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The Effect of Dilution with 10% Dextrose Solution on the Potency of Botulinum Toxin A

Jong Wook Ham[1], Jeong-Seung Kwon[2], Eunae Sandra Cho[3], Jong Hoon Choi[2,4]

[1]Department of Orthodontics and Cosmetic Dentistry, Dr. Ham's Dental and Botulinum Toxin Clinic, Seoul, Korea
[2]Department of Orofacial Pain and Oral Medicine, Yonsei Dental Hospital, Yonsei University College of Dentistry, Seoul, Korea
[3]Department of Oral Pathology, Oral Cancer Research Institute, Yonsei University College of Dentistry, Seoul, Korea
[4]Oral Science Research Center, Yonsei University College of Dentistry, Seoul, Korea

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Correspondence to:
Jong Hoon Choi
Oral Science Research Center, Yonsei University College of Dentistry, Department of Orofacial Pain and Oral Medicine, Dental Hospital of Yonsei University College of Dentistry, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Korea
Tel: +82-2-2228-3113
Fax: +82-2-393-5673
E-mail: jhchoij@yuhs.ac
https://orcid.org/0000-0003-3211-3619

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Purpose: The aim of this study was to compare the potency-stabilizing effects of two different diluents of botulinum toxin A (10% dextrose solution and 0.9% saline).

Methods: A mouse lethality bioassay was undertaken. Ninety mice were divided into experimental and control groups which received varying dosages in subgroups of 10. The experimental group was injected with botulinum toxin A diluted with 10% dextrose solution and the control group was injected with botulinum toxin A diluted with 0.9% saline. A 72 hours after intraperitoneal injection, the number of dead mice was counted to confirm median lethal dose (LD_{50}) of each group.

Results: The value of LD_{50} in the experimental group was approximately 0.131 mL (1.31 U) and the value of LD_{50} in the control group was approximately 0.107 mL (1.07 U). The potency preservation rate of the experimental group was estimated to be 93.5% and that of the control group was estimated to be 76.3%.

Conclusions: Dilution with 10% dextrose solution displayed less potency loss than 0.9% saline.

Key Words: Botulinum toxins, Type A; Glucose; Saline solution

INTRODUCTION

Botulinum toxin is a type of protein produced by *Clostridium botulinum* which acts on the presynaptic nerve endings and blocks acetylcholine secretion. The resulting muscle paresis effect is used clinically to treat a number of diseases related to overactive muscle activity.

Botulinum toxin is categorized immunologically into eight serotypes (A, B, C1, C2, D, E, F, and G), of which type A is recognized as having the highest efficacy. Botulinum toxin A was first isolated in the 1920's and its neurotransmitter-blocking activity was first documented in 1949. Type A toxin was first used for treating strabismus in monkeys in the 1970's and then in humans starting in 1981 [1].

Botulinum toxin A is sold in a freeze-dried state and must be diluted prior to clinical use. Since potency of the toxin gradually decreases after dilution, manufacturers recommend that it should be used within 4 hours after dilution with 0.9% saline solution.

Potency after dilution may be affected not only by time, but also by the type of diluent. Mclellan et al. [2] used a mouse lethality assay to show that botulinum toxin A diluted with saline lost twice as much potency than did toxin diluted with buffer solution containing 0.2% gelatin. Schantz and Johnson [3] reported a loss of toxin stability resulting from dilution of botulinum toxin A and that diluents containing gelatin or albumin could prevent such loss.

Most of the botulinum toxin products currently marketed in a powder form contain minute amounts of gelatin or albumin as a stabilizer and are diluted with 0.9% saline prior

to clinical use without any safety issues. However, when the toxin is diluted with gelatin or albumin solution instead of saline to lessen potency loss, unexpected problems, such as antibody production, viral cross-infections, and allergic reactions may occur [4]. Studies on safe and stable diluents that minimize potency loss during and after dilution are therefore needed.

This study examines the suitability of 10% dextrose solution, widely used as intravenous solution, as a diluent for botulinum toxin A by comparing changes in potency when the toxin is diluted with 10% dextrose solution and when it is diluted with 0.9% saline.

MATERIALS AND METHODS

1. Animals

A 7-to-8 week old female ICR mice weighing 18-22 g (G-Bio Co., Gwacheon, Korea) were used after at least 5 days of adjustment prior to the experiment. All animals were managed according to the strict protocols of Yonsei Center of Clinical Study. The experiment was approved by Yonsei Hospital Animal Experiment Ethics Committee (IACUC no. 2012-0068).

