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Protective Effects of *Citrus* Flavonoid Hesperidin in Enterocytes After Induction with TNF-α and IFN-ɣ Which Mimic the COVID-19 Disease

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Abstract

BACKGROUND/AIMS: Coronavirus disease (COVID-19) is caused by a virus and exhibits various symptoms such as cough, fever, and chills. Flavonoids have a potential inhibitory effect on coronaviruses. In this study, we determined the effects of hesperidin on enterocyte cells (IEC) after tumor necrosis factor (TNF)-α and interferon (IFN)-γ induction which mimics the severe acute respiratory therapy-coronavirus-2 (SARS-CoV-2) infection.

MATERIALS AND METHODS: The IEC-6 were treated with 50 ng/mL of TNF-α and 100 ng/mL of IFN-ɣ for 48 h to mimic inflammatory shock similar to COVID-19 disease. IEC-6 cells were cultured as control, COVID-19 disease mimic, hesperidin prophylactic, or treated groups. The cytotoxicity effect of hesperidin was analyzed using an MTT assay. Serum levels of TNF-α and interleukin (IL)8 were evaluated using ELISA. The distributions of TNF-α, IFN-ɣ, IL-1ß, Insulin-like growth factor-I, and caspase-3 were analyzed by indirect immunoperoxidase staining.

RESULTS: Both TNF-α and IL8 levels were higher in TNF-α and IFN-ɣ induction of enterocyte culture medium than in the control. Lesser immunoreactivity of TNF-α was detected in the treatment group which hesperidin applicate after TNF-α and IFN-ɣ combination. While IL-1 immunoreactivity was similar in both the hesperidin prophylactic and treatment groups, lesser immunoreactivity of TNF-α was observed in the hesperidin treatment group. Both IFN-ɣ and vascular endothelial growth factor A immunoreactivities were also decreased in the hesperidin treatment group.

CONCLUSION: We found that hesperidin had anti-inflammatory and cell protection effects in IEC after TNF-α and IFN-γ induction which mimics the model of SARS-CoV-2 infection. Therefore, hesperidin could be used to reduce gastrointestinal system symptoms in COVID-19 disease.

Keywords: COVID-19 disease, hesperidin, enterocytes, gastrointestinal tract, immunregulatory

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has infected millions of people worldwide, and its variant remains at risk of infection. There has been a lot of research against diagnostic kits for viruses, eradication and inactivation of them, control of the pandemic, and prevention of cellular damage in all organs. Because of the high mortality rate and easy spread of the viruses, there is still need for new treatment strategies to prevent the cells from attacking SARS-CoV-2.[1]

Angiotensin-converting enzyme 2 (ACE2) is the functional receptor of SARS-CoV-2, and its receptors have been demonstrated on alveolar epithelial cells, which are responsible for acute respiratory distress syndrome.[1] The intestinal epithelial cells, especially the enterocytes of

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the small intestine, also express ACE2 receptors. Rather than respiratory symptoms, coronavirus disease 2019 (COVID-19) patients also had gastrointestinal symptoms after 4,243 patients' meta-analysis.[2] ACE2 expression was more frequently observed in the ileum and colon than in the lung and was mainly expressed in the absorptive enterocytes of the ileum and colon, which offers a potential explanation for diarrhea observed in many COVID-19 patients.[3] It can explain gastrointestinal tract complaints such as diarrhea, nausea, vomiting, etc. and the formation of SARS-CoV-2 RNA in the feces of infected patients. The presence of ACE2 receptor in many organs such as the lung, small intestine, colon, kidney, spleen, brain, oral and nasal mucosa, etc. may explain the risk and multiorgan failure after SARS-CoV-2 infection.[1,4]

To protect the cells, including the lung, brain, bowel, etc., is also important to reduce cellular death and protect the cells from the effect of the viruses as well as SARS-CoV-2. The regeneration capacity of cells in all organs should be maintained during COVID-19 disease. Therefore, to treat or eliminate the viruses, a treatment needs to be developed.[5]

