Effect of the Refrigerator Storage Time on the Potency of Botox for Human Extensor Digitorum Brevis Muscle Paralysis

Mee Young Park,a Ki Young Ahn b

a Department of Neurology, College of Medicine, Yeungnam University, Daegu, Korea
b Ahn’s Plastic and Reconstructive Surgery Clinic and Botulinum Center, Daegu, Korea

Background and Purpose It is recommended that Botox be used within 5 hours of reconstitution, which results in substantial quantities being discarded. This is not only uneconomic, but also inconvenient for treating patients. The aim of this study was to determine the potencies of Botox used within 2 hours of reconstitution with unpreserved saline, the same Botox refrigerated (at +4ºC) 72 hours after reconstitution, and during the next 4 consecutive weeks (weeks 1, 2, 3, and 4). This comparison was used to determine the length of refrigeration time during which reconstituted Botox will maintain the same efficacy as freshly reconstituted toxin.

Methods Individual paralysis rates in the extensor digitorum brevis (EDB) compound muscle action potential (CMAP) amplitude and area were measured 1 week after injecting fresh reconstituted 2.5 MU of Botox on one side of the foot, and when the same quantity of Botox that had been refrigerated for a designated time (i.e., 72 h, or 1, 2, 3, or 4 weeks) into the other side of the foot. The EDB CMAP amplitude and area at 12 and 16 weeks postinjection were also measured to compare the efficacy durations in all five comparative groups.

Results Ninety-four volunteers were divided into five groups according to the refrigerator storage time of the second Botox injection. The paralysis of the EDBs was significant for each injection of Botox, both fresh and refrigerated, with no statistically significant differences between them, regardless of the refrigeration time. There was a tendency toward increased CMAP amplitude and area at 12 or 16 weeks postinjection (p<0.0001). The duration of effective muscle paralysis did not differ significantly throughout the 16-week follow-up period between all five groups.

Conclusions The potency of reconstituted Botox is not degraded by subsequent refrigeration for 4 weeks. However, there are definite concerns regarding its sterility, and hence its safety, since multiple withdrawals from the same vial over long periods can introduce bacterial contamination.

Key Words botox, potency, refrigeration.

Introduction Botulinum toxin type A (BoNT-A), an exotoxin that is generated by the anaerobic bacteria Clostridium botulinum, produces muscle paralysis by interacting with synaptosomal-associated protein 251,2 on the neuromuscular junction, blocking the secretion of acetylcholine. This effect may last for several months.3,4 The injection of a large amount of BoNT-A may lead to death due to paralysis of the respiratory muscles; however, if a small dose of refined BoNT-A is injected into specific muscles, its muscle-relaxing and paralyzing effects can be used as a remedy for areas of excessive muscle tension. Indeed, BoNT-A has been used for more than 20 years in treating patients with disorders related to excessive muscle tension. Indeed, BoNT-A has been used for more than 20 years in treating patients with disorders related to excessive muscle tension. Indeed, BoNT-A has been used for more than 20 years in treating patients with disorders related to excessive muscle tension.

Copyright © 2013 Korean Neurological Association
been widely used in the fields of plastic surgery, dermatology, neurology, and rehabilitation medicine. Its applications are also expected to increase in other areas.\textsuperscript{6-10}

While Botox has been used in various fields, there some disadvantages to be considered with regards to its storage. The package insert says that Botox should be kept at below \(-5^\circ\text{C}\) in a frozen dry state and should be used immediately after it is reconstituted. Furthermore, it is strongly recommended that re-freezing be avoided, and that any remaining solution should be stored in the refrigerator and used within 5 hours to prevent degeneration of its potency. Therefore, once it is reconstituted, any solution remaining of the diluted vial [which is typically 50 or 100 Mouse Units (MU)] should be discarded if it is not used in 5 hours. This may result in large financial losses, requiring clinicians to follow appropriate protocols to consume all of the 50 or 100 MU reconstituted at a time; one such protocol is to wait until they have sufficient patients to use the entire amount. In fact, except for special cases such as torticollis or cerebral palsy, most patients only need 10-30 MU. Therefore, five or more patients might be needed to consume all 50 or 100 MU at a time, which results in most clinicians storing it for more than 4 hours, possibly for up to 2-4 weeks after mixing, and using the refrigerated, reconstituted Botox without any accurate standards or guidelines.\textsuperscript{11} If the potency of the Botox is reduced, its efficacy would also be affected, and the treatment dose and the effective period should thus be changed. It is therefore critical to have information regarding any change in the potency of this toxin with refrigeration storage to enable a more accurate estimation of the required treatment dose and its effective period.

