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Antiviral effects of Korean Red Ginseng on human coronavirus OC43

Chi Hwan Jeong, Jisu Kim, Bo Kyeong Kim, Kang Bin Dan, Hyeyoung Min*

College of Pharmacy, Chung-Ang University, Seoul, 06974, South Korea

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ABSTRACT

Background: Panax ginseng Meyer is a medicinal plant well-known for its antiviral activities against various viruses, but its antiviral effect on coronavirus has not yet been studied thoroughly. The antiviral activity of Korean Red Ginseng (KRG) and ten ginsenosides against Human coronavirus OC43 (HCoV-OC43) was investigated in vitro.

Methods: The antiviral response and mechanism of action of KRG extract and ginsenoside Rc, Re, Rf, Rg1, Rg2-20 (R) and −20 (S), Rg3-20 (R) and −20 (S), and Rh2-20 (R) and −20 (S), against the human coronavirus strain OC43 were investigated by using plaque assay, time of addition assay, real-time PCR, and FACS analysis.

Results: Virus plaque formation was reduced in KRG extract-treated and HCoV-OC43-infected HCT-8 cells. KRG extract decreased the viral proteins (Nucleocapsid protein and Spike protein) and mRNA (N and M gene) expression, while increased the expression of interferon genes.

Conclusion: KRG extract exhibits antiviral activity by enhancing the expression of interferons and can be used in treating infections caused by HCoV-OC43.

1. Introduction

Severe acute respiratory syndrome-associated coronavirus-2 (SARS-CoV-2) is a highly pathogenic coronavirus and was responsible for causing the coronavirus disease in 2019 (COVID-19) which has been declared a pandemic worldwide. A few therapies, including vaccines, are available for COVID-19 treatment. Considering the outbreak of SARS-CoV-2, developing an effective treatment for the highly pathogenic virus is urgent. It is also essential to find broad-spectrum antiviral agents that effectively prevent or cure viral infection caused by the current reigning strain of coronavirus (CoV) and future-emerging strains of CoVs. However, manipulating highly pathogenic viruses, including SARS-CoV-2, for research must be performed under high biosafety level conditions. Accordingly, it is crucial to find alternatives to overcome the limitations of research accessibility [1].

Coronaviruses are enveloped viruses with a diameter of 120-160 nm, and the envelope derived from intracellular membranes contains multiple club-shaped spikes of a crown [2,3]. They have a capped, non-segmented, and linear, positive-sense single-stranded RNA genome of approximately 30 kb [2–4]. The genus Coronavirus belongs to the family Coronaviridae [5]. Based on genetic similarities, the coronavirinae subfamily comprises four genera: alpha, beta, gamma, and delta coronaviruses. Human coronaviruses (HCoV) are limited to the alpha and beta genera [6]. Alphacoronaviruses include HCoV-NL63 and HCoV-229E, while Betacoronaviruses contain HCoV-HKU1, SARS-CoV, MERS-CoV, and HCoV-OC43 [4]. HCoVs cause multiple respiratory diseases, and their severity varies from the mild common cold to life-threatening pneumonia [4,7].

HCoV-OC43 belongs to the same viral genus as SARS-CoV and SARS-CoV-2 [8]. HCoV-OC43 has been associated with common cold [9] and mild upper respiratory tract infections [10], and occasionally can cause a severe form of the disease in people with underlying respiratory conditions, infants, and the elderly [11,12]. The genome of HCoV-OC43 encodes several well-conserved motifs that match with SARS-CoV-2, and cross-reactivity between HCoV-OC43 and SARS-CoV-2 is reported, indicating that HCoV-OC43 might be used as a substitute for the study of SARS-CoV-2, which can only be conducted in a BSL-4 facility [13–16].

Panax ginseng Meyer has been shown to possess diverse physiological activities [17,18], and the beneficial effects of Korean Red Ginseng (KRG) in various diseases such as cancer, immune diseases, and neuronal diseases are well known [19–22]. The antiviral properties of *P. ginseng* have also been reported [23], and KRG extract and ginsenosides have been shown to exert antiviral activity

* Corresponding author. 84 Heukseokro, Dongjakgu, Seoul, 06974, South Korea.
E-mail address: hymin@cau.ac.kr (H. Min).

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against influenza virus, human immunodeficiency virus, norovirus, herpes simplex virus, and hepatitis B virus [24–29].

Most medicinal effects of ginseng are derived from ginsenosides, which are triterpene glycosides [30]. Ginsenosides can be divided based on the chemical structure of the aglycone part: the protopanaxadiol group (e.g., Rc, Rg3, Rh2) and the protopanaxatriol group (e.g., Re, Rf, Rg1, Rg2) [31]. Although it has been reported that *P. ginseng* possesses antiviral activities, the antiviral property of KRG and ginsenosides against coronavirus has not been elucidated [23–25,27]. Therefore, in this study, the antiviral activity of KRG and ginsenosides against HCoV-OC43 was investigated in vitro.

