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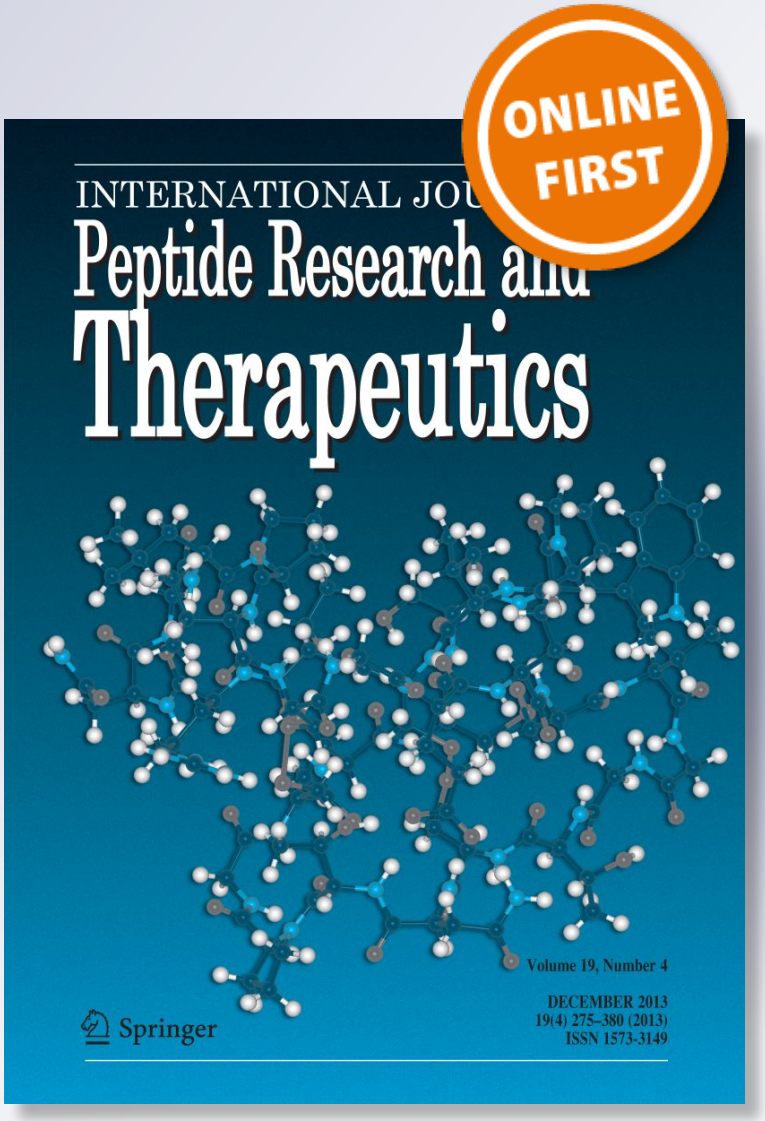

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The Human Cathelicidin LL-37, a Defensive Peptide Against Rotavirus Infection

Zohreh Hosseini[1] · Mohammad Bagher Habibi Najafi[1] · Masoud Yavarmanesh[1] · Angila Ataei-Pirkooh[2]

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Abstract
LL-37 is a 37 amino acid long cationic peptide belonging to the cathelicidin family of antimicrobial peptides. Limited investigations have shown the antimicrobial potency of LL-37 against different viral infections. We aimed to investigate the effect of the human cathelicidin peptide LL-37 on rotavirus infection, as a causative agent of severe gastroenteritis in children. After evaluation of LL-37 toxicity using methyl thiazolyl tetrazolium and neutral red uptake assays on the MA-104 cells, antiviral activity was assessed both by tissue culture infectious dose 50 (TCID50) and quantitative Real-Time PCR assays. Indirect immunofluorescence assay (IFA) and hematoxylin and eosin (H&E) staining were also performed to further confirm the inhibitory effects of the LL-37 on rotavirus. The viability maintained more than 90% up to the concentration of 50 µg/mL of peptide. LL-37 exerted its antiviral effect only when cells were pre-treated with peptide prior to rotavirus infection. 50 µg/mL of LL-37 could result in 3.36 log10 TCID50 reduction in virus titer ($p = 0.0001$), and an inhibition rate of 82.2% in copy number of rotavirus genomic RNA was obtained. IFA showed that the expression of rotavirus antigens in cells pre-treated with 50 µg/mL LL-37 is noticeably lower than the virus control. H&E assay also showed that the size and formation of inclusion bodies are decreased in rotavirus-infected cells pre-treated with 50 µg/mL LL-37 as compared to the virus control. Our findings suggested that the LL-37 peptide may interfere with viral attachment or act as an immune regulator, promoting innate immune responses.

Keywords Cathelicidin · LL-37 · Antiviral activity · Rotavirus · Antimicrobial peptides · Innate immunity · In vitro

Introduction

Antimicrobial peptides (AMPs) are short oligopeptides with a varying number of amino acids (from 4 to 100 amino acids) that are widely expressed in many organisms including mammals, arthropods and plants. Defensins and cathelicidins represent two major families of mammalian AMPs, and LL-37 is the sole member of the cathelicidin family of peptides expressed in humans (Bucki et al. 2010). Human cathelicidin peptide LL-37 is well-known conserved peptide of innate immunity, which has antimicrobial activities against a broad spectrum of pathogens such as gram-negative and gram-positive bacteria, parasite, fungi, and viruses (Bandurska et al. 2015). This peptide has immunomodulatory and adjuvant activities through acting as chemotactic for immune cells, also induces secretion of cytokines and chemokines (van Harten et al. 2018).