Natural products can be used to prevent infected cells with viruses, bacteri, etc. Therefore, they should be used as antiviral medicines, either directly or indirectly. A major functional flavanone in flavonoids, hesperidin (HD; 3,5,7- trihydroflavanone 7-rhamnoglucoside), can be isolated from lemons and other citrus fruits.[6] Anti-carcinogenic, anti-atherogenic, anti-hyperlipidemic, anti-diabetic, anti-inflammatory, anti-hypertensive, cardioprotective, membrane integrity agonist, caspase-3 and caspase-8 stimulant, apoptosis agonist, antibacterial, antiviral, etc. actions of hesperidin were also demonstrated.[7]

Hesperidin is one of the common compounds that interact with ACE2, TMPRSS2, GRP78, and AT1R, which are the most important receptors for SARS-CoV-2.[8,9] In addition, the antiviral activity of hesperidin demonstrated binding affinity to various SARS-CoV-2 protease domains.[10] Therefore, hesperidin may be a specific compound that binds both ACE2 and the receptor binding domain region of the spike protein of SARS-CoV-2.[11] In addition, hesperidin, baicalin, glycyrrhizic acid, and hyperoside were suggested to be key molecules related to traditional Chinese medicine formula.[9]

After SARS-CoV-2 infection, the release of cytokines, such as interleukin (IL)-1ß, IL-6, IL-12, IL-18, IL-33, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, transforming growth factor-ß, is triggered and causes damage to many organs, including the gastrointestinal system.[12] Kawaguchi et al.[13] demonstrated that hesperidin controls inflammatory and proinflammatory cytokine secretion in *in vitro* and *in vivo* studies. More recently, hesperidin has been shown to bind to cellular ACE2 and respond to anti-SARS- CoV-2 infection activity in an *in vitro* cell line model.[10,14] However, the mechanism of action of hesperidin against SARS-CoV-2-infected enterocytes remains unknown. In this study, we analyzed the effects of hesperidin on enterocytes cells after TNF-α and IFN-γ induction which mimic SARS-CoV-2 infection. This was an experimental study.

MATERIALS AND METHODS

Cell Line and Cell Culture

The enterocyte cell line (IEC-6, CRL-1592, ATCC) was grown in Dulbecco's Modified Eagle Medium containing 90% of fetal bovine serum and 1% streptomycin at 37 °C in a humidified 5% CO_2/95% incubator until 80% confluency. This study is an *in vitro* model and does not require the approval of the ethics committee or informed consent.

Measurement of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide

Cell viability was measured using an MTT assay as described previously.[15] The IEC-6 cell line (3,000 cells) in 96-well plates was treated with hesperidin in a dose-dependent manner for 24 or 48 h and then subjected to MTT assay (Sigma-Aldrich, Cat. No. M2003).

In vitro COVID-19 Mimic Model Consideration

To mimic inflammatory shock similar to COVID-19 disease, 3,000 IEC-6 cells per well in 96-well plates were treated with 50 ng/mL of TNF-α (315-01A-50UG, PetroTech) and 100 ng/mL of IFN-ɣ (315-05-100UG, PetroTech) for 48 h. Subsequently, the culture medium level of TNF-α (201-12-0083, SunRedBio) and IL8 (SRB-T-83151, SunRedBio) were evaluated with ELISA according to the manufacturer's protocol. Also, distributions of TNF-α and IL8 were also analyzed using indirect immunoperoxidase staining (see below).

Study Groups

IEC-6 cells were cultured in culture medium and used as the control group. Rest of the cells were other applicate with TNF-α and IFN-ɣ that was COVID-19 disease mimic group, or hesperidin applicate before or after TNF-α and IFN-ɣ combination and they were accepted prophylactic or treated groups, respectively. All culture experiments were performed in triplicate.

