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Apigetrin inhibits cytotoxicity and dysregulation of ACE2, IL1α and TGFβ expression induced by recombinant spike protein of SARS-CoV-2

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Abstract

SARS-CoV-2 virus is a novel coronavirus that was first identified in Wuhan, China in December 2019 and has caused an ongoing global health crisis. It has been a worldwide focus in research to understand viral pathogenesis and discover effective therapies. SARS-CoV-2 belongs to the same genus as the viruses responsible for Severe Acute Respiratory Syndrome (SARS) and the Middle East Respiratory Syndrome (MERS). Spike protein (SP) on SARS-CoV-2 plays a key role in the pathogenesis of SARS-CoV-2. The virus enters human cells via the binding of SP to the angiotensin-converting enzyme 2 (ACE2) on human cells. The binding of SP inhibits ACE2 function by reducing formation of angiotensin-(1-7), a compound that has inhibitory effects on inflammation. In addition, SARS-CoV-2 induces excessive proinflammatory cytokine production through various other signaling pathways such as the NFκB and NLRP3 inflammasome pathways.

Previous evidence showed that apigenin (APG), a plant phenolic compound, can bind to SP. However, whether or not apigetrin (APT), the glucoside conjugate of APG, can protect human cells against cell injury caused by SARS-CoV-2 is still unknown. Studies have shown that SARS-CoV-2 induced dysregulation of host cell ACE2 expression is one of the major pathophysiological factors of COVID-19 infection. Our current study demonstrated that recombinant SP significantly reduced ACE2 level in human neuronal cells in a dose dependent manner using ELISA assay. Interestingly, APT reversed the SP induced ACE2 downregulation in these cells. In this study, the effect of SP on cell proliferation and immune regulation was also investigated. Using MTT and LDH assays, I discovered that SP had a cytotoxic effect on these cells and significantly inhibited cell proliferation. This cytotoxic effect was mitigated by adding APT treatment. Furthermore, APT reduced SP induced cytokine production such as IL1α and TGFβ .

In sum, my study demonstrated that APT inhibited SARS-CoV-2 SP induced dysregulation of human cells and reduced its cytotoxic effects on cells. APT significantly upregulated ACE2 expression and inhibited the production of cytokines IL1α and TGFβ in the cells treated with SP. My study indicated that APT has potential to be a novel therapy for COVID-19 infection. More experiments to further elucidate molecular mechanisms of how APT modulates pathological effect of SP with different *in vitro* models including other human cell lines and *in vivo* animal models are currently being performed.

Keywords: Apigetrin; SARS-CoV-2; inflammation; Spike protein; ACE2

1. Introduction

SARS-CoV-2 is a novel coronavirus belonging to the *Betacoronavirus* genus, which also includes the coronaviruses responsible for severe acute respiratory syndrome (SARS) and the Middle East respiratory syndrome (MERS). SARS-CoV-2 is 96.2% identical to a bat coronavirus CoV RaTG13 and may have been transferred to humans through an unknown intermediate host [1]. It was first identified in Wuhan, China in December 2019 [2] and has caused an ongoing

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global health crisis (COVID-19) since its discovery. As of November 12th, 2020, there have been 52,331,462 confirmed cases and 1,287,966 deaths caused by SARS-CoV-2 infection globally, according to Johns Hopkins Coronavirus Resource Center.

1.1. Mechanisms of SARS-CoV-2 Entry into Human Cells

Human angiotensin-converting enzyme 2 (ACE2), which converts angiotensin II into angiotensin-(1-7), functions as the host receptor for SARS-CoV-2 [3] [4] [5]. Target cells of SARS-CoV-2 include the ones which express ACE2 such as alveolar epithelial type II cells and endothelial cells [6][7]. Various other receptors are also involved in viral infection. Transmembrane serine protease 2 (TMPRSS2), a protease found predominantly in prostate cells that influences several androgen receptor elements, and sialic acid receptors, which have structural and modulatory roles in addition to being the receptor for multiple pathogens, are suggested to play a role in the entry of SARS-CoV-2 [6] [8] [9]. Spike proteins (SP) on SARS-CoV-2 are activated by the protease TMPRSS2 and then attached to their receptors ACE2 on target cells [10]. Toll-like receptors also have an important role in viral infection as it detects pathogen-associated molecular patterns (PAMPs) and induces immune response by activation of NFκB pathway [11]. By downregulating ACE2, SARS-CoV-2 infection causes increased binding of angiotensin II to AT1 receptors, which can lead to vasoconstriction, enhanced inflammation and thrombosis, as well as increased pulmonary inflammation and coagulation [12].

1.2. Symptoms Associated with SARS-CoV-2 Infection

The most common symptoms from SARS-CoV-2 infection are nonspecific ones including headache or dizziness, as well as characteristic manifestations as fever or dry cough in lower respiratory tract infection [13] [14]. The infected patients can also develop gastrointestinal symptoms [15]. In addition to acute respiratory distress syndrome (ARDS), SARS-CoV-2 infection can also cause myocardial injury, as ACE2 dysregulation is also associated with cardiovascular disease [5] [16] [17]. In a retrospective study of 85 fatal COVID-19 cases, most patients died from multiple organ failure [18]. Patients above the age of 65 and patients with chronic conditions such as diabetes and hypertension have a greater risk of mortality [14]. Many patients with severe symptoms also express characteristics of macrophage activation syndrome or cytokine storm as well as high serum ferritin and D-dimer levels [7]. Compared to SARS and MERS, which had a fatality rate of 9.5% and 34.4% respectively, SARS-CoV-2 infection has a much lower fatality rate of 2.3% but seems to be more infectious [2].

1.3. Inflammatory Responses Caused by SARS-CoV-2 Infection

Cytokines are proteins secreted by cells which act on cells [19]. Cytokine storm involves the overproduction of proinflammatory cytokines as a part of an auto-amplifying immune response [20] [21]. In SARS-CoV and MERS infection, the release of antiviral factors interferons (IFNs) are delayed, hindering the body's ability to respond to infection. The N protein of SARS-CoV-2 regulates activity of IFNs [22]. Studies revealed that in SARS-CoV-2 infection, CD4 positive and CD8 positive T cells levels were low, while monocytes, macrophage and neutrophil levels were increased. This could explain the increased levels of proinflammatory cytokines during the infection [7] [23]. In a study of 1099 COVID-19 infected patients, 83.2% of them had lymphocytopenia, 36.2% had thrombocytopenia, and 33.7% had leukopenia. These abnormalities were more prominent in severe patients with systemic sepsis [20] [24]. Both the ratios of neutrophils to CD8 positive T cells and neutrophils to lymphocytes are able to predict the severity of SARS-CoV-2 infection [23].

