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ORIGINAL ARTICLE

Preparation of Equine Immunoglobulin F(ab')$_2$ against Smallpox and Evaluation of its Immunoprotective Effect

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Abstract

Objective: Smallpox, a severe infectious disease caused by the smallpox virus, causes a death rate as high as 30% within 15-20 days after infection. Therefore, development of anti-Smallpox product as a strategic reserve is urgently needed.

Methods: We prepared and tested pepsin-digested F(ab')$_2$ fragments of serum IgG from horses.

Results: Transmission electron microscopy indicated that the purified virus showed morphology consistent with VVTT. The titer was above 1.0×10^7 PFU/mL. The purity of the antigen exceeded 90%, according to HPLC. After purification and cleavage, the yield of the purified product F(ab')$_2$ was approximately 1.3%, its purity exceeded 90%, and the neutralizing antibody titer exceeded 1:3200. F(ab')$_2$ fragments had good preventive and therapeutic effects in mice at antibody doses of 5.2 mg/mL and 2.6 mg/mL. The viral loads of the drug-treated mice were suppressed to varying degrees, and the higher dose groups (5.2 and 2.6 mg/mL) showed a 2-3 fold lower viral load than that in the control group.

Conclusion: A process for producing equine immunoglobulin F(ab')$_2$ against VVTT was established. The prepared horse anti-smallpox immunoglobulin product had good neutralizing antibody effects on VVTT. The highly purified preparation may serve as a potential candidate for smallpox treatment.

Key words: smallpox, equine immunoglobulin F(ab')$_2$ fragments, needle-free, immunoprotective

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INTRODUCTION

Smallpox, a severe infectious disease caused by the smallpox virus, is transmitted among people and causes a death rate as high as 30% within 15–20 days after infection [1,2]. After the global eradication of smallpox was announced in 1980, smallpox vaccination was discontinued [3]. Currently, most people are unvaccinated, and vaccine–induced immunity in people vaccinated before 1980 is waning [4,5]. After the events of September 11, 2001 in America, concerns that terrorist organizations might use smallpox as a biologic weapon arose [6]. Smallpox virus is retained in high security biosafety laboratories in the United States and Russia, and recent biosafety accidents in high biosafety level laboratories have underscored the need to be fully prepared for smallpox outbreaks

[7]. Although chemosynthetic drugs are available for the treatment of patients with smallpox, they are not specific, and several key problems such as drug resistance and liver injury caused by long-term use are of concern [8]. The United States recently approved a non–replicating small-pox vaccine, but only for emergency use. Moreover, vaccine immunizations might not protect people who have already been infected with smallpox. Beyond vaccination for preventive purposes, a specific biological treatment drug providing comprehensive protection must be developed.

Passive immunotherapy with sera of animal origin has been used for more than 120 years to treat viral and bacterial infections, as well as drug intoxication [9]. Equine antisera, particularly $F(ab')_2$ fragments, are easily purified in large quantities and are relatively inexpensive for urgent prevention and treatment of infectious diseases, and have been demonstrated to be effective and safe, such as in the case of the rabies virus [10-12].

In this study, modern biopharmaceutical technology methods were used to optimize the production, purification, healthy horse immunization, preparation and purification of $F(ab')_2$ of horse immunoglobulin against vaccinia virus TianTan (VVTT). The purified immunoglobulin had a purity above 90%, on the basis of high performance liquid chromatography (HPLC). The neutralizing antibody titer of the product against VVTT exceeded 1:3200 in sensitive cells. The product had good protective effects in terms of both prevention and treatment in a sensitive animal model. According to test results from Joinn Laboratories (China) Co., Ltd., the safety of the product meets the Chinese Pharmacopoeia requirements. These results provide basic experimental data to support future clinical research.

METHODS

Virus, cells and animals
VVTT was provided by the Institute of Virology of the China Center for Disease Control and Prevention. It was originally isolated from the blister scabs of patients with smallpox in 1926 and obtained by successive generations of virus reduction. Specific-pathogen-free (SPF) chicken embryos were purchased from Beijing Meiliweitong Experimental Technology Co., Ltd. Primary chicken embryo fibroblasts were derived from freshly harvested, 9-11–day-old SPF chicken embryos. Cells were received in a suspension of DMEM containing 5% new-born calf serum. Vero cells were provided by the cell bank of the Beijing Institute of Microbial Epidemiology. Ten healthy horses, ranging in age from 3 to 5 years, were quarantined to meet the requirements of the Chinese Pharmacopoeia. Six-week-old BALB/c mice, weighing 18-20 g, were provided by SPF Experiment Animal Technology Co., Ltd. All animal experiments were approved by the Institutional Animal Welfare Committee (No. LL20220603).

