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Original article

Influence of vitamin A status on the antiviral immunity of children with hand, foot and mouth disease

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SUMMARY

Background & aims: Vitamin A (VA) deficiency has been shown to affect antiviral immunity and thus may be related to the progress and outcome of hand, foot and mouth disease (HFMD) in young children. Our objective was to determine whether children with HFMD associated with VA insufficiency displayed a decline in antiviral immunity.

Methods: 450 children with HFMD and 113 non-infected children were included in this study. Dietary investigations were performed using a 24-h dietary questionnaire. The serum concentrations of VA were measured by high-performance liquid chromatography. The serum levels of interferon-α (IFN-α) and enterovirus 71 (EV71) IgM antibodies were detected using an enzyme-linked immunosorbent assay (ELISA).

Results: The mean serum VA concentration for all patients was 0.73 ± 0.26 μmol/L, and 237 (52.7%) of them presented low concentrations (≤0.7 μmol/L). Both serum concentrations of VA and IFN-α in the patients with complications were significantly lower than in patients without complications ($P < 0.01$). The decreased concentrations of IFN-α and EV71-IgM were positively related to lower VA levels (correlation coefficient = 0.58 and 0.41, respectively, $P < 0.001$).

Conclusions: Most of the children with HFMD presented VA insufficiency, which was associated with their reduced immunity and more severe illness.

1. Introduction

Vitamin A (VA) deficiency has already been recognized as a risk factor for some viral infectious diseases, such as measles, in young children. Since 1987, the WHO have recommended VA treatment of children with measles.[1] Hand, foot, and mouth disease (HFMD) is a common viral infection that mostly affects children under four years of age, with typical pathological damage in the skin and mucous membranes.[2] Notably, VA plays important roles in the maintenance of mucous membrane health and adequate immune function, and VA insufficiency is most commonly found in young children from developing countries, including China.[3–5] Thus, whether the VA status affects the susceptibility and progress of HFMD in these young children is worth studying.

Several reports, including our previous studies, have revealed the widespread influence of VA and its metabolites on the development and function of the immune system in children,[6] including its effects on T- and B-cells,[7,8] antigen-presenting cells,[9] dendritic cells (DCs),[10] and other immune system components or structures. However, few studies have examined the effect of VA on the antiviral immunity of HFMD infection in children. Therefore, in this study, the dietary intake and serum concentrations of VA from children with HFMD were evaluated. The serum level of interferon-alpha (an important innate antiviral cytokine) and the production of the antiviral antibody for Entervirus 71 (a main pathogen that causes HFMD) were measured to determine whether the VA status of HFMD subjects was associated with poor immune function and/or illness severity.

Abbreviations: DCs, dendritic cells; DRIs, dietary reference intakes; EP Tube, Eppendorf micro–test tube; EV71, enterovirus 71; HFMD, hand, foot and mouth disease; IFN-α, interferon–α; RAE, retinol activity equivalents; VA, vitamin A.

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2. Materials & methods

This cross-sectional, observational study was conducted at the Children's Hospital of Fudan University between October 2010 and June 2011. In total, 450 patients with median (interquartile range) age of 25 (17–37) months were included in the study. All patients were diagnosed with HFMD according to the standardized clinical case definition published by the Ministry of Health of People's Republic of China.[11] A case was defined as a patient who had clinical symptoms of HFMD, typically vesicles on the hand or foot and oral lesions, with or without fever. Patients with HFMD should be hospitalized if there is evidence of central nervous system involvement (i.e., dispirited, drowsiness, headache, vomiting, irritability, convulsion and so on). The severity of the condition of patients with HFMD was classified according to the associated complications, including encephalitis, meningitis, meningoencephalitis, and so on. For comparison, a gender- and age- matched control group ($n = 113$) was recruited from among non-infected children. This study was approved by the Ethics Committee of the Children's Hospital of Fudan University. Parents were informed about the study, and written informed consent was obtained.

2.1. Sample collection and handling

Blood samples were obtained from the patients on admission and from non-infected children during healthcare visits to the Children's Hospital of Fudan University. Venous blood samples were taken by a registered nursing using a vacutainer needle and delivered into aluminum foil-wrapped tubes. To separate the serum, the blood sample was centrifuged at 3000 rpm for 3 min. Then, the serum was aliquoted in marked Eppendorf test tubes and frozen at −70 °C until assays to determine the VA and IFN-α levels were performed. Stool specimens were collected from patients at the time of hospitalization. Clinical data were collected by reviewing hospital records.

