Transplantation of Enterovirus 71 Virion Protein Particle Vaccine Protects Against Enterovirus 71 Infection in a Neonatal Mouse Model

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Background: Enterovirus 71 (EV71) is the pathogen most likely to cause HFMD in young children (1–5 years old). A small number of virion protein (VP) vaccine candidates are considered as the protective molecules in EV71 models. This study aimed to observe comprehensive immunogenicity for a promising EV71 vaccine depending on VP1 in neonatal mouse EV71 models.

Material/Methods: VP1 was isolated from patients and associated peptides were synthesized. EV71 particles were inactivated and mixed with Freund's complete adjuvant to prepare peptide vaccines. An EV71 vaccine was administered to establish the mouse model and the mice were infected with EV71. Hematoxylin and eosin staining was used to examine inflammatory response in EV71-infected neonatal mice. A semi-quantitative reverse transcription-polymerase chain reaction assay was performed to evaluate the levels of EV71 virus in skeletal muscle, small intestines, and brain tissues.

Results: Three peptides were selected from 20 VP1 peptides due to their exhibition of the highest immunogenicity. The peptide injection improved inflammation and decreased EV71 particle levels in muscle, small intestines, and brain tissues. The injection also decreased lesions in the small intestines of EV71-infected mice and protected brain tissues from the EV71 infection.

Conclusions: The present study confirmed the immuno-protective effects of VP1 vaccine transplantation in mice infected with EV71 virus. Our results provide valuable information that can be used in further studies investigating the specific mechanism of the anti-EV71 vaccine.

MeSH Keywords: Bacterial Vaccines • Enterovirus • Hand, Foot and Mouth Disease • Viral Structural Proteins

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Background

Hand, foot, and mouth disease (HFMD) is a type of febrile disorder mainly affecting infants and young children (1–5 years old) [1–3]. HFMD is always characterized by ulcers on the hands, mouth, and/or feet [4]. Enterovirus 71 (EV71) is the pathogen most likely to cause HFMD, which also affects the central nervous system [5]. EV71 belongs to the Picornaviridae family of the enterovirus genus [6]. Several other pathogens that cause HFMD have also been discovered, including coxsackievirus A16 (CVA16) and CVA6 [7]. HFMD is endemic in many Asian-Pacific countries, especially in China, Japan, Korea, Singapore, and Cambodia, which have suffered from the re-emergence of HFMD [8–12]. Especially in China, EV71 infection outbreaks in the past 10 years have been increasing [13]. In these cases, vaccines have been considered as the most efficient strategy for the treatment and prevention of HFMD [13]. However, the application of HFMD vaccine has also raised controversial and challenging issues. Therefore, there is a need for a type of vaccine for HFMD that does not affect other enteroviruses that cause HFMD [14]. Several studies [15,16] have created numerous methods to develop an effective and stable EV71 vaccine.

According to previous studies, virion proteins (VPs) are considered to be promising potential candidates for HFMD vaccines, as they are non-infectious particles [17]. Actually, VPs have been successfully used to develop vaccines against the hepatitis B virus and human papillomavirus (HPV) [18]. In recent years, HFMD vaccines were also designed by recombining the EV71 VPs, and it was demonstrated that these vaccines could neutralize the HFMD model [19]. In this investigation, the potential EV71 vaccine was established by employing VP1 as the antigen and evaluating the efficacy of the EV71 vaccine with a series of strategies, including etiological and pathological evaluation and lethal challenge analysis.

Material and Methods

Protein epitope identification

A total of 6 patients (male, n=3; female, n=3; age range, 1-5 years) with HFMD were recruited between January 2012 and December 2016 from the Third Affiliated Hospital of Zunyi Medical University (Zunyi, China). Blood samples were obtained and serum antibodies were examined using the EV71-IgG ELISA kit (cat. no. TW-Bio-E4381, R&D, MN, USA), based on the protocol of the manufacturer. ELISA was used to identify the best immunizing antigen for the EV71 vaccine using patients’ serum antibodies. In this study, the serum with the highest antibody titer was used to select the appropriate synthetic peptides after serum antibodies of the 6 samples were tested separately.

