FULL PAPER *Virology*

Adeno-Associated Viruses Serotype 2-Mediated RNA Interference Efficiently Inhibits Rabies Virus Replication *In Vitro* and *In Vivo*

Hong-Xia WU[1,2)#], Hua-Lei WANG[2)#], Xiao-Feng GUO[1)], Yu-Jiao YANG[2)], Jin-Zhu MA[2)], Tie-Cheng WANG[2)], Yu-Wei GAO[2)], Yong-Kun ZHAO[2)], Song-Tao YANG[2)] and Xian-Zhu XIA[2)]*

[1)]*College of Veterinary Medicine, South China Agricultural University, 483 Wushan Road, Guangzhou 510642, P. R. China*
[2)]*Military Veterinary Institute, Academy of Military Medical Sciences, 666 Liuyin Road, Changchun 130122, Jilin Province, China*

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ABSTRACT. To investigate the potential of adeno-associated viruses serotype 2 (AAV2)-mediated RNA interference (RNAi) as an antiviral agent against rabies, recombinant AAV2 vectors expressing siRNA targeting the nucleoprotein (N) gene of rabies virus (RABV) (rAAV-N796) were constructed and evaluated. When NA cells pretreated with rAAV-N796 were challenged with RABV, there was a 37.8 ± 3.4% to 55.1 ± 5.3% reduction in RABV virus titer. When cells pre-challenged with RABV were treated with rAAV-N796, there was a 4.4 ± 1.4 to 28.8 ± 3.2% reduction in RABV virus titer. Relative quantification of RABV transcripts using real-time PCR and Western blot revealed that the knockdown of RABV-N gene transcripts was based on the rAAV-N796 inoculation titer. When any NA cells were treated with rAAV-N796 before or after challenged with RABV, significant reduction in virus titer was observed in both administrations. Mice treated intracerebrally with rAAV-N796 exhibited 50 ± 5.3 and 62.5 ± 4.7% protection when challenged intracerebrally or intramuscally, respectively, with lethal RABV. When mice treated intramuscularly with rAAV-N796 were challenged intramuscularly with lethal RABV, they exhibited 37.5 ± 3.7% protection. When mice were intracerebrally and intramuscularly with rAAV-N796 24 hr after exposure to RABV infection, they exhibited 25 ± 4.1% protection The N gene mRNA levels in the brains of challenged mice with three different administrations were reduced (55, 68, 32 and 25%, respectively). These results indicated that AAV2 vector-mediated siRNA delivery *in vitro* in NA cells inhibited RABV multiplication, inhibited RABV multiplication *in vivo* in the mice brain and imparted partial protection against lethal rabies. So, it may have a potential to be used as an alternative antiviral approach against rabies.
KEY WORDS: AAV2 vectors, inhibit, N gene, RABV, siRNA.

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Rabies is a disease of the central nervous system that constantly produces victims in human beings as well as in animals. Rabies cases are reported worldwide, but are most prevalent in undeveloped countries, specifically in rural areas of Africa and Asia. No definitive and effective treatment has been established to cure the disease once the clinical stage has been reached, and only a small portion of clinical cases responds to intensive therapeutic management and survives [19]. Once the clinical symptoms of rabies appear, conventional post-exposure treatment is unsuccessful, because of its inability to cross the blood–brain barrier [9]. Thus, the current strategy to control rabies is pre-exposure prophylaxis by vaccine injection, but research efforts are focused on developing an effective, safe prophylactic and/or therapeutic tool against RABV infection.

RNAi mediated by small interfering RNAs (siRNAs) is a powerful technology allowing the silencing of genes with great specificity and potency. The RNAi strategy has already been successfully applied to inhibit the replication of many viruses, including hepatitis B virus (HBV) [13], dengue virus (DNV) [14], human immunodeficiency virus (HIV) [10], severe acute respiratory syndrome (SARS) coronavirus [11] and influenza virus [21]. Considering the potential of RNAi as an antiviral agent, few groups have evaluated RNAi-based antiviral agents against rabies [2, 8]. However, one of the potential limitations for applying this technology is developing an effective and targeted delivery tool.