SARS-CoV-2 infection can cause a rapid release of cytokines and chemokines, resulting in an excessive response from inflammatory cells [25]. The E protein on SARS-CoV-2 has ion-channel activity which contributes to the activation of the inflammasome complex. In an animal study, deletion of genes associated with the E protein on SARS-CoV-2 reduced levels of interleukin (IL)1β, IL6, and tumor necrosis factor (TNF) from inflammasome activation . Evidence supports that the severity of SARS-CoV-2 infection is related to the levels of proinflammatory cytokines [22]. IL6 concentrations were shown to be 2.9 times higher in severe cases of SARS-CoV-2 infection and highly predictive of severity of COVID-19 disease [23].

1.4. Inflammatory Response in COVID-19 Patients with Underlying Disease

Comorbid diabetes and uncontrollable hyperglycemia were predictors of severity of viral infection including SARS-CoV-2 and MERS. Hyperglycemia and insulin resistance in patients with diabetes contribute to synthesis of proinflammatory cytokines and oxidative stress, as well as other immune defects. In a retrospective study, around 10% of the patients with type 2 diabetes mellitus and COVID-19 suffered at least one episode of hypoglycemia (<3.9 mmol/L) and hypoglycemia has also been shown to increase activity of pro-inflammatory monocytes [26].

ACE2, the receptor for SARS-CoV-2, is also involved in metabolism, regulation of inflammation as well as glucose homeostasis [27]. ACE2 expression is reduced in patients with diabetes mellitus [28]. Serum ACE2 levels are elevated

in obese patients [29]. ACE2 is also a negative regulator of inflammation of epicardial adipose tissue associated with obesity [3]. Proinflammatory cytokine levels such as TNFα, IL6, IL18 are all elevated in obese people [30]. Chronic low-grade inflammation from obesity is associated with activation of NFκB signaling, which drives production of inflammatory cytokines and insulin resistance in obese people [30] [31].

Patients with chronic inflammatory diseases have been shown to have a higher risk of COVID-19 disease than the normal population. In addition, patients with systemic autoimmune or immune-mediated disease also showed a significant increase in risk, though patients with inflammatory arthritis or systemic lupus erythematosus did not [32]. In addition, patients with inflammatory bowel disease (IBD) are also not at additional risk of contracting COVID-19 disease, probably because the expression level of ACE2 and TMPRSS2 in these patients are comparable to patients without IBD [33].

People with allergic asthma and rhinitis have reduced expression of ACE2 in addition to increased expression of TMPRSS2 in nasal and airway epithelial cells, which could cause asthma to be a risk factor of contracting COVID-19. Currently, the data for asthma being a risk factor of COVID-19 is not conclusive. The studies from China have not yet identified asthma as a risk factor for severe COVID-19 [34].

1.5. SARS-CoV-2 Infection Induced Endotheliopathy and Coagulation

SARS-CoV-2 infection causes coagulopathy involved in thrombotic and microvascular complications and endotheliopathy. Fibrinolysis and pulmonary coagulation caused by SARS-CoV-2 infection is considered to be regulated by proinflammatory cytokines [35]. The heightened inflammatory responses, such as cytokine storm, vasculopathy, and NETosis may contribute to hypercoagulability [36]. Disseminated intravascular coagulation (DIC) which is particularly significant in the advanced stage of COVID-19, is also associated with sepsis [37]. In a study of 68 patients, endothelial cell and platelet activation markers were elevated considerably in ICU patients compared to non-ICU patients [38]. Patients with SARS-CoV-2 induced pneumonia also had higher platelet counts than those with non-SARS-CoV-2 pneumonia [35]. In addition, prothrombin time activity and antithrombin level were lower in SARS-CoV-2 infected patients compared to the control population, while D-dimer level was significantly higher in infected patients [39].

Antiphospholipid antibodies (aPL) are also reported in SARS-CoV-2 infected patients. aPL such as LAC, IgG and IgM 7 types of aCL and aβ2GPI are associated with antiphospholipid syndrome and hypercoagulability. In a study of 150 patients with severeSARS-CoV-2 infections, 43% showed relevant thrombotic complications. VWF levels were five times higher than the baseline [36].

1.6. Potential Treatments for SARS-CoV-2 Infection

Hydrochloroquine (HCQ) and chloroquine (CQ) have therapeutic effects against a number of RNA viruses such as Zika virus, Chikungunya-virus, SARS-CoV and MERS-CoV. They are important immunomodulatory agents used to treat inflammatory diseases. HCQ and CQ are proposed to target endosomal acidification. In addition, HCQ and CQ disrupt lysosome-endosome function and can inhibit SARS-COV-2 viral binding to ACE2 as well as regulate proinflammatory cytokines [40]. Remdesivir has also shown promising effects *in vitro* against coronaviruses, but clinical results are not conclusive [41]. Tocilizumab, a monoclonal antibody that acts against IL6, showed potential in ameliorating symptoms at early stages of cytokine storm and decreasing mortality [42].

E proteins of SARS-CoV-2 activate the NOD-, LRR-, and pyrin domain-containing 3 (NLRP3) inflammasome which causes the production of inflammatory cytokines, such as IL6 and TNF. This makes NLRP3 a possible target for COVID-19 treatments. Some studies show that activation of NLRP3 inflammasome is essential to pathogenesis of ARDS. Oridonin, Parthenolide, and Colchicine have all been shown to have NLRP3 inhibiting properties. In addition, parthenolide and Bay 11-7082, both NLRP3 and NFκB inhibitors, were shown to improve symptoms in SARS-CoV infected animals [43]. In a study on 56 flavonoids, flavone, 2',4'-dihydroxyflavone, 3',4'-dichloroflavone, apigenin (APG), kaempferol and quercetin significantly inhibited IL1β production through NLRP3 inflammasome pathway. APG regulated NLRP3 inflammasome activation through inhibition of phosphorylation of spleen tyrosine kinase/protein tyrosine kinase 2 (Syk/Pyk2) pathway [44].

