

Efficacy and Safety of a Recombinant Protein Vaccine against Botulinum Neurotoxin Serotype A

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Abstract : Botulinum neurotoxin serotype A (BoNT-A), produced by *Clostridium botulinum*, is one of the most potent biological toxins and is a significant bio-terrorism threat. Although recombinant subunit vaccines are considered promising vaccine candidates, their detailed immunogenic profile and comprehensive safety are not yet clearly characterized. In this study, we evaluated the efficacy and safety of a mutated recombinant subunit vaccine candidate, BA15, in a murine model. Immunogenicity was assessed by measuring total IgG titers, IgG subclass distribution (IgG1 and IgG2a), and antigen-induced Th1/Th2 cytokine secretion (IFN- γ , IL-4) from splenocyte cultures. Protective efficacy was determined by survival following a lethal BoNT-A challenge. Safety was rigorously evaluated via serial monitoring of body weight, food intake, neuromuscular function (grip strength and rotarod performance), and serum biochemical markers of hepatic, renal, and metabolic function. BA15 immunization elicited robust total IgG responses, with a mild predominance of IgG1 over IgG2a, indicating a slightly Th2-skewed but balanced humoral response. Splenocytes from immunized mice produced both IFN- γ and IL-4 upon antigen stimulation, reflecting concurrent Th1 and Th2 activation. BA15 vaccination conferred complete protection against the lethal BoNT-A challenge, with 100% survival and no clinical signs of botulism. Notably, intramuscular injection of BA15 was well-tolerated; no significant differences were observed in body weight, food intake, neuromuscular test performance, or serum biochemical parameters between the BA15 group and saline-injected control group, indicating a favorable safety profile. This work supports the translational potential of BA15 as a safe and effective subunit vaccine candidate for botulism prevention and addresses key preclinical requirements for further development.

Key words : Botulism, botulinum neurotoxin serotype A, efficacy, safety, vaccine

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1. Introduction

Botulinum neurotoxin serotype A (BoNT-A), produced by *Clostridium botulinum*, is one of the most potent toxins known to humans [1, 15]. BoNT-A can cause botulism, a life-threatening condition that results in flaccid paralysis, respiratory failure, and often death if not treated promptly [8]. While BoNT-A's high toxicity presents a significant threat to public health, it also holds therapeutic potential, as it is utilized in medical applications for the treatment of neurological disorders characterized by hyperactive cholinergic nerve [13].

Due to its extreme potency, ease of production, and prolonged duration of action, BoNT-A may be misused as a potential biological weapon [19]. As little as 1 ng/kg can be lethal to humans, if aerosolized or introduced into food or water, and its inclusion on the list of Category A bioterrorism agents reflects the urgent need for its prevention [5].

BoNT-A has a subunit structure comprising a 100-kDa heavy chain and a 50-kDa light chain, connected by a disulfide bond. The heavy chain can be further subdivided into two functional domains: the N-terminal domain and the C-terminal domain. The heavy chain C-terminal domain of BoNT-A, facilitates specific binding to its neuronal receptor, ganglioside, inducing toxin internalization via receptor-mediated endocytosis [10]. Once inside the neuron, the light chain of BoNT-A cleaves proteins involved in synaptic vesicle fusion, specifically SNAP-25, a component of the SNARE complex that is essential for the release of acetylcholine [2, 6]. This inhibition of neurotransmitter release results in the blockade of synaptic transmission, leading to muscle paralysis [12]. There is no approved anti-botulism vaccine until now, and a lack of an effective vaccine for populations at risk of botulism due to bioterrorism threats or infection suggests an urgent need for the development of a safe and effective vaccine.

In a previous study of our group, we constructed a mutated recombinant subunit vaccine candidate, BA15, which contains a point mutation, R1269A, within the ganglioside-binding site of the heavy chain C-terminal domain of BoNT-A [20]. However, its detailed immunogenic and comprehensive safety profiles are not yet characterized. In this study, we assessed the efficacy and safety of an anti-BoNT-A vaccine candidate BA15, focusing on the characterization of its immune response as well as its safety profiles.

2. Materials and Methods

2.1 Animal experiment

All animal procedures were approved by the Animal Care and Use Committee of the Agency for Defense Development (Approval No. ADD-IACUC-23-06). Female Balb/c mice, aged 6 weeks (Raon Bio, Seoul, Korea), were housed in groups of two or three per cage under standard conditions with free access to food and water. BA15 recombinant protein was provided by GEOBIOTECH Inc. (Daejeon, Korea). After a 1 week acclimatization period, the experimental group received an intramuscular injection, at their left thigh, of 10 µg BA15 recombinant protein dissolved in 100 µl saline with 10% (v/v) alhydrogel (InvivoGen, San Diego, CA, USA). The control group was injected with 100 µl saline with 10% (v/v) alhydrogel. Immunizations were administered at weeks 0, 2, and 4. Blood samples were collected through retro-orbital bleeding at weeks 2, 4, 6, and 9, followed by centrifugation to obtain serum for antibody titer measurement. Experimental schedule was shown in Fig. 1.

2.2 Antibody titer measurement

To measure the levels of specific antibodies, a 96-well plate was coated overnight at 4°C with 0.1 µg/well of BA15 recombinant protein in carbonate buffer (50 µl, pH 9.6). The wells were washed three times with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 5% skim milk for 2 hr at 37°C. After blocking, the wells were washed again and incubated with serially diluted serum samples for 2 hr at 37°C. Following this,