Viral vectors are one of the major vehicles used by scientists in gene therapy to get their sequences expressed in the proper host. Viral vectors, including adenovirus, retrovirus, lentivirus and adeno-associated virus (AAV), are undoubtedly efficient tools for gene delivery. They have been widely used in delivering various genes and siRNAs. More recently, Gupta *et al.* and Sonwane *et al.* reported that an adenoviral vector-mediated delivery of small hairpin (sh)RNAs targeting the RABV N or polymerase (L) mRNA led to a slight increase in survival of RABV-infected mice [7, 16, 20]. So far, no research group has reported whether AAV can work as an siRNA delivery tool and transport a siRNA targeting RABV into the cells or host to inhibit the RABV replication.

In this study, we constructed recombinant AAV vectors based on serotype 2 (rAAV2) expressing siRNA targeting the N gene of RABV. In *in vitro* and *in vivo* experiments, rAAV2 could efficiently inhibit the replication of RABV.

MATERIALS AND METHODS

Cells, viruses and animals: NA cells and BHK cells

*CORRESPONDENCE TO: XIA, X.-Z., Military Veterinary Institute, Academy of Military Medical Sciences, 666 Liuying Road, Changchun 130122, Jilin Province, China.
e-mail: xiaxianzhu@gmail.com

#These authors contributed equally to this paper.

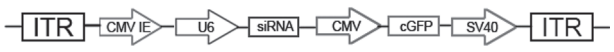

Fig. 1. Map of the rAAV-2 genome. cGFP and siRNA were flanked by ITRs. An siRNA transcript was produced by the U6 promoter, and a cGFP transcript was produced by the CMV promoter. CMV IE was the enhancer for the U6 promoter and CMV promoter.

were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Carlsbad, CA, U.S.A.) supplemented with 10% fetal bovine serum (FBS), 100 U/m*l* penicillin and 100 μg/m*l* streptomycin (Hyclone, Logan, UT, U.S.A.) at 37°C. The RABV virus standard (CVS)-11 strain was obtained from the Changchun Institute of Veterinary Science (Changchun, China). Female BALB/c mice (weighing 13–15 g; Changchun Institute of Biological Products, Changchun, China) were used to assess the antiviral activities of the rAAV2 *in vivo*. All experiments with the RABV virus were conducted in a biosafety level three laboratory facility at the Changchun Institute of Veterinary Science.

Plasmid construction: pAAV-hrGFP, pAAV-RC and pHelper are described in the information provided for the AAV Helper-Free System (Catalog #240071, Agilent Technologies, Santa Clara, CA, U.S.A.). siRNA-796 targeting the N gene was inserted into the pSilence2.1-U6 Hygro siRNA expression vector (Ambion, Austin, TX, U.S.A.) by BamHI and HindIII (ps-N796). The ps-N796 plasmid transported by a liposome could inhibit RABV replication *in vitro* and *vivo* [20]. The siRNA expressing box containing CMV, cGFP, U6 promoter and siRNA was amplified, and NotI was introduced into 5' and 3'. The siRNA-expressing box was digested with NotI and inserted into pAAV-hrGFP treated with NotI (pAAV-N796). The negative control was named with pAAV-Neg. The pAAV-N796 encoding the specific shRNAs is shown in Fig. 1.

AAV vector production: rAAV-N796 or rAAV-Neg vectors were produced by liposome-mediated co-transfection of pAAV-N796 or pAAV-Neg, pAAV-RC and pHelper in human embryonic kidney (HEK) 293 cells according to the protocol. Briefly, 2 hr before transfection, each 10-cm-diameter plate of human 293 cells (80% confluent) was fed 10 m*l* of fresh DMEM containing 10% FBS without antibiotics. A total of 25 mg of plasmid DNA were dissolved in 1 m*l* of Liposome 2000 (Invitrogen, Carlsbad, CA, U.S.A.) and then mixed and added to the cells after incubation for 25 min. At 4 to 6 hr after transfection, the medium was replaced with fresh DMEM containing 2% FBS and antibiotics. The cells and suspensions were harvested at 72 hr post infection. After low-speed centrifugation on a tabletop centrifuge, the cell pellets were resuspended in 1 m*l* of 100 mM NaCl–10 mM Tris-HCl (pH 8.5) and subjected to four cycles of freeze-thaw and removal of cell debris. The rAAV particles were then purified by HiTrap heparin column chromatography (Sigma, St. Louis, MO, U.S.A.). Peak virus fractions were collected and dialyzed against PBS containing 1 mM $MgSO_4$. Samples were then concentrated using a 100K-MicroSep centrifugal concentrator (Life Technologies, Carlsbad, CA, U.S.A.). Viral titer was quantified by real-time PCR using a TaqNan Universal PCR kit (Applied Biosystems, Foster City, CA, U.S.A.) with the forward primer 5'-TGCTGCTGCCC-GATAACC-3' and the reverse primer 5'-ATCACCCACG-GCATGGAC-3'.