The human cathelicidin LL-37 is a pleiotropic cationic host defense peptide (CHDP), produced by leukocytes (primarily neutrophils) and various epithelial cells, and found in a wide range of human tissues and bodily fluids (Ramos et al. 2011). Up-regulation of the peptide expression has reported in response to inflammation and infections (Barlow et al. 2010). Recent advances in synthesis of LL-37 by using different techniques have led to an increasing use of this peptide as an antimicrobial agent especially against many bacterial and viral pathogens.

Rotavirus is a non-enveloped and double-stranded RNA virus belonging to the family *Reoviridae* that is < 100 nm in diameter. The virus is one of the causative agent of severe

✉ Mohammad Bagher Habibi Najafi
habibi@um.ac.ir

✉ Angila Ataei-Pirkooh
anjilaataei@gmail.com

[1] Department of Food Science and Technology, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

[2] Department of Medical Virology, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran

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gastroenteritis among children < 5 years of age in both developing and developed countries (Desselberger 2014). According to recent published report, rotavirus caused nearly 258 million episodes of diarrhea and 128,500 deaths among children less than 5 years of age throughout the world in 2016 (Troeger et al. 2018). Transmission primarily occurs via the fecal–oral route, followed by contaminated foods, surfaces, hands, toys, and probably by the respiratory route (Desselberger 2014).

Rotaviruses infect and replicate within the mature enterocytes at the apex of the villus of the small intestine, often leading to severe watery diarrhea, fever, vomiting, and abdominal pain in children (Crawford et al. 2017; Kiulia et al. 2015). Since 2006, two live-attenuated rotavirus vaccines, the monovalent Rotarix (GlaxoSmithKline Biologicals, Belgium) and the pentavalent RotaTeq (Merck and Co, USA) have been licensed in more than 100 countries worldwide, and their use has dramatically decreased the rate of severe rotavirus gastroenteritis, especially in high-income and developed countries. However, in low- and middle-income or developing countries, the vaccines have been found to be considerably less potent (Burnett et al. 2018). Currently, treatment of viral diarrhea is based on intravenous fluid administration which has successfully led to reduce its duration and severity (Knipping et al. 2012). Until now, there is no specific drug available for the treatment of rotavirus infection and many significant challenges remain in the development of new antiviral agents.

To date, antiviral properties of LL-37 against a number of viruses has been reported including influenza A (Barlow et al. 2011; Tripathi et al. 2013), respiratory syncytial virus (Currie et al. 2013; Harcourt et al. 2016), adenovirus (Gordon et al. 2004), herpes simplex virus (Gordon et al. 2004, 2005), HIV-1 (Bergman et al. 2007), varicella zoster (Crack et al. 2012), hepatitis C virus (Matsumura et al. 2016), vaccinia virus (Howell et al. 2004), and dengue virus (Alagarasu et al. 2017), but no study addressed against rotavirus. So in the present study, we aimed to investigate the effects of LL-37 on rotavirus infection in vitro.

Materials and Methods

Cell Culture and Reagents

The epithelial rhesus monkey kidney cell line MA-104 was obtained from the cell bank of Department of Virology, Iran University of Medical Sciences. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Invitrogen, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Invitrogen, USA), 1% penicillin–streptomycin (Sigma-Aldrich, USA), 2.25 g/L sodium bicarbonate and 2 mM L-glutamine (Merck, Germany) at 37° in a humidified 5% CO_2 atmosphere.

LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) peptide (> 95% purity) was purchased from (Innovagen Co., Lund, Sweden) and used in all the experiments. For preparing 1000 µg/mL stock solution, 1 mg peptide was dissolved in 1 mL deionized distilled water, stored at − 70 °C until use.

Rotavirus Culture

Simian SA-11 rotavirus was also obtained from Virology Department of Iran University of Medical Sciences (Tehran, Iran), and propagated in MA-104 cells. For preparation of virus stock, MA-104 cell monolayer in 25-cm^2 flask (Nunc, Denmark) was incubated with 500 µL of trypsin-preactivated rotavirus suspension at a multiplicity of infection (MOI) of 1 at 37 °C. The virus inoculum was then replaced with serum-free DMEM, and the flask was incubated at 37 °C for an additional 48 h. The virus was then aliquoted into sterile cryovials and stored frozen at − 20 °C until use (Jolly et al. 2000). Virus was titrated using TCID50 method based on the Reed and Muench formula (Reed and Muench 1938), and was used for the next in vitro experiments at the titer of 100 TCID50/mL.

Cytotoxicity Assays

The toxicity of LL-37 peptide to MA-104 cells was evaluated using two assays: MTT and neutral red uptake assays.

