Reduced Potency After Refrigerated Storage of Botulitum Toxin A: Human Extensor Digitorum Brevis Muscle Study

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Abstract: To determine whether the potency of botulinum toxin A (BTA) decreases after being reconstituted with normal saline and stored in refrigerator, we injected one side of the extensor digitorum brevis muscle with 2.5 units of botulinum toxin A that had been immediately reconstituted with saline, and the contralateral side with identical material that had been reconstituted and stored in a refrigerator for preselected periods (1, 2, and 4 weeks) in 32 healthy volunteers. Mean compound muscle action potential amplitudes expressed as a percentage of the baseline healthy volunteers. Mean compound muscle action potential amplitudes expressed as a percentage of the baseline amplitude were more reduced in sides injected with immediately reconstituted BTA than in sides injected with BTA stored for 1 week or more (P < 0.05). No bacterial growth was observed in any stored BTA samples. Storage of reconstituted BTA at low temperatures may affect the potency of the toxin. Therefore, the use of BTA after refrigerated storage is not recommended. © 2006 Movement Disorder Society

Key words: botulinum toxin; potency; storage; reconstitution

Botulinum toxin type A (BTA) is a neurotoxin, which acts on cholinergic nerve terminals and inhibits the release of acetylcholine and hence causes transient muscle paralysis. This unique blocking effect of BTA on the neuromuscular junctions has facilitated its use in a number of neuromuscular dysfunctions, such as dystonia, spasticity, blepharospasm, spasmodic torticollis, and strabismus, and more recently for the treatment of palmar hyperhidrosis, hypersalivation, tension-type headache, muscular-origin pain, and fine wrinkle removal.1

Frequently, only a small amount of BTA, i.e., less than one vial (currently only available in a 100-unit vial from Botox, Allergan, Irvine, CA), is required for treating small muscles, for example, when treating pediatric patients, wrinkles or spasmodic dysphonia, or when BTA is combined with phenol for neurolysis.2 Given this situation and the cost of the material, clinicians sometimes try to recruit a number of patients within 4 hours of reconstituting BTA. (The current recommendation by Botox is to administer the product within 4 hours of reconstitution.)3 However, this is not always feasible. Therefore, many clinicians have resorted in practice to reusing residual toxin after refrigeration or freezing, which is contrary to the manufacturer’s guideline.3

The main concerns about reusing remaining toxin are reduced sterility and potency. Gartlan and Hoffman4 reported a degradation in the potency of BTA after refrigerated or frozen storage when assayed 2 weeks later in Swiss-Webster mice, whereas Garcia and Fulton5 and Sloop and colleagues6 reported that refrozen or refrigerated BTA did not lose its potency after comparable storage in their human clinical model. No problems have been reported in terms of sterility. However, the above studies were limited in that they used a mouse model and lacked objective measures or statistically meaningful numbers.6 Therefore, the purpose of this study was to evaluate the effect of storage on BTA potency in humans.

SUBJECTS AND METHODS

This was a prospective open-label randomized study. A total of 32 healthy volunteers (27 women, 5 men) with a mean age of 30.6 years (range, 19–44 years) were consecutively recruited. All enrolled subjects were in good general health. Neurological examinations indicated that all patients were free of central and peripheral nervous system anomalies, no patient had a history of neurological or musculoskeletal injury or disorder, and extensor digitorum brevis (EDB) muscles were confirmed normal. No medication was allowed either before 2 weeks or during the study. The experimental protocol was approved by the Institutional Review Board of Seoul National University Hospital and informed consent was obtained from all subjects.

Dilution Method

Botox was reconstituted with preservative-free normal saline (0.9% sodium chloride) at a concentration of 25 U/mL. Preservative-containing saline solutions, which include the bacteriostatic benzyl alcohol, were not used, because it is suspected that these may influence BTA potency regardless of storage.7,8 For this study, a con-
centration of 25 U/mL was selected because it is the concentration used at our hospital and frequently by other clinicians.9–11

**Injection**

After adding saline, the reconstituted material was divided into two aliquots. The first was injected within 2 hours and the second was refrigerated at 4°C for 1, 2, or 4 weeks. Subjects were injected with 2.5 units of freshly reconstituted Botox into one side of the EDB muscle (first aliquot) and this was followed by a injection with 2.5 units of Botox, which had been stored for a predetermined time, into the contralateral side (second aliquot) a predetermined time later (1, 2, and 4 weeks). Botox was prepared in a 1-mL tuberculin syringe. Subjects were randomly assigned to three groups. The first group (n = 11) was injected with 2.5 units of freshly reconstituted BTA into the EDB of one side and 1 week later was injected with the same number of units of BTA, which had been reconstituted at the time of the first injection and refrigerated for 1 week, into the EDB of the contralateral side. Similarly, the second (n = 10) and third group (n = 11) were injected with 2.5 units of freshly reconstituted BTA in one side, and with 2.5 units of stored material 2 or 4 weeks later into the contralateral side. Side orders were assigned randomly. Injections were performed under EMG guidance to ensure that the BTA was delivered to the target muscle. The same lot number of Botox was used in each of the two groups to reduce batch-to-batch variability.