Production, purification and identification of VVTT
Chicken embryo fibroblast cells were cultured at 37°C and cell monolayers were inoculated with VVTT at an MOI of 0.1 for 96-120 h; the virus was harvested within 24 h after whole cells were lysed. A 300 kD Millipore ultrafiltration membrane was used to concentrate the virus 100 × concentration. The virus was centrifuged at 30000 g through a sucrose gradient for 6 hours. Protein peaks were detected with Sepharose 4FF gel chromatography. A transmission electron microscope was used to observe the morphology of VVTT. Thioglycolate medium, nutrient agar slant medium and modified Martin medium were used for sterility tests. For mycoplasma tests, half fluid and half broth medium were used. Mice, chicken embryos and cells were inoculated in exogenous factor tests. HPLC was used for purity tests.

Preparation of antigens for immunity and production of raw plasma
In scheme 1, purified inactivated viral antigen (1:1) was mixed with an equal volume of ISA206 adjuvant, emulsified and used for immunization of horses with at least 10-point injection through the submaxillary and inguinal lymph nodes. The first four basic immune doses were 1, 2, 3 and 4 mg, with an interval of 21 days. The titer of neutralizing antibodies was determined 14 days after the last immunization. In scheme 2, purified viral antigen in phosphate buffered saline (PBS) was injected into the submaxillary and inguinal lymph nodes of horses at least 10 points. The first four basic immune doses were 5×10^7, 6×10^7, 7×10^7 and 8×10^7 PFU. The interval between vaccinations was 21 days, and the titer of neutralizing antibodies was determined by ELISA [8] 14 days after the last immunization (schema in S1 Fig).

Preparation and verification of $F(ab')_2$ products
The horse immunoglobulin $F(ab')_2$ product against smallpox virus was prepared in the GMP plant of Shanghai serum Biotechnology Co., Ltd. The protein concentration of immune plasma and $F(ab')_2$ was determined with Lowry assays [13]. The protein yield of the final product was calculated according to the initial volume of plasma and the final volume of $F(ab')_2$, and the purity of the final product $F(ab')_2$ was determined according to established product quality regulations [14].

Neutralization effect of horse anti smallpox virus immunoglobulin $F(ab')_2$ raw plasma and final product toward VVTT, on the basis of MTT assays
Vero cells were seeded in 96 well plates in 100 mL containing 2×10^4 cells/well, cultured at 37°C for 24 hours and grown into monolayers. The immunoglobulin $F(ab')_2$ of equine anti smallpox virus was diluted twice with DMEM; the initial titer was 1:800, with six gradients in total. VVTT at 1×10^5 PFU was diluted with serum-free DMEM and incubated at 37°C for 1 h, and 200 μL/well

was added to cells after the medium was removed. Each dilution was applied to four wells. The experimental groups comprised a normal cell control (without virus, without F(ab')$_2$); virus control (with virus, without F(ab')$_2$); and normal horse immunoglobulin control (with virus, with normal horse immunoglobulin). When cells in the virus control group were dead, MTT (0.25 mg/mL) was added at 200 mL/well for 4 h. The MTT was then aspirated, and termination solution (10% SDS + 0.01 mol/L HCl) at 200 ml/well was added and incubated for 8 h. The OD value was detected at 570 nm [15].

Preventive effect of equine anti smallpox immunoglobulin in animals

Twenty BALB/c mice were divided into five groups, with four mice per group. Four concentrations of anti-smallpox virus immunoglobulin were prepared through PBS dilution: 5.2, 2.6, 1.3 and 0.65 mg/mL. The same amount of normal saline was used as a control. Each mouse was administered 0.5 mL subcutaneously in the abdomen via a needle-free injection device (POK-V DART, Boker BioTe, China) according to the manufacturer's instructions. Six days later, VVTT at 1×10^6 PFU was injected intravenously. Mouse weight change, scab formation and death were observed. Peripheral blood was collected from the lateral tail vein on days 1, 3 and 6 after the viral challenge. Plaque assays were conducted as previously described to detect viral loads in peripheral blood.

