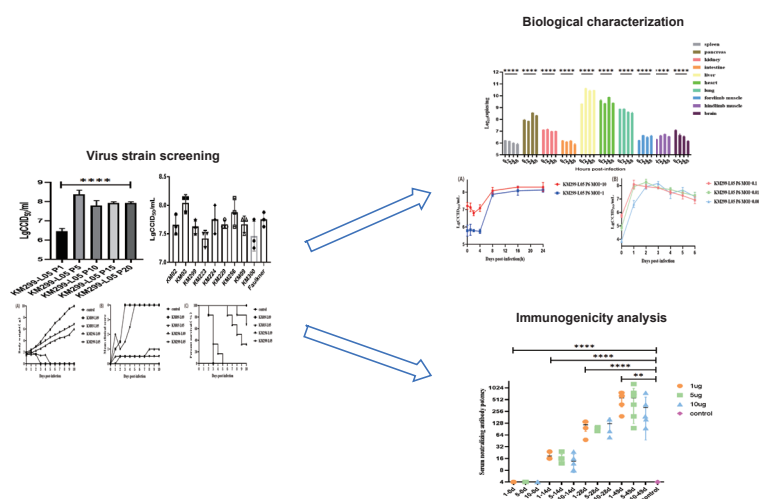




ORIGINAL ARTICLE

A New Vaccine Candidate Strain of Coxsackievirus B5 Selected on the Basis of its Biological Properties

Graphical abstract



Highlights

- The CVB5 candidate vaccine strain derived from a prevalent Chinese CVB5 strain was successfully screened on the basis of its biological properties.
- The inactivated CVB5 vaccine elicited neutralizing antibodies with a titer range from 1:384 to 1:768 against a variety of clinical CVB5 isolates, particularly the prototype strain. Maternal antibodies completely protected all tested suckling BALB/c mice against lethal challenge.
- Histopathological changes were observed in the liver, heart, pancreas, and lung tissues, and the highest viral loads were found in these tissues.

Authors

Yuhan Liu, Zhaoyang Chu, Ming Zhang, Junwei Chen, Jiansheng liu, Changzeng Feng and Shaohui Ma

Corresponding author

shaohuima70@hotmail.com (SM),
Tel.: +86-13116244041

Brief statement

The prevalent Chinese strain KM299-L05 was screened and subjected to phylogenetic analysis, sequence comparison, virulence assessment in BALB/c mice, Vero cell acclimation, and three consecutive rounds of plaque purification. An inactivated CVB5 vaccine derived from strain KM299-L05 produced neutralizing antibodies with titers ranging from 1:384 to 1:768 against a wide range of clinical CVB5 isolates, particularly the prototype strain, and maternal antibodies completely protected all examined suckling BALB/c mice against lethal challenge. Our results suggested that strain KM299-L05 has potential as a novel CVB5 vaccine candidate in China and provide a rationale for CVB5 vaccine development.

A New Vaccine Candidate Strain of Coxsackievirus B5 Selected on the Basis of its Biological Properties

Yuhan Liu^{1, #}, Zhaoyang Chu^{1, #}, Ming Zhang^{1, #}, Junwei Chen^{1, 2}, Jiansheng liu¹, Changzeng Feng¹ and Shaohui Ma^{1, *}

Abstract

Objective: Evidence indicates that coxsackievirus B5 (CVB5) can cause meningitis, and hand, foot, and mouth disease (HFMD), and is associated with type 1 diabetes. No effective drug or vaccine is available for treating CVB5 infections or controlling outbreaks.

Methods: The Vero-cell-adapted vaccine candidate strain KM299-L05 underwent comprehensive phylogenetic analysis, sequence alignment, assessment of virulence in BALB/c mice, Vero cell acclimation, three successive rounds of plaque purification, preparation of experimental vaccines, and immunogenicity evaluation.

Results: KM299-L05 was the most virulent strain in BALB/c mice. Its infectious titer reached $10^{8.0}$ CCID₅₀/mL in Vero cells, and it belonged to the Chinese epidemic genotype C3. An inactivated CVB5 vaccine derived from the KM299-L05 strain produced neutralizing antibodies with a titer range from 1:384 to 1:768 against a variety of clinical CVB5 isolates, particularly the prototype strain. Maternal antibodies fully protected all examined suckling BALB/c mice against lethal challenge.

Conclusion: Although the study was limited to neonatal mice, our findings suggested that the KM299-L05 strain has potential as a novel CVB5 vaccine candidate in China, and provide a rationale for the development of a CVB5 vaccine.

Key words: coxsackievirus B5, Vero cell, animal model, inactivated vaccine, immunogenicity

[#]These authors contributed equally to this work and share first authorship.

***Corresponding author:**

E-mail: shaohuima70@hotmail.com (SM), Tel.: +86-13116244041

¹Institute of Medical Biology, Chinese Academy of Medical Sciences, and Peking Union Medical College, Kunming 650118, PR China

²School of Life Sciences, Yunnan University, Kunming 650504, China

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INTRODUCTION

Coxsackievirus B5 (CVB5) is a member of the enterovirus B species, which belongs to the Picornaviridae family. CVB5 can induce hand, foot, and mouth disease (HFMD) [1], aseptic meningitis [2–4], acute flaccid paralysis [5], type 1 diabetes [6,7], and fatal infection. Since the HFMD outbreak in China in 2008, cases of HFMD caused by CVB5 have frequently been reported [8–10]. In 2009,

this virus was the main pathogenic cause of HFMD in Shandong, China [10]. Moreover, an increasing trend in CVB5 infection prevalence has been observed in recent years [11–13].

CVB5 is a non-enveloped, positive-strand RNA virus with a genome length of ~7,400 nucleotides. The genome contains an open reading frame and encodes a protein of 2,185 amino acids [1]. Cleavage of the polyprotein generates

three precursor proteins (P1, P2, and P3). P1 is divided into four structural proteins: VP1 to VP4. P2 and P3 are divided into seven non-structural proteins: 2A to 2C and 3A to 3D.

