



Original article

Epigallocatechin gallate (EGCG) combined with zinc sulfate inhibits Peste des petits ruminants virus entry and replication

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ABSTRACT

Despite the fact that the Peste des petits ruminants virus (PPRV) leads to high morbidity and mortality (up to 100%), antiviral drugs against PPRV are not available. The aim of this study was to estimate the dose of epigallocatechin gallate (EGCG) co-administered with zinc (II) ions as an antiviral agent against PPRV. Treatment of PPRV-infected Vero cells with EGCG and zinc sulfate (zinc II) was administered, and antiviral activities against PPRV in infected Vero cells was evaluated by determination of virus yields, expressed as logTCID₅₀/mL. Cytotoxicity was determined using the tetrazolium-based MTS test. Zinc sulfate at 1.1 mg/mL and EGCG at 25 μM showed low potentiated and potentiated antiviral activities against PPRV, respectively. These agents caused significant inhibition of PPRV in Vero cells ($p < 0.05$) with a reduction in logTCID₅₀/mL by up to 3-fold. The combination of EGCG (25 μM) and zinc sulfate (1.1 mg/mL) was observed to have strong antiviral activity ($p < 0.01$) against PPRV with a reduction in logTCID₅₀/mL of the virus up to 4-times without causing any host cell cytotoxicity. This study is the first one to prove that the zinc II has the capability of stimulating EGCG to inhibit *in vitro* PPRV entry. Moreover, this combination appears capable of reducing infection resistance by hindering viral adaptation.

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1. Introduction

The peste des petits ruminants virus (PPRV) is a member of the *Morbillivirus* genus in the family *Paramyxoviridae* and causes a contagious disease. It affects mainly small ruminants (Dundon et al., 2020). PPRV leads to heavy economic losses as a result of high rates of morbidity and mortality of up to 100% (Baron et al., 2011). PPRV outbreaks have occurred throughout Asia and Africa (Chan, 2010). The attenuated vaccine against PPRV is thermolabile. This property has proven to be a serious defect in terms of efficient use in endemic areas in addition to the lack of adequate coverage for the different emerging strains in the field. Therefore, the results from disease control programs have not been as successful as expected (Worrall et al., 2000; Kumar et al., 2013).

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Effective antiviral drugs for providing immediate protection and decreasing the risk of transmission and outbreaks in contact susceptible animals during epidemics are not available at this time. Therefore, the development of new treatments to combat this virus is necessary since antivirals can be used to bridge the period between vaccination administration and complete immunity (Goris et al., 2008; Kumar and Maherchandani, 2014). In addition, combination preparations lead to increased compliance, synergy, and increased efficacy in addition to reductions in side effects, costs, and drug-resistance (Saadh et al., 2021).

The major component in green tea is EGCG (Singh et al., 2011; Graham, 1992). EGCG has antiviral activity against diverse DNA viruses, including herpes simplex (Pradhan and Nguyen, 2018), adeno (Colpitts et al., 2014), human papilloma (He et al., 2013), and hepatitis B viruses (He et al., 2011), against (+)-RNA viruses, including hepatitis C virus (Calland et al., 2015; Tsai et al., 2019), Zika (Sharma et al., 2017), dengue (Vázquez-Calvo et al., 2017), and West Nile viruses (Vázquez-Calvo et al., 2017), and also against (-)-RNA viruses, such as human immunodeficiency (Hartjen et al., 2012), Ebola virus (Reid et al., 2014), and influenza viruses (Quosdorf et al., 2017).

Zinc has direct antiviral activity in addition to an indirect effect of stimulating antiviral treatments when co-administered with

such a treatment; one such example is the treatment for flu virus (Scott et al., 2019). Zinc is an integral part of many viral enzymes, such as proteases and polymerases, a finding that proves the importance of zinc in cellular regulation in terms of preventing virus outbreak and replication (Scott et al., 2019). Zinc combined with different antiviral agents has the potential to be used in many different medical and biological applications (Houston et al., 2017). Recently, the combination of zinc and epigallocatechin gallate or epigallocatechin-3-gallate (EGCG) has shown potential antiviral activity against the avian flu subtype, H5N1 (Saadh and Aldalaen, 2021). In this study we examined the *in vitro* the potentiated antiviral efficacy of zinc II combined with EGCG against PPRV.

