

Development of an Equine Antitoxin by Immunizing the Halla Horse with the Receptor-Binding Domain of Botulinum Neurotoxin Type A1

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Botulinum neurotoxins (BoNTs), produced by *Clostridium botulinum*, are the most toxic substances known. However, the number of currently approved medical countermeasures for these toxins is very limited. Therefore, studies on therapeutic antitoxins are essential to prepare for toxin-related emergencies. Currently, more than 10,000 Halla horses, a crossbreed between the native Jeju and Thoroughbred horses, are being raised in Jeju Island of Korea. They can be used for equine antitoxin experiments and production of hyperimmune serum against BoNT/A1. Instead of the inactivated BoNT/A1 toxoid, Halla horse was immunized with the receptor-binding domain present in the C-terminus of heavy chain of BoNT/A1 (BoNT/A1-HCR) expressed in *Escherichia coli*. The anti-BoNT/A1-HCR antibody titer increased rapidly by week 4, and this level was maintained for several weeks after boosting immunization. Notably, 20 μ l of the week-24 BoNT/A1-HCR(-immunized) equine serum showed an in vitro neutralizing activity of over 8 international units (IU) of a reference equine antitoxin. Furthermore, 20 μ l of equine serum and 100 μ g of purified equine F(ab')₂ showed 100% neutralization of 10,000 LD₅₀ in vivo. The results of this study shall contribute towards optimizing antitoxin production for BoNT/A1, which is essential for emergency preparedness and response.

Keywords: Equine antitoxin, botulinum neurotoxin type A, Halla horse

Introduction

In the targeted area, biological warfare agents exclusively affect living organisms, without damaging potentially

valuable non-living things. Botulinum neurotoxins (BoNTs) have been among the first candidates to be considered for bioterrorism because they can be easily manufactured, stored, and dispersed [1–4]. They can also have a ripple

effect mediated via social chaos.

Botulism, a fatal paralyzing disease, is caused by potent BoNTs produced by the anaerobic gram-positive spore-forming bacterium *Clostridium botulinum*. BoNTs are categorized into eight serotypes - A to H. Serotypes A, B, E, and rarely F and G cause disease in humans, while serotypes C and D can only cause disease in animals, including cattle and birds [5, 6]. BoNT/H is a novel botulinum toxin that is produced by the bivalent *Clostridium botulinum* strain, IBCA10-7060. This strain, which produces BoNT/B and BoNT/H, was isolated from an infant patient with botulism. BoNT/H is not neutralized by antitoxins to BoNT A-G [7]. Among the eight distinct exotoxins, BoNT/A is the most potent, followed by types B and F [8].

BoNTs are composed of a 50 kDa light chain (LC) and a 100 kDa heavy chain (HC), linked by a single disulfide bond. The N-terminal catalytic LC has zinc metalloprotease activity, while the HC contains two functional domains: a translocation domain and a receptor-binding domain [9, 10]. BoNTs can enter the human body through the respiratory system (inhalational botulism) [11], gastrointestinal tract (foodborne and infant botulism), and infected wounds (wound botulism) [12–14]. Upon infection, the circulatory system transports the BoNT to the peripheral nerves, where it mainly targets the neuromuscular junctions. In the neurons, BoNT/B, D, F, and G proteolyze VAMP [15–17], and BoNT/A, C, and E cleave SNAP-25 [18, 19]. This ultimately leads to the inhibition of acetylcholine exocytosis, thereby inducing neuromuscular paralysis [20].

Medical countermeasures against potential biothreats for mass destruction using BoNT/A are necessary. For the large volumes of high titer plasma to support large-scale antitoxin production, high yields of neutralizing antibodies should be generated rapidly in animals (*e.g.*, horses, sheep, and goats), and the resulting hyperimmune sera can be easily processed to make a product suitable for human use [21, 22]. BabyBIG consists of human serum-derived anti-BoNT polyclonal antibodies, and is approved by the FDA for the treatment of infant botulism types A and B [20, 23]. However, its use is limited for the treatment of adults due to its low potency and high cost [24]. Heptavalent botulism antitoxin (H-BATTM) is an antitoxin for BoNT serotypes A to G [25, 26], and was approved by the FDA in March 2013. H-BATTM is being supplied to the US government to be included in the Strategic National Stockpile (SNS). However, it is very expensive and available in limited quantities for exporting outside the US, which makes it necessary to produce antitoxins using local domestic

horses. In fact, the previous studies on antitoxins for BoNT/A have been reported consistently since 1980's [27, 28]. However, the first-line treatment for BoNT/A is produced only in the US, and exports are limited, making it difficult for the Republic of Korea to be prepared for emergency medical treatment for botulinum intoxication [29].

Currently, around 26,000 horses are being bred in the Republic of Korea, with 14,776 (57.2%) of them being bred on Jeju Island [30]. Horses bred on Jeju Island are subdivided into Jeju (6.1%), Thoroughbred (18.8%), and Halla (75.1%) horses. Halla horses are a crossbreed between the Jeju and Thoroughbred horses, and are used for horse racing, riding, and meat production [31]. Among more than 10,000 Halla horses in Jeju Island, some portion of them can be utilized for antitoxin production.

The purpose of this study was to examine the proof of concept for production of horse serum against botulinum toxins in emergency situations, and to investigate the possibility of producing an antitoxin against BoNT/A1 in the Korean domestic Halla horse using BoNT/A1-HCR, instead of using the inactivated toxoid or toxin which was used as the antigen to produce H-BAT. Collectively, our data using mouse and rabbit models confirm the antigenicity and safety of the selected antigens. We subsequently immunized a healthy, well-maintained Halla horse, and evaluated the *in vitro* and *in vivo* neutralization activities of our antitoxin.