At present, because no specific drug or vaccine is available for CVB5, a safe and effective vaccine is urgently needed. Only two forms of CVB5 vaccine are in development. The first is a hexavalent vaccine composed of formalin-inactivated whole CVB1–6, which has been shown to effectively prevent the onset of CVB-associated myocarditis and diabetes in experimental animal models [14], and to be tolerated and immunogenic in a phase 1 trial (NCT04690426) [15]. However, the CVB5 vaccine strain used was a prevalent foreign strain with a different genotype from the current prevalent Chinese strain. The other vaccine comprises virus-like particles (VLPs). The CVB5 VLP vaccine has been shown to induce relatively high levels of neutralizing antibodies and has provided robust immunoprotection in animal models [16]. For prevention of enterovirus infection, only attenuated or inactivated poliovirus vaccines [17], and an inactivated enterovirus A71 vaccine [18], are available on the market to date. However, these vaccines do not provide cross-protection against CVB5 [19]. Although attenuated live vaccines are also widely used, they pose safety concerns, because of their potential for reversion to viral virulence [20,21]. Therefore, inactivated vaccines remain an ideal form for the development of CVB5 vaccines. Since the prototype strain Faulkner was isolated in the USA in 1952 [22,23], CVB5 isolates have evolved into four genotypes [24]. Variations in cross-neutralization among enterovirus A 71 genotypes have been reported [25]. Whether the formalin-inactivated CVB5 vaccine currently under development can efficiently elicit neutralizing antibodies (NAbs) against other CVB5 genotype strains remains to be validated. To address this research gap, we selected the CVB5 vaccine strain associated with the Chinese epidemic for the development of an inactivated CVB5 vaccine in this study. Animal experiments demonstrated that the inactivated CVB5 vaccine exhibited relatively high levels of neutralizing antibodies among the same genotypes and conferred superior immunoprotection.

METHODS

Cells and viruses

From 2010 to 2022, CVB5 strains KM03 (MW762688), KM02, KM223, KM224, KM229, KM299, KM256, KM09 (JX843811), and KM300 were isolated from stool samples of patients with HFMD in Yunnan, China. CVB5 strains and CVB5 prototype strain Faulkner were stored at the Institute of Medical Biology, Chinese Academy of Medical Sciences, and Peking Union Medical College, China (S1 Table).

Vero cells (Vero 76, ATCC CRL-1587) were used to grow CVB5 and to produce CVB5 vaccines. The Vero cells were cultured in minimal essential medium (MEM) with 10% neonatal bovine serum (Gibco, Invitrogen,

USA), 2% glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C under a 5% CO₂ atmosphere.

Infectious titers

The infectious titers of the virus were tested with a microtissue culture method [26]. Serial 10-fold dilutions of the samples were inoculated into Vero cells (10⁴/well) in 96-well plates and cultured at 37°C in the presence of 5% CO₂ for 7 days. The 50% cell culture infectious dose (CCID₅₀) was quantified with the Reed–Muench method. One-way ANOVA was used to analyze various MOIs. Infectious titers of CVB5 stocks were quantified with the CCID₅₀ assay with the Reed–Muench method.

Plaque assays

Vero cells were seeded in six-well plates at 6.0×10^5 cells/well and incubated for 24 h, at 37°C under 5% CO₂. On the following day, 10-fold serial dilutions of the viral stock were prepared, and three dilutions from 10⁻³ to 10⁻⁵ were added to each well in duplicate (300 µL/well). After a 90-min adsorption at 37°C with 5% CO₂, the viral inoculum was aspirated, and cell monolayers were gently rinsed twice with 2 mL PBS per well. Subsequently, 3 mL semi-solid medium (1.5% methylcellulose in 1 × DMEM with 2% FBS and 1% penicillin–streptomycin) was added to each well. The plates were transferred to a 35°C, 5% CO₂ incubator for a 5-day observation period, during which plaque formation was monitored daily with light microscopy. When plaques with clearly defined cytopathic effect (CPE) foci emerged, 10–25 plaques of various sizes were selected under a light microscope with 10-µL tips. These plaques were cultured in Vero cells to produce CPEs. After culturing, the viral RNA was extracted from the cultured samples. The viral VP1 gene was amplified and sequenced with the primer pair B–OS and B–OAS. Finally, samples that exhibited rapid plaque formation and had sequences consistent with the parental virus strains, or only minor variations, were selected for the next round of plaque assays.

RT-PCR and sequencing

We amplified the VP1 sequences and the full-length genomes of CVB5 strains, as described previously [27]. Culture supernatants were used to extract viral RNA with an Axygen Body Fluid viral DNA/RNA Miniprep Kit (Axygen, Hanzhou, China). The full-length VP1 gene and full-length genome sequences were amplified with a PrimeScript One Step RT-PCR Kit Ver. 2 (Takara, Dalian, China) and subsequently sequenced with an ABI 3130 Genetic Analyzer (Applied Biosystems, USA). Primers used for amplification and sequencing are shown in S2 Table. Enterovirus types were identified with the Enterovirus Genotyping Tool available online at (<https://www.rivm.nl/mpf/typingtool/enterovirus/>).

Phylogenetic analysis and sequence alignment

Phylogenetic analyses were conducted in Molecular Evolutionary Genetic Analysis (MEGA) (7.0.26) software

(neighbor-joining method, 1,000 bootstrap replicates). The evolutionary distances were analyzed with the Kimura 2-parameter method [28]. The sequences were aligned with Geneious alignment in Geneious 9.0.2 software [cost matrix: 65% similarity (5.0/−4.0); gap open penalty 12, gap extension penalty 3, alignment type: global alignment with free and gaps] [29].

Animal infection

BALB/c mice 1–4 days old were injected intracerebrally (i.c.) with CVB5 strains (20 μ L per mouse) as previously described [30]. Control mice were injected i.c. with 20 μ L uninfected cell supernatant, then kept separately from virus-inoculated mice and monitored for 10 days. Each group included six to eight mice. Clinical symptoms were scored as follows: 0, healthy; 1, lethargy and inactivity; 2, weight loss; 3, weakness or paralysis of the limbs; and 4, dying or death [31].

Genetic stability of the KM299-L05 strain

The KM299-L05 strain underwent 20 successive passages to test for genetic stability. The full genomes of P1, P5, P10, P15, and P20 were amplified, sequenced, and aligned. Through the Reed–Muench method, the infectious titers of P1, P5, P10, P15, and P20 were determined.

