Botulinum Toxin A: Is it Really that Fragile a Molecule?

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BACKGROUND Botulinum toxin A is considered to be an extremely fragile molecule, with recommended usage of some reconstituted botulinum toxin A formulations within 4 hours of reconstitution. Extreme caution is recommended to avoid agitation of the vial.

OBJECTIVE To determine whether the effect of reconstituted botulinum toxin A is maintained even when it is agitated vigorously.

DESIGN Experimental, comparative, animal study.

PARTICIPANTS Sixty-four white Swiss Webster mice, aged 2 to 4 weeks, arranged in eight separate groups.

INTERVENTION Reconstituted onabotulinumtoxinA (2.5 U/0.1 mL) was placed securely in a battery-operated mechanized apparatus. The apparatus with the vial was placed in the refrigerator, and the apparatus continuously inverted and straightened the onabotulinumtoxinA vial 30 times a minute. Eight mice were each injected intraperitoneally with 1 U of the agitated onabotulinumtoxinA on days 1, 3, 5, 7, 14, 21, 28, and 42, to evaluate the efficacy of the continuously agitated onabotulinumtoxinA.

MAIN OUTCOME MEASURE Death of the mice, demonstrating efficacy of the onabotulinumtoxinA molecule.

RESULTS Half of each group of mice (4/8 mice) died within 48 hours of the injection (range 16–48 hours).

CONCLUSION The effect of botulinum toxin type A is maintained even when it is agitated vigorously for up to 6 weeks.

The authors have indicated no significant interest with commercial supporters.

OnabotulinumtoxinA is a sterile, vacuum-dried, purified botulinum toxin type A, produced from fermentation of Hall strain *Clostridium botulinum* type A grown in a medium containing casein hydrolysate, glucose, and yeast extract. It is purified from the culture solution by dialysis and a series of acid precipitations to a complex consisting of the neurotoxin and several accessory proteins.[1] It is approved for use in more than 70 countries worldwide. OnabotulinumtoxinA produces its therapeutic effect by acting selectively on peripheral cholinergic motor nerve endings to inhibit the release of the neurotransmitter acetylcholine at the neuromuscular junction.[2] This inhibition occurs as the neurotoxin cleaves synaptosomal-associated protein 25, a protein integral to the successful docking and release of acetylcholine from vesicles situated within nerve endings. On intramuscular injection at therapeutic doses, it produces partial chemical denervation of the muscle, resulting in a localized reduction in muscle activity.[1]

It is widely believed that onabotulinumtoxinA is a fragile molecule despite the lack of evidence to support this claim. Allergan, Inc. (Irvine, CA), the manufacturer of onabotulinumtoxinA (BOTOX), recommends slow injection of the diluent in the onabotulinumtoxinA vial, gentle mixing of the onabotulinumtoxinA with the saline by rotating the vial, and avoidance of any vigorous agitation of the

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reconstituted onabotulinumtoxinA vial. It is also recommended that the reconstituted onabotulinumtoxinA be used within 4 hours of reconstitution.[1]

The justifications behind the rigid guidelines for usage such as "slow injection of the diluent," the "gentle rotation of the vial,"[3,4] and the prescribed time limit within which onabotulinumtoxinA can be used once reconstituted[5–7] have individually been earlier investigated.

The objective of our study was to determine whether the efficacy of botulinum toxin type A is maintained even after being in a state of vigorous, continuous agitation for 6 weeks. We designed a mechanized device that holds the onabotulinumtoxinA vial securely and mechanically inverts and straightens the vial up to 30 times a minute continuously. This battery-operated mechanical device with the reconstituted onabotulinumtoxinA vial was kept in the refrigerator to prevent heat, a confounding variable, from altering the potency of the molecule. The main reason that we conducted this trial was to provide objective data regarding the fragility and the efficacy of the botulinum toxin molecule. Although other trials have tried to do the same in different ways, in our trial, we standardized the "vigorous reconstitution" by performing it mechanically and then tested the agitated, reconstituted onabotulinumtoxinA over a period of 6 weeks, hoping to lay to rest all speculation regarding the fragility of this molecule. Although we agree that this motion does not replicate the manner in which the toxin could conceivably be mixed, the possible extreme nature of continuous vigorous agitation was performed to first provide objective data and second lay to rest any speculation regarding the molecule's efficacy on usage after reconstitution even after 6 weeks, as well as after agitation, after reconstitution.

