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Cytokine Expression During the Early Response of A549 Cells to Infection with Influenza Virus, *Alphacoronavirus* and *Betacoronavirus*

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Abstract: The escalating incidence of coronavirus infections underscores the critical need for a comprehensive understanding of the differential impacts of Human Coronaviruses (HCoVs) belonging to the *Alphacoronavirus* and *Betacoronavirus* genera. This study investigated the varying effects of HCoV-229E (*Alphacoronavirus*), HCoV-OC43 (*Betacoronavirus*), and influenza A virus subtype H3N2 on A549 cell viability and cytokine expression. Results showed that H3N2 triggered a more potent cytokine response than HCoV-229E and HCoV-OC43, albeit without inducing apoptosis, HCoV-229E elicited a more potent cytokine response than HCoV-OC43, but HCoV-OC43 was more capable of inducing apoptosis. During the initial 24 h of infection, HCoV-229E and HCoV-OC43 led to significantly greater A549 cell viability reduction than H3N2, potentially attributed to their longer incubation periods. Additionally, the coronaviruses demonstrated enhanced infectivity. Meanwhile, H3N2 induced the production of IL-6, IL-8 and tumor necrosis factor-α. These results provide insights into the protracted incubation periods characteristic of coronaviruses and the cytokine storm observed during infection. Thus, emphasis is placed on the degree of the observed inflammatory response and how it might affect the severity of the condition. These findings provide valuable insights that may serve as a reference for future research on respiratory viruses. The observed distinctions in cell viability, cytokine expression, and infectivity demonstrate the nuanced dynamics of different coronaviruses and can inform the design of strategies for better managing and investigating these infections.

Keywords: *Alphacoronavirus*, *Betacoronavirus*, Cytokine, Influenza Virus, Respiratory Disease

Introduction

The recent surge in coronavirus infections demands heightened attention. Particularly, the present 2019 new coronavirus epidemic then the 2002 Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV-1) outbreak have left an indelible mark worldwide. Coronaviruses, which belong to Nidovirales, have a broad host range, from birds to mammals. Human Coronaviruses (HCoVs) are categorized into *Alphacoronavirus* and *Betacoronavirus* genera. *Alphacoronavirus* includes HCoV-229E and HCoV-NL63 (van der Hoek *et al*., 2004), while *Betacoronavirus* includes HCoV-OC43 (McIntosh *et al*., 1967), HCoV-HKU1 (Woo *et al*., 2005), SARS-CoV (Drosten *et al*., 2003; Ksiazek *et al*., 2003) and MERS-CoV (Zaki *et al*., 2012). In December 2019, the SARS-CoV-2 emerged, initiating the COVID-19 virus (Siddique *et al*., 2020). Meanwhile, amidst the direct spread of avian influenza viruses to individuals and the increase in neoantigen variants, the need to enhance diagnostic test quality has become evident.

While coronavirus infections typically present with mild upper respiratory tract symptoms common to influenza and coronavirus infections coronaviruses are notably associated with pulmonary fibroproliferative responses (Michalski *et al*., 2022) and pulmonary microthrombi (Iba *et al*., 2020). Earlier research has shown that the plasma concentrations of Tumor Necrosis Factor (TNF)-α, interleukin (IL)-6, and IL-8 increase substantially in reaction to influenza virus infection (Phung *et al*., 2011). Indeed, infections caused by

influenza A (H5N1, H3N2, H6N1, and H9N2) viruses and coronaviruses can cause systemic tissue damage mediated by immune responses (Morris *et al*., 2021). The well-established pivotal part of IL-6 in accelerating the cytokine storm further emphasizes the intricate immune response dynamics (Khadke *et al*., 2020). This response is implicated in diffuse tissue damage to airway vessels and subsequent TNF-α-induced infiltration of macrophages, neutrophils, fibrin deposition, and alveolar collapse. Consequently, Acute Respiratory Distress Syndrome (ARDS) develops (Murdaca *et al*., 2021).

This research investigated the effect of viral infections on lung epithelial cells, focusing on the *Alphacoronavirus* (HCoV-229E), *Betacoronavirus* (HCoV-OC43), and influenza A virus (H3N2). Unlike previous research that has predominantly explored the effects of each virus independently, we assessed and compared the survival of A549 cells when infected with similar and varying concentrations of each virus over different time intervals. MOI represents the ratio of virus particles to host cells, indicating how many virus particles are included in cultures.