Transduction with rAAV and infection with RABV in NA cells: NA cells were plated at 1×10^6 cells/well in 6-well plates and incubated overnight. The rAAV was inoculated into cells with different viral titers. After transduction for 2 hr, the medium was replaced with fresh DMEM containing 2% FBS and antibiotics. After 24 hr of transduction, the cells were infected with 0.01 MOI of the RABV CVS-11 virus for 48 hr. Another method was infection of cells with 0.01 MOI of the RABV CVS-11 virus for 6 hr and then inoculation with rAAV.

Viral titer assay: Viral titer was measured using 50% tissue culture infective dose ($TCID_{50}$) assays. Serial 10-fold dilutions of supernatants from the treated and viral samples were added onto a monolayer of BHK-21 cells in 96-well culture plates and incubated for two days. Virus concentrations were measured by direct fluorescent antibody staining (DFA), as described above, and the viral titer for each sample was calculated by the Reed–Muench method [15].

Real-time PCR: NA cells or mouse brains were harvested after RABV infection, and total RNA was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA was reverse transcribed into cDNA using AMV reverse transcriptase (Promega, Madison, WI, U.S.A.). The levels of N mRNA transcripts were determined by qPCR using Brilliant II SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA, U.S.A.) and the following gene-specific primers: forward primer (1168–1187 in genome) 5'-TCAAGAATATGAGGCGGCTG-3' and reverse primer (1356–1375 in genome) 5'-TGGACGGGCTTGAT-GATTGG-3' for CVS11-N (207 bp amplicon), and forward primer 5'-TGACAGGATGCAGAAGGAGA-3' and reverse primer 5'-GCTGGAAGGTGGACAGTGAG-3' for β-actin (86 bp amplicon). The PCR was carried out on an Mx 3000P System. The relative expression values of N gene were normalized to the expression value of the β-actin gene.

Western blotting: NA cells were harvested after 72 hr of RABV infection, and total protein samples were obtained by incubation in cell lysis buffer (Beyotime Biotech Inc., Nantong, China). Protein concentration was measured with a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, U.S.A.). Fifty microgram aliquots of total protein were resolved by sodium SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden). The resultant blots were probed with a mouse monoclonal antibody to RABV N protein (1:200) or a mouse monoclonal antibody to β-actin (1:2,000, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), followed by the horseradish peroxidase-conjugated goat anti-mouse IgG (1:2,000, Santa Cruz Biotechnology). Immunoreactive bands were detected with enhanced chemiluminescence reagent (Pierce).

Viral challenge in mice: Mice, in groups of eleven, were anesthetized with dry ice and inoculated intracerebrally or intramuscularly (in masseter muscle) with the rAAV (2

