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Anti-tembusu virus of capsid-targeted viral inactivation delivered by lentiviral vector *in vivo*

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ABSTRACT

Tembusu virus (TMUV) is a member of genus *flavivirus*, which mainly causes decrease in production in egg ducks and neurological symptom in meatducks, causing serious economic losses to the poultry industry. Recently, the commercialized TMUV vaccines are mainly the WF100 live vaccine and the attenuated live vaccine (FX2010–180P), so it is particularly important to find new methods to combat TMUV. The capsid-targeted viral inactivation (CTVI) strategy is based on a viral core protein and an exogenous factor that can destroy viral DNA or RNA. Lentivirus vectors are an effective tool for transferring the recombinant lentiviruses to target cells and are a promising system for efficient gene delivery. This study injected recombinant lentivirus carrying the Cap-SNase and Cap-Linker-SNase fusion proteins into duck early embryos at 10^9 TU/mL, achieving widespread expression of the fusion proteins in duck embryo tissues. After TMUV infection, the symptoms of the ducks in the Cap-SNase and Cap-Linker-SNase groups were significantly alleviated to the 1640 group. Pathological sections showed that compared with the 1640 group, the pathological damage in the Cap-SNase and Cap-Linker-SNase groups was greatly alleviated, and the virus loads in the feces, blood and tissues of Cap-SNase or Cap-Linker-SNase groups were significantly lower than those in the 1640 group. The results indicate that the Cap-SNase or Cap-Linker-SNase fusion proteins delivered by lentivirus have anti-TMUV effect. This study combines lentiviral vectors with CTVI strategy for the first time, which could be a simple and practical technology to treating human or animal diseases or biomedical animals.

1. Introduction

Together with Dengue virus (DENV) (Roy and Bhattacharjee, 2021), Japanese encephalitis virus (JEV) (Zhu et al., 2023), Zika virus (ZIKV) and yellow fever virus (YFV), duck TMUV (DTMUV) is also a member of the genus Flavivirus, and is transmitted by arthropod vectors such as mosquitoes, midges, ticks and sandflies (Schnettler et al., 2012; Polonio and Peron, 2021). TMUV mainly affects egg production in breeder ducks causing high fever, reduced feed intake, and egg production losses. It can even lead to ataxia and paralysis in commercial meat ducks (Zhang et al., 2019), it can cause infection in ducks of all ages and breeds. Its genome is about 11 kb and contains only an open reading frame (ORF) that encoded a single polyprotein, which is processed into three structure proteins (capsid protein, Cap; precursor membrane, prM; envelope glycoprotein, E) and seven nonstructural (NS) proteins (NS1, NS2A/B, NS3, NS4A/B, 2 K, NS5) that are degraded by viral and host proteases (Qu et al., 2023).

The Cap protein is responsible for genome packaging and formation of the nucleocapsid (Saeedi and Geiss, 2013). Capsid-targeted viral inactivation (CTVI) has been applied to different types of DNA or RNA viruses (Zhang et al., 2016), such as retroviruses, hepadnaviruses, caulimoviruses, circoviruses, flaviviruses (Zhang et al., 2019; Pang et al.,

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2013). *Staphylococcus aureus* nuclease (SNase) is a non-specific phosphodiesterase secreted extracellular by pathogenic *gold Staphylococcus aureus*, which has strong degrading effects on single or double-stranded DNA or RNA (Wang et al., 2010). SNase is heat-resistant and does not lose its activity even after being treated at 100 ℃ for 10 min, so it is also known as thermostable nuclease. However, the activity of SNase is strictly regulated by the concentration of Ca^{2+} ions. The concentration of Ca^{2+} in human blood and other extracellular fluids is generally in the millimolar range, which activates the activity of SNase to exert its hydrolysis function. However, the concentration of Ca^{2+} in cell is generally in the nanomolar range, which is far lower than the concentration required for nuclease activity. Therefore, the nuclease does not exhibit enzymatic activity, i.e., the SNase is no cytotoxic (Zhang et al., 2016). Recent studies have shown that SNase can be used as an antiviral agent in the CTVI strategy for treatment of neoplastic and viral diseases (Zhang et al., 2019; Pang et al., 2013; Zhou et al., 2010). It has achieved certain results and shown promising prospects, thus becoming one of the most widely used antiviral drugs and favored by researchers.

In recent years, the development of lentiviral vectors has shown great potential for overcoming limitations, and the transfer of recombinant lentivirus has been proven to be an efficient method of transgene delivery. Therefore, it becomes a new effective tool for developing transgenic livestock at low cost and high efficiency in the field of transgenic animals (Chen et al., 2016). In this study, the CTVI strategy was combined with lentiviral vectors to explore the effect of SNase against DTMUV.