Growth kinetics of KM299-L05 P6 strain

Vero cells (90–95% confluent monolayers) were inoculated with the KM299-L05 P6 strain at an MOI of 0.001, 0.01, or 0.1. The viral inocula were removed after 1 h of adsorption at 37°C. Subsequently, the cells were washed three times with PBS, 1 mL MEM was added, and the cells were incubated at 35°C under 5% CO₂. Infected cells were collected at regular intervals (every 24 hours) over a period of 6 days. After three freeze–thaw cycles, viral samples were stored at −80°C. Three replicate tests were conducted per time point.

Histopathology and immunohistochemistry

Histopathology and immunohistochemistry analyses were performed as previously described [26]. A comparison of CVB5 sensitivity in 1–4-day-old BALB/c mice indicated that 1-day-old BALB/c mice were the most sensitive to CVB5 (data not shown); therefore, these mice were used in subsequent experiments. The mice were inoculated with the KM299-L05 P6 strain ($10^{6.05}$ CCID₅₀/20 μ L), and dying mice were anesthetized and killed. Tissues were removed, fixed with 4% formalin, dehydrated, permeabilized, and embedded with paraffin. After hematoxylin and eosin staining, tissue sections were examined histopathologically. For immunohistochemistry, tissue sections were dewaxed, dehydrated, and boiled for 15 min, then incubated overnight with rabbit polyclonal anti-CVB5 antibody (1:500 dilution, Institute of Medical Biology, Chinese Academy of Medical Sciences, Yunnan, China). After being washed, the samples were incubated with goat anti-rabbit secondary antibody at room temperature for

50 min, then visualized with a histochemical DAB color development kit (Servicebio, Beijing, China).

CVB5 viral loads in various organs

Quantitative real-time PCR was performed to determine the viral loads in various tissues from 1-day-old BALB/c mice inoculated with KM299-L05 P6, as previously reported [26]. RNA standards were transcribed from the clone plasmid pMDTM18-TCVB5. After viral challenge, at intervals of 6, 12, 24, and 48 h, tissues were removed and weighed. These tissues included the blood, spleen, lung, pancreas, liver, kidney, heart, small intestine, forelimb and hindlimb muscle, and brain. Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Viral RNA quantification was performed with a One Step PrimeScriptTM RT-PCR Kit for Perfect Real Time PCR (Takara Dalian, China) and the following specific primers: CVB5-qP-F: 5′-TAATACGACTCACTATAGGGTGCAGATCAGCATGCGTCTT-3′; CVB5-qP-R: 5′-GCTGCCATCATTCACGTGTC-3′.

Additionally, a CVB5 probe (6-FAM-CCCTAGTGTGTTCTGGACTGAAGGGA-BHQ1) was used. A standard curve was established with the equation $Y = -3.30X + 48.42$, $R^2 = 0.99$. The assay's detection threshold was determined to be 3.99 copies/ μ L through analysis of CVB5 standard samples.

Preparation of inactivated CVB5 vaccine

Inactivated CVB5 vaccine was prepared as previously reported [26,32,33]. The CVB5 KM299-L05 P6 strain (MOI 0.01) was inoculated into Vero cells and incubated at 35°C for 2–3 days. When a CPE of 90% was observed, the culture medium was harvested through three freeze–thaw cycles and filtered through a 0.45- μ m filter, mixed with 4,000:1 formaldehyde (Sigma-Aldrich, St. Louis, MO, USA), and inactivated for 7 days at 37°C. The viral inactivation was verified through three successive passages in Vero cells. When no CPE was observed after three passages in Vero cells, the inactivated viral solution was concentrated with a 100-kDa PES membrane (Millipore, Billerica, MA, USA), then purified in Capto Core 400 gels. Vaccine concentration was determined with a PierceTM BCA Protein Assay Kit (Rockford, IL, USA). The inactivated CVB5 experimental vaccine was formulated from M199 culture and aluminum.

Immunogenicity of the inactivated CVB5 vaccine

Four-week-old female BALB/c mice were randomly assigned to four groups of five mice each. On days 0, 14, and 28, three groups were immunized i.p. with 1, 5, or 10 μ g inactivated CVB5 vaccine in 100 μ L [29]. The negative control group was immunized i.p. with 100 μ L adjuvant and M199 mixture. Blood samples were collected on days 0, 14, 28, and 49 post-immunization. After immunization, the clinical signs of all mice were monitored daily.

NAb titer was calculated with the Reed–Muench method, as previously reported [26]. Collected sera were inactivated at 56°C for 30 min. Subsequently, 50 µL of various diluted antiserum samples were mixed with 50 µL 100 CCID₅₀ CVB5 (Faulkner, KM299-L05, KM09-L09, and KM300) in 96-well plates in three replicates. Vero cells (10⁴/well) were added to the 96-well plates after incubation at 37°C for 2 h, then incubated at 37°C for 7 days. Vero cells and antiserum served as controls. Virus back-titration was performed as follows: 50 µL CVB5 virus (Faulkner, KM299-L05, KM09-L09, and KM300) was diluted 10-fold in four replicates per dilution, and mixed with 50 µL MEM (without fetal bovine serum). Subsequently, 10⁴ Vero cells were added to the 96-well plates. If the infectious titers of these viruses were within the range of 32–320 CCID₅₀/50 µL after 7 days, the experiment was considered valid. One-way ANOVA was used to compare vaccine doses.

Maternal antibody protection

For evaluation of maternal antibody protection, 4-week-old female BALB/c mice (two per group) were i.p. injected with 10 µg inactivated CVB5 vaccine in 100 µL three times in a 2-week interval. The control group was injected with 100 µL adjuvant and M199 mixture. When the female mice were pregnant, a third immunization was administered. After birth, 1-day-old BALB/c mice were injected i.c. with 10^{6.05} CCID₅₀/20 µL of the homologous CVB5 KM299-L05 strain. Clinical signs including weight were monitored daily in all mice and scored.

Statistical analysis

The data analyses were performed in GraphPad Prism 9.0.0 (San Diego, CA, USA). Survival and mortality were evaluated with Mantel–Cox log rank software. Other analyses (tissue viral loads and NAb) were performed with one-way ANOVA, and $p < 0.05$ was considered to indicate significance.