MTT Assay

The MTT assay was performed according to a modification of the standard method described by Mosmann (Mosmann 1983). Briefly, MA-104 cells were seeded in a flat-bottomed 96-well polystyrene coated plate (SPL Lifesciences, South Korea) and were incubated at 37 °C for 24 h in a 5% CO_2 incubator. Different concentrations of LL-37 peptide ranged from 0.1 to 50 µg/mL were added to the plate in triplicate. After 48 h of incubation, 10 µL of MTT reagent was added to each well and was further incubated for 3 h. Formazan crystals formed after 3 h in each well were dissolved in 50 µL of DMSO solution (Bio-Idea, Iran) and the plates were read immediately in a microplate reader (Hiperion MPR 4+, Germany) at 550 nm (Tavakoli and Hashemzadeh 2019).

Neutral Red Uptake Assay

Briefly, MA-104 cells were seeded at a density of 1.5×10^5 cells/well in a 96-well plate. After 24 h incubation at 37 °C in a 5% CO_2 incubator, spent media was discarded and the different concentrations of LL-37 peptide (0.1 to 50 µg/mL)

were added. The plate was then incubated for 72 h at 37° in a humidified incubator with a 5% CO_2 environment. Following the incubation period, the medium was discarded and serum-free media containing neutral red was then added to each well and incubated for 3 h. Incorporated dye was then liberated from the cells by adding of dye release agent to each well. The plate was placed on a shaker for 15 min after which the optical density at 550 nm was determined on a microplate reader (Fotakis and Timbrell 2006).

Assessment of Antiviral Activities

Different methods were used to treat the cell monolayers to assess the effect of LL-37 peptide on inhibition of rotavirus infectivity. To determine the antiviral activity of the LL-37 peptide before, during, and after viral adsorption, cell pre-treatment, co-treatment, and cell post-treatment assays were performed, respectively (Tavakoli et al. 2018).

Virucidal Activity

To evaluate direct effect of peptide on rotavirus particles, 100 µL of viral suspensions (100 TCID50) were mixed with 100 µL of different non-cytotoxic concentrations of the peptide for 3 h at 37 °C in a humidified 5% CO_2 atmosphere. The suspension was then added to the MA-104 cell monolayers and incubated again for 1 h at 37 °C. The added suspensions were aspirated, and the cells were washed three times with PBS to remove non-absorbed viruses, overlaid with fresh serum-free DMEM, and incubated at 37 °C for 48 h.

Cell Pre-treatment Assay

The monolayers of MA-104 cells seeded in 96-well plates were treated with different concentration ranges of LL-37 peptide below their cytotoxic levels for 3 h at 37 °C. Culture medium containing peptides was then removed from wells and the cells were washed three times with PBS. Afterwards, the cells in each well were infected with 100 µL of rotavirus suspensions (100 TCID50/mL) in the absence of LL-37 and incubated for 1 h at 37 °C. Viral inoculum was then removed and fresh serum-free DMEM was added, and the plates were further incubated for 48 h at 37 °C.

Co-treatment Assay

MA-104 cell monolayers in 96-well plates were washed with PBS and infected with a mixture of 0.1 mL 100 TCID50/mL of rotavirus and 100 µL of peptide at its non-toxic concentrations for 1 h at 37 °C. After incubation, the cells were washed twice with PBS to remove non-penetrated viruses, overlaid with fresh serum-free DMEM, and incubated for 48 h at 37 °C in a humidified 5% CO_2 atmosphere.

Cell Post-treatment Assay

A monolayer of MA-104 cells in a 96-well plate were infected with 100 µL of 100 TCID50/mL of rotavirus suspension and incubated for 1 h at 37 °C in a humidified 5% CO_2 incubator. The cells were then washed three times with PBS to remove unattached viruses, and different non-cytotoxic concentrations of the peptide in fresh serum-free DMEM were added to MA-104 cells and further incubated at 37 °C for 48 h. In all assays, cell control and virus control were included under the same conditions. For all experiments, rotavirus was quantified by TCID50 and quantitative real-time PCR (qPCR) assays.

Quantitative Real-Time PCR (qPCR)

To measure the rotavirus viral load, a quantitative system using real-time PCR assay was performed. The rotavirus viral RNA was extracted from the lysate using the AccuPrep Viral RNA Extraction Kit (Bioneer, South Korea) according to the manufacturer's recommendations. Isolated RNA was then converted to cDNA using cDNA Synthesis Kit (YTA, Yekta Tajhiz Azma, Iran) according to the manufacturer's instruction, stored at −70 °C until further analysis. The forward and reverse primer sequences targeting VP6 gene of rotavirus SA-11 were 5′-CGA ATG GCT GTG CAT TCG GG-3′ and 5′-CAG CTG ACG GGG CAA CTA CA-3′, respectively, with amplicon size of 225 bp. The real-time PCR was performed in a final volume of 16 µL reaction including 8 µL of the SYBR Green PCR master mix 2× (Yekta Tajhiz, Iran), 10 pmol/µL of each primer, 50 ng of template DNA, and the rest of ddH_2O. The assay was performed using the Rotor-Gene Q instrument (Qiagen, Germany) under the following conditions: 5 min activation of Taq DNA polymerase at 95 °C, followed by 40 cycles of 20 s at 95 °C, 45 s at 60 °C.