2. Materials and methods

2.1. Cells, animals, plasmids and virus

HEK293T cells were cultured in 1640 medium with 10 % fetal bovine serum (FBS, Gibco, Gaithersburg, MD, USA). All cells were maintained in a 5 % CO_2 humidified incubator at 37 ℃. 20 duck embryos were obtained from the Sichuan Agricultural University farm. The lentiviral packaging plasmid (PHBLV-3Flag-ZsGreen) was purchased from Hanbio, the helper plasmids (psPAX2, PMD2.G) were gifts from Dr. Gao Xinghong, and the recombinant lentiviral plasmids (PHBLV-Cap-SNase and PHBLV-Cap-Linker-SNase) were constructed previously. The DTMUV CQW1 strain (Genbank accession number: KM233707.1) was preserved in our laboratory.

2.2. Antibodies and reagents

The polyclonal antibodies against mouse anti-SNase serum was prepared in our lab. Flag-Tag mouse monoclonal antibodies, horseradish peroxidase (HRP)- labeled goat anti-mouse IgG, and fluorescein isothiocyanate (FITC)-tagged goat anti-mouse secondary antibodies were purchased from Beijing Biodragon Immunotechnologies Co., Ltd. and Proteintech, respectively. β-tubulin was purchased from Abmart.

2.3. Lentivirus packaging, concentration and titer determination

HEK293T cells were seeded in 10 cm dishes with a density of 10^5 cells/mL, and co-transfected with lentiviral core plasmids (PHBLV-3Flag-ZsGreen, PHBLV-Cap-SNase or PHBLV-Cap-Linker-SNase) and-packaging helper plasmids (psPAX2, PMD2.G) at a mass ratio 2:2:1 using Lipofectamine™ 3000. The lentiviral particles were collected at 24 h, 48 h, 72 h post-transfection. The supernatant containing lentivirus particles was filtered through a 0.22 μm syringe filter, concentrated at 2500 r/min for 1 h at 4 ℃ using an ultrafiltration device (Millipore), 200 μl of the concentrate was taken stored at −80 ℃ for later use.

To determine the lentivirus titer, HEK293T cells cultured in 96-well plates were infected with recombinat lentivirus diluted in 10-fold gradients.Wells with 30 % cell count fluorescence were selected for each group, and the lentivirus titer was calculated the formula: Titer (TU/mL) = Total number of cells × fluorescence× 10^3 percentage / volume of the virus original liquid (μl).

2.4. Lentivirus injection and duck embryos incubation

The 1-day-old duck embryos were divided into 4 groups, with 5 embryos in each group: 1640 group, lentivirus-PHBLV group, lentivirus-Cap-SNase group, lentivirus-Cap-Linker-SNase group. The recombinant lentivirus of each group were sterile injected into the allantoic cavity of 7-day-old duck embryos at a dose of 1×10^9 TU and then sealed with paraffin and incubated in an incubator until the duck hatched.

2.5. Detection of target protein expression in duck embryos

The brain, heart and liver tissues of the duck embryos were collected 96 h after infection with lentivirus. The collected tissues were crushed using a homogenizer, and 200 μl of each tissue was centrifuged collect the tissue cells. The tissue proteins were extracted with strong RIPA lysis buffer, heat treated for 10 min, separated by 10 % SDS-PAGE, and transferred onto PVDF membranes. The membranes were blocked with 5 % skim milk at room temperature for 1 h, and then incubated with Flag-tag mouse monoclonal antibody and β-tubulin overnight at 4 ℃. The second antibody was incubated for 1 h at room temperature, and the proteins were visualized using enhanced chemiluminescence (ECL) and quantified using Quantity One (Bio-Rad, 140 California, USA).

2.6. Growth status, body weight monitoring and histopathological observations of ducklings

From the birth of the ducklings, one duckling with similar weight was randomly selected from each group for weight monitoring, with monitoring conducted once one week for 5 weeks in each group. Ducklings were inoculated with 4×10^5 $TCID_{50}$ of DTMUV at 7 days of age. Samples were collected 13 days post-infection, including duck feces, whole blood and tissue samples (heart, liver, spleen, lung, kidney, brain). Tissues samples were cut into approximately $1.0 \times 1.0 \times 0.3$ cm^3 pieces for section preparation, fixed with 4 % paraformaldehyde, and the remaining tissues were stored at −80 ℃ for later use.

2.7. Detection of the anti-DTMUV effect of fusion protein in ducklings

50 mg of spleen and brain tissues from each group were weighed and then broken with crusher in 250 μl PBS, and the tissue suspension was taken. 750 μl Trizol reagent was added for RNA extraction, then performed reverse transcription, following the instructions. The feces and blood were also extracted with Trizol.

All cDNA was test by RT-qPCR with DTMUV Cap gene primers for analysis of the virus copy number. The Cap gene primers were as follows: forward 5' -AGGTTTGTGCTGGCTCTAC-3' and reverse 5'-TGTTTGGTCGCCTCATT-3'. The PCR conditions were as follows: denaturation at 95 ℃for 3 min, followed by 39 cycles of denaturation at 95 ℃ for 10 s, 62.5 ℃ for 30 s, melting curve from 65 to 95 ℃ for 3 s.