2.2. Dietary investigation

Daily food intake was investigated using a 24-h dietary questionnaire, and the VA intake was enumerated in retinol activity equivalents per day (μg RAE/day). The VA intake of patients with HFMD was compared with Chinese Dietary Reference Intake (DRI), which is an age-based measure.[12] Dietary intake and food consumption data were analyzed by a dietician using the Nutrient Elements Calculator V1.6, based on the China food composition tables and developed by the Institute of Food Safety and Nutrition at China's Center for Disease Control and Prevention.

2.3. Serum vitamin A levels

VA levels of the serum samples were analyzed by high-performance liquid chromatography according to the method proposed by Driskell[13] with some modifications, using a Waters 2487 dual lambda absorbance detector. VA was detected at 315 nm. Serum was pipetted into an Eppendorf Micro Test Tube (EP Tube). Ethanol was added to the serum, and then the tubes were vortexed for 60 s. Hexane was added, and the mixture was vortexed for 120 s (the tube was bounced as it was being vortexed to ensure thorough mixing of the two layers). Then the tubes were centrifuged for 10 min. A 500-μL aliquot of the top layer fluid was transferred to another EP tube. These tubes were placed in a room-temperature water bath, and the hexane was evaporated with a stream of nitrogen. The residue was redissolved in 100 μL of methanol and vortexed for 10 s. Then, 20 μL of the solution was injected into the chromatography system.

2.4. Serum IFN-α concentration

The expression of IFN-α protein was determined using a human IFN-α ELISA kit (eBioscience, USA) according to the manufacturer's instructions. This involved adding 60 μL of dilution buffer and 40 μL of serum from each sample to microplate wells. Next, 50 μL of horseradish peroxidase-conjugated antibodies were added to all wells. Microplate strips were then covered and incubated for 2 h at room temperature (18–25 °C). After washing 3 times with washing buffer, 100 μL of tetramethylbenzidine substrate solution were added to all wells, which were then incubated at room temperature for 10 min. Then, 100 μL of stop solution was added to each well. The optical density was detected at a wavelength of 450 nm on a microplate reader (Wellscan MK3, Labsystems, Finland). A standard curve ranging from 7.8 to 500 pg/ml was constructed, using serial dilutions of a human IFN-α standard provided with the kit. Samples with values greater than the negative control but <7.8 pg/ml were assigned values based on extrapolation of the standard curve.

2.5. Detection of human enterovirus 71

The diagnosis of human enterovirus 71 was performed by fluorescence-based quantitative real-time PCR (qRT-PCR). Viral RNA was extracted from the stool specimens using an RNA Extraction Kit (DAAN Gene Co., Ltd., China), according to the manufacturer's instructions. The LightCycler RNA amplification hybridization probe kit (DAAN Gene) was used in this study. The test kit allows for one-step qRT-PCR to be performed using the LightCycler instrument (MJ Research, USA). The reaction mix in the hybridization probe kit contains a mixture of hybridization probe, primer and dNTP mix. Each reaction was performed in a reaction capillary by mixing the reagents, followed by spinning the mixture down briefly with the help of a centrifuge (Eppendorf, Germany). Each reaction contained 5 μL of RNA, 15 μL of reaction mix, 2 μL of reverse transcriptase and 3 μL of Taq enzyme. After reverse transcription at 40 °C (25 min) and initial denaturation at 94 °C (3 min), amplification was performed in 40 cycles at 93 °C for 15 s and 55 °C for 45 s.

2.6. Detection of IgM anti-EV71

EV71-IgM antibodies were detected using a human enterovirus 71-type IgM antibody ELISA kit (IBL, Germany). Briefly, 40 μL of dilution buffer and 10 μL of serum samples from each sample were added to microplate wells, and then 50 μL of horseradish peroxidase-conjugated antibodies was added and mixed gently, then incubated at 37 °C for 60 min. After washing 5 times with washing buffer, 50 μL of chromogen solution A and chromogen solution B, respectively, were added to each well. Samples were then incubated at 37 °C for 15 min. Stop solution (50 μL) was then added to each well, and optical density was measured at a wavelength of 450 nm within 15 min. A standard curve ranging from 0 to 80 ng/ml was constructed.

2.7. Statistical analysis

Statistical operations were performed with the SPSS Statistical Package, version 13.0. Descriptive statistics were presented as mean ± standard deviation (SD) and a median (interquartile range) for normally and non-normally distributed data, respectively. Student's *t*-test and ANOVA were used for continuous variables with normal distributions, when appropriate. The Mann–Whitney *U* test was used for continuous variables without a normal distribution, whereas the Chi-squared test was used for the analysis of categorical data. Pearson correlation coefficients or Spearman rank correlations were performed to test for significant associations