The recombinant plasmid pGH-SA11-VP6 containing rotavirus VP6 gene was constructed as a template. A 225 bp DNA segment spanned the sequences between the forward and reverse primers was amplified and cloned into the pGH vector, and prepared as a lyophilized powder. All procedures were performed by the Generay Biotech (Shanghai, China). A stock solution was prepared by dissolving 4 µg of the template in 40 µL of dilution buffer to yield a final concentration of 100 ng/mL. The cDNA concentration was determined using a NanoDrop spectrometer (Thermo Scientific, USA). The number of copies of DNA template was then estimated using a program available at the website: http://cels.uri.edu/gsc/cndna.html. The stock solution was serially diluted tenfold and used as templates

to construct standard curves. Given to the known copy number of the standards, the copy number of rotavirus in the unknown samples was calculated.

Indirect Immunofluorescence Assay (IFA)

Confluent MA-104 cells grown on sterile glass coverslips (Nunc, Denmark) in a 24-well plate were incubated with 200 µL of 100 TCID50/mL rotavirus suspension at 37 °C for 1 h in a humidified 5% CO_2 incubator. The virus inocula were then removed from the wells, and the cells were washed three times with PBS. The maximum non-cytotoxic concentration of LL-37 peptide (50 µg/mL) suspended in serum-free DMEM containing 4 µg/mL trypsin were then added to the wells and the plate was incubated at 35 °C with 5% CO_2. The cell and virus controls were also included in this experiment. After 16 h incubation, the cells were fixed with cold acetone (4 °C) for 15 min, and the fixed cells were overlaid with rabbit anti-rotavirus polyclonal sera as primary antibodies, followed by incubation for 45 min at 37 °C. The cells were washed three times with PBS, and were then overlaid with goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) (Dako, Germany), followed by incubation at 37 °C for 30 min. In the next step, the cells were washed again three times with PBS and coverslips were mounted on slides with glycerol buffer. Eventually, the cells were visualized under the Olympus BH2-RFCA fluorescence microscope (Tokyo, Japan).

Hematoxylin and Eosin (H&E) Staining

MA-104 cells were grown to confluence on sterile glass coverslips (Nunc, Denmark) in a 24-well plate. 100 µL of 50 µg/mL stock of LL-37 peptide was added to each well 3 h before virus inoculation. After pre-treatment with the peptide, the virus inoculum was removed from the wells, the monolayers were washed with PBS, and replaced with 100 µL of 100TCID50 rotavirus suspension. For H&E staining, the coverslips were collected 6–8 h after infection and rinsed twice with PBS. The cells were fixed by cytology fixative solution for 1 h, washed with PBS, and stained with hematoxylin for 10 min. The cells were washed with PBS again and rinsed once in 1% acid-alcohol solution. Then, the cells grown the coverslips were placed into a saturated solution of lithium carbonate Li_2CO_3 for 30 s. After washing with PBS, the cells were stained with a 1% eosin solution for 30 s. Final washing with PBS was performed and the cells were rinsed twice with ethanol. Finally, the coverslips were mounted onto microscope slides to be viewed from observation of forming inclusion bodies.

Statistical Analysis

Data represent the mean of three independent experiments. One-way analysis of variance (ANOVA) was used to analyze statistical difference between groups, followed by the Dunnett's post hoc test (Dunnett 1955). Statistical analyses of observational data and graphs-drawing were performed using the GraphPad Prism software package, version 7.0 (GraphPad Software, Inc., San Diego, CA, USA) and p values < 0.05 were considered significant.

Results

Cytotoxicity of LL-37 peptide was evaluated in MA-104 cells using the MTT and neutral red uptake assays. Figure 1 shows the viability assays data estimated as the percentage of viable cells relative to control. According to the MTT and neutral red results, viability maintained more than 90% up to the concentration of 50 µg/mL of LL-37 peptide. According to these results, the concentrations of LL-37 peptide that exhibited cytotoxic activity $\leq 10\%$ were used for the subsequent antiviral assays. Significant statistically differences ($p < 0.05$) in cytotoxicity were observed in range of 20–50 µg/mL for LL-37 peptide in comparison with cell control in both assays.

According to our time-of-addition study, LL-37 peptide did not have any antiviral activity against rotavirus in cell post-treatment and co-treatment assays. Furthermore, no virucidal activity was found against rotavirus by the peptide at any concentrations. It is interesting that inhibitory effects were only visible in cell pre-treatment assay.

In the pre-treatment assay, different concentrations of the LL-37 peptide were treated with confluent monolayer MA-104 cells and incubated for 3 h before rotavirus infection. The morphological changes of the LL-37-untreated rotavirus-infected MA-104 cells (virus control) was compared with the various concentrations of LL-37-pretreated rotavirus-infected cells with light microscopy. It was obvious that rotavirus CPE could be decreased by increasing the concentration of the LL-37 peptide or, in other words, in a dose-dependent manner. As indicated in Fig. 2, rotavirus showed a distinct cytopathic effect characterized by cell rounding, detachment of cells from the monolayer, and lytic foci. TCID50 was then calculated by CPE assay.