2.8. Pathological observation of the tissues

The brain, spleen, liver, heart, lung, and kidney tissues fixed with 4 % paraformaldehyde were embedded in paraffin, stained with HE, and made into pathological sections. a digital pathological scanner was used to scan the tissue sections, the pathological changes in each group was observed and analyzed.

2.9. Statistical analysis

All data were analyzed using GraphPad Prism 6.0 software (GraphPad software, La Jolla, CA, USA). Statistical significance was carried out using Student's *t*-test. Data were expressed as the mean standard

deviation (SD). * , * *, * ** , * ** * indicate statistical significance (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ****: $P < 0.0001$).

3. Results

3.1. Lentivirus titer determination

The concentrated lentivirus titer was tested using HEK293T cells, and obvious green fluorescence was observed in the cells at 36 h, as shown in Fig. 1. In the group infected with 10 μl of lentivirus, fluorescence rate of the cells was about 100 %, while in the groups with 1 μl and 0.1 μl gradients, the fluorescence rates were about 50 % and 30 %, respectively.

The lentivirus titer was determined using HEK293T cells, which were cultured in 96-well plates and infected with lentivirus diluted at a 10-fold gradient. The wells with a fluorescence ratio of 30 % were selected as the cells for each group.

The lentivirus titer was calculated according to the formula: Titer (TU/mL) = Total cell number × fluorescence percentage × 10^3/ Virus original liquid volume (μl). The lentivirus titer was approximately 10^9 TU/mL (Table 1).

3.2. Detection of fusion protein expression in duck embryo

The visceral tissues of the slow virus infected duck embryos were collected for Western Blot analysis. The results are shown in Fig. 2. The bands of brain, heart and liver were between 35 and 40 kDa, which is consistent with the expected size.

Table 1
Calculation of lentiviral titers.

Groups	Numbers of cells	Percentage fluorescence (%)	Viral stock volume (μl)	Virus titer (TU/ mL)
PHBLV−3Flag-ZsGreen	0.41×10^6	30	10^{-1}	1.23×10^9
PHBLV-Cap-SNase	0.39×10^6	30	10^{-1}	1.17×10^9
PHBLV-Cap-Linker-SNase	0.35×10^6	30	10^{-1}	1.05×10^9

3.3. Growth performance, body weight monitoring, and histopathological observation of ducklings

The ducks in the 1640 and lentivirus-PHBLV groups showed depression after infection with DTMUV, mainly as neurological symptoms, as shown in Fig. 3A, characterized by unsteady gait, falling, lateral recumbency, head and neck tilt. The ducks in the lentivirus-Cap-SNase and lentivirus-Cap-Linker-SNase groups had soft, glossy feathers and a normal physique. Additionally, the ducks in the other two groups, 1640 and lentivirus-PHBLV, had messy feathers and a smaller physique. The body weights of ducks in each group were monitored as shown in Fig. 3B. After infection with DTMUV, the body weights of ducks in 1640 and lentivirus-PHBLV groups were significantly lower than those in the