2. Materials and methods

2.1. Cell line, virus, Korean Red Ginseng extract, and ginsenosides

The human colon cell-8 (HCT-8) cell line was obtained from Korean Cell Line Bank (Seoul, Korea). HCT-8 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Welgene, Gyeongsan, Gyeong-buk, Korea), 25 mM HEPES (Gibco, Waltham, MA, USA), 100 U/mL penicillin/streptomycin (Welgene). The cells were incubated at 37°C in a 5% CO_2 humidified air environment. HCoV-OC43 was obtained from Korea Bank for Pathogenic Viruses (Seoul, Korea). KRG extract was provided by the Korea Ginseng Corporation (Buyeo, Chung-nam, Korea). For KRG extract preparation, fresh ginseng roots were prepared and processed by steaming and drying to make red ginseng in red ginseng manufacturing factory of Korea Ginseng Corporation. Washed fresh ginseng roots were steamed for 4 hours while slowly raising its temperature from 50 °C to 98 °C and then firstly dried at 60~70 °C for 15 hours. Thereafter, secondary drying process was performed in a closed chamber at 50°C for 5 days to resulting the red ginseng roots. To prepare red ginseng extract, the root was sequentially extracted 7 times at 87°C for 12 hours with distilled water. The extracted water was combined followed by filtering and concentrating process. The KRG extract contains ginsenoside Rg1 (1.74 mg/g), Re (1.88 mg/g), Rf (1.33 mg/g), Rg2 (S) (1.21 mg/g), Rb1 (7.9 mg/g), Rc (2.83 mg/g), Rb2 (2.58 mg/g), Rd (1.04 mg/g), Rg3 (S) (2.06 mg/g), Rg3 (R) (0.93 mg/g), and Rh1 (0.89 mg/g). Ginsenosides Rc, Re, Rf, Rg1, Rg2 -20 (R), and −20 (S), Rg3 -20 (R) and −20 (S), and Rh2 -20 (R) and −20 (S) were purchased from Ambo Institute (Daejeon, Korea). −20 (R) and −20 (S) represent the isomer designation for carbon 20 of each ginsenoside.

2.2. Cell viability assay

HCT-8 cells were seeded in 96-well plates (5×10^4 cells) and treated with 100 μg/mL KRG or 100 μM ginsenoside for 7 days. The cells were then treated with 10 μL MTT solution (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37°C in 5% CO_2 for 4 h. This step was followed by adding 100 μL of 0.04 N HCl-isopropanol to dissolve the formazan crystals. The cell viability was quantified at an absorbance of 540 nm using an E_{max} microplate reader (Molecular Devices, Sunnyvale, CA, USA)

2.3. Plaque assays

HCT-8 cells were seeded at 5×10^5 cells/well in 12-well plates. After 24 h of culture, virus stock solutions were added to the cells and allowed to incubate for 60 min at 37°C, rocking every 15 min for 1 h. HCT-8 cells were washed once in PBS and overlaid with overlay medium (complete DMEM containing 0.6% methylcellulose). KRG or ginsenoside was added into the overlay medium at 100 μg/mL and 100 μmol/mL, respectively. After 7 days of culture, the cells were fixed and stained with 2% crystal violet (Sigma-Aldrich) in 20% ethanol overnight. The plaques were counted to determine the antiviral effect of KRG.

2.4. Time-of-addition assays

HCT-8 cells (5×10^5 cells) were seeded in 12-well plates. KRG (25, 50, 100 μg/mL) was added to the HCT-8 cells at 24 h before HCoV-OC43 infection (pre-treatment), during infection (co-treatment), and after removal of the virus (post-treatment). The infected cells were cultured for 7 days at 37°C in 5% CO_2, and a plaque assay was performed.

2.5. Quantitative real-time PCR (qRT-PCR)

HCT-8 cells (1×10^6 cells) were seeded in 6-well plates. They were treated with 25, 50, and 100 μg/mL of KRG or 5 μM of remdesivir (Hunan Hua Teng Pharmaceutical Co., Ltd, Changsha, Hunan, China) for 1–4 days. After washing, the cells were treated with RNAiso (Takara Bio Inc., Kusatsu, Shiga, Japan), and total RNA was obtained according to the manufacturer's protocol. The cDNA was synthesized and reverse-transcribed from 3 μg of total RNA. qRT-PCR was performed with a CFX Connect™ RT-PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The amplification condition was an initial denaturation (95°C, 10 min), followed by amplification cycles (40 cycles of 95°C, 10 s; 65°C, 15 s; 72°C, 30 s).

2.6. Quantification of viral RNA copy number

According to the manufacturer's instructions, the viral RNA was isolated using a QiaAMP viral RNA purification kit (Qiagen, Hilden, Germany). qRT-PCR was performed using TOPreal™ qPCR 2X PreMIX (Enzynomics, Daejeon, Korea). For RNA quantification of HCoV-OC43 nucleoprotein (N protein) and matrix protein (M protein), N and M protein genes were amplified by PCR, digested with BamHI and EcoRI restriction enzymes (Enzynomics), and inserted into the MDH1-PGK-GFP_2.0 vector (Addgene, Watertown, MA, USA). The HCoV-OC43 RNA copy number was calculated by a standard curve of viral RNA concentrations. The primer sequences are listed in Table 1.

