Absence of Bacterial or Fungal Growth in Vials of Reconstituted Botulinum Toxin Type A After Storage

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Abstract

Background: Botulinum toxin type A (BTX-A; Botox) is supplied as individual freeze-dried preparations that should be administered within 24 hours after reconstitution. To avoid wasting this expensive drug, some physicians have resorted to storing vials of reconstituted BTX-A beyond the recommended duration. However, there is insufficient evidence to indicate that the sterility of previously reconstituted BTX-A is maintained during storage.

Objectives: The authors sought to determine whether bacterial and/or fungal proliferation occurred in vials of reconstituted BTX-A and subsequent storage of the remaining solution under refrigeration for 4 weeks.

Methods: A portion of the contents of 88 consecutive 100-U vials of BTX-A was administered aseptically to 108 patients for essential blepharospasm, hemifacial spasm, or facial rejuvenation. The vials were then stored for 4 weeks in a refrigerator, after which the contents were transferred to various media (blood agar, chocolate agar, Sabouraud agar, brain-heart infusion medium, and thioglycolate broth) and assessed for bacterial and/or fungal growth by standard methods.

Results: None of the BTX-A vials contained detectable bacterial or fungal contamination after 4 weeks of storage.

Conclusions: Storing vials of reconstituted BTX-A for 4 weeks after administration to patients was not associated with detectable growth of bacteria or fungi.

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Botulinum toxin (BTX) is a potent neurotoxin produced by Clostridium botulinum that functions by blocking the release of acetylcholine at the neuromuscular junction of cholinergic nerves. C. botulinum synthesizes 7 structurally similar but antigenically distinct serotypes of BTX, denoted A, B, C, D, E, F, and G. In 1973, Scott et al1 described the administration of serotype A of BTX (BTX-A) as treatment for strabismus. Subsequently, authors have applied BTX-A to facial rejuvenation and to treat essential blepharospasm, hemifacial spasm, synkinesis, spasmodic dysphonia, torticollis, hyperhidrosis and chronic migraine.2-7

Prepared BTX-A (ie, onabotulinumtoxinA, Botox) is easily denatures, and the manufacturer (Allergan Inc, Irvine, CA) recommends storage in a refrigerator at ≤5°C. BTX-A is supplied as 100-U vials of a freeze-dried crystal-line complex without preservatives and must be reconsti-tuted with preservative-free sterile saline before injection. Once reconstituted, the manufacturer recommends administering BTX-A within 24 hours. However, the typical patient does not require the entire contents of a BTX-A vial, and even if the physician chooses the off-label practice of applying the contents of a vial to 2 or more patients, it may be unlikely that the patients could undergo treatment within the 24-hour interval. Consequently, waste

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associated with BTX-A treatment is common. The high cost of BTX-A and results of several studies indicating that the drug retains efficacy for longer than the recommended period have led physicians, especially those administering BTX-A for cosmetic purposes, to split BTX-A vials among patients and store the remaining reconstituted product for > 24 hours. However, there is insufficient evidence to indicate that the sterility of previously reconstituted BTX-A is maintained during storage.

In the present study, we sought to determine whether bacterial and/or fungal proliferation occurred in reconstituted vials of BTX-A after clinical administration of an aliquot and subsequent storage under refrigeration for 4 weeks.

**METHODS**

Eighty-eight consecutively 100-U vials of BTX-A (onabotulinumtoxinA, Botox; Allergan Inc) were assessed for fungal and/or bacterial contamination according to a protocol approved by the Institutional Review Board of the Federal University of São Paulo.

Seventy patients were treated with reconstituted BTX-A under aseptic conditions for essential blepharospasm or hemifacial spasm (group 1: 68 patients each treated from a separate vial) or for facial rejuvenation (group 2: 2 patients treated successively with 20 vials total). Two of the senior investigators (M.H.O. and A.E.S.) performed all steps involved in preparing, administering, and the BTX-A. Immediately before application, the rubber top of each vial was punctured with a 22-G needle, and the BTX-A preparation was reconstituted with 2.0 mL of normal saline without preservatives. With the 22-G needle still attached to the vial, an insulin syringe was connected, and a volume of reconstituted BTX-A was aspirated according to the patient’s clinical condition and individual response. The insulin syringe then was disconnected from the 22-G needle and attached to an insulin needle.