Few studies have investigated the effects of storage methods and times on Botox potency,\textsuperscript{12-18} and they have yielded inconsistent findings, making it very difficult to obtain coherent information. The Institutional Review Board of our hospital approved the present prospective study, which aimed to determine how long reconstituted Botox can be stored in a refrigerator and maintain the same efficacy as if it had been used within 5 hours of its initial reconstitution in saline solution. To this end, experimental periods that are commonly used in normal clinical studies were used, such that the potency of Botox was tested after refrigerator storage for 72 hours and then 1, 2, 3, and 4 weeks after reconstitution. The same amounts of freshly reconstituted Botox and the same sample but after a period of refrigeration was injected into each subject’s opposing extensor digitorum brevis muscles (EDBs; i.e., on opposite feet) at regular intervals, and the paralysis rates of each subject were measured at 1, 12, and 16 weeks postinjection to allow comparison of fresh and refrigerated Botox.

The findings of this study will contribute to effective Botox treatments and should resolve some of the current disadvantages related to its use, as well as achieving cost effectiveness by determining the maximum refrigerator storage period that ensures the same effect of freshly reconstituted Botox.

**Methods**

**Subjects**

This was a rater-blinded randomized study. In total, 94 healthy volunteers (67 men and 27 women) were divided into 5 groups. To avoid the age-related differences in paralysis rates between subjects, only subjects in their twenties were allowed to enroll. None of the enrolled healthy subjects had any previous experience with BoNT-A and were free of both central and peripheral nervous system diseases, and had no history of musculoskeletal injuries. Their EDB muscles were confirmed as being normal before the study, with subjects exhibiting a more than 50% difference in the amplitude of mean compound muscle action potential (CMAP) between the EDBs on the two feet during the nerve conduction study (NCS) performed simultaneously before Botox injection were excluded.

Subjects were randomly assigned to one of five groups, and each EDB was also randomly assigned to either freshly reconstituted (control group) or refrigerator-stored (test group) Botox in all subjects. Each subject in each of the five study groups was injected with fresh toxin into the EDB on one side and refrigerated toxin from the same vial into the EDB on the opposite foot (Fig. 1). To avoid any differences attributable to the specific vial, the same vial was used in each study group, thus limiting the number of subjects 20 for each study group (20 subjects×2.5 MU×2 sides EDB=100 MU).

**Dilution and injection**

The EDB was used as a test muscle because it is readily differentiated from the other foot muscles and is not normally used as much as other foot muscles so that even if it is paralyzed, it would be asymptomatic or cause only minor inconvenience. In addition, NCSs on the EDB are generally considered reliable.

Botox (Allergan Pharmaceuticals) was injected into a spot marked on the EDB muscle where the amplitude and area of the CMAP would be measured before and after injection; the mark placed on the EDB was maintained for a further week to minimize any errors in the subsequent NCS tests for assessing EDB paralysis. The injection was given with a 29-gauge insulin needle with the foot in the same posture as in the NCS.

The EDB muscle on one foot was injected with 2.5 MU/0.1 mL Botox that had been immediately reconstituted with 4 mL of normal saline (fresh toxin; control side), and that on the contralateral foot was injected with the same amount of the same toxin, from the same vial, but which had been stored in a refrigerator (below \(+4^\circ\text{C}\)) for a designated test period (i.e., 72
hours, or 1, 2, 3, or 4 weeks) after first reconstitution.

**Measurement and analysis**

To measure the effect of refrigerator storage time on the potency of Botox, muscle paralysis rates—as assessed by the CMAP amplitude and area—were compared with baseline measurements using NCSs at 1 week postinjection, which was the maximum time point of the effect. In addition, the effect of Botox refrigerator storage time on the duration of the paralysis was evaluated by measuring the CMAP of each of the five storage time groups at 12 and 16 weeks postinjection and compared to those measured preinjection (Fig. 1).

The EDBs were considered to have been paralyzed if there was a decrease of more than 20% in the CMAP amplitude and area. Conversely, the effect was considered to have faded if these parameters had recovered to more than 80%. The degree of paralysis of the EDB was calculated using the following formula:

\[
\text{Paralysis} (\%) = \frac{\text{CMAP amplitude (area) of EDB before injection} - \text{CMAP amplitude (area) of EDB after injection}}{\text{CMAP amplitude (area) of EDB before injection}} \times 100
\]

In the NCS, Counterpoint MK2 (Dantec, Skorlunde, Denmark) electromyography was used with filter settings of 5 Hz and 5 kHz. A ground electrode (a 10-mm-diameter surface electrode) was placed on the anterior tibialis, and a reference electrode was placed on the outside of the foot about 6 cm from a recording electrode (recording site). A supramaximal stimulus was applied to the ankle area about 6-8 cm from the recording site to stimulate the peroneal nerve, yielding a maximum CMAP. For the test reliability, NCSs were repeated five times and the maximum duplicable value was accepted. The skin temperature was maintained at \( \geq 32^\circ\text{C} \) during the test. All NCSs were performed by the same investigator who was blinded to the injection condition.

SPSS (version 10.0, Chicago, IL, USA) software was used for statistical analysis of the results. Changes in the paralysis rate over time and comparison of paralysis rates between the control and test groups were detected using repeated-measurement two-factor analysis. Comparison analysis for age, stimulus intensity, and paralysis rate among the five refrigeration period groups was achieved by ANOVA verification. The data are presented as mean \( \pm \) SD values, and the level of statistical significance was set at \( p < 0.05 \).

