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Original Article

A single dose of recombinant adenoviral vector rabies vaccine expressing two copies of glycoprotein protects mice from lethal virus challenge

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Abstract

Introduction: Rabies is a fatal infectious disease, that poses a major public health threat in developing countries. With an annual death toll of approximately 59,000, more than half of which are children, an urgent need exists for a safe, affordable, and effective preventive measure against rabies virus infection.

Methodology: A recombinant rabies vaccine called Ad5-dRVG was constructed by introducing two copies of the rabies virus glycoprotein into a human adenoviral vector. Virus-neutralizing assays and virus challenge experiments were employed to evaluate the Ad5-dRVG vaccine.

Results: Our findings demonstrate that a single dose of Ad5-dRVG, administered either intramuscularly or orally, elicited significantly stronger immune responses than Ad5-RVG. Moreover, both vaccines provided complete protection in mice. Notably, the vaccine exhibited remarkable efficacy even at low doses, suggesting potential cost reduction in production.

Conclusions: The development of the Ad5-dRVG recombinant rabies vaccine represents a significant advancement in rabies prevention. Its enhanced immunogenicity, demonstrated efficacy and potential cost savings make it a promising candidate for widespread use.

Key words: Rabies; glycoprotein; adenovirus; recombinant rabies vaccine.

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Introduction

Rabies, a fatal neurological disease, is caused by the rabies virus. The virus can infect different species of mammals including humans, livestock, and wild animals. Its virion structure consists of an enveloped, bullet-shaped morphology. The viral genome is composed of a negative-sense single-stranded RNA, encoding five distinct viral proteins: nucleoprotein, phosphoprotein, matrix protein, glycoprotein, and RNA-dependent RNA polymerase. Out of these proteins, the glycoprotein is the only one exposed to the surface of the virion particle and is demonstrated to be the key element of immunogenicity, making it the most pivotal immunogen of rabies vaccines [1]. Once the rabies virus enters the body, it infects the peripheral nerves and subsequently migrates into the central nervous system of the brain, causing fatal encephalitis and myelitis. Infected individuals experience a range of symptoms, starting with flu-like manifestations during the incubation period. As the disease progresses, severe neurological symptoms arise due to the progressive nature of the encephalomyelitis [2]. Tragically, these symptoms ultimately lead to the death of the infected individuals. The incubation period of rabies typically spans approximately 2-3 months, although it can vary considerably, ranging from as brief as 1 week to as long as 1 year. The duration depends on factors such as the site of virus entry and the viral load. Unfortunately, once symptoms appear, death typically occurs within a narrow timeframe. For individuals with the furious or paralytic forms of rabies, the average survival time after the onset of symptoms is usually between 6 and 11 days. This leaves very little time for effective interventions or therapeutic options, making treatment options extremely limited [3]. Once clinical symptoms appear, this disease is virtually 100% fatal [4]. This neglected zoonosis causes the death of an estimated 59,000 people each year, with more than half of them in children. The burden of rabies is closely associated with the level of the sociodemographic index [5]. Significant progress has been made globally in terms of social and economic development, leading to a notable decline in both the incidence and mortality rates of rabies. Nonetheless, in specific regions characterized by lower and middle-income levels, such as Asia and Africa, rabies remains a persistent and grave threat to human life. In 2019, India recorded the highest number of rabies-related fatalities, with over 5,200 reported cases. Following

India, Nigeria ranked third with 1,295 cases, and Pakistan ranked fourth with 1,198 cases. Ethiopia secured the fifth position, reporting 921 cases, while China stood at sixth place with 719 cases. Remarkably, only 34 countries worldwide have achieved the elimination of rabies by implementing effective vaccination strategies [5,6].

Vaccination is strongly recommended for individuals at high risk of exposure or those who have been exposed to the rabies virus. According to the WHO, over 29 million people worldwide receive post-bite vaccinations. This is estimated to prevent hundreds of thousands of rabies-related deaths annually. The most widely used rabies vaccines are purified cell culture and embryonated egg-based vaccines [6]. From a geographical standpoint, 85% of supply is concentrated in two countries – China and India – with the largest manufacturers mostly producing cell culture vaccines. However, the high cost and required repeated vaccination seriously hinder the use and acceptance of the current rabies vaccine in developing countries [7]. Managing rabies exposure, where the average cost of rabies post-exposure prophylaxis (PEP) is currently estimated at an average of US$ 108 (along with travel costs and loss of income) can be a catastrophic financial burden on affected families whose average daily income may be as low as US$ 1–2 per person [8]. Globally, the economic burden of dog-mediated rabies is estimated at US$ 8.6 billion per year. Thus, the development of novel affordable, safe, and effective vaccines becomes a top priority for rabies control in developing areas.

The inactivated vaccine is a well-established technology that has significantly improved the safety of vaccines. However, it does have limitations in terms of its ability to generate robust and long-lasting immune responses. The low cost of attenuated vaccines makes them an attractive vaccination principle, but such vaccines require the availability of an attenuated strain. Moreover, safety concerns exist as they may pose risks to individuals with compromised immune systems and can potentially revert to wild-type virulence, a safer approach is still desired [9]. While PEP is commonly used to control rabies in many countries, the optimal strategy would involve implementing pre-exposure prophylaxis (PrEP) targeting high-risk groups and conducting vaccination for domestic dogs and wild animals, which may carry the rabies virus. In such cases, an orally delivered vaccine has the potential to be widely accepted in the rabies PrEP campaign or formulated as bait to control rabies in wild animals. The development of alternative vaccine technologies is crucial to address the prohibitive cost of currently available cell-culture-derived vaccines [10], prevent vaccine supply shortages [11], and facilitate vaccine distribution.

Virus-vectored vaccines have emerged as a highly versatile category of vaccines that address the needs mentioned earlier. Different vector types have been explored for vaccination purposes, including influenza virus, lentivirus, adeno associated virus, yellow fever virus, etc. [12–15]. However, none of these have been deemed ideal for vaccine use due to suboptimal characteristics such as genetic stability, safety, immunogenicity, cloning capacity, or production convenience and cost. Adenovirus (Ad) and poxvirus vectors have shown remarkable potential to become the gold standard of virus vector vaccines [16].

Viral vector-based rabies vaccines have shown promise for both veterinary and human use [7,17]. A recombinant vaccinia virus expressing the glycoprotein of the rabies virus was tested as an oral rabies vaccine [18]. This vaccine is formulated as oral bait and has demonstrated the ability to induce protective immunity in several wild animals. However, it has been associated with the risk of infection and allergies in humans (Centers for Disease Control Prevention, 2013). Adenovirus, another popular vaccine vector, has been widely utilized in vaccine development for various diseases due to its ability to stimulate robust cellular, humoral, and mucosal immune responses in both animals and humans. Replication-competent human adenovirus type 5 (Ad5) expressing rabies glycoprotein, known as AdRG1.3 or ONRAB, was developed as an oral vaccine bait by Artemis Technologies Inc (Guelph, Canada) [19]. Since 2006, several million doses of ONRAB have been distributed in Canada and have shown encouraging results in field efficacy assessment conducted on skunks and raccoons, without causing significant human contact or public safety concerns [20,21].