Indirect Immunoperoxidase Staining

The cells from all groups were fixed with 4% paraformaldehyde (1.04004.0800, Merck) at room temperature for 30 min, after washing with phosphate-buffered saline (PBS, PBS404.100, Bioshop) 0.1% Triton X-100 (A4975,0100, Applichem) for permeabilization at 4 °C for 15 min. The cells from all groups were incubated with 3% H_2O_2 (1.08597.2500, Merck, Germany) for 5 min and then with blocking solution (TA-125-UB, ThermoFisher Scientific, USA) for 1 h at room temperature. Primary antibodies against TNF-α (rabbit polyclonal, BT-AP09103, BT-LAB), IFN-ɣ (rabbit polyclonal, 15365-1-AP, Proteintech), IL-1ß (rabbit polyclonal, ABP51611, Abbkine), insulin-like growth factor (IGF)-I (rabbit polyclonal, sc-9013, Santa Cruz), VEGFA (rabbit polyclonal, sc-152, Santa Cruz), and caspase-3 (rabbit polyclonal, BT-AP01199, BT-LAB) were incubated at 4 °C overnight. After washing with PBS, biotinylated goat anti-rabbit/mouse IgG (TP-125-UB, ThermoFisher Scientific) and then peroxidase-conjugated streptavidin (TS-125-UB, ThermoFisher Scientific, USA) were incubated for 30 mi. Diaminobenzidine (DAB, TA-125-HD, ThermoFisher Scientific) was applied to the cells for 5 min, and Mayer's hematoxylin (TA-125-MH, ThermoFisher Scientific) was used for counterstaining. Slides were covered with mounting medium (DMM-125, Spring Bioscience) and viewed under a light microscope (BX43, Olympus, Japan).

Statistical Analysis

The data are expressed as mean ± standard deviation in the assay. Additionally, the Graph Pad Prism 7 software was used for analysis, and $p<0.05$ was considered statistically significant. Mann-Whitney U tests were used for data analyzes

RESULTS

The inhibitory effect of hesperidin on the proliferation of IEC-6 cells was determined using the MTT assay in our study. After the MTT assay, IEC-6 cell viability was similar to or without hesperidin application in a dosage-dependent manner. The double cell proliferation level was detected 100 µM dosage for 48 h, and this concentration was used for the rest of the study (Figure 1). After the MTT data analysis, we found no significant differences between the groups.

The levels of TNF-α and IL8 in the culture medium were measured using ELISA. TNF-α and IL8 levels were higher in the model culture medium than in the control. Therefore, a 48-h incubation of enterocytes with TNF-α and IFN-ɣ combination was used (Figure 2).

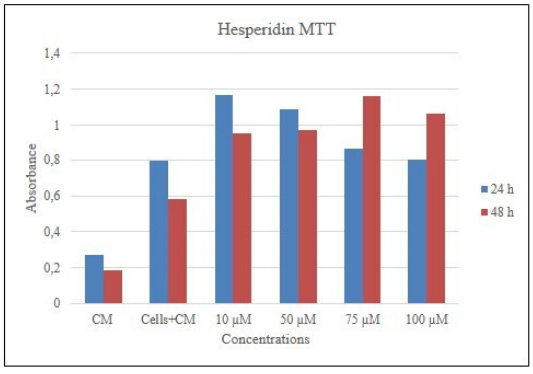

Figure 1. Cytotoxicity of hesperidin on IEC-6 cells. Hesperidin was not inhibiting cell proliferation of IEC-6 cells at all concentrations (10 µM, 50 µM, 75 µM and 100 µM) for 24 and 48 h. Cytotoxicity was determined in MTT assays. CM: culture medium.