Apigetrin (APT), the glucoside conjugate of APG, lowers production and mRNA expression of TNFα and IL6 in addition to suppressing NFκB in LPS stimulated BV-2 cells [44] [45]. APT also reduced levels of proinflammatory cytokines such as ILlβ, TNFα and IL6 in mice with acute otitis media, attributed to suppression of toll-like receptor 4 (TLR4)/NFκB pathway [46]. TLR4 is involved in the SARS-CoV-2 induced inflammatory response and has also been shown to have the highest protein-protein interactions with the SARS-CoV-2 spike protein [47]. mRNA levels of C/EBPα, PPARγ, SREBP1c, FAS, ROS, and proinflammatory cytokines were reduced after treating cells with APT [45]. NFκB inhibitors also reduced

NLRP3 priming in a dose dependent manner [48]. Based on previous studies, I hypothesize that APT can reduce SARS-CoV-2 SP induced cytotoxicity and dysregulation of cytokine productions, which can potentially attenuate the effects of coagulation and sepsis in patients with severe COVID-19 disease.

2. Material and methods

2.1. Cells and Cell Culture

SK-N-SH (ATCC) is a human neuroblastoma cell line and RAW 264.7 (ATCC) is a Abelson-murine-leukemia-virus-transformed macrophage cell line. Cells were placed in DMEM (Mediatech Inc.) and then incubated at 37°C in a 5% CO2 environment. Media was changed weekly under a sterilization hood.

To transfer cells, 4 mL of trypsin-EDTA (0.25%) (Fisher Scientific) was used to detach cells from the container. cells were left in trypsin-EDTA for 4 min. Pipettes and filtered aerosol resistant pipette tips (Fisher Scientific) were used. 4 mL of DMEM was then added to neutralize Trypsin-EDTA. Contents were centrifuged at 3000 rpm for 4 min. Trypsin and old media were removed and 30 mL new media added to the container with the cell pellet. The contents in the container were homogenized.

To place cells into 6-well and 96-well plates (Corning Inc.), 3 mL of DMEM media was added to cells and transferred according to previous mentioned procedure. Cell solution was divided and added to the wells on the plates.

2.2. Chemical Dilution

1 mg of apigetrin (Sigma-Aldrich Inc.) was originally dissolved in 1 mL of distilled deionized water and was further diluted in microcentrifuge tubes to different concentrations. The dilutions were labeled and kept at room temperature.

2.3. MTT Assay

For 96-well plate preparation, SK-N-SH cells were removed from their cell culture flasks, suspended and homogenized in DMEM media. 30 µl of the cell suspension solution was added to each well. After the 96-well plate was incubated for two hours to allow the newly placed cells to seed, the cells were treated with various concentrations of SP and APT. 10µL of each chemical solution was added and then cells were incubated for 24 hours. MTT stock solution was prepared by dissolving 10 mg of yellow MTT powder (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) (Fisher Scientific) with 2 mL of phosphate buffer solution (PBS). Stock solution was vortexed to allow complete solubilization. 30 µL of MTT solution was dispensed into each well. Cells were incubated at 37°C for an hour to allow formazan crystals to form. After incubation, 80 µL of DMSO (Fisher Chemical) was added to break down cellular membranes and dissolve the formazan product. To ensure solubilization of formazan, the plate was placed in an incubator for 10 min. Solution for each well was analyzed spectrophotometrically at 595 nm using a microplate reader.

2.4. Cell Survival Rate

Using colorimetric data obtained from MTT assay, cell survival rates were calculated and t-tests were conducted to determine if the effects of apigetrin on cell survival were significant. Cell survival rates were determined from the following equation:

((Array Average) / (Control Average)) x 100 = Percent survival rate

The percent decreases of cell survival rates were calculated by subtracting the percentage of survival rate for the treatment groups from the percentage of the survival rate for the control group.

2.5. LDH Assay

LDH assay kit obtained from ThermoScientific. 96- well plates containing SK-N-SH cells were prepared and treated with chemicals following the same protocol mentioned for the MTT assay preparations. 0.6 mL of LDH buffer and substrate mix powder were dissolved in 11.4 mL of distilled pure water. For total LDH, 10 µL of lysis buffer was added to two rows from the first MTT plate and cells were incubated for 1 hour. 50 µl of media was removed from each well and were placed into a new 96 well plate. 30 µl of the LDH substrate solution was then dispensed into each well containing only treatment media. Air bubbles were removed. Absorbance was read from the plate twice at 490 nm for the actual colorimetric reading, and 655 nm to assess background noise in the reading. Seeded cells were used for duplicate MTT assays with respective procedures. To measure the released LDH, 30 µL of LDH substrate was added to the solution.

Solution was then incubated for 30 minutes at room temperature and out of direct light. 30 µL of stop solution was then added. Air bubbles were removed and the solution was analyzed spectrophotometrically twice at 490 nm for the actual colorimetric reading, and 655 nm to assess background noise in the reading.

2.6. ELISA Assay

ELISA assay kit was acquired from Bolster Biological Technology. Reagents were brought to room temperature prior to the experiment. To prepare cell samples, media was removed from a 6-well plate. 1000 µL of PBS was added to each well and the plate was placed in the freezer for 20 minutes. Samples were then thawed for 10 minutes at room temperature and freeze/thaw cycle was repeated for two additional times to ensure accurate measurement of cytokines. 100 µL of standard, control, or sample were added to each well and 100 µL of sample diluent buffer was added to zero wells. There were two replicates for each standard, control and sample. The plate was sealed and incubated for 90 minutes at 37°C. Plate sealer was taken off and liquid was removed from the plate and remaining liquid was removed by inverting the plate on a paper towel. 100 µL of 1x Biotinylated Anti-Human IL1α was added to each well. Cover was placed back and the plate was incubated for 90 minutes at 37°C. Plate was washed 3 times with the 1 x wash buffer. Liquid was discarded using the same procedure as previously mentioned and 300 µL of the 1x wash buffer was added to each assay well. This step was repeated twice. 100 µL of 1x Avidin-Peroxidase Complex was added to each well and the plate was covered with plate sealer and incubated for 90 minutes at 37°C. The plate was washed 5 times with the 1x wash buffer. Liquid was discarded using the same procedure as previously mentioned and 300 µL of the 1x wash buffer was added to each assay well. This step was repeated 4 times. 90 µL of the color developing reagent was added to each well. The plate was covered with plate sealer and incubated at 37°C for 25 minutes. 100 µL of stop solution at 37°C was added to each well. O.D. absorbance was read with a microplate reader at 450 nm 30 minutes after the stop solution was added. Standard curve was made to determine the concentration of samples from absorbance. A similar procedure was used to perform an ELISA assay to test measure TGFβ production.