In previous studies, it has been observed that E1-deleted replication-deficient adenovirus exhibits improved safety and efficacy compared to replication-competent adenovirus [22]. Ad5 has been widely used in vaccine research, including vaccines that have been approved for human use during the COVID-19 pandemic, such as those developed by CanSino Biologics in China. Rabies vaccine based on E1-deleted Ad5 has been developed and yielded promising results in rodents and canines [23]. Additionally, a chimpanzee adenovirus vector rabies vaccine was developed and tested in beagles, followed by human trials to assess safety and efficacy [24,25].

In this study, a novel recombinant rabies vaccine was generated by inserting two copies of rabies virus glycoprotein into a human serotype 5 adenovirus vector and evaluated the immunogenicity and protective capacity of the vaccines. The results confirmed that the Ad5-dRVG vaccine significantly improved rabies glycoprotein expression in vitro. Furthermore, both intramuscular and oral administration of the vaccine induced virus neutralizing antibody responses in mice that exceeded the threshold of 0.5 IU/mL established by the WHO. These findings indicate that Ad5-dRVG is a promising candidate for a safe and effective rabies vaccine against rabies infection.

Methodology

Vaccines, cells, and viruses

The human serotype 5 adenovirus genome (NCBI Reference Sequence: AC_000008.1) was synthesized at Tsingke Biotechnology Co., Ltd. (Chengdu, China). E1 and E3 regions were deleted (deletion of the E1 region was from bp 560 to 4031, E3 region from 27858 to 30839). The replication-deficient recombinant Ad5 vector was used to construct recombinant Ad5 vaccines encoding codon-optimized full-length RVG or dRVG of Rabies CVS strain (GenBank accession number AJ506997). The transgene cassette of full-length RVG and dRVG has been inserted by homologous recombination in the E1 region. The human CMV promoter drives the transcription of the transgene, and the bovine growth hormone (BGH) poly(A) sequence is downstream to the transgene stop codon, forming a recombinant adenovirus genome. These recombinant adenovirus genomes were linearized and transfected into HEK293A cells (Thermo Fisher Scientific, MA, USA) to rescue the recombinant adenovirus, which was further propagated and purified by chromatography using POROS™ 50 HQ anion exchange resin (Thermo Fisher Scientific, MA, USA) and Capto Core 700 multimodal chromatography resin (Cytiva, MA, USA) refers to the user's manual. Viral particle (vp) measurements of adenovirus stocks were conducted using 260-nm absorbance [26]. HEK293A cells were maintained in complete Dulbecco's modified Eagle's medium (Cytiva, MA, USA) supplemented with 10% fetal bovine serum (HyClone, Utah, USA) and cultured at 37 °C and 5% CO_2. BHK-21 cells (ATCC CCL-10) were grown in an EMEM medium (Thermo Fisher Scientific, MA, USA), supplemented with 10% fetal bovine serum. Rabies virus strain CVS-11 (GenBank accession number AJ506997) was propagated and maintained in mouse brains at Chengdu Kanghua Biological Products Co. Ltd.

Western blotting

HEK293A cells were pre-plated in 6-well plate, followed by infected with 1×10^9 vp of Ad5-RVG or Ad5-dRVG. Forty-eight hours post-infection, cells were lysed, and supernatants and cell pellets were collected. Protein samples were separated by 12% SDS-PAGE and analyzed by Western blotting with rabbit anti-G of rabies virus polyclonal antibody. Goat anti-mouse IgG-horseradish peroxidase (HRP) antibodies were used as secondary antibodies. The membranes were developed by ECL chemiluminescent substrate (Thermo Fisher Scientific, MA, USA).

Mice and Immunization

Female BALB/c mice at 6 to 8 weeks of age were randomly divided into 5 groups (n = 16). Groups 1 and 2 were immunized intramuscularly (i.m.), and groups 3 and 4 were immunized orally (i.o.), group 5 serves as control and was orally instilled with Ad5-empty. Ad5-RVG and Ad5-dRVG were diluted in PBS. Mice were immunized with 10^{10} vp (100 μL) of vaccine or sham vaccine through i.m. or i.o. route.

For i.o. administration, vaccines were concentrated to 20 μL and mixed with 20 μL RedDog nourishing cream (Guangdong, China) and then fed to the animals. Extra food and water were prohibited for the next 2 hours after feeding. The sera were collected as indicated in Figure 2A.

Challenge

On day 60 after vaccination, the animals were challenged with 20 □L of 50 folds of the 50% lethal dose of RABV (LD_{50}) by i.m. inoculation. Rabies virus strain CVS-11 was used as the challenge virus and diluted with sterile phosphate-buffered saline. Mice were observed daily for signs of disease or death until 30 days after the challenge. All animal experiments with rabies virus challenge were conducted under a biosafety level 2 (BSL2) facility at Sichuan University (Chengdu, China).