2. Dilution and Injection of Botulinum Toxin

One hundred units (U) of botulinum toxin A products containing 0.5 mg human serum albumin stabilizer (Botox; Allergan Inc., Dublin, Ireland) were used with each diluent. A 20% dextrose solution (Huons Co., Seongnam, Korea) was diluted to 10% for the experimental group. A 0.9% saline solution (Dai Han Pharm Co., Seoul, Korea) was used for the control group. Injections were carried out using 0.5 mL insulin syringes (Omnican 50; B-Braun, Melsungen, Germany) via the left abdominal cavity. All dilutions and injections of Botox were carried out by one operator.

3. Preliminary Study (Additional Injection of 10% Dextrose Solution in Control Group)

In a preliminary study, effects of 10% dextrose additional injection following the injection of botulinum toxin A diluted with 0.9% saline solution were studied. Additional injection of 0.15 mL of 10% dextrose solution resulted in a pronounced reduction in mortality rate from 90% to 60%, while 0.20 mL additional injection resulted in only minor reduction of mortality rate from 100% to 90% (Table 1). These results suggest that dextrose solution may act as an energy source, though only a small amount can significantly reduce rates of mortality in 20 g mice.

4. Experimental Protocols

For the control group, a total of 40 mice, 10 for each of the 4 dosages, were used. One hundred U Botox were diluted with 10 mL of 0.9% saline solution and doses of 0.10 mL (1.0 U), 0.15 mL (1.5 U), 0.18 mL (1.8 U), and 0.20 mL (2.0 U) were injected into 10 mice each and the same amount of 10% dextrose solution was given with additional injection.

For the experimental group, a total of 50 mice were used. Ten were used for each of the 5 different dosages. One hundred U Botox were diluted with 10 mL of 10% dextrose solution. Five different dosages of 0.075 mL (0.75 U), 0.080 mL (0.80 U), 0.100 mL (1.0 U), 0.125 mL (1.25 U), and 0.150 mL (1.50 U) were given to 10 mice each.

To confirm no effect for either diluent solution on the mortality rate, either 0.20 mL of 0.9% saline or 0.20 mL of 10% dextrose solution were given to 10 mice each via injection into the left abdominal cavity.

5. Calculation of Lethal Dose (LD_{50})

Assuming no potency loss immediately after dilution, in a 10 mL solution dilution of 100 U Botox, 0.10 mL injection is equivalent to 1 mouse unit. One mouse unit translates to a potency at median LD_{50} in a 20 g mouse.

In order to assess the potency of botulinum toxin A using the approximate LD_{50} of the control and experimental groups, record was made of the number of mice dead 72 hours after abdominal injections. Mortality rates and LD_{50}, using the Miller and Tainer method, were calculated [5].

Table 1. Mortality rate after botulinum toxin A injection with or without additional dextrose solution injection

Botulinum toxin A diluted with 0.9% saline dilution	Mortality rate (%) after 72 h with no further injection of 10% dextrose solution (n=10)	Mortality rate (%) after 72 h with 10% dextrose solution injection (n=10)
0.15 mL (1.5 U)	90	60
0.20 mL (2.0 U)	100	90

RESULTS

1. Mortality Rate and Conversions for LD_{50} in the Control Group (0.9% Saline)

A total of 40 mice were used for the control group. Ten were used for each of the 4 different dosages. Mortality rates after 72 hours were 20% for the 0.10 mL (1.0 U) dosage and 90% for 0.18 mL (1.8 U) (Table 2). For probit analysis, log-doses corresponding to the X-axis were calculated and the mortality rates were converted to probit for Y-axis values (Table 3). Conversion of mortality rate to probit was done using the probit conversion table [6].

2. Mortality Rate and Conversions for LD_{50} in the Experimental Group (10% Dextrose Solution)

A total of 50 mice, 10 for each of the 5 dosages, were used for the experimental group. Mortality rates after 72 hours were 60% for the 0.1 mL (1.0 U) dosage, showing much higher potency than that of the control group (Table 4). For the 0.075 mL (0.75 U) dosage the mortality rate was 0%, so a correction using the formula 100 (0.25/n) with n=10, 100 (0.25/10)=2.5 was required before probit conversion (Table 5) [7].