The peptide was evaluated in vitro for antiviral property against rotavirus with TCID50 method. There was statistically significant difference between the control and all experimental groups (p value = 0.0001), except at the concentration of 0.1 µg/mL LL-37 (p value = 0.69). The inhibitory effect of 50 µg/mL LL-37 was the most remarkable among all concentrations, so that pre-treatment of rotavirus with the concentration of 50 µg/mL LL-37 led to 3.36 $\log_{10}$

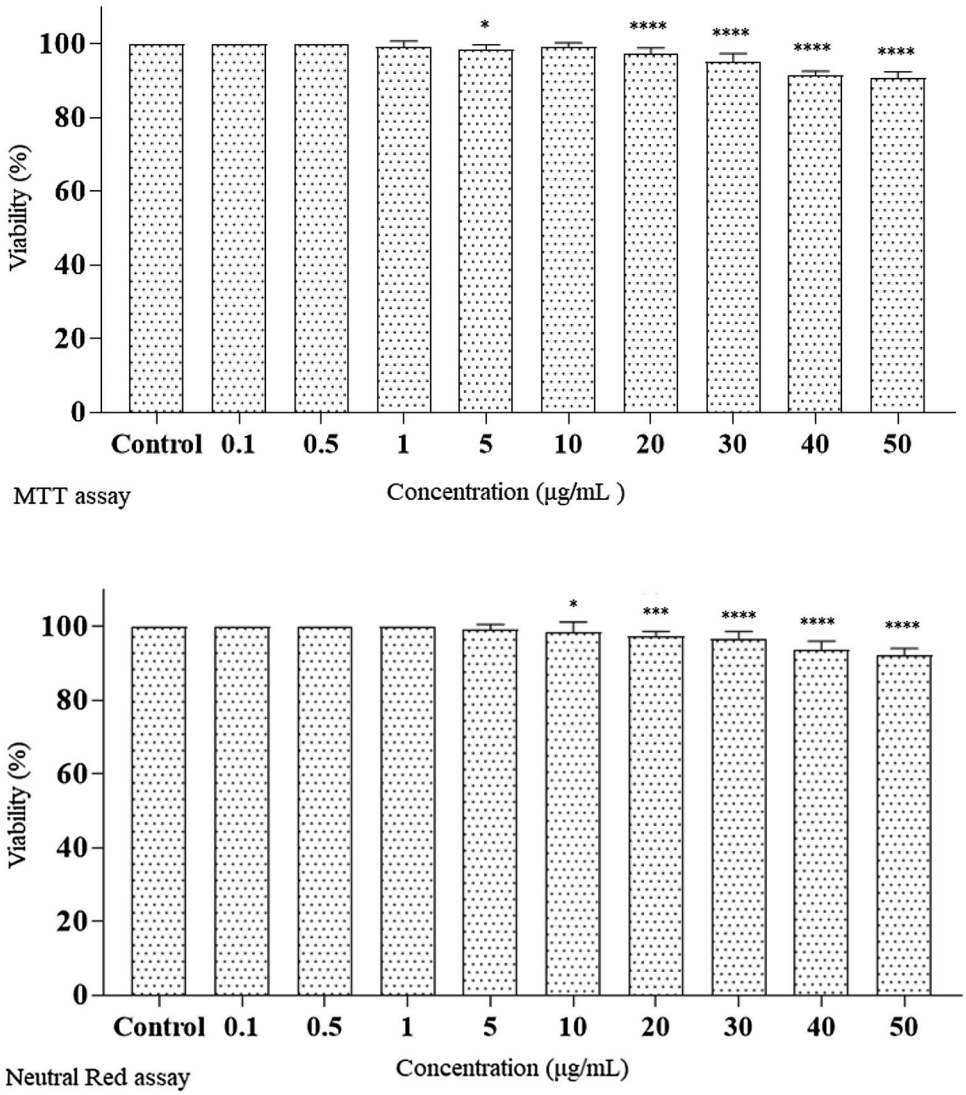

Fig. 1 Cytotoxicity of LL-37 on MA-104 cells using the MTT and neutral red assays. The viability maintained more than 90% up to the concentration of 50 µg/mL of LL-37 peptide. Statistically significant differences in cytotoxicity were calculated in range of 20–50 µg/mL of LL-37 peptide compared to the cell control. Data shown represent the mean of three independent experiments. *Represent statistically significant difference ($p < 0.05$). ***Represent statistically significant difference ($p = 0.0007$). ****Represent statistically significant difference ($p = 0.0001$)

TCID50 reduction in virus titers when compared to the virus control group (p value = 0.0001) (Table 1).

The quantitative real-time PCR assay was further performed in parallel to confirm the TCID50 results. As shown in the Fig. 3, the results of the real-time PCR demonstrated that LL-37 peptide at a concentration of 50 µg/mL could remarkably decrease the number of copies of rotavirus genomic RNA with an inhibition rate of 82.2% compared to untreated control.

IFA was used to determine and confirm the inhibitory activity of LL-37 peptide on replication and antigen expression of rotavirus in cell culture. The rotavirus infected MA-104 cells on coverslips were pre-treated with the most inhibitory concentration of LL-37 (50 µg/mL) resulted from previous antiviral screening assays. Negative and positive controls were included in the assay for comparisons. As shown in Fig. 4, the intensity of fluorescence signals in MA-104 cells treated with LL-37 (50 µg/mL) was noticeably weaker than that of virus control group. This observation shows that the expression of rotavirus antigens in MA-104 cells pre-treated with LL-37 (50 µg/mL) was noticeably lower than the untreated infected cells, indicating the strong antiviral activity of LL-37 against rotavirus.