3. Results

3.1. Protective Effect of APT on Cell Survival and Proliferation of the Cells Treated with Recombinant SP

Recombinant SP inhibited neuronal cell proliferation in a dose dependent manner. In SK-N-SH cells from a neuroblastoma cell line, treatment with SP at concentrations of 1, 10 and 100 µM decreased cell survival by 12, 26 and 43% respectively **(Fig. 1A)**. APT treatment alone at both 1 µM and 10 µM slightly reduced cell survival by ~ 3%. In comparison, the addition of APT at 1 µM and 10 µM to the cells treated with SP at 10 µM significantly lowered the decreased cell survival from 26% to 6 and 4%, in a dose dependent manner **(Fig. 1B)**.

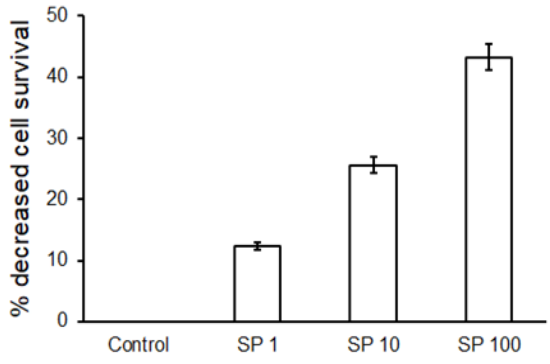

Figure 1A

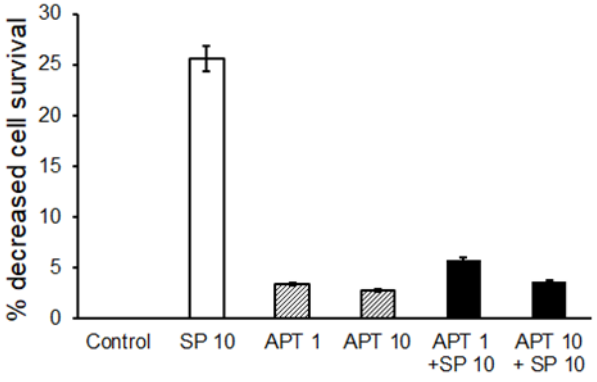

Figure 1B

Figure 1 Comparison of percentages of decreased cell survival in the cells treated with SP and APT. A. Decreased cell survival in SK-N-SH cells caused by SP treatment at different concentrations (1 µM, 10 µM and 10 µM) for 24 hours was determined by MTT assay. The percentages of cytotoxicity in the treatment groups were compared to the control group without treatment. B. The similar experiment was performed in the cells with SP treatment at 10 µM, APT at 1 µM and 10 µM, SP at 10 µM combined with APT at 10 µM. The results were statistically significant with $p < 0.05$. Each experiment was repeated independently for 3 times with similar results.

SP treatment also caused dose dependent cytotoxicity in SK-N-SH cells. Treatment with SP at concentrations of 1, 10 and 100 μM increased cytotoxicity by 9, 16 and 22% respectively (Fig. 2A). APT treatment alone at both 1 μM and 10 μM increased cytotoxicity 6 and 7% respectively. In comparison, the addition of APT at 1 μM and 10 μM to the cells treated with SP at 10 μM lowered the increased cytotoxicity from 18% to 6 and 9% respectively (Fig. 2B).

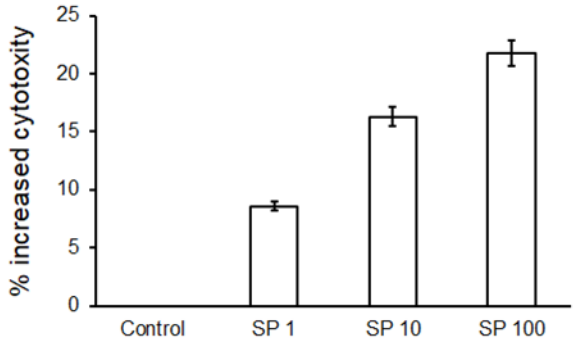

Figure 2A

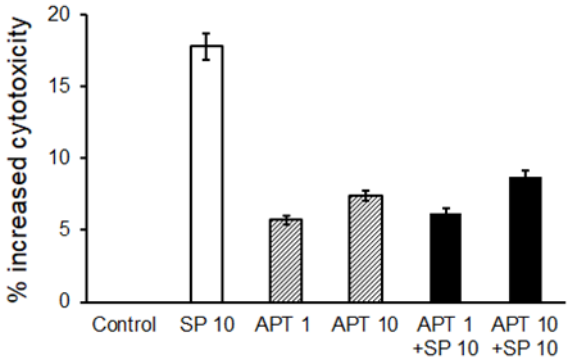

Figure 2B

Figure 2 Comparison of percentages of increased cytotoxicity in the cells treated with SP and APT. A. Cytotoxicity in SK-N-SH cells caused by SP treatment at different concentrations (1, 10 and 10 μM) for 24 hours was determined by LDH assay. The percentages of cytotoxicity in the treatment groups were compared to the control group without treatment. B. The similar experiment was performed in the cells with SP treatment at 10 μM, APT at 1 μM and 10 μM, SP at 10 μM combined with APT at 10 μM. The results were statistically significant with $p < 0.05$. Each experiment was repeated independently for 3 times with similar results.

3.2. Effects of APT on Production of ACE2, IL1α and TGFβ in the Cells Treated with Recombinant SP

SP treatment significantly downregulated ACE2 expression in SK-N-SH cells in a dose dependent manner. SP treatment at concentrations of 1 and 10 μM significantly lowered the ACE2 expression levels by 37 and 72% respectively. On the contrary, the cells treated with APT at 1 μM and 10 μM significantly increased ACE2 expression level by 16% and 43% respectively in a dose dependent manner. The decreased ACE2 expression in the cells treated with SP at 1 μM and 10 μM was attenuated by APT treatment at 1 μM and 10 μM to 12 and 19% respectively **(Fig. 3)**.