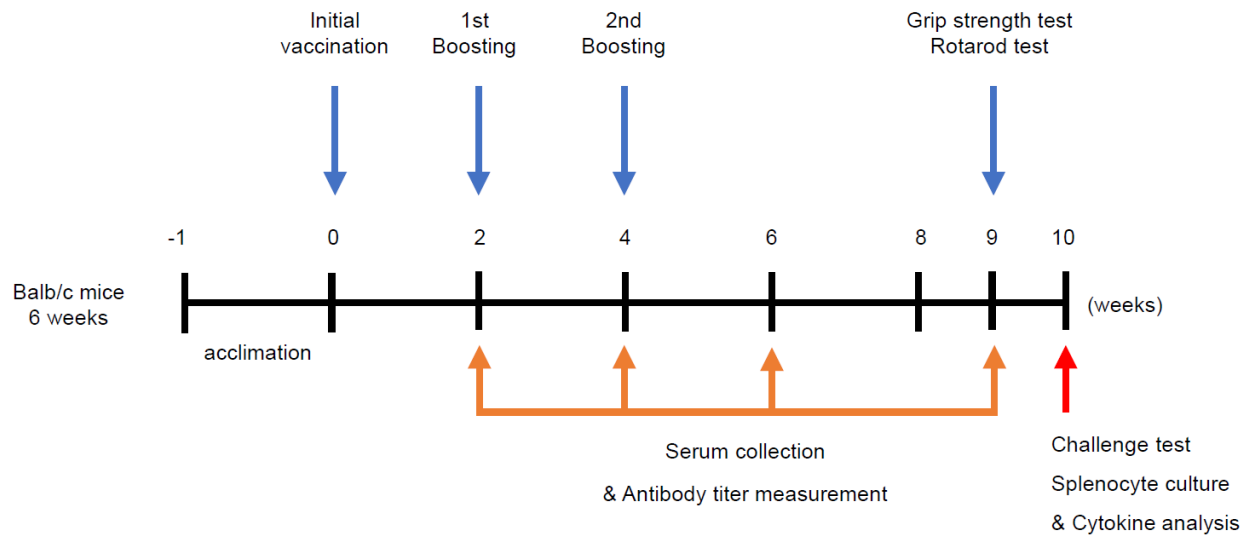


Fig. 1. Experimental schedule.

the wells were incubated with peroxidase-conjugated anti-mouse IgG, IgG1, or IgG2a (1:1,000 in PBS-T) for 1 hr at 37°C. After washing, color development was induced with tetramethylbenzidine (Abcam, Cambridge, UK) and stopped with 3 M HCl. Optical density (OD) was measured at 450 nm using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The cutoff value was set as the mean OD plus three standard deviations from serum samples of non-immunized mice at a 1:100 dilution. The antibody titer was defined as the $\log_{10}$ of the highest dilution showing OD above the cutoff.

2.3 Splenocyte culture and cytokine analysis

At week 10, the spleens of mice were aseptically removed, and single cell suspensions were prepared by passing the organ through a 200- μ m nylon mesh sieve. Splenocytes were resuspended in ACK buffer (0.15 M ammonium chloride, 10 mM potassium bicarbonate, and 0.1 mM EDTA, pH 7.4), washed twice with RPMI 1640 media, and cultured in RPMI 1640 media supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin and 50 μ M β -mercaptoethanol at 37°C under 5% CO₂. For analysis of cytokine production, splenocytes (4×10^6 cells/ml) were restimulated for 24, 48, or 72 hr with 1.25 μ g/ml of BA15 recombinant protein. At the end of restimulation, cell culture supernatants were harvested and analyzed for IFN- γ and IL-4 using ELISA, as described previously [11].

2.4 Challenge test

To assess the protective efficacy of the BA15 vaccine candidate, BA15-immunized and control mice were challenged with a lethal dose of BoNT-A. Mice were immunized as described in section 2.1. At week 10, each mouse was intraperitoneally injected with 1.6 ng/head of BoNT-A, which corresponds to a 20 $\times$ LD₅₀ dose, based on our preliminary study where the LD₅₀ of BoNT-A in mice was determined to be 0.08 ng/head via intraperitoneal injection. Survival was monitored every 4 hr up to 24 hr and then daily for 14 days.

2.5 Safety test

To evaluate the safety profile of BA15 vaccine candidate, several physiological parameters were monitored over the course of the study. Body weight and food intake were measured weekly. At week 9, muscle strength was assessed using a grip strength meter (Bio-GS3, Bioseb, Vitrolles, France). Each mouse under-

went six trials, with 1-min rests in between. The highest and lowest scores were excluded, and the remaining values were averaged [18]. Motor coordination was evaluated using a rotarod tester (JD-A-07TSM, Jeung Do Bio, Seoul, Korea), where mice were placed on a rotating rod accelerating from 3 to 30 rpm over 5 minutes, and the retention time was recorded [9]. After these assessments, blood samples were collected through heart puncture and analyzed for serum biochemistry, including alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine, total protein, glucose, total cholesterol, and triglycerides using a serum biochemistry analyzer (AU480, Beckman Coulter, Brea, CA, USA).

2.6 Statistical analysis

All data are expressed as mean $\pm$ standard deviations. Statistical differences between groups were assessed using an unpaired *t*-test, with a significance threshold of $p < 0.05$. All analyses were performed using GraphPad Prism, version 6 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1 Humoral immune responses induced by BA15

To assess the immunogenicity of the BA15 antigen, we measured the levels of serum antibodies specific to BA15 in immunized mice. Total IgG as well as IgG1 and IgG2a isotypes were analyzed to characterize the kinetics and subclass distribution of the humoral immune response. As shown in Fig. 2, BA15-immunized mice showed an increase in specific IgG levels over the course of the study, while control group maintained only baseline antibody titers. The average $\log_{10}$ titers of IgG at 4, 6, and 9 weeks after immunization were 2.4, 3.4, and 2.8, respectively (Fig. 2A) and significantly higher than those of control group ($p < 0.0001$ by unpaired *t*-test, Fig. 2B). This significant elevation in antigen-specific IgG confirms that BA15 is a strongly immunogenic antigen when delivered via intramuscular route.

To evaluate the Th1/Th2 polarization of the antibody response, IgG subclass levels were analyzed. IgG1 is generally associated with Th2-type responses, while IgG2a reflects Th1-type immune bias [3]. As shown in Fig. 2C, BA15 immunization induced a robust increase in IgG1 titers over time. The average $\log_{10}$ titers of IgG1 at 4, 6, and 9 weeks after immunization were 3.0, 3.4, and 3.6 respectively (Fig. 2C) and significantly higher than those of control group ($p < 0.0001$ by unpaired *t*-test, Fig. 2D). IgG2a levels were also monitored over time (Fig. 2E). In BA15-immunized mice, a steady increase in IgG2a was observed at a lower magnitude than IgG1. Nevertheless, significant elevation over control was noted. The average $\log_{10}$ titers of IgG2a at 6, and 9 weeks after immunization were 1.4, and 2.2 respectively (Fig. 2E) and significantly higher than those of control group ($p < 0.0001$ by unpaired *t*-test, Fig. 2F).

The ratios of the average $\log_{10}$ titers of IgG1/IgG2a were 2.4 at week 6 and 1.6 at week 9, which showed that IgG1 induction is higher than IgG2a, and Th2-type immune response is stronger than Th1-type response. BA15 also elicits a Th1-type antibody response, albeit to a lesser extent than Th2.