3. Calculation of LD_{50} in the Control (0.9% Saline) and Experimental (10% Dextrose Solution) Group

LD_{50} of the control group was approximately 0.107 mL (1.07 U) (Fig. 1) and LD_{50} of the experimental group was 0.131 mL (1.31 U) (Fig. 2). Regression analysis results from the control and experimental groups yielded a p-value of less than 0.05, indicating statistical significance with a 95% confidence level.

Injection of diluent-only resulted in zero mortality for 0.2 mL of 0.9% saline and 0.2 mL of 10% dextrose solution, confirming no effect for either diluent solution on the mortality rate.

DISCUSSION

Numerous studies have shown that potency of botulinum toxin stored in a diluted form gradually decreases [8-10]. Even the dilution process itself contributes to potency loss [2]. This potency loss may be due to a lack of stabilization function in 0.9% saline solution used as a diluting agent.

Table 2. Number of dead mouse and mortality rate in the control group

Botulinum toxin A dosage (U)	Number of dead mouse after 72 h	Mortality rate (%)
0.10 mL (1.0 U)	2	20
0.15 mL (1.5 U)	6	60
0.18 mL (1.8 U)	9	90
0.20 mL (2.0 U)	9	90

Table 3. Conversions for LD_{50} in the control group

Dose (U)	Log dose	Mortality rate (%)	Probits
1.0	0	20	4.16
1.5	0.1761	60	5.25
1.8	0.2553	90	6.28
2.0	0.3030	90	6.28

LD, lethal dose.

Table 4. Number of dead mice and mortality rate in the experimental group

Botulinum toxin A dosage	Number of dead mice after 72 h	Mortality (%)
0.075 mL (0.75 U)	0	0
0.080 mL (0.80 U)	2	20
0.100 mL (1.00 U)	6	60
0.125 mL (1.25 U)	8	80
0.150 mL (1.50 U)	8	80

Table 5. Conversions for LD_{50} in the experimental group

Dose (U)	Log dose	Mortality rate (%)	Corrected %[a]	Probits
0.75	−0.1249	0	2.5	3.04
0.80	−0.0969	20	20	4.16
1.00	0	60	60	5.26
1.25	0.0969	80	80	5.84
1.50	0.1761	80	80	5.84

LD, lethal dose.
[a]Corrected % Formula for 0 and 100% mortality.
For 0% dead: 100 (0.25/n). For 100% dead: 100 (n-0.25/n).

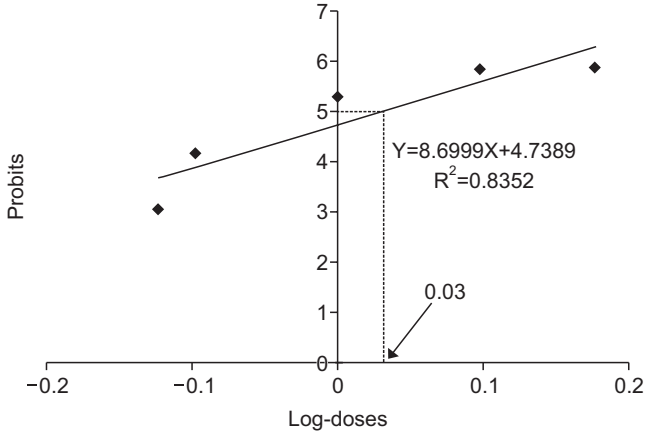

Fig. 1. LD_{50} calculation of control group. Using regression equation Y=8.6999X+4.7389, X is calculated when Y is 5, and anti-LogX (Y=5) is 1.07 U.

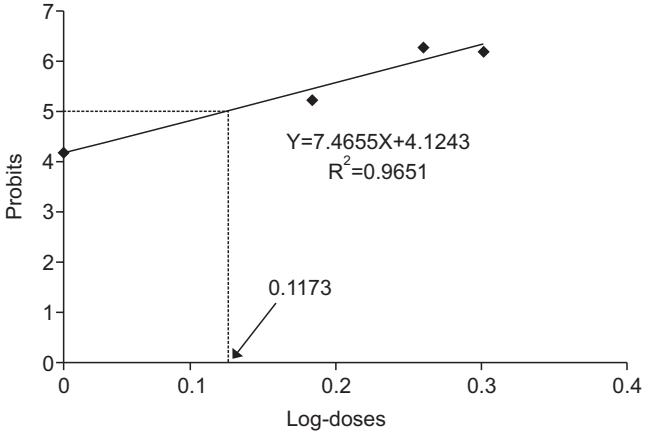

Fig. 2. LD_{50} calculation of experimental group. Using regression equation Y=7.4655X+4.1243, X is calculated when Y is 5, and anti-LogX (Y=5) is 1.31 U.