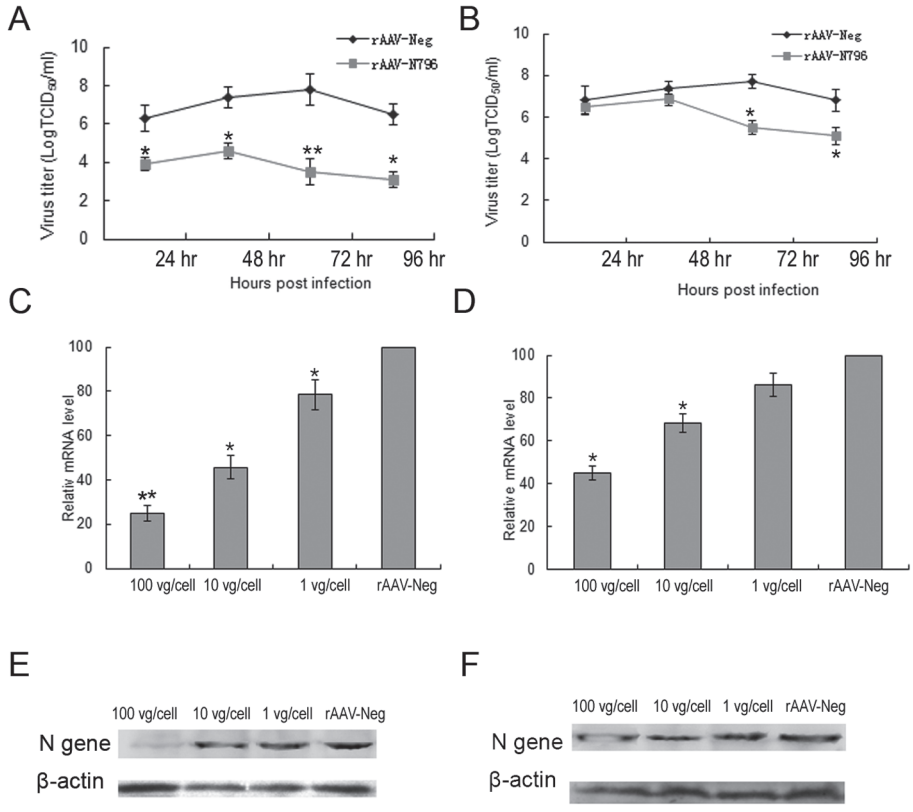

Fig. 2. Inhibition of RABV multiplication in NA cells treated with rAAV2. (1) NA cells were first treated with rAAV-N796 and rAAV-Neg (A), respectively, and then challenged with 0.01 MOI of the RABV-CVS-11 strain. Other NA cells were first challenged with 0.01 MOI of RABV and then treated with rAAV-N796 and rAAV-Neg (B). At different time points post challenge, the virus titer of RABV was detected. (2) NA cells were firstly treated with rAAV-N796 and rAAV-Neg, respectively, with the titer of 1, 10 and 100 vg/cell and then challenged with 0.01 MOI of RABV. At 72 hr post infection, the cells were collected for assay of the N gene mRNA level (C) and protein expression level (E). In another group of NA cells, the cells were first challenged with RABV and then treated with the same titers of rAAV-N796 and rAAV-Neg mentioned above. At 72 hr post infection, the cells were collected for assay of the N gene mRNA level (D) and protein expression level (F). The data shown in A, B, C and D represent mean ± SD for three independent experiments. *P<0.05, **P<0.01 between rAAV-N796 and rAAV-Neg (A, B); *P<0.05, **P<0.01 between different inoculation titer of rAAV-N796 and rAAV-Neg (C, D). Three independent experiments yielded consistent results in E and F.

$\times 10^{12}$ vg/kg). After 48hr, the mice were infected with 10 LD_{50} of RABV CVS-11 for intracerebral challenge and 20 LD_{50} of RABV CVS-11 for intramuscular challenge at the same inoculation site as the rAAV inoculation site. The third group of mice was inoculated intracerebrally with the rAAV and then challenged with 20 LD_{50} of RABV CVS-11 intramuscularly (in masseter muscle) more than 48 hr after inoculation with rAAV (2×10^{12} vg/kg). The forth group of mice was infected with RABV intramuscularly and inoculated with rAAV-N796 or rAAV-Neg intracerebrally and intramuscularly after infection for 24 hr. At 6 days post infection with RABV CVS-11, three mice from each group were anesthetized, and their brains were collected for assay of virus titer; the other mice were observed for 21 days for survival.

All animal researches were conducted under the guidance of the CDC's Institutional Animal Care and Use Committee and in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility. The animal research in our study was approved by the Jilin Province Animal Disease Control Center.

Statistical analysis: Statistical significance of differences between experimental groups was determined through the use of the paired, nonparametric Student's *t*-test. P<0.05 was consider a significant difference.