3.2 Cellular immune responses induced by BA15

To evaluate the cellular immune response elicited by intramuscular injection of BA15, we analyzed cytokine production by splenocytes isolated from mice at week 10 post-immunization. Splenocytes isolated from each mouse were stimulated with BA15 antigen, and the secretion of IFN- γ , a Th1 cytokine, and IL-4, a Th2 cytokine [4], was measured at 24, 48, and 72 hr post-stimulation. In the BA15-immunized group, IFN- γ production increased progressively over the 72-hr stimulation period (Fig. 3A). At 72 hr, the mean IFN- γ level of BA15 group reached 2.73 pg/ml which is significantly higher than that of control group (0.61 pg/ml) (Fig. 3G). This trend indicates antigen-specific activation of Th1-type T cells in the BA15 group. Similarly, IL-4 se-

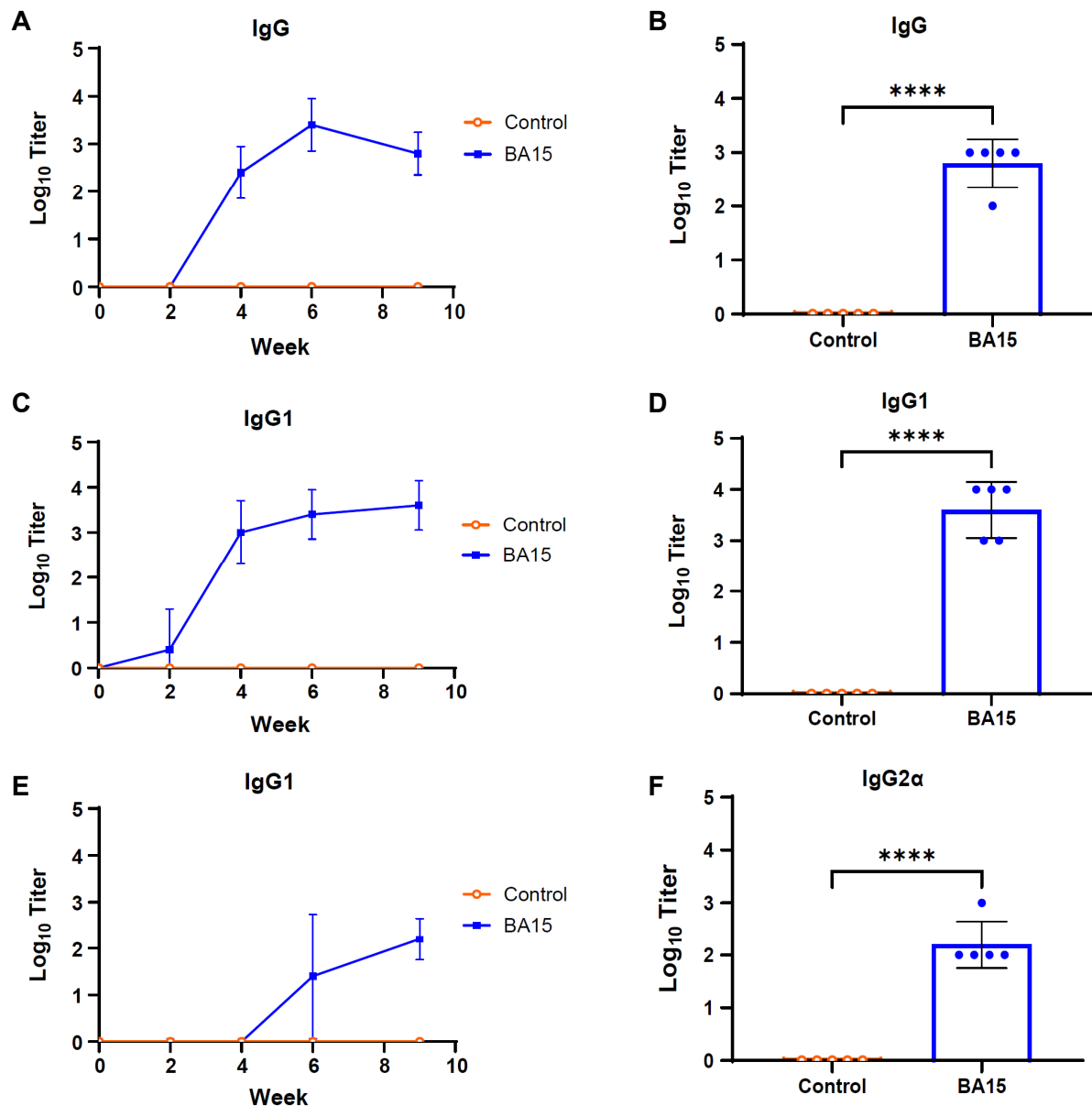


Fig. 2. Antibody production in mice immunized with BA15 injection. (A) Time course of IgG antibody titers in mice immunized by intramuscular injection of antigen (BA15) or saline (Control). (B) Comparison of IgG antibody titers between groups at week 9. (C) Time course of IgG1 antibody titers in experimental groups. (D) Comparison of IgG1 antibody titers between groups at week 9. (E) Time course of IgG2a antibody titers in experimental groups. (F) Comparison of IgG2a antibody titers between groups at week 9. Data are presented as mean $\pm$ standard deviation ($n=5$). *** $p < 0.0001$ by unpaired t -test.

cretion was elevated in splenocytes from BA15-immunized mice, over the 72-hr stimulation period (Fig. 2B). At 72 hr, the mean IL-4 level of BA15 group reached 15.53 pg/ml which is significantly higher than that of control group (5.20 pg/ml) (Fig. 3H). The simultaneous elevation of IFN- γ and IL-4 suggests that BA15 immunization induces a mixed Th1/Th2 immune response, which is advantageous for effective immunity against toxins such as BoNT-A.