2.7. Fluorescence-activated cell sorting (FACS) analysis

HCT-8 cells (1×10^6 cells) were seeded in a 6-well plate. After 24 h of culture, HCT-8 cells were infected by HCoV-OC43 for 1 h. After

Table 1
Primers Sequences

Primer	Direction	Sequence (5′ – 3′)
GAPDH	Fwd	AATGGTGAAGGTCGGTGTGAAC
	Rev	GAAGATGGTGATGGGCTTCC
N	Fwd	AGCAACCAGGCTGATGTCAATACC
	Rev	AGCAGACCTTCCTGAGCCTTCAAT
N (for cloning)	Fwd	AATTATGGATCCAGCAACCAGGCTGATGTCAATACC
	Rev	ATAGTCGAATTCAGCAGACCTTCCTGAGCCTTCAAT
M	Fwd	GGCTTATGTGGCCCCTTACT
	Rev	GGCAAATCTGCCCAAGAATA
M (for cloning)	Fwd	ATATCTGGATCCGGCTTATGTGGCCCCTTACT
	Rev	GCGCCTGAATTCGGCAAATCTGCCCAAGAATA
IFN-α	Fwd	GTGCTCAGCTGCAAGTCAAG
	Rev	TTATCCAGGCTGTGGGTCTC
IFN-β	Fwd	ACCAACAAGTGTCTCCTCCA
	Rev	GTAGTGGAGAAGCACAACAGG
IFN-γ	Fwd	TCTTGGCTGTTACT GCCAGGACCCA
	Rev	TCAGCCACTTCGTGCCG GTCTTC
MxA	Fwd	CAACCTGTGCAGCCAGTATG
	Rev	GTCCTGCTCCACACCTAGAG

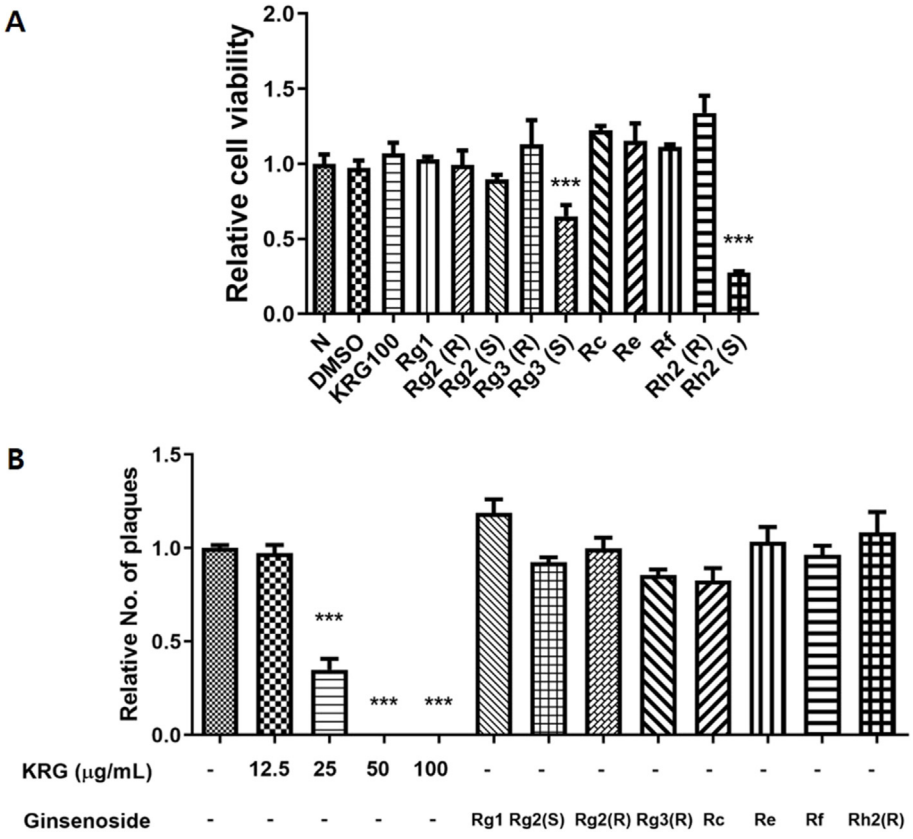

Fig. 1. Cytotoxicity and antiviral effect of KRG and ginsenosides. For cytotoxicity, HCT-8 cells were treated with KRG (100 μg/mL) or each of the 10 different ginsenosides (100 μM) for 7 days, and cell viability was measured by the MTT assay (A). For testing antiviral effect, plaque formation assay was performed (B). Cell viability is shown as relative values in each treatment compared to the untreated control group (N). HCoV-OC43 plaque formation levels were represented as the relative number of plaques compared with that obtained from DMSO-treated control (B). The data are represented as mean ± SEM of three independent experiments with similar results. One-way ANOVA plus Tukey's multiple comparison test was applied.

