Inhibition of Enterovirus 71 by Selenium Nanoparticles Loaded with siRNA through Bax Signaling Pathways

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ABSTRACT: Enterovirus 71 (EV71) is the principal pathogen leading to severe cases of hand, foot, and mouth disease (HFMD). Specific drugs for EV71 are not discovered currently. Small interfering RNA (siRNA) provides a promising antiviral treatment pathway, but it is difficult to cross cell membranes and is easy to degrade. Nanoparticles are promising for their carrying capacity currently. In this study, the siRNA targeting EV71 VP1 gene was loaded with selenium nanoparticles (SeNPs) and surface decorated with polyethylenimine (PEI) (Se@PEI@siRNA). Se@PEI@siRNA showed a remarkable interference efficiency in the nerve cell line SK-N-SH and prevented the cells to be infected. The mechanism study revealed that Se@PEI@siRNA could lighten the extent of SK-N-SH cells for staying in the sub-G1 phase. Activation of Bax apoptosis signaling was restrained either. Taken together, this study demonstrated that Se@PEI@siRNA is a promising drug against EV71 virus.

INTRODUCTION

Enterovirus 71 (EV71) is a major pathogen that causes hand, foot, and mouth disease (HFMD). HFMD has been breaking out widely in recent years in the Asia Pacific region.1 It is a common communicable disease that usually affects children under 5 years old. Children suffering from this disease manifest fever or herpes. It is self-limited in general, but a minority of children infected by EV71 develop severe complications in the nervous system due to its neurotropism, such as aseptic meningitis, brain stem encephalitis, and neurogenic pulmonary edema, leading to fatal cases.2−4 EV71 belongs to the genus Enterovirus within the family Picornaviridae. It is a single-stranded positive-sense RNA virus, encoding VP1, VP2, VP3, and VP4 four structural proteins and seven nonstructural proteins. VP1 is the most important one since neutralizing epitopes of EV71 chiefly lie on the protein.5,6 VP1 is verified to be related to virus titer and the emergence of severe clinical cases during EV71 infection. Currently, there is no effective medicine for HFMD. The three vaccines against EV71 launched so far are univalent and have deficiencies, for instance, a short duration of immunological effects, no immune body production, adverse immune reaction, and high cost.7 Hence, it is significant for new drug research against EV71. EV71 infection can induce apoptosis in host cells. Bax is a key protein in the Bcl-2 family, which induces apoptosis. To explore the relationship between EV71 infection and Bax protein in nerve cells is important to clarify the pathogenic mechanism of severe cases with EV71 infection.

RNA interference (RNAi) is a post-transcriptional gene silencing process mediated by double-stranded RNA with gene sequence specificity. It ubiquitously exists in eukaryotes for resisting virus invasion, inhibiting activity of transposons, and regulating gene expression. Since small interfering RNA (siRNA) induces RNAi in mammals, it has been used widely in biomedicine research and drug development. In virus research, siRNA is designed to target genes of virus or vital virus receptors in cells to interfere with viral replication and suppress infection, such as EV71 virus, human immunodeficiency virus, hepatitis virus, influenza virus, respiratory syncytial virus, dengue virus, and so on.8,9 It has been reported that siRNA targeted 3’UTR, 2C, 3C, 3D, 2A, and 5’UTR genes of EV71 blocked the viral replication.10,11 Nevertheless, the application of siRNA is still facing challenges since siRNA is easy to degrade in a physiological environment and is difficult to pass through the cell membrane or release to the cytoplasm. As a cationic polymer, polyethyleneimine (PEI) can compress the negative nucleic acids and help them pass through the cell membrane, reaching a high transfection efficiency. However, toxicity of PEI is still a major limiting factor for application.

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Therefore, it is important to find out promising vectors for siRNA delivery. With the rapid development of nanotechnology, functionalized nanomaterials with unique chemical and physical properties have emerged as promising alternatives for treatment of disease.\textsuperscript{12} Nanoparticles are good drug delivery vectors due to their small particle size leading to stable structures and are easier to enter cells.\textsuperscript{13} Inorganic nanoparticles, including silver, gold, iron, silica, titanium dioxide, and selenium nanoparticles, have been used as drug or siRNA delivery vectors in biomedical research.\textsuperscript{14,15} Among them, selenium nanoparticles modified with PEI and loaded with siRNA have been verified to downregulate the gene level effectively.\textsuperscript{16} In addition, antiviral drugs loaded with selenium nanoparticles present a stronger ability in inhibiting H1N1 influenza virus infection.\textsuperscript{17,18} Inhibition of enterovirus A71 by selenium nanoparticles, which interferes with the JNK signaling pathway, was reported by Li et al.\textsuperscript{19} In this study, siRNA targeted VP1 gene of EV71 was synthesized. Cells were transfected with the siRNA carried by selenium nanoparticles modified with PEI before EV71 infection. Subsequently, cell viability and virus titer were detected after transfection efficiency was checked. The relationship between the siRNA-loading selenium nano-system and apoptosis induced by EV71 infection was further explored.