RESULTS

Antiviral activity of the rAAV-N796 in NA cells: To evaluate the antiviral effects of rAAV-N796 targeting the conserved sequences of the CVS11-N gene, the rAAV-N796 and a negative control (rAAV-Neg) were respectively inoculated

into NA cells with 10 vg/cell. The transduction efficiency of rAAV in NA cells was more than 90% (result not shown). After transduction for 24 hr, the cells were infected with 0.01 MOI of the RABV CVS-11 virus. At the different time points, the cells supernatants were collected for analysis of RABV titer. As shown in Fig. 2A, the virus titer in the rAAV-N796 group was significantly lower ($P<0.05$) than that in the rAAV-Neg group at each time point. At 72 hr post infection, the virus titer ($TCID_{50}$/m*l* of 3.5 ± 0.65) in the rAAV-N796 pretreatment group was reduced by nearly 10^4 times ($P<0.01$) compared with the rAAV-Neg group ($TCID_{50}$/m*l* of 7.8 ± 0.82). When RABV infection was before rAAV transduction, no significant difference was observed at 24 and 48 hr post infection. Only at 72 and 96 hr post infection (Fig. 2B) was the RABV titer reduced significantly ($P<0.05$). These results indicated that the inoculation of rAAV-N796 before RABV infection could inhibit the virus replication well, but that inoculation of rAAV-N796 after RABV infection inhibited virus replication only at later time points.

To check whether the antiviral activity of the rAAV-N796 in NA cells depended on the inoculation titer of rAAV-N796, as show in Fig. 2C and 2E, the NA cells were transduced with different titers of rAAV-N796 (1, 10 and 100 vg/cell) and were infected with 0.01 MOI of the RABV CVS-11 virus after transduction for 24 hr. At 72 hr post infection, the cells were collected for assay of N gene mRNA level and protein level. The results showed that the N gene mRNA level was reduced by 78.54 ± 6.8, 45.61 ± 5.3 and 25.10 ± 3.5%, respectively, compared with the rAAV-Neg group. When NA cells were infected with RABV firstly and then transduced with different titers of rAAV-N796 (1, 10 and 100 vg/cell) (Fig. 2D and 2F), a significant lower N gene mRNA level ($P<0.05$) was observed in higher rAAV-N796 titer groups (10 and 100 vg/cell), but not in the lower rAAV-N796 titer group (1 vg/cell). The N protein level also showed the similar result. The higher the rAAV-N796 titer was, the lower the N protein level was, which indicated that the antiviral activity of the rAAV-N796 in NA cells depended on the titer of rAAV-N796.

Collectively, these results demonstrated that the rAAV-N796 could significantly inhibit replication of the CVS-11 virus in NA cells and that the antiviral activity from pretreatment of rAAV-N796 was better than that with treatment after infection.

Antiviral activity of rAAV-N796 in RABV-infected mice: To test whether the inhibition of RABV replication observed *in vitro* for rAAV-N796 was adequate for protection *in vivo*, we used an established BALB/c mouse model of RABV. To test whether rAAV-N796 could protect mice from RABV infection in the brain, as shown in Fig. 3A, the mice were first intracerebrally treated with rAAV-N796 and were then intracerebrally challenged with a lethal dose (10 LD_{50}) of CVS-11 48 hr later. Compared with the rAAV-Neg-treated mice, all of the rAAV-N796-treated mice showed fewer clinical signs of disease and lower morbidity. Moreover, the mouse survival rates in the rAAV-N796-treated groups showed higher survival rates than the rAAV-Neg-treated mice. At 21 days post-challenge, the mouse survival rate in the rAAV-N796-treated groups was 50 ± 5.3%, and all the mice in the rAAV-Neg-treated groups died at 18 days post-challenge.

