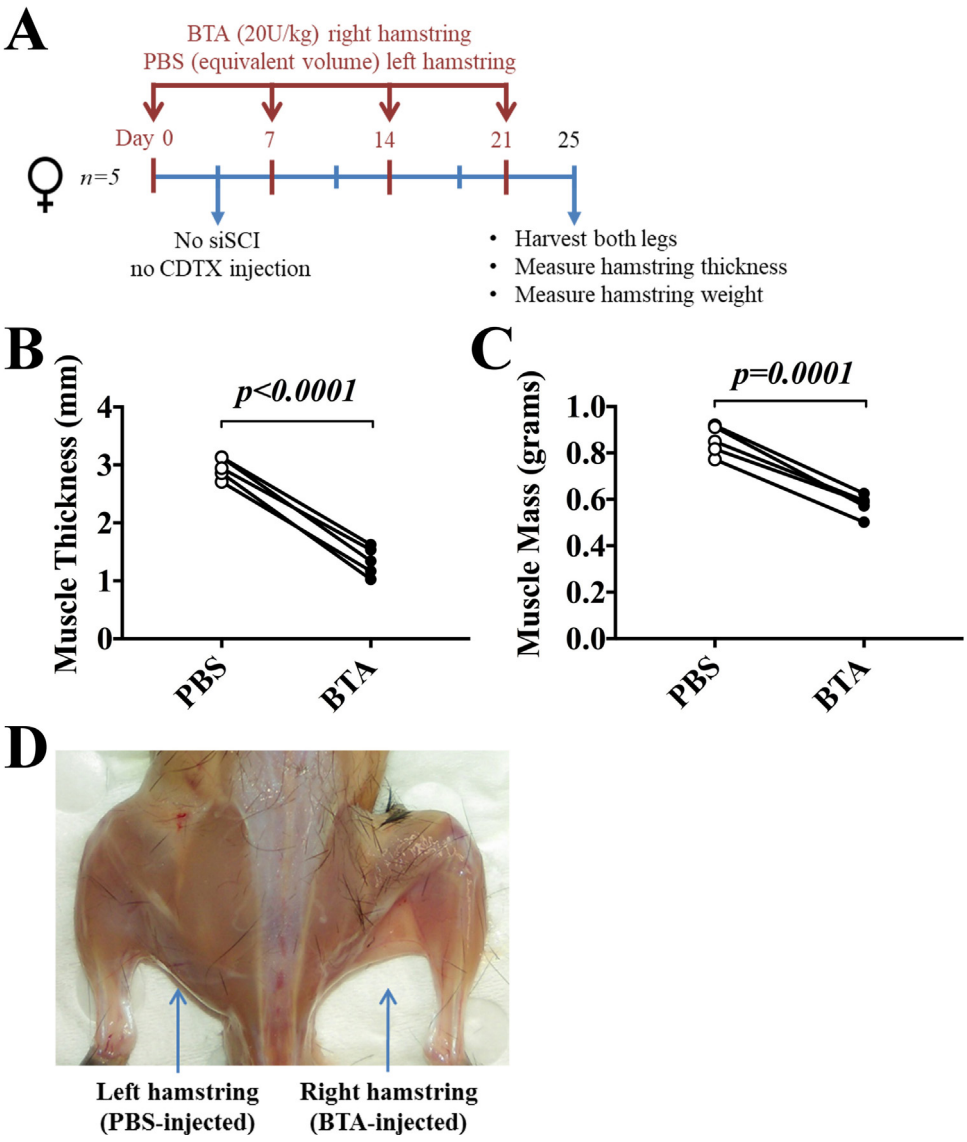

Fig. 2. Effect of BTA injections on muscle thickness and mass. A. Injection schedule of BTA in right hamstring muscle and PBS in left hamstring muscle. B. Thickness and (C) mass of left and right hamstrings at 25 days after the primary injection of BTA or PBS. Paired muscles for each individual mouse are joined by a line. Significance was calculated a paired *t* test ($n = 5$ mice). D. Anatomical difference between the right (BTA-injected) and left (PBS- injected) hamstrings.

injected with BTA was 1.34 mm (IQR 1.10–1.58), 2.2 fold lower ($P < 10^{-4}$) than that of contralateral hamstrings injected with PBS (2.95 mm [2.79–3.13]) (Fig. 2B and Table 1). Hamstrings from both sides were collected and their mass was measured (Fig. 2C). Median mass was significantly lower for BTA-injected than PBS-injected hamstring muscles (0.59 g [0.54–0.61] vs. 0.85 g [IQR 0.7–0.92], $P = 10^{-4}$). Therefore, BTA treatment was effective in reducing hamstring mass and thickness.