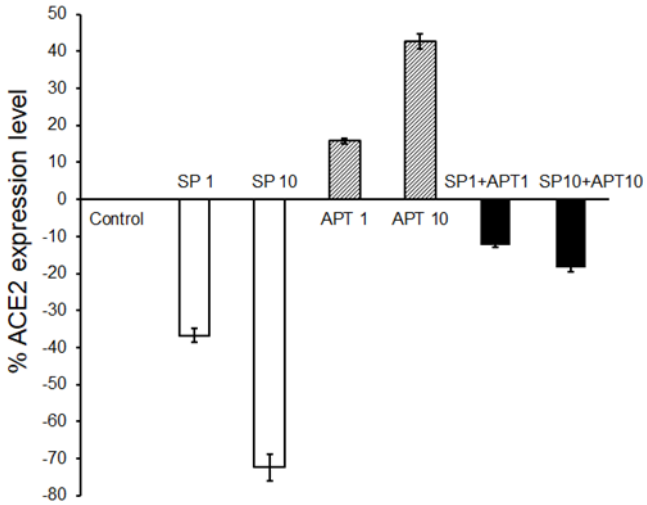

Figure 3 Comparison of percentages of ACE2 expression level in the cells treated with SP and APT. SK-N-SH cells were treated with SP and APT separately at different concentrations at 1 μM and 10 μM for 24 hours. In a separate experiment, the cells were treated with APT combined with SP at different concentrations (SP at 1 μM combined with APT at 1 μM, SP at 10 μM combined with APT at 10 μM). The ACE2 levels in both experiments were measured by ELISA. The percentages of ACE2 expression level in the treatment groups were compared to the control group without

treatment. The results were statistically significant with p <0.05. Each experiment was repeated independently for 3 times with similar results.

SP treatment significantly increased proinflammatory cytokine IL1α production in RAW264.7 cells in a dose dependent manner. SP treatment at concentrations of 1 and 10 μM significantly increased IL1α production by 18 and 38% respectively. APT treatment alone at 1 and 10 μM resulted in a slightly increased IL1α production of ~2%. The increased IL1α production in the cells treated with SP at 1 μM and 10 μM was significantly reduced to 9 and 12% by APT treatment at 1 μM and 10 μM respectively **(Fig. 4)**.

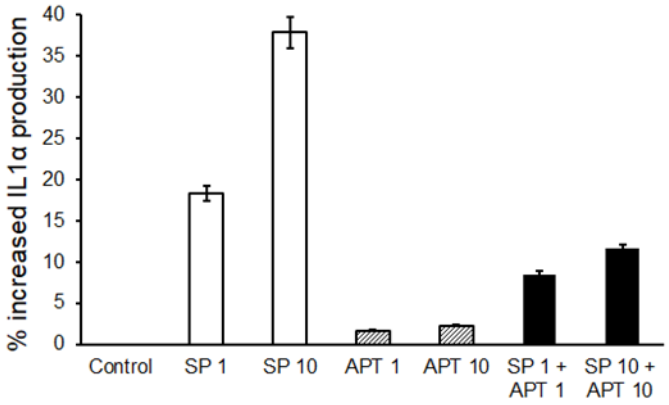

Figure 4 Comparison of percentages of IL1α production in the cells treated with SP and APT. RAW264.7 cells were treated with different concentrations of SP and APT respectively, as well as with APT combined with SP at different concentrations (SP at 1 μM combined with APT at 1 μM, SP at 10 μM combined with APT at 10 μM). IL1α levels were measured by ELISA. The percentages of IL1α level in the treatment groups were compared to the control group without treatment. The results were statistically significant with p <0.05. Each experiment was repeated independently for 3 times with similar results.

SP treatment also significantly increased production of cytokine TGFβ in RAW264.7 cells in a dose dependent manner. SP treatment at concentrations of 1 and 10 μM significantly increased TGFβ production by 25 and 42% respectively. APT treatment alone at 1 and 10 μM resulted in a slightly increased TGFβ production of 3 and 5% respectively. The increased TGFβ production in the cells treated with SP at 1 μM and 10 μM was significantly reduced to 7 and 10% by adding APT treatment at 1 μM and 10 μM respectively **(Fig. 5)**.

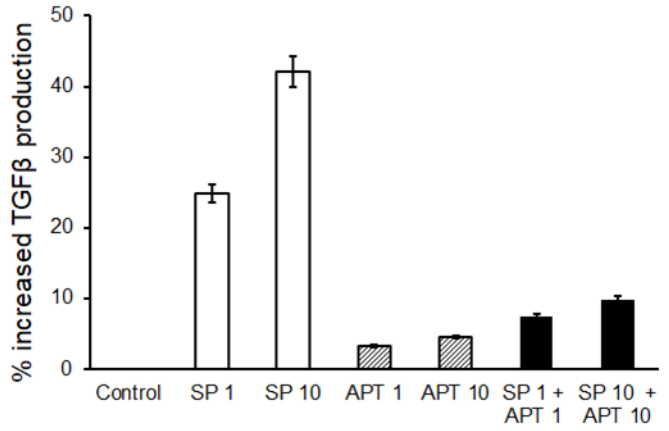

Figure 5 Comparison of percentages of TGFβ production in the cells treated with SP and APT. RAW264.7 cells were treated with different concentrations of SP and APT respectively, as well as with APT combined with SP at different concentrations (SP at 1 μM combined with APT at 1 μM, SP at 10 μM combined with APT at 10 μM). TGFβ levels were measured by ELISA. The percentages of TGFβ level in the treatment groups were compared to the control group without treatment. The results were statistically significant with p <0.05. Each experiment was repeated independently for 3 times with similar results.

4. Discussion

SP treatment at different concentrations of 1, 10, 100 μM were used to determine dose dependent effect. An obvious dose dependent cytotoxic effect was observed on cell survival and cytotoxicity of the cells treated with SP. Therefore, SP treatment at 10 μM was chosen in later experiments as this concentration was shown to be effective as well as affordable. Both MTT assays and LDH assays were used to investigate the protective effect of APT treatment on SP induced cytotoxic effects and the results obtained from the two methods were consistent. The current study was performed *in vitro* using 2 different cell lines, SK-N-SH cells originated from human neuroblastoma cells and RAW264.7 cells originated from mice macrophages. Given the fact that protective effects of APT treatment against SP were observed in these two phylogenetically distant cell lines, it would be reasonable to suspect that a biologically fundamental process might be involved in the underlying molecular mechanism and therefore this protection exists across different species and cell types.