IEC: Enterocyte cells

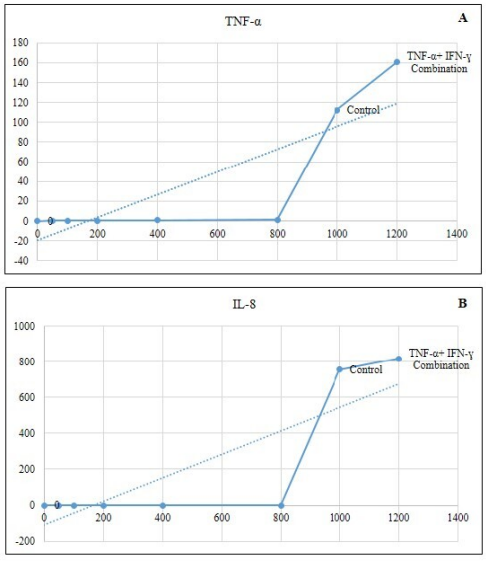

Figure 2. ELISA results from control and TNF-α + IFN-ɣ combination application IEC-6 cell culture mediums. Both TNF-α (A) and IL8 (B) levels were higher after TNF-α + IFN-ɣ combination application than in the culture mediumof control cells. The level of standards is shown as dotted lines.

IEC: Enterocyte cells, TNF: Tumor necrosis factor, IFN: Interferon

Lesser TNF-α immunoreactivity was detected in the treatment group which hesperidin applicate after TNF-α and IFN-ɣ combination (Figure 3). Moreover, with hesperidin application after TNF-α + IFN-ɣ combination, the IFN-ɣ immunoreactivity was moderate and distributions of IFN-ɣ was less than that in the hesperidin prophylactic group (Figure 4).

Immunoreactivity of IL-1ß was similar in both the hesperidin prophylactic (Figure 5C) and hesperidin treatment (Figure 5D) groups. However, strong IL-1 immunoreactivity was detected TNF-α + IFN-ɣ combination group (Figure 5B). In our study, cytoplasmic precipitation of IGF-1 was detected in the hesperidin prophylactic group (Figure 6C); this precipitation was not detected in other hesperidin treatment groups (Figure 6).

The immunoreactivity of VEGFA was decreased in hesperidin application after TNF-α + IFN-ɣ combination (treatment) group (Figure 7D) when compared with the other groups' VEGFA immunoreactivity

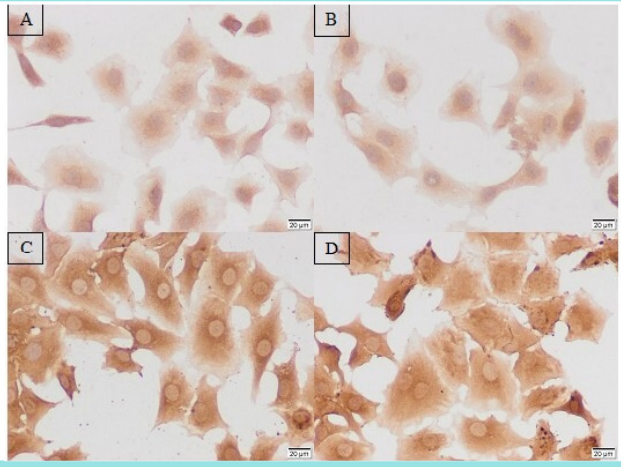

Figure 3. Distributions of TNF-α immunoreactivity on control (A), TNF-α + IFN-ɣ combination (B), hesperidin prophylactic (C), and hesperidin treatment (D) groups. Scale bars: 20 µm.

TNF: Tumor necrosis factor, IFN: Interferon

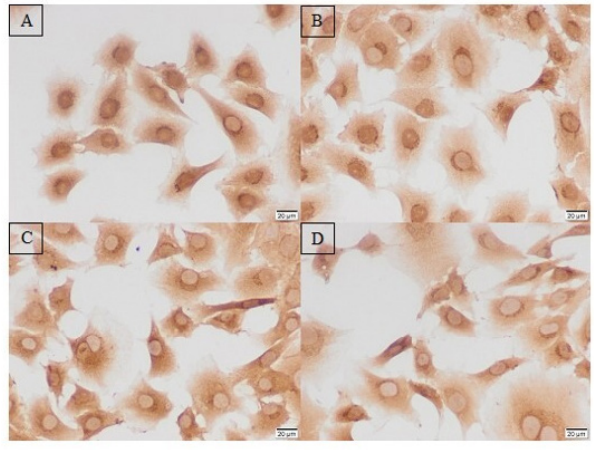

Figure 4. Distributions of IFN-ɣ immunoreactivity on control (A), TNF-α + IFN-ɣ combination (B), hesperidin prophylactic (C), and hesperidin treatment (D) groups. Scale bars: 20 µm.