Accession numbers

The full VP1 gene sequences of CVB5 strains in this study have been deposited in GenBank under accession numbers PQ553574–PQ553580. The full genome sequences of generations 1, 5, 10, 15, and 20 of KM299-L05 and KM03-L05 have been deposited in GenBank under accession numbers PQ553581–PQ553585.

RESULTS

Phylogenetic analysis of the VP1 sequences

According to the VP1 segment, differences of >15 nucleotides were defined as genotypes, whereas differences of ≤7.5% were defined as sub-genotypes. CVB5 strains were divided into four genotypes, A, B, C, and D. The C genotype was further divided into three sub-genotypes, C1, C2, and C3, whereas the D genotype was divided into four sub-genotypes, D1, D2, D3, and D4. Genotype A

was represented solely by the Faulkner prototype strain. Genotype B included isolates from the Netherlands in 1996; an isolate from South Korea in 2000; isolates from Shandong, China, in 1998; and isolates from Taiwan, China, in 1995 and 2002–2003. The C1 and C2 sub-genotypes were represented by isolates from India in 2007–2009 and an isolate from Central Asian countries in 1990–1992. Additionally, the C3 sub-genotype included most of the endemic strains in mainland China during 2002–2022, as well as several isolates from other countries, including France and South Korea in 2005 and Russia in 2013. The D1 sub-genotype included isolates from 1987 to 1992 in Europe, Oceania, and North America. The D2 sub-genotype included isolates from 1970 to 1987 in Europe and North America, whereas the D3 sub-genotype included isolates from South America and Europe collected between 2005 and 2019, as well as several isolates from China, specifically Yunnan, Guangdong, Fujian, and Shandong, from 2017 to 2018. Additionally, the D4 sub-genotype included only isolates from Brazil collected in 2014–2015.

Primary characteristics of viral isolates

Nine CVB5 strains adapted well to Vero cells, and their infection titers ranged from 10^{7.46} to 10^{8.04} CCID₅₀/mL (S1 Fig). The KM03 strain exhibited the highest value, whereas the KM223 strain showed the lowest value. All nine CVB5 isolates belonged to the C3 genotype, which consists of epidemic strains in mainland China (Fig 1). The clinical isolates exhibited high similarity in their complete VP1 sequences, with nucleotide sequence identity ranging from 94.0% to 100%, and amino acid sequence identity between 97.8% and 100%. Compared with the CVB5 prototype Faulkner strain, these isolates showed nucleotide sequence identity of 78.3–78.5% and amino acid sequence identity of 85.7–86.4% in the entire VP1 region.

Virulence of four CVB5 strains

Four CVB5 Vero-cell-adapted strains (KM03, KM299, KM256, and KM09) with high infectious titers (>10^{7.5} CCID₅₀/mL) (S1 Fig) were screened and subjected to plaque purification. Their virulence in 2-day-old BALB/c mice was evaluated after i.c. injection of 20 µL of the original viral solution per mouse. These mice were monitored for clinical symptoms for 7 days (Fig 2). Consistent with the parental virus strains, the KM299-L05 and KM03-L05 clones showed the highest virulence levels.

Genetic stability of KM299-L05 and KM03-L05 strains

A comparison of the full genome sequences of the different generations (P1, P5, P10, P15, and P20) revealed two amino acid substitutions in the VP4 (1C→G for P5 and P15) and VP1 (274L→I for P5 to P20) proteins in the KM299-L05 strain (Table 1). The nucleotide and amino acid sequence identity among the full genomes of these offspring strains of KM299-L05 was 99.8%–99.9%

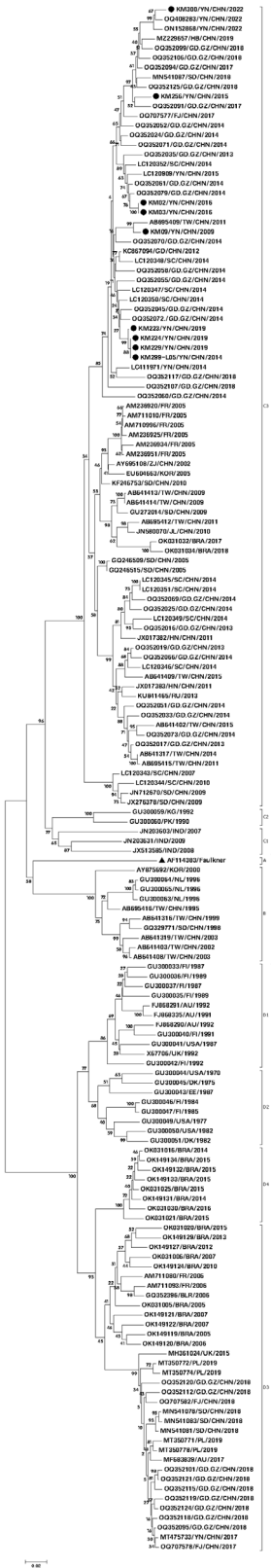


FIGURE 1 | Phylogenetic analysis based on the full VP1 gene sequence (849 nt) of CVB5, including seven isolates from this study and 151 representative CVB5 strains from GenBank. The parameters for the phylogenetic test were as follows: bootstrap method with 1,000 replications and the Kimura 2-parameter model. The analysis showed a bootstrap value >70%. • CVB5 prototype strain, ▲ CVB5 strain isolated in this study.

and 99.9%–100%, respectively. The infectivity titers of P5, P10, and P15 were $>10^{8.0}$ CCID₅₀/mL, whereas that of P1 generation was $10^{6.50}$ CCID₅₀/mL. However, for the KM03-L05 strain, five amino acid substitutions were found in the VP1 (132Q→R for P15 and P20, 277L→I for P5 to P20), VP2 (193C→G for P10 and P15), and 2C (34V→G for P10, 50L→V for P15) proteins (S3 Table). Although the KM03-L05 strain had a higher infection titer than KM299-L05, it bore five mutations in amino acids across different generations of the virus, whereas KM299-L05 had only a two-site mutation. This finding indicated that KM299-L05 was genetically more stable in Vero cells. Therefore, the KM299 strain was selected for further study.