**Results**

The 72-hour, and 1-, 2-, 3-, and 4-week Botox refrigeration groups comprised 20 (18 men and 2 women), 18 (12 men and 6 women), 20 (11 men and 9 women), 19 (13 men and 6 women), and 17 (13 men and 4 women) subjects, respectively. Thus, 94 subjects (67 men and 27 women) participated in this study.

The age of the entire cohort was \( 24.0 \pm 2.3 \) years, and the electrical stimulation intensity did not differ significantly between the fresh-toxin group (61.2 \( \pm \) 2.7 mV) and the refrigerated-toxin group (61.3 \( \pm \) 3.3 mV) (Table 1). Representative NCS
Potency of Refrigerator-Stored Diluted Botox

CMAP results before and after Botox injection are presented in Fig. 2. The mean EDB CMAP amplitudes in the fresh-toxin and refrigerated-toxin groups at baseline were 9.8±3.6 mV (range, 8.9-10.7 mV) and 10.4±3.9 mV (9.6-10.7 mV), respectively; the difference between the groups was not significant. The mean EDB CMAP amplitudes and areas at baseline, and weeks 1, 12, and 16 postinjection for paralysis rating of both the fresh toxin (control) and toxin refrigerated for 72 hours, or 1, 2, 3, or 4 weeks (test) are listed in Table 2.

There was no difference in the individual paralysis rates of the CMAP amplitude at 1 week postinjection between the fresh-toxin group (control, 48.5±17.1%) and all of the refrigerated-toxin groups (test, 48.4±15.7%). Furthermore, there were no statistical differences between the control and all five test groups, which shows that there was meaningful EDB paralysis with both the fresh toxin and the variously refrigerated toxin at 1 week post-Botox injection (p<0.0001). Similarly, testing of the paralysis rate the fresh-toxin control group and all of the individual test groups (i.e., 72 hours, and 1, 2, 3, and 4 weeks of refrigeration) revealed no significant differences between group pairs (p>0.05), decreasing equally over time, with recovery from muscle paralysis beginning after 16 weeks. However, with the exception of group 1 (Table 2), most of the EDBs were still paralyzed by a decrease of more than 20% at 16 weeks compared with the baseline (Table 2). Nevertheless, the paralysis rate did not differ significantly between the control groups and all five test groups, and thus it appears that the potency of Botox was not affected even after refrigeration at +4ºC for 4 weeks after initial reconstitution in normal saline solution. Indeed, not only was the potency of the reconstituted, 4-week refrigerated Botox the same as that of freshly reconstituted Botox with respect to the EDB paralysis rate, but also the effect of the refrigerated toxin appears to have lasted as long as that of the freshly diluted toxin. Furthermore, since the 1-, 2-, 3-, and 4-week refrigerated-toxin groups exhibited a paralysis rate of more than 20% in the 16th week postinjection, the effect lasted for up to 16 weeks (Table 2).

The average paralysis rates of the CMAP area yielded similar results (Table 3). None of the subjects experienced any particular side effects or reported any subjective symptoms or muscle pain throughout the test.

**Discussion**

BoNT-A is a neurotoxin that can be generated by rotten canned food, and causes botulism. In 1895, 34 persons in Belgium who had eaten “off” ham exhibited muscle paralysis. Professor Ermengem isolated the bacteria from the remaining food

---

Table 1. Mean values of age of the entire cohort and electrical stimulation intensities

| Group | Volunteer | Age (years) | Stimulation intensity (mV) |
|-------|-----------|-------------|---------------------------|
|       |           |             | Control | Test |
| 72 hrs | 23.7±1.1  | 63.0±6.4    | 64.0±7.1 |
| 1 wk   | 26.4±3.2  | 62.0±3.4    | 61.7±3.0 |
| 2 wks  | 22.3±1.6  | 60.3±1.1    | 60.0±2.5 |
| 3 wks  | 23.1±1.2  | 60.0±1.2    | 61.0±2.1 |
| 4 wks  | 24.6±2.0  | 60.0±1.3    | 60.0±1.9 |
| Mean   | 24.0±2.3  | 61.1±2.7    | 61.3±3.3 |

Control: fresh toxin, hrs: hours, Test: refrigerated toxin, wk: week.