TNF: Tumor necrosis factor, IFN: Interferon

(Figure 7). In our study, cellular damage of the IEC-6 cells was analyzed by the distribution of caspase-3. Weak immunoreactivity of caspase-3 was detected in all groups (Figure 8). Because similar caspase-3 immunoreactivity was observed in all groups, it was thought that apoptotic cell death was triggered in IEC-6 cells after TNF-α + IFN-ɣ application, but hesperidin had no effect on the control of caspase-3 secretion (Figure 8).

DISCUSSION

SARS-CoV-2 is responsible for millions of infections and deaths worldwide. Research has shown that ACE2 is a crucial functional SARS-CoV-2 receptor for gaining cellular entry into target cells. In addition, the ACE2 receptor is widely found in various organs such as the lung, small intestine, liver, and oral and nasal mucosa.[3,16] Therefore, blocking the ACE2 receptor can decrease the infection risk and protect cells against SARS-CoV-2. However, curative treatment is not available against viruses.

Hesperidin is a bioactive polyphenolic compound that displays numerous biological activities such as anti-inflammatory and antioxidant properties.[17] It has been used as an herbal medicine for a long time because of its high safety profile after oral intake.[18] Recently, Cheng et al.[19] showed that hesperidin suppressed the infection by blocking SARS-CoV-2 binding to the ACE2 receptor and inhibiting ACE2 protein expression. Additionally, Kandeil et al.[14] reported a hesperidine inhibitory effect on the viral replication of SARS-CoV-2 at the early stage of virus infection. On the other hand, to the best of our knowledge, there is no study regarding the effects of hesperidin on SARS-CoV-2 infection-related digestive symptoms.

Immune system hyperactivation in COVID-19 patients increases proinflammatory cytokines that induce inflammatory cell death, tissue damage, and multi-organ failure.[20] According to the Karki et al.[21] protocol, to analyze the model after incubation with TNF-α and IFN-ɣ, the levels of TNF-α and IL8 were investigated in culture media after 48 h of incubation. In our study, both TNF-α and IL8 levels were higher in the model culture medium than in the control; therefore, 48 h incubation of enterocytes with TNF-α and IFN-ɣ combination was decided.

TNF-α, IFN-γ and IL-1ß are crucial cytokines that play a critical role in SARS-CoV-2 infection-related organ damage. Case studies have shown that patients who have chronic, immune-inflammatory diseases such as inflammatory bowel disease (IBD) or Crohn's disease and are

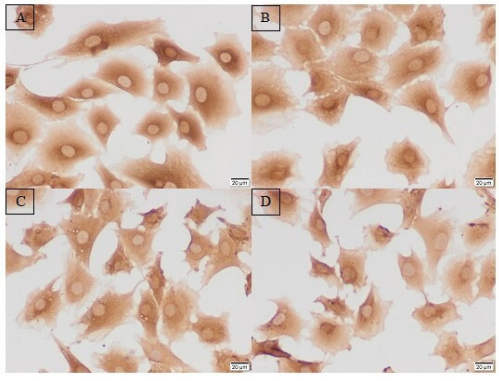

Figure 5. Distributions of IL-1 immunoreactivity in the control (A), TNF-α + IFN-ɣ combination (B), hesperidin prophylactic (C), and hesperidin treatment (D) groups. Scale bars: 20 µm