Growth characteristics of the KM299-L05 P6 strain

To determine the growth features of the working seed stock KM299-L05 P6, we inoculated Vero cells at 0.001, 0.01, or 0.1 MOI. With an MOI of 0.1, the infectivity titers of KM299-L05 P6 peaked at 24 h post-infection ($10^{7.96}$ CCID₅₀/mL) and exhibited the best growth kinetics. However, under a moderate MOI (0.01), the infectivity titers peaked at 48 h post-infection ($10^{8.25}$ CCID₅₀/mL). Under a low MOI (0.001), the infectivity titers reached a maximum of $10^{8.17}$ CCID₅₀/mL. The infectivity titers decreased gradually for all three MOIs on day 3 after infection (Fig 3B). An MOI of 0.01 facilitated downstream processes for the production of inactivated CVB5.

Histopathological and immunohistochemical analyses of KM299 P6-infected mice

Histopathological examination revealed severe cellular necrosis accompanied by inflammatory infiltration in the pancreas, brain, and liver in infected mice. Punctate or focal necrosis was noted in the heart and limb muscles, whereas numerous renal tubular epithelial cells displayed cytoplasmic loosening (Fig 4A). No pathological changes were detected in other tissues, including the spleen, lungs, and intestines. Immunohistochemical analysis confirmed the presence of CVB5 antigens in the pancreas, brain, liver, forelimb muscles, kidneys, and intestines (Fig 4B). However, no viral antigens were found in tissues such as the heart, spleen, or lungs (data not shown). In the control groups, neither histopathological abnormalities nor viral antigens were observed.

CVB5 viral loads in various organs in KM299-L05 P6-infected mice

A viral load of 10^6 copies/mg was detected in various organs 6 h after infection. The CVB5 copy number in the pancreas, liver, heart, and lungs markedly increased after 6 h and reached 10^8 copies/mg, exceeding the levels in other organs ($p < 0.0001$). Hematoxylin and eosin staining and immunohistochemistry were consistent with this finding (Fig 5).

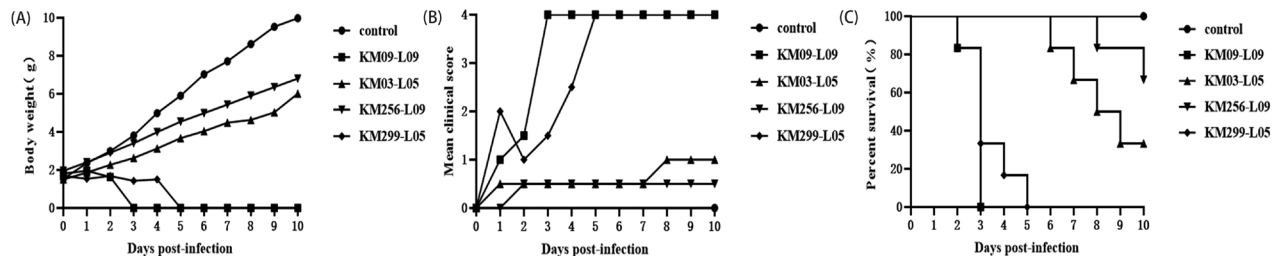


FIGURE 2 | Virulence of four CVB5 isolates in BALB/c mice. Two-day-old BALB/c mice were injected intracerebrally with KM03, KM299, KM256, and KM09 CVB5 isolates at $10^{6.05}$ TCID₅₀/20 μ L/mouse. Control groups were injected with the same volume of virus-free cell supernatant. (A) Body weight, (B) mean clinical score, and (C) percentage survival.

TABLE 1 | Amino acid changes in the whole genome sequences of various KM299-L05 offspring viruses.

Virus	Amino acid site	
	VP4-1	VP1-274
KM299-L05 P1	C	L
KM299-L05 P5	C	I
KM299-L05 P10	G	I
KM299-L05 P15	G	I
KM299-L05 P20	C	I

Note: Amino acid positions are referenced to the KM299-L05 P1 strain. Amino acid abbreviations are: C (cysteine), L (leucine), G (glycine), and I (isoleucine).

Immunogenicity of inactivated CVB5 vaccine

When 4-week-old female BALB/c mice were vaccinated three times with inactivated CVB5 vaccine prepared from the KM299-L05 P6 strain, high levels of NAbs were induced (Fig 6). A marked increase in NAb levels was observed in the 1 μ g and 5 μ g groups 21 days after the third vaccination, reaching 1:552 and 1:565, respectively. However, the NAb level of the 10 μ g group was 1:309. The best dose was determined to be 1 μ g. In vaccinated mice, no abnormalities were observed, thus indicating that the vaccine was safe. However, within-group comparisons of the NAb potency elicited by the three doses of vaccine in inoculated BALB/c mice were not statistically significant according to one-way ANOVA. A statistically significant difference ($p < 0.0001$) was observed between the control group and each of the dose groups. Various clinical CVB5 isolates (KM299-L05, KM09-L09, and KM300) as well as the CVB5 prototype Faulkner strain were significantly neutralized by the antiserum against the inactivated CVB5 vaccine. Antiserum induced by 1 μ g vaccine neutralized these viruses with a titer range from 1:384 to 768. The neutralization titers against the prototype Faulkner strain were lowest. These findings indicated that active immunization with the inactivated CVB5 vaccine provided adequate protection against disease caused by CVB5.

Protective maternal antibodies

To assess the protective effects of maternal antibodies in neonatal mice, we inoculated 4-week-old female BALB/c mice ($n = 2$ per group) three times with the inactivated CVB5 vaccine. After delivery, 1-day-old mice were challenged i.c. with KM299-L05 ($10^{6.05}$ CCID₅₀/20 μ L). The vaccine group showed normal weight, no clear symptoms, and a 100% survival rate. However, the control group showed severe clinical symptoms after KM299 challenge, and all mice died at 3 days post-challenge. The new inactivated CVB5 vaccine induced maternal antibodies against CVB5, which conferred 100% protection against CVB5 challenge in newborn mice.

