Multicenter, Double-Blind Study of the Efficacy of Injections With Botulinum Toxin Type A Reconstituted Up to Six Consecutive Weeks Before Application

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BACKGROUND. It is recommended that botulinum toxin be used immediately or within 2 weeks after its reconstitution because its efficacy might be compromised by prolonged storage.

OBJECTIVES. To evaluate the efficacy of botulinum toxin type A (BTX-A) reconstituted over 6 consecutive weeks for the treatment of glabellar frown lines.

METHODS. Four vials of BTX-A were reconstituted each of 7 days over a period of 6 weeks, totaling 28 vials, corresponding to seven reconstitution dates. During this period, the BTX-A was stored according to the manufacturer’s instructions. On the day after the last reconstitution, all of the reconstituted vials were injected in patients from four dermatologic centers taking part in this study. A total of 88 patients were treated on the same day and were followed every 2 weeks for 4 months. All patients were photographed at all stages. A number of professionals assessed the efficacy of reconstituted BTX-A based on the reduction of the maximum frowning capacity of the treated muscles.

RESULTS. Of the 88 patients who were selected, 3 were excluded. Three forms of evaluation were applied, and no statistically significant differences were found in the results presented.

CONCLUSION. BTX-A may be applied up to 6 weeks after reconstitution without losing its effectiveness. Other factors, which are probably individual, may influence the response to BTX-A injections.

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THE BOTULINUM toxin type A (BTX-A; Botox) produced by Allergan has proven therapeutic action in the treatment of glabellar frown lines1–8 and has been approved by the Food and Drug Administration for this indication. The glabellar region has frequently been used for research when assessing the efficacy and safety of BTX-A.3,5,8,11

Some precautions should be taken in order to achieve effective, long-lasting results from BTX-A application. Among them, the recommendations for reconstitution stand out. BTX-A should be reconstituted in 0.9% saline solution without preservatives.2,3,12–18 It is recommended, among other things, to avoid shaking, bubbling, and storing the vial for a long period of time.12,13,17,19 The manufacturers of the commercially available BTX-A products recommend that they should be used within the first 4 hours after reconstitution when using the BTX-A produced by Allergan (Botox)17 or within 1 hour after reconstitution when using the BTX-A produced by Speywood (Dysport).18 Using the reconstituted BTX-A after a period of time longer than that recommended may jeopardize the efficacy of the products. However, studies have shown that Botox does not have its potency altered even when it is refrigerated or refrozen for up to 1 week20 or for up to 2 weeks after reconstitution.15,21,22 Klein23 has reported that some investigators use BTX-A for 7 to 30 days after its reconstitution, which is also reported by Khawaja and Hernandez-Perez.24 Alam et al.25 in their study, mention that Aoki, a researcher working for Allergan, has reported that reconstituted botulinum toxin preserved in saline and kept in refrigeration is stable for up to 5 weeks. However, Fagien and Brandt26 observed a subtle, although significant, decrease in the potency of botulinum toxin that had been reconstituted 48 hours previously.

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This study was carried out in four dermatologic centers using BTX-A produced by Allergan (Botox). All of the toxin used in this study was reconstituted over a period of 6 weeks and was applied into the glabellar region of individuals of different genders and ages. The aim of the study was to assess the efficacy of BTX-A reconstituted beyond the time limits recommended by the manufacturers and until now found in the literature.

Methods

In the first stage of this study, representing 6 weeks, a single professional (V.L.B.S.) was responsible for reconstituting, labeling, and storing the vials of BTX-A. This professional did not participate in the other stages. Each 7 days, four vials of BTX-A were reconstituted over a 6-week period, resulting in seven predetermined reconstitution dates: 43, 36, 29, 22, 15, 8, and 1 day before the application day. On each date, the four reconstituted vials were labeled with color-coded tags to indicate the date of reconstitution. The reconstituted vials were stored in a refrigerator at a constant temperature of 4°C.2,12 The nonreconstituted vials were stored in the freezer according to the manufacturer’s instructions.17 The color code for the reconstitution dates was known to only the single professional responsible and was maintained confidential until the end of the study. Seven different colored-labeled vials were sent to each of the four study centers 1 day before the application date.