To test whether rAAV-N796 could protect mice from RABV infection in the masseter muscle, as shown in Fig. 3B, the mice were intramuscularly treated with rAAV-N796 and then intramuscularly challenged with a lethal dose (20 LD_{50}) of RABV CVS-11 48 hr later. The results showed that there was partial protection (37.5 ± 3.7%) against virulent RABV challenge in mice pretreated with rAAV-N796 and no protection in mice pretreated with rAAV-Neg. To check whether intracerebral inoculation with rAAV-N796 could protect mice from intramuscular infection with RABV, as shown in Fig. 3C, the mice were first intracerebrally treated with rAAV-N796 and were then intramuscularly challenged with a lethal dose (20 LD_{50}) of RABV CVS-11 48 hr later. Treatment of mice with rAAV-N796 demonstrated 62.5 ± 4.7% protection, and all the mice treated with rAAV-Neg died at 21 days post-challenge. The mice that survived did not develop symptoms and remained healthy for the entire period of experimentation. Moreover, at 6 days post challenge, we checked the N gene mRNA level in the brain for the three administrations. The results showed (Fig. 3E) that N gene mRNA level in the rAAV-N796-treated mice brain was significantly decreased. Also, the N gene mRNA level in the intracerebral pretreatment group was lower than that in the intramuscular pretreatment group, which agreed with the result pertaining to mouse survival rate.

Post-exposure vaccination is obligatory in all cases as treatment after human exposure to infected animal bites. Regarding whether rAAV-N796 could protect mice exposed to RABV infection within 24 hr, the mice were first intramuscularly challenged with a lethal dose (20 LD_{50}) of RABV CVS-11 and were then injected with rAAV-N796 intracerebrally and intramuscularly 24 hr later. The results (Fig. 3D) showed that there was a partial protection (25 ± 4.1%) and no protection in mice treated with rAAV-Neg. The N gene mRNA level in the rAAV-N796-treated mouse brain was significantly decreased compared with the control (Fig. 3E).

So, these results provided evidence that rAAV-N796 treatment could inhibit RABV replication and conferred partial protection against lethal rabies challenge.

DISCUSSION

Rabies is one of the oldest and most fatal viral diseases known to mankind, and clinical rabies, in which the virus invades the central nervous system, is incurable [17]. There is a need for development of an effective anti-rabies approach. RNA interference (RNAi) has emerged as a potential therapeutic strategy to suppress virus replication *in vitro* and *in vivo* without damaging the host cell. However, one of the potential limitations for applying this technology is developing an effective and targeted delivery tool.

In this study, we developed a universal siRNA agent that would recognize different RABV strains by using the N gene, which is the most highly conserved gene among the five RABV genes [21]. Moreover, we selected the most conserved sequences of the N transcript for use as RNAi targets.