TNF: Tumor necrosis factor, IFN: Interferon, IL: Interleukin

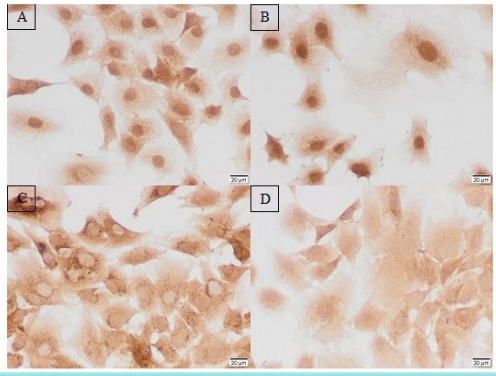

Figure 7. Distributions of VEGFA immunoreactivity in the control (A), TNF-α + IFN-ɣ combination (B), hesperidin prophylactic (C), and hesperidin treatment (D) groups. Scale bars: 20 µm.

TNF: Tumor necrosis factor, IFN: Interferon, VEGFA: Vascular endothelial growth factor-A

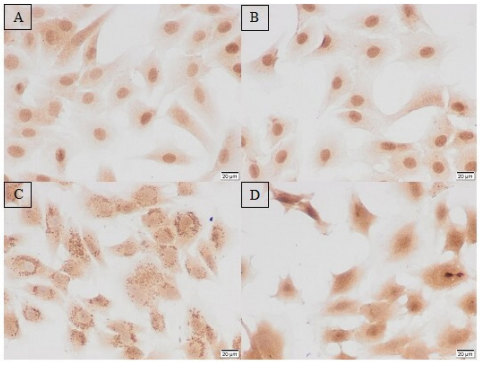

Figure 6. Distributions of IGF-I immunoreactivity in the control (A), TNF-α + IFN-ɣ combination (B), hesperidin prophylactic (C), and hesperidin treatment (D) groups. Scale bars: 20 µm.

TNF: Tumor necrosis factor, IFN: Interferon

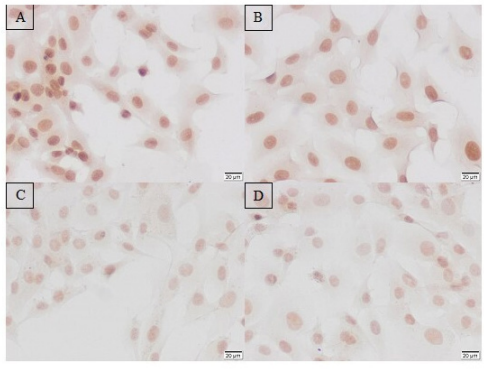

Figure 8. Distributions of caspase immunoreactivity in the control (A), TNF-α + IFN-ɣ combination (B), hesperidin prophylactic (C), and hesperidin treatment (D) groups. Scale bars: 20 µm.

TNF: Tumor necrosis factor, IFN: Interferon,

on anti-TNF-α therapy tends to have a mild course after SARS-CoV-2 inflammation.[22,23] These results suggest that prophylactic anti-cytokine therapy may also be beneficial in SARS-CoV-2 infection.

The anti-inflammatory properties of hesperidin are due to its inhibition of different pro-inflammatory mediators.[24] It was reported that hesperidin significantly reduced inflammatory mediators IL-1ß and TNF-α in various studies.[25] Therefore, distributions of the TNF-α, IFN-ɣ, IL-1ß was investigated. In our study, lesser immunoreactivity of TNF-α was detected in the treatment group which hesperidin applicate after TNF-α and IFN-ɣ combination.

Recent studies have demonstrated that higher expressions of IFN-γ in serum is an important predictor of the severity and prognosis of SARS-CoV-2-infected patients.[5,21] In addition, anti-SARS-CoV-2 plant-derived phenolic compounds such as hesperidin, which decrease inflammatory cytokines, were reported. Hesperidin is a natural compound that could target the binding interface between the ACE2 receptor and SARS-CoV-2 Spike.[11] It has been reported that hesperidin strongly binds to the RNA-dependent RNA polymerase active site, which catalyzes the replication of SARS-CoV-2 RNA.[26] In our study, with hesperidin application after TNF-α + IFN-ɣ combination, the IFN-ɣ immunoreactivity was moderate and distributions of IFN-ɣ was less than that in the hesperidin prophylactic group.