**Measurement**

Peroneal motor nerve conduction model developed by Sloop and colleagues6 was used to quantify the extent of muscle paralysis following BTA injection by measuring the amplitude of compound muscle action potential amplitude (CMAP) of EDB muscles. The EDB muscle was selected because it is seldom used for daily functions, and because the CMAPs of EDBs are less variable than facial or hand muscles.12,13

CMAPs were measured using disposable surface electrodes (11-mm diameter) from onset to peak. The peroneal nerve was supramaximally stimulated 8 cm proximally to the recording electrode using a standard nerve conduction technique. For recording, the standard tendon–mid muscle belly montage was used to maximize CMAPs. A rectangular pulse of 0.1 ms duration was delivered. Filters were set at 2 Hz and 10 KHz, the sensitivity was 2 mV per division, and the sweep speed used was 2 milliseconds per division. Temperature was measured and maintained at above 32°C. All subjects were examined using a Sapphire electromyography machine (Medelec, Surrey, UK). We obtained CMAPs on three occasions per side: before injection and 1 and 4 weeks after injection. The maximum amplitude recorded during three consecutive measurements on each occasion was used for the statistical analysis. Baseline CMAPs, side-injected with stored material, were obtained just prior to injection to avoid concerns of a remote effect from the fresh side to the stored side. Stimulation and recording sites were marked with indelible ink to minimize variability, and all nerve conduction studies were performed by the same investigator who was blinded to the injection.

**Statistical Analysis**

CMAP % was expressed as a percentage of the amplitude of the baseline CMAP. The effects of refrigerated storage on BTA potency were analyzed using a repeated measures of ANOVA (ANOVARM) incorporating time (1 and 4 weeks after injection) and side (side of injection: fresh vs. stored BTA). Baseline comparisons were performed using the paired t test. A sample size estimation based on the available data14,15 revealed 9 subjects per group with a statistical power of 80% and the 0.05 level for detecting the relevant difference in CMAPs between sides. SPSS version 12.0 was used for the statistical calculations.

To assess sterility, three samples were taken from each BTA preparation at the time of injection for routine aerobic and anaerobic bacterial cultures. These samples were cultured for 4 weeks and sterility was qualitatively evaluated.

**RESULTS**

CMAP amplitudes before injection were not significantly different between the two sides (P > 0.05 by paired t test). In subjects injected with material stored for 1 week, CMAP %, expressed as a percentage of the amplitude of the baseline, was reduced to 40.6% ± 6.0% (mean ± standard error) after 1 week, to 50.6% ± 8.4% after 4 weeks following the injection of fresh material, to 55.9% ± 5.6% after 1 week, and to 64.0% ± 11.1% after 4 weeks for 1-week–stored material. ANOVARM showed no significant effect of side [F(1,10) = 2.68; P = 0.133] and time [F(1,10) = 3.26; P = 0.101] in the absence of a significant interaction side × time [F(1,10) = 0.049; P = 0.830].

In the 2-week storage group, ANOVARM showed a significant effect of side [F(1,9) = 10.79; P = 0.009], but not time [F(1,9) = 4.47; P = 0.064]. However, in the 4-week storage group, ANOVARM revealed no significant effect of side [F(1,10) = 2.90; P = 0.120] or time [F(1,10) = 3.38; P = 0.096].
In the 1-week and 4-week storage groups, the CMAP % of fresh BTA-injected sides tended to be lower than those of stored BTA-injected sides, but this was not statistically significant. Therefore, we compared CMAP % values of sides injected with fresh with those injected with stored BTA in all 32 subjects. In this comparison, CMAP % was 42.9% / H11006 2.9% after 1 week and 49.6% / H11006 3.9% after 4 weeks after fresh BTA, and these were 57.4% / H11006 3.9% after 1 week and 65.8% / H11006 5.9% after 4 weeks after injecting stored BTA, which showed a significant side [F(1,31) = 10.5; P = 0.003] and time [F(1,31) = 11.6; P = 0.002] effect, in the absence of a significant interaction side × time [F(1,31) = 0.20; P = 0.657] by ANOVA RM (Table 1; Fig. 1). In terms of sterility, no aerobic or anaerobic bacterial growth was detected in any sample at the level of 10⁵ organism growth after 1 month of culture.