Virus neutralization assay

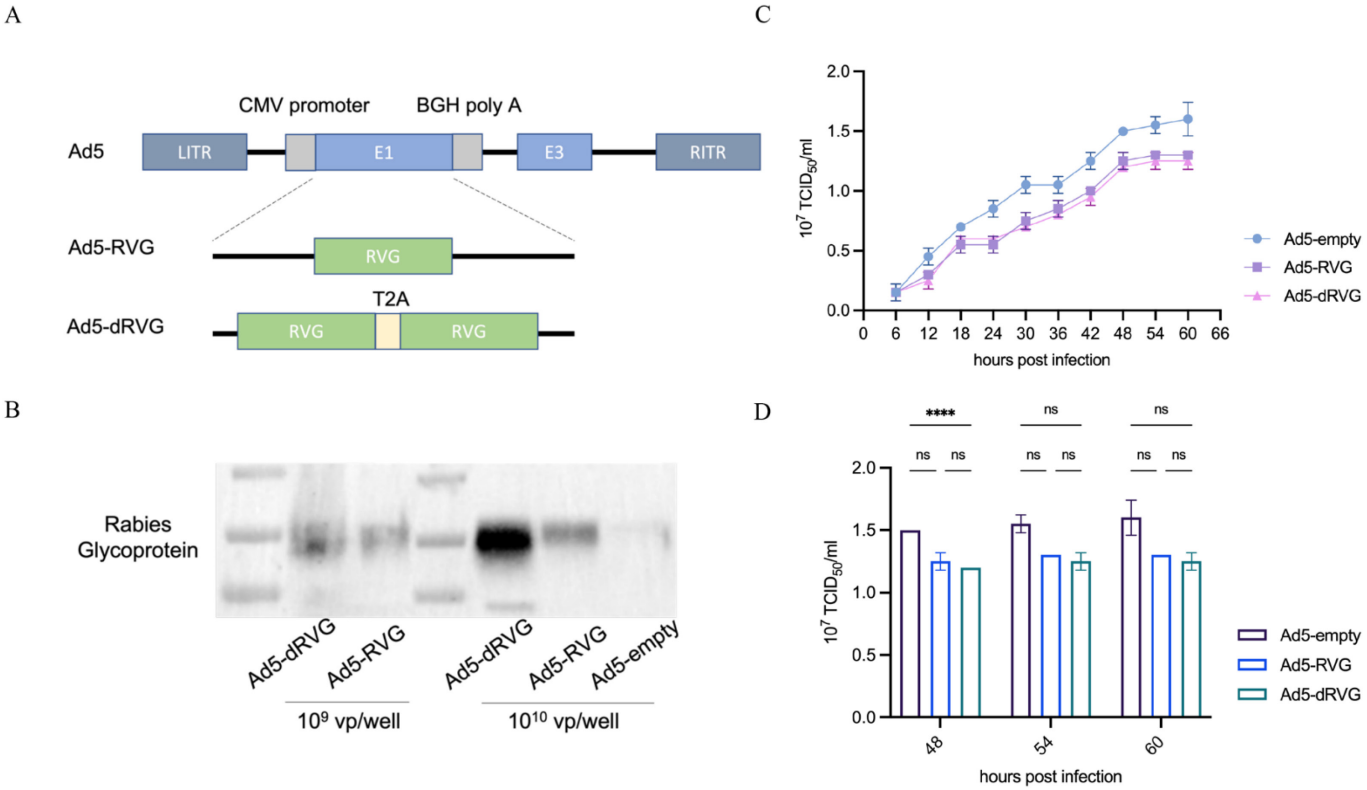
Rabies virus neutralizing antibodies (RVNA) were measured using a validated, WHO-approved test, fluorescent antibody virus neutralization (FAVN) test as previously described [27]. Briefly, 3-fold serial dilutions of standard serum (0.5 IU/mL) and test serum samples were prepared in quadruplicate in a multi-well plate and mixed with 100 $TCID_{50}$ (50 μL) of CVS-11. After incubation at 37 °C in a humidified 5%, CO_2 incubator for 1 hour, a 50 μL suspension containing 2×10^4 BHK-21 cells was added, and the incubation was

continued for 48 hours. The cells were fixed at 4 °C by treatment with 80% acetone for 30 min and stained with FITC-labeled anti-RABV-N monoclonal antibodies (Veterinary Research Institute, Changchun, China). Fluorescence was observed using ultraviolet microscopy, and the RVNA titers were calculated using the Spearman-Karber formula. According to guidelines from the World Health Organization, an RVNA titer of 0.5 IU/mL is adequate to provide full protection.

ELISPOT assay

To detect antigen-specific T lymphocyte responses, rabies-specific T cell responses in mice splenocytes were determined by a standard IFN-γ ELISPOT assay. Spleens of vaccinated BALB/c mice were harvested at 2 weeks post immunization and splenocytes were isolated. Flat-bottom, 96-well plates were precoated with 10 μg/mL anti-mouse IFN-γ Ab (BD Biosciences, USA) overnight at 4 °C and then blocked for 2 hours at 37 °C. After blocking the plates, mouse splenocytes were plated in duplicate at 400,000 cells per well. Cells were stimulated overnight with 2 μg/mL final concentration of RVG peptide pools consisting of 15-mer sequences with 11-amino acid overlaps to cover the sequence of rabies glycoprotein. Phytohemagglutinin (PHA) (ChemGen, Shanghai, China) was added as a positive control. Cells incubated without stimulation were employed as a negative control. After 24 hours of incubation, plates were developed with biotinylated anti-mouse IFN-γ antibody, conjugated streptavidin–alkaline phosphatase (BD Biosciences, San Jose, CA) and with 1-Step NBT/BCIP solution (Thermo Fisher Scientific, Rockford, IL). When the colored spots were intense enough to be visually observed, the development was stopped by thoroughly rinsing samples with deionized water. The numbers of the spots were determined using an automatic ELISPOT reader and image analysis software (Cellular Technology Ltd.). A positive ELISPOT response was at least 50 specific spots/million splenocytes on at least one peptide pool and three times the number detected in the negative control wells.

Figure 1. Construction and characterization of Ad5 vector vaccines.



(A) Schematic demonstration of antigen constructs of full-length RVG and dRVG. LITR, left inverted terminal repeat; RITR, right inverted terminal repeat; T2A, *Thosea asigna* virus 2A self-cleaving peptides. (B) Western blot analysis of rabies glycoprotein on lysates of 293T cells infected with 10^9 and 10^{10} viral particles (vp) of Ad5-RVG and Ad5-dRVG. 48 hours after infection cells were harvested and 20 mg of total cell lysates were used for WB analysis. 10^{10} vp of Ad5 vector infected cell lysates were used as the negative control, and GAPDH was used as a loading control. (C) 10^{10} vp of Ad5-RVG or Ad5-dRVG vaccines were inoculated into HEK293A cells, cell cultures were collected at different time points post infection, data are 10^7 $TCID_{50}$/mL. (D) virus titers were analyzed at 48, 54 and 60 hours post infection. *p* values were analyzed with t-test (ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$).

Statistical analysis

Data are expressed as the means ± standard errors of the means (SEM). For all analyses, *p* values were analyzed with one-way or two-way ANOVA with multiple comparisons or unpaired t-tests. All graphs were generated with GraphPad Prism software v9.1.1 (GraphPad, CA, USA).

Results

In the study, two replication-incompetent adenovirus vector-based vaccines were created, each carrying a different number of copies of the full-length glycoprotein of the rabies virus. The first vaccine, designated as Ad5-RVG, contained one copy of the glycoprotein, while the second vaccine, designated as Ad5-dRVG, carried two copies of the glycoprotein. These vaccines were developed and utilized for further evaluation of their immunogenicity and protective capacity against rabies infection (Figure 1A). The expression cassettes were inserted into the E1 region of an Ad5 vector with E1 and E3 deletion. Western blot was performed to confirm antigen expression in HEK293T cells infected with the recombinant adenovirus. Results show that all these antigens were detected in the cell lysates, where the antigen expression of Ad5-dRVG was significantly higher than Ad5-RVG (Figure 1B). Virus titer was measured every 6 hours after inoculating 10^{10} viral particles of the vaccine in 6 well plates. Data show virus titer reached its highest-level 48~60 hours after infection, the insertion of the transgenes doesn't significantly affect virus yield (Figure 1C, D). These results indicate the recombinant adenovirus was successfully constructed and the expression of rabies glycoprotein was consistent with our predictions. The Ad5-dRVG construct is potentially an optimal candidate for adenovirus vector rabies vaccine due to the potential advantage of requiring a lower vaccine dose, as the presence of multiple copies of the glycoprotein in the construct could enhance the immune response.