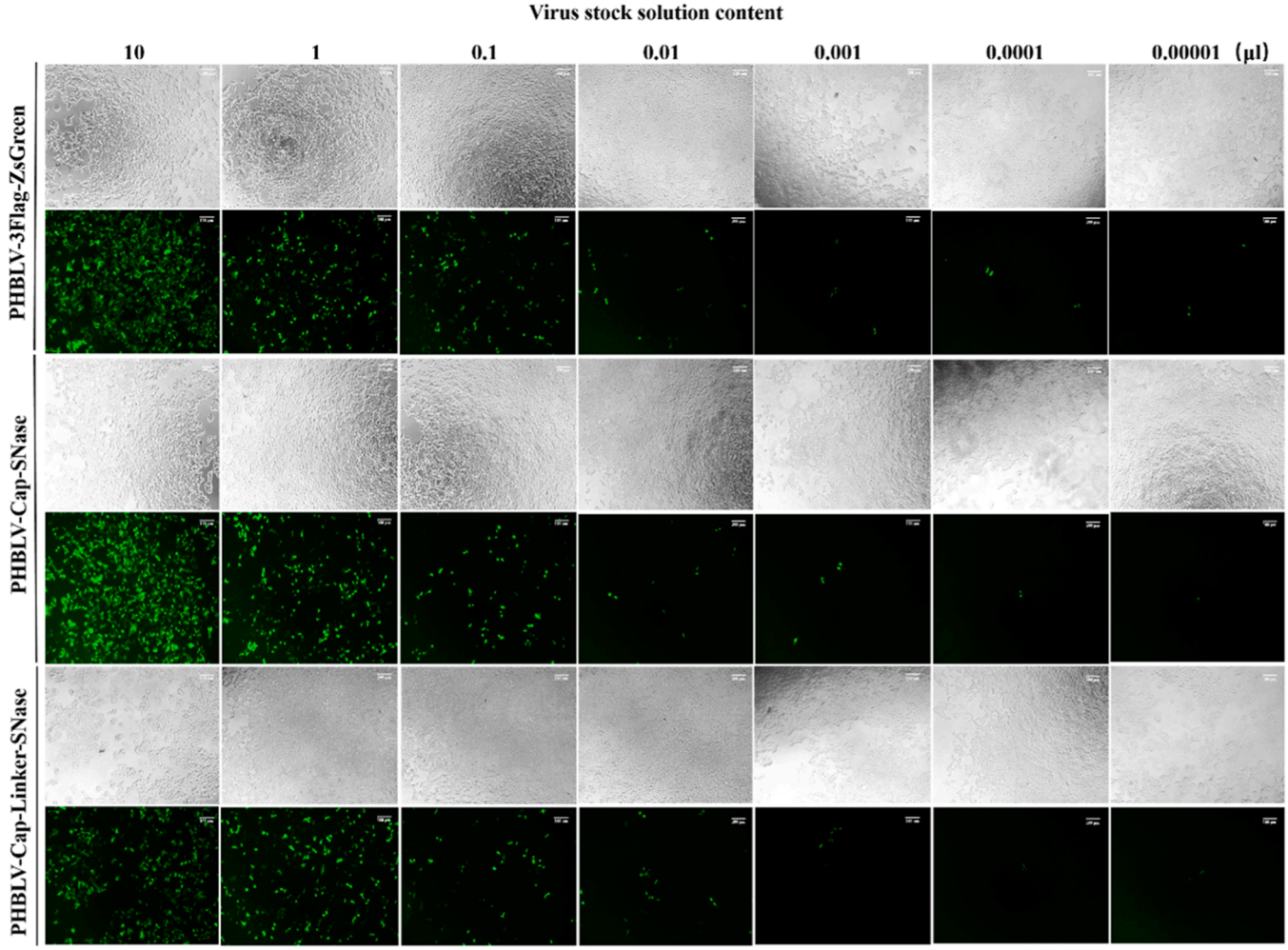

Fig. 1. Lentivirus titer assay fluorescence observation.

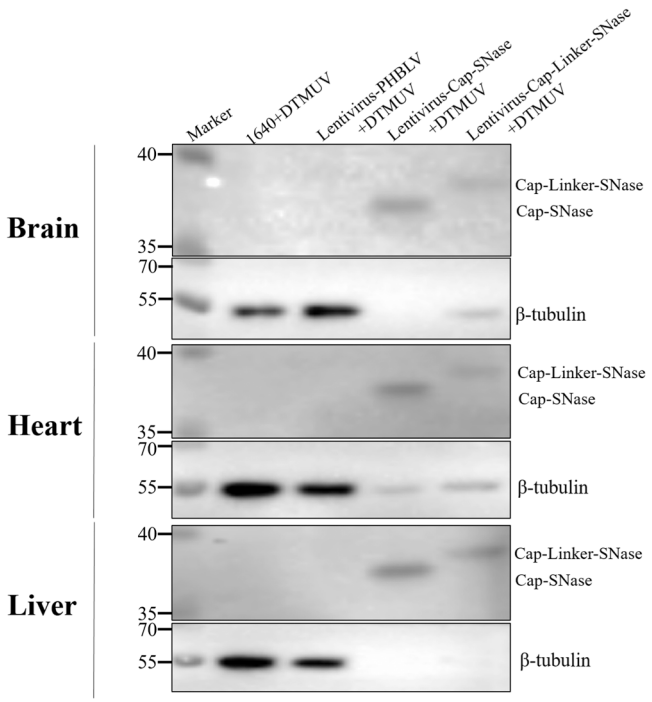

Fig. 2. Detection of target protein expression in duck embryo tissue.

lentivirus-Cap-SNase and lentivirus-Cap-Linker-SNase groups, with retarded growth. The diseased ducks died mostly because of difficulty in drinking and eating due to nervous system symptoms. Gross pathological examination of the dead ducks, as shown in Fig. 3C, revealed that the ducks in the 1640 and the lentivirus-PHBLV groups had mild edema of the meninges, increased blood vessels on the surface of the meninges, and hemorrhage of the meninges. The brain tissues of the lentivirus-Cap-SNase and lentivirus-Cap-Linker-SNase groups were light pink, with a small amount of blood on the surface of the meninges. The spleens and livers of the 1640 and lentivirus-PHBLV groups were significantly smaller than those of the lentivirus-Cap-SNase and lentivirus-Cap-Linker-SNase groups. The spleen of the PHBLV group was congested and dark red, with lentivirus-Cap-SNase being dark red and lentivirus-Cap-Linker-SNase being light red, all of normal size. The surface of duck liver in the 1640 and lentivirus-PHBLV groups showed spotted necrotic lesions and small area of yellowish-brown stove.