The primary objectives included evaluating cell viability, assessing cytotoxicity levels, and analyzing cytokine production in response to viral infection. To this end, we performed a comparative analysis and discerned distinct patterns or differences in the cellular responses induced by an *Alphacoronavirus*, *Betacoronavirus*, and influenza A virus H3N2. This approach offers a more thorough comprehension of the effects of respiratory viruses on human lung epithelial cells, elucidating crucial aspects, including overall cell health, cytotoxic impact, and inflammatory responses. By elucidating the similarities and disparities in the effects of these viruses, valuable insights are provided into the nuanced interactions between respiratory viruses and human lung epithelial cells. Hence, this research deepens our understanding of the comparative pathogenicity and host cell responses associated with *Alphacoronaviruses*, *Betacoronaviruses*, and influenza A virus H3N2. These findings will inform the development of improved strategies to effectively combat these respiratory infections.

Materials and Methods

Materials

Dulbecco's Modified Eagle Medium (DMEM), added with 10% Fetal Bovine Serum (FBS) were purchased from Corning (Glendale, Arizona, USA). penicillin/streptomycin solution were purchased from genDEPOT (Houston, TX, USA). Trypsin was purchased from Thermo Fisher Scientific (Seoul, Korea). Quanti-MAX WST-8 Cell Viability Assay Kit and Quanti-LDH PLUS Cytotoxicity Assay Kit were obtained from Biomax (Guri-si, Gyeonggi-doi, Korea). The human premixed multi-analyte kit for cytokines IL-6, IL-8, and TNF-α assay reagents were purchased from Biotechne (MN, USA). The A Flex station 3 multi-mode microplate reader was utilized to detect all absorbance purchased from molecular devices (San Jose, CA, USA)

Virus Culture

The HCoV-229E (KBPV-VR-9) and then the HCoV-OC43 (KBPV-VR-8) strains were procured after the Korea Bank for Pathogenic Viruses (Seoul, Republic of Korea). The Influenza A (H3N2) virus (subtype A (H3N2) NCCP 43228) was found in the National Culture Collection for Pathogens. To generate stock viruses, HCoV-229E, HCoV-OC43, and H3N2 were propagated in specific cell lines: VeroE6 for HCoV-229E, HCT-8 for HCoV-OC43 and MDCK for H3N2. The Korean Cell Line Bank in Seoul provided these cell lines. The propagation process involved infecting the respective cell lines with the viruses, followed by cultivation at 37°C in a 5% CO_2 atmosphere for 24 h to facilitate virus infection. Subsequently, cell supernatants were gathered at 1000× g for 6 min to eliminate cell debris. The clarified supernatants were aliquoted at 20-25°C and kept at -80°C for later exploitation. For details on the VeroE6, HCT-8, and MDCK culture conditions during virus propagation, please refer to the specific protocols or references provided by the Korean cell line bank. This ensures transparency and replicability of the experimental procedures undertaken in the study.

Cell Culture

Korean cell line bank A549 human lung alveolar carcinoma epithelial cells were nurtured in Dulbecco's Modified Eagle Medium (DMEM), added with 10% Fetal Bovine Serum (FBS; Corning, Glendale, Arizona, USA) and 100× penicillin/streptomycin solution (genDEPOT, Houston, TX, USA). The culture was maintained at 37°C in an environment with 5% CO_2 humidity. A549 cells were seeded in 96-well plates at a concentration of 1×10^4 cells/well at 37°C in a 5% CO_2 atmosphere for 24 h. The A549 cells were passage 12.

Viral Infection of Cells

HCoV-OC43 or HCoV-229E was introduced into A549 cells at a thickness of 1×10^4 cells/well, with Multiplicity of Infection (MOI) options of 0.1, 1.0, or 10.0. Meanwhile, A549 cells were infested with H3N2 at an MOI of 0.001, 0.01, or 0.1 in the occurrence of supplemented trypsin (Thermo Fisher Scientific, Seoul, Korea). Cultures were incubated for up to 24 h post-infection. There are many papers on the previous research on the influenza virus and A549 lung epithelial cells with a diversity of contamination of MOI 0.01, MOI 0.1, and MOI 10. H3N2 at the same concentration (MOI of 0.1) caused massive cell death; therefore, a relatively low MOI

was used for the experiment. For cells that were not infected, the medium was switched with DMEM alone and the cells served as the control group.