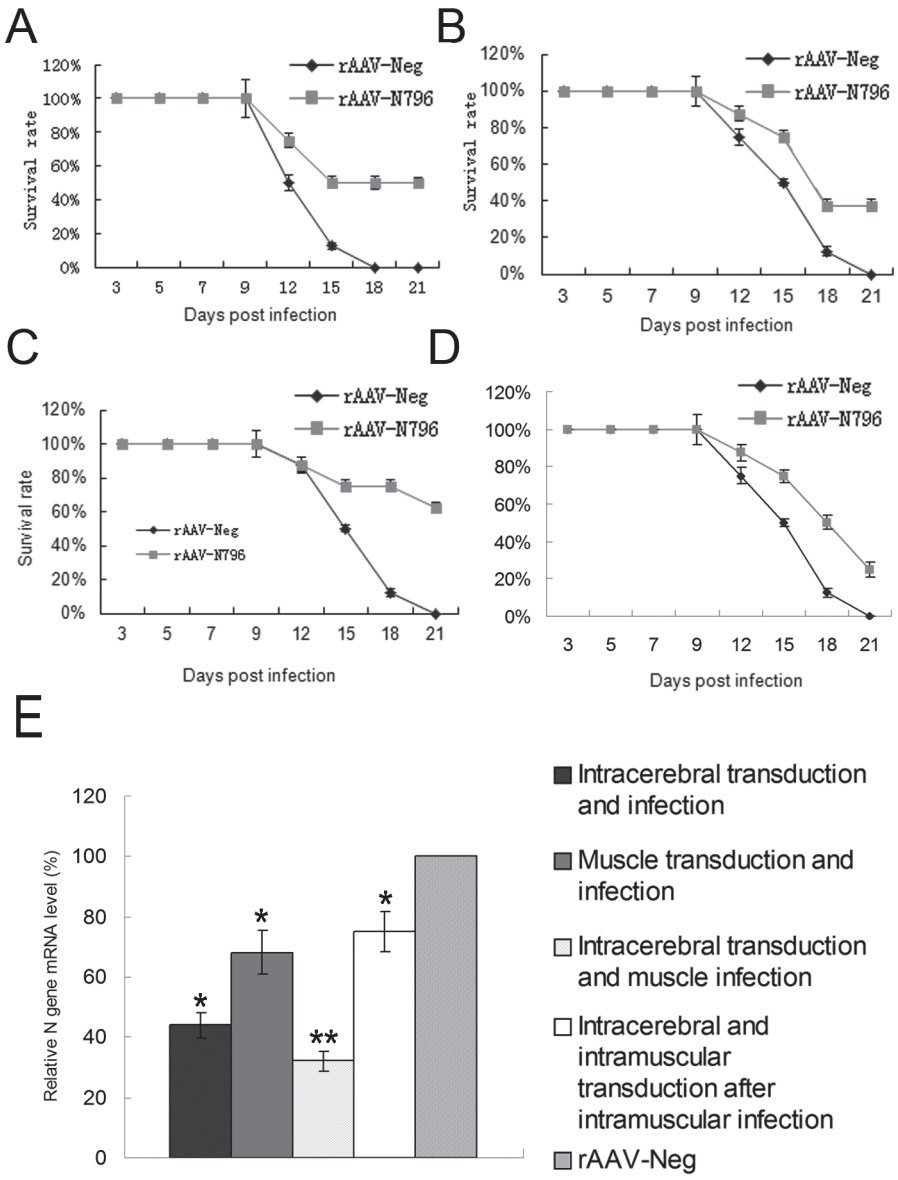

Fig. 3. Treatment with rAAV-N796 before and after challenge increased survival of RABV-infected mice. Mice (n=11/group) were treated intracerebrally with rAAV-N796 or rAAV-Neg and intracerebrally challenged with 10 LD_{50} of lethal CVS-11 (A) or muscularly challenged with 20 LD_{50} (C) 24 hr later. The third of group mice (n=11) was treated muscularly with rAAV-N796 or rAAV-Neg and muscularly challenged with 20 LD_{50} of lethal CVS-11 24 hr later at the same site (B). The forth group of mice (n=11) was muscularly challenged with 20 LD_{50} of lethal CVS-11 and treated with rAAV-N796 or rAAV-Neg by intracerebral and muscular routes 24 hr later (D). The challenged mice were observed for 21 days, and the survival percent was calculated. At 6 days post challenge, three mouse brains from each group were collected for analysis of the N gene mRNA level (E). The data shown represent the mean ± SD for three independent experiments. *P<0.05, **P<0.01 between rAAV-N796 and rAAV-Neg.

The siRNA of rAAV-N796 recognized the sequence-5'-GAGGAAGAGATAAGAAGAA-3' [position 796–814 of the N gene open reading frame (ORF)]. The sequence is very conserved in most of RABV strains. So, rAAV-N796 should be effective against RABV strains other than CVS-11 strain.

Viral vectors used as the delivery tool for siRNA are one of the major vehicles used by scientists in gene therapy to get their sequences expressed in the proper host. Viral vectors, including adenovirus, retrovirus and lentivirus, are undoubtedly efficient tools for gene delivery. They have been

widely used in delivering various genes and siRNAs. But, these viral vectors could stimulate host immune response and produce a specific antibody against viral vectors, which would restrict the use again in the same host. In addition to unwanted activation of the immune response, the safety concerns also involve off-targeting effects.