3.3 BA15 confers protection against lethal BoNT-A challenge

To evaluate the protective efficacy of the BA15 injection against BoNT-A, BA15-immunized and control mice were challenged with a lethal dose of BoNT-A and monitored for survival. Survival was assessed daily

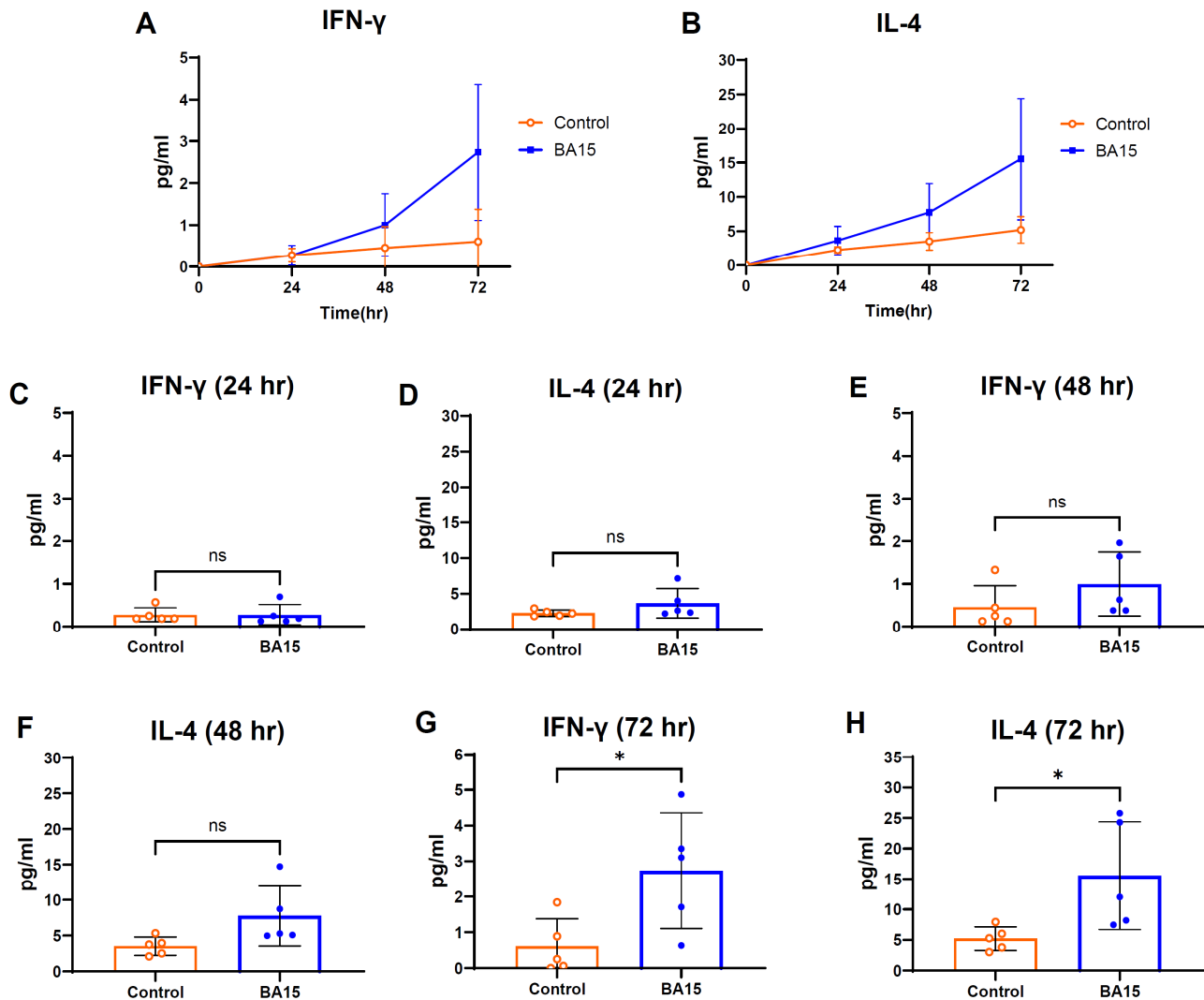


Fig. 3. Cytokine production of splenocytes isolated from mice immunized with BA15. (A) Time course of IFN- γ production of splenocytes isolated from mice immunized by intramuscular injection of antigen (BA15) or saline (Control) after antigenic stimulation up to 72 hr. (B) Time course of IL-4 production of splenocytes isolated from mice immunized by intramuscular injection of antigen (BA15) or saline (Control) after antigenic stimulation up to 72 hr. (C) Comparison of IFN- γ production of splenocytes after 24 hr of antigenic stimulation. (D) Comparison of IL-4 production of splenocytes after 24 hr of antigenic stimulation. (E) Comparison of IFN- γ production of splenocytes after 48 hr of antigenic stimulation. (F) Comparison of IL-4 production of splenocytes after 48 hr of antigenic stimulation. (G) Comparison of IFN- γ production of splenocytes after 72 hr of antigenic stimulation. (H) Comparison of IL-4 production of splenocytes after 72 hr of antigenic stimulation. Data are presented as mean $\pm$ standard deviation ($n=5$). ^{ns}not significant, * $p<0.05$ by unpaired t -test.

for 14 days post-challenge when the study concluded, as no further changes were anticipated. All mice in the BA15-immunized group survived the entire 14-day observation period following BoNT-A challenge, indicating complete protection conferred by the vaccination. In contrast, 100% of the mice in the control group succumbed to BoNT-A challenge within 16 hr post-challenge (Fig. 4). These findings clearly demonstrate the protective efficacy of BA15 injection against systemic BoNT-A exposure. No signs of neurological dysfunction such as paralysis and respiratory distress were observed in the BA15-immunized mice, whereas control mice exhibited characteristic symptoms of botulinum intoxication—flaccid paralysis and rapid mortality. The complete protection offered by BA15 injection strongly correlates with its robust humoral response, high IgG titers including IgG1 and IgG2a subclasses. These antibodies are known to play a critical role in neutralizing botulinum neurotoxin by blocking receptor binding or internalization into peripheral neurons. Given the stringent nature of the

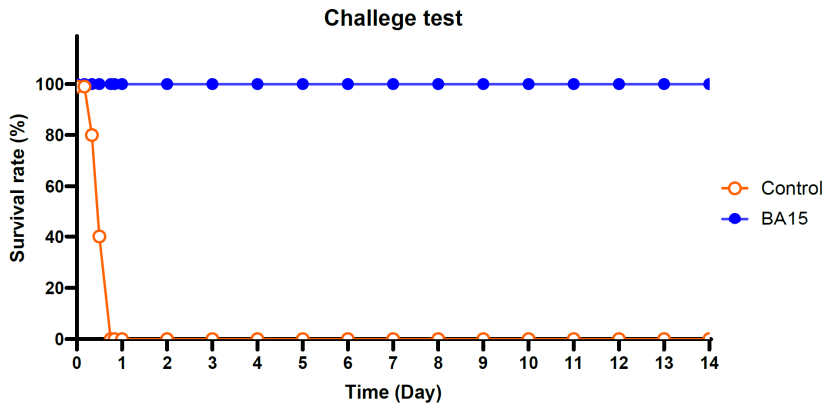


Fig. 4. Survival rates of mice after challenge with BoNT-A. Mice were immunized by the intramuscular injection of antigen (BA15) or saline (Control), and then challenged with BoNT-A. Survival rates were measured for 14 days in each experimental group (n=5).