2. Materials & methods

2.1. Reagents and virus

PPRV vaccine Nigeria 75/1 strain was used from Jordan Bio-Industries Centre (JOVAC, Amman, Jordan). EGCG (purity $\geq 95\%$) was obtained from Sigma-Aldrich (Saint Louis, MO). Virus handling and experiments were conducted under guidelines of Biosafety Level 3 (Devendra et al., 2014).

2.2. Virus propagation as control

The working seed of the PPRV vaccine Nigeria 75/1 strain was diluted in 1 mL of Dulbecco's Modified Eagle's Medium (DMEM) from Sigma-Aldrich (St. Louis, USA) without serum. This liquid was mixed with African green monkey kidney cells (Vero cells) suspended in complete DMEM with 10% fetal bovine serum (FBS) from Sigma Aldrich (St. Louis, USA) to provide at least 0.001 TCID₅₀ per cell. Cell culture dishes were filled with this virus/cell mixture (around 2×10^7 Vero cells per 175 cm² dish) and incubated at 37 °C. The cytopathic effect (CPE) was detected for the cultured Vero cells. DMEM was changed every two days during which time the proportion of FBS was reduced to 2% once the Vero cell monolayer became confluent. The virus was harvested for the first time when the CPE reached 40%–50%. The harvest from the viral suspension was stored at –70 °C. Every 2 days the sequent harvesting was performed until the CPE reached 70%–80% at which time the final freezing of the culture dishes was done. Two freeze–thaw cycles were used for all viruses collected in the T75 flask after which the viral suspension was collected in one sterile bottle to form a single batch, which was used as control (OIE, 2008). The TCID₅₀/mL was determined.

2.3. Virucidal reduction assay

The PPRV solution that was as described above containing a titer of $10^{5.9}$ TCID₅₀/mL was incubated at 4 °C with different concentrations of EGCG for 2 h to monitor antiviral activity that was expressed as a log reduction. This PPRV was treated with EGCG and mixed with Vero cells that were suspended in DMEM to provide at least 0.001 TCID₅₀ per cell. The Vero cells were then inoculated with virus, and the remainder of the procedure was similar to that used in testing of viral propagation. The TCID₅₀/mL was determined from that point forward. Also, several antiviral compounds were tested to determine their antiviral effects on PPRV and then compared with the results of the control.

2.4. Pre-treatment of Vero cells with EGCG

This Vero cells suspended in complete culture medium were treated with EGCG for 2 h at 4 °C. Cell culture dishes were filled with this virus/cell mixture (around 2×10^7 Vero cells in a

175 cm² dish), and after this period, the medium containing the drug was removed. The cell suspension was washed 3 X with phosphate-buffered saline (PBS) to eliminate any trace of EGCG after centrifuging the cell and washing them. The treated cells were then inoculated with the virus and the remainder of the procedure was similar to that used in the test of virus propagation.

The TCID₅₀/mL was determined from that point forward. Also, several antiviral compounds were tested to determine their antiviral effects on PPRV and compare the results with the control.

2.5. 50% cell culture infectious (TCID₅₀/mL) assay

The presence of the virus was confirmed by CPE, and a titration was performed. Viral TCID₅₀ titers were found by inoculating 200 μ L of 6-fold virus dilutions into the microtiter plate to which 100 μ L Vero cell suspension had already been dispensed in all wells in the microtiter plate. For each dilution, 10 wells were used for exact estimation of the titer. Also, 200 μ L of (DMEM + 2% FBS) was dispensed into uninoculated wells as the negative control. The microtiter plate was incubated at 37 °C and 5% CO₂ for 9 to 10 days. Microtiter plates were checked daily for cytopathic effects. The experiment was repeated if any CPEs were observed before day 3 post-inoculation or if any CPEs were observed in control wells. The TCID₅₀/mL was calculated based on the method of Reed and Muench (Reed et al., 1938; Piercy et al., 2010).

2.6. Cytotoxicity

CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) was used for determination of cytotoxicity (Promega, Southampton, UK). First, trypsinization of Vero cells monolayers (80% confluent) in a T75 flask (Greiner BioOne, Stonehouse, UK) was done and after washing, cells were reconstituted in DMEM in each well of the 96-well-plate. Cells were seeded to obtain 2.5×10^3 cells per well and then incubated for 24 h at 37 °C under 5% CO₂. The culture media was removed by aspiration, cells were then washed 3X with PBS, and immediately replaced with the dilutions of test materials after which cells were incubated for 24, 48, and 72 h. CellTiter 96® Aqueous One Solution was added to each cell well to determine cell viability. The plate was placed in an incubator for 1 h at 37 °C under 5% CO₂. The absorbance was recorded at 492 nm. The absorbance of the blank was subtracted from each sample reading, and the mean absorbance for the control cells was considered a value of 100% (n = 3).