Therapeutic effect of equine anti smallpox immunoglobulin in vivo

Twenty BALB/c mice were divided into five groups, with four mice per group. Each mouse was injected intravenously with 1×10^6 PFU VVTT. Six days later, each mouse was administered 0.5 mL via needle-free injection subcutaneously in the abdomen (5.2, 2.6, 1.3 or 0.65 mg/mL); the same amount of normal saline was used as a control. Peripheral blood was collected from the lateral tail vein on days 1, 3 and 6 after the immunoglobulin injection. Plaque assays [16] were conducted.

Safety evaluation

According to the requirements of the Chinese Pharmacopoeia, Joinn Laboratories (China) Co., Ltd. performed sterility testing, pyrogen testing, abnormal toxicity testing, general pharmacological testing, acute toxicity testing, immune toxicity testing, hemolysis testing and vascular stimulation testing on the final product of horse anti-smallpox virus immunoglobulin F(ab')$_2$.

Statistical analysis

Statistical analyses were performed in Prism 9.0. Data are presented as mean values $\pm$ SD or SEM. Two-way ANOVA for repeated measures was used to compare three or more groups. $p < 0.05$ was considered significant.

RESULTS

Production, purification and detection of VVTT

Transmission electron microscopy indicated that the purified virus conformed to the morphology of the vaccinia virus (Fig 1A). The purified virus was sterile and had no mycoplasma contamination or exogenous factors. The purity of the purified antigen was found to exceed 90% by HPLC (Fig 1B). The antigens for immunization met the standards of the Chinese Pharmacopoeia.

Yield and purity of the final product F(ab')$_2$

The F(ab')$_2$ product of horse anti-smallpox virus immunoglobulin was tested (Fig 2). The yield of the purified F(ab')$_2$ product was approximately 1.3%, and the purity exceeded 90%.

Determination of the neutralization effect of F(ab')$_2$ toward VVTT, according to MTT assays

The prepared horse anti-smallpox immunoglobulin product had good neutralizing antibody effects toward VVTT, and the neutralizing antibody titer was 1:3200 (Table 1).

Preventive effect of F(ab')$_2$ in animals

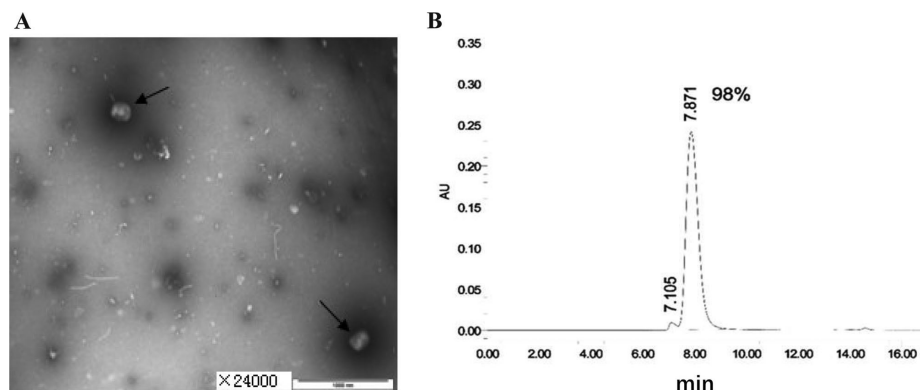Four groups of mice in the prevention experiment group were administered immunoglobulin F(ab')$_2$ via

FIGURE 1 | Detection results of purified antigens of vaccinia virus TianTan strain. (A) Transmission electron microscopy showing that the purified virus conformed to the morphology of vaccinia virus. (B) HPLC test results. The purity of the purified antigen was 98.4%.
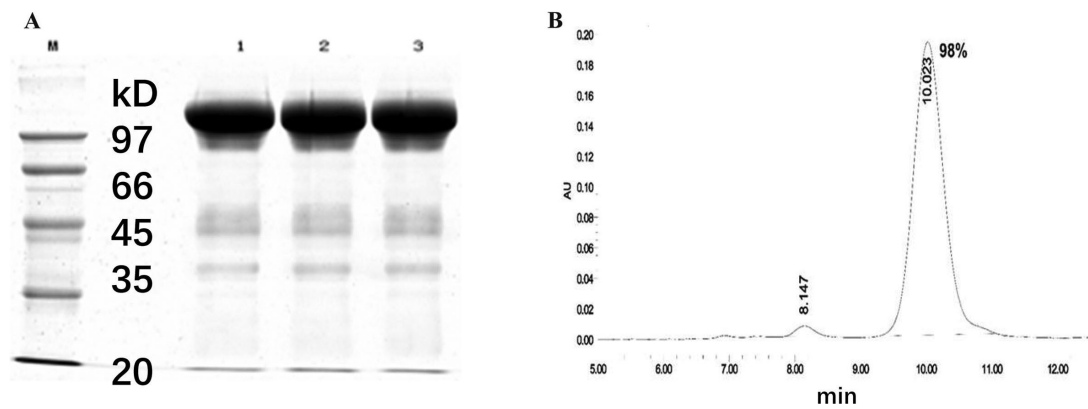
FIGURE 2 | Test results of horse anti smallpox virus immunoglobulin F(ab')$_2$. (A) SDS-PAGE of equine purified F(ab')$_2$. Lanes 1-3, purified F(ab')$_2$. (B) HPLC of purified F(ab')$_2$, with the F(ab')$_2$ peak indicated.