In this study, APT treatment was shown to reduce SP induced cytotoxic effects on cells. The underlying mechanism is still unclear. Notably, APT treatment alone resulted in mild cytotoxicity to the cells as well as mildly elevated IL1α and TGFβ production in the cells treated. These effects may result from the regulatory effects of APT on leukocytes [46]. Also, APG has been shown to have anti-cancer effects [49], which may also be applicable to APT. A study to further elucidate the underlying molecular mechanism of APT is warranted to better understand possible adverse reactions associated with APT treatment.

SP mediates the entry of SARS-CoV-2 into human cells. SARS-CoV-2 infection also results in downregulation of ACE2 which can cause increased inflammation as a result of increased binding of Angiotensin II to AT1 receptors [12]. In this study, APT treatment alone was shown to upregulate ACE2 expression levels. Downregulation of ACE2 expression induced by SP treatment was significantly ameliorated by combined APT treatment. The mechanism of how APT treatment can upregulate ACE2 in these conditions is still unclear. Another study has shown that APG has a high binding affinity to SP [50]. APG was also shown to upregulate ACE2 expression in the kidney of hypertensive mice [51]. Kidney cells express significant amounts of ACE2 and acute kidney injury has been reported in patients with severe SARS-CoV-2 infections [12] [52]. It is promising to use APT treatment to relieve COVID-19 symptoms associated with dysregulation of ACE2 expression including acute kidney injury.

Severe SARS-CoV-2 infection is also characterized by increased TNFα as well as IL1α [53] [54]. TNFα was shown to induce production of proinflammatory cytokines such as IL1α, which then initiates a self-sustaining loop of inflammation [55] [56]. The effects of APT treatment to reduce IL1α production can be used to oppose the effects of upregulation of TNFα in SARS-CoV-2 infection as well as prevent further production of proinflammatory cytokines which can lead to the life threatening cytokine storm.

This study also revealed that APT treatment was able to suppress the increase of SP induced TGFβ production. TGFβ is an important cytokine associated with pulmonary fibrosis, a central component of SARS pathology [57] [58]. Studies have also found that SARS-CoV-2 infection resulted in increased TGFβ production [58]. APG has been shown to reduce TGFβ induced activation of NFκb and inhibit TGFβ induced fibroblast activity [59]. Therefore, APT treatment might have potential to not only treat the acute inflammatory response caused by SARS-CoV-2 infection, but also prevent chronic complications such as pulmonary fibrosis associated with it.

5. Conclusion

The current study revealed that APT treatment can attenuate cytotoxicity induced by SARS-CoV-2 infection. It also ameliorates certain dysregulatory effects of SP on proinflammatory cytokines. APT treatment has a potential to prevent cell injury and cytokine storm associated with SARS-CoV-2 infection. More experiments to further elucidate underlying molecular mechanisms of how APT modulates pathological effect of SP with different *in vitro* models including other human cell lines and *in vivo* animal models are currently being performed.

References

[1] Guo YR, Cao QD, Hong ZS, Tan YY, Chen SD, Jin HJ, Tan KS, Wang DY, Yan Y. The origin, transmission and clinical therapies on coronavirus disease 2019 (COVID-19) outbreak - an update on the status. Military Medical Research. 2020; 7(1): 11.

[2] Petrosillo N, Viceconte G, Ergonul O, Ippolito G, Petersen E. COVID-19, SARS and MERS: are they closely related?. Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2020; 26(6): 729–734.

[3] Patel VB, Basu R, Oudit GY. ACE2/Ang 1-7 axis: A critical regulator of epicardial adipose tissue inflammation and cardiac dysfunction in obesity. Adipocyte. 2016; 5(3): 306–311.

[4] Bourgonje AR, Abdulle AE, Timens W, Hillebrands JL, Navis GJ, Gordijn SJ, Bolling MC, Dijkstra G, Voors AA, Osterhaus AD, van der Voort PH, Mulder DJ, van Goor H. (2020). Angiotensin-converting enzyme 2 (ACE2), SARS-CoV-2 and the pathophysiology of coronavirus disease 2019 (COVID-19). The Journal of pathology. 2020; 251(3): 228–248.

[5] Li X, Ma X. Acute respiratory failure in COVID-19: is it "typical" ARDS?. Critical care (London, England). 2020; 24(1): 198.

[6] Sardu C, Gambardella J, Morelli MB, Wang X, Marfella R, Santulli G. Hypertension, Thrombosis, Kidney Failure, and Diabetes: Is COVID-19 an Endothelial Disease? A Comprehensive Evaluation of Clinical and Basic Evidence. Journal of clinical medicine. 2020; 9(5): 1417.

[7] Soy M, Keser G, Atagündüz P, Tabak F, Atagündüz I, Kayhan S. (2020). Cytokine storm in COVID-19: pathogenesis and overview of anti-inflammatory agents used in treatment. Clinical rheumatology. 2020; 39(7): 2085–2094.

[8] Shen LW, Mao HJ, Wu YL, Tanaka Y, Zhang W. TMPRSS2: A potential target for treatment of influenza virus and coronavirus infections. Biochimie. 2017; 142: 1–10.

[9] Varki A. Sialic acids in human health and disease. Trends in molecular medicine. 2008; 14(8): 351–360.

[10] Mousavizadeh, L., & Ghasemi, S. (2020). Genotype and phenotype of COVID-19: Their roles in pathogenesis. *Journal of microbiology, immunology, and infection = Wei mian yu gan ran za zhi*, S1684-1182(20)30082-7. Advance online publication.

[11] Kawai T, Akira S. Signaling to NF-kappaB by Toll-like receptors. Trends in molecular medicine. 2007; 13(11): 460–469.

[12] Verdecchia P, Cavallini C, Spanevello A, Angeli F. The pivotal link between ACE2 deficiency and SARS-CoV-2 infection. European journal of internal medicine. 2020; 76: 14–20.

[13] Shi H, Han X, Jiang N, Cao Y, Alwalid O, Gu J, Fan Y, Zheng C. Radiological findings from 81 patients with COVID-19 pneumonia in Wuhan, China: a descriptive study. The Lancet. Infectious diseases. 2020; 20(4): 425–434.