Materials and Methods

Materials

pET-28a vector was purchased from Novagen (UK). The 0.45 µm MF membrane filters were purchased from Millipore (USA). The Ni-NTA resin column was purchased from Qiagen (Germany) and thrombin was purchased from GE Healthcare (UK). Alhydrogen adjuvant was purchased from InvivoGen (USA). Goat anti-mouse antibody (IgG-HRP), Goat anti-rabbit antibody (IgG-HRP), Ganglioside, and Anti-beta-actin antibody were purchased from Santa Cruz Biotechnology (USA). Aluminum hydroxide gel (0.6 mg/ml) was bought from Brenntag (Denmark). Casein, EDC, and Ethanolamine were obtained from Sigma Aldrich (USA). HRP-conjugated donkey anti-horse IgG was purchased from Jackson ImmunoResearch (USA). Neuro-2a cells (CCL-131) were purchased from ATCC (USA). MEM media, FBS, and antibiotics were bought from GE Healthcare Life Sciences/Hyclone (USA). Botulinum antitoxin equine type A (standard antitoxin) was bought from NIBSC (UK). RIPA buffer was obtained from GenDEPOT (USA). Anti-SNAP-25 was purchased from Biolegend (USA). BCA protein kit and Goat-anti

mouse HRP were purchased from Thermo Fisher Scientific (USA), and Pierce™ F(ab')₂ Preparation Kit, and Sulfo-NHS buffer were also purchased from Thermo Fisher Scientific (USA). Western Lighting™ PLUS-ECL was bought from PerkinElmer (USA). MabSelect SuRe resin was bought from GE Healthcare Life Sciences (USA). BoNT/A1 was prepared by the Agency for Defense Development in South Korea.

Production of the BoNT/A1-HCR

The amino acid sequences of BoNT/A1 were obtained from the ExpASY, a bioinformatics resource portal (P10845). Full-length BoNT/A1-HCR gene (amino acid 876 through 1296) was chemically synthesized (Bioneer, South Korea), and cloned into the pET-28a vector. The BoNT/A1-HCR gene-containing plasmid was transformed into the *E. coli* BL21(DE3) cells. When OD₆₀₀ of the *E. coli* culture reached 0.6 ± 0.1, the cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 6 h at 30°C. The cells were harvested by centrifugation at 4,000 ×g for 20 min, and resuspended in lysis buffer (50 mM sodium phosphate, 300 mM NaCl, and 10 mM imidazole, pH 8.0). BoNT/A1-HCR was purified using the His-tagged protein purification protocol, as described previously [32]. Complete cell lysis was achieved by sonication, and the lysate was treated with 5 μg/ml DNase I. Cell debris was removed by centrifugation at 10,000 ×g for 30 min. The supernatants were filtered through a 0.45 μm MF membrane filter, and applied to a Ni-NTA resin column for 1 h at 4°C. Then, the column was washed with washing buffer (50 mM sodium phosphate, 300 mM NaCl, and 20 mM imidazole pH 8.0), and the resin-bound BoNT/A1-HCR was eluted by addition of elution buffer increasing concentrations of imidazole (30–250 mM). The eluted protein was analyzed on a 12% SDS-PAGE, and dialyzed overnight with PBS at 4°C to remove imidazole. Dialyzed BoNT/A1-HCR protein was treated with thrombin (10 units/1 mg) for 12 h at 4°C. Cleaved protein was purified by a Ni-NTA resin packed affinity chromatography column. Flow through, 20 mM, and 30 mM eluted fractions were dialyzed with PBS overnight at 4°C. Protein concentration was determined by the BCA protein kit.

The purity of BoNT/A1-HCR was analyzed by reverse-phase HPLC (Agilent 1260 Infinity II HPLC System, Agilent, USA). The 600 μg sample was injected into the 4.6 × 250 mm C4 column (VyDAC, USA). The protein was eluted from the column using a linear gradient from 0% buffer A (0.1% TFA) to 100% buffer B (100% acetonitrile, 0.1% TFA). The separation was performed at ambient temperature, 1 ml/min, and was monitored at a wavelength of 280 nm. The peaks in the chromatogram were integrated, and area under the BoNT/A1-HCR peak was reported as the percentage of the total area under all peaks.

Immunization of Mice and Rabbits Using BoNT/A1-HCR

Six-week-old female Balb/c mice (*n* = 5) were immunized with the BoNT/A1-HCR. For vaccination, BoNT/A1-HCR (the antigen) was dissolved in PBS, and emulsified in 2% alhydrogel adjuvant in a 1:1 proportion. Mice were intramuscularly injected with

100 μl of emulsion containing 10 μg of BoNT/A-HCR three times at two-week intervals. The control group mice (*n* = 5) received the adjuvant only.

Two female New Zealand white rabbits (Doo Yeol Biotech., Korea) weighing 2–3 kg were intramuscularly administered 300 μg of the BoNT/A1-HCR in 100 μl of PBS (pH 7.4) emulsified with 100 μl of Freund's Complete Adjuvant (FCA) at week 0. Subsequently, the rabbits were injected intramuscularly with the same volume of BoNT/A1-HCR antigen emulsified with 100 μl of Freund's Incomplete Adjuvant (FIA) five times every two weeks. Approximately 50 μl mouse blood was collected by retro-orbital bleeding after anesthesia using Ketamine (100 mg/kg) and Xylazine (10 mg/kg), and also about 200 μl of rabbit blood was collected from the marginal ear vein. The collected blood was separated and serum was stored at –20°C until further use. The Institutional Animal Care and Use Committee of the Agency for Defense Development approved the mouse and rabbit experiments (ADD-IACUC-15-21).