AAV vectors have been highly successful in overcoming these disadvantages. They are currently among the most frequently used viral vectors for gene therapy. Twelve human serotypes of AAV (AAV-1 to AAV-12) and more than 100 serotypes from nonhuman primates have been discovered to date [4]. The characterization of new AAV serotypes has revealed that they have different patterns of transduction in diverse tissues [3]. AAV has become increasingly common as a vector for use in human clinical trials, such as immunodeficiency disease [5], muscle directed gene therapy [6] and lung disease [12]. AAV-2-based rAAV vectors can transduce muscle, the liver, the brain, the retina and the lungs [4]. rAAV2 has emerged as a vector of choice for gene transfer to the central nervous system, because of its strong neuronal tropism and lack of pathogenicity in mammals [4]. Moreover, AAV-2 has been shown to induce a weak cell-mediated immune response. This may be attributed to AAV inefficiently infecting mature dendritic cells (DC) [22]. So, in this study, we made use of an AAV-2 vector to deliver the siRNA targeting the RABV N gene. The results in the *in vitro* (NA cells) and *in vivo* (mice brain and muscle) experiments showed that AAV-2 can work as a good siRNA delivery tool.

The AAV-2 genome is a linear, single-stranded DNA of 4.7 kb [1]. After infection, second-strand synthesis is needed. So, the gene expression time would be later than other double-stranded DNA virus vectors. In the *in vitro* experiment, if rAAV-N796 treatment was later than RABV infection, the antiviral effect was not displayed at 24 and 48 hr post infection, but was displayed at 72 and 96 hr post infection (Fig. 2B). The reason for this may be ascribe to the later gene expression. After the AAV-2 synthesizes the second-strand, shRNA begins to be transcribed by the U6 promoter. During the time of second-strand synthesis, the replication of RABV was not inhibited, and the virus has replicated for several cycles. The RABV titers of rAAV-N796 and rAAV-Neg treatment groups were both increased with the same efficiency. So, the antiviral effect was not observed in early infection.

Clinical rabies, the CNS form of the disease, can be treated with anti-rabies RNAi through elimination of RABV from the infected neurons by delivering anti-rabies siRNA effectively to the neurons [17]. rAAV2 has emerged as a vector of choice for gene transfer to the central nervous system [3]. So, in the *in vivo* experiment, the antiviral effect of intracerebral inoculation with rAAV-N796 (50 and 62% survival, respectively) was better than with intramuscular inoculation (38% survival). Whether the transduction efficiency in muscle is higher than in the brain needs to be investigated further. The survival rate of the intracerebral inoculation and infection group (50% survival) was lower than that of the intracerebral inoculation and intramuscular infection group (63% survival). After infection with RABV, the time until symptoms were shown was associated with the site of infection. Once RABV is introduced through the skin or mucous membrane, it replicates in the myocytes for hours or weeks and then migrates to nerves. If RABV was introduced into the brain, it would replicate quickly. During the process of RABV entry into brain from masseter muscle, rAAV-N796 has more time to transduce the neural cells and produce more siRNA than direct intracerebral infection. So, the antiviral effect of intracerebral inoculation and intramuscular infection was better than that of intracerebral inoculation and infection.

The antiviral activity of the rAAV-N796 was present after exposure to RABV infection 24 hr later. However, the antiviral activity of rAAV-N796 would be lost, if the mice were injected with rAAV-N796 after showing the symptoms (data not shown). To retain the antiviral activity of rAAV-N796 after exposure to RABV infection, the time of injection with rAAV-N796 should be within 24 hr.

The antiviral activity of rAAV-N796 in NA cells depended on the titer of rAAV-N796. In the *in vivo* experiment, rAAV-N796 pretreatment did not achieve complete protection, which may be associated with the inoculation dose of rAAV-N796. The biggest rAAV titer achieved in our lab through co-transfection in HEK293 cells is 4×10^{11} vg/m*l*. So, a better method to produce rAAV particles needs to be established. The production of rAAV vectors in insect cells via the use of recombinant baculovirus technology has proven to be an efficient and scalable means of rAAV production. The yield of genome-containing particles produced per Sf9 cell approached 5×10^4; thus, 1,000 m*l* of cultured Sf9 cells produced the equivalent of between 500 to 1,000 × 175 cm^2 flasks of 293 cells. This robust system provides a simple, cost-effective method for AAV vector production [18]. However, to achieve complete protection, other siRNAs targeting the RABV gene, the rAAV titer and the timing of therapeutic administration should be assessed to generate an optimal treatment strategy [20].

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