SARS-CoV-2 infection activates the NLRP3 inflammasome, leading to the secretion of active IL-1ß and IL-18 and the initiation of a cytokine storm.[27] IL-1ß stimulates Th-17 and IL-6 immune response, however IL-18 induces IFN-γ producing by Th-1 lymphocytes.[28] In this study, the immunoreactivity of IL-1ß was similar in both the hesperidin prophylactic and hesperidin treatment groups. Strong IL-1ß immunoreactivity was detected TNF-α + a IFN-ɣ combination group. IL-1ß is activated after SARS-CoV-2 infection then TNF-α and IFN-ɣ secretion are stimulated. For this reason, hesperidin may affect different steps of infection.

According to the immunocytochemistry results, application of hesperidin after TNF-α + IFN-ɣ combination, which was a COVID-19 disease mimic model, controlled the secretion of TNF-α and IFN-ɣ. Application of hesperidin either before or after TNF-α + IFN-ɣ combination, the secretion of IL-1ß was also controlled. Cheng et al.[19] demonstrated that hesperidin was proven to prevent cytokine storm by inhibiting the expression of proinflammatory cytokines. Our results also indicated that application of hesperidin after TNF-α + IFN-ɣ shock, secretion of cytokines, which were especially responsible for the SARS-CoV-2 cytokine storm, was controlled on IEC-6 enterocytes. The inhibitory effect of hesperidin on viral replication of SARS-CoV-2 was demonstrated at the early stage of virus infection.[14] In this study, we demonstrated the prophylactic and treatment role of hesperidin in enterocytes after inflammatory shock, which is similar to COVID-19 disease.

Immune gut homeostasis plays an important role in determining the course of IBD and infection caused by SARS-CoV-2. The improvement of colitis after hesperidin treatment is related to the inhibition of pro-inflammatory cytokines TNF-α, IL-6, IL-1ß, and IL-33 in the colon.[29] The regulatory balance of inflammatory cytokines in the gut is important for lung commensal microorganisms. Therefore, treatment or preventive therapy that controls cytokine diversity in the gut should be considered in COVID-19 disease. Hesperidin may be used for this purpose.

Bioinformatics data analyzes demonstrated that the immunoregulatory effects of hesperidin or glucosyl hesperidin in treating COVID-19 were targeting TNF-α, IGF-I, vascular endothelial growth factor (VEGF) A, etc.[12,30] IGF-1 can stimulate inflammatory cytokine secretion, and IGF-1 level is one of the possible immune system regulators. Hazrati et al.[31] showed that IGF-1 was suspected to modulate inflammation and was related to COVID-19 infection in a severe form. In fact, higher IGF-1 concentrations were associated with a lower risk of COVID-19 infection mortality.[31] In our study, cytoplasmic precipitation of IGF-1 was detected in the hesperidin prophylactic group, but this precipitation was not detected in the hesperidin treatment group.

VEGFA is a pro-nociceptive and angiogenic factor. It has been shown that VEGFA levels increase in bronchial alveolar lavage fluid from SARS-CoV-2-infected patients.[32] The immunoreactivity of VEGFA was decreased in the hesperidin application after TNF-α + IFN-ɣ combination (treatment) group when compared with the other groups VEGFA immunoreactivity.

Trigger of inflammation and failure of immune response are the most common side effects of SARS-CoV-2. Therefore, understanding the biological process that controls homeostasis is important for controlling cellular damage after virus infection. Our study demonstrated that IGF-I and VEGFA distribution decreased after hesperidin application on IEC-6 cells. Therefore, hesperidin, other than cytokine controlling in enterocytes, may also regulate the secretion of growth factors.