The present research was approved by the Ethics Committee of the Third Affiliated Hospital of Zunyi Medical University. All of the patients involved in this study provided written informed consent and approved this study.

EV71 isolation and peptide synthesis

The human EV71 strain was isolated from the 6 patients using the following processes. 1) Sample pretreatment of throat swab: Put throat swab in virus preservation solution and stir 40 times to wash down the virus adhered to the swab and cells containing the virus. The preservation solution was then collected and centrifuged at 4°C and 10 000 rpm for 20 min. The supernatant was retained and filtered for sterilization. 2) Virus culture and isolation: Take 0.4 ml of virus supernatant, and inoculate it on the healthy human embryo rhabdomyoma (RD) cells with a full monolayer. The RD cells were cultured with 5% CO₂ at 37°C. The growth and pathological effects of RD cells were observed by microscopy every day. When 80%-90% of RD cells demonstrated the pathological effect, cells and maintenance fluid were harvested and centrifuged and the supernatant was retained.

Then, the genome of EV71 was isolated using the TaKaRa MiniBest Viral RNA/DNA Extraction Kit (Cat. No. 9766) following the manufacturer’s protocol.

Using the above isolated EV71 genome, the amino acid (aa) sequence of EV71 was divided into 20 groups (20 aa/group except for the Peptide 20 group) (Table 1).

Inactivation of EV71 particles

Inactivated EV71 particles were prepared as described previously [20]. The inactivated EV71 particles were quantified by western blot analysis as previously described [21]. Briefly, total protein was extracted using Triton X-100 diluted in phenylmethylsulfonyl fluoride (Beyotime Biotech., Shanghai, China) at a final dosage of 100 μg/ml. The protein concentrations were quantified using a BCA kit (Sigma-Aldrich, St. Louis, MO, USA). Then, a total of 0.2 μg protein was added to each lane of 15% SDS-PAGE (Beyotime Biotech.). The SDS-PAGE produced protein in lanes was transferred onto the polyvinylidene difluoride membranes (PVDF, Amersco, Inc., Framingham, MA, USA). Then, the PVDF membranes were blocked using 5% non-fat milk in PBS containing 0.05% Tween-20 (ShineGene, Molecular Biotech, Inc., Shanghai, China) for 1 h at 37°C. The PVDF membranes were treated using the mouse anti-human EV71 antibody (1:1000, Cat. No. ab169442) and mouse anti-human β-actin antibody (1:2000, Cat. No. ab8224) at 4°C overnight. Subsequently, the PVDF membranes were treated using HRP-conjugated rabbit anti-mouse IgG (1:1000, Cat. No. ab6728) at 37°C for 2 h. All of the above antibodies were purchased
Table 1. Amino acid sequences for the synthesized peptides (1–20).

| Peptide number | Amino acid sequences                        |
|----------------|--------------------------------------------|
| 1              | GDRVADVIESSGIDSVSRAL                      |
| 2              | VSRALTHALPAPTRGNTQVS                      |
| 3              | NTOYSSHRLDTGKVPALQAA                      |
| 4              | ALQAAEIGASSNASDESMIE                      |
| 5              | ESMIETCRVLNSHTAETTL                      |
| 6              | AETTLDSFFSRLAVGEIDL                     |
| 7              | GIDLPLEGTNPNPGYANWD                      |
| 8              | YANWDDITGYAQMRRKVEL                      |
| 9              | RKVELFTYMRFDAETFVAC                      |
| 10             | TVFACPTPGEVPPQLQYMF                      |
| 11             | LQYMPVPGAKPDSRESLA                       |
| 12             | RESLAWQTATNPSPVFKLSD                     |
| 13             | VKLSDPPAQPVSVPFMPSASA                    |
| 14             | SPASAVQWPYDGYPTFGEHK                     |
| 15             | FEGHELQKQDELYGACPNNMM                    |
| 16             | PNNNMGTSVRTGTSKSY                       |
| 17             | SKSKYPLUVRIYMRKHKVRA                     |
| 18             | KHVRAWIPHMRNQNQYLFKA                     |
| 19             | YLPKANPNVYGATNTKPOVS                     |
| 20             | PTGVSRTAITTL                             |

from Abcam Biotech. (Cambridge, MA, USA). Finally, the western blot bands were visualized with Pierce™ ECL Plus Western Blotting Substrate (Cat. No. 32132; Pierce, Waltham, MA, USA). The TCID<sub>50</sub> was evaluated as described in a previous study [22].