Cell Viability Assay

The effects of HCoV-OC43, HCoV-229E, or H3N2 on cell feasibility were utilizing the Quanti-MAX WST-8 cell viability assay kit (Biomax, Guri-si, Gyeonggi-doi, Republic of Korea). Different Multiplicities of Infection (MOIs) of HCoV-OC43 or HCoV-229E were applied to A549 cells at a concentration of 1×10^4 cells/well. A Flex station 3 multi-mode microplate reader (molecular devices, San Jose, CA, USA) was utilized to detect absorbance at 450 nm. Cell viability changes were assessed in triplicate using cells without infection as an unfavorable control. The vitality of the cells was measured in comparison to the negative control cells using the identical media.

Lactate Dehydrogenase Assay

When cells experience cytotoxicity or necrosis, the plasma membrane integrity is compromised, leading to the release of the cytoplasmic enzyme, Lactate Dehydrogenase (LDH) into the surrounding media. Therefore, the Quanti-LDH PLUS cytotoxicity assay kit (Biomax) was utilized to check the release of LDH from A549 in the media. A colorimetric test was used to determine the LDH levels. 100 µL aliquots of the cell culture were taken out and set on fresh plates. After adding 100 µL of LDH reaction mixture to each well and letting it sit for 0.5 h, the samples' absorbance was estimated at 490 nm by molecular devices' flex Station 3 multi-mode microplate reader. Every experiment was run three times. Cytotoxicity was expressed relative to the basal LDH release in uninfected cells and FBS-free medium. The amount of LDH released by the HCoV-OC43-, HCoV-229E-, or H3N2-infected cells were infected as a portion of that released by the uninfected cells.

Cytokine Analysis Via Enzyme-Linked Immunosorbent Assays

The levels of released cytokines, as well as IL-6, IL-8, and TNF-α, were restrained in the cell culture supernatants from HCoV-229E-, HCoV-OC43, and H3N2-infected cells, as well as control cells, using the Human Premixed multi-analyte kit (Biotechne; Minneapolis, MN, USA). All tests were carried out three times to evaluate intra-group variation and guarantee data accuracy. The Flex Station 3 multi-mode microplate reader measured absorbance at 450 nm. Standard curves for each cytokine were used to compute the levels of IL-6, IL-8, and TNF-α; the findings were expressed in pg/mL. A cytokine assays were expressed relative to the percentage of non-treated control.

Statistical Analysis

The experiments were independently repeated twice. Statistical analyses were implemented by SAS form 9.4 and One-Way Analysis of Variance (ANOVA). Assessments among three groups were performed by ANOVA with Dunnett's tests. Outcomes are said as the mean ± sem of the mean. The thresholds for statistical impact were $^{**}p<0.01$ and $^{*}p<0.05$.

Results

The viability of A549 cells infected with H3N2 was greater than that of cells spread with HCoV-OC43 or HCoV-229E (Fig. 1). H3N2 was predicted to cause membrane damage from an MOI of 0.01. We observed time-dependent effects on cell viability; after 24 h, HCoV-OC43 at an MOI of 0.1, 1 and 10 reduced cell viability by 32.4% ($p = 0.0032$), 59.5% ($p = 0.0001$) and 59.1% ($p = 0.0003$), correspondingly (Fig. 1a). HCoV-229E at an MOI of 0.1, 1, plus 10 reduced cell viability by 1.3% ($p = 0.1956$), 23.4% ($p = 0.0241$) and 55.3% ($p = 0.0004$), correspondingly (Fig. 1b). H3N2 at an MOI of 0.1 reduced cell viability by 22.3% ($p = 0.0043$; Fig. 1c).

Exposure to HCoV-229E, HCoV-OC43, or H3N2 for up to 24 h augmented LDH levels in the culture supernatants of infected cells in a time- and concentration-dependent way. LDH release increased by 10.0, 24.8 and 27.6% in cells exposed to HCoV-OC43 at an MOI of 0.001, 0.01 and 0.1, correspondingly, at 24 h (Fig. 2a). LDH release increased by 1.2, 3.6 and 20.1% in cells exposed to HCoV-229E at an MOI of 0.001, 0.01 and 0.1, correspondingly, at 24 h (Fig. 2b). LDH release increased by 0.8%, 18.2% and 36.0% in cells exposed to H3N2 at an MOI of 0.001, 0.01 and 0.1, correspondingly, at 24 h (Fig. 2c).