### DISCUSSION

The Swiss-Webster mouse bioassay, based on median lethal intraperitoneal dose (LD50) determination, is traditionally used to evaluate BTA potency. Using this method, Gartlan and colleagues⁴ observed a 43.9% loss in potency when Botox was reconstituted with normal saline and stored in a refrigerator at 4°C for 12 hours. However, this assay may not provide sufficient information regarding the actual potency of BTA in humans for several reasons. First, it is a method of measuring animal death and not a method of measuring muscle weakness and therefore cannot accurately predict potency in terms of weakness, which is closely related to clinical response. Second, mouse response to BTA probably differs from that of humans: BTA susceptibility is known to be species-dependent.¹⁶ Third, the route of BTA administration in the same species can also affect results.¹⁷ Therefore, our purpose was to evaluate the efficacy of the stored BTA with human clinical model.

Contrary to our findings, Garcia and Fulton⁵ found that the therapeutic effect of BTA stored in a refrigerator for as much as 30 days was equivalent to that of freshly reconstituted toxin. However, in this study, they employed a subjective clinical observation method. Recently, a human model for evaluating BTA potency was proposed by Sloop and colleagues.⁶ They too reported that the potency of BTA reconstituted with normal saline and stored at 4°C for 2 weeks was equal to that of freshly reconstituted toxin. Although the concept of their study was novel, it had some limitations. For example, they only tested four subjects, and they compared the potency of BTA stored for only 2 weeks with freshly reconstituted toxin. Researchers at Allergan also claim that BTA

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**TABLE 1. CMAP% of peroneal nerve for sides injected with fresh or stored Botox**

<table>
<thead>
<tr>
<th></th>
<th>1 week after injection</th>
<th>4 weeks after injection</th>
<th>Statistics ANOVA&lt;sub&gt;RM&lt;/sub&gt;</th>
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<tr>
<td></td>
<td>Side Effect</td>
<td>Time Effect</td>
<td>Side × Time</td>
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<tr>
<td>1-week storage group (n = 11)</td>
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<td></td>
<td></td>
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<tr>
<td>Fresh side (%)</td>
<td>40.6 ± 6.0</td>
<td>50.6 ± 8.4</td>
<td>0.133</td>
</tr>
<tr>
<td>Stored side (%)</td>
<td>55.9 ± 5.6</td>
<td>64.0 ± 11.1</td>
<td>0.009</td>
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<tr>
<td>2-week storage group (n = 10)</td>
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<tr>
<td>Fresh side (%)</td>
<td>38.2 ± 2.8</td>
<td>46.5 ± 3.6</td>
<td>0.009</td>
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<tr>
<td>Stored side (%)</td>
<td>55.7 ± 6.5</td>
<td>61.6 ± 7.4</td>
<td>0.120</td>
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<tr>
<td>4-week storage group (n = 11)</td>
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<tr>
<td>Fresh side (%)</td>
<td>49.4 ± 5.0</td>
<td>51.3 ± 7.3</td>
<td>0.120</td>
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<tr>
<td>Stored side (%)</td>
<td>60.3 ± 8.3</td>
<td>71.3 ± 11.6</td>
<td>0.003</td>
</tr>
<tr>
<td>All subjects (n = 32)</td>
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<tr>
<td>Fresh side (%)</td>
<td>42.9 ± 2.9</td>
<td>49.6 ± 3.9</td>
<td>0.003</td>
</tr>
<tr>
<td>Stored side (%)</td>
<td>57.4 ± 3.9</td>
<td>65.8 ± 5.9</td>
<td>0.657</td>
</tr>
</tbody>
</table>

CMAP were expressed as a percentage of the amplitude of the baseline. Values are means ± standard errors. Values in Statistics ANOVA<sub>RM</sub> are P values.

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**FIG. 1.** Comparison of CMAPs for fresh-Botox–injected sides and stored-Botox–injected side. CMAPs are expressed as percentages of baseline amplitude in the respective sides. CMAP % was more attenuated in side treated with fresh Botox than in side treated with stored Botox. ANOVA<sub>RM</sub> revealed significant effect of side (P = 0.003) and time (P = 0.002). Bar represents standard error.
is stable in refrigerated storage for 5 weeks after being reconstituted with preserved saline.\textsuperscript{18}

According to the results of the present study, all stored BTA showed more reduction in potency than fresh BTA. However, only 2-week–stored group showed a statistically significant reduction, and 1- and 4-week–stored groups showed statistically nonsignificant reductions. A possible explanation for these statistical contradictory findings might result from large CMAP variability. It is also probable that stored BTA might have similar potency initially but the duration of action may be affected.\textsuperscript{19}

Although we did not assay the antibody in this study, it is probable that refrigerated storage of BTA may induce higher antigenicity due to autofragmentation of the toxin.\textsuperscript{20} Therefore, it should be discarded if more than 4 hours elapse after reconstitution.