BoNT-A challenge, the 100% survival rate in the BA15 group confirms that the antigen-induced immune response was not only immunogenic but also functionally protective, satisfying a key criterion for vaccine efficacy in preclinical development.

3.4 Effects of BA15 on physiological parameters

To assess the safety of BA15 immunization, a comprehensive evaluation of general health status was conducted in mice. Parameters included body weight, food intake, muscle strength and motor coordination. Body weight measurements were conducted weekly from the initiation of immunization. As shown in Fig. 5A, both BA15-injected and control groups demonstrated similar patterns of gradual weight gain, with no overt signs of growth retardation or weight loss. At week 8, average body weights (mean $\pm$ SD) were 20.44 ± 1.15 g in

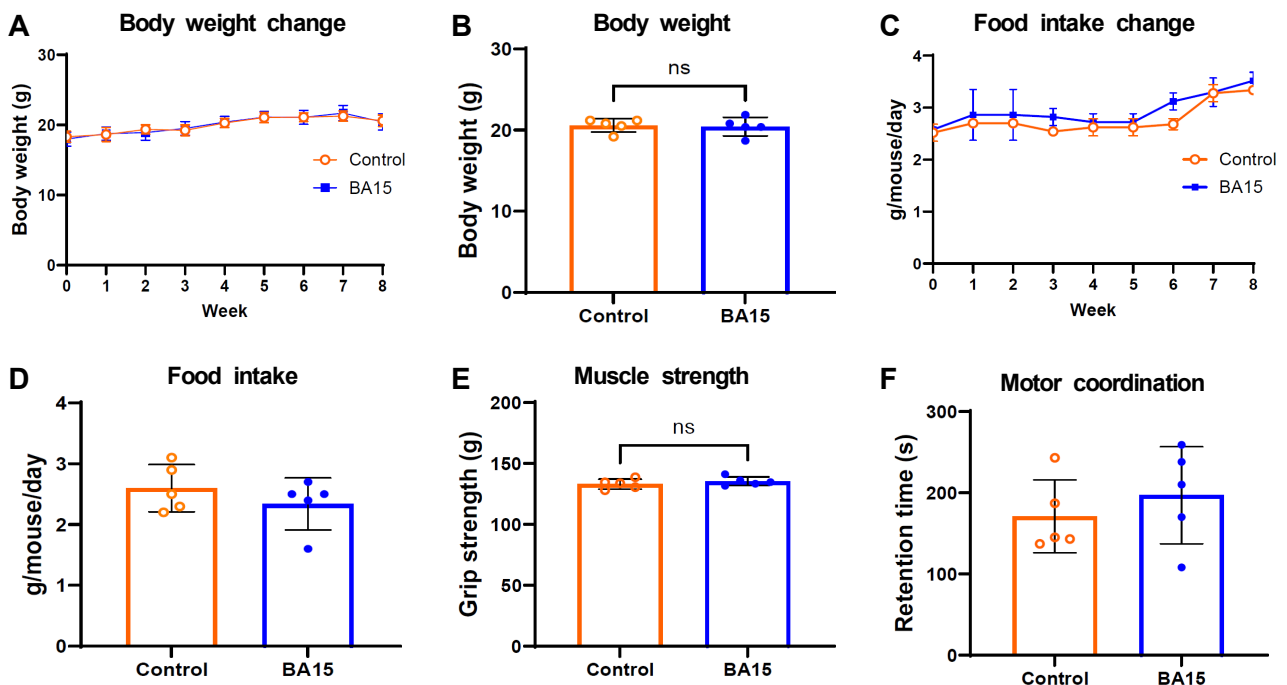


Fig. 5. Effects of BA15 on physiological parameters. (A) Time course of body weight change in mice immunized by intramuscular injection of antigen (BA15) or saline (Control). (B) Comparison of body weight between groups at week 8. (C) Time course of food intake change in mice immunized by intramuscular injection of antigen (BA15) or saline (Control). (D) Comparison of food intake between groups at week 8. (E) Comparison of muscle strength measured by a grip strength test between groups at week 8. (F) Comparison of motor coordination measured by a rotarod test between groups at week 8. Data are presented as mean $\pm$ standard deviation (n=5). ^{ns}not significant by unpaired *t*-test.

BA15 group, and 20.58 ± 0.83 g in control group, which were not statistically different ($p > 0.05$ by unpaired t -test, Fig. 5B). Food consumption was measured weekly. Fig. 5C depicts that both groups maintained steady levels of daily food intake, with no evidence of anorexia or feeding changes. At week 8, BA15 group consumed 2.34 ± 0.43 g/day per mouse, while control group consumed 2.60 ± 0.39 g/day per mouse, which were not statistically different, showing that BA15 immunization did not impair appetite or feeding behavior (Fig. 5D). Grip strength test, a measure of muscle strength, was conducted at week 9 using a grip strength meter. Grip strength of BA15 group was 135.40 ± 3.59 g, while that of control group was 133.10 ± 4.69 g, which were not statistically different (Fig. 5E). No evidence of muscle weakness, neurotoxicity, or fatigue-like symptoms was observed in BA15-immunized mice. To evaluate potential effects of BA15 on motor coordination and balance, a rotarod test was performed at week 9. Retention time on a rotarod of BA15 group was 197.0 ± 59.9 s while that of control group was 171.0 ± 44.9 s, which were not statistically different (Fig. 5F). All mice in the BA15 group exhibited normal performance, indicating no impairment of motor function. Collectively, the absence of statistically significant differences in body weight, food consumption, muscle strength, and motor coordination between groups strongly indicate that BA15 immunization is well tolerated, and BA15 does not exert any adverse effects in mice.