**Peptide vaccine preparation**

The obtained peptides were dissolved in 0.9% NaCl and adjusted to a concentration of 1 mg/ml. Subsequently, the peptide vaccine solution was prepared following the 1:1 (v/v) mixture of peptides and Freund's complete adjuvant (Cat. No. P2036, Beyotime Biotech., Shanghai, China).

**Establishment of a mouse model and EV71 infection**

We obtained 18 healthy 6-8-week-old Balb/C mice (female, n=12; male, n=6; weight range, 150–200 g) from HFK Bioscience Biotech. (Beijing, China). Mice were housed under the conditions of 24–25°C, 50–60% humidity, and a light/dark cycle of 12/12 h. The mice had free access to food and water. The mice were divided into 6 groups (2 females and 1 male in each group) to produce newborn mice (suckling mice). The mice in 3 of the 6 groups were administered synthesized peptide 2, synthesized peptide 4, and synthesized peptide 8, followed by mating and reproduction, to obtain the newborn mice (as the experimental groups). Then, the newborn mice were divided into a Peptide 2 group (n=4), a Peptide 4 group (n=4), and a Peptide 8 group (n=4) for subsequent experiments. The mice in the other 3 groups were not administered any peptides, were allowed to mate and reproduce, and the newborn mice were used as the control groups. The newborn mice were divided into a Newborn group (newborn mice without any treatment, n=4), a Newborn peptide group (newborn mice received peptide only), and a Newborn EV71 group (newborn mice received an EV71 infection).

The EV71 infection mouse model was established as described in a previous study [23], with a few modifications. According to the Reed-Muench method, the specific value or titer of EV71 was 10<sup>4.5</sup>/0.1 ml. A total of 5×10<sup>5</sup> TCID<sub>50</sub> EV71 particles were intraperitoneally injected into each newborn mouse. One day after EV71 infection, neonatal mice were killed and the skeletal muscle, small intestines, and brain tissues were extracted for testing. The peptides (0.1 ml/mouse) were intraperitoneally injected and re-administered 1 week later. Finally, the mice in each group were fed together and were able to mate freely.

All of the animal experiments or tests were approved by Ethics Committee of the Third Affiliated Hospital of Zunyi Medical University.

**HE staining assay**

The skeletal muscle, small intestines, and brain tissues were treated with 4% paraformaldehyde at 4°C for 24 h. Then, the tissues were embedded in paraffin (Beyotime Biotech.) and cut into sections 4 µm thick. Tissue slices were subsequently deparaffinized as previously described [23]. The tissue sections were used for HE staining assay as previously described [24]. Briefly, the above tissue sections were stained with hematoxylin for 10 min, followed by eosin staining for 30 s. Both hematoxylin and eosin staining were conducted at room temperature. The stained sections were imaged with an inverted fluorescence microscope (Model IX70; Olympus, Tokyo, Japan) and analyzed using the Medical Image Analysis System (HMIAS22000; Wuhan Champion Image Technology Co., Ltd., Wuhan, China).

**Quantitative real-time (qRT-PCR)**

RNAs in the skeletal muscle, small intestines were extracted with 1 ml TRIzol reagent (Thermo Scientific Pierce, Rockford, IL, USA). Samples were centrifuged at 10 000 x g for 5 min at 37°C and the supernatants were stored for subsequent experimentation. Subsequently, total RNA was precipitated using 1 ml isopropanol and washed twice in 70% ethanol. The RNA precipitations were dissolved in diethyl pyrocarbonate (Sigma-Aldrich; Merck).
KGaA) pre-treated water. RNAs were reverse-transcribed into the complementary DNA (cDNA) using the Superscript III First-Strand Synthesis system (Western Biotech. Inc., Chongqing, China) following the manufacturer’s protocol. PCR assay was conducted to examine EV71 virus and β-actin mRNA expression. PCR assay was then conducted with a Takara SYBR Green PCR kit (Cat. No. DRR820A; Takara, Dalian, China) using T100 RT-PCR cycler (Bio-Rad., Hercules, CA, USA). The following listed primers were used for the amplifications:

EV71 forward: 5'-CAAGCACTTCTGTTTCCCCGG-3' and reverse: 5'-ACCCAGAGTAGTCGG TTCCGC-3',

β-actin forward: 5'-CCCATCTATAGGGTTACGC-3' and reverse: 5'-TTTAATGTCACGCACGATTTC-3'.

The PCR assay underwent the following processes: Initial denaturation for 5 min at 95°C, then 30 cycles of 95°C for 30 s, 58°C for 45 s, and 72°C for 30 s. The final extension step was 72°C for 10 min. The DNA products were run on a 1.5% agarose gel containing ethidium bromide (Beyotime Biotech.).

**Figure 1.** Peptide injections decrease EV71 particle levels in muscle, small intestines, and brain tissues. (A) ELISA immunogenicity analysis for the synthesized peptides (1–20). Lanes 1–20 represent the synthesized peptides. (B) cDNA bands for EV71 distribution in tissues. Lanes 1, 2, and 3 show the level of EV71 virus in skeletal muscle, small intestines, and brain tissues, respectively. (C) Quantification and statistical analysis of relative mRNA expression of the EV71 virus by a semi-quantitative RT-PCR assay. **P <0.01 vs. the Newborn EV71 group. EV71 – enterovirus 71.
DNA bands were visualized using a gel imager and bands were quantified using LightCycler® 96 software (version 1.1.0.1320; Roche Applied Science, Penzberg, Germany) with β-actin as the internal reference gene.

**Statistical analysis**

Data are expressed as mean±standard deviation (SD) and were analyzed with professional SPSS software 19.0 (SPSS, Inc., Chicago, IL, USA). Tukey’s post hoc test-validated one-way analysis of variance (ANOVA) was conducted to analyze statistical differences among multiple groups. *P* values less than 0.05 were regarded as statistically significant.

**Results**

**Three peptides were selected from VP1**

Serum samples from patients with high serum antibody concentrations (>35.71 ng/ml) were used for the current study. Among the 20 synthesized peptides truncated from the VP1 protein, ELISA assay revealed that the immunogenicity was stronger in peptide 2, peptide 4, and peptide 8 compared with the other peptides, and these 3 were used for immunizing mice (Figure 1A; *P*<0.05). Therefore, the above 3 peptide vaccine immune groups were named as Peptide 2, Peptide 4 and Peptide 8.

**Peptide injections decreased EV71 particle levels in muscle tissues**

The EV71 virus gene distribution in skeletal muscle, small intestines, and brain tissues were determined using semi-quantitative RT-PCR (Figure 1B). The semi-quantitative RT-PCR results also demonstrated that the EV71 virus levels in skeletal muscle, small intestines, and brain tissues of the Peptide 2 group, Peptide 4 group, and Peptide 8 group were remarkably lower than in the Newborn EV71 group (Figure 1C, all *P*<0.01).

**Peptide injections ameliorated inflammation and decreased EV71 particle levels in muscle tissues**

The HE staining indicated that the Newborn EV71 group exhibited a severe inflammatory response with myo-necrosis,
myo-degeneration, and muscle fiber atrophy (Figure 2). The muscle tissue samples in the Peptide 2, 4, and 8 groups appeared more defined and there was less inflammation compared with the Newborn EV71 group (Figure 2). The muscle condition in the Newborn peptide group was similar to that of the Newborn group.

**Peptide injections decreased the number of lesions in small intestines of EV71-infected mice**

HE staining was also used to examine the lesions in the small intestines of the EV71-infected mice. The results indicated that there were fewer lesions in the Peptide 2 group, Peptide 4 group, and Peptide 8 group compared with that in the Newborn EV71 group (Figure 3).