The cellular death and other side effects of SARS-CoV-2 viruses. The apical enterocyte membrane and epithelial tight junctions are the barrier of the intestinal epithelium. After alterations or destruction of the intestinal epithelium induce enterocyte apoptosis.[33] In our study, cellular damage of the IEC-6 cells was analyzed by the distribution of caspase-3. Weak immunoreactivity of caspase-3 was detected in all the groups. Because similar caspase-3 immunoreactivity was observed in all groups, it was thought that apoptotic cell death was triggered in IEC-6 cells after TNF-α + IFN-ɣ application, but hesperidin had no effect on the control of caspase-3 secretion. After inflammatory shock, caspase-1-dependent cell death is observed by inflammatory shock; therefore, in our study, cell death by caspase-3 may not have been triggered.

Study Limitations

The main limitation of this study was that more infection-related antibodies could have been studied to investigate the protective effects of hesperidin against the SARS-CoV-2 infection model. The protective effects of hesperidin are also needed in *in vivo* models.

CONCLUSION

The genetic diversity of SARS-CoV-2 results in a higher rate of widespread infection. After infection with SARS-CoV-2, the cytokine storm responds to the uncontrolled overproduction of soluble markers of inflammation. Therefore, therapeutic strategies and control of the side effects of the viruses are needed.

During the pandemic, it has been observed that patients might present or develop various GI symptoms during COVID-19. The detection of SARS-CoV-2 in fecal samples is essential for clinical practice, particularly for patients with atypical symptoms, and should be performed when COVID-19 patients are leaving the hospital to confirm viral clearance. The relationship between the digestive system and COVID-19 should be further explored in future related studies. The composition of balanced

gut microbiota has a major influence on the effectiveness of lung immunity.[34]

Hesperidin, diosmin, and rutin are widely available in pharmaceutical stores under various trade names and can be derived from various natural nutritive foods reported to have antiviral properties. The GO biological process result showed that the 45 targets of hesperidin were involved in a series of biological process which are mainly involved in the regulation of immune response, inflammation and virus infection, such as the regulation of production of molecular mediator of immune response, positive regulation of leukocyte migration, cytokine production involved in immune response, production of molecular mediator involved in inflammatory response, and virion attachment to the host cell.[30] Both hesperidin and glucosyl hesperidin had a great impact on immune, inflammation, and viral infection induced by COVID-19 according to systematic pharmacological analysis.[12] On the basis of *in silico* screening, hesperidin was also predicted to target the interaction site between SARS-CoV-2 Spike and ACE2 receptors, thus blocking the entry of the virus into human lung cells. Therefore, hesperidin could be a promising prophylactic drug against COVID-19.[35] According to the research aimed to screen drugs with high affinity to bind ACE2 and SARS-CoV-2 proteins, hesperidin was always at the top of the bioactive antiviral com-pounds.[8,10] Consistently, the present research concluded the remarkable immunoregulatory effects of hesperidin in treating COVID-19.

MAIN POINTS

- Hesperidin had anti-inflammatory and cell protection effects in enterocyte cells after tumor necrosis factor (TNF)-α and interferon (IFN)-γ induction which mimics the model of severe acute respiratory therapy-coronavirus-2 infection.
- IGF-I and vascular endothelial growth factor-A (VEGFA) distribution decreased after hesperidin application to enterocyte cells-6 cells. Hesperidin, other than cytokine control in enterocytes, may also regulate the secretion of growth factors.
- The immunoreactivity of VEGFA was decreased in hesperidin application after the TNF-α + IFN-ɣ combination (treatment) group.

ETHICS

Ethics Committee Approval:

Informed Consent: This study is an in vitro model and does not require the approval of the ethics committee or informed consent.

Peer-review: Externallay and internally peer reviewed.

Authorship Contributions

Surgical and Medical Practices: U.Ö., H.K.E., D.A.Ç., Concept: H.S.V., Design: U.Ö., H.S.V., D.A.Ç., Data Collection and/or Processing: U.Ö., H.S.V., E.B., H.K.E., Analysis and/or Interpretation: H.S.V., H.K.E., Literature Search: H.S.V., E.B., D.A.Ç., Writing: U.Ö., H.S.V., E.B., H.E.K., D.A.Ç.

DISCLOSURES

Conflict of Interest: No conflict of interest was declared by the authors.

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