infection, HCT-8 cells were washed once in PBS to remove the uninfected virus. The cells were cultured with 25, 50, 100 μg/mL of KRG and 5 μM of remdesivir for 4 days. After 4 days of infection, HCT-8 cells were harvested by trypsinization and washed in PBS. The cells were fixed and permeabilized for 15 min at 4°C with fixation/permeabilization working solution diluted from fixation/permeabilization concentrate (eBioscience, San Diego, CA, USA) with fixation/permeabilization diluent (eBioscience). Afterward, samples were incubated for 2 h at 4°C with primary anti-HCoV-OC43 antibody (Merck, Rahway, NJ, USA) or anti-HcoV-OC43 S antibody (Cusabio, Houston, TX, USA). Subsequently, samples were incubated for 1 h with Alexa Fluor 488 labeled goat Anti-Mouse IgG (Cell Signaling, Danvers, MA, USA) for anti-HCoV-OC43 antibody or Alexa Fluor 488-conjugated goat Anti-rabbit IgG (H + L), F(ab')2 Fragment (Cell Signaling) for anti-HcoV-OC43 S antibody. Cells were resuspended in PBS and analyzed by FACS Calibur (Becton Dickinson, San Diego, CA, USA). Data were analyzed by CellQuest Pro software (Becton Dickinson).

2.8. Statistics

All experiments were repeated at least three times, and data are presented as mean ± standard deviation. For statistical analysis, one-way ANOVA plus Tukey's multiple comparison test was applied using Prism 5.0 (GraphPad Software, San Diego, CA, USA). $p < 0.05$ was considered statistically significant. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$.

3. Results

3.1. Cytotoxic effects of KRG extract and ginsenosides

To evaluate the cytotoxicity of KRG extract and ginsenosides in HCT-8 cells, KRG and ginsenosides were added to HCT-8 cells for 7 days, and cytotoxicity was measured using an MTT assay. KRG and all ginsenosides except Rg3 (S) and Rh2 (S) showed no significant cytotoxic effects (Fig. 1A). Therefore, subsequent experiments were conducted with KRG and the eight ginsenosides devoid of cytotoxicity.

3.2. Antiviral activity of KRG and ginsenosides against HCoV-OC43 in HCT-8 cells

To test the antiviral activity of KRG and ginsenosides, the number of plaques formed in HCT-8 cells infected with HCoV-OC43 was counted following treatment with KRG or the eight ginsenosides. Compared with the DMSO control, the plaque formation in HCT-8 was significantly decreased in KRG-treated cells at 25, 50, and 100 μg/mL. However, none of the ginsenoside-treated groups had antiviral activity at 100 μM against HCoV-OC43 infection (Fig. 1B).

3.3. Identification of the step of virus life cycle affected by KRG

A time-of-addition assay was implemented to identify the step of the virus life cycle at which KRG exerts its antiviral activity