In the second stage of this study, representing 16 weeks, 88 patients of both genders and ranging from 19 to 79 years of age (mean age 46.41) were selected in four participating centers. These exclusion criteria were considered: pregnant women; people with muscular, rheumatic, neurologic, or psychiatric diseases; patients having taken aminoglycoside antibiotics and anti-inflammatory agents within the last 4 weeks; and those with known hypersensitivity to human albumin or botulinum toxin.2,24,26–28 Three patients were excluded. The remaining 85 patients signed a free postinformed consent form.4,28

The BTX-A used in the study was produced by Allergan (Botox), a 100 U/vial, reconstituted in 1 mL of sterile 0.9% saline solution without preservatives,17 which was applied with 3-mL insulin syringes (Becton-Dickinson Canada Inc., Mississauga, Ontario, Canada) and 21-gauge needles2,12,13,29 and resulting in a concentration of 100 U per 1 mL.2,12,13,26 All vials containing the product belonged to the same manufacturer’s batch (C373) and were valid up to March 2005. Bubbling and excessive handling of the vials were avoided, as recommended.2,12,30

A single professional from each of the four participating dermatologic centers was responsible for injecting the BTX-A from the seven color-coded labeled vials on the application day, 1 day after the last vials were reconstituted. On the application day, all patients were photographed at rest and during maximum contraction of the procerus and corrugator supercilii muscles, which are responsible for the glabellar frown lines. The vial to be used for application was randomly selected and was recorded in a specific register.

BTX-A was applied into three sites according to the study protocol: one at each corrugator muscle, one at the point in the brow area along a vertical line originating from the inner eye canthus, and one at the procerus muscle, in the middle of an imaginary “X” between the brows and the inner eye canthus (Figure 1). Male patients received 9 U into each site, totaling 27 U, and female patients received 8 U at each site, totaling 24 U. For the application, a Becton-Dickinson Ultra Fine II 0.3-cc syringe with a short needle was used.14,29

Patients were followed every 2 weeks over a period of 4 months, and the results were recorded in the following three forms: (1) clinical and photographic evaluation, with Kodak-Ektachrome ISO 100 film, at maximum voluntary frowning (frequency: at each visit); (2) evaluating physician’s and patient’s perceptions (frequency: at each visit); and (3) blind and independent analysis of the photographs by the participating physicians as well as by two other invited physicians who were not involved in the other study phases (at separate sessions).

In the third stage of the study, 3 months after the end of the second stage, the results obtained from the
blind analysis were evaluated by all of the investigators and two other collaborators at a joint session. A simultaneous exhibition using two Kodak 4400 carousel projectors was carried out. First, the initial photographs (Day 1) at rest were exhibited in order to evaluate the presence of wrinkles at rest and then those during maximum voluntary contraction of the muscles in the glabella were exhibited in order to evaluate muscle contraction capacity. Immediately afterward, the photograph with the maximum contraction was kept on the left, and the control photographs were exhibited on the right in a decreasing chronological order of visits, beginning from the comparative control at 120 days and successively (105, 90, 75, etc.), as illustrated in Figure 2.

Figure 2. Comparative photographic sequence of the same patient. (A) Forty-three days of reconstitution. (B) Day 1 of reconstitution.
Evaluators filled out an evaluation report according to the following criteria: (1) the presence or the absence of wrinkles or furrows on the glabella, resulting from contraction of the corrugator supercilii and procerus muscles at rest, on the first day of the study, and the classification of the wrinkle that is formed during contraction of the muscles in the glabella, before botulinum toxin application, as small, medium, or large; and (2) motility degree of the muscles in the glabella at all visits compared with Day 1 of the study in four categories: unaltered (0), slightly reduced (1), moderately reduced (2), and greatly reduced (3).