In the next stage, effects of the concentration of 50 µg/mL LL-37 peptide against rotavirus were examined by H&E staining. Formation of inclusion bodies in the cytoplasm of MA-104 cells is a characteristic morphological feature of rotavirus infection. This staining was performed to further confirm inhibitory activity of LL-37 on rotavirus in the pre-treatment assay. As shown in the Fig. 5, formation of inclusion bodies has notably decreased in the presence of peptide in comparison to the absence of peptide (positive control).

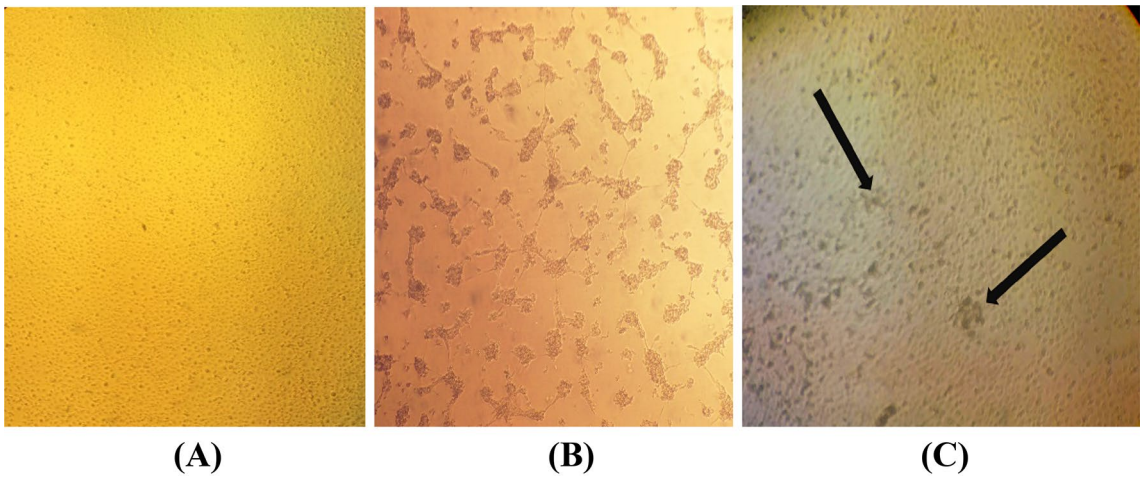

Fig. 2 Inhibition of rotavirus-induced cytopathic effects in MA-104 cells in the pre-treatment assay. **a** Cell control; **b** virus control; **c** rotavirus-infected cells pre-treated with 50 µg/mL of LL-37 peptide. As shown in the figure, rotavirus resulted in distinct cytopathic effects characterized by cell rounding, detachment of cells from the monolayer, and lytic foci. Cytopathic effects could be decreased in LL-37 pre-treated MA-104 cells infected with rotavirus. Black arrows show the cytopathic effects of rotavirus in LL-37 pre-treated MA-104 infected cells

Table 1 The result of TCID50 in the pre-treatment assay

	Concentration (µg/mL)	Log10 TCID50/mL	*p* value
Cell control	–	–	–
Virus control	–	6.5	–
LL-37 peptide	0.1	6.4	0.6
	0.5	6.2	0.0001*
	1	5.2	0.0001*
	5	5	0.0001*
	10	3.8	0.0001*
	20	3.7	0.0001*
	30	3.6	0.0001*
	40	3.5	0.0001*
	50	3.1	0.0001*

Data shown represent the mean of three independent experiments

*Represent statistically significant difference

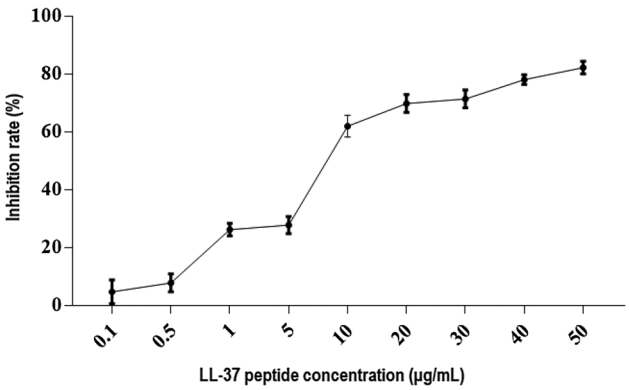

Fig. 3 The inhibitory rate of the LL-37 peptide against rotavirus replication determined by real-time PCR in cell pre-treatment assay. The quantitative real-time PCR assay was done to investigate the inhibitory effect of LL-37 peptide on rotavirus viral load. As presented in the figure, LL-37 peptide at the concentration of 50 µg/mL could significantly decrease the number of copies of rotavirus genomic RNA, with an inhibition rate of 82.2% compared to untreated control

Discussion

In the current study, two techniques were applied to determine the antiviral activity of the LL-37 peptide on replication of rotavirus. The first technique was TCID50 and the second one was Real-Time PCR assay. In pre-treated cells, rotavirus replication was inhibited by 3.36 log10 steps; however, this value was not compatible with the reduction of rotavirus RNA by less than 2 log10 steps. The discrepancy between the results of TCID50 and Real-Time PCR assays can be justified by this explanation that the basis of these two techniques is different. It should be noted that TCID50 assay is an operator-dependent method, and requires technical skills and relies on microscopic examination to observe virus induced-cytopathic effects. Therefore, it is more susceptible to errors. While molecular methods such as Real-Time PCR has been shown to be more specific and sensitive for the detection of viral infection.