TABLE 1 | Neutralization effects of horse anti smallpox virus purified F(ab')$_2$ against vaccinia virus TianTan strain, according to MTT assays.

Antibody dilution	OD value (570 nm)	
	Purified equine F(ab')$_2$[a]	Normal horse immunoglobulin control[a]
1/800	1.684±0.568	0.726±0.220
1/1600	1.781±0.327	0.798±0.210
1/3200	1.521±0.507	0.640±0.295
1/6400	1.029±0.600	0.565±0.212
1/12800	0.960±0.493	0.653±0.282
1/25600	0.793±0.190	0.587±0.517
Normal cell control[b]	1.763±0.467	
Virus infected cell control[b]	0.663±0.374	

[a]The F(ab')$_2$ equine immunoglobulin against smallpox virus and a normal horse immunoglobulin control (with virus, with normal horse immunoglobulin) were both diluted twice with DMEM. The initial titer was 1:800, with six gradients in total. The vaccinia virus TianTan strain at 10^5 PFU was diluted with serum-free DMEM, and then 200 mL/well was successively added to wells after the medium was removed. Each dilution was applied to four wells.
[b]Normal cell control (without virus, without F(ab')$_2$), virus control (with virus, without F(ab')$_2$).
When the virus was in control cell lesion, MTT (0.25 mg/mL) at 200 mL/well was added and incubated for 4 h. The MTT was then aspirated, and termination solution (10% SDS + 0.01 mol/L HCl) at 200 mL/well was added and incubated for 8 h. The OD value at 570 nm was determined with a plate reader.

needle-free injection. Mice in the antibody dose groups of 5.2 mg/mL and 2.6 mg/mL showed the lowest body weights on the 6th day after VVTT challenge, and their weights subsequently gradually increased to normal. Mice in the antibody dose groups of 1.3 mg/mL and 0.65 mg/mL showed a decrease in weight and a subsequent increase, but it was not clear (Fig 3A). The horse anti–smallpox virus immunoglobulin had good preventive effects in mice. In the PBS control group, the mice began to show pockmark on their limbs on the 6th day after the VVTT challenge, which spread to the entire body by the 8th day, and were associated with poor life status. At the end of the experiment, no body weight recovery or scab disappearance was observed. As shown in Fig 3B, the viral titer in the peripheral blood in mice in the model group was significantly higher after the challenge. In contrast, the viral loads were suppressed in mice in the prevention group, and higher dose groups (5.2 and 2.6 mg/mL) showed diminished viral loads, by 2–3 fold. Viral proliferation was significantly inhibited in the higher dose group.