At an MOI of 0.1, H3N2, HcoV-OC43, and HcoV-229E stimulated the production of large amounts of IL-6, IL-8, and TNF-α at 24 h post-infection. These cytokines are essential in the inflammatory response, immune resgulation, and cell proliferation. They are secreted by epithelial cells and can serve as markers of various biological functions (Li *et al*., 2020). Cells exposed to HCoV-OC43 at an MOI of 0.1 for 24 h produced 7.9 pg/mL IL-6, 581.9 pg/mL IL-8, and 1.2 pg/mL TNF. Cells exposed to HCoV-229E produced 63.9 pg/mL IL-6, 1736.4 pg/mL IL-8, and 4.8 pg/mL TNF. Cells exposed to H3N2 produced the highest amounts of IL-6 (1118.9 pg/mL), IL-8 (2836.0 pg/mL), and TNF-α (26.4 pg/mL). Cells exposed to H3N2 produced the highest amounts of IL-6, IL-8 and TNF-α ($p = 0.0012$ for IL-6 after 12 h and $p = 0.0072$ at 24 h; $p = 0.0039$ for IL-8 after 12 h and $p = 0.0041$ at 24 h; and $p = 0.0074$ for TNF-α after 12 h), followed by those of cells exposed to HCoV-229E and HCoV-OC43 (Fig. 3).

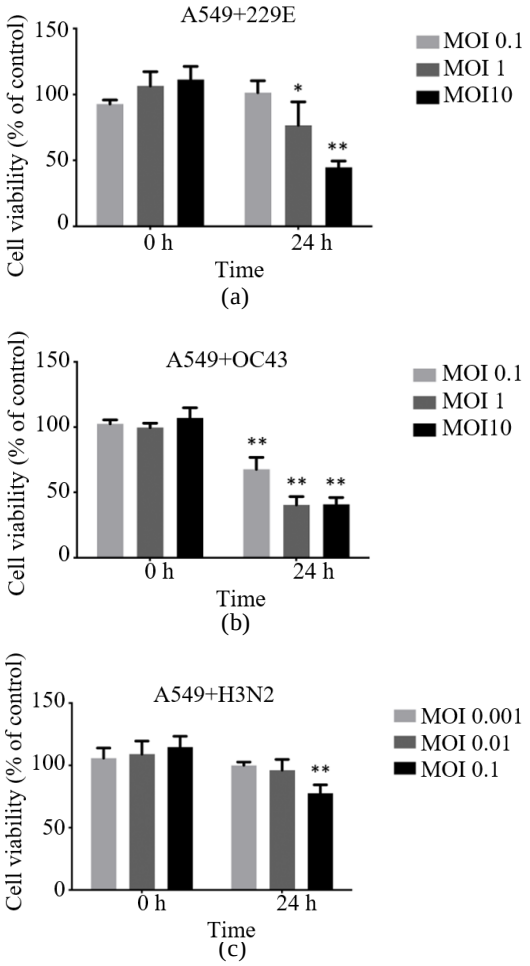
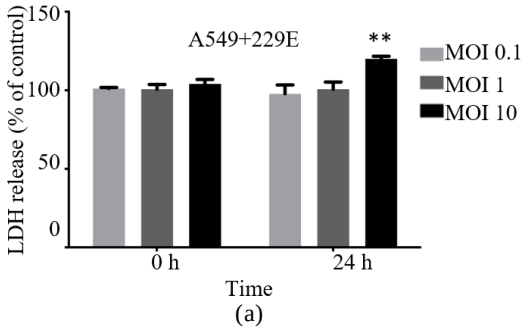


Fig. 1: Viability of A549 cells after infection with; (a) OC43 and (b) 229E at an MOI of 0.1, 1.0, and 10.0, or; (c) H3N2 at an MOI of 0.001, 0.01, and 0.1 in the presence of trypsin 24 h post-infection the data is shown as the average ± standard deviation of three separate studies. Utilizing the WST-8 assay, the inhibitory effect on cell proliferation was computed by comparing the absorbance of the virus-exposed cells' culture media at 450 nm with that of the control group. Abbreviations: 229E, HCoV-229E; OC43, HCoV-OC43; MOI, the multiplicity of infection. The three independent experiments, each conducted in triplicate, are represented in the data. **p<0.01 and *p<0.05 in relation to the control