Each patient in group 1 received a single treatment with a separate insulin needle and syringe. Each patient in group 2 received several treatments at intervals of 0 to 7 days. Between treatments, vials containing reconstituted BTX-A were stored in sealed plastic containers in a refrigerator at 5°C, and metal and rubber caps were removed from vials for the subsequent treatment; this method is common in clinical practice.

Upon completion of each treatment, the aspiration needle was withdrawn, and each vial was placed in a plastic bag and stored for 4 weeks at 3°C to 5°C on a shelf of an unlocked, multipurpose medication refrigerator in a clean utility room. The refrigerator door was opened and closed as needed for routine office practice, and access to the refrigerator for other supplies was not restricted.

After 4 weeks of storage, the vials were assessed for the presence of bacteria and/or fungi. Adherence to all routine quality-control measures of the Department of Ophthalmology at our institution, including comparison with reference strains (ATCC, Manassas, VA) and sensitivity tests, was ensured throughout the study. A senior microbiologist (M.C.Z.Y.) removed the caps and aspirated 0.1-mL (5-U) aliquots of the vial contents. From these aliquots, 10-μL volumes were transferred dropwise with calibrated inoculation loops to the surfaces of plates containing blood agar, chocolate agar, and Sabouraud agar and incubated under anaerobic conditions with a GasPak container (BD, Franklin Lakes, NJ). The presence of homogeneous growth in the solid culture medium at the site of seeding was presumed to indicate contamination of the corresponding vial.

**RESULTS**

The remaining contents of the 88 vials of reconstituted BTX-A, refrigerated for 4 weeks after utilization for facial rejuvenation or treatment of essential blepharospasm or hemifacial spasm, yielded no detectable bacterial or fungal contamination via any testing method employed.

**DISCUSSION**

BTX-A is a costly product supplied as individual vials that contain more drug than is typically administered to a single patient in a given treatment session. The preparation of BTX-A employed in this study (onabotulinumtoxinA, Botox) is packaged in 100-U vials. However, patients undergoing treatment for essential blepharospasm or hemifacial spasm require substantially less, between 30 U and 50 U per visit. Given the recommendations of the manufacturer (Allergan, Inc) that BTX-A should be applied within 24 hours after reconstitution, large amounts of this drug are often discarded in clinical practice.

It is common for physicians to perform the off-label practices of storing reconstituted BTX-A for more than the recommended 24 hours and splitting vial contents to treat multiple patients. Assessments of stored, reconstituted BTX-A indicated that the toxin’s activity is retained during storage periods ranging from 24 hours to 6 weeks.

In 1998, while the manufacturer had recommended administering BTX-A within 4 hours after reconstitution,
Klein\textsuperscript{11} polled physicians who applied the drug for aesthetic purposes and found that 23\% limited the storage period of the diluted material to 24 hours, 8\% stored it for up to 4 days, 46\% stored it for up to 1 week, 8\% stored it for up to 10 days, and 15\% stored it for up to 2 weeks.\textsuperscript{11} A consensus panel in 2010\textsuperscript{12} evaluated adverse outcomes of BTX-A administered after the recommended storage period and found that the drug could be applied safely 2 to 3 weeks after reconstitution. The results of an online survey of BTX-A utilization among members of the American Society for Dermatologic Surgery\textsuperscript{13} indicated that most physicians (68.6\%) routinely stored BTX-A for >1 week after reconstitution.

Although finding of various studies\textsuperscript{5,8-10} indicate that reconstituted BTX-A retains efficacy during storage for several weeks, there is insufficient evidence that sterility is maintained for this duration. Regardless of potency, contamination remains a concern for physicians who treat patients with reconstituted BTX-A after the recommended 24-hour period.