3.4. Analysis of tissue lesions caused by DTMUV infection in ducks

In order to further analyze the pathological symptoms of DTMUV infected ducks affected by the fusion protein, tissue pathological sections were made and HE staining was performed. As shown in Fig. 4A, the brain tissue results showed that the meninges were ruptured with a large amount of red blood cells flowing after DTMUV infection in the 1640 and lentivirus-PHBLV groups, while the symptoms of hyperglobulinemia caused by meninge rupture were significantly relieved after DTMUV infection in the lentivirus-Cap-SNase and lentivirus-Cap-Linker-SNase groups. As shown in Fig. 4B, the red and white pulp boundaries were indistinct in the 1640 +DTMUV group, with an increased white pulp area, while the white pulp area was reduced in the lentivirus-PHBLV group, with a significantly red pulp area. DTMUV infection can lead to an increase in the number of red blood cells in the red pulp area, resulting in splenic hemorrhage congestion. In the lentivirus-Cap-SNase and lentivirus-Cap-Linker-SNase groups, the pulp area and the pulp border were obviously red white, and the symptoms were significantly alleviated. Observation of liver tissue as shown in Fig. 4C revealed that the liver presented with fatty degeneration, with obvious fat vacuoles, cytoplasm of hepatocytes had vacuoles of varying sizes, with clear edges. Some of the vacuoles were large, squeezing the nucleus to side, while the rate of vacuole formation in the lentivirus-

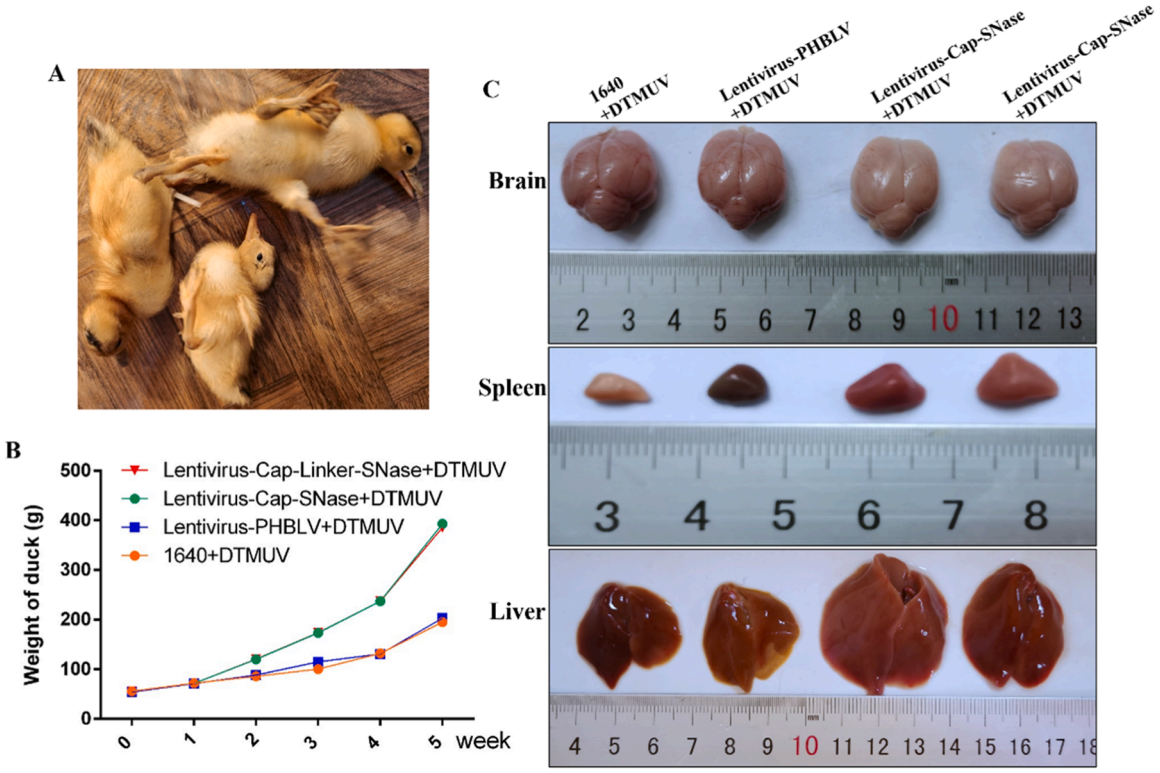

Fig. 3. Effects of fusion protein on growth status, body weight and tissues of ducks infected with DTMUV. A. Effect of DTMUV infection on the growth status of ducks. B. Monitoring of the body weight of ducks. C. Macroscopic histopathological observation of duck anatomy.