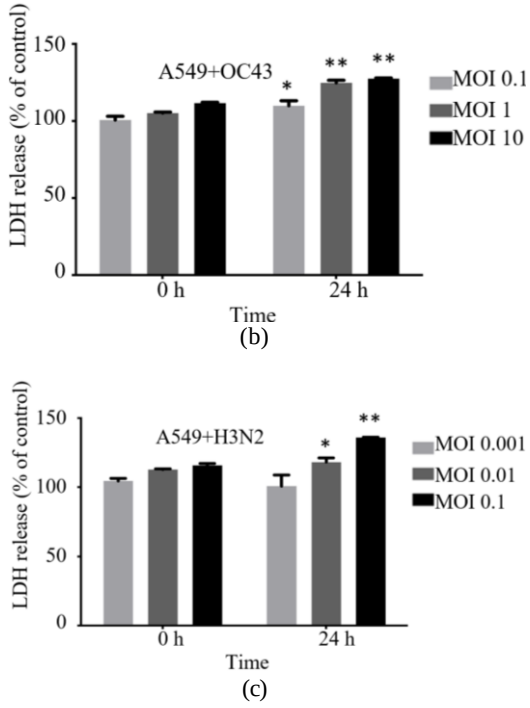


Fig. 2: LDH released from A549 cells after infection with; (a) OC43 and (b) 229E at an MOI of 0.1, 1.0, and 10.0, or; (c) H3N2 at an MOI of 0.001, 0.01 and 0.1 in the presence of supplemented trypsin 24 h post-infection. The data is revealed as the average ± standard deviation of 3 separate studies. Using a culture medium of virus-exposed cells non-virus-exposed cells served as the control the LDH release was determined spectrophotometrically. Abbreviations: 229E, HCoV-229E; OC43, HcoV-OC43; LDH, lactate dehydrogenase; MOI, multiplicity of infection. The three independent experiments, each conducted in triplicate, are represented in the data. **p<0.01 and *p<0.05 in relation to the control

Discussion

Cell viability was lower in A549 cells infected with HCoV-OC43 than with HCoV-229E, whereas the same concentration of H3N2 did not cause an important reduction in cell viability. The effects of the three viruses on cell viability were time and concentration-dependent for 24 h after infection. Although a small amount of H3N2 promoted the release of LDH, it did not reduce cell viability. H3N2 at the same concentration (MOI of 0.1) caused massive cell death; therefore, a relatively low MOI was used for the experiment. In future experiments, data from influenza viruses at the same MOI can be used to analyze the effects on A549 cell viability and cytotoxicity in the initial phase of infection.

The release of LDH induced by the three viruses increased with time. HCoV-OC43 had a greater effect on A549 cells than HCoV-229E, as reflected by the LDH values. LDH release at 24 h after infection was significantly

greater for HCoV-229E than for HCoV-OC43 at an MOI of 1. The LDH values of cells uncovered to HCoV-229E and HCoV-OC43 were 104.7 and 123.6% compared with those in the control group, respectively (Fig. 2). This difference may be attributed to the different mechanisms underlying the pathogenesis of *Alphacoronavirus* and *Betacoronavirus* infections. However, in A549 cells exposed to H3N2 at an MOI of 0.1, the LDH value after 24 h was 136.1% that of the control group, markedly higher than for cells transmit infection with HCoV-229E or HCoV-OC43 at a MOI of 10 at 24 h. A549 cells transmit infection with H3N2 at an MOI of 0.1 at 24 h exhibited a greater change in the amount of LDH released than cells infected with HCoV-229E or HCoV-OC43 at a MOI of 10 at 24 h. Within 24 h, H3N2 induced greater cytotoxicity than either of the coronaviruses (Fig. 1). Therefore, H3N2 triggers greater cytotoxicity in the initial phases of infection, which may be consistent with the inhibition of pro-apoptotic signals in coronavirus-infected cells. This finding can be applied to the design of virus detection kits or colloidal gold methods.