The results of our study demonstrated no bacterial or fungal contamination after treatment of patients with BTX-A and subsequent refrigeration of the reconstituted product for 4 weeks. Table 2 summarizes published findings of BTX-A administration and storage and compares them with results of the present study. Alam et al\textsuperscript{2} suggested that vials of exotoxin reconstituted with benzyl alcohol–preserved saline could safely be stored in a refrigerator for up to 7 weeks and that multiple aliquots of BTX-A could be

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**Table 1. Culture Media Utilized to Detect Bacterial and/or Fungal Growth in Vials Containing Botulinum Toxin Type A**

<table>
<thead>
<tr>
<th>Medium Name</th>
<th>Medium Type</th>
<th>Site of Preparation</th>
<th>Incubation Parameters</th>
<th>Microorganism(s) Supported</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood agar</td>
<td>Solid</td>
<td>In-house with 5% sheep blood</td>
<td>35°C for 7 d; ambient atmosphere; observe daily</td>
<td>Most aerobic and facultative anaerobic bacteria; coccus and bacillus; Gram negative and positive</td>
</tr>
<tr>
<td>Chocolate agar</td>
<td>Nonselective enriched solid</td>
<td>In-house with 5% sheep blood, heated to release the components of red blood cells</td>
<td>35°C for 7 d; 5%-10% CO\textsubscript{2}; observe daily</td>
<td>Exigent and or microaerophilic bacteria; nutritionally fastidious bacteria (eg, Streptococcus and Haemophilus spp)</td>
</tr>
<tr>
<td>Sabouraud agar</td>
<td>Solid</td>
<td>Oxoid\textsuperscript{a}</td>
<td>25°C for 30 d; ambient atmosphere; observe daily</td>
<td>Fungi</td>
</tr>
<tr>
<td>Thioglycolate broth</td>
<td>Nutrient-enriched liquid</td>
<td>Oxoid\textsuperscript{a}</td>
<td>35°C for 10 d; 5%-10% CO\textsubscript{2}; observe daily</td>
<td>Aerobic, microaerophilic, and anaerobic bacteria</td>
</tr>
<tr>
<td>Brain-heart infusion medium</td>
<td>Nutrient-enriched liquid</td>
<td>Oxoid\textsuperscript{a}</td>
<td>35°C for 7 d; ambient atmosphere; observe daily</td>
<td>Most aerobic microorganisms</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Manufacturer is located in Basingstoke, UK.

**Table 2. Summary of Studies Addressing Contamination in Stored Vials of Botulinum Toxin Type A**

<table>
<thead>
<tr>
<th>Reference</th>
<th>No. of Vials</th>
<th>Preparation of BTX-A</th>
<th>Reconstitution Liquid</th>
<th>Recipients</th>
<th>Storage Duration (Location)</th>
<th>Culture Media</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alam et al\textsuperscript{2}</td>
<td>127</td>
<td>Onabotulinum toxina</td>
<td>Preserved saline</td>
<td>Patients</td>
<td>Up to 7 wk (R)</td>
<td>Thioglycolate broth</td>
<td>No bacterial contamination</td>
</tr>
<tr>
<td>Menon et al\textsuperscript{3}</td>
<td>11</td>
<td>Abobotulinum toxina</td>
<td>Not stated</td>
<td>Patients</td>
<td>Up to 4 h at RT then 5-7 d (R)</td>
<td>Blood agar, chocolate agar, Sabouraud agar, and nutrient broth</td>
<td>No bacterial/ fungal contamination</td>
</tr>
<tr>
<td>Jabor et al\textsuperscript{8}</td>
<td>1</td>
<td>Onabotulinum toxina</td>
<td>Nonpreserved saline</td>
<td>Experimental rabbit model</td>
<td>Up to 12 wk (F)</td>
<td>Not stated</td>
<td>No bacterial contamination</td>
</tr>
<tr>
<td>Hexsel et al\textsuperscript{14}</td>
<td>8</td>
<td>Onabotulinum toxina</td>
<td>Nonpreserved saline</td>
<td>Patients</td>
<td>14 wk for 7 vials, 15 wk for 1 vial (NS)</td>
<td>Blood agar and Sabouraud agar</td>
<td>No bacterial/ fungal contamination</td>
</tr>
<tr>
<td>Present study</td>
<td>88</td>
<td>Onabotulinum toxina</td>
<td>Nonpreserved saline</td>
<td>Patients</td>
<td>4 wk (R)</td>
<td>Blood agar, chocolate agar, Sabouraud agar, brain-heart infusion medium, and thioglycolate broth</td>
<td>No bacterial/ fungal contamination</td>
</tr>
</tbody>
</table>