In this study, we demonstrated that LL-37 exerts its antiviral effect only when MA-104 cells are treated with peptide prior to rotavirus infection. In fact, cell pre-treated with LL-37 has led to a remarkable reduction in production of infectious virions at a dose-dependent fashion. So that

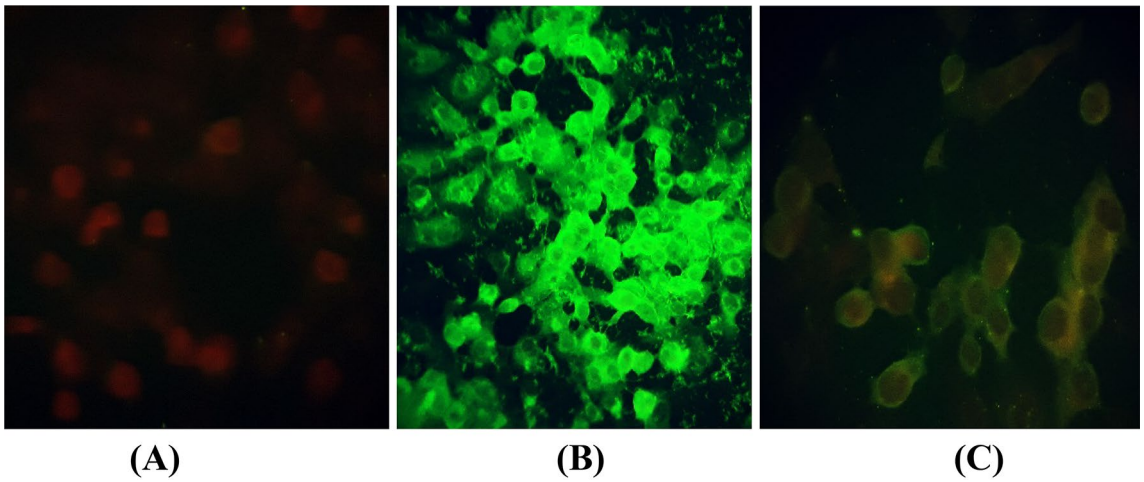

Fig. 4 Effect of LL-37 peptide on rotavirus antigens expression (IFA assay). **a** Cell control, **b** Virus control, **c** Infected cells pre-treated with LL-37 (50 µg/mL). The cells were fixed, and incubated with rabbit anti-rotavirus polyclonal serum, followed by incubation with goat anti-rabbit IgG conjugated to FITC antibody. Immunofluorescence was visualized by fluorescence microscope. Intensity of fluorescence signals in pre-treated MA-104 cells with LL-37 was remarkably weaker than that of virus control, demonstrating the strong inhibitory effect of LL-37 on the expression of rotavirus antigens

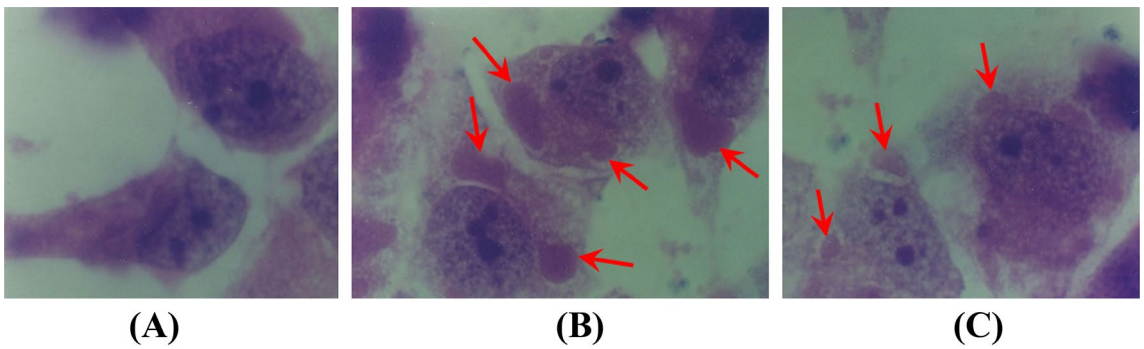

Fig. 5 Effect of LL-37 peptide on rotavirus-induced inclusion bodies (H&E assay). **a** Cell control, **b** Virus control, **c** Infected cells pre-treated with LL-37 (50 µg/mL). Red arrows show the inclusion bodies. The figure shows that the size and formation of inclusion bodies has been decreased in rotavirus-infected cells pre-treated with 50 µg/mL LL-37 compared to the virus control

addition of 50 µg/mL of LL-37 had a maximum inhibitory effect on virions production (Table 1 and Fig. 3). The replication of rotavirus occurs in membrane-free cytoplasmic inclusion bodies termed viroplasms, which consist of two non-structural proteins, NSP2 and NSP5. Replication of viral dsRNA and early stages of viral morphogenesis such as packaging of viral particles take place in these inclusions (Carreño-Torres et al. 2010; Cheung et al. 2010). Our findings show that the size and formation of inclusion bodies has been decreased in rotavirus-infected cells pre-treated with 50 µg/ml LL-37 compared to the virus control (Fig. 5), suggesting that LL-37 at the maximum concentration used could inhibit the replication of rotavirus. However, no inhibitory effect was detected after cell exposure to virus and also, virucidal activity was not demonstrated at any LL-37 peptide concentrations used. Our hypothesis is that peptide binds to rotavirus receptor on the cell surface and subsequently, leads to prevention of virus entry into host cells. Sialic acids are known as important cellular receptors for SA-11 rotaviruses. In previous studies, it has been documented that antimicrobial peptides such as LL-37 can interact with receptors on the cells containing sialic acids like erythrocytes (Hou et al. 2013). Another explanation for decreased viral replication during pre-treatment of cells with LL-37 may be attributed to internalization and active cellular uptake of peptide. In this way, LL-37 can provide a protective antiviral state in pre-treated epithelial cells.