Table 1. Animal grouping and immunization. Groups of female BALB/c mice (n = 16) were immunized with Ad5-RVG, Ad5-dRVG, or Ad5-empty vaccines by intramuscular injection or oral feed.

Group	Animal no.	Vaccine	Dose/route
1	16	Ad5-RVG	10^{10} vp i.m
2	16	Ad5-dRVG	10^{10} vp i.m
3	16	Ad5-RVG	10^{10} vp i.o
4	16	Ad5-dRVG	10^{10} vp i.o
5	16	Ad5-empty	10^{10} vp i.m

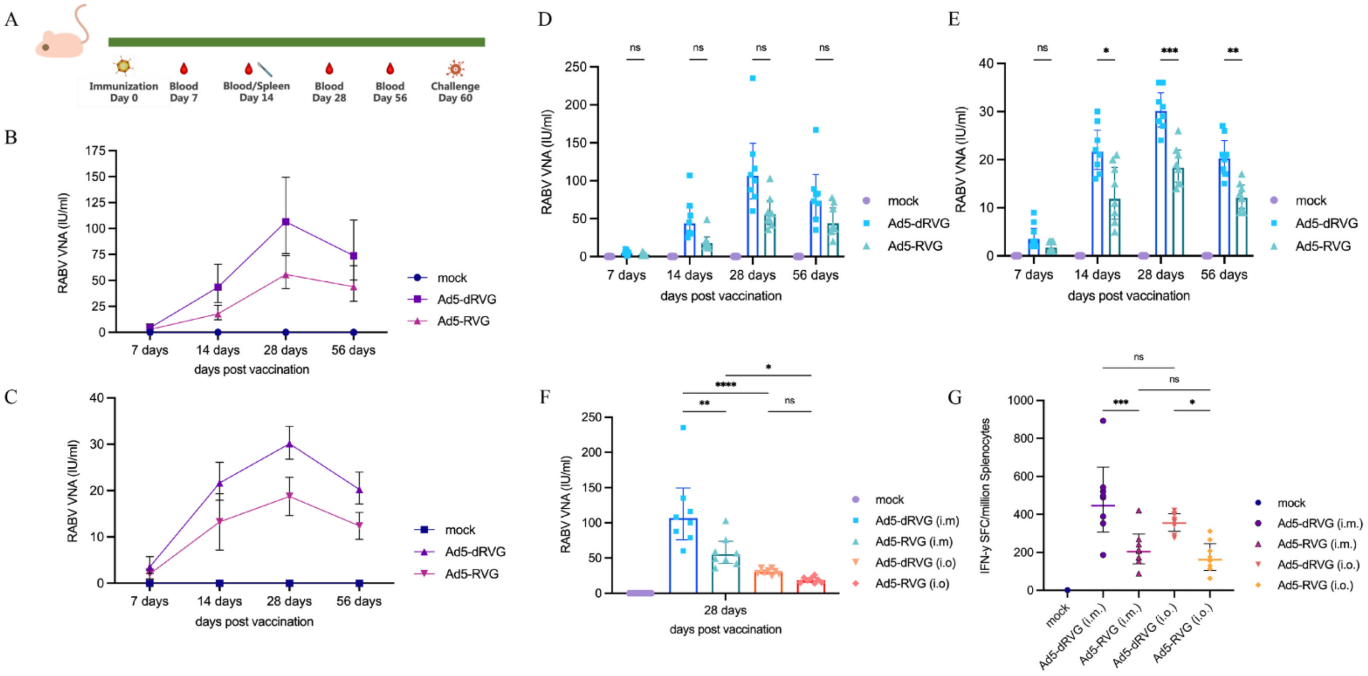


Figure 2. (A) Schedule of animal immunization, blood, and challenge. Female BALB/c mice (n = 16/group) were immunized with one dose of 1×10^{10} vp of Ad5 vaccines through the i.m or i.o route, respectively. Sera from half of the mice were collected on days 7, 14, 28, and 56 post immunization. Spleens from half of the mice were collected two weeks post immunization. Rabies virus challenge experiment was performed on day 60 post immunization. (B-F) RVNA kinetics upon single vaccination with Ad5-RVG and Ad5-dRVG. Female BALB/c mice (n = 8/group) were primed at day 0 and were bled on day 7, 14, 28, and 56. Serum from each animal was tested for detection of rabies neutralizing antibody by FAVN method and expressed as IU/mL. Data are shown as group means ± SEM. (B, D) i.m vaccination humoral response. (C, E) i.o vaccination humoral response. (F) Humoral immune response on day 28. (G) Characterization of the cellular immune responses. BALB/c mice were immunized with 1×10^{10} vp Ad5-RVG, Ad5-dRVG, or mock (Ad5-empty). Mice splenocytes were isolated and analyzed by ELISPOT assays to evaluate the IFN-γ secretion of splenocytes after rabies G peptides stimulation. Data are means ± SEM. *p* values were analyzed with t-test (ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

To evaluate the immunogenicity of the recombinant vaccines, groups of BALB/c mice (n = 16/group) were immunized with 1×10^{10} vp of Ad5-RVG and Ad5-dRVG vaccines via intramuscular (i.m) injection or intra-oral feed (i.o) (Table 1). Ad5-empty was instilled into mice mouths as the control group. Blood samples were collected on days 7, 14, 28, and 56 post-immunizations (Figure 2A). The serum-neutralizing endpoint titers against rabies virus were measured by FAVN method. Half of the animals (8 mice in each group) were sacrificed on day 14 after vaccination and the T-cell responses were measured by IFN-γ ELISPOT.

Humoral immune responses

In the intramuscular immunization group mice, mice sera were collected on days 0, 7, 14, 28, and 56 to measure the neutralizing antibody against rabies virus (RVNA). The RVNA can be detected as soon as 7 days post-immunization and continued to increase until day 28 in vaccine groups. 28 days after immunization with the vaccines, both the Ad5-RVG and Ad5-dRVG groups elicited strong neutralizing antibodies against rabies (Figure 2B) (GMT 106.5 IU/mL for Ad5-dRVG group and 55.8 IU/mL for Ad5-RVG group). Rabies neutralization antibody responses continue to maintain at a high level during the whole investigation window.