2.7. Statistical analysis

Data analysis was performed using the GraphPad Prism package and SPSS software. Differences among the studied groups were determined based on one-way ANOVA followed by Tukey's multiple comparisons as post-hoc test. $P < 0.05$ was considered significant.

3. Results

3.1. Inhibitory effects of EGCG on PPRV

The PPRV was proliferated in the Vero cells as a feasible quantity. LogTCID₅₀/mL was determined after cells were treated with EGCG at different concentrations of 0, 10, 25, 50, 100, and 200 μ M as shown in Fig. 1. The reduction in logTCID₅₀/mL value at 25 μ M EGCG was 3-times when compared with the control.

The decrease in logTCID₅₀/mL is proportional to the increase in the concentration of the EGCG. Concentrations of $>25 \mu$ M EGCG did

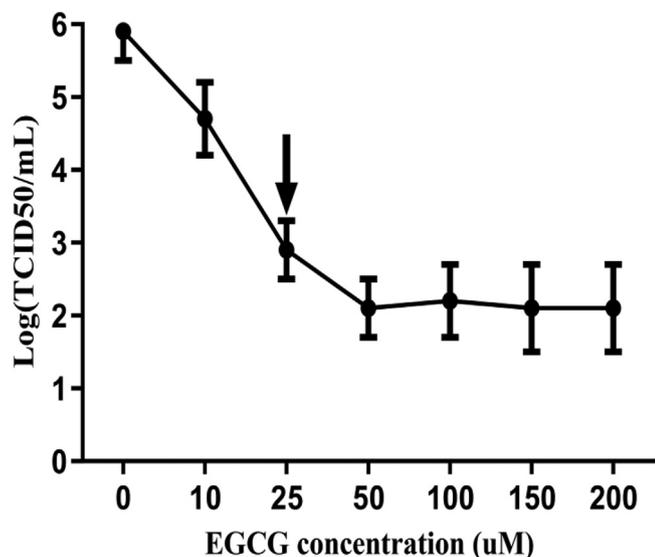


Fig. 1. Inhibition of Peste des petits ruminants virus entry and replication in Vero cells at different epigallocatechin gallate concentrations. The vero cells that were suspended in Dulbecco's Modified Eagle's Medium infected with PPRV which incubated at 4 °C with different concentrations of epigallocatechin gallate for 2 h to monitor antiviral activity that was expressed as a log reduction of TCID₅₀/mL.

not lead to a significant decrease in the in logTCID₅₀/mL (Fig. 1). Therefore, in the next set of assays, 25 μM of EGCG was used.

3.2. Virucidal effect of EGCG when combined with different levels of zinc sulfate

The increase in zinc sulfate concentration shows a direct relationship to the stimulation of the antiviral effect of EGCG, which caused a decrease in the value of logTCID₅₀/mL of the PPRV. The logTCID₅₀/mL values for PPRV when treated with 25 μM of EGCG alone and when co-administered with 1.1 mg/mL of zinc sulfate (Zn II) were 2.9 ± 0.4 and 1 ± 0.4, respectively. Also, in Fig. 2, zinc sulfate concentrations > 1.1 mg/mL did not produce any further effects.

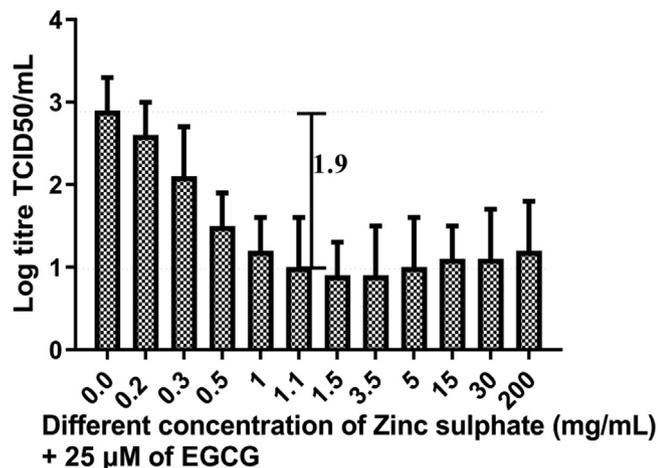


Fig. 2. The impact of different zinc sulfate concentrations (mg/mL) on the potentiated antiviral activity of epigallocatechin gallate (25 μM). The vero cells that were suspended in Dulbecco's Modified Eagle's Medium infected with PPRV which incubated at 4 °C with constant concentration of epigallocatechin gallate (25 μM) and different zinc sulfate concentrations (mg/mL) for 2 h to monitor antiviral activity that was expressed as a log reduction of TCID₅₀/mL.

