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Dose-dependent Effect of Berberine on SARS-CoV-2 Spike Protein Induced Inflammatory Host Cell Response

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Abstract

The novel Coronavirus disease 2019 (COVID-19) is the cause behind the current global pandemic, capable of inducing pneumonia-like symptoms and lung failure. A key pathway involved in the pathogenesis of COVID-19 is NF-kB, responsible for controlling the expression of pro-inflammatory cytokines such as TNF-alpha and IL-6. Berberine is a compound commonly found in plants belonging to the Berberis family which has previously been researched as an anti-inflammatory drug. The use of berberine has been shown to suppress both TNF-alpha expression and NF-kB activity, suggesting its potential as a treatment for the inflammatory symptoms of COVID-19. This experiment focuses on the dose-dependent effect of berberine on cells treated with COVID-19 spike proteins, which has previously been shown to upregulate the expression of inflammatory cytokines. Cell viability of SK-N-SH (neuroblastoma) and CCD 841 CoN (colon) cells were monitored by MTT and LDH assays after 24 hour treatment with the spike protein. TNF-alpha and IL-6 cytokine concentrations in CCD 841 CoN were quantified using ELISA assays. The presence of spike proteins appeared to significantly diminish the viability of both cell lines, an effect which berberine managed to reverse dose-dependently. In addition, the ELISA detected a near threefold increase in both inflammatory cytokines after treatment with spike proteins in the CCD 841 CoN cells. Berberine managed to ameliorate this, lowering the concentrations down to near-untreated levels. These results display the damaging effects of the COVID-19 spike protein alone on cells, demonstrated by the decrease in viability and increased inflammatory cytokine levels. Furthermore, they support the anti-inflammatory effect of berberine in regards to these cytokines as berberine was able to increase viability and reduce cytokine production. Coupled with the viability assays, these results suggest that berberine may be either an effective treatment for COVID-19 inflammation or a possible component of a treatment.

1.0 Introduction

1.1 COVID-19

The emergent viral disease COVID-19 is the cause of the global pandemic beginning in 2019. The virus induces pneumonia-like symptoms, including respiratory failure and lung inflammation. To date, COVID-19 has infected over 14 million and caused over 600 thousand

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deaths globally (WHO, 2020). The numbers have continued to rise, owing to the highly infectious nature of this virus, devastating both global health and economy. The virus can be transmitted via human to human contact. COVID-19 features asymptomatic cases and contagiousness before visible symptoms, making early detection and transmission prevention difficult. Symptoms include pneumonia, lymphopenia, nausea, fever, etc (Zheng, 2020). To date, no effective vaccines or drugs have been discovered. It has become an international health and safety concern, requiring adequate and immediate research attention (Sun et al., 2020).

The novel COVID-19 is remarkably similar to SARS-CoV, based on studies done on the genomes, cell entry mechanisms, and human cell receptor usage. Structural proteins of COVID-19 are genetically similar to those found in SARS-CoV (Ahmed et al., 2020). Spike proteins, in particular, have a 76%-78% sequence similarity for the overall protein and 73%-76% similarity for the receptor binding domain RBD. Both viruses belong to the beta-genus category of the Coronavirus family and have high RBD affinity for the same receptor, ACE2 (Wan et al., 2020). COVID-19 and SARS-CoV rely on the same enzyme (TMPRSS2) for cell entry

Understanding the infection mechanism of SARS-CoV-2 is integral to devising a proper treatment for this virus. COVID-19 composed of four main structural proteins, identified as spike (S) proteins, membrane (M) glycoproteins, small envelope (E) glycoproteins, and nucleocapsid (N) proteins. S proteins are what mediate the entry of SARS-CoV-2 (Astuti and Ysrafil, 2020).

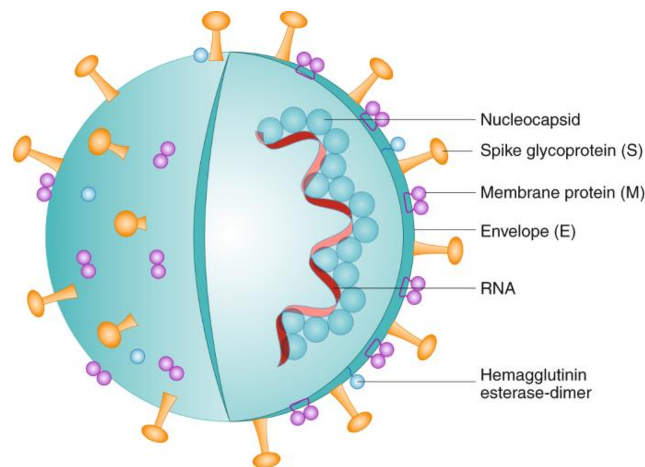

Figure 1. Diagram of COVID-19's structure. Portrays multiple proteins and RNA. Obtained from Nature (Florindo et al., 2020).

The S protein appears as a trimer on the COVID-19 envelope, composed of 3 S1 heads and an S2 stalk. For entry to occur, the S protein must be activated proteolytically at the boundary between S1 and S2 so that the S1 portion dissociates from the S2, which undergoes a conformational change. This alteration allows for a fusion of membranes between the virus and the cell (Shang et al., 2020). COVID-19 specifically uses ACE2 (angiotensin-converting enzyme 2) as an entry receptor, due to the affinity of its receptor-binding domain (RBD) for ACE2. The high affinity of

COVID-19's RBD for ACE2 suggests that cells with a higher quantity of ACE2, such as lung alveolar cells and small intestinal cells, will be the main potential targets of this virus (Zheng, 2020; Shang et al., 2020). COVID-19's S protein, after binding to ACE2, will be activated by the cell surface protease TMPRSS2, inducing the conformational changes that allow it inside the cell. COVID-19 then releases the genetic material mRNA, which is then translated into proteins by the ribosome. The new virus is then assembled within the cell before release via exocytosis (Astuti and Ysrafil, 2020).