The neutralizing antibody response showed a similar pattern in i.o groups, while the overall antibody level was lower than in the i.m groups. RVNA reached its peak on day 28 in both vaccine groups (Figure 2C) (GMT 30.1 IU/mL for Ad5-dRVG group and 18.4 IU/mL for Ad5-RVG group).

In the intramuscular groups, neutralizing antibody titers elicited by the two vaccines are not significantly different, while in i.o groups, antibody titer induced by Ad5-dRVG is significantly higher when compared with the Ad5-RVG group (Figure 2D, E). When comparing the peak RVNA in the groups, intramuscular Ad5-dRVG stands out dramatically, Ad5-dRVG and Ad5-RVG induced similar humoral immune responses in i.o. groups (Figure 2F).

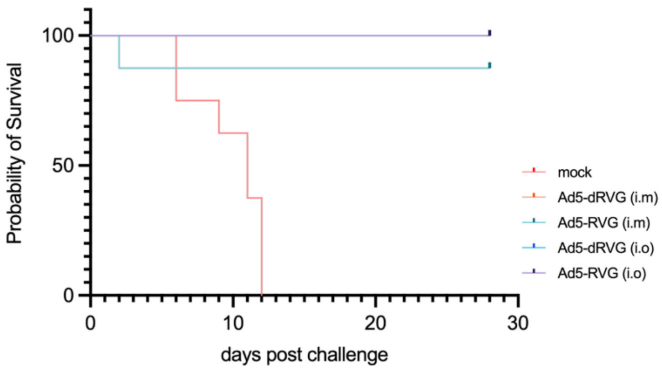
Cellular immune responses

To characterize the cellular immune responses induced by the Ad5-dRVG vaccine, three groups of immunized BALB/c mice were euthanized on day 14. Splenic lymphocytes were harvested, stimulated with an overlapping 15-mer peptide pool spanning the rabies virus glycoprotein, and analyzed by IFN-γ enzyme-linked immunospot (ELISPOT). ELISPOT analysis revealed the robust T cell responses for mice vaccinated with vaccines via the i.m. or i.o. route (Figure 2G). Intramuscular and oral routes induced similar cellular immune responses, and Ad5-dRVG vaccine induced significantly stronger cellular immune responses than Ad5-RVG vaccine.

Survival after challenge

BALB/c mouse is a proper model for rabies virus infection. On day 60, the mice were challenged with lethal rabies challenge virus standard strain for further protection efficacy evaluation. The survival ratio was monitored daily until 28 days after the virus challenge. All the mice in the placebo group appeared with rabid symptoms after 3 days and were all dead on the 14th day after the challenge (mice were euthanized the day rabies-associated symptoms appeared). In the i.m groups, all the mice in vaccine groups remained uninfected, except one in the Ad5-RVG group died of non-rabies reasons. In the oral groups, all the mice survived during the whole monitoring period (Figure 3).

Figure 3. Protective efficacy of AdC5 vaccines.



Female BALB/c mice (n = 8/group) received one dose of AdC5-RVG or Ad5-dRVG or sham vaccine via the i.m or i.o route. 60 days post vaccination, mice were challenged with $50LD_{50}$ of Rabies CVS-11. Animals were monitored daily for survival. Data are survival percentages.

Discussion

The complete elimination of rabies from human society is unlikely to occur quickly due to the presence of lyssaviruses in numerous wild animals. The WHO is committed to intensify efforts towards the elimination of dog-mediated human rabies by 2030, a goal that has been achieved in several regions, including developing and developed regions [28,29]. Vaccination of pet dogs and wild animals along with PrEP for people who live in rabies-prevalent areas are effective means of avoiding human rabies. Nevertheless, the current inactivated rabies vaccines for PrEP are not cost-effective in these areas due to their low-income status. Additionally, conducting mass vaccination campaigns

for wild animals presents logistical challenges and feasibility concerns [30]. The development of cheaper and more immunogenic vaccines that are stable and can be administered without injection becomes necessary.

Adenovirus vector vaccines are a genetic vaccine format that may address the shortcomings of current rabies vaccine technologies. Benefits are the induction of balanced and enduring immunity as demonstrated for anti-tumor and prophylactic vaccination and storage at elevated temperatures [31,32]. In addition, it is a consensus that production for adenovirus vaccines is cost-effective and easy to scale up.

Ad5 belongs to subgroup C adenoviruses and is an important tool for vaccine development, which allows for high yield production in HEK 293 cell lines containing the E1 region, thus being an ideal vaccine platform for neglected diseases. An E1-deleted replication-deficient Ad5 vector expressing rabies virus glycoprotein was initially developed as a rabies vaccine, which has been tested in mice via different administered route and could provide full protection at a low dose against lethal challenge of rabies virus [33,34]. It has also been tested in non-human primates and a small group of people, whereby a single immunization with a moderate dose of this vaccine resulted in sustained titers of rabies virus neutralizing antibodies [35].

To improve vaccine efficacy and further reduce the production cost, an E1/E3-deleted Ad5 vector expressing two copies of glycoprotein of rabies virus was generated to enhance antigen expression. In this study, we conducted a comparison of the immune response and protective efficacy between two vaccines, Ad5-RVG and Ad5-dRVG, using different immunization routes. In both i.o and i.m animal groups of the two vaccines, humoral and cellular responses to rabies virus were effectively elicited. High levels of antibody could be retained for about four weeks and then gradually decline but remained at a high-level significantly above the WHO reference until 56 days post-immunization. As anticipated, Ad5-dRVG induced a higher immune response than Ad5-RVG.

While RABV is extensively studied, 15 additional lyssavirus species exhibit genetic and antigenic divergence. Among these lyssaviruses, only RABV is capable of infecting multiple hosts, whereas the others are exclusively associated with bats [36]. Studies on lyssavirus neutralization have demonstrated that antibodies against RABV also confer protection against ARAV, ABLV, BBLV, DUVV, EBLV-1, EBLV-2, IRKV, KHUV, and GBLV. However, the specific level of neutralizing antibodies required for each individual lyssavirus species remains unknown [37]. These viruses share phylogenetic and antigenic similarities with classical RABV, and the 10 lyssaviruses in this group are further classified as phylogroup I based on their genetic and antigenic relationships. Within the Lyssavirus genus, two additional phylogroups have been defined. In vivo vaccine-challenge experiments have indicated that the antibody response generated by current rabies vaccines may not produce sufficient neutralizing antibodies to protect against viruses belonging to the other two phylogroups of lyssavirus [38–42]. Based on this knowledge and considering the rarity of non-RABV lyssavirus infections, the development of a "multi-valent rabies vaccine" may not be an urgent priority. However, it is worth exploring the concept proposed in this study, which involves generating a recombinant adenovirus vaccine expressing heterologous glycoproteins. This direction presents an opportunity to expand our research.