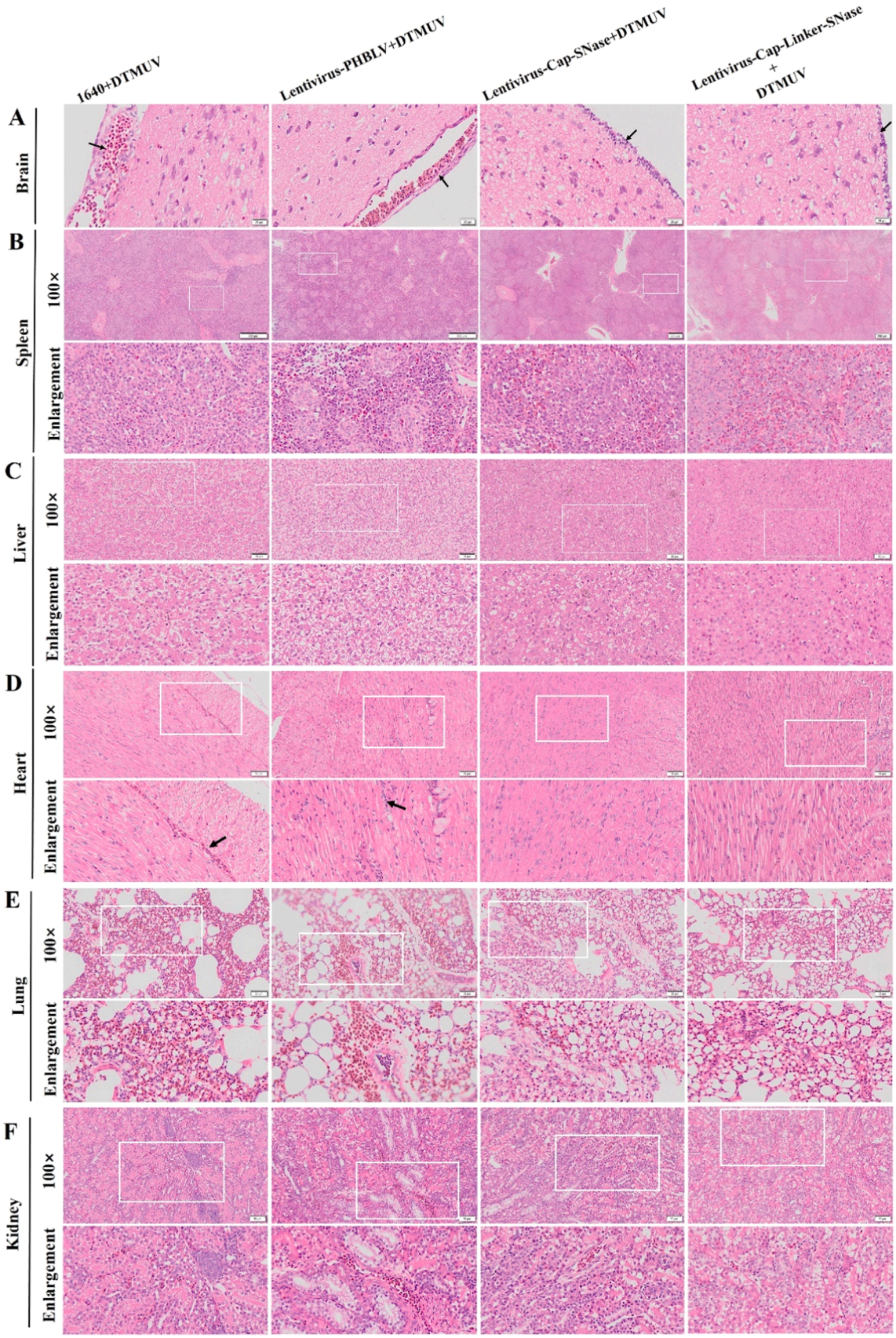

Fig. 4. The histopathological effects of DTMUV infection. A-F: Pathological changes in brain, spleen, liver, heart, lung, and kidney tissues after DTMUV infection.

Cap-SNase group were slightly slower than that in the first two groups. The lipovacuole symptoms in the lentivirus-Cap-Linker-SNase group were significantly alleviated compared with the other three groups. As shown in Fig. 4D, the hearts in the 1640 group showed mild hemorrhagic symptoms, and the lentivirus-PHBLV group showed heart atrophy and aggregation, while no abnormalities were found in the hearts of the lentivirus-Cap-SNase and lentivirus-Cap-Linker-SNase groups. The lung (Fig. 4E) and kidney (Fig. 4F) tissues of each group also had hemorrhages, but theages in the 1640 and lentivirus-PHBLV groups were more obvious than those in the lentivirus-Cap-SNase and lentivirus-Cap-Linker-SNase groups.