It should be reiterated here that none of the participants in this stage were aware of the significance of the color-coded labels.

Statistical Data Analysis

Several statistical tests were applied with the goal of identifying possible relationships between the different variables. The Kruskal–Wallis test was employed in the statistical analysis of the data obtained in the study because it permits the comparison of more than one variable in just one treatment. The critical \( P \) value used was lower than or equal to 0.05. Data were loaded in a Microsoft Excel 7.0 spreadsheet for Windows 98, transferred to a database, and were subsequently assessed using the software for statistical analysis SPSS 8.0.

Results

Of the 88 patients in the study, 3 were excluded. Of the remaining patients, 13 were included in group 1 (received BTX-A reconstituted 1 day before), 14 in group 2 (received BTX-A reconstituted 8 days before), 12 in group 3 (received BTX-A reconstituted 15 days before), 12 in group 4 (received BTX-A reconstituted 22 days before), 12 in group 5 (received BTX-A reconstituted 29 days before), 10 in group 6 (received BTX-A reconstituted 36 days before), and 12 in group 7 (received BTX-A reconstituted 43 days before), with a mean of 12.14 patients per group. Eighty-one patients attended the first evaluation at 15 days (95.2%), and 65 patients completed the evaluation at 120 days (76.4%). Considering losses, only those patients that missed the last evaluation or more than two consecutive evaluations were considered as such. There was no statistically significant difference in loss between the groups; the highest loss was in group 5, with six patients, and the lowest losses were in groups 2 and 3, with one patient.

As for the genders, 11 (12.9%) male patients and 74 (87.1%) female patients were included. Fitzpatrick standards for skin phototypes were used, and patients were classified as follows: 2 patients (3.2%) belonging to phototype I, 22 (34.9%) belonging to phototype II, 23 (36.5%) belonging to phototype III, 15 (23.8%) belonging to phototype IV, and 1 patient belonging to phototype V.

Five patients had minor bleeding during the application. One patient reported erythema at the application site. One patient experienced worsening of pre-existing unilateral eyelid ptosis, which resolved spontaneously within approximately 30 days. One patient reported a decrease in visual acuity in the first 3 days.

Three distinct evaluations of the responses to these applications of Botox were analyzed: (1) The perception of the physician who performed the application was evaluated at 15, 30, 45, 60, 75, 90, 105, and 120 days after injection when the muscle motility at the injected area was verified; (2) the perception of the patient regarding motility at the injected area, on those same evaluation days; and (3) the average of each one of the six evaluators’ perceptions, in a joint session at the end of the study, with regard the presence of wrinkles and the intensity of maximum voluntary contraction through the photographs taken on the evaluation days, compared with maximum voluntary frowning at the first day.

The Kruskal–Wallis statistical analysis test was employed. No statistically significant difference, in terms of motility and efficacy, was found in the results of the three ascertainments cited previously here. This can be observed in accordance with the significance value, statistic \( P \), expressed in Table 1 and also with the results obtained with Botox from different dates of reconstitution, showed in the Figure 2A,B.

| Table 1. Significance Values, Critical \( P \), of the Different Ascertainments, on the Evaluation Dates, Based on the Kruskal–Wallis Test (\( P < 0.05 \)) |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Day 15          | Day 30          | Day 45          | Day 60          | Day 75          | Day 90          | Day 105         | Day 120         |
| Motility as evaluated by the observer | 0.549           | 0.332           | 0.616           | 0.815           | 0.891           | 0.97            | 0.884           | 0.53            |
| Motility as evaluated by the patient  | 0.221           | 0.295           | 0.481           | 0.485           | 0.697           | 0.471           | 0.88            | 0.844           |
| Motility as jointly evaluated         | 0.082           | 0.132           | 0.431           | 0.928           | 0.237           | 0.792           | 0.699           | 0.643           |
Figures 3–5 show the comparison of the averages of the evaluating physician’s perceptions, the patient’s perceptions, and the analysis at a joint session.