In this study, we observed the unfortunate death of one mouse in the muscular immunization group. It is important to note that this adverse event was unlikely caused by the virus challenge. First, the mice in the muscular immunization group did not show any rabid symptoms before their death compared to the mice in the PBS group. Second, before the mice were infected with the rabies virus, the serum-neutralizing antibody titer was substantially higher than 0.5 IU/mL, which represents theoretically total protection against rabies. Third, previous studies have shown that when mice are challenged with the rabies virus, they usually develop symptoms and die 5-12 days after infection [29,34,42–46]. In our study, the mice died on the second day after infection, which is too soon for the virus to attack the central nervous system. However, immunostaining is needed to confirm this hypothesis. Since the mouse managed to produce neutralizing antibodies, it is unlikely that the mouse had an underlying immunodeficiency condition that may have predisposed it to a higher risk of adverse events. Furthermore, it is important to acknowledge the inherent variability in experimental animals, including genetic factors, individual responses, and susceptibility to stress. Even within a controlled experimental setting, mice can exhibit heterogeneous responses to handling due to innate biological differences. Therefore, it is crucial to consider the possibility that the unfortunate death of the mouse may have resulted from a combination of genetic predisposition, individual variability, and other unknown factors. Despite the unfortunate occurrence, the findings from the

remaining mice in the immunization group support the efficacy and safety of the vaccine.

Needle phobia is an important issue for about 20% of adults and children and can make immunizations stressful [47,48]. In addition, accidental needle sticks are a serious problem in both developed and developing countries (World Health Organization. State of the World's Vaccines and Immunization (World Health Organization, Geneva, 1996)). Unsafe and improper use of syringes also causes overwhelming disease transmission. Needle-free method of vaccine immunization is safe, cost-friendly, easy to manipulate, and well-accepted, thus being recommended by WHO [49].

In a previous study, it was observed that oral immunization using adenoviral vector vaccines failed to activate immune responses against the rabies virus primarily due to low pH conditions in animal gastric juice [24,46]. In our study, the vaccine was mixed with viscous bait in a small volume and prevented the animals from food or water intake for several hours. By doing so, it is ensured that the adenovirus can be released and fully in contact with the oral cavity mucosal. The results demonstrated that the Ad5-dRVG improved antigen expression and induced a higher immune response than Ad5-RVG. Furthermore, by delivering the vaccine through oral feeding with a small volume and utilizing a viscous formulation, the contact time between the vaccine and the mucosal surfaces was extended. This approach effectively circumvented the destructive impact of gastric acid, which could compromise the vaccine's efficacy.

It is known that RVNA is the most important indicator in evaluating whether a rabies vaccine is effective [50]. In the case of post-exposure prophylaxis (PEP), where achieving a serum threshold of 0.5 IU/ml of RVNA within two weeks after vaccination is crucial [51]. The Ad5-dRVG demonstrates a rapid onset of RVNA production. This characteristic positions Ad5-dRVG as a suitable vaccine candidate for PEP. However, further investigation is required in PEP animal models to confirm its efficacy.

One of the common concerns about human adenovirus vectors is the presence of pre-existing neutralizing antibodies. Previous studies have indicated that these pre-existing antibodies are significantly lower in mucosal than in blood. Additionally, intranasal administration of adenovirus vaccines induced fewer neutralizing antibodies against adenovirus itself. Furthermore, repeated vaccination dose not boost the antibody titer in these cases. These findings suggests that mucosal administration may help overcome the pre-existing antibody problem [52]. Our study shows similar results that both i.m and i.o administration induced adenovirus neutralizing antibody, while in i.o groups antibody titer of adenovirus is notably lower compared with the i.m group (data not shown).

In conclusion, our study demonstrates that the adenovirus vector rabies vaccine Ad5-dRVG is effective in activating the immune response and providing complete protection against lethal rabies virus challenge when administered via both intramuscular and intra-oral routes. This vaccine shows potential in addressing the various requirements for protecting humans from rabies disease. By incorporating the vaccine into bait, it holds the potential to reduce rabies circulation in wildlife populations. This approach can improve compliance and enhance the successful implementation of rabies control programs.

Conclusions

Our work have demonstrated the potential of using human serotype 5 adenovirus vectors as effective tools for antigen delivery in the development of rabies vaccines. We successfully developed novel recombinant rabies vaccines, Ad5-RVG and Ad5-dRVG, by cloning one or two copies of the rabies virus glycoprotein into a human adenoviral vector. Comparative analysis revealed that a single dose of either intramuscular or oral immunization with Ad5-dRVG resulted in significantly stronger immune responses compared to Ad5-RVG. Both vaccines demonstrated the ability to provide complete protection in mice. Moreover, our additional investigations revealed that Ad5-dRVG achieved complete protection even at a very low dose, which has the potential to significantly reduce the production cost of the rabies vaccine. This finding further supports the cost-effectiveness and accessibility of using adenovirus vectors for rabies vaccination.