Protein stabilizers, such as albumin or gelatins in a powder form are added by manufacturers to minimize the potency loss, but their stabilizing effects start to decrease as soon as diluting saline is added.

This study compares the potency-stabilizing effect of two different diluents of botulinum toxin A (10% dextrose and 0.9% saline) by examining the mortality rate of mouse and their LD_{50}.

Yun et al [11]. studied potency loss after saline dilution of two kinds of botulinum A toxins. They found that a 1 U injection per mouse resulted in 0% mortality after 72 hours while a 2 U injection resulted in nearly LD_{50}, indicating pronounced potency loss after dilution. Manufacturers of botulinum toxins recommend mild dilution to avoid potency loss, but some studies have showed no clinical difference between vigorous dilution and mild dilution methods [12,13]. However, these studies did not use LD_{50}. Operators of the present study decided to use mild dilution method to exclude potential potency loss due to mechanical force.

LD_{50} values were 1.31 units for the 0.9% saline control group and 1.07 units for 10% dextrose experimental group, indicating 1.22 times more stabilizing effect in the dextrose dilution. Provided that the potency of the toxin was 100 U before dilution, the potency of 1 vial would be 76.3 U (100/1.31) for the control group and 93.5 U (100/1.07) for the experimental group. This indicates a potency retention rate of 76.3% for the control group and 93.5% for the experimental group. The higher potency retention of the experimental group might be a result of the function of dextrose as a protein stabilizer. Dextrose concentration may affect the extent of stabilization [14]. Due to the stronger stabilization effect of the 10% dextrose solution, potency loss can be reduced by using dextrose solution instead of saline as a diluent.

Although measurement of mouse LD_{50} is generally considered an accurate and reliable method for assessing the potency of botulinum toxin [15], alternative methods are currently being investigated in order to reduce the large number of mice sacrificed by the LD_{50} bioassay.

In 1959, British biologists Russell and Burch published their seminal paper, *The principles of humane experimental technique*, which introduced ethical principles for animal experiments that are colloquially referred to as the '3 Rs': Replacement, Refinement and Reduction. Experiments using botulinum toxin on animals follow these principles. This study adhered to the principle of 'reduction' by trying to minimize the number of mice used. Other methods that may meet the '3 Rs' requirements include mouse flaccid paralysis assay (mouse abdominal ptosis assay), hind limb paralysis assay, endopeptidase assay, and compound muscle action potential (CMAP) assay [16-18].

The systematic effect of mouse mortality rates may not accurately reflect local therapeutic effects. Dysport, for example, requires a dose 3 to 4 times greater than that of Botox for similar results on muscles, which suggests that mouse LD_{50} alone would not be an appropriate indicator of human therapeutic effects. Mortality rate after 1 U injection

was 20% and 60% for the control and experimental groups respectively, yet it is difficult to conclude that actual clinical efficacy would be three-fold. Similarly, the difference of 22% in LD_{50} between the two groups may not correspond to a 22% difference in clinical results after muscular injection. Abdominally injected dextrose solution may also act as an energy source, contributing to the reduced mortality rate. Individual variability in reaction to abdominal injection may also affect the results.

This study is a preliminary experiment to investigate the effects of different diluent solutions on local efficacy of botulinium A toxin. More specific comparison of local effects may require CMAP assay, mouse flaccid paralysis assay, or histological study as in Kim et al. [19].

This study compares the potency-stabilizing effect of two different diluents of botulinum toxin A (10% dextrose and 0.9% saline) by examining the mortality rate of mouse and its LD_{50}. The experiment group, injected with dextrose solution, displayed less potency loss than the control group injected with a diluent of saline solution. These results suggest potential for improvement on the shortcomings of commonly used diluting methods. Further studies may need to compare the clinical efficacy of dextrose-diluted botulinum toxin.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

ORCID

Jong Wook Ham
https://orcid.org/0000-0002-1502-0675
Jeong-Seung Kwon
https://orcid.org/0000-0003-4584-7355
Eunae Sandra Cho
https://orcid.org/0000-0002-0820-3019
Jong Hoon Choi
https://orcid.org/0000-0003-3211-3619

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