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Transcutaneous immunization *via* rapidly dissolvable microneedles protects against hand-foot-and-mouth disease caused by enterovirus 71

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ABSTRACT

Recent large outbreaks of hand-foot-and-mouth disease (HFMD) have seriously affected the health of young children. Enterovirus 71 (EV71) is the main causative agent of HFMD. Herein, for the first time, rapidly dissolvable microneedles (MNs) loaded with EV71 virus-like particles (VLPs) were evaluated whether they could induce robust immune responses that confer protection against EV71 infection. The characteristics of prepared MNs including hygroscopy, mechanical strength, insertion capacity, dissolution profile, skin irritation and storage stability were comprehensively assessed. EV71 VLPs remained morphologically stable during fabrication. The MNs made of sodium hyaluronate maintained their insertion ability for at least 3 h even at a high relative humidity of 75%. With the aid of spring-operated applicator, EV71 MNs (approximately 500 μm length) could be readily penetrated into the mouse skin *in vivo*, and then rapidly dissolved to release encapsulated antigen within 2 min. Additionally, MNs induced slight erythema that disappeared within a few hours. More importantly, mouse immunization and virus challenge studies demonstrated that MNs immunization induced high level of antibody responses conferring full protection against lethal EV71 virus challenge that were comparable to conventional intramuscular injection, but with only 1/10th of the delivered antigen (dose sparing). Consequently, our rapidly dissolving MNs may present as an effective and promising transcutaneous immunization device for HFMD prophylaxis among children.

1. Introduction

Hand-foot-and-mouth disease (HFMD), characterized by fever, mouth ulcers and vesicles mainly on palms and soles, is an acute enterovirus infection that commonly affects infants and young children [1]. Over the past decades, several large epidemics of HFMD in the Asia-Pacific region have caused significant morbidity and mortality, making it a substantial burden to public health [2,3]. The major causative pathogens of HFMD are enterovirus 71 (EV71) and coxsackievirus A16 (CA16), while infection by EV71 is more often responsible for HFMD cases with severe neurological complications and even death [2,4]. Therefore, most effort in HFMD vaccine development is against EV71 [5,6]. Among various forms of EV71 vaccines, inactivated whole-virus vaccine has progressed fastest due to its high immunogenicity and mature manufacture technology, and the first inactivated EV71 prophylactic vaccine was approved by the China Food and Drug Administration on 3 December 2015 [7–9]. In addition, recombinant virus-like particles (VLPs), as another promising vaccine candidate formed by self-assembly of structural proteins, mimic the conformation of native viruses but lack viral genes, thus demonstrating superior safety as compared with conventional inactivated or attenuated vaccine [7,10,11].

Vaccination might be the most effective way for prevention and control of infectious disease. Currently, most vaccines including inactivated-EV71 are administered intramuscularly (IM) or subcutaneously (SC) by injections with needles and syringes, which possess several inherent drawbacks (pain, medical personnel requirement, needle-related diseases or injuries, and cold chains for storage and distribution, *etc.*) [12–14]. Besides, injection vaccination primarily delivers antigen to muscle or subcutis regions where neither is particularly rich in immunologically sensitive cells compared to the skin layers [15,16]. To overcome these limitations, development of needle-free, easy-to-use, and efficient vaccination strategies is urgently needed, especially during a pandemic. Transcutaneous immunization (TCI) offers a promising novel alternative due to attractive immunological characteristics of skin. With abundant professional antigen-presenting cells (APCs),

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such as Langerhans cells in epidermis and dermal dendritic cells in dermis, direct delivery of immunogen to these APCs could induce potent immune responses [14,17–19].

To breach skin's barrier imposed primarily by the outermost *stratum corneum* layer, a range of physical technologies, including iontophoresis, electroporation, liquid/powder jet injections, ultrasound and thermal ablation, have been extensively investigated [15,20,21]. Such approaches, however, employ sophisticated equipment, complex vaccination protocols, and may cause pain similar to standard needle injection. Microneedles (MNs), in contrast, are simple, miniaturized needles that could painlessly penetrate the *stratum corneum* barrier to access the underlying immunocompetent cells. These minimally invasive devices can potentially be manufactured cost-effectively and enable self-administration without special training, thereby mitigating the financial and logistical challenges posed by traditional vaccination strategy [22–24]. Previous studies have reported that a variety of vaccines, of which influenza vaccine is a representative antigen, administrated by MNs elicited comparable or superior immune reactions to standard parenteral inoculations, with some showing dose-sparing effects [22,25–27]. Moreover, dissolving MNs composed of water-soluble polymers or sugars dissolve upon insertion into skin, leaving no biohazardous sharps waste after use [28,29]. Recently, our group have fabricated rapidly dissolving MNs using low-molecular-weight sodium hyaluronate (HA) and assessed the feasibility of transdermal delivery of exenatide for type 2 diabetes mellitus therapy. The *in vivo* pharmacokinetics and hypoglycemic effects results showed that prepared MNs were equally effective to SC injections, and thus might present a credible, non-injected option for other biotherapeutics [30].

In the current study, EV71 recombinant VLPs were selected as the model antigen to explore the feasibility of TCI using dissolving MNs, and whether MNs could elicit robust immune responses that confer protection against EV71 infection. After fabrication of EV71 VLPs-loaded MNs using micromold casting method, the characteristics of prepared MNs including hygroscopy, mechanical strength, *in vivo* insertion capacity, dissolution profile, skin irritation and storage stability were then investigated. Finally, mouse immunization and virus challenge studies were carried out to compare the vaccination efficacy of our dissolving MNs to that of conventional immunization systems. To the authors' knowledge, this is the first report translating the use of dissolving MNs for EV71 immunization, which may serve as an excellent and effective vaccination alternative for HFMD prophylaxis among children.