**Discussion**

This study evaluated an 85-patient sample, with mean age of 46.41 years, homogeneous regarding the phototype, and predominantly female. This sample is coherent with that of people who go to practices in order to make use of Botox for cosmetic purposes.

Each patient has particularities regarding their facial muscles, and they need individualized cosmetic treatment. However, each of the muscles in the glabella in different individuals may require more than one application site and different doses to reach the same efficacy on paralysis.\(^{31,32}\) However, a comparative study such as this requires standardization of some items. Standardization of application sites for all of the patients and the doses according to the gender was preferred, which was higher for male patients. It is consensual that men have more potent muscles and thus need higher doses for treatment.\(^{5,33,34}\)

Typically, the dose predicted for the glabella is 25 U—distributed into five sites\(^{2,3,13,35}\)—or 20 or 25 U—distributed into five or seven sites (depending on the wrinkle type and intensity) for women, and 35 U into seven sites for men.\(^{5,33}\)
Although Gartland and Hoffman found in an in vitro study a 69.8% loss in potency when Botox was reconstituted, immediately frozen, and then assayed 2 weeks later and a statistically significant degradation in potency after refrigerator storage for 12 hours, 36 in this study, it has been demonstrated that with correct sterile handling and storage under refrigeration12,13,17 using Botox within up to 6 weeks after reconstitution does not significantly alter the detectable clinical response, whether from the patient’s or the observer’s point of view. The relevance of this lies in the fact that it is possible to fractionate doses from the same Botox vial, which may be stored for a longer period of time; this facilitates the clinical practice of physicians who work in specialties that use very low doses.37,38 The reduction of Botox activity, with motility and wrinkles returning over the study time, was clear and may be evidenced in the three forms of evaluation illustrated in Figures 1–3. Such a reduction is well known in clinical practice and in the studies of efficacy of the product2,3,13 and could be considered as a suggestive indirect indication that the forms of evaluation used showed enough sensitivity to detect alterations in motility and in the pattern of wrinkles. It should be highlighted that the methods used for the evaluation of results are subjective. To obtain objective results, the use of electromyographic recording,13,39 an invasive procedure that requires some additional financial investment and that is painful for the patient, thus diminishing the compliance of patients with the study, would be necessary.

However, as for the reduction of motility in the treated areas, compared with the initial motility, no statistically significant differences were found. These results may be observed in Table 1, where P<0.05 is considered. This assessment proves that vials of reconstituted BTX-A can be preserved in refrigeration and stored in optimal conditions for up to 6 weeks.

Few complications or adverse effects have been reported to date.7,40 This study verified the occurrence of few unwanted effects.

Some differences in the duration of effect were observed for some patients who received BTX-A from the same vial, reconstituted on the same date, and applied with the same dose. From this, we could infer that there is the possibility that individual factors may influence the response to BTX-A application.31,32,41,42 Because this was not the objective of the study, comparisons to ascertain this fact have not been performed.

Conclusion

This study showed that BTX-A applied up to 43 days after reconstitution does not present significant differences in terms of efficacy, in accordance with the used methods. Differences observed in the responses of patients who received the same BTX-A, reconstituted on the same date, show that other, perhaps individual factors may interfere with the response.

References


Figure 5. Average of the perceptions of the six evaluators in the study, at a joint session, about the presence of wrinkles in the photographs taken on the evaluation days (15, 30, 45, 60, 75, 90, 105, and 120 days after injection).


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Commentary

For some time, there has been evidence that the potency of BTX-A does not decline as rapidly as some authors have suggested. Garcia and Fulton were the first to publish evidence that the toxin was effective for longer than the 4 hours printed on the label. There have been oral reports of potency being maintained for 5 weeks after reconstitution with preserved saline, using the mouse lethality assay.

Now Hexsel et al. present a well-designed study that demonstrates clinical effectiveness is unchanged up to 6 weeks. Clinicians can be reassured that this further evidence supports our using BTX-A for periods longer than those on the label.

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