A number of studies have shown the antimicrobial effects of LL-37 against various bacterial strains such as klebsiella pneumoniae (Smeianov et al. 2000), neisseria gonorrhoeae (Bergman et al. 2005), and staphylococcus aureus (Travis et al. 2000). Additionally, LL-37 is effective

in killing common bacteria causing oral infections (Lee et al. 2010; Tanaka et al. 2000). Most studies have suggested disruption of bacterial membrane is the main mode of action of LL-37. It has been documented that LL-37 is bound to the lipid bilayers in the α-helical structure, which leads to increased permeability of the bacterial membranes (Lee et al. 2011).

Antiviral properties of LL-37 against some viral pathogens have been investigated in several studies. Tripathi et al. assessed the inhibitory effect of LL-37 on influenza A virus and their results have shown that the peptide was more effective when the virus and LL-37 were pre-incubated prior to viral infection of cells. They have suggested that LL-37 directly interacts with the virus, leading to inactivation of infectious virions (Tripathi et al. 2013). In another study conducted by Barlow et al., LL-37 exhibited significant antiviral actions against influenza A in a mouse model (Barlow et al. 2011). The peptide could lead to decrease in severity of disease and replication of influenza. They suggested that LL-37 interact with influenza virion directly rather than through receptor-based mechanisms. Similarly, in another study by Alagarasu et al., it has been reported that pre-incubation of dengue virus type 2 with LL-37 resulted in a significant decrease of infectivity compared to the virus control, and treatment of Vero E6 cells before and after viral infection had no effect on the amount of virion (Alagarasu et al. 2017). They concluded that the peptide directly interacts with the virus, leading to viral receptor occupancy and subsequently, inhibition of viral entry and infection. Currie et al. demonstrated that LL-37 peptide has significant antiviral effect against respiratory syncytial virus (RSV) (Currie et al. 2013). They suggested that LL-37 is able to inhibit virus-induced cell death in epithelial cultures, remarkably decrease levels of virion particles into the culture medium, and limit spread of RSV infection. The maximum antiviral effect was observed when LL-37 was pre-incubated with the virus, or added simultaneously. Direct interaction of LL-37 with viral particles and epithelial cells proposed as a possible antiviral action of the peptide. In accordance with these findings, pre incubation of hepatitis C virus (HCV) with LL-37 resulted in significant reduction in infectivity titer in a dose-dependent manner (Matsumura et al. 2016).

It should be noted that all experiments described above were performed on enveloped viruses. As an explanation for these observations, the lipid composition of the viral envelope may be susceptible to LL-37, causing envelope disruption. Similar findings have demonstrated in studies performed on bacteria in which antimicrobial activity of LL-37 was mediated by permeabilization of bacterial membranes, as mentioned above. However, rotavirus is a non-enveloped virus, the suggested mechanism for inhibition of enveloped viruses by LL-37 is probably different from that of non-enveloped viruses. It hypothesizes that there are other factors that may play important roles in viral inhibition by LL-37 peptide.

Increasing evidence indicates that LL-37 can act as potent immune regulators, chemokines or chemokines production inducers. This peptide is produced by leukocytes and epithelial cells immediately after infection, and considered as a chemoattractant for monocytes, neutrophils, dendritic and T-cells (Lai and Gallo 2009). Innate immune response is considered as a first line of defense against rotavirus, which limits viral replication and infection into the host (Holloway and Coulson 2013). Regarding to these facts, we hypothesize that LL-37 is able to inhibit rotavirus infection by promoting the innate immune responses. This hypothesis is supported by our results in which, pre-incubation of cell with the peptide before viral exposure could resulted in maximum antiviral effect against rotavirus infection.

Conclusion

In conclusion, our study was the first attempt to investigate the effects of the LL-37 peptide on rotavirus infection in vitro. Cytotoxic effects of LL-37 peptide on MA-104 cells were evaluated using the MTT and neutral red uptake assays, and viability maintained more than 90% up to the concentration of 50 µg/mL. Pre-treatment of cells with LL-37 before viral exposure has resulted in significant reduction in production of infectious virions at a dose-dependent manner. This finding can be explained by two possibilities: (a) LL-37 binds to rotavirus receptor on the cell surface, leading to prevention of virus entry into the host cells; (b) LL-37 is able to inhibit rotavirus infection by promoting the innate immune responses as a potent immune regulator. Certainly, our study have some limitations. For example, we did not used the human rotavirus strain and the human adenocarcinoma cell line HT 29 instead of the SA-11 strain and MA-104 cell line, respectively. Furthermore, our results need to be confirmed using animal models.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Research Involving Human and Animal Rights This article does not contain any studies with human participants or animals performed by any of the authors.

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