To our knowledge, this is the first report of BTA injection for NMJ blockage on NHO formation. Interfering in NMJ signaling led to enhanced NHO formation in a mouse model of SCI-induced NHO.

Our study has a few limitations. First, we could not assess muscle spasticity in our mouse model before and after BTA injection. Assessment of muscle overactivity in mice is challenging because of small animal size and lower limb joint deformation after paraplegia. Muscle atrophy has been found a common BTA side effect of denervation [11]; we quantified hamstring thickness and mass to assess its efficacy instead of spasticity. We confirmed that BTA induced amyotrophy because the thickness of BTA-injected mouse muscles was reduced by more than 50% and mass was reduced by 30% versus muscles injected with PBS. We do not know whether reducing muscle overactivity directly affected NHO development. Further experiments should be performed, such as injecting BTA in other muscles accessible to clinical assessment (Disease Activity Score response) [8], such as the gastrocnemius muscle. However, BTA is currently used to treat focal muscle overactivity by blocking NMJ in human pathology, so in our model, neurosignaling and subsequent muscle overactivity was abolished by BTA injection. Thus, for reducing muscle overactivity in humans by using BTA, we found major and global muscle atrophy in the right BTA-injected hind limb versus the contralateral side in all mice, which may exacerbate NHO volume.

Second, apart from blocking neuromuscular signaling, BTA has other important biological effects including inflammation, satellite cell activation, oxidative stress, atrophy and metal cation imbalance [12]. Repeated intramuscular BTA treatment causes profound and persistent loss of muscle function and altered muscle structure. Pingel et al. [13] demonstrated that high doses of BTA

greatly altered muscle structural composition, and prolonged BTA treatment upregulated the inflammatory cytokines interleukin 6 and transforming growth factor β1 in BTA-treated muscles [13]. Furthermore, BTA activated macrophages dependent on Toll-like receptor 2, thereby leading to enhance secretion of pro-inflammatory mediators such as nitric oxide and transforming growth factor α. Consequently, structural and immune changes induced by BTA injection may aggravate inflammation and muscle damage induced by CTDX injection in our model, which may further exacerbate NHO formation.

Third, we used a high dose of BTA to maintain the blockage of NMJ during the experiment. Fortuna et al. suggested that most of adverse effects induced by BTA injection into muscle are caused by the primary injection and that multiple injections of BTA do not produce cumulative adverse effects [11]. Therefore, we believe that over-dosage is not a concern in our study. Furthermore, our BTA treatment protocol, while aggressive, involves injecting BTA once a week according to the work of Aoki et al. [8] in order to maintain efficient blockage of NMJ throughout the experiment. NHO development may occur concomitant with muscle deterioration in fibrosis because NHO requires cell progenitors to develop under inflammatory conditions. Indeed, the mouse model of SCI-induced NHO requires acute muscle damage induced by the injection of CDTX from *N. pallida* snake venom. BTA injection probably increases muscle deterioration and contributes to NHO development.

The characterization of muscle overactivity as a risk factor or a consequence on NHO formation is still ambiguous. Elucidating the exact role of muscle spasticity during NHO formation is challenging in humans, because mainly retrospective and heterogeneous studies have been performed. Therefore, we used the first mouse model of SCI-induced NHO. This model effectively mimics human pathology and provides continuing understanding of NHO pathogenesis [7]. Our study showed that BTA injection in a context of SCI increased NHO size. Although our study is only observational and further mechanistic studies are needed, our findings question the status of muscle overactivity as a causal factor of NHO developing after CNS damage in humans.

Disclosure of interest

The authors declare that they have no competing interest.

Acknowledgements

This work was supported in part by the Mater Foundation (grant no. 1101620) from the National Health and Medical Research Council of Australia (NHMRC) to JPL, DWH and FG, and by the Assistant Secretary of Defense for Health Affairs through Spinal Cord Injury Research Program (award no. W81XWH-15-1-0606) from the US Department of Defence. JPL was supported by Research Fellowship 1136130 from the NHMRC. The authors also acknowledge the scientific and technical assistance of Dr. Brian Tse from the Translational Research Institute Preclinical Imaging Facility, which is supported by Therapeutic Innovation Australia (TIA). TIA is supported by the Australian Government through the National Collaborative Research Infrastructure Strategy Program. MS was supported in part by ORPEA-CLINEA society and a grant from the Collège Français des Enseignants universitaires de Médecine Physique et de Réadaptation (COFEMER).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at: https://doi.org/10.1016/j.amp.2018.09.005.

