Original Article

In vitro inhibition of *Rotavirus* multiplication by copper oxide nanoparticles

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ABSTRACT

Group A rotaviruses are the most common cause of gastroenteritis in children under five years of age worldwide. Rotavirus gastroenteritis can be related to mild to severe diarrhea in children and in some cases, can lead to death due to severe dehydration. Approximately 146,480 people die annually from rotavirus infection worldwide, and most of these deaths occur in low-income countries in Africa and Asia. Since there are no specific effective drugs to treat rotavirus infections, and infected patients can only be treated supportively, new antiviral agents need to be developed. Copper oxide nanoparticles (CuO NPs) have a wide range of applications in the magnetic and electrical industries, as well as in biology. The antiviral activity of nanoparticles (CuO NPs) is well documented. This study aimed to investigate the antiviral effect of CuO NPs on rotaviruses. The cytotoxic effects of CuO NPs on MA-104 cells were examined by methyl thiazolyl tetrazolium assay. In addition, the anti-rotavirus activity of CuO NPs was evaluated by $TCID_{50}$ and real-time polymerase chain reaction PCR assay. Our results showed that exposure of rotavirus-infected cells to various non-toxic concentrations of CuO NPs did not cause a decrease in viral titer, compared to the control. However, the virucidal effect of CuO NPs on rotavirus was observed at concentrations of 80 and 100 µg/ml (P<0.001). Our study suggested that CuO NPs had significant antiviral activity against rotavirus replication. However, the exact mechanism of anti-rotavirus activity of CuO NPs remained unknown. According to the virucidal assay, it appears that the loss of capsid integrity and genome disruption in the presence of CuO NPs are possible mechanisms of its anti-rotavirus activity.

Keywords: 50% tissue culture infectious dose, Copper oxide nanoparticles, Nanoparticle, Rotavirus

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1. Introduction

Group A rotaviruses are the leading cause of gastroenteritis in children under five years of age worldwide. These viruses are transmitted by fecal-oral transmission through contaminated water or food or by human-to-human contact (1). The prevalence of this viral infection is higher during the cold season and in areas with poor sanitation. Rotavirus gastroenteritis can be associated with mild to severe diarrhea in children, and in some cases, can lead to death due to severe dehydration. Annually, approximately 146,480 people die from rotavirus infection worldwide, and most of these deaths occur in low-income countries in Africa and Asia (2).

The main goal in treating rotavirus gastroenteritis is to replace fluid and electrolyte losses due to vomiting and diarrhea. Since there are no specific effective drugs to treat rotavirus infections, and only supportive treatment is available to infected patients, new antiviral agents need to be developed (3).

Recent advances in nanotechnology have paved the way for the development of new drugs (4). Nanoparticles are tiny particles with a diameter of 100 nm or less produced by nanotechnology. Due to their unique properties compared to bulk materials, they can be used in various fields of science, such as biology, pharmaceuticals, medicine, gold industry, diagnosis, and the treatment of diseases (5). Due to their strong surface adsorption and potential to bind to various components, these nanoparticles can be imparted with various new biological and antimicrobial properties by manipulating their surface (6, 7). Therefore, these new nanocomposites can be used in the fight against various diseases (8).

Copper oxide nanoparticles (CuO NPs), similar to other metal oxide nanoparticles, have various applications in the magnetic and electrical industries; however, their antimicrobial and biocidal properties have attracted the attention of biologists (9). Copper has strong antiviral properties, and studies have shown that it can neutralize various viral infections, such as hepatitis C virus (HCV) (10), human immunodeficiency virus 1, herpes simplex virus type 1 (HSV-1) (11), measles, infectious bronchitis virus, influenza virus A (12), and poliovirus (13). This study aimed to investigate the antiviral effects of CuO NPs on rotaviruses, which are among the most important viruses threatening the health of children.

2. Materials and Methods

Preparation and characterization of nanoparticles

CuO NP powder was obtained from US Research Nanomaterials (USA; Product Number: US3070). Nanoparticles were suspended in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, USA) and sonicated to prevent aggregation. Finally, they were serially diluted and prepared at different concentrations. The structure of the nanoparticles was studied by X-ray diffraction (XRD) and electron microscopy.