[14] Yuki K, Fujiogi M, Koutsogiannaki S. COVID-19 pathophysiology: A review. Clinical immunology (Orlando, Fla.). 2020; 215: 108427.

[15] Rothan HA, Byrareddy SN. The epidemiology and pathogenesis of coronavirus disease (COVID-19) outbreak. Journal of autoimmunity. 2020; 109: 102433.

[16] Babapoor-Farrokhran S, Gill D, Walker J, Rasekhi RT, Bozorgnia B, Amanullah A. (2020). Myocardial injury and COVID-19: Possible mechanisms. *Life sciences*. 2020; 253: 117723.

[17] Jin Y, Yang H, Ji W, Wu W, Chen S, Zhang W, Duan G. Virology, Epidemiology, Pathogenesis, and Control of COVID-19. Viruses. 2020; 12(4): 372.

[18] Du Y, Tu L, Zhu P, Mu M, Wang R, Yang P, Wang X, Hu C, Ping R, Hu P, Li T, Cao F, Chang C, Hu Q, Jin Y, Xu G. (2020). Clinical Features of 85 Fatal Cases of COVID-19 from Wuhan. A Retrospective Observational Study. American journal of respiratory and critical care medicine. 2020; 201(11): 1372–1379.

[19] Zhang JM, An J. Cytokines, inflammation, and pain. International anesthesiology clinics. 2007; 45(2): 27–37.

[20] Tisoncik JR, Korth MJ, Simmons CP, Farrar J, Martin TR, Katze MG. Into the eye of the cytokine storm. Microbiology and molecular biology reviews: MMBR. 2012; 76(1): 16–32.

[21] Chousterman, B. G., Swirski, F. K., & Weber, G. F. (2017). Cytokine storm and sepsis disease pathogenesis. *Seminars in immunopathology*, *39*(5), 517–528.

[22] Tufan A, Avanoğlu Güler A, Matucci-Cerinic M. COVID-19, immune system response, hyperinflammation and repurposing antirheumatic drugs. Turkish journal of medical sciences. 2020; 50(SI-1): 620–632.

[23] Kuppalli K, Rasmussen AL. (2020). A glimpse into the eye of the COVID-19 cytokine storm. EBioMedicine. 2020; 55: 102789.

[24] Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui D, Du B, Li LJ, Zeng G, Yuen KY, Chen RC, Tang CL, Wang T, Chen PY, Xiang J, Li SY. China Medical Treatment Expert Group for Covid-19 (2020). Clinical Characteristics of Coronavirus Disease 2019 in China. The New England journal of medicine. 2020; 382(18): 1708–1720.

[25] Ye Q, Wang B, Mao J. The pathogenesis and treatment of the `Cytokine Storm' in COVID-19. The Journal of infection. 2020; 80(6): 607–613.

[26] Hussain A, Bhowmik B, do Vale Moreira NC. COVID-19 and diabetes: Knowledge in progress. Diabetes research and clinical practice. 2020; 162: 108142.

[27] Drucker DJ. Coronavirus Infections and Type 2 Diabetes-Shared Pathways with Therapeutic Implications. Endocrine reviews. 2020; 41(3): bnaa011.

[28] Pal R, Bhadada SK. COVID-19 and diabetes mellitus: An unholy interaction of two pandemics. Diabetes & metabolic syndrome. 2020; *14*(4): 513–517.

[29] Emilsson V, Gudmundsson EF, Aspelund T, Jonsson BG, Gudjonsson A, Launer LJ, Lamb JR, Gudmundsdottir V, Jennings LL, Gudnason V. (2020). ACE2 levels are altered in comorbidities linked to severe outcome in COVID-19. medRxiv : the preprint server for health sciences. 2020; 06.04.20122044.

[30] Shah A, Mehta N, Reilly MP. (2008). Adipose inflammation, insulin resistance, and cardiovascular disease. JPEN. Journal of parenteral and enteral nutrition. 2008; 32(6): 638–644.

[31] Zahid H, Simpson ER, Brown KA. Inflammation, dysregulated metabolism and aromatase in obesity and breast cancer. Current opinion in pharmacology. 2016; 31: 90–96.

[32] Pablos JL, Abasolo L, Alvaro-Gracia JM, Blanco FJ, Blanco R, Castrejón I, Fernandez-Fernandez D, Fernandez-Gutierrez B, Galindo-Izquierdo M, Gonzalez-Gay MA, Manrique-Arija S, Mena Vázquez N, Mera Varela A, Retuerto M, Seijas-Lopez A, RIER investigators group. Prevalence of hospital PCR-confirmed COVID-19 cases in patients with chronic inflammatory and autoimmune rheumatic diseases. Annals of the rheumatic diseases. 2020; *79*(9): 1170–1173.

[33] Burgueño JF, Reich A, Hazime H, Quintero MA, Fernandez I, Fritsch J, Santander AM, Brito N, Damas OM, Deshpande A, Kerman DH, Zhang L, Gao Z, Ban Y, Wang L, Pignac-Kobinger J, Abreu MT. Expression of SARS-CoV-2 Entry Molecules ACE2 and TMPRSS2 in the Gut of Patients With IBD. Inflammatory bowel diseases. 2020; 26(6): 797–808.

[34] Kimura H, Francisco D, Conway M, Martinez FD, Vercelli D, Polverino F, Billheimer D, Kraft M. Type 2 inflammation modulates ACE2 and TMPRSS2 in airway epithelial cells. The Journal of allergy and clinical immunology. 2020; 146(1): 80–88.e8.

[35] Yin S, Huang M, Li D, Tang N. Difference of coagulation features between severe pneumonia induced by SARS-CoV2 and non-SARS-CoV2. Journal of thrombosis and thrombolysis, 1–4. Advance online publication. 2020.

[36] Fei Y, Tang N, Liu H, Cao W. Coagulation Dysfunction. Archives of pathology & laboratory medicine. 2020; 144(10): 1223–1229.

[37] Guevara-Noriega KA, Lucar-Lopez GA, Nuñez G, Rivera-Aguasvivas L, Chauhan I. Coagulation Panel in Patients with SARS-CoV2 Infection (COVID-19). Annals of clinical and laboratory science. 2020; 50(3): 295–298.