\section*{RESULTS AND DISCUSSION}

\textbf{Preparation and Characterization of Se@PEI@siRNA.} The TEM observation in Figure 1A showed that SeNPs, Se@PEI, and Se@PEI@siRNA presented spherical structures. Figure 1B shows the image of the Tyndall effect of Se@PEI@siRNA. Among them, selenium nanoparticles modified with PEI and loaded with siRNA have been verified to downregulate the gene level effectively.\textsuperscript{16} In addition, antiviral drugs loaded with selenium nanoparticles present a stronger ability in inhibiting H1N1 influenza virus infection.\textsuperscript{17,18} Inhibition of enterovirus A71 by selenium nanoparticles, which interferes with the JNK signaling pathway, was reported by Li et al.\textsuperscript{19} In this study, siRNA targeted VP1 gene of EV71 was synthesized. Cells were transfected with the siRNA carried by selenium nanoparticles modified with PEI before EV71 infection. Subsequently, cell viability and virus titer were detected after transfection efficiency was checked. The relationship between the siRNA-loading selenium nano-system and apoptosis induced by EV71 infection was further explored.
Figure 1C further verified that diameters of SeNPs and Se@PEI were approximately 200 and 100 nm, respectively, while Se@PEI@siRNA was about 80 nm. The small size of Se@PEI@siRNA contributed to its stable nanostructure, which made it easy to pass through the cell membrane. As shown in Figure 1D, the zeta potential of SeNPs alone was $-25$ mV, and it increased to 6 mV after modification with PEI and reached 12 mV when loading siRNA, explaining the higher stability of Se@PEI@siRNA.

Interference Efficiency and Cell Viability. SK-N-SH cells were transfected with SeNPs, Se@PEI, and Se@PEI@siRNA for 12 h before EV71 infection. In Figure 2A, the nucleic acid level of EV71 VP1 gene fell to 27% in cells treated with Se@PEI@siRNA before infection compared to cells without any treatment. Moreover, the expression amount of VP1 protein depressed in that group either. These results suggested that proliferation of EV71 was inhibited by Se@PEI@siRNA, and siRNA targeting EV71 VP1 got a good interference efficiency when loaded with selenium nanoparticles. The cytotoxic effect of SK-N-SH cells was detected by the MTT assay. As Figure 2B indicates, cell viability without any treatment before infection was 39%, while cells treated with SeNPs and Se@PEI were 42 and 36%, respectively. In addition, cells transfected with Se@PEI@siRNA reached 68% significantly. This assay presented that Se@PEI@siRNA effectively resisted the EV71 proliferation.

Suppression of Apoptosis by Se@PEI@siRNA. Flow cytometric analysis was performed to evaluate the antiviral mechanisms of Se@PEI@siRNA. In Figure 3, cells in sub-G1 rose to 24.6% proportion when SK-N-SH cells were infected with EV71 without treatment. However, cells treated with Se@PEI@siRNA before infection reduced the sub-G1 proportion to 10.0%. These data indicated that SK-N-SH cells were protected by Se@PEI@siRNA from EV71-virus-induced apoptosis.

Inhibition of the Apoptotic Signaling Pathway. SK-N-SH cells were transfected with Se@PEI@siRNA and then infected with EV71. Proteins related to apoptosis were detected by Western blot. The Bax protein was known to induce apoptosis increased when infected with EV71. Meanwhile, as seen in Figure 4, the expression reduced when cells were treated with Se@PEI@siRNA before infection. Bax is an important apoptosis-promoting protein in the Bcl-2 family. This result illustrated that Se@PEI@siRNA resists EV71 infection by inhibiting the Bax signaling pathway.

CONCLUSIONS

In conclusion, the nanocompound (Se@PEI@siRNA) comprising selenium nanoparticles and the siRNA targeting EV71 VP1 gene was synthesized. The siRNA showed a higher interference efficiency, which was more than 70% against the EV71 VP1 gene in the nerve cell line SK-N-SH when loaded with Se@PEI. Se@PEI@siRNA increased the cell viability to prevent EV71 virus infection. This may be associated with the decrease of cells in the sub-G1 phase due to treatment with Se@PEI@siRNA. EV71 infection can induce apoptosis in host cells. Bax is a key protein in the Bcl-2 family that induces apoptosis. The apoptosis signaling induced by Bax was restrained. Altogether, this study illustrates that Se@PEI@siRNA can effectively prevent the nerve cell line SK-N-SH cells from apoptosis induced by EV71 virus infection through the Bax protein signaling pathway.