Cell culture and virus preparation

The rhesus monkey kidney cell line (MA-104) was provided by the cell bank of the virology department of Iran University of Medical Sciences, Tehran, Iran. Cells were cultured in high glucose DMEM containing 10% fetal bovine serum (Gibco, USA), 2 mM sodium pyruvate (Merck, Germany), 2 mM L-glutamine (Merck, Germany), and 100 IU/ml penicillin at 37°C in a humidified environment with 5% CO_2. Simian SA-11 rotavirus was obtained from the Research Center for Pediatric Infectious Diseases affiliated with Iran University of Medical Sciences. The titer of viable viruses was determined using the standard 50% tissue culture infectious dose ($TCID_{50}$).

Cytotoxicity assay and determination of cell viability

The cytotoxic effect of CuO NPs on MA-104 cells was evaluated using a methyl thiazolyl tetrazolium (MTT) assay. Briefly, 1.5×10^4 MA-104 cells/well were seeded in a 96-well microplate and incubated at 37°C for 24 h. Subsequently, various concentrations of CuO NPs (20 to 400 µg/ml) were added to the microplate in triplicate and incubated for 48 h at 37°C. A volume of 10 µ l MTT solution (5 mg/ml) was added to each well, and the plate was incubated at 37°C for 3 h. In the end, the MTT reagent was removed and 50 µl dimethyl sulfoxide was added to each well and the plate was shaken at room temperature for 10 min. Finally, the microplate was read at 550 nm using a microplate reader, and the percentage of viable cells was calculated for each concentration compared to control cells.

Evaluation of antiviral activities

Assay for post-treatment of cells

After MA 104 cells were 80% confluent in a 96-well microplate, they were infected with 100 $TCID_{50}$/ml rotavirus and incubated at 37°C for 1 h. Phosphate-buffered saline was used to wash the cells and remove extracellular virus. Different non-toxic concentrations of CuO NPs were added to the virus-infected cells and incubated for 48 h at 37°C under 5% CO_2 conditions. Finally, infectious rotavirus titer and viral load were calculated using standard $TCID_{50}$ and real-time polymerase chain reaction (PCR) methods, respectively.

Virucidal assay

MA-104 cells were incubated at 37°C for 24 h to produce a confluent monolayer in a glass plate in a humidified 5% CO_2 incubator. CuO NPs were diluted in serum-free DMEM, and concentrations of 80 and 100 µg/ml CuO NPs were used for the virucidal assay. CuO NPs at concentrations of 80 and 100 µg/ml were mixed with an equal volume (400 µl) of a viral solution containing 100 $TCID_{50}$ of rotavirus. The mixture was incubated at 37°C for 1 h. After incubation, 100 µl of the mixture was added in triplicate to a 96-well plate containing confluent MA-104 cell monolayers. After incubation at 37°C for 1 h, the mixture was removed, and the cells were fed with serum-free DMEM containing 1.5 g/ml trypsin and incubated at 37°C for 48 h. Infectious rotavirus titer and viral load were then calculated using standard $TCID_{50}$ and real-time PCR methods, respectively.

Quantitative real-time polymerase chain reaction

RNA from the cell culture supernatant was extracted using the viral RNA/DNA extraction kit (GeneAll, Korea) according to the instructions of the manufacturer. The extracted RNA was transcribed into cDNA using the SinaClon First Strand cDNA synthesis kit (SINACLON) according to the manufacturer's protocols. The 225 bp region of the SA-11 rotavirus VP6 gene was used to confirm rotavirus. The sequences of the forward and reverse primers were 5′-CGA ATG GCT GTG CAT TCG GG-3′ and 5′- CAG CTG ACG GGG CAA CTA CA-3′, respectively (14). Real-time PCR was performed in a 25 µl reaction containing 12.5 µl of SYBR Green PCR Master Mix 2× (Ampliqon, Denmark), 4µl of cDNA, 10 pmol of each primer, and the remaining $ddH2_O$. The PCR assay was performed under the following conditions: initial denaturation at 95°C for 5 min, followed by 40 cycles of 20 s at 95°C and 45 s at 60°C.