Mouse and Rabbit Antibody Titer Determination Using ELISA

The anti-BoNT/A1-HCR antibodies in the sera of mice or rabbits were measured by indirect ELISA. Microplates were coated with the BoNT/A1-HCR protein (0.5 μg/ml) in PBS and incubated for 1 h at 37°C. Next, the plates were washed with the washing buffer (0.1% Tween 20-containing PBS), and blocked with 1% BSA-containing PBS. Serum samples diluted from 1:100 to 1:6,400 with two-fold serial dilutions in the blocking buffer (100 μl/well) were added to the plates. After 1 h incubation at 37°C, each well was washed four times with washing buffer. The goat anti-mouse IgG antibody or goat anti-rabbit IgG antibody was diluted (1:2,000) in the blocking buffer, added to each well (100 μl/well), and incubated for 30 min. Following the washing step, the plates were treated with 100 μl/well of 3,3',5,5'-tetramethylbenzidine (TMB) solution (substrate) for 30 min in the dark at 25°C. The reaction was stopped by adding 100 μl/well of 1 N H₂SO₄, and the absorbance of the reaction products was measured at OD₄₅₀. The endpoint of titration was determined as described previously [29]. For calculation of endpoint titer, the optimum cut-off value for this method was determined by calculating the average absorbance values of negative control sera of mouse or rabbit. The negative control sample in mouse titer analysis was the serum from mice injected with only adjuvant, and that of rabbits was the pre-immune serum. The background absorbance values of pre-immune serum from mice and rabbits were 0.3 and 0.15, respectively. Therefore, we set the cut-off value to 0.3, and immune serum titers were calculated using the highest sample dilution with an OD₄₅₀ reading greater than 0.3.

Vaccination of Horse Using BoNT/A1-HCR

A five-year-old female Halla horse weighing 200–300 kg served as the experimental animal, and the animal was maintained under the supervision of a veterinarian, according to the Guidance of Livestock Production announced by the Ministry of Food and

Drug Safety (MFDS 2016-110). The Halla horse was immunized with the BoNT/A1-HCR antigen, and aluminum hydroxide gel (0.6 mg/ml) was used as the adjuvant. The adjuvant was mixed with 3 mg of BoNT/A1-HCR antigen in a 1:1 ratio, to achieve a final concentration of 1 mg/ml, and 3 ml of this mixture was injected to the Halla horse. The antigen was injected three times at 2-week intervals, followed by four times at 4–5 week intervals. Injection was performed subcutaneously on the right side of the cervix, and blood for assay was collected from the left jugular vein before vaccination. The blood sample was allowed to be coagulated naturally in a conical tube, and the serum was separated by centrifugation. The Halla horse experiment was performed following the protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Jeju National University (Jeju, Republic of Korea), where the animal experiment was conducted (Approval ID: 2015-0008).

Horse Antibody Titer Determination Using ELISA

To develop and optimize an indirect ELISA protocol for titration of the anti-BoNT/A1-HCR antibodies in horse serum, dilution factor for the HRP-conjugated anti-horse IgG, reaction time, temperature of samples/antibodies, and washing steps were optimized. For coating wells with antigen, microplates (Corning Inc., NY, USA) were incubated with 100 µl of antigen-containing carbonate–bicarbonate buffer (1 µg/ml, pH 9.6) for 16 h at 25°C. The plates were blocked with blocking buffer (PBS containing 1% casein). The 100 µl serum samples, diluted from 1:100 to 1:6,400 with two-fold serial dilutions in the blocking buffer, were added to the plates, incubated for 1 h at 37°C, and subsequently washed five times with the 300 µl/well of washing buffer (PBS containing 0.5% Tween 20). The HRP-conjugated donkey anti-horse IgG was diluted (1:30,000) with the blocking buffer, added to each well with 100 µl, and incubated for 30 min at 37°C. Following washing, 100 µl/well of TMB solution was added to the samples and incubated for 30 min in the dark at RT. The reaction was stopped by adding 100 µl/well of 1 N H₂SO₄, and the absorbance of the reaction products was measured at OD₄₅₀. For titration, the optimum cut-off value for this method was determined by calculating the average absorbance values of pre-immune horse serum. The optimum cut-off value was 0.15 and the titer was calculated from the highest dilution of sample with an OD₄₅₀ reading of greater than 0.15 [29].

In Vitro Neutralization Assay against BoNT/A1 Toxin

The BoNT/A1 toxin neutralizing efficacy was evaluated by estimating the cleavage of SNAP-25 in the Neuro-2a cells [33, 34]. The Neuro-2a cells were seeded at a density of 1 × 10⁵ cells/well in a 24-well plate with MEM media (10% FBS, 1% antibiotics). The cells were treated with 50 µg/ml ganglioside for 24 h. After the treatment of ganglioside, the cells were changed with toxin and equine serum diluted MEM media. Four hundred microliters of 20 µg/ml BoNT/A1 toxin (1.75 × 10⁵ LD₅₀/ml) and equine serum at varying concentrations were diluted in MEM media (10% FBS,

1% antibiotics), and incubated for 1 h at 37°C. After the incubation, the toxin and equine serum diluted media were added to the cells. Neuro-2a cells treated with the toxin and equine serum samples were cultured for 2 days, and the cells were harvested for western blot analysis. The cells were centrifuged at 4,000 ×g for 5 min, washed with fresh PBS, and the harvested cells were lysed using RIPA buffer for 30 min at 4°C. After the incubation, the lysed cells were centrifuged at 13,000 ×g, 4°C for 30 min, and the supernatants were collected. Next, 3 µg of whole cell lysate was loaded onto a 12% polyacrylamide gel, and transferred to nitrocellulose membrane by electroblotting. The membrane was blocked with 6% skim milk, and then washed with washing buffer (0.1% Tween 20-containing TBS). After washing, the membrane was incubated with the anti-SNAP-25 or anti-beta-actin at a 1:5,000 dilution for 1 h at 25°C. Membranes were then washed and hybridized with the HRP-conjugated goat anti-mouse IgG antibodies at 1:15,000 dilution for 1 h at 25°C. After additional washing of membranes, the bound antibody was detected with Western Lighting PLUS-ECL. For SNAP-25 cleavage assay, the negative controls were untreated Neuro-2A cells and Neuro-2A cells treated with 20 µg/ml (1.7 × 10⁵ LD₅₀/ml) BoNT/A1 toxin. Standard antitoxin was used as the positive control.