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References

1. Kieny MP, Lathe R, Drillien R, Spehner D, Skory S, Schmitt D, Wiktor T, Koprowski H, Lecocq JP (1984) Expression of rabies virus glycoprotein from a recombinant vaccinia virus. Nature 312: 163–6. doi: 10.1038/312163a0.
2. Murphy FA (1977) Rabies pathogenesis. Arch Virol 54: 279–97. doi: 10.1007/BF01314774.
3. Hemachudha T, Ugolini G, Wacharapluesadee S, Sungkarat W, Shuangshoti S, Laothamatas J (2013) Human rabies: neuropathogenesis, diagnosis, and management. Lancet Neurol 12: 498–513. doi: 10.1016/S1474-4422(13)70038-3.
4. Brunker K, Mollentze N (2018) Rabies virus. Trends Microbiol 26: 886–887. doi: 10.1016/j.tim.2018.07.001.
5. Gan H, Hou X, Wang Y, Xu G, Huang Z, Zhang T, Lin R, Xue M, Hu H, Liu M, Cheng ZJ, Zhu Z, Sun B (2023) Global burden of rabies in 204 countries and territories, from 1990 to 2019: results from the global burden of disease study 2019. Int J Infect Dis 126: 136–144. doi: 10.1016/j.ijid.2022.10.046.
6. Lodha L, Manoor Ananda A, Mani RS (2023) Rabies control in high-burden countries: role of universal pre-exposure immunization. The Lancet Regional Health - Southeast Asia. doi: 10.1016/j.lansea.2023.100258
7. Wang C, Dulal P, Zhou X, Xiang Z, Goharriz H, Banyard A, Green N, Brunner L, Ventura R, Collin N, Draper SJ, Hill AVS, Ashfield R, Fooks AR, Ertl HC, Douglas AD (2018) A simian-adenovirus-vectored rabies vaccine suitable for thermostabilisation and clinical development for low-cost single-dose pre-exposure prophylaxis. PLoS Negl Trop Dis 12: e0006870. doi: 10.1371/journal.pntd.0006870.
8. Plotkin SA (2000) Rabies. Clin Infect Dis 30: 4–12. doi: 10.1086/313632.
9. Nakagawa K, Nakagawa K, Omatsu T, Katayama Y, Oba M, Mitake H, Okada K, Yamaoka S, Takashima Y, Masatani T, Okadera K, Ito N, Mizutani T, Sugiyama M (2017) Generation of a novel live rabies vaccine strain with a high level of safety by introducing attenuating mutations in the nucleoprotein and glycoprotein. Vaccine 35: 5622–5628. doi: 10.1016/j.vaccine.2017.08.050.
10. Rupprecht CE, Nagarajan T, Ertl H (2016) Current status and development of vaccines and other biologics for human rabies prevention. Expert Rev Vaccines 15: 731–49. doi: 10.1586/14760584.2016.1140040.
11. Parize P, Dacheux L, Larrous F, Bourhy H, French network of antirabies clinics (2018) The shift in rabies epidemiology in France: time to adjust rabies post-exposure risk assessment. Euro Surveill 23: 1700548. doi: 10.2807/1560-7917.ES.2018.23.39.1700548.
12. Arroyo J, Miller CA, Catalan J, Monath TP (2001) Yellow fever vector live-virus vaccines: West Nile virus vaccine development. Trends Mol Med 7: 350–4. doi: 10.1016/s1471-4914(01)02048-2.
13. Gerlach T, Elbahesh H, Saletti G, Rimmelzwaan GF (2019) Recombinant influenza A viruses as vaccine vectors. Expert Rev Vaccines 18: 379–392. doi: 10.1080/14760584.2019.1582338.
14. Milone MC, O'Doherty U (2018) Clinical use of lentiviral vectors. Leukemia 32: 1529–1541. doi: 10.1038/s41375-018-0106-0.
15. Wang D, Tai PWL, Gao G (2019) Adeno-associated virus vector as a platform for gene therapy delivery. Nat Rev Drug Discov 18: 358–378. doi: 10.1038/s41573-019-0012-9.
16. Fougeroux C, Holst PJ (2017) Future prospects for the development of cost-effective adenovirus vaccines. Int J Mol Sci 18: 686. doi: 10.3390/ijms18040686.
17. Ertl HC (2009) Novel vaccines to human rabies. PLoS Negl Trop Dis 3: e515. doi: 10.1371/journal.pntd.0000515.
18. Weyer J, Rupprecht CE, Nel LH (2009) Poxvirus-vectored vaccines for rabies-a review. Vaccine 27: 7198–201. doi: 10.1016/j.vaccine.2009.09.033.
19. Knowles MK, Nadin-Davis SA, Sheen M, Rosatte R, Mueller R, Beresford A (2009) Safety studies on an adenovirus recombinant vaccine for rabies (AdRG1.3-ONRAB) in target and non-target species. Vaccine 27: 6619–26. doi: 10.1016/j.vaccine.2009.08.005.
20. Rosatte RC, Donovan D, Davies JC, Allan M, Bachmann P, Stevenson B, Sobey K, Brown L, Silver A, Bennett K, Buchanan T, Bruce L, Gibson M, Beresford A, Beath A, Fehlner-Gardiner C, Lawson K (2009) Aerial distribution of ONRAB baits as a tactic to control rabies in raccoons and striped skunks in Ontario, Canada. J Wildl Dis 45: 363–74. doi: 10.7589/0090-3558-45.2.363.
21. Fehlner-Gardiner C, Rudd R, Donovan D, Slate D, Kempf L, Badcock J (2012) Comparing ONRAB(R) AND RABORAL V-RG(R) oral rabies vaccine field performance in raccoons and striped skunks, New Brunswick, Canada, and Maine, USA. J Wildl Dis 48: 157–67. doi: 10.7589/0090-3558-48.1.157.
22. Tatsis N, Ertl HC (2004) Adenoviruses as vaccine vectors. Mol Ther 10: 616–29. doi: 10.1016/j.ymthe.2004.07.013.
23. Wang Y, Xiang Z, Pasquini S, Ertl HC (1997) The use of an E1-deleted, replication-defective adenovirus recombinant expressing the rabies virus glycoprotein for early vaccination of mice against rabies virus. J Virol 71: 3677–83. doi: 10.1128/JVI.71.5.3677-3683.1997.
24. Wang X, Fang Z, Xiong J, Yang K, Chi Y, Tang X, Ma L, Zhang R, Deng F, Lan K, Zhou D (2019) A chimpanzee adenoviral vector-based rabies vaccine protects beagle dogs from lethal rabies virus challenge. Virology 536: 32–38. doi: 10.1016/j.virol.2019.07.022.
25. Fooks AR, Banyard AC, Ertl HCJ (2019) New human rabies vaccines in the pipeline. Vaccine 37 Suppl 1: A140–A145. doi: 10.1016/j.vaccine.2018.08.039.
26. Sweeney JA, Hennessey JP (2002) Evaluation of accuracy and precision of adenovirus absorptivity at 260 nm under conditions of complete DNA disruption. Virology 295: 284–8. doi: 10.1006/viro.2002.1406
27. Cliquet F, Aubert M, Sagne L (1998) Development of a fluorescent antibody virus neutralisation test (FAVN test) for the quantitation of rabies-neutralising antibody. J Immunol Methods 212: 79–87. doi: 10.1016/s0022-1759(97)00212-3.
28. Belotto AJ (2004) The Pan American Health Organization (PAHO) role in the control of rabies in Latin America. Dev Biol (Basel) 119: 213–6.
29. Napolitano F, Merone R, Abbate A, Ammendola V, Horncastle E, Lanzaro F, Esposito M, Contino AM, Sbrocchi R, Sommella A, Duncan JD, Hinds J, Urbanowicz RA, Lahm A, Colloca S, Folgori A, Ball JK, Nicosia A, Wizel B, Capone S, Vitelli A (2020) A next generation vaccine against human rabies based on a single dose of a chimpanzee adenovirus vector serotype C. PLoS Negl Trop Dis 14: e0008459. doi: 10.1371/journal.pntd.0008459.