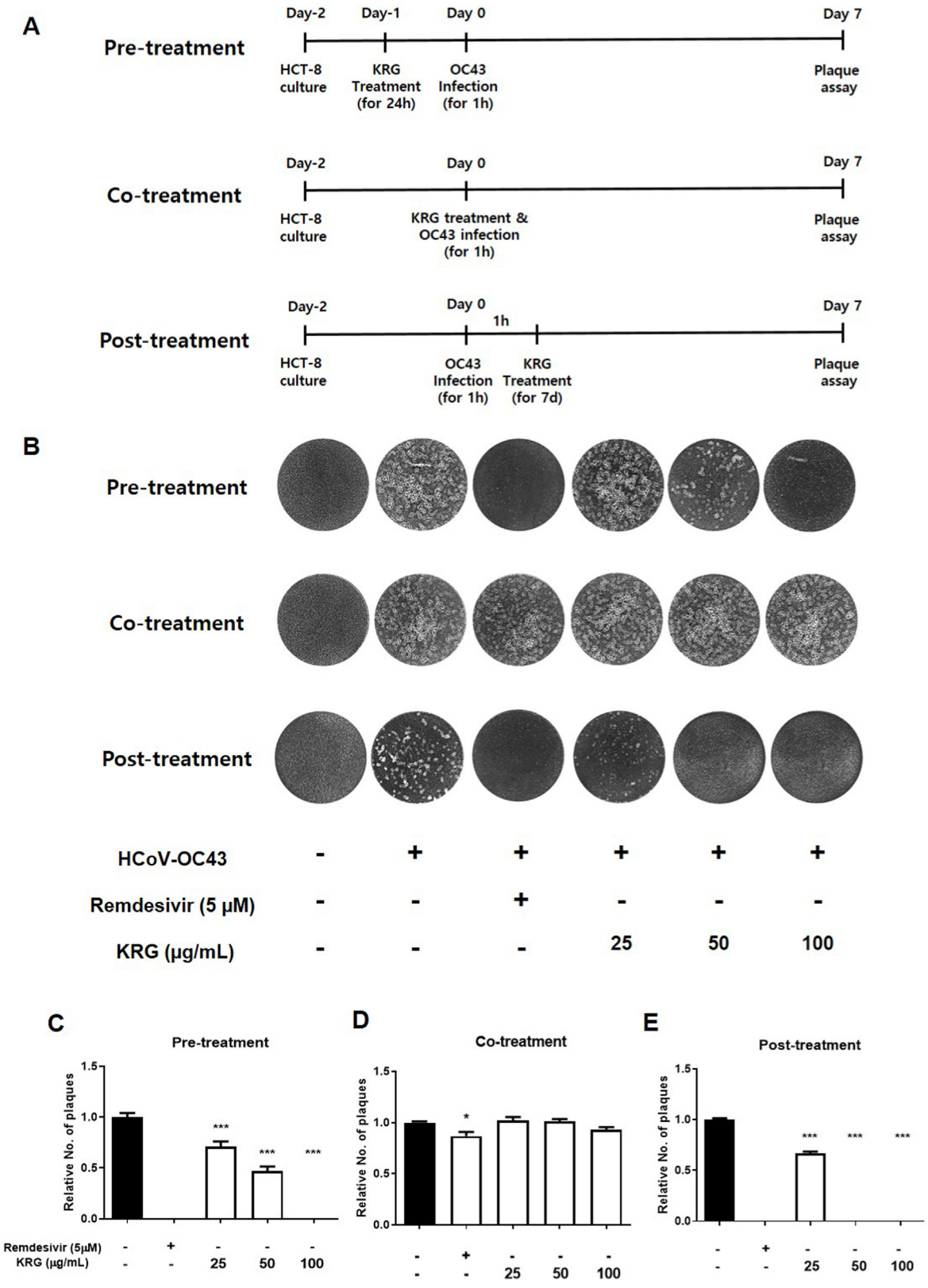

Fig. 2. Time of addition assay to determine the antiviral activities of KRG. HCT-8 cells were infected with HCoV-OC43, and KRG was added to the cells at 24 h before, during, or after HCoV-OC43 infection. The overall scheme of the time-of-addition assay (A). Plaque assay image of HCoV-OC43-infected HCT-8 cells with or without KRG treatment (B). The plaques of pre-, co-, and post-KRG-treated cells were quantified at 7 dpi (Fig. C–E). The data are representative of three experiments with similar results.

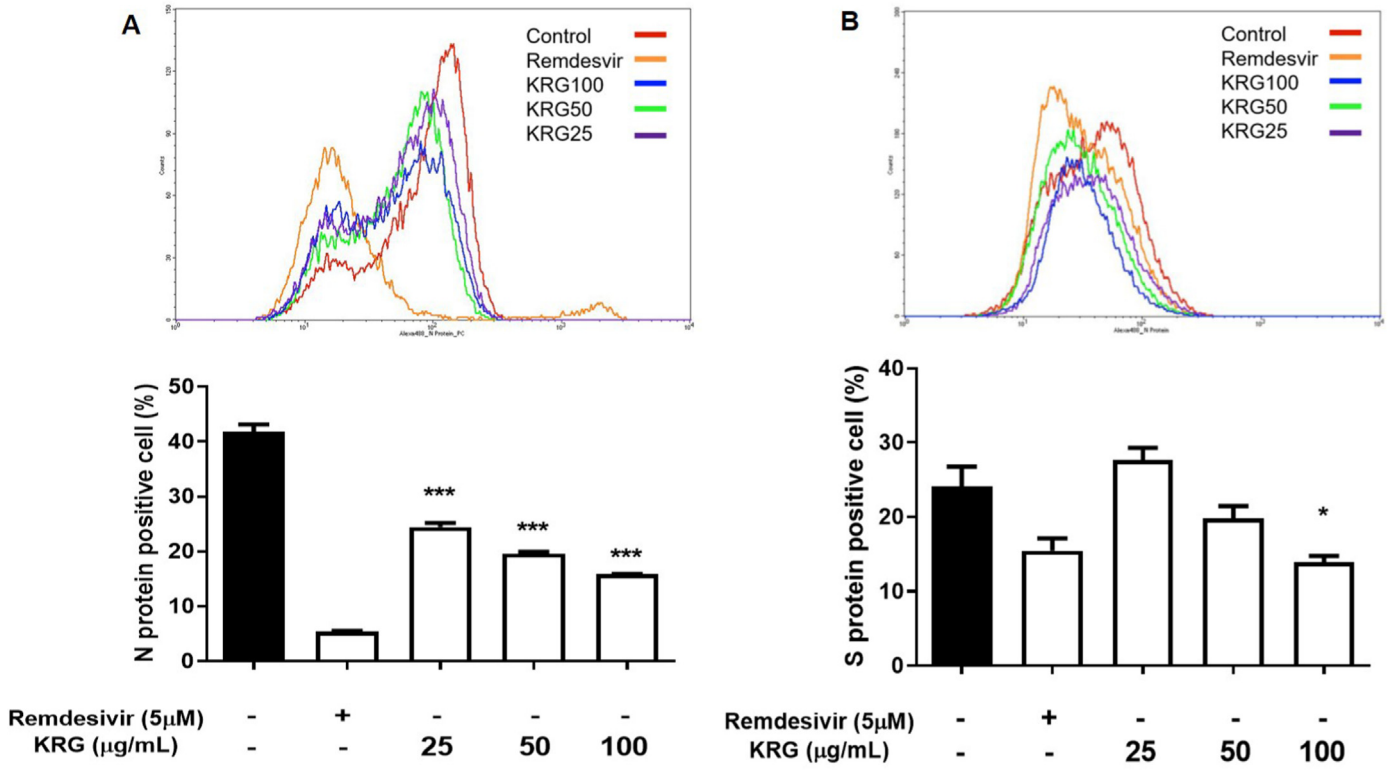

Fig. 3. Expression of HCoV-OC43 nucleoprotein (N protein) and spike protein (S protein) in HCT-8 cells. The cells were infected with HCoV-OC43 followed by KRG treatment, and the expression of N protein (A) and S protein (B) was analyzed by flow cytometry at 4 dpi. The FACS plot is representative of three experiments with similar results.