Preparation of F(ab')₂

Saturated ammonium sulfate solution (4.1 M, 25°C) was slowly added to 20 ml of horse serum until the concentration in the solution reached 70%, while the temperature was maintained to 4°C. Precipitated protein was separated through centrifugation at 4°C, 5,000 ×g, for 30 min. Subsequently, the pellet was resuspended in MabSelect SuRe resin buffer A (20 mM sodium phosphate, 150 mM NaCl, pH 7.2), bound to MabSelect SuRe resin, washed in 5 column volumes of buffer A, and eluted in 10 column volumes of 100% buffer B (0.1 M sodium citrate, pH 3.0) in a linear gradient. Before the elution, the tube in which the elution fraction would be collected was prepared by placing 1.0 M Tris-HCl (pH 8.0) for one-tenth of the elution volume. The elution fraction buffer was changed to pepsin digestion buffer (20 mM sodium acetate, pH 4.0), and pepsin treatment was conducted. F(ab')₂ was prepared using a Pierce F(ab')₂ Preparation Kit according to the manufacturer's instructions. To determine the extent of digestion and confirm the presence and purity of F(ab')₂, the purified F(ab')₂ was analyzed on a 8% SDS-PAGE under non-reducing conditions. The F(ab')₂ was dialyzed for 12 h at 4°C with PBS (pH 7.4) and then concentrated by Viva-spin. The final product was stored at –80°C until use.

Affinity Measurements of F(ab')₂

Affinity studies of the purified F(ab')₂ were carried out using biolayer interferometry by the Octet Red System (Pall ForteBio LCC, USA). All steps were performed at 30°C with shaking at 1,500 rpm in a 96-well plate containing 200 µl per well. Amine-reactive second generation biosensors were activated by the EDC and sulfo-NHS buffer. The 10 µg/ml of ligand, BoNT/A1-HCR,

was loaded into the activated Amine Reactive 2nd Generation (AR2G) Biosensor which was then moved to the quenching step. After the quenching in the ethanolamine, the ligand-loaded biosensor reacted with the purified equine F(ab')₂ concentration dependently (0.32, 8, 40, 200, and 1,000 nM). The ligand-loaded biosensors were in the association step about 300 sec, and it moved to the dissociation step. Association and dissociation were measured as changes over time in light interference, and curves are presented after subtraction of parallel measurements from unloaded biosensors. The data fitting and K_D value were analyzed by the Data Analysis 9.0 program supplied by Pall ForteBio. For the data fitting, a heterogeneous ligand model was used.

In Vivo Protection Assay

A bioassay using mice was performed to evaluate the in vivo protection by BoNT/A1 toxin, as described previously [35]. Briefly, 0.1 ml of the BoNT/A1 toxin mixture (1 LD₅₀ = 0.114 ng/mouse, 1.14 µg/0.1 ml) was prepared for 10,000 LD₅₀ assays with PBS (pH 7.4), and the pre-immune serum was used as the negative control. Next, 0.1 ml of toxin neutralization assay samples were prepared by mixing 10,000 LD₅₀ of toxin with 10% equine BoNT/A1 serum or purified F(ab')₂ in PBS (pH 7.4), and the toxin and antitoxin mixture was incubated for 1.5 h at room temperature. For the calculation of the amounts of 1 IU of the purified F(ab')₂,

F(ab')₂ was prepared in a dose step-down manner (500, 300, and 100 µg/mouse) and was mixed with 10,000 LD₅₀ BoNT/A1 toxin. After completion of the incubation, the incubation samples were administered into Balb/c mice ($n = 5-6$ /group) with intraperitoneal injection and mice were monitored for seven days for intoxication symptoms or death after the injection. All animal experiments were performed following the protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the Agency of Defense Development, where the animal experiments were conducted (approval ID: ADD-IACUC-16-05/ADD-IACUC-16-28).

Results

Preparation of BoNT/A1-HCR and Immunization of Mice and Rabbits

Recombinant BoNT/A1-HCR, amino acid 876 through 1296 was expressed as a N-terminal His-tagged fusion protein in *E. coli* BL21(DE3) [36]. After thrombin cleavage of the His-tag, recombinant BoNT/A1-HCR was confirmed by the presence of a protein with the expected molecular weight (50 kDa) using SDS-PAGE followed by Coomassie blue staining (Fig. 1B). To confirm the purity of BoNT/A1-