To produce the standard positive control, the VP6 gene of rotavirus was cloned into the pGH plasmid. The recombinant plasmid pGH-SA11-VP6 was prepared as a lyophilized powder. All procedures were performed by Generay Biotech (Shanghai, China). To prepare the standard positive control, 4 µg of the lyophilized plasmid was dissolved in 40 µl dilution buffer. Finally, tenfold serial dilutions were used as templates to generate standard curves.

Plotting of log transcript R2 copy number against Cq values showed a linear correlation with an R2 value of 0.9774, and the efficiency of the singleplex assay was 102%. The sensitivity and specificity of the assay were calculated using standard procedures (Dawson & Trapp, 1994). The known copy number of the standards was used to calculate the number of rotaviruses in the unknown samples.

Statistical analysis

Because the data were considered the mean of three separate groups, a one-way analysis of variance (ANOVA) was performed to calculate the statistical difference. All analyses and graphs were generated using SPSS and GraphPad Prism programs, and p-values of less than 0.05 were considered statistically significant.

3. Results

Characterization of nanoparticles

XRD analysis showed that the crystal structure and position, as well as the relative intensity of all CuO NPs diffraction peaks, were similar to the standard CuO pattern reported previously (Figure 1). Field emission scanning electron microscope and (transmission electron microscopy) TEM images of CuO NPs showed that the morphological shape of CuO NPs was almost spherical and had an average diameter of 50 nm (Figure 2).

Cytotoxicity test

The cytotoxic effect of CuO NPs on MA-104 cells was determined by the MTT method. As shown in figure 3, the viability of MA-104 cells exposed to concentrations ranging from 20 to 400 µg/ml CuO NPs was examined. The results demonstrated that with an increase in CuO NPs concentration to 140 µg/ml, the viability of cells decreased to 71%, compared to control

cells (*P*=0.001). The CuO NP concentrations that showed a cytotoxic effect of less than 10% (80 and 100 µg/ml CuO NP) were used for subsequent antiviral assays.

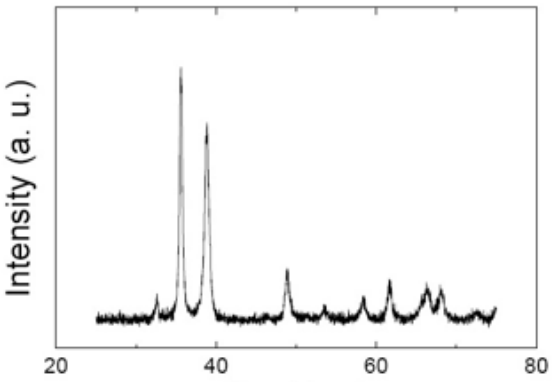
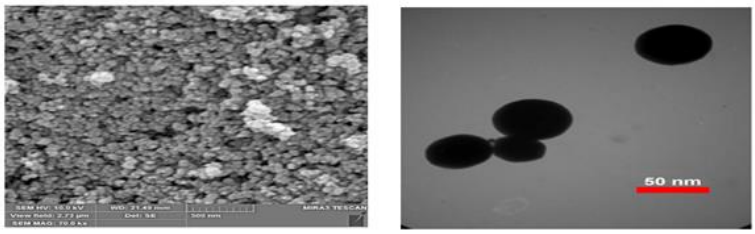


Figure 1. Powder X-ray Diffraction Pattern of CuONPs. All diffraction peaks were well-matched with CuO standard XRD pattern.



A **B**

Figure 2. FE-SEM image of CuONPs (A); TEM image of CuONPs (B).