Materials and Methods

This was an experimental, comparative animal study conducted in 64 white Swiss Webster mice. The authors confirm adherence to the ARVO Statement for the Use of Animals in Research (http://www.arvo.org/eweb/dynamicpage.aspx?site=arvo2&webcode=AnimalsResearch).

The Appolo Hospitals' Institutional Review Board approved this trial.

A 100-U vial of onabotulinumtoxinA was reconstituted with 4 mL of 0.9% nonpreserved saline (2.5 U/0.1 mL) and immediately placed securely in a mechanized, battery-operated apparatus (Figure 1). The apparatus with the vial was placed in the refrigerator. The apparatus was designed such that it could invert and straighten the reconstituted onabotulinumtoxinA vial 30 times a minute. The purpose of this constant state of motion was to maintain a continuous agitated state of the reconstituted onabotulinumtoxinA and to subject the molecule to maximum possible agitation and motion stress. To evaluate the efficacy of the onabotulinumtoxinA, eight groups of eight Swiss Webster female mice (total = 64), aged 2 to 4 weeks, were each injected intraperitoneally with 1 U of onabotulinumtoxinA on days 1, 3, 5, 7, 14, 21, 28, and 42. The mice were all weighed before injection of the onabotulinumtoxinA.

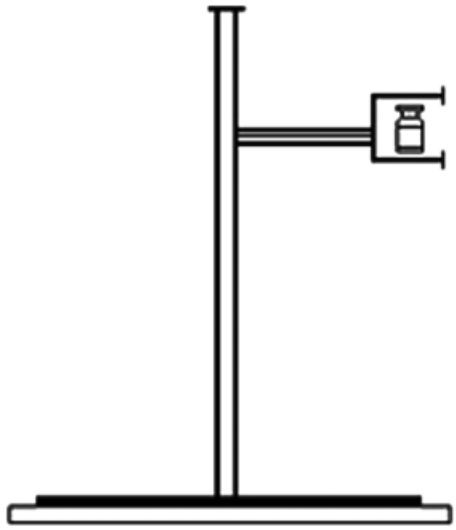

Figure 1. A line diagram of the device used to invert and straighten the reconstituted onabotulinumtoxinA vial 30 times a minute.

On each of the aforementioned days, the vial was removed from the apparatus in the refrigerator, and after the removal of 1 U of onabotulinumtoxinA each in eight different syringes, the vial was immediately placed back into the apparatus in the refrigerator (within an average of a minute), and the oscillations were restarted. One unit was injected into each mouse of the group, using a different syringe for each mouse.

Results

The toxicity of onabotulinumtoxinA is traditionally expressed in biologic mouse units (U), which is a unit of bioactivity or potency and not of weight or volume. One mouse unit is equal to the median lethal intraperitoneal dose or the amount of intraperitoneally injected toxin needed to kill 50% (LD50) of a group of female Swiss Webster mice that weigh 18 ± 22 g each.[2] Eight mice were kept in a batch on each testing day. On each of the occasions when onabotulinumtoxinA was injected into the group of eight mice, 50% of the group of mice (4 of 8) died within 48 hours of the injection (range 16–48 hours) (complete results in Table 1), on all the testing days, demonstrating the maintained potency of the injected unit of onabotulinumtoxinA.