MATERIALS AND METHODS

Materials. A human neuroblastoma cell line (SK-N-SH) was bought from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Dulbecco’s modified eagle medium (DMEM) and fetal bovine serum (FBS) were procured from Gibco. EV71 virus was isolated and stored in Guangzhou Women and Children’s Medical Center (GenBank...
Accession FJ360545.1). Na2SeO3 and ascorbic acid (VC) from Sigma were used in the study. The one-step qPCR kit was acquired from Takara. SiRNAs and primers were designed and synthesized by Sangon Biotech, China. The VP1 monoclonal antibody was bought from Abnova. Bax and β-actin monoclonal antibodies were acquired from Cell Signaling Technology.

**Synthesis of Se@PEI@siRNA.** 0.1 M Na2SeO3 solution, 0.05 M VC solution, and ultrapure water were mixed at a volume ratio of 1:8:91 followed by 60 min of stirring and overnight dialysis in ultrapure water. Then 1.2 nmol of siRNA and 37.92 mg of PEI were added into 20 mL of solution prepared above. The Se@PEI@siRNA compound was purified through centrifugation at 8000 rpm for 20 min. The sequence of VP1 siRNA is as follows: sense 5′ CACAUUCGAGAAGCAAAATT 3′ and anti-sense 5′ UUUUGUUCUCGGAAGUGGTT 3′. The sequence of negative control is as follows: sense 5′ UUUCUGAAGCGUCACGGUTT 3′ and anti-sense 5′ ACUGUAGACGUCUCCGAGAAT 3′.

**Characterization of Se@PEI@siRNA.** Morphology of Se@PEI@siRNA was observed by transmission electron microscopy (TEM). The Tyndall effect was studied in a dark room with a laser pointer. Size distributions and zeta potentials of SeNPs, Se@PEI, and Se@PEI@siRNA were determined by Cell Signaling Technology.

**Cells and Infection.** SK-N-SH cells were cultured in DMEM with 10% FBS. Cells were plated for 24 h and then adsorbed by EV71 for 1 h. The supernatant was removed, and DMEM with 2% FBS was added. The 50% tissue culture infective dose (TCID50) was calculated using the Reed-Muench method. EV71 used was at a titer of 100 TCID50/mL. SK-N-SH cells were transfected with Se@PEI@siRNA for 12 h followed by EV71 infection.

**Transfection with Se@PEI@siRNA.** SK-N-SH cells were seeded in 6-well plates for 5 × 10^5 cells per well. 24 h later, cells were treated with Se@PEI@siRNA. 12 h later, the cells were infected with EV71. After 48 h, the transfection efficiency was detected by qPCR and Western blot.

**MTT Assay.** Cell viability was determined by the MTT assay. Briefly, 7 × 10^3 cells were incubated in a well of 96-well cell culture plate for 24 h. After siRNA transfection and virus infection, 5 mg/mL MTT solution was added and incubated for 4 h before 150 μL of dimethyl sulfoxide was added. The OD value was measured at 570 nm.

**Polymerase Chain Reaction.** The mRNA level of EV71 was tested by qPCR. SK-N-SH cells were transfected with Se@PEI@siRNA and infected with EV71. qPCR was performed with a one-step kit according to the instrument. Primers are as follows: EV71 VP1 gene forward primer 5′ GGAGATGGTGATGGGATTTC 3′ and reverse primer 5′ GAAGATGGTGATGGGATTTC 3′. The sequence of the probe was CCATAGCAGCAGAACCCTTCC. GAPDH forward primer 5′ GAAGATGGTGATGGGATTTC 3′ and reverse primer 5′ GAAGATGGTGATGGGATTTC 3′.

**Cell Cycle Assay.** The cell cycle assay was performed as described previously. After treatment with Se@PEI@siRNA for 12 h, SK-N-SH cells were infected with EV71. After 48 h, cells were harvested and resuspended in ice cold 70% ethanol for fixation. After incubation with propidium iodide for 30 min, cells were measured by flow cytometry. Cell cycle phase quantification was analyzed using ModFit LT 3.1 software.

**Western Blot.** SK-N-SH cells transfected with Se@PEI@siRNA were infected with EV71 for 48 h. EV71 VP1, Bax, and β-actin monoclonal antibodies were used for incubation.

**Statistical Analysis.** GraphPad Prism 7.0 software was used for data analysis. Student’s t test and one-way analysis of variance were used to evaluate the significance of the data. Differences were considered statistically significant at P < 0.05 or P < 0.01 (**).
Author Contributions
Z.L. and Y.L. contributed equally to the work.

Author Contributions
Y.L. and Z.L. designed the study. T.X., M.G., C.W., and M.Z. analyzed the experimental data and drafted the manuscript. Ha.C., J.K., W.L., and Y.Z. carried out the experiments. T.L., Y.C., and Hu.C. participated in the design. B.Z. refined the manuscript and coordination. All authors read and approved the final manuscript.

Notes
The authors declare no competing financial interest.

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■ ABBREVIATIONS
EV-A71: enterovirus 71; HFMD: hand, foot, and mouth disease; NPs: nanoparticles; Se: selenium; TEM: transmission electron microscopy

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