30. Chulasugandha P, Khawplod P, Havanond P, Wilde H (2006) Cost comparison of rabies pre-exposure vaccination with post-exposure treatment in Thai children. Vaccine 24: 1478–82. doi: 10.1016/j.vaccine.2005.03.059.
31. Kozarsky KF, Wilson JM (1993) Gene therapy: adenovirus vectors. Curr Opin Genet Dev 3: 499–503. doi: 10.1016/0959-437x(93)90126-a.
32. Lasaro MO, Ertl HC (2009) New insights on adenovirus as vaccine vectors. Mol Ther 17: 1333–9. doi: 10.1038/mt.2009.130.
33. Chen H, Xiang ZQ, Li Y, Kurupati RK, Jia B, Bian A, Zhou DM, Hutnick N, Yuan S, Gray C, Serwanga J, Auma B, Kaleebu P, Zhou X, Betts MR, Ertl HC (2010) Adenovirus-based vaccines: comparison of vectors from three species of adenoviridae. J Virol 84: 10522–32. doi: 10.1128/JVI.00450-10.
34. Xiang Z, Gao G, Reyes-Sandoval A, Cohen CJ, Li Y, Bergelson JM, Wilson JM, Ertl HC (2002) Novel, chimpanzee serotype 68-based adenoviral vaccine carrier for induction of antibodies to a transgene product. J Virol 76: 2667–75. doi: 10.1128/jvi.76.6.2667-2675.2002.
35. Xiang ZQ, Greenberg L, Ertl HC, Rupprecht CE (2014) Protection of non-human primates against rabies with an adenovirus recombinant vaccine. Virology 450–451: 243–9. doi: 10.1016/j.virol.2013.12.029.
36. Marston DA, Banyard AC, McElhinney LM, Freuling CM, Finke S, de Lamballerie X, Muller T, Fooks AR (2018) The lyssavirus host-specificity conundrum-rabies virus-the exception not the rule. Curr Opin Virol 28: 68–73. doi: 10.1016/j.coviro.2017.11.007.
37. Evans JS, Selden D, Wu G, Wright E, Horton DL, Fooks AR, Banyard AC (2018) Antigenic site changes in the rabies virus glycoprotein dictates functionality and neutralizing capability against divergent lyssaviruses. J Gen Virol 99: 169–180. doi: 10.1099/jgv.0.000998.
38. Hanlon CA, Kuzmin IV, Blanton JD, Weldon WC, Manangan JS, Rupprecht CE (2005) Efficacy of rabies biologics against new lyssaviruses from Eurasia. Virus Res 111: 44–54. doi: 10.1016/j.virusres.2005.03.009.
39. Badrane H, Bahloul C, Perrin P, Tordo N (2001) Evidence of two Lyssavirus phylogroups with distinct pathogenicity and immunogenicity. J Virol 75: 3268–76. doi: 10.1128/JVI.75.7.3268-3276.2001.
40. Horton DL, Banyard AC, Marston DA, Wise E, Selden D, Nunez A, Hicks D, Lembo T, Cleaveland S, Peel AJ, Kuzmin IV, Rupprecht CE, Fooks AR (2014) Antigenic and genetic characterization of a divergent African virus, Ikoma lyssavirus. J Gen Virol 95: 1025–1032. doi: 10.1099/vir.0.061952-0.
41. Evans JS, Horton DL, Easton AJ, Fooks AR, Banyard AC (2012) Rabies virus vaccines: is there a need for a pan-lyssavirus vaccine? Vaccine 30: 7447–54. doi: 10.1016/j.vaccine.2012.10.015.
42. Stading B, Ellison JA, Carson WC, Satheshkumar PS, Rocke TE, Osorio JE (2017) Protection of bats (*Eptesicus fuscus*) against rabies following topical or oronasal exposure to a recombinant raccoon poxvirus vaccine. PLoS Negl Trop Dis 11: e0005958. doi: 10.1371/journal.pntd.0005958.
43. Kim HH, Yang DK, Nah JJ, Song JY, Cho IS (2017) Comparison of the protective efficacy between single and combination of recombinant adenoviruses expressing complete and truncated glycoprotein, and nucleoprotein of the pathogenic street rabies virus in mice. Virol J 14: 122. doi: 10.1186/s12985-017-0789-2.
44. Li J, Liu Q, Liu J, Wu X, Lei Y, Li S, Zhao D, Li Z, Luo L, Peng S, Ou Y, Yang H, Jin J, Li Y, Peng Y (2022) An mRNA-based rabies vaccine induces strong protective immune responses in mice and dogs. Virol J 19: 184. doi: 10.1186/s12985-022-01919-7.
45. Chen Z, Zhou M, Gao X, Zhang G, Ren G, Gnanadurai CW, Fu ZF, He B (2013) A novel rabies vaccine based on a recombinant parainfluenza virus 5 expressing rabies virus glycoprotein. J Virol 87: 2986–93. doi: 10.1128/JVI.02886-12.
46. Vos A, Neubert A, Pommerening E, Muller T, Dohner L, Neubert L, Hughes K (2001) Immunogenicity of an E1-deleted recombinant human adenovirus against rabies by different routes of administration. J Gen Virol 82: 2191–2197. doi: 10.1099/0022-1317-82-9-2191.
47. Nir Y, Paz A, Sabo E, Potasman I (2003) Fear of injections in young adults: prevalence and associations. Am J Trop Med Hyg 68: 341–4.
48. Breau LM, McGrath PJ, Craig KD, Santor D, Cassidy KL, Reid GJ (2001) Facial expression of children receiving immunizations: a principal components analysis of the child facial coding system. Clin J Pain 17: 178–86. doi: 10.1097/00002508-200106000-00011.
49. Mitragotri S (2005) Immunization without needles. Nat Rev Immunol 5: 905–16. doi: 10.1038/nri1728.
50. Beran J, Honegr K, Banzhoff A, Malerczyk C (2005) Potency requirements of rabies vaccines administered intradermally using the Thai Red Cross regimen: investigation of the immunogenicity of serially diluted purified chick embryo cell rabies vaccine. Vaccine 23: 3902–7. doi: 10.1016/j.vaccine.2005.03.007.
51. World Health O (2018) Rabies vaccines: WHO position paper, April 2018 - recommendations. Vaccine 36: 5500–5503. doi: 10.1016/j.vaccine.2018.06.061.
52. Jeyanathan M, Fritz DK, Afkhami S, Aguirre E, Howie KJ, Zganiacz A, Dvorkin-Gheva A, Thompson MR, Silver RF, Cusack RP, Lichty BD, O'Byrne PM, Kolb M, Medina MFC, Dolovich MB, Satia I, Gauvreau GM, Xing Z, Smaill F (2022) Aerosol delivery, but not intramuscular injection, of adenovirus-vectored tuberculosis vaccine induces respiratory-mucosal immunity in humans. JCI Insight 7: e155655. doi: 10.1172/jci.insight.155655.

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