[38] Goshua G, Pine AB, Meizlish ML, Chang CH, Zhang H, Bahel P, Baluha A, Bar N, Bona RD, Burns AJ, Dela Cruz CS, Dumont A, Halene S, Hwa J, Koff J, Menninger H, Neparidze N, Price C, Siner JM, Tormey C, Lee AI. Endotheliopathy in COVID-19-associated coagulopathy: evidence from a single-centre, cross-sectional study. The Lancet. Haematology. 2020; *7*(8): e575–e582.

[39] Han H, Yang L, Liu R, Liu F, Wu KL, Li J, Liu XH, Zhu CL. (2020). Prominent changes in blood coagulation of patients with SARS-CoV-2 infection. Clinical chemistry and laboratory medicine. 2020; 58(7): 1116–1120.

[40] Esposito S, Noviello S, Pagliano P. Update on treatment of COVID-19: ongoing studies between promising and disappointing results. Le infezioni in medicina. 2020; 28(2): 198–211.

[41] Wang Y, Zhang D, Du G, Du R, Zhao J, Jin Y, Fu S, Gao L, Cheng Z, Lu Q, Hu Y, Luo G, Wang K, Lu Y, Li H, Wang S, Ruan S, Yang C, Mei C, Wang Y, Wang C. (2020). Remdesivir in adults with severe COVID-19: a randomised, double-blind, placebo-controlled, multicentre trial. Lancet (London, England). 2020; 395(10236), 1569–1578.

[42] Khiali S, Khani E, Entezari-Maleki T. (2020). A Comprehensive Review on Tocilizumab in COVID-19 Acute Respiratory Distress Syndrome. Journal of clinical pharmacology, 10.1002/jcph.1693. Advance online publication.

[43] Shah A. Novel Coronavirus-Induced NLRP3 Inflammasome Activation: A Potential Drug Target in the Treatment of COVID-19. Frontiers in immunology. 2020; 11: 1021.

[44] Lim HS, Kim OS, Kim BY, Jeong SJ. Apigetrin from Scutellaria baicalensis Georgi Inhibits Neuroinflammation in BV-2 Microglia and Exerts Neuroprotective Effect in HT22 Hippocampal Cells. Journal of medicinal food. 2016; 19(11): 1032–1040.

[45] Hadrich F, Sayadi S. (2018). Apigetrin inhibits adipogenesis in 3T3-L1 cells by downregulating PPARγ and CEBP-α. Lipids in health and disease. 2018; 17(1): 95.

[46] Guo H, Li M, Xu LJ. Apigetrin treatment attenuates LPS-induced acute otitis media though suppressing inflammation and oxidative stress. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie. 2019; 109: 1978–1987.

[47] Choudhury A, Mukherjee S. In silico studies on the comparative characterization of the interactions of SARS-CoV-2 spike glycoprotein with ACE-2 receptor homologs and human TLRs. Journal of medical virology, 10.1002/jmv.25987. Advance online publication. 2020.

[48] Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, Fernandes-Alnemri T, Wu J, Monks B. G, Fitzgerald KA, Hornung V, Latz E. Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. Journal of immunology (Baltimore, Md: 1950); 2009; *183*(2): 787–791.

[49] Xu L, Zhang Y, Tian K, Chen X, Zhang R, Mu X, Wu Y, Wang D, Wang S, Liu F, Wang T, Zhang J, Liu S, Zhang Y, Tu C, Liu H. Apigenin suppresses PD-L1 expression in melanoma and host dendritic cells to elicit synergistic therapeutic effects. Journal of experimental & clinical cancer research: 2018; *CR* 37(1): 261.

[50] Rane Jitendra Subhash, Chatterjee Aroni, Kumar Abhijeet, Ray Shashikant. Targeting SARS-CoV-2 Spike Protein of COVID-19 with Naturally Occurring Phytochemicals: An in Silco Study for Drug Development. ChemRxiv. Preprint. 2020.

[51] Sui H, Yu Q, Zhi Y, Geng G, Liu H, Xu H. [Effects of apigenin on the expression of angiotensin-converting enzyme 2 in kidney in spontaneously hypertensive rats]. Wei Sheng Yan Jiu. 2010 Nov; 39(6): 693-6, 700.

[52] Gabarre P, Dumas G, Dupont T, Darmon M, Azoulay E, Zafrani L. Acute kidney injury in critically ill patients with COVID-19. Intensive care medicine. 2020; 46(7): 1339–1348.

[53] Hadjadj J, Yatim N, Barnabei L, Corneau A, Boussier J, Smith N, Péré H, Charbit B, Bondet V, Chenevier-Gobeaux C, Breillat P, Carlier N, Gauzit R, Morbieu C, Pène F, Marin N, Roche N, Szwebel TA, Merkling SH, Treluyer JM, Terrier B. Impaired type I interferon activity and inflammatory responses in severe COVID-19 patients. *Science (New York, N.Y.)*. 2020; *369*(6504): 718–724.

[54] Shaath H, Vishnubalaji R, Elkord E, Alajez NM. Single-Cell Transcriptome Analysis Highlights a Role for Neutrophils and Inflammatory Macrophages in the Pathogenesis of Severe COVID-19. Cells. 2020 Oct 29; 9(11): E2374.

[55] Bauer D, Mazzio E, Soliman K. Whole Transcriptomic Analysis of Apigenin on TNFα Immuno-activated MDA-MB-231 Breast Cancer Cells. *Cancer genomics & proteomics*. 2019; *16*(6), 421–431.

[56] Di Paolo NC, Shayakhmetov DM. Interleukin 1α and the inflammatory process. Nature immunology. 2016; 17(8): 906–913.

[57] Saito A, Horie M, Nagase T. TGF-β Signaling in Lung Health and Disease. International journal of molecular sciences. 2018; 19(8): 2460.

[58] Ojo AS, Balogun SA, Williams OT, Ojo OS. Pulmonary Fibrosis in COVID-19 Survivors: Predictive Factors and Risk Reduction Strategies. Pulmonary medicine. 2020; 6175964.

Yang H-W, Kim H-J, Park J-H, Shin J-M, Lee H-M. Apigenin alleviates TGF-β1-induced nasal mucosa remodeling by inhibiting MAPK / NF-kB signaling pathways in chronic rhinosinusitis. PLoS ONE. 2018; 13(8): e0201595.