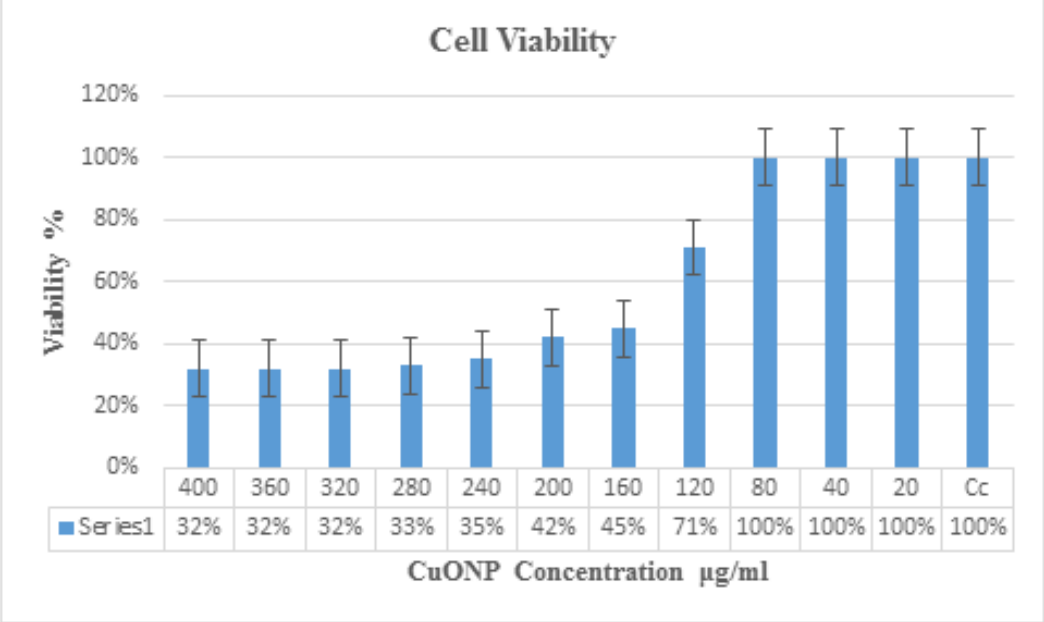
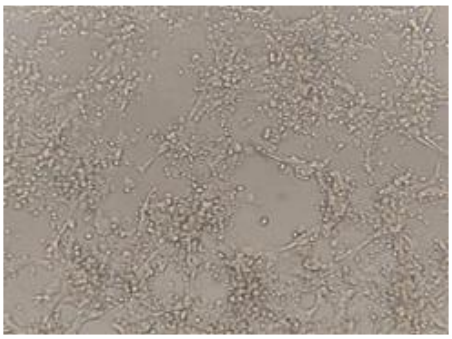


Figure 3. Cytotoxicity of CuONPs on MA-104 cells

Evaluation of antiviral activity

To investigate the effect of CuO NPs on rotavirus infection, a morphological comparison was performed between MA-104 cells untreated with CuO NPs and infected with rotavirus (virus control) and MA-104 cells treated with CuO NPs and infected with rotavirus. The effect of CuO NPs on the production of viable rotavirus was evaluated using the $TCID_{50}$ method. According to the $TCID_{50}$ results, the exposure of rotavirus-infected cells to concentrations of 80 and 100 µg/ml CuO NPs did not result in a decrease in cytopathic effect (CPE) inhibition compared with the virus control (Figure 4). The virucidal effect of CuO NPs on rotavirus was observed at concentrations of 80 and 100 µg/ml and resulted in a decrease in viral titer of 1.5 and 2.5 log10 $TCID_{50}$, compared to the viral control (P<0.001) (Figure 5). Quantitative real-time PCR was performed to evaluate the effect of CuO NPs on rotavirus viral load. As shown in figure 6, neutralization of rotavirus with 80 and 100 µg/ml CuO NPs resulted in 73% and 92% inhibition rates, respectively. In contrast, no decrease in viral load and no CPE inhibition was observed in rotavirus-infected cells.

A



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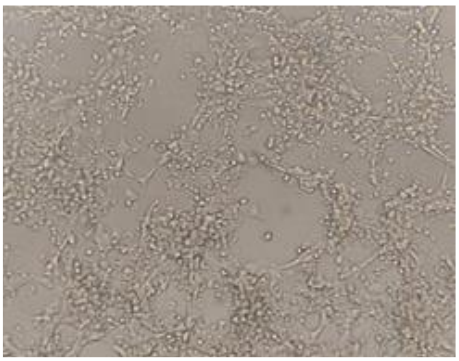

D

Figure 4. No Inhibition of Rotavirus-induced cytopathic effects on MA-104 cells in the presence of CuONPs (Post-treatment method). (A) Cell control; (B) Virus control; (C) Rotavirus-infected cells treated with 80 µg/ml of CuONPs; (D) Rotavirus-infected cells treated with 100 µg/ml of CuONPs.