Therapeutic effect of equine anti smallpox immunoglobulin F(ab')$_2$ in animals
On the 6th day after intravenous injection of VVTT, the bodies of the five groups of mice began to develop pox, and the four groups of mice injected with immunoglobulin F(ab')$_2$ showed weight changes. The weights of mice in the experimental group after injection of immunoglobulin F(ab')$_2$ at 5.2 mg/mL, 2.6 mg/mL or 1.3 mg/mL slowly recovered to baseline levels, whereas mice with 0.65 mg/mL treatment recovered slowly until the 8th day after injection of immunoglobulin F(ab')$_2$ and showed
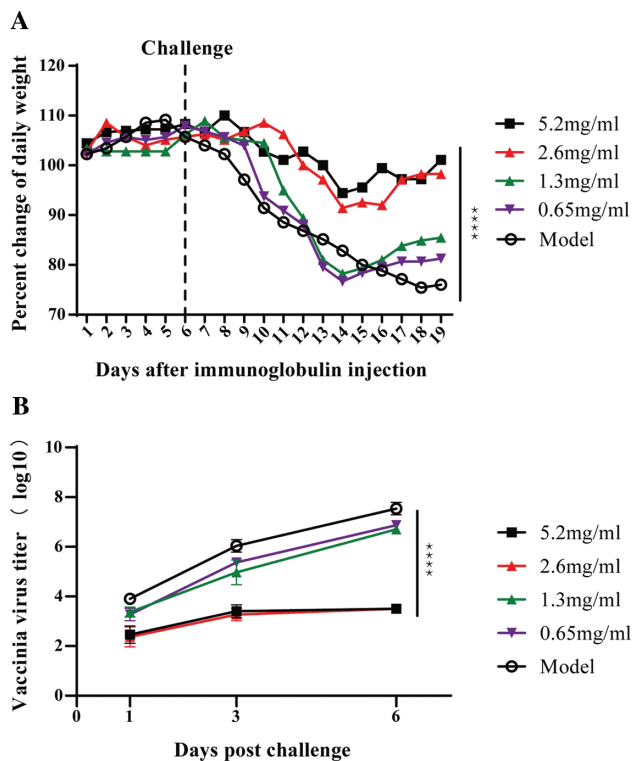
FIGURE 3 | (A) Change in average body weight in mice treated with horse anti smallpox virus immunoglobulin F(ab')₂ in the prevention experiment. Four concentrations of horse anti-smallpox virus immunoglobulin were prepared by PBS dilution: 5.2, 2.6, 1.3 and 0.65 mg/mL. The same amount of normal saline was used as a control. After each mouse was administered 0.5 mL subcutaneously with a needle-free device, it was challenged intravenously with VVTT at 1×10^6 PFU. The weight changes in mice are shown. (B) Determination of viral load in mouse peripheral blood at various time points post challenge. Results expressed as mean ± standard; *$p < 0.05$; ***$p < 0.001$; and ****$p < 0.0001$.

FIGURE 4 | (A) Changes in average body weight in mice treated with horse anti smallpox virus immunoglobulin F(ab')₂. Twenty BALB/c mice were divided into five groups. Each mouse was injected intravenously with 1×10^6 PFU of VVTT and observed for 1 week. Four concentrations of horse anti smallpox virus immunoglobulin were prepared by PBS dilution (5.2, 2.6, 1.3 and 0.65 mg/mL). Each mouse was injected with 0.5 mL subcutaneously with a needle-free device, and the same amount of normal saline was used as a control. The weight changes in mice are shown. (B) Determination of viral loads in mouse peripheral blood at various time points after treatment. Results expressed as mean ± standard; *$p < 0.05$; ***$p < 0.001$; and ****$p < 0.0001$.

some scabs. However, mice in the PBS group began to show scabs on their limbs on the 6th day after the VVTT challenge, which spread to the entire the body by the 8th day, and were associated with poor life status. At the end of the experiment, neither weight recovery nor scab formation had disappeared (Fig 4A). As shown in Fig 4B, the viral loads of the treated mice were suppressed to varying degrees with respect to that in the control group. The decrease was more significant in the high-dose group.