DISCUSSION

For the production of a new vaccine, the immunological traits of a candidate virus must be fully characterized. Since the prototype Faulkner strain was isolated in 1952 in the USA, CVB5 strains have evolved into four genotypes [24]. Differences in cross-neutralization have been reported among the various enterovirus genotypes. Vaccines prepared from a specific CVB5 genotype have been found to induce strong neutralizing antibody responses against the corresponding genotype virus [25]. An inactivated hexavalent CVB vaccine has been found to induce the production of NAbs against all CVB serotypes, including CVB5 [14]. However, the CVB5 strain used for the vaccine was isolated from Finland [14] and belonged to the D genotype. Currently, the Chinese CVB5 strains are divided into C and D genotypes. However, the C genotype is the predominant strain in China, according to the analysis of CVB5 sequences available in the GenBank. Our study identified cross-neutralization differences between Chinese strains and the CVB5 prototype Faulkner strain. The nine CVB5 isolates in this study belonged to the C genotype. For enteroviruses, the VP1 protein is the main antigenic determinant [34]. The average difference in the amino acid sequences of the full VP1 protein between the nine CVB5 isolates and other Chinese strains available in GenBank was 1.95%, whereas the differences with respect to the prototype strain or isolates from other countries were greater (4.5% and 2.7%, respectively).

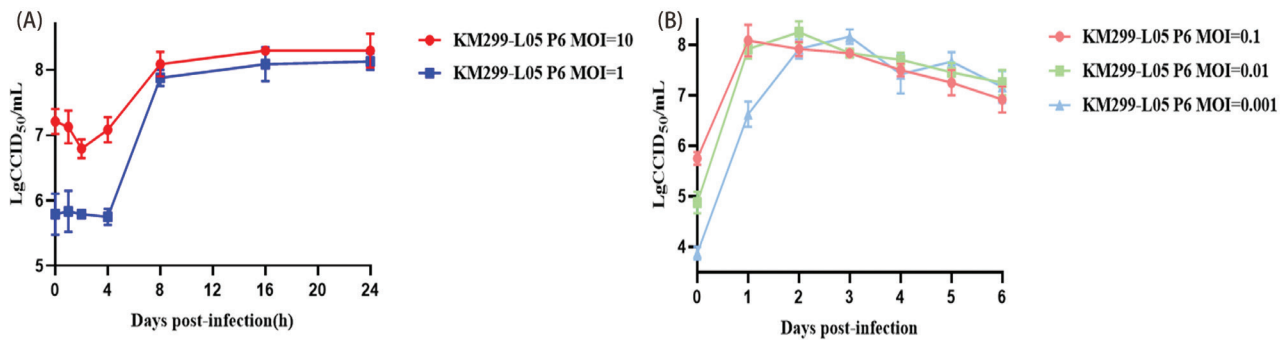


FIGURE 3 | Growth curve of KM299-L05 in Vero cells. The infectious titers for KM299-L05 P6 were determined with the Reed–Muench method at 1, 2, 3, 4, 5, and 6 days post-infection with MOIs of 0.001, 0.01, and 0.1. Data are presented as mean $\pm$ SEM.

These findings indicated that the CVB5 clinical isolates from this study should be optimally screened as potential candidates for a new CVB5 vaccine in China. In addition, differences in neutralization potency among genotypes might be attributable to genetic variation.

The enterovirus 71 and poliovirus vaccines are frequently made from Vero cells. Analysis of the virulence of eight CVB5 Vero-cell-adapted strains in BALB/c mice and their infectious titers in Vero cells indicated that the KM299 strain was the most virulent and was well adapted in Vero cells ($10^{7.8}$ CCID₅₀/mL). KM299-L05 was subsequently selected after plaque purification. After serial passaging of KM299-L05, the infection titers of KM299-L05 P5, P10, P15, and P20 all remained above $10^{7.5}$ CCID₅₀/mL, whereas that of P1 was $10^{6.50}$ CCID₅₀/mL. The KM299-L05 strain showed minimal mutations (VP4-1C→G and VP1-274L→I) across passages. VP4 is an internal protein that maintains the stability of the EV capsid, and its core sequence elicits cross-protective antibodies [35]. In contrast, VP1 is a surface capsid protein that plays key roles in virulence and antigenicity [34,35]. We observed that NAbS induced by the KM299-L05 P6-based CVB5 inactivated vaccine neutralized the KM299-L05 P5, KM299-L05 P10, KM299-L05 P15, and KM299-L05 P20 strains (titers 1:640–1:768). However, further studies are needed to assess potential virulence effects. In addition, the five KM299-L05 offspring isolates maintained 99.9%–100% genomic amino acid sequence identity, thereby demonstrating strong genetic stability. Animal models are important tools in vaccine research, and several CVB5-mouse models have been reported. After i.p. inoculation of 3–4-week-old C3H/HeJ male mice, CVB5 (VR-185) induced acute pancreatitis without myocarditis. The liver cleared CVB5 RNA more rapidly than the pancreas and heart [36]. In 3–4-week-old BALB/c mice infected i.p. with CVB5, only the heart and lungs have been reported to exhibit histological changes [37]. Moreover, in 3-day-old BALB/c mice challenged i.p. with the HFMD clinical isolate CV-B5/JS417, pathological changes have been observed in the cerebral cortex, cardiomyocytes, and hindlimb muscles [38]. A mouse-adapted strain of CVB5

(AFP229/GD/CHN/2017) administered intracranially to ICR neonatal mice has been found to result in pathological changes in the muscle, brain, and myocardium [16]. In our study, a 1-day-old BALB/c mouse model was established. After i.c. challenge with KM299-L05 at a dose of $10^{6.05}$ CCID₅₀/20 μ L, the mice showed weight loss; hair loss; neurological symptoms, such as trembling or hunching; or even death. Histopathological changes were observed in the liver, heart, pancreas, and lung tissues, and the highest viral loads were found in those tissues. Previous studies have shown that CVB5 infection can lead to myocarditis [39,40], type 1 diabetes, pneumonia, and neonatal hepatitis in humans [41,42]. Therefore, this model should be applicable to studies on the mechanism of CVB5 infection, and the evaluation of vaccines and drugs. The CVB5 mouse model indicated that the pathogenic characteristics varied according to the viral clinical isolate, inoculation method, animal species or age, and environmental source.