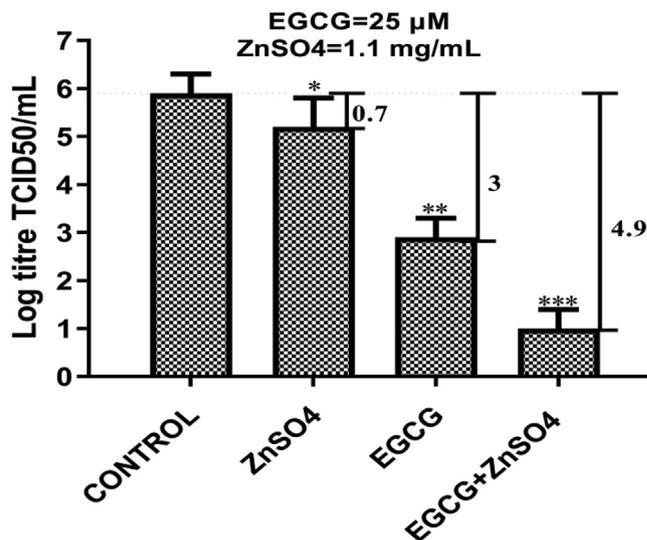


Fig. 3. Log TCID₅₀/mL of Peste des petits ruminants virus as control, Peste des petits ruminants virus treated with 1.1 mg/mL of zinc sulfate, 25 μM of epigallocatechin gallate, or 25 μM of epigallocatechin gallate + 1.1 mg/mL of zinc sulfate. (**p* > 0.05, ***p* < 0.05, ****p* < 0.01).

3.3. Virucidal effect of zinc Sulfate, EGCG, and EGCG with zinc sulfate

The viral logTCID₅₀/mL value in Vero cells was 5.9 in the control. At 1.1 mg/ml of zinc sulfate, the virus logTCID₅₀/mL was 5.2 ± 0.6 without significant antiviral activity (*p* > 0.05). At 25 μM of EGCG, logTCID₅₀/mL was 2.9 ± 0.4 with significant antiviral activity (*p* < 0.05). When 25 μM of EGCG and 1.1 mg/mL of zinc sulfate were combined, logTCID₅₀/mL was 1.0 ± 0.4 and showed highly significant antiviral activity (*p* < 0.01). The virucidal log reduction effect was 4.9-times that of the control with strongly significant antiviral activity (*p* < 0.01) as shown in Fig. 3.

3.4. Cytotoxicity

Using the tetrazolium-based MTS proliferation assay, cytotoxicity in Vero cells was evaluated at 50 μM of EGCG, 1.1 mg/mL of zinc

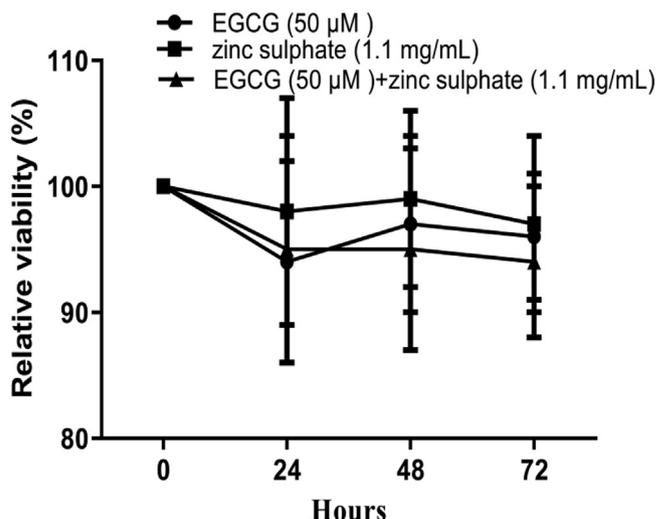


Fig. 4. The percentage of viable cells after treating the cells with (1) epigallocatechin gallate (50 μM), (2) zinc sulfate (1.1 mg/mL), and (3) epigallocatechin gallate (50 μM) coadministered with zinc sulfate (1.1 mg/mL) in Vero cells. No cytotoxic impact for these compounds at this concentration was found.

sulfate, or a combination of 50 μM of EGCG and 1.1 mg/mL of zinc sulfate. No cytotoxic effects were observed for any of these compounds at 24, 48, and 72 h ($p > 0.05$). In addition, no reduction in the level of viable Vero cells when compared with the control was found (Fig. 4).