The statement of IL-6 and IL-8 from virus-infected A549 cells exhibited different patterns than uninfected A549 cells. The issue of IL-6 after H3N2 infection was positively correlated with time; the full level of IL-6 is observed during a late infection period. The release of IL-6 from A549 cells transmitted infection with HCoV-229E at an MOI of 1 also manifested at a later stage (24 h), whereas a slight change in IL-6 expression was observed in A549 cells infected with HCoV-OC43 (Fig. 3a). Similar results were observed for TNF-α and IL-8 expression (Fig. 3b). The release of IL-8 after influenza virus infection at an MOI of 1 was positively correlated with time. The issue of IL-8 from A549 cells after HCoV-OC43 or HCoV-229E infection at an MOI of 1 also positively correlated with time. HCoV-229E infection induced a higher level of IL-8 release than observed with H3N2 or HCoV-OC43 (Fig. 3c). Changes in cytokine expression were observed within 24 h of infection, indicating that in the short term, cytokine levels were positively correlated with time. The changes induced by the coronaviruses were smaller in magnitude than those induced by the influenza virus. Moreover, the cytokine release after the HCoV-229E infection was lower than that after the HCoV-OC43 infection, indicating that the two viruses have differences in cytotoxicity and inflammatory responses.

The incubation period refers to the time after initial exposure to a pathogen before clinical signs and symptoms become apparent. According to Subbarao and Mahanty (2020), the virus replicates its genome throughout this time, attaches itself to cells, and spreads to other cells. While SARS-CoV-2 takes 4.5-5.8 days to incubate, influenza A (H1N1) viruses only take about 1-2 days (Murdaca *et al*., 2021). Host cells become stuck in an antiviral state, release pro-inflammatory cytokines, and prevent their cellular translation when infected with the virus. However, a lot of respiratory viruses prevent apoptosis and obstruct signaling (Zhang *et al*., 2020). This induces an antiviral state in neighboring cells, preventing the efficient clearance of virus-infected cells and promoting viral replication (Lauer *et al*., 2020). The current research examined the amounts of IL-6 in HCoV-229E or HCoV-OC43-infected cells were lower at 24 h post-infection than in cells infected with H3N2. This reduction in IL-6 release prevents the secretion of regulatory cytokines, resulting in immune system overstimulation and a cytokine storm, which may result in organ failure (Dolmatova *et al*., 2021).

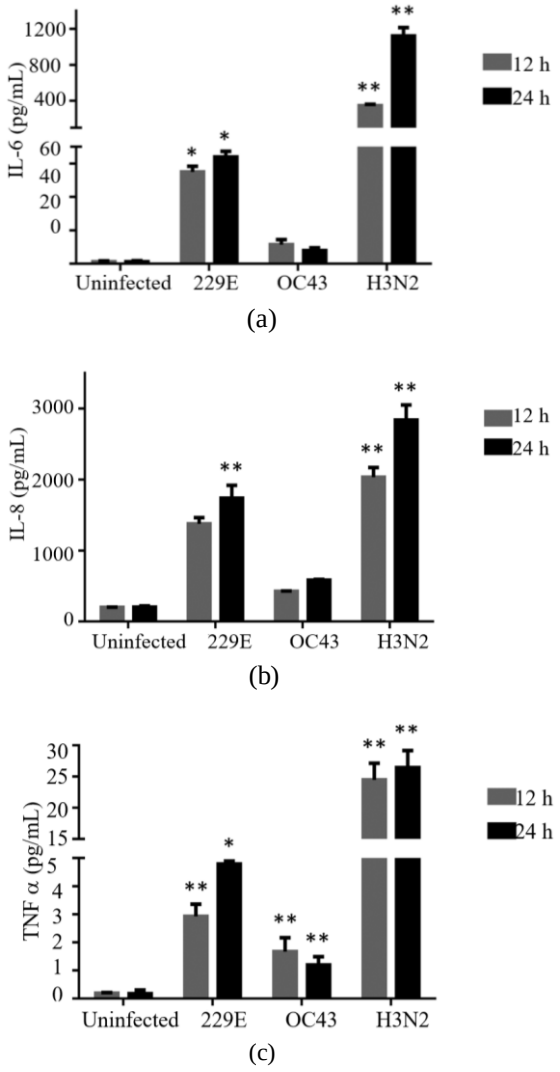


Fig. 3: Cytokine levels released from A549 cells after infection with H3N2, OC43, and 229E at an MOI of 0.1 24 h post-infection. For every treatment group, the statistics are exposed as the mean ± standard deviation of three separate studies. Abbreviations: 229E, HCoV-229E; OC43, HCoV-OC43; IL, interleukin; TNF, tumor necrosis factor. The three independent experiments, each conducted in triplicate, are represented in the data. **p<0.01 and *p<0.05 in relation to the control