![Fig. 2. Extensor digitorum brevis (EDB) muscle compound muscle action potential amplitudes and areas of representative subject who was injected 2.5 MU/0.1 mL fresh Botox and 1 week refrigerated Botox on each side of the EDB muscles. There are meaningful EDB paralysis with both fresh and refrigerated toxins at 1, 12, and 16 weeks post injection.](image-url)
and dead bodies, and 2 years later disclosed that it was botulism caused by the bacteria *Clostridium botulinum*. Since then, crystallized BoNT-A has been isolated and refined for medical applications. Clinically, after animal experiments conducted by Dr. Scott in 1973, it was first used for treating patients with extracranial muscle strabismus, and later mainly for neurological diseases. Finally, in 1989, BoNT-A, a form of BoNT-A, was formally approved by the United States Food and Drug Administration (US FDA) as a treatment for strabismus, facial spasm, blepharospasm, and facial nerve disorders. The United States Food and Drug Administration approved the toxin for the treatment of cervical dystonia in 2000, glabellar frown lines in 2002, hyperhydrosis in 2004, upper limb spasticity in adults and chronic migraine in 2010, and more recently (in 2011), urinary incontinence in adults with neurological conditions including multiple sclerosis and spinal cord injury. Botox is currently approved for clinical use in more than 70 countries in the world, and has various medical and cosmetic indications.

Botulinum toxin type A blocks the neuromuscular junction to produce muscle paralysis by inhibiting acetylcholine release in the vesicular membrane, but its degree of reaction may differ according to the clinical issue. Several factors affect the position of EDB CMAP: extensor digitorum brevis compound muscle action potential, FT: fresh toxin, hrs: hours, RT: refrigerated toxin, wk: week.

**Table 2.** Mean CMAP amplitudes at baseline, and weeks 1, 2, 16 post Botox injection (2.5 MU/0.1 mL) for paralysis rating of both the fresh toxin (control) and toxin refrigerated for 72 hours, or 1, 2, 3, or 4 weeks (test) (mean mV±SD)

| Group | Pre & post injection | FT | RT | FT | RT | FT | RT | FT | RT | FT | RT |
|-------|----------------------|----|----|----|----|----|----|----|----|----|----|
| Pre-injection | 10.4±4.6 | 10.1±4.1 | 8.9±3.2 | 9.6±4.7 | 10.5±3.4 | 10.7±3.6 | 10.7±3.7 | 10.1±2.5 | 9.7±3.8 | 10.4±3.6 |
| Post-injection (1 wk) | 5.0±2.2 | 5.6±2.8 | 4.5±2.0 | 5.1±2.9 | 4.8±2.7 | 5.5±2.9 | 5.7±2.4 | 5.6±2.3 | 5.3±2.4 | 5.2±2.5 |
| % paralysis | 49.8±11.6 | 45.8±15.1 | 48.0±19.0 | 46.4±17.8 | 53.3±19.1 | 48.3±15.1 | 45.8±13.4 | 44.2±15.2 | 44.5±15.9 | 49.5±19.3 |
| Post-injection (12 wks) | 7.5±2.8 | 8.0±4.0 | 5.9±2.5 | 6.7±3.7 | 7.0±3.4 | 8.0±2.8 | 6.4±2.4 | 5.8±2.1 | 5.9±2.4 | 6.1±2.0 |
| % paralysis | 24.0±21.1 | 23.3±17.5 | 29.2±22.9 | 30.1±19.3 | 32.9±22.8 | 24.3±16.7 | 38.5±15.5 | 42.1±14.2 | 36.6±18.7 | 38.2±19.0 |
| Post-injection (16 wks) | 8.2±2.5 | 8.8±3.8 | 6.4±2.4 | 7.3±3.6 | 7.2±3.1 | 7.9±3.0 | 6.7±2.4 | 6.4±1.8 | 6.3±2.6 | 6.3±2.4 |

There was no difference in the individual paralysis rates of the CMAP amplitude 1 week post injection between the fresh-toxin groups (control) and all of the refrigerated-toxin groups (test). Furthermore, there were no statistical differences between the control and all five test groups, which shows that meaningful EDB paralysis with both the fresh toxin and the variously refrigerated toxin at 1 week post Botox injection (p=0.0001). Similarly, testing of the paralysis rate the fresh toxin control group and all of the individual test groups (i.e., 72 hours, and 1, 2, 3, and 4 weeks of refrigeration) revealed no significant differences between group pairs (p>0.05), decrease equally over time, with recovery from muscle paralysis beginning after 16 weeks. % paralysis is average value of individual percent paralysis of EDB CMAP amplitude in each subject.

* p<0.0001.

EDB CMAP: extensor digitorum brevis compound muscle action potential, FT: fresh toxin, hrs: hours, RT: refrigerated toxin, wk: week.

**Table 3.** EDB CMAP areas at baseline, and weeks 1, 12, and 16 post Botox injection (2.5 MU/0.1 mL) for paralysis rating of both the fresh toxin (control) and toxin refrigerated for 72 hours, or 1, 2, 3, or 4 weeks (test) (mean ms±mV±SD)