BTX-A, botulinum toxin type A; F, freezer; R, refrigerator; RT, room temperature.
removed and applied to patients during that period. In contrast to our study, Alam et al\(^2\) reconstituted the toxin with preserved saline and assessed contamination by transferring vial contents to thioglycolate broth only. Reconstitution with preserved saline is expected to enable more prolonged maintenance of sterility, but the manufacturer recommends preservative-free sterile saline. In addition, microorganisms that were unsupported by thioglycolate broth would have been missed by these authors.\(^9\) In a study of outpatients treated with BTX-A (abobotulinumtoxinA, Dysport; Ipsen, Slough, UK) from 11 consecutive bottles, Menon et al\(^3\) found that the bottles exposed to room temperature for up to 4 hours and subsequently stored in a refrigerator at 3°C to 5°C for 5 to 7 days remained sterile when aseptic precautions were taken during treatment. In an experimental study, Jabor et al\(^9\) observed no bacterial contamination in BTX-A reconstituted without preservatives, injected into rabbits, and subsequently stored for up to 12 weeks. However, these authors evaluated only 1 vial and did not specify the culture media utilized to detect contamination.\(^9\) Huxel et al\(^14\) performed microbiologic analyses of the contents of 8 vials of BTX-A reconstituted with unpreserved saline and stored for 14 weeks (7 vials) or 15 weeks (1 vial). These authors observed no growth of microorganisms in blood agar or Sabouraud agar.\(^14\)

It is challenging to compare the present study with existing literature on this topic because all such studies have involved different methodologies and sample sizes. However, the findings from all of these studies support that reconstituted BTX-A remains free of contamination by bacteria and/or fungi for 1 to 14 weeks, assuming aseptic precautions are taken.\(^2,3,9,14\)

The authors of the present study do not recommend utilizing reconstituted BTX-A beyond the 24-hour period indicated by the manufacturer. This was a pilot study undertaken to assess whether fungal and/or bacterial contamination could be detected after administration of some amount of reconstituted BTX-A and subsequent storage for 4 weeks. To our knowledge, this study is the first to apply the contents of numerous, consecutive BTX-A vials to several types of culture media for the assessment of bacterial and/or fungal growth. However, this study does have limitations. Group 2 comprised only 20 vials, and contamination may have been detected if more samples had been included. The evaluation of vials stored for longer than 4 weeks with analysis for contamination at weekly intervals would provide a more comprehensive depiction of bacterial and fungal contamination over time. Finally, the accumulation of other contaminants in reconstituted BTX-A vials, including other toxins or viruses, was not assessed in this study and has not been noted previously in the literature. In future studies, our research group plans to examine reconstituted BTX-A for viruses and other contaminants in conjunction with a team in the Ophthalmic Laboratory Division.

**CONCLUSIONS**

Reconstituted BTX-A that remained after application to patients (under aseptic conditions) and was refrigerated for 4 weeks did not yield detectable bacterial or fungal contamination. Subsequent studies are needed to assess a larger number of vials (especially in Group 2) of stored reconstituted BTX-A contents, especially those applied in facial rejuvenation, and to evaluate longer storage periods and the potential growth of other contaminants. The results of these studies may support the storage of reconstituted BTX-A for longer than the 24-hour period recommended by the manufacturer.

**Disclosures**

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**Disclaimer**

The authors of this article discuss storing reconstituted vials of onabotulinumtoxinA (Botox Cosmetic), a practice that is contrary to the recommendations in the manufacturer’s insert (Allergan, Inc, Irvine, CA). Publication of this article does not constitute endorsement of this practice by The American Society for Aesthetic Plastic Surgery, the *Aesthetic Surgery Journal*, or the publisher.

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