References

[1] Almangour W, et al. Recurrence of heterotopic ossification after removal in patients with traumatic brain injury: a systematic review. Ann Phys Rehabil Med 2016;59:263–9.
[2] Reznik JE, et al. Prevalence and risk-factors of neurogenic heterotopic ossification in traumatic spinal cord and traumatic brain injured patients admitted to specialised units in Australia. J Musculoskelet Neuronal Interact 2014;14:19–28.
[3] Genet F, et al. Troublesome heterotopic ossification after central nervous system damage: a survey of 570 surgeries. PLoS One 2011;6:e16632.
[4] Bargellesi S, et al. Occurrence and predictive factors of heterotopic ossification in severe acquired brain injured patients during rehabilitation stay: cross-sectional survey. Clin Rehabil 2018;32:255–62.
[5] Wharton GW, Morgan TH. Ankylosis in the paralyzed patient. J Bone Joint Surg Am 1970;52:105–12.
[6] Synnot A, et al. Interventions for managing skeletal muscle spasticity following traumatic brain injury. Cochrane Database Syst Rev 2017;11 [Cd008929].
[7] Genet F, et al. Neurological heterotopic ossification following spinal cord injury is triggered by macrophage-mediated inflammation in muscle. J Pathol 2015;236:229–40.
[8] Aoki KR. Preclinical update on BOTOX® (botulinum toxin type A) – purified neurotoxin complex relative to other botulinum neurotoxin preparations. Eur J Neurol 1999;6:S3–10.
[9] Warner SE, et al. Botox induced muscle paralysis rapidly degrades bone. Bone 2006;38:257–64.
[10] Torossian F, et al. Macrophage-derived oncostatin M contributes to human and mouse neurogenic heterotopic ossifications. JCI Insight 2017;2.
[11] Fortuna R, et al. Changes in contractile properties of muscles receiving repeat injections of botulinum toxin (Botox). J Biomech 2011;44:39–44.
[12] Mukund K, et al. Systems analysis of transcriptional data provides insights into muscle's biological response to botulinum toxin. Muscle Nerve 2014;50:744–58.
[13] Pingel J, et al. Injection of high dose botulinum-toxin A leads to impaired skeletal muscle function and damage of the fibrilar and non-fibrilar structures. Sci Rep 2017;7:14746.

Marjorie Salga[a,b,d], Hsu-Wen Tseng[d,e], Kylie A. Alexander[d,e], Beulah Jose[d], Cedryck Vaquette[f,g], Charlotte Debaud[a], Laure Gatin[a,c], François Genêt[a,b,d,*], Jean-Pierre Levesque[d,e,**]

[a]*END: ICAP U1179 Inserm, UFR des Sciences de la Santé–Simone Veil, Université Versailles Saint-Quentin-en-Yvelines, 78180 Montigny-le-Bretonneux, France*
[b]*CIC-IT 1429, Department of Physical Medicine and Rehabilitation, Hôpital Raymond-Poincaré, AP–HP, 92380 Garches, France*
[c]*CIC-IT 1429, Department of Orthopedic Surgery, Hôpital Raymond-Poincaré, AP–HP, 92380 Garches, France*
[d]*Stem Cell Biology Laboratory, Cancer Care and Biology Program, Mater Research, University of Queensland, Woolloongabba, Queensland, Australia*
[e]*School of Medicine, University of Queensland, Herston, Queensland, Australia*
[f]*Center in Regenerative Medicine, Institute of health of Biomedical Innovation, Queensland University of Technology, Kelvin Grove, Queensland, Australia*
[g]*School of Dentistry, University of Queensland, Herston, Queensland, Australia*

*Corresponding author. Service de Médecine Physique et de Réadaptation, CHU R.-Poincaré, 104, boulevard R.-Poincaré, 92380 Garches, France
**Co-corresponding author at: Stem Cell Biology Laboratory, Mater Research Institute, University of Queensland, Translational Research Institute, 37, Kent street, 4102 Queensland, Woolloongabba, Australia
E-mail addresses: francois.genet@rpc.aphp.fr (F. Genêt)., jp.levesque@mater.uq.edu.au (J.-P. Levesque).

Received 16 September 2018
Accepted 6 January 2019