| Group | Pre & post injection | FT | RT | FT | RT | FT | RT | FT | RT | FT | RT |
|-------|----------------------|----|----|----|----|----|----|----|----|----|----|
| Pre-injection | 18.3±7.2 | 20.3±7.6 | 16.3±6.9 | 18.0±8.6 | 20.6±6.9 | 20.6±7.1 | 21.4±8.4 | 18.8±4.5 | 17.1±5.9 | 20.1±6.5 |
| Post-injection (1 wk) | 8.5±3.8 | 10.1±4.5 | 6.9±4.0 | 9.2±5.6 | 9.0±5.5 | 10.7±5.2 | 10.4±5.1 | 8.9±4.5 | 8.6±3.8 | 9.0±4.7 |
| % paralysis | 50.9±21.6 | 46.0±24.3 | 55.2±25.1 | 46.6±30.0 | 57.2±21.0 | 49.4±16.4 | 53.0±15.8 | 46.4±19.7 | 47.5±19.4 | 54.6±18.3 |
| Post-injection (12 wks) | 13.4±5.1 | 15.6±7.0 | 10.7±5.5 | 13.2±7.6 | 13.2±7.3 | 15.8±5.9 | 11.7±4.7 | 9.9±4.0 | 9.7±3.7 | 10.2±3.3 |
| % paralysis | 21.5±30.2 | 19.1±26.9 | 29.2±31.3 | 26.5±35.0 | 36.7±26.2 | 27.5±16.1 | 42.6±21.0 | 46.4±19.7 | 40.7±20.8 | 47.0±16.1 |
| Post-injection (16 wks) | 14.3±3.9 | 16.4±5.5 | 12.1±4.9 | 13.7±6.4 | 13.6±6.1 | 14.3±5.6 | 12.7±5.1 | 10.8±4.4 | 11.0±3.6 | 11.4±3.2 |
| % paralysis | 14.7±24.6 | 13.9±17.9 | 16.4±33.8 | 20.5±23.2 | 31.7±27.5 | 28.1±23.8 | 36.6±24.8 | 40.5±27.1 | 33.1±18.1 | 41.9±10.6 |

There was no difference in the individual paralysis rates of the CMAP area 1 week post injection between the fresh-toxin groups (control) and all of the refrigerated-toxin groups (test). Furthermore, there were no statistical differences between the control and all five test groups, which shows that meaningful EDB paralysis with both the fresh toxin and the variously refrigerated toxin at 1 week post Botox injection (p<0.0001). Similarly, testing of the paralysis rate the fresh toxin control group and all of the individual test groups (i.e., 72 hours, and 1, 2, 3, and 4 weeks of refrigeration) revealed no significant differences between group pairs (p>0.05), decrease equally over time, with recovery from muscle paralysis beginning after 16 weeks. % paralysis is average value of individual percent paralysis of EDB CMAP area in each subject.

* p<0.0001.

EDB CMAP: extensor digitorum brevis compound muscle action potential, FT: fresh toxin, hrs: hours, RT: refrigerated toxin, wk: week.
tency of BoNT-A: the number or type of receptors against BoNT-A in the presynapse,20–23 the degree of dilution,24 the method of injection,25,26 the presence of antibodies against BoNT-A,27,28 and the actual potency of the toxin in the vial.29–31 In addition, the status of the muscle to be injected can affect the degree of response. When an electrical stimulus is applied to the muscles in animals32–34 or humans35 that have been injected with BoNT-A to raise the activity of muscle, the degree of paralysis is actually increased. Thus, BoNT-A is more effective in hyperactive spastic or dystonic abnormal muscle than in normal muscle.31,36 Thus, patients with hyperactive muscles improve more after the first treatment than after the second.

It is therefore clear that studies into the precise measurement of potency under similar conditions are needed to determine effective treatments. There are objective methods of measuring the potency of BoNT-A in both animals (e.g., glycogen dyeing,24 a method of comparing the contraction power of the white mouse gastrocnemius,37 and LD50 studies38) and humans (e.g., measuring M-wave amplitude and CMAP using electromyography39–42).

In one study, injection of Botox into the abdominal cavity of Swiss Webster mice and subsequent measurement of LD50 revealed no difference in potency with up to 6 hours of refrigerator storage (+6°C) after mixing with saline. However, after 12 hours, the potency of the toxin had reduced by 43.9%, and after 2 weeks of freezing storage (-70°C), it had decreased by 69.8%.39 Nonetheless, the findings of animal experiments on BoNT-A potency regardless LD50 cannot be extrapolated to verify the degree of clinical responses in humans. Since the LD50 method uses fatal amounts of the toxin and provides information about toxicity, it cannot definitively reflect muscle paralysis. In addition, the sensitivity to BoNT-A may vary between species.39 Furthermore, because the LD50 dose differs according to the location of the BoNT-A injection, for example abdominally or intradermally,43 the muscle paralysis rate does not necessarily increase in proportion to the LD50 dose.44,45 Moreover, the LD50 dose of Botox and another form of BoNT-A, Dysport, did not produce the same clinical responses.44,45