DISCUSSION

Smallpox is one of the most infectious and pathogenic diseases worldwide. Because the smallpox virus has high infectivity and lethality, and countries have stopped vaccinating populations, humans have lost the protection of smallpox virus immunity; therefore, the smallpox virus has become a powerful biological weapon [6]. At present, there are two places worldwide: the FDA/US Centers for Disease Prevention and Control in Atlanta and the Russian National Center for Virology and Biotechnology Research [7,8]. In recent years, safety accidents have
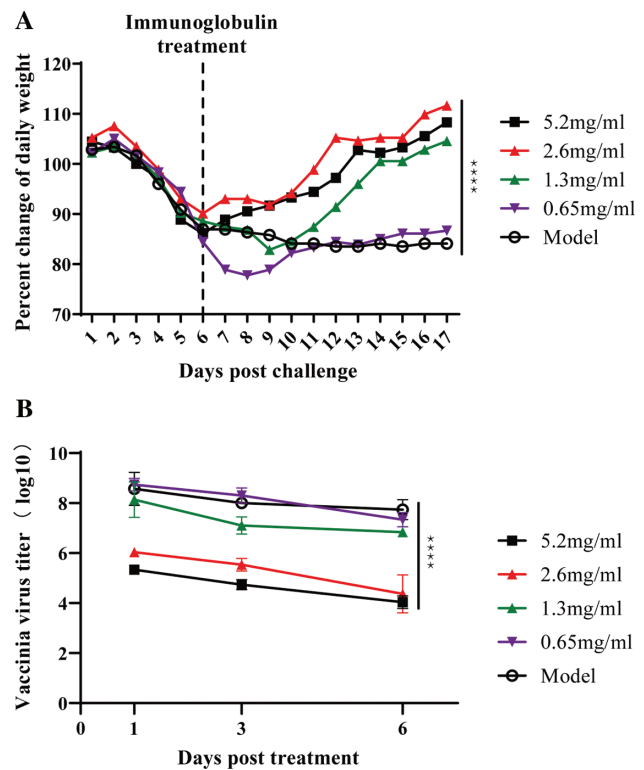
occurred in research centers, thus prompting concerns regarding biosafety. Although a chemosynthetic drug, cidofovir, is available for patients with smallpox, its efficacy is limited, and its adverse effects are strong. To date, no specific drug for smallpox is available. In view of the current biosafety situation, the United States approved a non-replicating smallpox vaccine in 2019, in case of accidents. However, most people in China do not have immunity to smallpox. With the increase in overseas interests and the expansion of foreign exchange, the smallpox virus and similar monkeypox virus have become potential threats to biological security.

Therefore, studying specific drugs for smallpox has become an urgent and important social need. In the struggle against infectious diseases in human history, one of the most effective and economical mitigation means has been passive immunotherapy with antiserum, which remains critical in the treatment and prevention of important infectious diseases. The advantages of traditional horse serum are that 1. the large-scale production process is established, simple and convenient; 2. the antibody is cleaved by a gastric enzyme and retains the F(ab')₂ functional group, thus

removing most of the IgG molecule and Fc fragments that cause adverse effects, and yielding a product with high stability and affinity; and 3. the heterogeneity of horse serum to humans is weaker than that of other animals. Its disadvantages are low purity of immunoglobulin $F(ab')_2$ and a 1% rate of adverse effects [17].

The Beijing Institute of Microbiology and Epidemiology and Shanghai Serum Biotechnology Co., Ltd. have successfully developed anti-smallpox immuno-globulin $F(ab')_2$ products with notable advantages. The purity of the final products can exceed 90%, and the rate of adverse effects can be decreased from approximately 1% to <0.01% [18].

The core of therapeutic antibody research and development involves the selection and preparation of immune antigens. VVTT is a virus isolated from the blister scabs of smallpox patients in 1926, obtained by successive generations of virus reduction. The use of VVTT has eradicated smallpox in China, thus indicating an immunoprotective effect against the smallpox virus. The technology for large-scale culture, purification and identification of VVTT is well developed. We chose chicken embryo fibroblasts to culture the smallpox virus. After density gradient centrifugation and chromatography purification, we prepared qualified antigens, whose purity exceeded 80%. The morphology conformed to the characteristics of the smallpox virus and met the requirements of the Chinese Pharmacopoeia for antigens for immunization.

We initially chose two programs: live virus immunization or inactivated vaccine plus adjuvant immunization of healthy horses. Although both can achieve immune effects, considering the biological safety concerns and high cost of the live virus, we finally chose the inactivated vaccine plus adjuvant program.

The bottleneck in the development of products for the prevention and control of smallpox virus and other virulent pathogens relies on high biosafety level laboratories and smallpox virus. With VVTT, we established two methods to overcome this bottleneck: a cell infection model and an animal infection model. Although the two models cannot completely replace the model established by the smallpox virus, considering that the immune protection mechanism is the same, they may serve as an important and useful reference [19].

This study innovatively used a needle-free injection device for immunoglobulin delivery, which elicited the same immunoprotective effects as needle-based injection. The needle-free injection device is simple, fast and portable, and is suitable for use in a wide range of vaccinations and battlefield self-help medical systems. In addition, it avoids the pain and needle-phobia caused by needle injection [20].

In summary, we successfully prepared equine anti-smallpox immunoglobulin $F(ab')_2$ products by using current modern biopharmaceutical technology. This product is expected to become a life-saving drug against the smallpox virus. Clinical approval has been obtained, and intensive clinical trials are in progress.

CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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