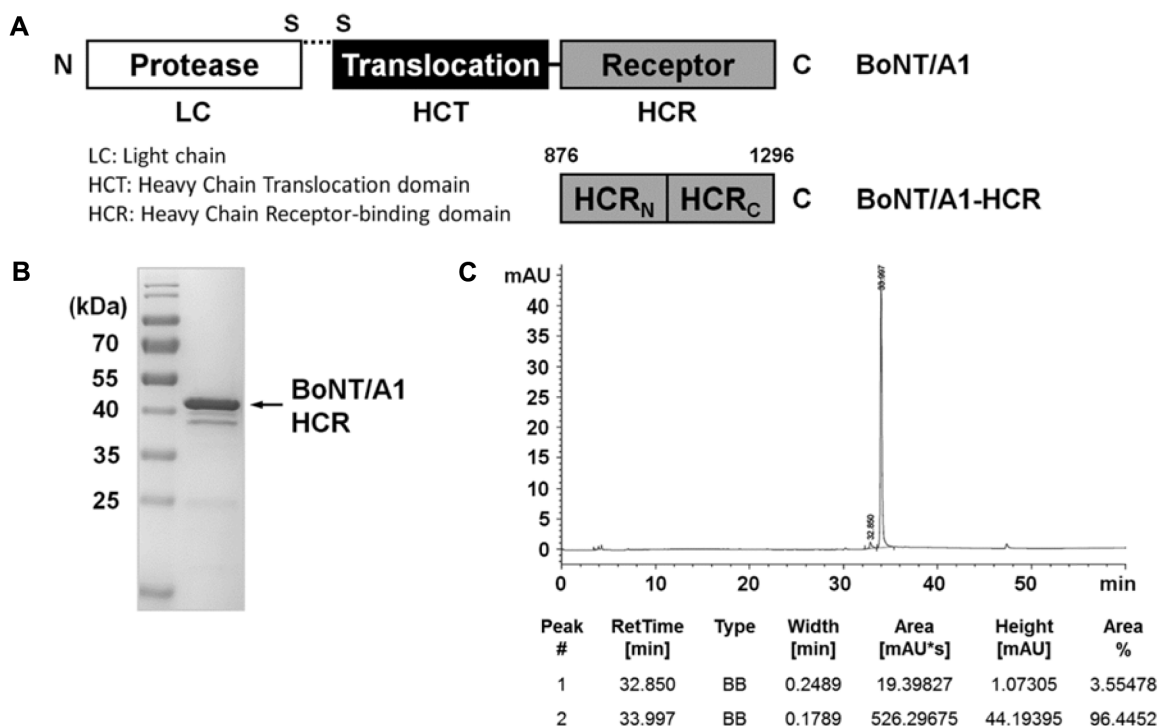


Fig. 1. Purity analysis of the purified BoNT/A1-HCR.

The proteins were purified using Ni-NTA resin and the His-tag was removed using thrombin. (A) A schematic diagram of BoNT/A1 and BoNT/A1-HCR. (B) SDS-PAGE analysis of the purified BoNT/A1-HCR. Lane 1, standard molecular-weight markers; lane 2, BoNT/A1-HCR (10 µg) under reducing conditions. (C) Reverse phase-HPLC analysis. BoNT/A1-HCR (30 µg) was analyzed on the C4 column. Detection was performed at OD280.

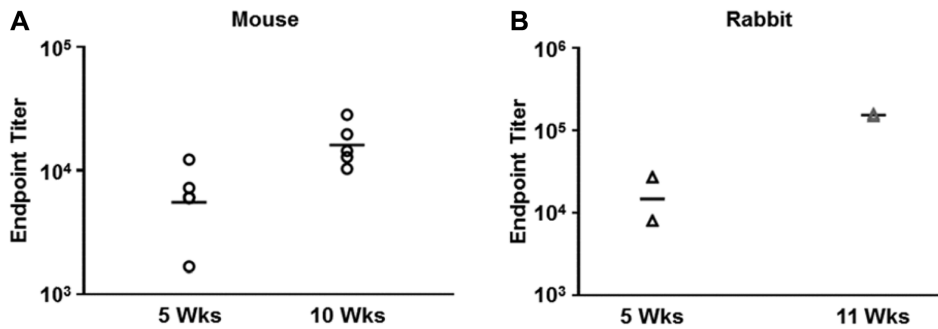


Fig. 2. BoNT/A1-HCR-specific antibody titer in mice (A) and rabbits (B).

Mice ($n = 5$ /group) and rabbits ($n = 2$ /group) were immunized three times, and six times, respectively. Serum samples from immunized animals were collected at different time points, and the specific responses of anti-BoNT/A1-HCR antibody were assayed by ELISA. Antibody level from each animal is presented as endpoint titers in ELISA, and symbols showed individual values. The bars represent geometric mean titers of each group.

HCR, reverse-phase HPLC analysis was performed using a C4 column. The peak of BoNT/A1-HCR was observed at 33.9-min retention time. The result from reverse-phase HPLC analysis indicated that the purified BoNT/A1-HCR had 96% purity (Fig. 1C).

To analyze the immune response-inducing ability of BoNT/A1-HCR, we applied our BoNT/A1-HCR to Balb/c mice ($n = 5$ /group), and rabbits ($n = 2$ /group). In this analysis, the titers of anti-BoNT/A1-HCR IgG in serum from BoNT/A1-HCR-immunized mice and rabbits were time-dependently increased, and remained high until 10 weeks for mice (Fig. 2A) and 11 weeks for rabbits (Fig. 2B). However, no anti-BoNT/A1-HCR IgG was detected in any adjuvant-treated control animals (data not shown).

These data indicated that our BoNT/A1-HCR could induce BoNT/A1-HCR-specific antibodies in mice and rabbits.

Immunization of Halla Horse with BoNT/A-HCR

Before immunization, the hematological parameters of Halla horses were evaluated to assess their clinical health and suitability for immunization (data not shown). Subsequently, the horses were injected with the BoNT/A1-HCR antigen according to the immunization schedule. After immunization, equine serum sample was collected at different time points over 24 weeks, and the antibody titer against BoNT/A1-HCR was measured using indirect ELISA. The titer increased rapidly on week 4, and remained close to 1,000 for the next 20 weeks (Table 1). In order to test the boosting effects of additional vaccination, we monitored the changes in antibody titers after administration of the boosts. The titers of weeks 13 and 22 decreased compared to those observed after the previous injections, since

immunization was not performed on week 10 or 20. The antibody titers increased after additional boost vaccination; however, the titers did not increase to the level observed at week 4. These data indicated that BoNT/A1-HCR injection in Halla horse induced an immune response with antibody production.