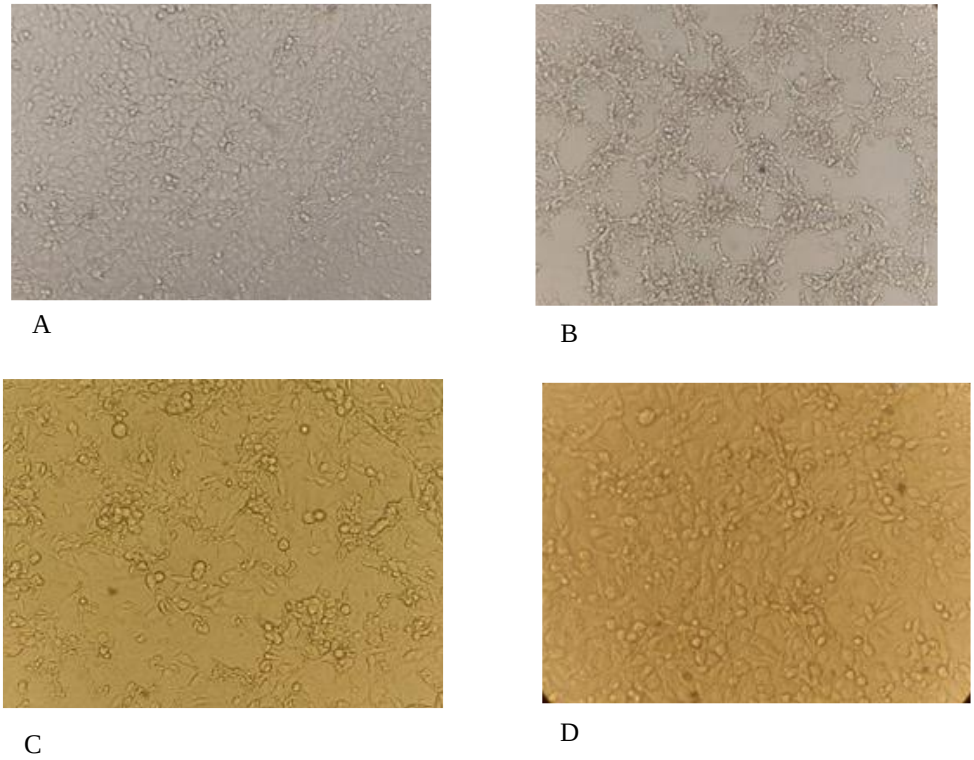

Figure 5. Inhibition of Rotavirus-induced cytopathic effects on MA-104 cells in the presence of CuONPs (virucidal method). (A) Cell control; (B) Virus control; (C) Rotavirus-infected cells treated with 80 and (D) 100 µg/ml of CuONPs.

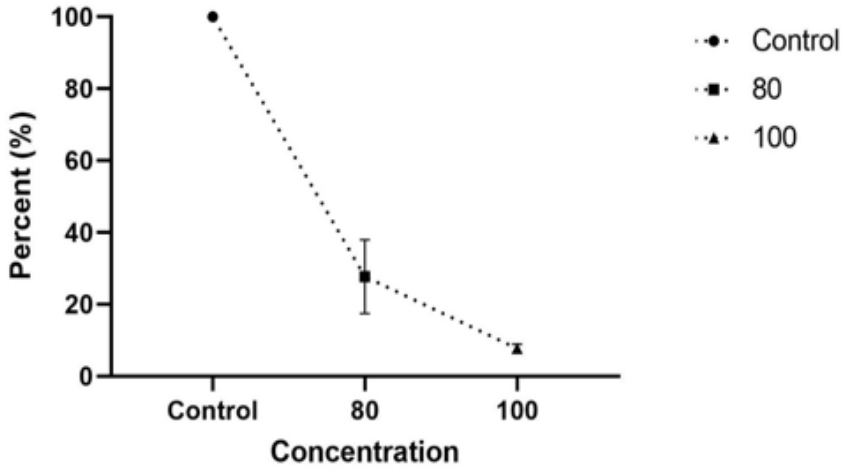

Figure 6A. The impact of CuONPs against Rotavirus viral load, determined by real-time PCR. CuONPs at 80 and 100 µg/mL concentrations led to 73% and 92% inhibition rates, respectively