Vaccines are the most effective means of preventing and controlling infectious diseases, and NAbS are a key indicator of vaccine efficacy. NAbS bind viruses and neutralize their infectivity. In this study, mice immunized with inactivated CVB5 produced NAbS capable of neutralizing three heterologous CVB5 strains. Protective antibodies from immunized adult female mice were transferred to their offspring, thus conferring immunity against lethal CVB5 challenge, whereas control mice developed clinical symptoms and succumbed to infection. These results demonstrated that the inactivated CVB5 vaccine effectively protected newborns against CVB5 infection. Previous studies have reported that CVB5 VLP and inactivated vaccines elicit anti-CVB5 NAbS with average titers of 1:160 and 1:265, respectively. In contrast, the KM299-L05-based vaccine in this study induced higher NAb titers. The NAb titer reflects antibody concentration, and higher titers correlate with prolonged persistence in the body. However, an anti-polio NAb titer $\geq 1:8$ confers clinical protection [43], whereas an EV71 inactivated vaccine titer $\geq 1:32$ is considered a potential protective threshold [44]. Notably, the CVB5 strain used in the hexavalent vaccines is a foreign-prevalent strain differing

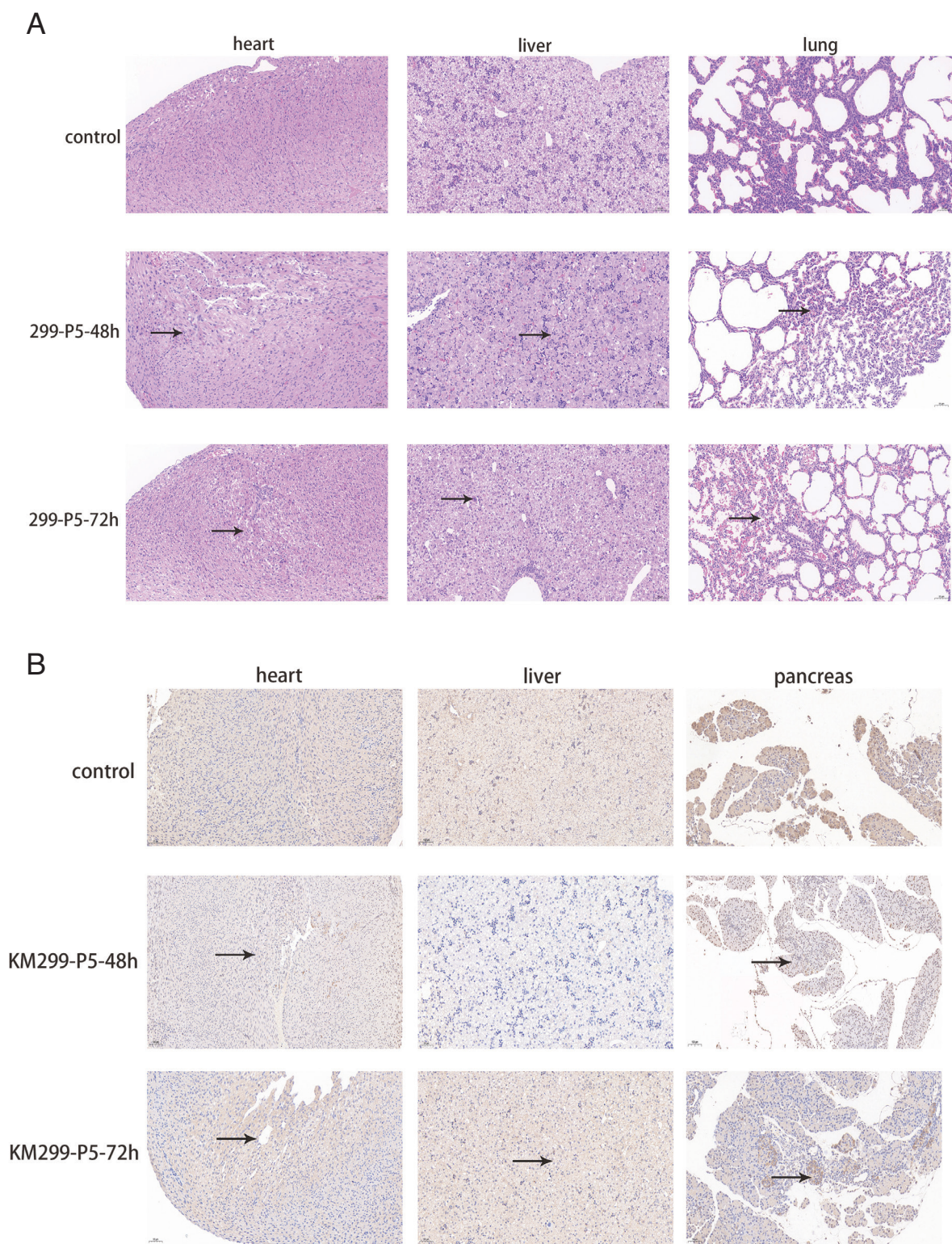


FIGURE 4 | Histopathology (A) and immunohistochemistry (B) of tissues from KM299-L05 P6-infected 3-day-old BALB/c mice (x100). Three-day-old BALB/c mice were i.c. infected with $10^{6.05}$ CCID₅₀/20 μ L KM299-L05P6.

from the dominant genotype in China. Given the demonstrated immunogenicity and safety of licensed enterovirus vaccines (e.g., inactivated polio and EV71 vaccines), the

inactivated CVB5 vaccine is a promising candidate for further development. Our findings provide a strong foundation for future CVB5 vaccine research.