It is therefore clearly desirable to use human muscle for the analysis of BoNT-A potency. Among the studies in which human subjects were tested, NCSs have been used to measure paralysis rates on the EDB muscle. Another two inconsistent findings have been reported regarding EDB paralysis rates when Botox was kept in the refrigerator or freezer. In one study, mean CMAP amplitudes expressed as a percentage of the baseline amplitude were more reduced on sides injected with immediately reconstituted Botox than on sides injected with reconstituted Botox stored for 1 week or more,14 while another study found no difference in the potency after 2 weeks between reconstituted Botox stored in a refrigerator or a freezer.13 The NCS is a good and objective method of assessing potency, but both of these NCSs had limitations. For example, Sloop et al’s45 study included a sample that was too small (four subjects) to enable the findings to be generally accepted, and failed to compare consecutively and consistently by refrigeration period, since it compared freshly mixed toxins at only one time period without monitoring their effective duration.14

In other potency studies looking at the effect of BoNT-A on facial wrinkle elimination, the reports stated no change of potency after 2 weeks,16 1 month,17 and even up to 6 weeks18 of refrigeration. However, these three studies16–18 also pose their own limits, for example by making only subjective measurements based on the clinical effects on facial wrinkles (improvements), not implementing a double-blind study,16,17 or by measuring the effect on removal of forehead wrinkles relative to the refrigeration period, but without using a control group using same vial.18 It has therefore been difficult to obtain consistent information.

On the other hand, the BoNT-A responsiveness test designed by Sloop et al.13,14 using NCSs on the EDB analyzes the relationship between dose-response and potency. This is considered an easy and reliable test, as it is used for diagnosing patients who do not respond to the treatment because they possess antibodies against BoNT-A,44,45 or to analyze potency according to the serotype of BoNT-A.13,46

Accordingly, the present study used a minimum dose of Botox (2.5 MU) to paralyze human EDBs;18 the toxin was injected into the midbelly of the EDB, close to the motor endplate, in order to maximize the paralysis rate of the muscle.47 The tracing test was conducted 1 week postinjection, which is considered to be the point of maximum paralysis.48 Due to the possibility that each vial has a slightly different potency [there is thought to be a potential 80–125% difference (corresponding to the 95% confidence interval) in potency from vial to vial (2.2.9. Botox validation assay for KDFA approval)], the same vial was used for each group to improve the accuracy of the experiment.

The tracing tests performed 1 week postinjection in the present study revealed that all control and test groups exhibited significant EDB paralysis. According to Hamjian and Walker,48 there was no muscle atrophy for 42 days after injecting BoNT-A into the EDB, and so muscle atrophy might not affect the CMAP of the tested muscle just 1 week postinjection. If elimination of wrinkles is the direct effect of the paralysis of muscle contraction, the correction of the square-shaped mandible or calf muscle hypertrophy49 is based on the secondary muscle atrophy that occurs after muscle paralysis. According to previous reports4,49 the effect of removing facial wrinkles was found within 2 weeks, while the thickness of the masseter muscle was maximally reduced after 3 months. This may support the pos-
sibility of differential mechanisms of action, such as disuse atrophy, being responsible for the observed results.

In the present study the maximum individual paralysis rates ranged from 44.5±15.9% to 53.3±19.1%. However, any slightly difference in the potency of the drug in each vial or the diffusion effect from the volume increase after dilution may cause differences in the paralysis rates between the groups.\(^{31}\)

As found in the present study, the SD of the EDB paralysis rate, as assessed by measuring the CMAP area, was larger than that of the CMAP amplitude. Thus, the CMAP amplitude is a more subjective measure than the CMAP area (Table 2 and 3); this is because of mechanical limitations.

The manufacturer recommends that Botox be reconstituted in unpreserved saline and used within 5 hours of dilution, after which it should be disposed of. In real applications it is normally re-deep-frozen or refrigerated from 8 hours to 2 weeks, or up to 4 weeks after dilution, with uncertainty regarding any possible reduction of potency or contamination.\(^{11}\) Moreover, toxin reuse should be avoided since the chemical structure may have changed, and proteins can be denatured during the storage and the re-freezing process.\(^{15}\) Clearly, then, accurate analyses of Botox potency with refrigeration storage are required.

The results of the present study demonstrate that if Botox is diluted with unpreserved normal saline solution and kept in the refrigerator at below +4°C, its potency can be ensured for at least 4 weeks. There is thus no need to increase the dose of the toxin within that 4-week period, and it can be used with the confidence that its potency will not have decreased during that time, since we have shown that the response to refrigerated solutions lasted for 16 weeks, the same as for freshly diluted solutions. However, it is important that the refrigerator is maintained at a temperature of no more than +4°C, because higher temperatures may influence the potency.

The possibility of contamination should also be considered when storing and reusing the same Botox vials. In my own experience of 10 years in medical practice I have never had a case in which a patient has been infected from a refrigerated solution from which I have distributed several doses from the same vial to different patients. Therefore, if it is stored in a refrigerator at below +4°C without removing the rubber lid from the bottle, and appropriate care is taken after it is diluted to prevent it from being contaminated by organisms, Botox diluted with unpreserved normal saline can be considered to be fresh and safe for at least 4 weeks.

We anticipate that the results of this study will be helpful in various clinical treatment fields where Botox is used, and that they will act as guidelines for clinicians to build economic and effective protocols.