Discussion

OnabotulinumtoxinA is supplied in a single-use vial, each vial containing 100 U of *C. botulinum* type A neurotoxin complex, which must be reconstituted with 0.9% nonpreserved sodium chloride solution, the only recommended diluent according to the package insert, although most clinicians now use sodium chloride solution preserved with benzalkonium alcohol. A number of trials[8,9] have reported equivalent success with preserved saline and have also described that patients experienced less discomfort during the injections with preserved saline, which has been attributed to the fact that the preserved saline is pH balanced. We used nonpreserved saline in this trial for reconstitution, but we do not believe that the alternative use of preserved saline would have affected the observations in any way. The recommended method of dilution states that one must draw the required amount of diluent in a syringe and slowly inject it into the onabotulinumtoxinA vial, the vial is to be gently rotated, and the date and time of reconstitution are to be recorded on the space on the label. Allergan, Inc. also mentions that onabotulinumtoxinA is supplied only as a single-use vial, meant for use within 4 hours after reconstitution. It is believed that foam formation and vigorous agitation may render onabotulinumtoxinA solution vulnerable to surface denaturation caused by bubbles or that bubbles may cause toxin dispersion in the surface of the vial,[3] but the current study shows that this "gentle rotation" is not necessary after the diluent has been added.

TABLE 1. Complete Data of Mortality of Mice

	Hours:Minutes After Injection Until Death			
Day	*Mouse 1*	*Mouse 2*	*Mouse 3*	*Mouse 4*
1	19:14	21:30	27:55	43:57
3	23:44	25:48	27:16	45:42
5	22:29	27:42	31:23	40:46
7	20:46	22:12	29:10	47:37
14	25:43	30:19	36:46	44:56
21	28:05	34:40	39:32	46:12
28	16:03	19:12	28:13	39:49
42	24:10	29:15	38:16	39:13

Trinidade and colleagues,[3] in their study, created foam by rapidly shaking the vial after reconstitution of onabotulinumtoxinA. This trial involved masked observers who examined photographs of patients who were treated for glabellar and periocular wrinkles with gently reconstituted onabotulinumtoxinA on one side of the face and rapidly reconstituted onabotulinumtoxinA with foam formation on the other side of the face. Kazim and Black[4] conducted a prospective, double-blind, randomized study with subjects who were injected with gently reconstituted onabotulinumtoxinA on one side of the forehead and vigorously reconstituted onabotulinumtoxinA on the other side. The vigorous reconstitution, in this study, was achieved by placing the vial of reconstituted

onabotulinumtoxinA on the Vortex Touch Mixer Model 232 (GeneQ, Montreal, Canada) at the maximum speed of 10 for 30 seconds. Eyebrow excursion was measured in millimeters before injection, 1 week after injection, and every month after injection up to a total of 6 months. The authors concluded that the effect of botulinum toxin type A is maintained and has the same duration of action when it is reconstituted vigorously as when it is reconstituted gently. Trinidade and colleagues[3] made no mention of how long the vial was shaken, whereas Kazim and Black[4] performed the vigorous reconstitution for only 30 seconds. In our trial, we standardized the "vigorous reconstitution" by performing it mechanically and then tested the reconstituted onabotulinumtoxinA over 6 weeks, hoping to lay at rest all speculation regarding the fragility of this molecule.

The directions for usage further state that unopened vials of onabotulinumtoxinA can be stored in a refrigerator (2–8°C) for up to 36 months for the 100-U vial or up to 24 months for the 50-U vial. Administration of onabotulinumtoxinA within 4 hours of reconstitution is also recommended, and during this period, reconstituted onabotulinumtoxinA should be stored in a refrigerator (2–8°C).[1] As mentioned earlier, onabotulinumtoxinA is supplied in a single-use vial, with each vial containing 100 U. Therefore, if in any one sitting less than 100 U is used, there is a potential for substantial wastage of the reconstituted solution. Hui and Lee[10] reported that 2 weeks of refrigeration does not appear to significantly affect the time of onset or efficacy of botulinum toxin in the treatment of lateral periorbital rhytides. In our study, we found that the reconstituted onabotulinumtoxinA solution was efficacious even after 6 weeks in refrigeration at 4°C. These findings are compatible with those found by Hexsel and colleagues.[5] It has been demonstrated that reconstituted onabotulinumtoxinA may be frozen, thawed, and injected without losing its potency for up to 6 months, with efficacy equivalent to that of freshly prepared onabotulinumtoxinA,[11] although none of these studies, which examined how long onabotulinumtoxinA can be stored after reconstitution without a loss in efficacy, make any mention of vigorous mechanical reconstitution or agitation of the vial. In centers where clinicians require low doses of onabotulinumtoxinA, the practice of disposing of a partly used vial of onabotulinumtoxinA 4 hours after reconstitution certainly will lead to economic losses. This is of further relevance in developing countries, where onabotulinumtoxinA is an expensive drug, and the cost of this wasted solution is passed on to patients.