2. Materials and methods

2.1. Preparation and fluorescent labeling of EV71 VLPs

Recombinant EV71 VLPs were produced using the baculovirus-insect cell expression system as reported previously [11]. For visualization the distribution of EV71 VLPs in needle shafts and mouse skin, VLPs were fluorescently labeled with amine-reactive Cy5. To perform labeling, EV71 VLPs diluted in PBS buffer (500 μL, 2.0 mg/mL) was incubated with Cy5-*N*-hydroxysuccinimide dye (OPE Tech., Shanghai, China) in 0.1 M bicarbonate buffer at room temperature for 1 h, with gentle rotation. During incubation, *N*-hydroxysuccinimide ester moieties readily reacted with the amine groups of the VLPs proteins to form stable dye-VLPs conjugates (Fig. S1). Excess unincorporated dye was removed using a Zeba™ spin desalting column (3 kDa MWCO, Thermo Fisher Scientific, IL, USA) by centrifugation at 1000 ×*g* for 2 min. The resulting Cy5-labeled EV71 VLPs (Cy5-EV71 VLPs) were quantified by Bradford assay before using.

2.2. Fabrication of EV71 VLPs-loaded dissolving MNs

The EV71 VLPs-loaded dissolving MNs were fabricated using micromold casting method as described previously with minor modifications [30]. Briefly, polydimethylsiloxane (PDMS, Sylgard® 184, Dow Corning Corp., Midland, USA) micromolds were first inversely replicated from a stainless steel master structure that consisted of 15 × 15 pyramidal needles (with approximately 550 μm height, 180 μm width at base and 500 μm interspacing). Twenty milligrams of low-molecular-weight HA (5.1 kDa, Bloomage Freda Biopharm Co., Ltd., Jinan, China) was dissolved in 0.2 mL of EV71 VLPs or Cy5-EV71 VLPs solution (approximately 0.65 mg/mL) to serve as the casting material, which was added to the surface of the mold and centrifuged to fill the cavities. After pipetting excess solution and centrifuging to facilitate drying, a 50% (w/w) HA solution was dispensed onto the mold and centrifuged to form the backing layer. The EV71 VLPs-loaded dissolving MNs patches were then dried in a sealed desiccator at room temperature and detached from the micromolds.

After fabrication, the obtained MNs were immersed and dissolved in 0.2 mL of PBS to evaluate the structural stability of VLPs by transmission electron microscopy (TEM) and dynamic light scattering (DLS) [31]. The reconstituted solution was negatively stained with 0.75% uranyl acetate, and TEM images were acquired on a Tecnai G2 Spirit microscope (FEI Company, USA) operated at 120 kV with a magnification of 100,000×. Particle size distribution of VLPs was measured by DLS using a Zetasizer Nano ZS particle analyzer (Malvern Instruments Ltd., UK). Meanwhile, the native VLPs solution with similar concentration was also analyzed as control.

To examine the distribution of antigen in MNs, Cy5-EV71 VLPs-loaded MNs were scanned from needle tips to shaft bases using a Zeiss LSM 710 confocal microscope (Carl Zeiss MicroImaging, Germany) under excitation at 646 nm [32]. Images were acquired in the *xy*-plane (*i.e.* parallel to the base plate of MNs) along *z*-direction at an interval of 20 μm.

2.3. Quantification of EV71 VLPs in MNs by enzyme-linked immunosorbent assay (ELISA)

A sandwich ELISA was performed to quantify EV71 VLPs loaded in MNs. Purified EV71 VLPs quantified by Bradford assay were used as the standard samples. VLPs were extracted from each MNs patch with 1.0 mL of PBS followed by vortex mixing to release the encapsulated antigen (VLPs) completely. The ELISA was carried out as described previously with slight modifications [11]. Briefly, each well on the 96-well ELISA plates was coated with 50 μL of 1:5000 diluted rabbit anti-EV71 antiserum (homemade) in PBS and incubated overnight at 4 °C. Then and after each of the following steps, plates were washed three times with PBST (PBS containing 0.05% Tween-20). All the samples were diluted with PBST plus 1% non-fat milk. Consecutively, the plates were blocked with 5% nonfat milk in PBST (200 μL/well) for 2 h at 37 °C and incubated with 50 μL/well of diluted samples, standards or controls (blank MNs without loading VLPs) for 2 h at 37 °C, followed by incubation with 50 μL/well of the EV71-specific D5 monoclonal antibody (0.2 μg/mL) for 1.5 h, and then with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (diluted 1:5000, 50 μL/well) for 1 h at 37 °C. For color development, 50 μL/well of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added and reacted for 5–10 min; then, 50 μL/well of 1 M H_3PO_4 was added to stop the reaction. Absorbance was determined at 450 nm using a 96-well plate reader. Then, a calibration curve of EV71 VLP standard samples in correlation with OD_{450} values was created and used to calculate the amount of VLPs encapsulated in MNs.

2.4. Hygroscopic and mechanical properties of VLPs-loaded HA MNs

EV71 VLPs-loaded MNs were placed at a high relative humidity (RH) of 75%, which was maintained through the use of saturated NaCl solution [33]. Subsequently, incubated MNs were removed at set intervals, and their water contents and mechanical strengths were measured as reported previously [30]. Briefly, water contents in MNs were determined by loss on drying method that measuring mass changes due to