4. Discussion

Viral infections leading to the death of sheep and livestock at a rate that can reach 100% can cause devastating economic losses (Bora et al., 2020). Although vaccines are available as effective methods for controlling a PPRV, a vaccine does not provide immediate protection during epidemics. Moreover, effective antiviral treatments against PPR are not available at this time (Khandelwal et al., 2014).

We first assessed the antiviral activity of EGCG by incubating the PPRV with different concentrations of this agent. This compound has strong antiviral activity against PPRV with a significant correlation ($p < 0.05$). At 25 μM of EGCG, the drug showed the capability to inhibit virus entry, and we demonstrated a 3-time reduction in the $\log\text{TCID}_{50}/\text{mL}$ value. Based on non-linear regression, we calculated the EC_{50} of EGCG to be 18.3 μM . The highest antiviral activity of EGCG that inhibited viral entry was observed at 50 μM with a 3.8-times reduction in the $\log\text{TCID}_{50}/\text{mL}$. Moreover, PPRV was shown to be more sensible than the avian influenza virus (H5N1). The highest level of viral entry inhibition was observed at 60 μM of EGCG with a 4-time reduction in the $\log\text{EID}_{50}/\text{mL}$ (Saadh and Aldalaen, 2021). This finding is consistent with different studies demonstrating that EGCG has the potential to inhibit different enveloped and nonenveloped viral growth (Saadh and Aldalaen, 2021; Marinova-Petkova et al., 2016; Jae-Min et al., 2005).

EGCG is characterized as a general viral inhibitor that suppresses a wide range of viruses. This compound works mainly during the early stages of infection by acting indirectly on cellular proteins or directly by acting on the virus protein particle; therefore, the mechanism by which EGCG works occurs via inhibition of virus entry by impairing virus attachment, effects on the host cell that might affect the fusion with the cell membrane of the virus, or both of these mechanisms (Steinmann et al., 2013; Bruno et al., 2016). The interpretation of these results indicates that EGCG has the capability of binding to the surface of the viral envelope by distorting the lipids on the envelope and thus induces virion destruction. Also, the EGCG can act by covering and preventing viral attachment to target cell receptors (Williamson et al., 2006). EGCG can also be inhibited by the activity of hemagglutination (Govindarajan et al., 1997).

Our results demonstrate that the antiviral activity of EGCG is due to a direct effect on the PPRV particle but not from an indirect effect after interacting with host cells.

Despite the fact that EGCG is a natural compound, studies have found toxicity associated with this compound at high concentrations (Lambert et al., 2006; Fanguero et al., 2016). Therefore, this study aimed to stimulate antiviral activity when using low EGCG concentrations and combining them with zinc sulfate.

Zinc II possessed limited antiviral activity against several viruses (Scott et al., 2019), the antiviral activity of zinc II against PPRV was shown to be reduced 0.7-times with respect to the $\log\text{TCID}_{50}/\text{mL}$ but without any significant correlation. However, due to the dual effect when the combination of 25 μM EGCG and 1.1 mg/mL of zinc sulfate was used, the antiviral activity was increased when compared with either EGCG or zinc II used alone, demonstrating a 4.9-times reduction in the $\log\text{TCID}_{50}/\text{mL}$ with significant correlation ($p < 0.01$). This finding proves that the most likely possibility for the antiviral activity was an EGCG interaction

directly with the viral shell (Williamson et al., 2006; Kaihatsu et al., 2018) This study confirms that zinc II ions stimulate EGCG to interact with the glycoprotein envelope of the virus and then destroy it. Moreover, zinc possesses inhibitory protease and polymerase activities, both of which inhibit viral replication or the viral maturation process of the viral assembly protein in addition to physical processes, such as virus attachment, infection, and uncoating (Scott et al., 2019).

5. Conclusions

This study demonstrating that EGCG in combination with zinc II inhibits virus entry into Vero cells; thus, this combination has potential as an antiviral therapeutic agent against PPRV.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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