(Fig. 2A). When KRG was added to the cells during virus infection (co-treatment), no protection against viral replication was observed. However, the addition of KRG at 24 h before (pre-treatment) and after (post-treatment) HCoV-OC43 infection significantly reduced the number of viral plaques dose-dependently (Fig. 2B and C). The reduction of viral plaques against HCoV-OC43 infection was more prominent upon exposure of cells to KRG post-treatment than pre-treatment (Fig. 2B and C).

3.4. Inhibition of HCoV-OC43 replication by KRG

To assess the inhibitory effects of KRG on HCoV-OC43 replication, the presence of viral nucleocapsid (N) protein and spike (S) protein in HCT-8 cells was measured by flow cytometry. The cells were infected with HCoV-OC43, and the culture medium was replaced with a fresh medium containing KRG to remove unbound virus particles. After 7 days post-infection (dpi), the expression of N protein was analyzed by flow cytometry. KRG treatment decreased the expression of N and S proteins in a dose-dependent manner (Fig. 3), indicating that KRG significantly affects virus replication in HCT-8 cells.

In addition, to confirm the antiviral effect of KRG on HCoV-OC43 replication, HCT-8 cells were treated with KRG after viral infection, and the culture supernatants were harvested at 3 and 4 dpi. The abundance of HCoV-OC43 N and M protein mRNA in the supernatant was then measured by qRT-PCR. KRG treatment significantly reduced the expression of viral N protein mRNA (Fig. 4A and B), confirming that KRG blocks the replication of HCoV-OC43 in HCT-8 cells. In addition, viral matrix (M) protein mRNA expression was also decreased by KRG treatment (Fig. 4C and D).

3.5. Induction of antiviral gene expression by KRG treatment

To confirm the antiviral activity of KRG, the levels of IFN genes (IFN-α, IFN-β, and IFN- γ) and MxA were evaluated in HCT-8 cell lysates 4 dpi. KRG treatment after virus infection augmented the mRNA levels of IFN-α, IFN-β, and IFN- γ (Fig. 5A-C). MxA mRNA expression was not increased by both virus infection and KRG treatment (Fig. 5D). These data suggest that KRG suppresses viral infection by inducing the expression of antiviral cytokines such as interferons in HCoV-OC43-infected HCT-8 cells.

4. Discussion

Antiviral effects of ginsenosides have been demonstrated in previous studies. Ginsenosides Re, Rf, and Rg2 were found to protect the host from coxsackievirus and rhinovirus 3 infections [27], while ginsenoside Rg3 significantly inhibited hepatitis B virus secretion [32]. In addition, ginsenoside Rb1 suppressed the replication of various viruses including SARS-CoV [24,33–35]. However, no study reports the antiviral activity of ginsenosides against HCoV-OC43. Thus, this study assessed the antiviral effect of KRG extract and ginsenosides against HCoV-OC43. The data showed that KRG extract considerably reduced the number of HCoV-OC43 plaques dose-dependently (Fig. 1B), but none of the ginsenosides tested (ginsenosides Rc, Re, Rf, Rg1, and each isoform of Rg2, Rg3, and Rh2) showed antiviral activities against HCoV-OC43.

To find the steps of the HCoV-OC43 life cycle that could be affected by KRG extract, KRG extract was added to HCT-8 cells at three time points: after (post-treatment), during (co-treatment), and 24 h before (pre-treatment) virus infection. Our data demonstrated that KRG extract did not directly interact with virus particle and block the viral entry, but inhibited viral replication, as shown