**Conflicts of Interest**

The authors have no financial conflicts of interest.

**Acknowledgements**

This research was supported by the Yeungnam University research grants in 2011.

**REFERENCES**

1. Blasi J, Chapman ER, Link E, Brunz T, Yamasaki S, De Camilli P, et al. Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. *Nature* 1993;365:160-163.
2. Schiavo G, Santucci A, Dasgupta BR, Mehta PP, Jontes J, Benfenati F, et al. Botulinum neurotoxins serotypes A and E cleave SNAP-25 at distinct COOH-terminal peptide bonds. *FEBS Lett* 1993;333:99-103.
3. Duchen LW. An electron microscopic study of the changes induced by botulinum toxin in the motor end-plates of slow and fast skeletal muscle fibres of the mouse. *J Neurol Sci* 1971;14:87-90.
4. Molgo J, Comella JX, Angaut-Petit D, Pecot-Dechavassine M, Tabti N, Faille L, et al. Presynaptic actions of botulinum neurotoxins at vertebrate neuromuscular junctions. *J Physiol (Paris)* 1990;84:152-166.
5. Brin MF. Botulinum toxin: new and expanded indications. *Eur J Neurol* 1997;4:559-565.
6. Ahn KY, Park MY, Park DH, Han DG. Botulinum toxin A for the treatment of facial hyperkinetic wrinkle lines in Koreans. *Plast Reconstr Surg* 2000;105:778-784.
7. Carruthers J, Carruthers A. Botulinum toxin (botox) chemodenervation for facial rejuvenation. *Facial Plast Surg Clin North Am* 2001;9:197-204, vii.
8. Park MY, Ahn KY, Jung DS. Botulinum toxin type A treatment for contouring of the lower face. *Dermatol Surg* 2003;29:477-483; discussion 483.
9. Brin MF, Binder WJ, Blitzzer A, Schenrock L, Pogoda JM. Botulinum toxin type A for pain and headache. In: Brin MF, Hallett M, Jankovic J. *Scientific and Therapeutic Aspects of Botulinum Toxin*. Philadelphia, PA: Lippincott Williams & Wilkins, 2002.
10. Naumann M, Lowe NJ. Botulinum toxin type A in treatment of bilateral primary axillary hyperhidrosis: randomised, parallel group, double blind, placebo controlled trial. *BMJ* 2001;323:596-599.
11. Klein AW. Dilution and storage of botulinum toxin. *Dermatol Surg* 1998;24:1179-1180.
12. Garlant MG, Hoffman HT. Crystalline preparation of botulinum toxin type A (Botox): degradation in potency with storage. *Otolaryngol Head Neck Surg* 1993;108:135-140.
13. Sloop RR, Cole BA, Escutin RO. Human response to botulinum toxin injection: type B compared with type A. *Neurology* 1997;49:189-194.
14. Paik NJ, Seo K, Eun HC. Reduced potency after refrigerated storage of botulinum toxin A: human extensor digitorum brevis muscle study. *Mov Disord* 2006;21:1759-1763.
15. 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;48:249-253.
16. Yang GC, Chiu RJ, Gillman GS. Questioning the need to use Botox within 4 hours of reconstitution: a study of fresh vs 2-week-old Botox. *Arch Facial Plast Surg* 2008;10:273-279.
17. Garcia A, Fulton JE Jr. Cosmetic derenervation of the muscles of facial expression with botulinum toxin. A dose-response study. *Dermatol Surg* 1996;22:39-43.
18. Hessel DM, De Almeida AT, Rutowitz M, De Castro IA, Silveira VL, Gobatto DO, et al. Multicenter, double-blind study of the efficacy of injections of botulinum toxin type A reconstituted up to six consecutive weeks before application. *Dermatol Surg* 2003;29:523-529; discussion 529.
19. Dolman CE. Botulism as a world problem. In: Lewis KH, Cassel K Jr.
Botulinum. Cincinnati, OH: U.S. Dept. of Health, Education, and Welfare, Public Health Service, 1964.
20. Hambleton P. Clostridium botulinum toxins: a general review of involvement in disease, structure, mode of action and preparation for clinical use. J Neurol 1992;239:16-20.
21. Jessell TM, Kandel ER. Synaptic transmission: a bidirectional and self-modifiable form of cell-cell communication. Cell 1993;72:Suppl 1-30.
22. Montecucco C, Schiavo G. Tetanus and botulism neurotoxins: a new group of zinc proteases. Trends Biochem Sci 1993;18:324-327.
23. Schiavo G, Rossetto O, Benfenati F, Pouilain B, Montecucco C. Tetanus and botulinum neurotoxins are zinc proteases specific for components of the neurotoxocytosis apparatus. Annu N Y Acad Sci 1994;710:65-75.
24. Shaari CM, Sanders I. Quantifying how location and dose of botulinum toxin injections affect muscle paralysis. Muscle Nerve 1993;16:964-969.