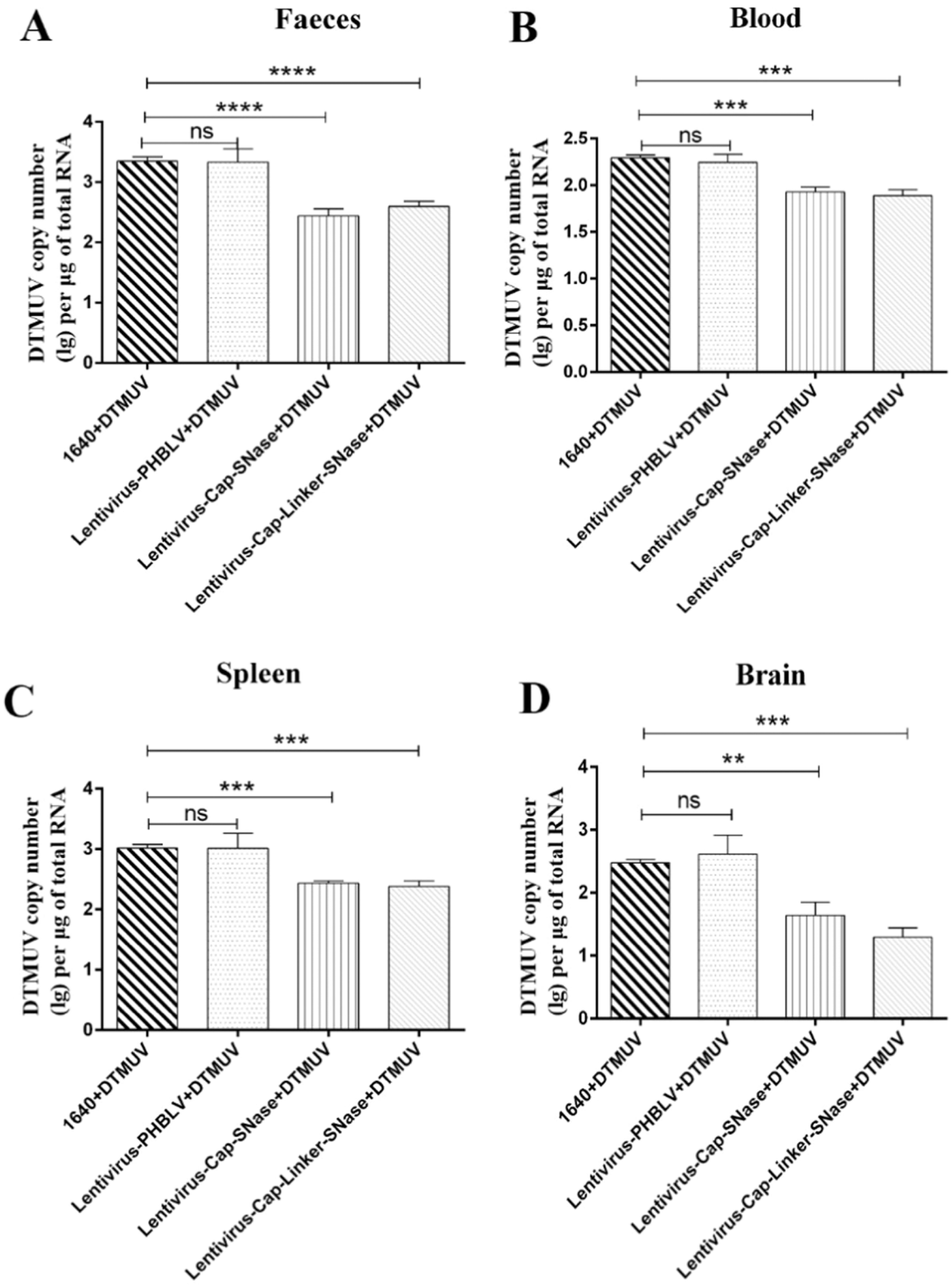

Fig. 5. Effect of the fusion protein on DTMUV virus in feces, blood and tissue.

3.5. Effect of fusion protein on DTMUV in stool, blood, spleen and brain

After DTMUV infection, ducks excreted green feces, and the fecal virus copy number was detected, as shown in Fig. 5A. Compaired with the 1640 group, the virus content in the lentivirus-PHBLV group was not significant ($P = 0.8638$), but the fecal virus copy number in the lentivirus-Cap-SNase and lentivirus-Cap-Linker-SNase groups was significantly lower than that in 1640 group ($P < 0.0001$).

The above pathological sections show that the fusion proteins Cap-SNase and Cap-Linker-SNase can alleviate the target organ tissue lesions caused by DTMUV, which may be due to the fusion proteins affecting viral replication. To test this hypothesis, we also measured the viral load in the blood in ducks of each group, as shown in Fig. 5B, there was no difference in viral load in blood between the 1640 and lentivirus-PHBLV groups ($P = 0.4069$), while the viral load in the lentivirus-Cap-SNase or lentivirus-Cap-Linker-SNase was significantly lower than that of the 1640 group ($P < 0.0005$).

Since the main target organs of DTMUV are spleen and brain tissues, we investigated the viral copy number of them. As shown in Fig. 5 C/D, no significant difference in spleen and brain viral load was observed between the 1640 and lentivirus-PHBLV group ($P = 0.9565$ and 0.4743, respectively). The viral loads in the spleen tissues of the lentivirus-Cap-SNase and lentivirus-Cap-Linker-SNase groups were significantly lower than those in the 1640 group (spleen: $P < 0.0001$, $P < 0.0005$, respectively), brain: $P < 0.0024$ and $P < 0.0002$, respectively).