In Vitro Neutralizing Efficacy of BoNT/A1 Antitoxin

The neutralizing efficacy of the week 24 serum from the immunized horse was evaluated in vitro using the SNAP-25 protein cleavage assay and the Neuro-2a cells. Standard antitoxin was used as the standard for comparison of the neutralizing efficacy of the experimental serum. The reported data showed that one international unit (IU) of the standard antitoxin neutralized approximately 10,000 intraperitoneal mouse LD₅₀ of the BoNT/A1 toxin [35, 37]. The 1:1 ratio of cleaved and uncleaved SNAP25 was found

Table 1. The vaccination schedules of BoNT/A1-HCR to Halla horse and the titer of horse serum after 24 weeks of immunization.

Week	Blood collection	Vaccination	Antibody titer
0	○	○	0
2	○	○	635
4	○	○	1,420
8	○	○	1,130
10	○	-	1,035
13	○	○	876
18	○	○	798
20	○	-	1,016
22	○	○	898
24	○	○	1,108

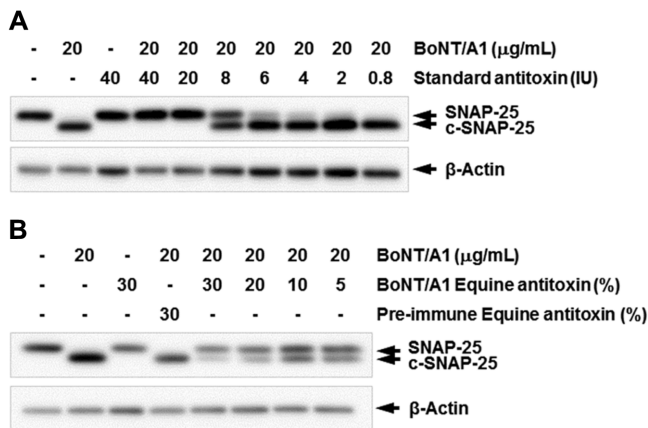


Fig. 3. In vitro neutralizing assay for BoNT/A1 antitoxin.

The Neuro-2a cells were seeded at a density of 1×10^5 cells/well in a 24-well plate. The cultured cells were treated with ganglioside for 1 day. Then, the culture medium was changed into the indicated BoNT/A1 and standard antitoxin (A) or equine anti-toxin-containing media (B). Before treating cells, the mixtures of BoNT/A1 and standard antitoxin or equine antitoxin were incubated for 1 h at 37°C. After two days of treatment, cells were collected and analyzed by western blot. (A) Inhibition of BoNT/A1-mediated SNAP-25 cleavage by co-incubation with 20 µg/ml of standard antitoxin. (B) Inhibition of BoNT/A1-mediated SNAP-25 cleavage during co-incubation with BoNT/A1-HCR-immunized equine serum.

in the 8 IU standard antitoxin treatments. The 20 µg/ml BoNT/A1 toxins were neutralized completely in the 20 IU

standard antitoxin treatments (Fig. 3A). To test the in vitro neutralizing efficacy of our BoNT/A1-HCR equine serum, BoNT/A1 toxin (20 µg/ml) and equine serum at various concentrations were mixed and allowed to react. The Neuro-2a cells were treated with the pre-incubated mixture and the protective effect of the mixture against SNAP-25 protein cleavage was examined. Cleavage of SNAP-25 protein decreased with the increasing concentration of equine serum, which suggested neutralization of the BoNT/A1 toxin by the BoNT/A1-HCR equine serum (Fig. 3B). It was estimated that a 5% BoNT/A1 equine antitoxin (20 µl serum/400 µl reaction sample) had an in vitro neutralizing activity of > 8 IU.

Characterization of Purified F(ab')₂ as Antitoxin

To obtain F(ab')₂ from the immunized horse serum, 70% ammonium sulfate precipitation was performed, followed by total IgG purification using affinity chromatography with MabSelect SuRe resin. Purified IgG was fragmented to F(ab')₂ by pepsin digestion (Fig. 4A). In order to determine the F(ab')₂-binding affinity of BoNT/A1-HCR, the affinity constant (K_D) for antibodies binding to BoNT/A1-HCR was measured (Fig. 4B). BoNT/A1-HCR was loaded on a biosensor and the various concentrations of F(ab')₂ were reacted with the BoNT/A1-HCR-loaded biosensor. Kinetic measurements were done through association (K_{on}) and dissociation (K_{off}) rate constants of F(ab')₂ [38–40]. The K_D value of F(ab')₂ was determined from the ratio of K_{off}/K_{on} ,