In the early stage of infection, although HCoV-OC43 did not induce the expression of inflammatory factors or reduce cell viability, greater cytotoxicity was observed. This may be due to only the RNA and protein coat being replicated during the early stages of infection (Rakowska *et al*., 2021). Moreover, delayed apoptosis and greater toxicity may be caused by viral attachment; LDH levels are altered by changes in the cell membrane when the virus injects its genetic material into the cell (Wei *et al*., 2022). However, during the early stages of infection, innate cellular immunity, early antiviral reaction gene transcription, and cell series arrest in the S stage are all decreased by HCoV-229E. (Friedman *et al*., 2021). This could explain why A549 cells exhibit HCoV-OC43-induced cytotoxicity. The proteins on the illness membrane fuse with the mass cell membrane when HCoV-OC43 attaches to receptors on the cell side, discharging the viral genetic material (RNA) into the host cell's cytoplasm. The infection uses the biological equipment of the host cell for protein synthesis. Through RNA polymerase, the RNA of the virus is replicated to generate more viral genetic material.

Immunity to HCoV-OC43 enables previously infected host cells to suppress reinfection with the same virus, limiting viral replication and spread in host cells. Cells secrete low levels of interferon and great levels of pro-inflammatory cytokines (Ye *et al*., 2020). Interestingly, this was more apparent in experiments with H3N2 than with HCoV-OC43. Cytokine levels may have disrupted cellular processes without inducing apoptosis. In addition to the effects of the novel coronavirus on the lungs, neurological signs can take many forms, from generic symptoms such as headaches, myalgia, exhaustion, and taste or smell problems to particular disorders (Desai *et al*., 2021). HPAI H5N1 and WSN viruses attacked substantially greater numbers of cells in SK-N-SH and U87-MG cells than H3N2 or pH1N1 illnesses did. The infection of different neural cells by influenza viruses also showed differences (Siegers *et al*., 2019). This warrants further investigation and verification in future studies.

Previous studies have compared data on cell viability, cytotoxicity, and inflammatory factors during infection with RSV (Ishioka *et al*., 2011), at A549 MOIs of 0.1, 1, 10. Meanwhile, HCoV-229E, HCoV-OC43, and H3N2 both often cause pneumonia. Hence, the current comparative study identified factors that may cause co-infection with influenza. However, there is a lack of data on simultaneous infection with these viruses.

Pathogenic characteristics were observed in A549 cells following infection with the coronaviruses or influenza. The induced cytokine production levels for IL-6, IL-8, and TNF-α from highest to lowest were for influenza virus, *Alphacoronavirus*, and *Betacoronavirus*. These findings can be adopted as a reference parameter for clinical diagnosis and treatment.

HCoV-OC43 can be used as a copy of SARS-CoV-2 in environmentally stable tests (Owen *et al*., 2021). However, certain limitations were noted in this study. The MOI range was limited; thus, cells infected with a wide range of MOI will be evaluated in our future work. Moreover, we plan to study the impact of more complex environmental factors on viral infection and the simultaneous infection of dissimilar cell types by *Alphacoronavirus*, *Betacoronavirus*, and influenza.

Conclusion

HCoV-OC43 elicits greater cytotoxicity than HCoV-229E in A549 lung epithelial cells within 24 h of infection and results in lower cell viability. Furthermore, HCoV-OC43 induces an inflammatory response earlier than HCoV-229E. H3N2 also induces an earlier inflammatory response than HCoV-229E or HCoV-OC43 and increased secretion of TNF-α, IL-6, and IL-8. This may account for the longer incubation period of coronaviruses and the manifestation of lung inflammation and tumors. These results serve as a foundation for ongoing studies on respiratory diseases and a reference for designing viral detection methods and related treatments.

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Author's Contributions

Qian Wen Wang: Participated in all experiments, coordinated the data analysis, and contributed to the writing of the manuscript.

Jae Kyung Kim: Designed the research plan and organized the study.

Ethics

The Dankook University Institutional Review Board Committee gave its approval to the research being conducted (No. 2019-12-007). The Declaration of Helsinki was followed in every aspect of this investigation.

Competing Interests

There are no competing interests, according to the authors.

Data, Material and/or Code Availability

Upon inquiry, the corresponding author will provide the data needed to verify the study's conclusions.

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