In conclusion, this study demonstrates that onabotulinumtoxinA is a molecule stable enough to retain its efficiency for up to 6 weeks after reconstitution. The practice of disposing of a vial of onabotulinumtoxinA 4 hours after reconstitution should be abolished. To the best of our knowledge, there is no study that has investigated the stability of onabotulinumtoxinA, in terms of retaining its potency after vigorous agitation, and how long the onabotulinumtoxinA molecule can sustain this state of mechanical agitation without losing its efficacy. We would like to further state that the onabotulinumtoxinA molecule is not only stable enough to withstand agitation immediately after reconstitution, but is also stable enough to retain its efficacy after being in a state of continuous agitation for as long as 42 days (6 weeks).

Conclusion

OnabotulinumtoxinA is an extremely stable molecule, and vigorous agitation does not impair its potency, even after 6 weeks.

References

1. Botox (OnabotulinumtoxinA) cosmetic (botulinum toxin type A) purified neurotoxin complex [package insert]. Irvine, CA: Allergan, Inc.

2. Beedetto AV. The cosmetic uses of botulinum toxin type A. Int J Dermatol 1999;38:641–55.

3. Trindade De Almeida AR, Kadunc BV, Di Chiacchio N, Neto DR. Foam during reconstitution does not affect the potency of botulinum toxin type A. Dermatol Surg 2003;29: 530–2.

4. Kazim NA, Black EH. Botox (OnabotulinumtoxinA): shaken, not stirred. Ophthal Plast Reconstr Surg 2008;24:10–2.

5. Hexsel DM, De Almeida AT, Rutowitsch M, et al. Multicenter, double-blind study of the efficacy of injections with botulinum toxin type A reconstituted up to six consecutive weeks before application. Dermatol Surg 2003;29:523–9.

6. Yang GC, Chiu RJ, Gillman GS. Questioning the need to use Botox (OnabotulinumtoxinA) within 4 hours of reconstitution: a study of fresh vs 2-week-old Botox (OnabotulinumtoxinA). Arch Facial Plast Surg 2008;10:273–9.

7. Sloop RR, Cole BA, Escutin RO. Reconstituted botulinum toxin type A does not lose potency in humans if it is refrozen or refrigerated for 2 weeks before use. Neurology 1997;48:249–53.

8. Alam M, Dover JS, Arndt KA. Pain associated with injection of botulinum A exotoxin reconstituted using isotonic sodium chloride with and without preservative: a double blind, randomized controlled trial. Arch Dermatol 2002;138:510–4.

9. Garcia A, Fulton JE Jr. Cosmetic denervation of the muscles of facial expression with botulinum toxin. A dose-response study. Dermatol Surg 1996;22:39–43.

10. Hui JI, Lee WW. Efficacy of fresh versus refrigerated botulinum toxin in the treatment of lateral periorbital rhytids. Opthal Plast Reconstr Surg 2007;23:433–8.

11. Parsa AA, Lye KD, Parsa FD. Reconstituted Botulinum Type A Neurotoxin: clinical efficacy after long-term freezing before use. Aesth Plast Surg 2007;31:188–91.

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