The results of the present study also give rise to the question of how long BTA retains its potency in its unreconstituted form, which could be investigated using the model described herein. Preservative-containing saline has been used to store reconstituted BTA.\textsuperscript{5,6,21} Such saline usually contains benzyl alcohol as a bacteriostatic, in addition to 0.9\% sodium chloride.\textsuperscript{22,23} However, it has been suggested that potency of BTA deteriorates more rapidly when it is reconstituted with preservative-containing saline, due to the effect of the benzyl alcohol on BTA. In the study by Alam and colleagues,\textsuperscript{18} no differences were found in terms of the potency of BTA reconstituted with preserved or normal saline. In the present study, we used normal saline to reconstitute BTA in accordance with the manufacturer’s guidelines to avoid a possible confounding effect by the preservative.

The findings of the study are limited in scope, e.g., to clinical effectiveness in terms of treating large muscles at higher doses. Also, different muscles might behave somewhat differently according to muscle size, frequency of use, type of motion, etc. Moreover, a decay in CMAP does not necessarily translate to potential loss of clinical effect.

We believe that most clinicians do not want to freeze or refrigerate reconstituted product for future use or to use preservative saline for reconstitution because of its presumed bacteriostatic and pain-reducing effect.\textsuperscript{18} In conclusion, the potency of BTA was reduced when it is stored at low temperatures after being reconstituted with saline. The use of reconstituted BTA after the recommended 4-hour expiration is not recommended.

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REFERENCES

Predictive Value of Transcranial Sonography in the Diagnosis of Parkinson’s Disease

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Abstract: Transcranial sonography (TCS) is increasingly applied in the diagnosis of Parkinson’s disease (PD), but investigator bias may influence the results of examination. Blinding the sonographer to the clinical diagnosis of 42 PD patients and 35 controls, we obtained a positive predictive value of 85.7% and a negative predictive value of 82.9% in the diagnosis of PD solely by interpreting the results of TCS, indicating that TCS is a valuable additional tool in the diagnosis of PD.

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Key words: transcranial sonography; Parkinson’s disease; validity of diagnosis; predictive value

Transcranial sonography (TCS) is increasingly applied in the diagnosis of Parkinson’s disease (PD) and its differentiation from atypical parkinsonian syndromes. An area of increased echogenicity at the anatomical site of the substantia nigra (SN) above the 90th percentile of the healthy population has been found in more than 90% of PD patients. However, as in every ultrasound examination, knowledge of the diagnosis by the examiner may bias the results. In order to determine the validity of TCS findings in the diagnosis of PD, we blinded in this study the sonographer to the possible diagnosis of PD by veiling the person investigated except of two small holes at the temporal bone window for the ultrasound probe and one for breathing to exclude any visual clues indicating the diagnosis of PD.

PATIENTS AND METHODS

Patients

Eighty-five individuals gave informed consent to participate in the study. Of these, seven had to be excluded because bone windows were insufficient for the assessment of SN echogenicity. Of the remaining, 42 (15 women, 27 men; mean age, 64.6 ± 9.7 years) were clinically diagnosed as PD according to the U.K. Brain Bank Criteria. Patients were recruited from the department’s ward and outpatient clinic. Patients with no evidence for any kind of neurodegenerative disorder and healthy spouses of the patients (n = 35; 14 women, 21 men) were included as controls. Average age of the control group was 59.2 ± 12.3 years. Difference in age between the groups was not statistically significant (U test: P > 0.05). Exclusion criteria for the study was evident tremor at rest that could not be hidden by veiling or placing the respective arm under the back or leg of the patient and any evidence for another basal ganglia disorder, including multiple-system atrophy (MSA), progressive supranuclear palsy (PSP), dementia with Lewy bodies (DLB), and corticobasal ganglionic degeneration (CBGD).

Blinding

All participants were seated on a comfortable chair that supported the neck in a slightly angled position to mask possible rigidity of the neck and covered with sheets and blankets to hide any abnormal postures or tremor by a person who was not engaged in the ultrasound examination. The head including the face was veiled by a sheet in order to hide obvious cues for the diagnosis except of two small holes at the temporal bone window for the ultrasound probe and one for breathing (Fig. 1). Moreover, the examination took place in a darkened room.

TCS Examination

For TCS, a phased-array ultrasound system equipped with a 2.5 MHz transducer with an axial resolution of approximately 0.7 mm and a lateral resolution of about 3 mm (Elegra; Siemens, Erlangen, Germany) was used. The examination was performed through a preauricular acoustic bone window with a penetration depth of 16 cm and a dynamic range of 45 dB as described previously. The SN was identified within the butterfly-shaped structure of the mesencephalic brainstem as clearly as possible, scanning from both temporal bone windows, then the area of hyperechogenic signals in the SN region was encircled and measured.

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