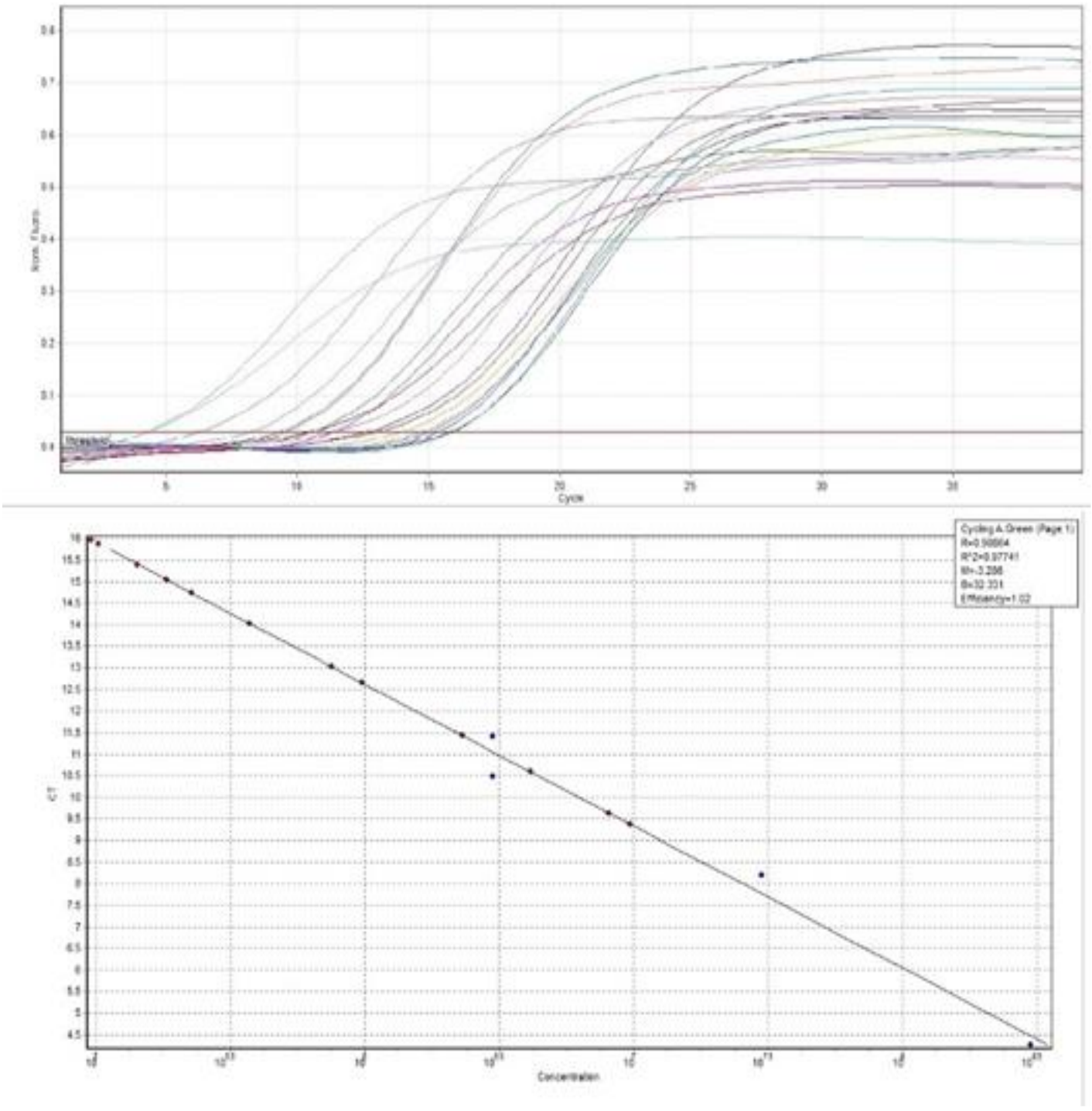

Figure 6B. Amplification and standard curves of the realtime PCR test. The standard curve shows the Cq value versus the log copy number fitted with a regression line. The R2 value and the efficiency of the test were 0.9774 and 102% ,respectively