25. Borodic GE, Cozzolino D, Ferrante R, Wiegener AW, Young RR. Innervation zone of orbicularis oculi muscle and implications for botulinum A toxin therapy. Ophthal Plast Reconstr Surg 1991;7:54-60.
26. Borodic GE, Pearce LB, Smith K, Joseph M. Botulinum A toxin for spasmodic torticollis: multiple vs single injection points per muscle. Head Neck 1992;14:33-37.
27. Hambleton P, Cohen HE, Palmer BJ, Melling J. Antitoxins and botulinum toxin treatment. BMJ 1992;304:959-960.
28. Zuber M, Sebald M, Bathien N, de Recondo J, Rondot P. Botulinum A toxin activity: evaluation of the lethality assay. Toxicol Appl Pharmacol 1984;69:1464-1468.
29. Greene P. Potency of frozen/thawed botulinum toxin type A in the treatment of torsion dystonia. Otolaryngol Head Neck Surg 1993;109:968-969.
30. First ER, Pearce LB, Borodic GE. Dose standardisation of botulinum toxin. Lancet 1994;343:1035.
31. Pickett AM, Hambleton P. Dose standardisation of botulinum toxin. Lancet 1994;344:474-475.
32. Hughes R, Whaler BC. Influence of nerve-ending activity and of drugs on the rate of paralysis of rat diaphragm preparations by Clostridium botulinum type A toxin. J Physiol 1962;160:221-233.
33. Eleopra R, Tugnoli V, De Grandis D. The variability in the clinical effect induced by botulinum toxin type A: the role of muscle activity in humans. Mov Disord 1997;12:89-94.
34. Kim HS, Hwang JH, Jeong ST, Lee YT, Lee PK, Suh YL, et al. Effect of muscle activity and botulinum toxin dilution volume on muscle paralysis. Dev Med Child Neurol 2003;45:200-206.
35. Glocker FX, Guschlbauer B, Lücking CH, Deuschl G. Effects of local injections of botulinum toxin on electrophysiological parameters in patients with hemifacial spasm: role of synaptic activity and size of motor units. Neurosci Lett 1995;187:161-164.
36. Hesse S, Jahnke MT, Luecke D, Mauritz KH. Short-term electrical stimulation enhances the effectiveness of Botulinum toxin in the treatment of lower limb spasticity in hemiparetic patients. Neurosci Lett 1995;201:37-40.
37. Hold SJ, Fogg SG, Anderson RL. Botulinum A toxin injection. Failures in clinical practice and a biomechanical system for the study of toxin-induced paralysis. Ophthal Plast Reconstr Surg 1990;6:252-259.
38. Sloop RR, Esclatn RO, Matus JA, Cole BA, Peterson GW. Dose-response curve of human extensor digitorum brevis muscle function to intramuscularly injected botulinum toxin type A. Neurology 1996;46:1382-1386.
39. Kauffmann JA, Way JF, Jr, Siegel LS, Sellin LC. Comparison of the action of types A and F botulinum toxin at the rat neuromuscular junction. Toxicol Appl Pharmacol 1985;79:211-217.
40. Frueh BR, Felt DP, Wojno TH, Musch DC. Treatment of blepharospasm with botulinum toxin. A preliminary report. Arch Ophthalmol 1984;102:1464-1468.
41. Borodic GE, Cozzolino D. Blepharospasm and its treatment, with emphasis on the use of botulinum toxin. Plast Reconstr Surg 1989;83:546-554.
42. Pearce LB, Borodic GE, First ER, MacCallum RD. Measurement of botulinum toxin activity: evaluation of the lethality assay. Toxicol Appl Pharmacol 1994;128:69-77.
43. Pearce LB, Borodic GE, Johnson EA, First ER, MacCallum R. The median paralysis unit: a more pharmacologically relevant unit of bulbaric activity for botulinum toxin. Toxicon 1995;33:217-227.
44. Kessler KR, Benecke R. The EBD test--a clinical test for the detection of antibodies to botulinum toxin type A. Mov Disord 1997;12:95-99.
45. Gordon PH, Gooch CL, Greene PE. Extensor digitorum brevis test and resistance to botulinum toxin type A. Muscle Nerve 2002;26:828-831.
46. Housek MK, Sheean GL, Lees AJ. Further studies using higher doses of botulinum toxin type F for torticollis resistant to botulinum toxin type A. J Neurol Neurosurg Psychiatry 1998;64:577-580.
47. Inagi K, Schulte E, Ford CN. An anatomic study of the rat larynx: establishing the rat model for neuromuscular function. Otolaryngol Head Neck Surg 1998;118:74-81.
48. Hamjian JA, Walker FO. Serial neurophysiological studies of intramuscular botulinum-A toxin in humans. Muscle Nerve 1994;17:1385-1392.
49. Park MY, Ahn KY. Botulinum toxin a for the treatment of hyperkineticic wrinkle lines. Plast Reconstr Surg 2003;112(5 Suppl):148S-150S.