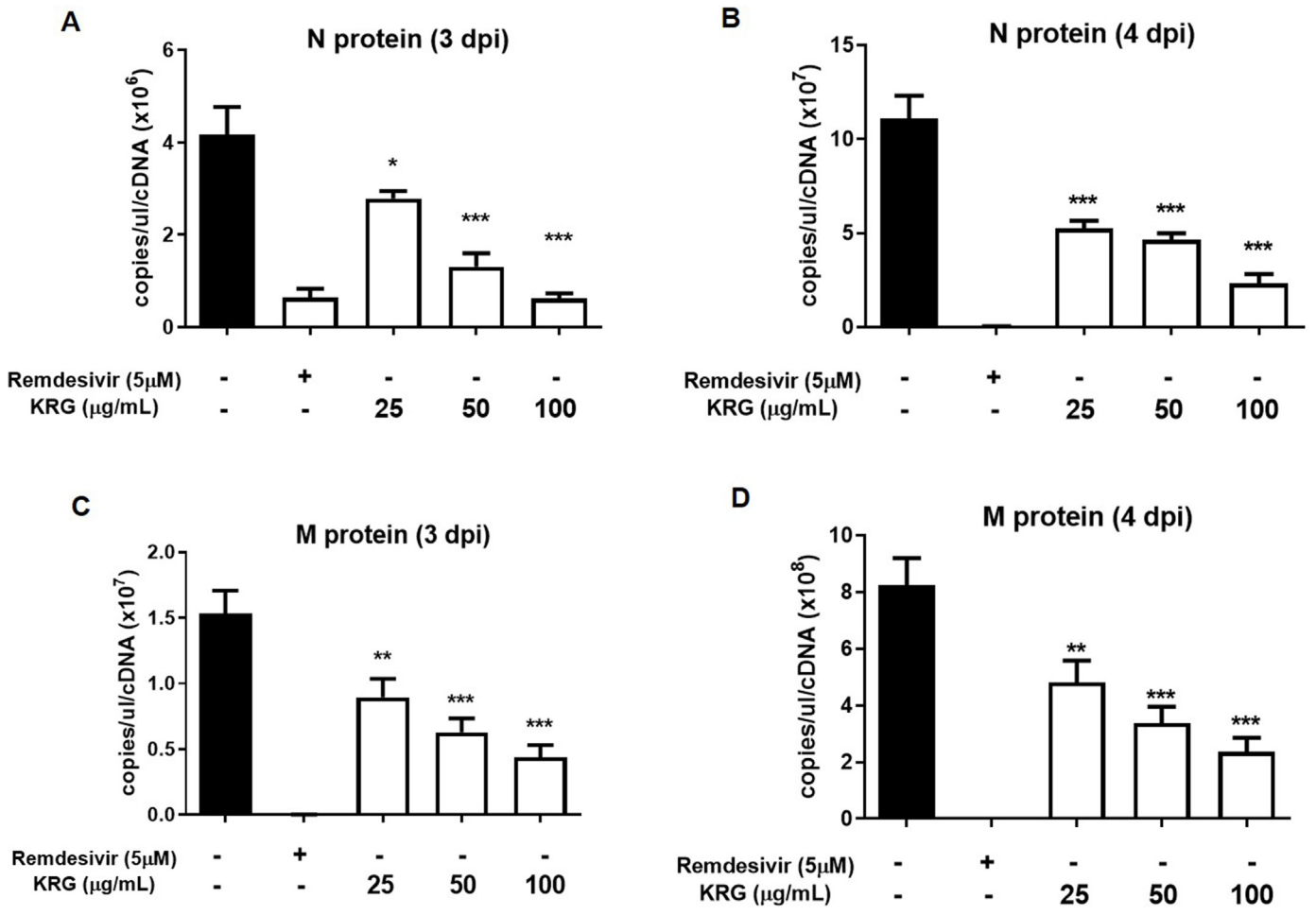

Fig. 4. Decrease in nucleoprotein (N) and membrane protein (M) mRNA levels in KRG-treated HCT-8 cells. HCT-8 cells were treated with 25, 50, and 100 μg/mL KRG for 4 days after infection with HCoV-OC43, and the culture supernatant was harvested at 3 dpi and 4 dpi. The viral RNA was obtained from the culture supernatant of HCoV-OC43-infected cells, and the mRNA level of HCoV-OC43 N protein (A, B) and M protein (C, D) was examined by qRT-PCR.

by the decrease in plaque numbers in the pre- and post-treatment groups (Fig. 2). Considering the anti-viral effect of pre-treated KRG extract, KRG can be expected to have preventive effect on HCOV-OC43 infection as well as inhibition of virus replication.

In addition, to confirm the antiviral effect of KRG extracts, the mRNA and protein levels of HCoV-OC43 *N* gene were examined in HCoV-OC4-infected HCT-8 cells. The nucleocapsid N protein is a structural protein forming a helical capsid while binding to genomic RNA, and plays a critical role in CoV replication [36,37]. KRG extract treatment reduced N protein expression in HCT-8 cells and decreased mRNA expression of N protein at 3 and 4 dpi. The membrane protein (M) is a type III transmembrane glycoprotein and most abundantly present in the CoV particle. It plays a central role in virus assembly. The data demonstrated that KRG extract significantly also decreased the mRNA level of M protein. Furthermore, the expression of spike protein was also reduced by KRG extract treatment.

To investigate the effects of KRG on the host antiviral response, the production of IFN genes was measured in HCoV-OC43-infected HCT-8 cells. Type I and II IFNs are important cytokines induced upon viral infections [38,39], and they promote an antiviral state that resists viral spreading in uninfected cells and replication in infected cells [40]. The mRNA levels of IFN-α, IFN-β, and IFN-γ were increased in the lysates of virus-infected cells at 4 dpi, and the increase in IFN was more prominent in the KRG-treated groups (Fig. 5). However, the mRNA level of MxA was not improved at 4 dpi. MxA is an IFN-inducible gene product, and MxA induced by type I IFNs (IFN- α and IFN- β) can block viral replication. Beidas and Chehadeh have reported that M and N proteins of HCoV-OC43 downregulated the expression of antiviral genes associated with the type I IFN and NF-κB signaling pathways [41]. In addition to the direct increase in IFN response, KRG treatment may promote IFN response by decreasing M and N proteins.