4. Discussions

TMUV mainly causes severe hemorrhage of the follicles in egg-laying ducks, with obvious turbidity of the egg yolk, which leads to decrease in egg production. In meat ducks, it manifested as neurological symptoms, mild cerebral hemorrhage, testicular hemorrhage, atrophy, decreased sperm quality in males, resulting in low fertilization rates. CTVI is a novel antiviral strategy based on protein that has been successfully applied to several types of viral species. In flavivirus genus, CTVI has been successfully applied to JEV (Pang et al., 2013) and DENV (Qin and Qin, 2006). It takes advantage of the key viral protein fused with exogenous molecules such as nucleases, lipases, proteases, and scab antibodies (Zhang et al., 2016). The Cap protein of *flaviviride* is responsible for packaging the genome and capsidation, thereby synchronously assembling the exogenous molecules into the virus particles, specifically and efficiently destroying the virus components from the inside. The activity of SNase is regulated by Ca^{2+} concentration, which is generally in the millimolar level in human blood and other extracellular body fluids, activating SNase to perform its enzymatic function. However, in intracellular, the Ca^{2+}concentration is nanomolar range, far below the required for SNase nuclease activity, so the SNase does not exhibit enzymatic activity. That is to say, it is no cytotoxic, making SNase a suitable candidate for CTVI. In the CTVI strategy, the capsid protein serves as the carrier for SNase, it can efficiently prevent immune escape mutants (Zhou et al., 2010). Because the fusion protein can incorporate into the host genome, thus no specific antigens are produced. When the viruses replicate and assemble in the host cells, the fusion proteins are incorporated into virions during packaging. The capsid protein binds the viral RNA to form the nucleocapsid, so the SNase in the fusion protein can degrade the viral RNA from within, evading the cap specific immunity (Zhang et al., 2016). Theoretically, the nuclease can efficiently disable virus particles even when only one nuclease molecule is incorporated into the virion (Zhou et al., 2010). Animal transgenesis is one of the most powerful technical advancements. To date, various transgenic species have been generated by lentiviral transgenesis, including mice, fish, chickens, pigs, non-human primates, cows and sheep (Milone and O'Doherty, 2018). The first transgenic pigs were produced by lentivirus injecting into zygote, with a production rate of 19–33 % (Hofmann et al., 2003). Animal retroviral vectors have been widely used in research and clinical trials, and are powerful tools for modifying eukaryotic cell genes, with application prospects in human and animal gene therapy (Wang et al., 2021).

In this study, we first combine the CTVI strategy with lentivirus vector for anti-TMUV research, and detected the target genes in brain, heart and liver tissues of duck embryos, indicating that the lentvirus infected the duck embryos successfully. And the fusion proteins with the addition of flexible linker can better maintain the function of their respective proteins, and it shows better antiviral effect. The variation in the infection scores might be related to the animal species and lentiviral titer injected. We then executed the lentivirus at 10^9 TU/mL. The mortality rate of the lentivirus-infected duck was not significantly from that of the non-infected duck, indicating that the lentivirus did not interfere with the development of ducks. Lentivirus-based vectors have entered clinic trial as promising vehicles for antiviral gene therapy or vaccine applications. CTVI strategy with lentivirus vector has higher antiviral efficacy compared with other methods.

Authors' contributions

Xingcui Zhang conceived and designed of the study, execute the experiment and wrote the paper; Renyong Jia conceived the study and designed the experiments; Ning Luo and Hui Ni helped complete the experiments; Mingshu Wang, Shun Chen, Dekang Zhu, Mafeng Liu, Xinxin Zhao, Qiao Yang, Ying Wu, Shaqiu Zhang, Zhongqiong Yin, Juan Huang, Bin Tian guided the experiments, helped analyse the data and edited the English language in the manuscript; and Anchun Cheng was responsible for revising the manuscript critically for expert content. All authors read and approved the final manuscript.

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CRediT authorship contribution statement

Xinxin Zhao: Supervision. **Mafeng Liu:** Writing – review & editing, Supervision. **Dekang Zhu:** Supervision. **Shun Chen:** Supervision. **Bin Tian:** Supervision, Conceptualization. **Mingshu Wang:** Supervision. **Juan Huang:** Writing – review & editing, Supervision. **Anchun Cheng:** Project administration. **Bo Jing:** Software. **Hui Ni:** Software, Resources. **Zhongqiong Yin:** Writing – review & editing, Supervision. **Ning Luo:** Software, Resources. **Shaqiu Zhang:** Supervision, Investigation. **Xingcui Zhang:** Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Ying Wu:** Writing – review & editing, Supervision. **Renyong Jia:** Funding acquisition, Conceptualization. **Qiao Yang:** Methodology.

Declaration of Competing Interest

The authors have no competing interests to declare.

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