3.5 Effects of BA15 injection on serum biochemical parameters

To further assess the systemic safety and potential organ toxicity of BA15 immunization, serum biochemical parameters were analyzed at week 10 post-immunization. Blood samples were collected from all mice via cardiac puncture, and serum was subjected to standard clinical chemistry analysis. Results were summarized in Table 1 and Fig. 6. Alanine transaminase (ALT) and aspartate transaminase (AST) are sensitive indicators of hepatocellular injury. Alkaline phosphatase (ALP) levels reflect biliary tract function and general hepatic metabolism. All hepatic enzymes remained within physiological ranges, and no significant differences were observed between groups (Fig. 6A-6C), indicating no hepatotoxicity induced by BA15. Blood urea nitrogen (BUN) and creatinine are canonical markers of glomerular filtration and renal excretory function. No significant differences were observed, suggesting normal renal function in BA15-immunized mice (Fig. 6D-6E). Total protein reflects nutritional status and systemic inflammation. Glucose level assesses glycemic homeostasis. Total cholesterol and triglyceride levels provide insights into lipid metabolism. No significant abnormalities or inter-group differences were found (Fig. 6F-6I), supporting the notion that BA15 does not perturb systemic metabolism or nutritional balance. The comprehensive serum chemistry profile revealed no evidence of organ toxicity in BA15-immunized mice. All measured parameters remained within normal physiological limits, and no statistically significant changes were noted compared to the saline-injected controls. These findings collectively support the safety of the BA15 and its potential suitability for future translational development as a botulinum vaccine candidate.

Table 1. Serum biochemical parameters in experimental groups

Parameter	Control	BA15	Normal range
ALT (U/l)	38.3 (32-45)	34.7 (30-40)	17-77
AST (U/l)	196.3 (115-280)	191.7 (166-207)	54-298
ALP (U/l)	84.2 (79-90)	81.2 (70-89)	35-96
BUN (mg/dl)	18.5 (16.9-20.7)	16.3 (13.6-21.9)	8-33
Creatinine (mg/dl)	0.1 (0.007-0.14)	0.1 (0.12-0.14)	0.057-0.76
Total protein (g/dl)	4.8 (4.64-5.03)	4.8 (4.55-5.05)	3.5-7.2
Glucose (g/dl)	80.2 (28-113)	71.2 (50-105)	62-175
Total cholesterol (mg/dl)	106.7 (84-118)	119.5 (108-131)	80-219
Triglyceride (mg/dl)	58.7 (35-111)	79.3 (41-115)	3-193

Data are shown as average (minimum-maximum).

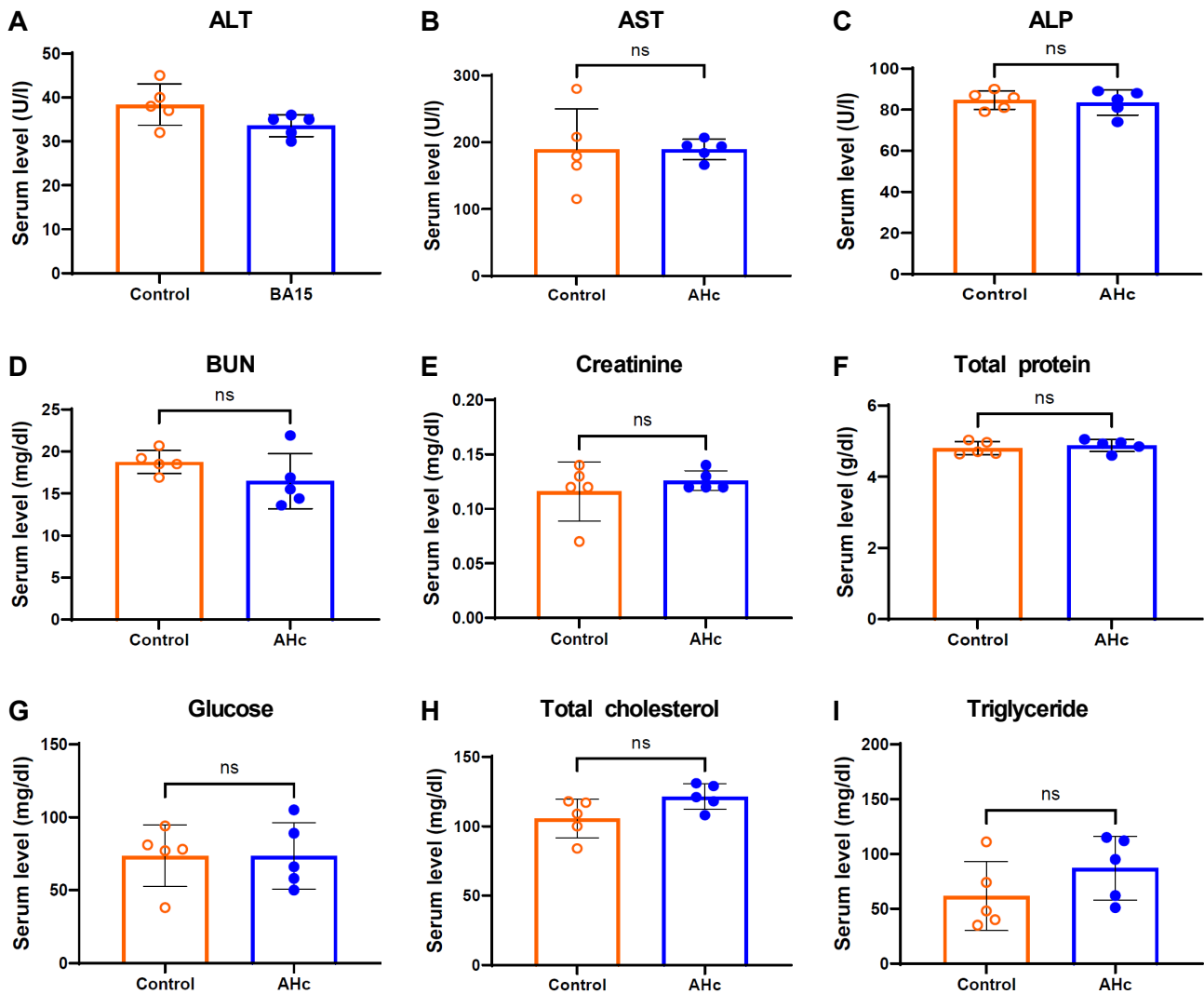


Fig. 6. Effects of BA15 on serum biochemical parameters. Mice were immunized by intramuscular injection of antigen (BA15) or saline (Control) and serum levels of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine, total protein, glucose, total cholesterol, and triglyceride were compared between groups at week 9. Data are presented as mean $\pm$ standard deviation ($n=5$). ^{ns}not significant by unpaired t -test.