Although ginsenosides are well known active ingredients of KRG, ten individual ginsenoside tested in this study did not show any antiviral effect on HCoV-OC43. Since KRG extract consists of numerous compounds, the combination of multiple ingredients present in KRG extract may exert synergistic effect on HCoV-OC43. In addition, further research will be required to investigate the antiviral effects of other active constituents of KRG such polyacetylenes, phenolic compounds, and polysaccharides, and ginsenosides on HCoV-OC43 [42].

In conclusion, this study indicates that KRG extract can impede the progression of CoV infection by enhancing virus-induced IFN responses. Further investigation would require evaluating the antiviral effects of KRG on SARS-CoV-2.

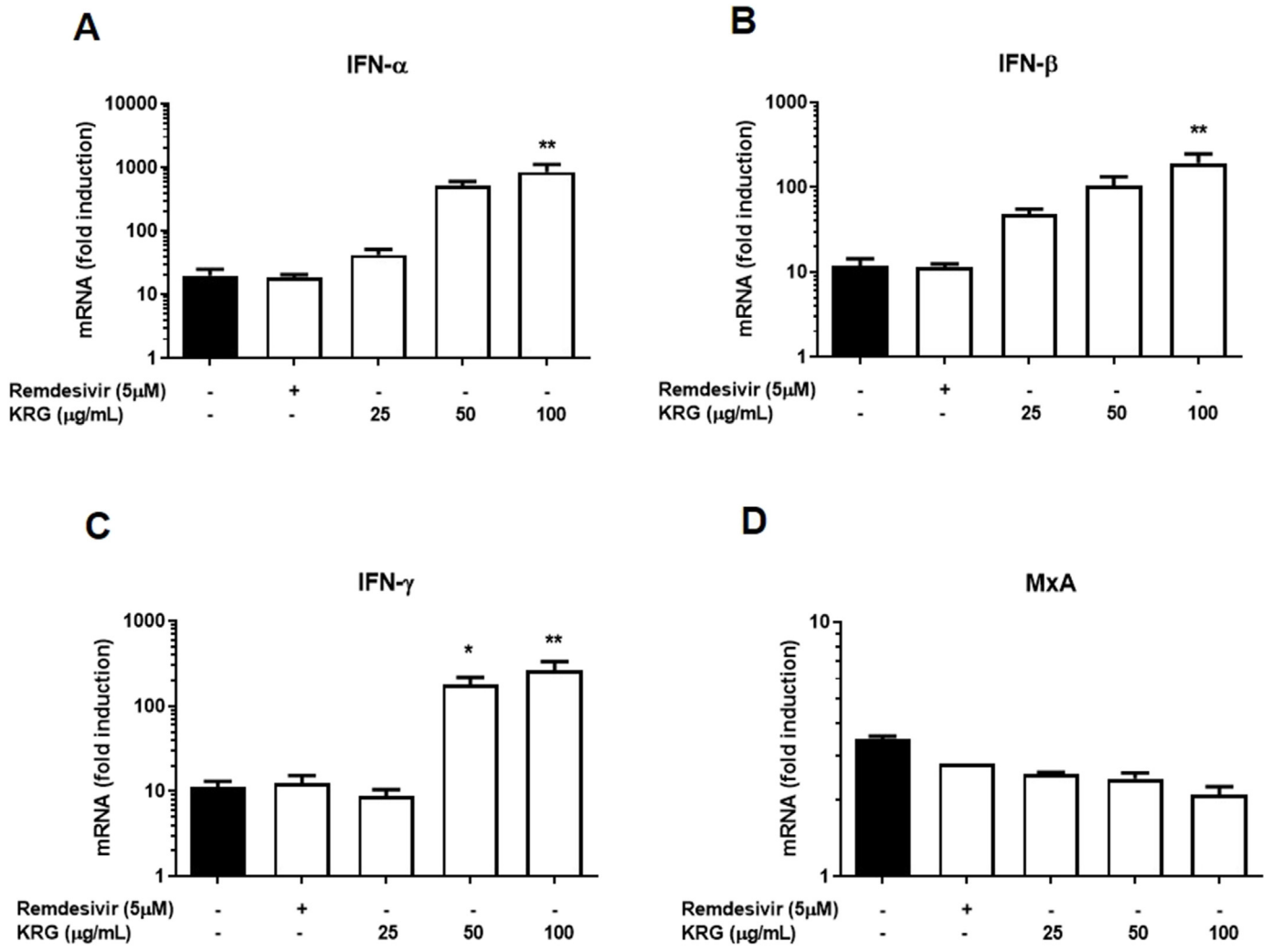

Fig. 5. Changes in the expression of IFN-α, IFN-β, IFN-γ, and MxA in KRG-treated, HCoV-OC43-infected HCT-8 cells. Total RNA was extracted from HCT-8 cells at 4 dpi, and the mRNA expression levels of IFN-α (**A**), IFN-β (**B**), IFN-γ (**C**), and MxA (**D**) were measured by qRT-PCR. GAPDH was used for data normalization.

Declaration of competing interest

All authors have no conflicts of interest to declare.

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