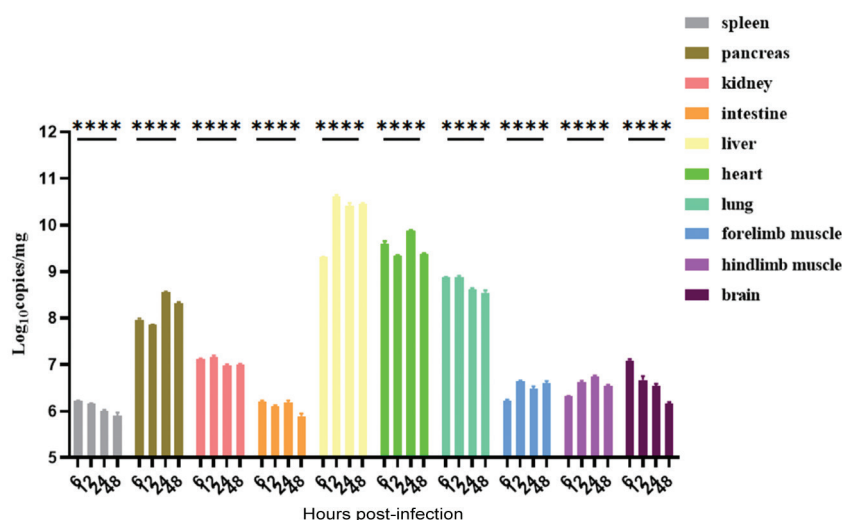


FIGURE 5 | Viral load in various organs in 1-day-old BALB/c mice infected by the CVB5 strain KM299-L05. Viral loads were identified with qRT-PCR and compared with the standard curves from 10-fold serial dilutions of the CVB5 transcript. Data are shown as mean $\pm$ SEM (3 mice/group) and were analyzed with one-way ANOVA (**** $p < 0.0001$).

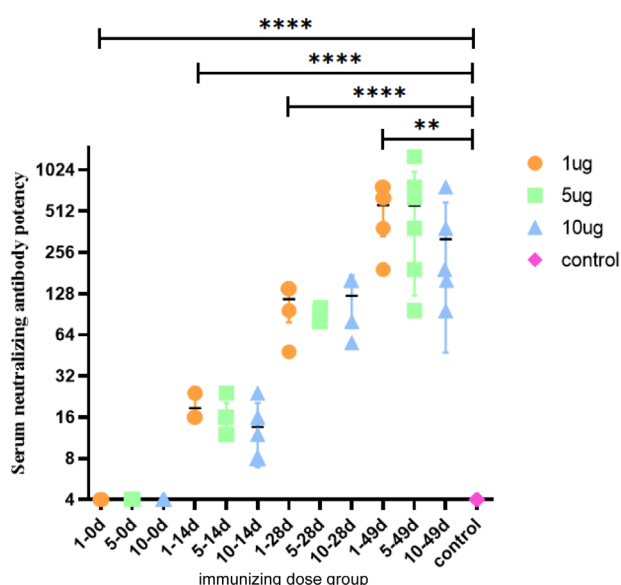


FIGURE 6 | Dynamic distribution of the neutralizing antibody responses induced by three inactivated CVB5 vaccine doses at various immunization intervals in BALB/c mice. Four-week-old female BALB/c mice (five mice per group) were vaccinated with 1, 5, or 10 μ g inactivated CVB5 vaccine and received two booster vaccinations at a 2-week interval. Blood samples were collected at days 0, 14, 28, and 49. The negative control group was immunized i.p. with 100 μ L adjuvant and M199 mixture. Data are mean $\pm$ SEM and were analyzed with one-way ANOVA (** $p < 0.05$ and **** $p < 0.0001$).

This study has several limitations. First, neonatal mice have immature immune systems, which might not fully replicate the immune response in older children or adults. Second, clinical signs in neonatal mice are not easily observed or assessed. Third, in CVB5 maternal antibody protection tests, NABs against CVB5 were not induced

by the suckling mice themselves, and were passed to the fetus through the placenta and breast milk. CVB5 infection occurs primarily in children younger than 5 years, similarly to other enteroviruses. Additionally, CVB5 sensitivity was observed only in 1-day-old suckling mice. Therefore, the results might not translate to older children or adults. These limitations could be overcome through use of other animal models, such as non-human primates, or alternative methods.

The large-scale production of vaccine candidates involves key steps such as viral amplification in large-scale Vero cell bioreactors, formaldehyde inactivation, purification, and formulation. Critical quality attributes to be considered for future clinical development include safety, potency, and stability. Notably, the KM299-L05 candidate demonstrated excellent genetic stability and broad-spectrum neutralizing activity against multiple CVB5 strains, and is based on a scalable Vero cell platform. These characteristics collectively support its suitability for clinical translation.

In conclusion, the CVB5 candidate vaccine strain derived from a prevalent Chinese CVB5 strain was successfully screened. Our results highlight the potential of new inactivated CVB5 vaccines, on the basis of their good immunogenicity and immunoprotection. However, the present study was validated only in mice, and further research in non-human primates is necessary. Nevertheless, this study provides a solid basis for further development of an inactivated CVB5 vaccine and holds promise for the development of a vaccine platform for multivalent CVBs.

DATA AVAILABILITY STATEMENT

All primary findings and contributions from this investigation are presented within the main article or its supplementary materials. For any additional information or clarification, interested parties may contact the corresponding author directly.

ETHICS STATEMENT

The experimental animals were obtained from the Institute of Medical Biology, Chinese Academy of Medical Sciences, China [SCXK (Dian) K2022–0002], and the animal experiments were conducted in the experimental facilities of the Experimental Animal Centre, Institute of Medical Biology, Chinese Academy of Medical Sciences, China [SYXK (Dian) K2022–0006]. The experimental design was based on the Reduction, Refinement, and Replacement (3R) principles for the use of experimental animals, and the research protocol was approved by the Animal Ethics Committee of the Institute of Medical Biology, Chinese Academy of Medical Sciences (DWLL202108001). The animals received humane care throughout the experimental process. In addition, neonatal mice were provided with comfortable bedding and nutrition during rearing. When operating on neonatal mice, we avoided use of traumatic instruments to the greatest extent possible. We separated the mother mice and neonatal mice and performed euthanasia in the experimental operation room to avoid discomfort to the mother mice. The neonatal mice were handled gently and returned to their mothers as soon as possible after completion of the operation.

AUTHOR CONTRIBUTIONS

LYH, CZY, and ZM share equal primary authorship for this study. The manuscript was drafted by LYH and CZY. MSH and LJS provided critical revisions and edited the text. ZM and FCZ were responsible for data management and analysis. All co-authors have reviewed and given their approval to the final version of this manuscript.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

SUPPLEMENTARY MATERIAL

Supplementary Material can be downloaded from https://zoones-journal.org/wp-content/uploads/2025/06/zoones20250004_Suppl.zip

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