4. Discussion

Rotaviruses are the leading cause of severe acute gastroenteritis in children and represent one of the most serious public health problems worldwide. Although rotavirus diarrhea is a severe and common disease in children, there is no effective treatment, and most treatments are supportive. Therefore, drug development for this disease is an area of great interest to researchers. Recently, nanoparticles have been used in research as antiviral agents. CuO NP is one of the effective compounds against viral infections. Due to the antiviral and biocidal properties of CuO NP, the effect of CuO NP on rotavirus infection was investigated in this study. Few studies have investigated the effect of CuO NP on viral infections. Tavakoli et al. investigated the *in vitro* antiviral activity of CuO NP against HSV-1. They reported that CuO NPs inhibited HSV infection when added to the cell after virus adsorption. Moreover, a concentration of 100 µg/ml CuO NPs could reduce viral titer by 83% (11). In another study by Hang et al., it was found that CuO NPs inhibited HCV entry into Huh7 cells. Previous studies have shown that CuO NPs are a potential new antiviral agent for the treatment of viral infections (10).

We investigated the antiviral activity of CuO NPs against rotavirus in two ways. First, after the virus was taken into the cell (post-treatment method), and second, by neutralizing the virus with the nanoparticles before injecting it into the cell (neutralization method). After the rotavirus enters the host cell, the replication cycle of the virus takes place in the cytosol of the infected cells. It appears that CuO NPs in the post-treatment method do not affect these stages of virus replication.

On the other hand, in the neutralization method, our results showed that the concentrations of 80 and 100 µg/ml CuO NP had a virucidal effect and could neutralize the infectivity of the virus. It seems that nanoparticles damage the capsid and kill the virus. The antiviral activity was confirmed by $TCID_{50}$ and real-time PCR assays. The limitation of this study was that the exact mechanism of the effect of CuO NPs on virus particles was still unknown; therefore, this requires further investigation. Secondly, since culturing human rotavirus in cell culture was difficult, we used a simian rotavirus strain. The antibacterial role of CuO NPs was confirmed in several studies. Reactive oxygen species production, lipid peroxidation, cell integrity damage, protein oxidation, and bacterial DNA degradation are the main mechanisms of antibacterial properties of CuO NPs (15, 16). The exact mechanism of the antiviral activity of copper nanoparticles is not known. However, the results of a study by Warnes showed that copper could inhibit murine norovirus infectivity by affecting the genome, especially the genome-linked virion protein coding region. In another study, Warnes reported that copper could disrupt the integrity of the capsid of noroviruses. In the mentioned study, the exact mechanism of the antiviral activity of CuO NPs was not investigated. Nevertheless, the virucidal assay suggested that the loss of capsid integrity and genome disruption in the presence of CuO NPs were possible mechanisms of antiviral activity against rotaviruses (17, 18). The findings of our study demonstrated that CuO NPs had significant antiviral activity against rotavirus infections. However, the exact mechanism of the antiviral activity of CuO NPs against rotaviruses remained unknown. According to the virucidal assay, it seems that the loss of capsid integrity and genome disruption in the presence of CuO NPs are possible mechanisms of antiviral activity against rotaviruses.

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Authors' Contribution
Study concept and design: S.H.M and M.H
Acquisition of data: M.H, A.K, Z.H, S.J.K, A.T
Analysis and interpretation of data: S.H.M, S.J.K, and A.T
Drafting of the manuscript: M.H
Critical revision of the manuscript: S.H.M and A.T
Statistical analysis: A.T and M.H

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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