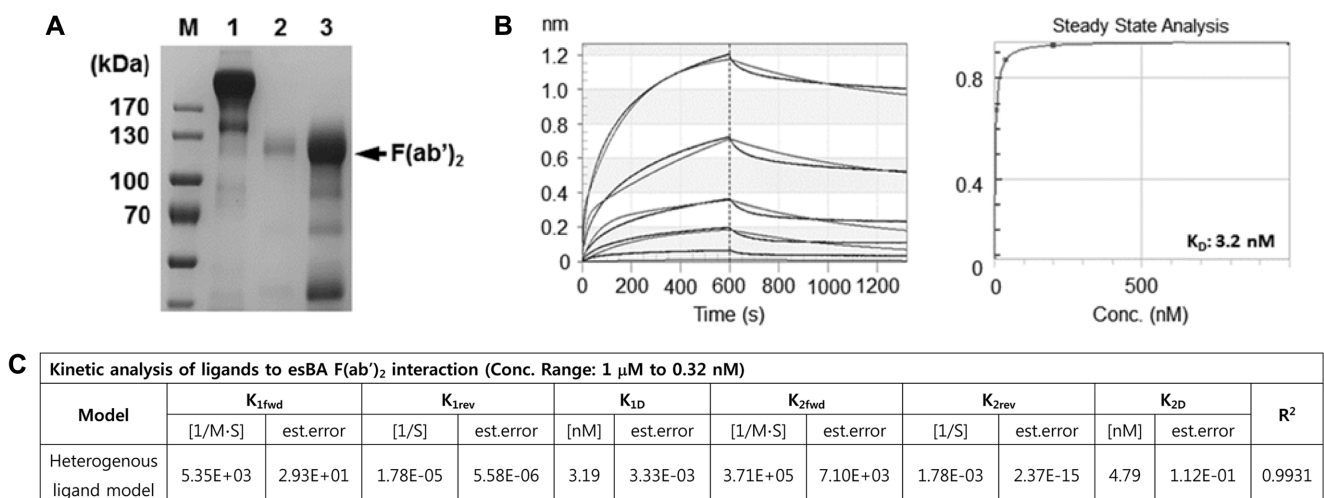


Fig. 4. Purification of F(ab')₂ from BoNT/A1-HCR-immunized equine serum and in vitro binding affinity analysis for purified F(ab')₂. (A) The samples were analyzed with 10% SDS-PAGE under non-reducing conditions. Lane 1, purified IgG before the pepsin treatments; lane 2, wash fraction after the pepsin treatments; lane 3, final F(ab')₂ purification product. (B) In vitro binding affinity was analyzed by Octet Red 384. The kinetics of association and dissociation for 0.32, 8, 40, 200, and 1,000 nM of F(ab')₂ to BoNT/A1-HCR (10 µg/ml). The Y axis indicates the changes in wavelength depending on ligand and analyte binding. K_D value was 3.2 nM in steady-state analysis.

Table 2. In vivo protection of BoNT/A1-HCR antitoxin.

Control ^a	BoNT/A1-HCR serum ^b	Numbers of survived mice (%)		
		F(ab') ₂ (500 µg/mouse) ^c	F(ab') ₂ (300 µg/mouse) ^c	F(ab') ₂ (100 µg/mouse) ^c
0/5 (0)	5/5 (100)	6/6 (100)	5/5 (100)	6/6 (100)

^a10% pre-immune equine serum was incubated with 10,000 LD₅₀ toxin.

^b10% BoNT/A1-HCR-immunized equine serum at 24 weeks with 10,000 LD₅₀ toxin.

^cThe purified F(ab')₂ from BoNT/A1-HCR-immunized equine serum at 24 weeks with 10,000 LD₅₀ toxin.

and results showed the best fits using the heterogeneous ligand model. The values of K_{D1} and K_{D2} were 3.16 nM and 4.79 nM, respectively. K_{on} and K_{off} values were shown in Fig. 4C.

In Vivo Neutralizing Efficacy of the BoNT/A Antitoxin

To evaluate the in vivo neutralizing efficacy, the BoNT/A1 toxin (10,000 LD₅₀), and equine serum immunized with BoNT/A1-HCR at 24 weeks (10% dilution in 200 µl) or F(ab')₂ (500 µg/mouse) were mixed, and incubated for 1.5 h at room temperature. Next, the mixture was administered intraperitoneally to mice, and lethality was recorded for seven days. The control group was injected with pre-immune equine serum. All the mice was injected with BoNT/A1-HCR-immunized serum or F(ab')₂ survived and showed normal behavior. In particular, 500 µg of the purified F(ab')₂ antitoxin protected mice exposed to 10,000 LD₅₀ of BoNT/A1 toxin. To calculate the amounts of 1 IU of the purified F(ab')₂, the mice were injected with F(ab')₂ in a dose step-down manner (500, 300, and 100 µg/mouse). The results showed that the group injected with 100 µg F(ab')₂ was resistant against the 10,000 LD₅₀, and that the 100 µg purified F(ab')₂ had above 1 IU activity in mice (Table 2). As expected, all the mice in the control group died within 24 h of the toxin challenge. These results demonstrated that the Halla horses immunized with BoNT/A1-HCR produced protective IgG antibodies against BoNT/A1, and the final antitoxin products of F(ab')₂ had in vivo neutralizing activities.

Discussion

Antitoxins derived from the sera of hyperimmunized horses have a long history [41–43], and have been used for neutralizing toxins from microorganisms. In this study, antitoxin production against BoNT/A1 using Halla horse bred on Jeju Island was investigated.

BoNTs are protein toxins, which are associated with a high mortality rate, when inhaled or ingested even in small

amounts [3], therefore, antitoxin therapy is indispensable for the treatment of botulism as well as appropriate antibiotic therapy. For this reason, various antitoxins against botulinum toxins have been developed persistently. BabyBIG, is a human-derived antitoxin against serotypes A and B, and approved for use in infants and toddlers. However, the efficacy of BabyBIG is merely 1–4% of BAT-AB, Sanofi Pasteur's botulinum antitoxin. H-BAT (Emergent Biosolutions Inc.) is a polyclonal hyperimmune product manufactured from BoNT toxoids or toxin-immunized equine, and employed to treat botulism in adults or children. Also, this product contains antitoxins against seven botulinum toxin serotypes (A-G). Hence, H-BAT is currently the only treatment for botulism poisoning, and can be used in adults and pediatric patients [24, 25, 44]. However, the use of H-BAT is mostly restricted within the US, thus, the access to botulinum antitoxins under emergency situations is limited in other countries.