**Peptides protected brain tissues from EV71 infections**

To investigate the effects of peptides on the central nerve system, the brain tissues were isolated, and HE staining was performed to observe the inflammatory response and lesions. The results demonstrated that EV71-infected newborn mice (without peptide injection) exhibited an inflammatory response and several lesions in the brain tissues (Figure 4). The Peptide 2 group, Peptide 4 group, and Peptide 8 group exhibited less inflammatory infiltration and fewer lesions compared with the Newborn EV71 group (Figure 4).

**Discussion**

Several enteroviruses are associated with the incidence of HFMD in clinical practice, including EV71, CVA16, and CVA6 [4–6]; therefore, it is necessary to discover a broadly applicable neutralizing vaccine. In fact, humans are considered to be the only natural host for the enteroviruses [5]. According to previous studies [25–27], most vaccine research is focused on EV71 virus and the newborn immuno-competent mouse to develop small-animal in vivo models. Cao et al. [18] reported that neutralizing antibodies induced by VPs could neutralize the homologous live EV71 virus. A previous study [28] reported that virus-like particles (VLPs) could also induce IgGs
against the EV71 virus. However, the neutralization titers of VLPs were lower compared with the inactivated EV71 virus. VP1 is an important antigenic epitope, which plays a critical role against the EV71 virus [29]. A previous study [16] applied the VP1 residues of 210-220 to trigger the anti-EV71 vaccine; however, the immunogenicity was not very high. In the present study, VP1 was employed as the antigen to trigger the immunogenicity of an anti-EV71 vaccine.

The antibody response and the cellular immune response serve important roles in human immunity [30,31], which can trigger marked immune responses [31]. It is found that EV71 virus easily produces high levels of maternal antibody. The incidence rate of HFMD is lower in children under 1 years old, which may be related to the higher level of maternal antibody in newborns. The maternal antibody of mammals is mainly transmitted to newborn through the placenta and colostrum, so that it can provide immune protection. After newborn animals ingest colostrum, they can obtain maternal antibody immediately, and this improves the resistance of newborn animals. Therefore, when an animal model of vaccine immunization was established, mother mice were immunized by vaccine so that the offspring mice could obtain maternal antibody through placenta and milk. Therefore, the vaccine had an indirect immune-protective effect on the newborn mice. In summary, the adult mice were administered synthetic peptides and reproduced, providing the suckling mice used in this study.

In our study, among serum samples of 6 patients, the serum with the highest antibody titer was selected and the binding of serum and synthetic peptide was assessed by indirect ELISA. The highest binding rate of the synthetic peptide to EV71 IgG antibody was found. This synthetic peptide demonstrated the highest specificity; therefore, we used the synthesized peptide to immunize mice. The present study demonstrated that immunization with a VP1-synthesized vaccine could trigger an effective protective response to an EV71 infection in a small number of tissues in the mouse model, including the skeletal muscle, small intestines, and brain tissues. These findings were consistent with the effect of a number of other VP1-associated vaccines, including the HPV and influenza viruses [32,33].

Figure 4. Peptides protect brain tissues from EV71 infections. Hematoxylin and eosin staining of brain tissues in the (A) Peptide 2, (B) Peptide 4, (C) Peptide 8, (D) Newborn, (E) Newborn peptide, and (F) Newborn EV71 groups. EV71 – enterovirus 71.
A previous study reported that the muscle cells are the most important sites for EV71 proliferation in patients living in Fuyang, China [24]. EV71 is presumed to infect the CNS through peripheral nerves; therefore, CNS injury may also be responsible for the EV71-infected mouse mortalities [34]. The present study also performed a pathological analysis of the small intestines to observe the immune-protective effects in vivo levels. The VP1 vaccines could prevent EV71 from triggering serious injury to the body and improved the recovery from injury caused by EV71 infection.

**Conclusions**

This study confirmed the immuno-protective effects of VP1 vaccine against EV71 infection in a mouse model and provides a valuable clue in the investigation of specific mechanisms of the anti-EV71 vaccine.

**Conflict of interests**

None.

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