4. Discussion

In this study, we demonstrated that intramuscular immunization with BA15—a recombinant fragment of the C-terminal heavy chain domain of BoNT-A containing a point mutation R1229A in its ganglioside binding site—elicits a robust and balanced immune response in mice, confers complete protection against lethal BoNT-A challenge, and does so without inducing detectable adverse physiological or biochemical effects. These results collectively highlight the translational potential of the intramuscular injection of BA15 as a vaccine candidate for botulism prevention.

Our results showed that BA15 immunization induced strong total IgG responses, with significantly elevated titers of both IgG1 and IgG2a subclasses (Fig. 2). The IgG1/G2a ratios of 1.6 to 2.4 suggests a mildly Th2-skewed response, which is typically associated with effective neutralization of extracellular antigens, such as botulinum neurotoxins. However, the concurrent induction of IgG2a also suggests Th1-type activation, which may contribute to antibody class switching, B cell memory development, and long-term immunological protection [3, 4]. This subclass distribution indicates that BA15 can elicit both Th1 and Th2 immune components,

though Th2-type is stronger than Th1-type immune response.

Supporting this result, splenocytes from BA15-immunized mice produced significant levels of both IFN- γ and IL-4 upon antigenic stimulation (Fig. 3). IFN- γ is a hallmark cytokine of Th1-type responses and facilitates isotype switching to IgG2a and the activation of cellular immunity, and IL-4 promotes Th2-type response and IgG1 production, which is crucial for toxin neutralization [3, 4]. The simultaneous upregulation of these cytokines indicates a functionally balanced Th1/Th2 immune response, enhancing the protection against BoNT-A.

The ultimate measure of a vaccine's efficacy is its ability to confer protection against pathogen exposure. In our BoNT-A challenge model, all mice immunized with BA15 survived the lethal dose without signs of paralysis or distress, whereas all control mice succumbed within 16 hr (Fig. 4). This results in survival outcomes provides strong preclinical evidence that BA15-induced immunity is protective, not just immunogenic.

In addition to its immunogenicity and protective efficacy, BA15 exhibited a highly favorable safety profile. Over the immunization period, there were no significant differences in body weight, food intake, muscle strength, or motor coordination between BA15 and control groups. These parameters collectively assess general health status, neuromuscular function, and potential behavioral toxicity. Data shown in Fig. 5 support the physiological tolerability of the BA15 antigen. Moreover, serum biochemical analyses revealed no significant differences in markers of hepatic (ALT, AST, ALP) or renal (BUN, creatinine) function, nor in metabolic indices (total protein, glucose, cholesterol, triglycerides). These findings indicate that BA15 immunization does not perturb critical organ systems or induce toxicity (Fig. 6 and Table 1).

Currently, the primary prevention method against botulism relies on the use of heptavalent antitoxins against BoNT, typically derived from immunized horses. These antitoxins, however, have limitations, including variability in potency, short half-lives, and adverse reactions such as hypersensitivity, pyrexia, tachycardia, bradycardia, anaphylaxis, and blood pressure increase [14]. A formalin-inactivated pentavalent anti-BoNT toxoid vaccine had been used to vaccinate people who are at high risk of contracting botulism in the United States, which was discontinued in 2011 due to losing potency and side effects [17]. Recombinant subunit vaccines may present a safer alternative, though no vaccine is approved until now. Previous studies on recombinant BoNT-A vaccine conducted by our group [20] or others [7, 16, 21] have focused primarily on immunogenicity such as serum total IgG level and protective efficacy after challenge test. This study, however, provides detailed immunological profiling of humoral and cellular immune response including the levels of IgG subclasses, IgG1 and IgG2a (Fig. 2C-2F) as well as the induction of Th1/Th2 cytokine, IFN- γ and IL-4, from splenocytes (Fig. 3). This study also provides assessments of neuromuscular function measured by grip strength and rotarod tests as well as general safety markers including serum biochemical markers, body weight, and food intake (Fig. 5 and 6, Table 1), thereby thoroughly ruling out physiological or behavioral toxicity which were largely unaddressed in previous studies, directly supporting further human vaccine development.

While the present study provides compelling preclinical evidence, it has several limitations. The sample size (n=5 per group) was limited, and larger animal study may be necessary. The duration of immunity was not explored; future studies should include long-term follow-up to determine the persistence of protective responses. This study is a single-dose study, and multiple-dose study is necessary to find dose-response. In addition, further investigations into cross-protection against other BoNT serotypes (e.g., B, E and F) will be necessary for translating these findings into a broadly applicable human vaccine.

Conclusion

Taken together, our results establish that intramuscular immunization with the BA15 vaccine candidate induces a balanced humoral and cellular immune response, provides complete protection against BoNT-A challenge, and exhibits a good safety profile in mice. These findings lay the groundwork for further development

of BA15 as an effective and safe strategy for preventing botulism.

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The Conflict of Interest Statement

These authors declare that they have no conflicts of interest with the contents of this article.