In the present study, we immunized Halla horse with BoNT/A1-HCR to obtain an antitoxin against BoNT/A1 consisting of neutralizing antibodies. The heavy chain domain of BoNTs has been used as a vaccine for botulism, and could induce high levels of neutralizing antibodies to efficiently protect against BoNTs. In addition, it is shown to be a good immunogen for antitoxins in mice and non-human primates [45–48]. The previous reports showed that BoNT/A-heavy chain (amino acid 868 through 1296), BoNT/C-heavy chain (425 C-terminal amino acid of the heavy chain), and BoNT/D-heavy chain (413 C-terminal amino acid of the heavy chain) could induce immune responses in horses [48, 49]. In general, inactivated botulinum toxin was used as an antigen for the production of BoNT/A1 antitoxin, such as H-BAT, because the production of toxoid is expensive, and the preparation involves a hazardous detoxification process [45, 46]. BoNT/A toxin is produced by *Clostridium botulinum* as a form of complex with non-toxic non-hemagglutinin A (NTNHA), hemagglutinin (HA)-17, HA-33, and HA-70, under regulations with high biosafety levels, and the yield

of toxin production is relatively poor (~1 mg/l) [45, 50, 51]. Therefore, current equine polyclonal hyperimmune product includes antibodies against NTNHA and HAs. We used BoNT/A1-HCR (amino acid 876 through 1296) as antigens, instead of BoNT/A toxoid, to avoid the risks in large-scale production of toxin and manufacturing issues according to the biosafety regulations. Furthermore, it was estimated that BoNT/A1-HCR could be good immunogen in Halla horses in previous studies [48, 49].

After the immunization of BoNT/A1-HCR in Halla horse, the highest antibody titer was obtained at week 4, and subsequently a titer was maintained as around 1,000. In the case of rabbits, the highest antibody titer was accomplished up to 150,000 after five injections of BoNT/A1-HCR (Fig. 2B). These results are appeared to be due to rabbits producing antibodies with higher titer compared to other animal species [52, 53]. Moreover, this difference in titer between horse and rabbit might have occurred by the difference in adjuvant, antigen doses, immunization schedule, or species. However, the serum from BoNT/A1-HCR-immunized Halla horse had a competent neutralization activity against BoNT/A1 toxins regardless of the relatively low titer compared to that of brown horses. The equine serum from week 24 displayed neutralizing efficacy *in vitro*, and neutralized 10,000 LD₅₀ of BoNT/A1 in the mouse protection assay corresponding to the standard of international units. It was estimated that 20 µl of the equine serum had > 1 IU of neutralizing activity *in vivo* (> 50 IU/ml).

BoNT/A consists of four subtypes from BoNT/A1 to BoNT/A4, and the amino acid variation between them is about 15% or less [54]. These variants have been reported to affect binding affinities of the BoNTs to anti-BoNT/A monoclonal antibodies [55, 56]. In the previous study, a high dose vaccination of BoNT/A HCR protected mice from challenge by heterologous BoNT/A subtype A1-A4 [57]. Therefore, our BoNT/A1-HCR equine serum is expected to contain polyclonal antibodies against BoNT/A1, and could protect against BoNT/A2-A4. However, the neutralization efficacy may be dependent on the variation in amino acid sequence of BoNT/A subtypes.

To fit the data, we used the heterogeneous ligand model (Fig. 4B). The heterogeneous ligand model is appropriate to match the actual curves because polyclonal antibodies could bind different sites on the same ligand molecule [58]. In this analysis, our purified F(ab')₂ revealed 3.16 nM of K_{D1} and 4.79 nM K_{D2} values. In former studies, monoclonal antibodies against BoNT/A toxin showed K_D values from 10⁻¹¹ (10 pM) to 10⁻⁸ (10 nM) [56, 59]. The K_D value of a

monoclonal antibody might be higher than that of a polyclonal antibody, because the K_D value of a monoclonal antibody was derived from a single epitope, whereas the K_D value of a polyclonal antibody was from the mixture of a number of epitopes [60].

Several reports suggested that the combination of serotype-specific monoclonal antibodies showed strong synergic effects corresponding to ~400-fold enhancement in their neutralizing activity against BoNTs, and these results suggested that the combination of antibodies can be a considerable strategy for synergic neutralization effect against BoNT/A [61–64]. In our neutralization analysis, due to the absence of the Fc region, the Fc-mediated clearance of antibody-toxin complexes from the serum for synergic neutralization effect may not be revealed in our equine F(ab')₂ antitoxin treatments. However, no differences were observed in the *in vivo* neutralizing activity at the same concentrations when we compared the neutralizing activity between IgG and F(ab')₂ in mice (data not shown). Therefore, the synergic neutralizing effect of our equine F(ab')₂ antitoxins may exert through the simultaneous binding of interfering polyclonal antibodies to multiple cell binding sites on the BoNT/A1 toxin, and block the entry of BoNT/A toxin into cells [62, 63].

The toxicities caused by equine antiserum are mainly due to hypersensitivity reactions (*e.g.*, anaphylaxis, urticarial, serum sickness), so the equine antitoxins have been partially despeciated by enzymatic cleavage of the allogenic Fc region [20, 65, 66]. We also cleaved the Fc regions to minimize the toxicity of our equine antitoxin through the pepsin treatment (Fig. 4). However, the purified F(ab')₂ contained a small residual of intact IgG that may retain hypersensitivity reactions [67]. Despite the risks of immediate hypersensitivity and sensitization to equine antitoxins, the development of equine antitoxins is important because of the usage of emergent drugs to save the patients exposed to the toxin.

In conclusion, we developed BoNT/A1 equine antitoxin in Halla horse which is well known for its superior immunity and resistance against diverse diseases using BoNT/A1-HCR as an antigen. Our results demonstrate that BoNT/A1-HCR is useful for producing BoNT/A1 equine antitoxin, and it could produce antitoxin from Korean domestic horses. Finally, our findings provide an effective protocol for producing antitoxin in Halla horses, which may contribute to the establishment of a system for emergency treatment using horse sera to cope with various infectious diseases that may occur in the Republic of Korea.

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Conflict of interest

Jun Young Choi and Young Kee Shin own some stocks in ABION Inc., and Jun Young Choi, Na Young Kim, and Kyungeui Park hold stock options in ABION Inc.

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