References

1. Arnon, S. S., Schechter, R., Inglesby, T. V., Henderson, D. A., Bartlett, J. G., Ascher, M. S., Eitzen, E., Fine, A. D., Hauer, J., Layton, M., Lillibridge, S., Osterholm, M. T., O'Toole, T., Parker, G., Perl, T. M., Russell, P. K., Swerdlow, D. L. and Tonat, K.; Working Group on Civilian Biodefense. 2001. Botulinum toxin as a biological weapon: Medical and public health management. *JAMA* **285**,1059–1070.
2. Blasi, J., Chapman, E. R., Link, E., Binz, T., Yamasaki, S., De Camilli, P., Südhof, T. C., Niemann, H. and Jahn, R. 1993. Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. *Nature* **365**, 160–163.
3. Bretscher, P. A. 2014. On the mechanism determining the Th1/Th2 phenotype of an immune response, and its pertinence to strategies for the prevention, and treatment, of certain infectious diseases. *Scand. J. Immunol.* **79**, 361–376.
4. Bretscher, P. A. 2022. The role of cytokines in determining the Th1/Th2 phenotype of an immune response: Coherence of the T cell response and the cytokine implementation hypothesis. *Scand. J. Immunol.* **95**, e13110.
5. Cenciarelli, O., Riley, P. W. and Baka, A. 2019. Biosecurity threat posed by botulinum toxin. *Toxins (Basel)* **11**, 681.
6. Chen, S. and Barbieri, J. T. 2011. Association of botulinum neurotoxin serotype A light chain with plasma membrane-bound SNAP-25. *J. Biol. Chem.* **286**, 15067–15072.
7. Choi, E. S., Pyo, S. W., Kim, S. H., Jeon, J. H., Rhie, G. E., Yun, M. R., Yi, H. and Chung, Y. S. 2025. Development of a recombinant fusion vaccine candidate against lethal clostridium botulinum neurotoxin types A and B. *Vaccines (Basel)* **13**, 39.
8. Corsalini, M., Inchingolo, F., Dipalma, G., Wegierska, A. E., Charitos, I. A., Potenza, M. A., Scarano, A., Lorusso, F., Inchingolo, A. D., Montagnani, M. and Santacroce, L. 2021. Botulinum neurotoxins (BoNTs) and their biological, pharmacological, and toxicological issues: A scoping review. *Appl. Sci.* **11**, 8849.
9. Deacon, R. M. 2013. Measuring motor coordination in mice. *J. Vis. Exp.* **29**, e2609.
10. Kumar, R. and Singh, B. R. 2025. Botulinum toxin: A comprehensive review of its molecular architecture and mechanistic action. *Int. J. Mol. Sci.* **26**, 777.
11. Lee, S. H., Lee, S., Chae, C. and Ryu, D. Y. 2014. A recombinant chimera comprising the R1 and R2 repeat regions of *M. hyopneumoniae* P97 and the N-terminal region of *A. pleuropneumoniae* ApxIII elicits immune responses. *BMC Vet. Res.* **10**, 43.
12. Lu, B. 2015. The destructive effect of botulinum neurotoxins on the SNARE protein: SNAP-25 and synaptic membrane fusion. *PeerJ.* **3**, e1065.
13. Matak, I. and Lacković, Z. 2014. Botulinum toxin A, brain and pain. *Prog. Neurobiol.* **119–120**, 39–59.
14. Parrera, G. S., Astacio, H., Tunga, P., Anderson, D. M., Hall, C. L. and Richardson, J. S. 2021. Use of botulism antitoxin heptavalent (A, B, C, D, E, F, G)–(Equine) (BAT®) in clinical study subjects and patients: A 15-year systematic safety review. *Toxins (Basel)* **14**, 19.
15. Rossetto, O. and Montecucco, C. 2019. Tables of toxicity of botulinum and tetanus neurotoxins. *Toxins (Basel)* **11**, 686.
16. Shi, D. Y., Liu, F. J., Li, Z. Y., Mao, Y. Y., Lu, J. S., Wang, R., Pang, X. B., Yu, Y. Z. and Yang, Z. X. 2022. Development and evaluation of a tetravalent botulinum vaccine. *Hum. Vaccin. Immunother.* **18**, 2048621.
17. Sundeen, G. and Barbieri, J. T. 2017. Vaccines against botulism. *Toxins (Basel)* **9**, 268.

18. Takeshita, H., Yamamoto, K., Nozato, S., Inagaki, T., Tsuchimochi, H., Shirai, M., Yamamoto, R., Imaizumi, Y., Hongyo, K., Yokoyama, S., Takeda, M., Oguro, R., Takami, Y., Itoh, N., Takeya, Y., Sugimoto, K., Fukada, S. I. and Rakugi, H. 2017. Modified forelimb grip strength test detects aging-associated physiological decline in skeletal muscle function in male mice. *Sci. Rep.* **7**, 42323.
19. Tatu, L. and Feugeas, J. P. 2021. Botulinum toxin in WW2 German and allied armies: Failures and myths of weaponization. *Eur. Neurol.* **84**, 53-60.
20. Yu, C. H., Song, D. H., Choi, J. Y., Joe, H. E., Jeong, W. H., Hur, G. H., Shin, Y. K. and Jeong, S. T. 2018. A mutated recombinant subunit vaccine protects mice and guinea pigs against botulinum type A intoxication. *Hum. Vaccin Immunother.* **14**, 329-336.
21. Yu, Y. Z., Li, N., Zhu, H. Q., Wang, R. L., Du, Y., Wang, S., Yu, W. Y. and Sun, Z. W. The recombinant Hc subunit of *Clostridium botulinum* neurotoxin serotype A is an effective botulism vaccine candidate. *Vaccine* **27**, 2816-2822.

초록 : 보툴리눔 신경독소 A형에 대한 재조합 단백질 백신의 효력 및 안전성 연구

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보툴리눔 신경독소 A형(BoNT-A)은 *Clostridium botulinum*에 의해 생성되는 가장 강력한 생물학적 독소 중 하나로, 생물학적 테러에 악용될 수 있는 중대한 위협 요소이다. BoNT-A에 대한 재조합 단백질 백신은 유망한 백신 후보로 고려되고 있지만, 그 면역원 프로파일과 안전성에 대한 구체적인 특성은 아직 명확하게 규명되지 않고 있다. 본 연구에서는 돌연변이 재조합 단백질 백신 후보인 BA15 근육주사의 효능과 안전성을 마우스 모델에서 평가하였다. 면역원성은 마우스 혈청내의 총 IgG 농도 및 IgG 아형 분포(IgG1과 IgG2a)와 비장 세포 배양액 내의 Th1/Th2 사이토카인(IFN- γ , IL-4) 농도를 측정하여 평가하였다. 백신의 보호 효능은 BoNT-A 복소의 복강주사에 대한 생존율을 통해 측정되었다. 안전성은 체중, 식이 섭취, 신경근 기능(그립 강도 및 로타로드 수행), 간, 신장 및 대사 기능에 대한 혈청 생화학적 마커를 통해 평가되었다. BA15는 강력한 총 IgG 항체 형성을 유도했으며, IgG1이 IgG2a에 비해 약간 우세하게 나타났다. 이는 약간 Th2 편향되었으나 균형 잡힌 체액성 면역 반응을 나타낸다. BA15로 면역화된 마우스의 비장 세포는 항원 자극 시 IFN- γ 와 IL-4를 모두 생성하여 Th1과 Th2면역이 동시에 활성화된 것을 나타내었다. BA15 백신은 BoNT-A 독소 복강주사에 대한 완벽한 보호 효과를 나타내어, 마우스들은 100% 생존율을 보였고 보툴리즘의 임상 증상은 나타나지 않았다. BA15를 근육주사한 실험군과 생리식염수를 근육주사한 대조군 사이에서 체중, 식이 섭취, 신경근 기능, 혈청 생화학적 파라미터에서 유의미한 차이가 관찰되지 않아 안전성이 우수함을 나타내었다. 이 연구는 BA15가 보툴리즘 예방을 위한 안전하고 효과적인 재조합 단백질 백신으로 개발될 수 있는 가능성을 제시하고 있다.