1.2 Immune Response and Symptoms

Inflammation plays a significant part in the cell's immune response to COVID-19 (Chu et al., 2020). Inflammation is characterized as the body's response to damage, responsible for clearing pathogens and repairing damaged cells. This defense mechanism, however, is also the cause behind various human diseases when uncontrolled. The process of inflammation includes the triggering of inflammatory pathways within the immune cells via chemokines released by the damaged cells. These pathways mediate the production of pro-inflammatory cytokines. Uncontrolled inflammation causes overexpression of these inflammatory cytokines, leading to a phenomenon known as a "cytokine storm" in which (Chen et al., 2017)

A prominent pathway involved in this inflammation cycle is the Nuclear factor kappa B pathway, commonly referred to as NF-kB, which is responsible for transcriptional regulation of the production of proinflammatory cytokines such as IL-6 and TNF-alpha in immune cells. NF-kB is known to be canonically (naturally) activated by multiple cytokine receptors, including TNF receptors and IL-1 beta receptors (Liu et al., 2017). Both moderate and severe cases of COVID-19 resulted in the upregulation of pro-inflammatory cytokines such as IL-6, TNF-alpha, etc (Song et al., 2020). In COVID-19 patients, it was noted that the severity of hypercytokinemia (cytokine storm) was reflective of the disease severity. Plasma concentrations of cytokines such as TNF-alpha, IL-2, IL-7 etc. were much higher in ICU patients as opposed to non-ICU patients (Catanzaro et al., 2020). From previous data, the overexpression of NF-kB was also reported to play a role in the inflammatory diseases induced by SARS-CoV. NF-kB inhibition in SARS-CoV infected mice appeared to reduce lung pathology and inflammation, displaying the integral role of the pathway (DeDiego et al., 2014). Judging by the sequence and structure similarities between SARS-CoV and COVID-19, NF-kB exacerbation may also be the cause of inflammatory and autoimmune diseases observed in COVID-19.

Another symptom associated with the induction of COVID-19 is lymphopenia, with a negative correlation between disease severity and T-lymphocyte count. It's been suggested that the severity of lymphopenia in a patient corresponds to the severity of COVID-19. Patients with severe COVID-19 and in the ICU had a lower lymphocyte count than their non-severe counterparts. (Huang and Pranata, 2020). Despite the significant decrease in T-lymphocyte count, severe cases of COVID-19 displayed over-activation of T-cells along with increased expression of T-cell inhibitory particles, indicating T-cell exhaustion (Song et al., 2020). It may be possible that the decrease in T-cells contributes to the exhaustion. Previous research indicates that the cause of this lymphopenia may be the influx of pro-inflammatory cytokines. TNF-alpha,

specifically, promoted activated cell death in human T-cells via upregulation of death receptor pathways such as FasL and TRAIL (Zhang et al., 2017). It was noted that age played a factor as well, as elders tended to have an increased concentration of TNF-alpha already present in their blood plasma. The apoptosis of CD8+ and CD4+ cells were shown to positively correlate with the influx of TNF-alpha (Gupta 2002). As mentioned previously, the pathway NF-kB mediates the transcription and production of pro-inflammatory cytokines such as TNF-alpha. Deducing from this information, the upregulation of pathway NF-kB could be a factor in the lymphopenia in COVID-19 patients. Consequently, the downregulation or inhibition of NF-kB may be an effective treatment for COVID-19.

1.3 Berberine's Effect on Inflammation

Berberine is an isoquinoline alkaloid, derived from plants belonging to the *Berberis* family. Previous experiments showed the potential of berberine in treating intestinal infections, managing metabolic diseases, and having therapeutic effects on cancer via various pathways and phosphorylation (Singh and Mahajan, 2013). A prominent characteristic of berberine is the anti-inflammatory effect it induces in immune cells via downregulation of pro-inflammatory cytokine production. In a study done with LPS (lipopolysaccharide) treated porcine epithelial cells, LPS was noted to upregulate the expression of pro-inflammatory cytokines. Within berberine treated cells, the expression was shown to be suppressed. The expression of multiple proteins involved in the NF-kB pathway was also shown to be downregulated, which suggests that berberine has an inhibitory effect on NF-kB (Zhu et al., 2020). When tested on LPS-treated RAW 264.7 (immune) cells, berberine reduced the level of TNF-alpha and IL-6 cytokines in serum via inhibition of pathway NF-kB, possibly through Sirt1 activation (Zhang et al., 2017). A similar study with LPS-treated mice and berberine showed similar results. Berberine appeared to dose-dependently decrease the mRNA expressions of TNF-alpha and IL-6. A Western blot determined that berberine was capable of suppressing the NF-kB pathway activated by LPS (Wang et al., 2018). Summing from these studies, it would be reasonable to conclude berberine has the ability to alleviate the pro-inflammatory effects of LPS, potentially via the suppression of the NF-kB pathway and of pro-inflammatory cytokine expression. As such, berberine may be a viable treatment for inflammatory COVID-19 symptoms.

1.7 Hypothesis and Aim of research

The goal of this study is to evaluate the effect of berberine (administered in dissolved liquid form) on cell lines SK-N-SH (neuroblastoma) and CCD 841 CoN treated with COVID-19 spike proteins. Judging by previous experiments, Berberine may prove to be a viable treatment for COVID-19 symptoms.

2.0 Materials and Methods

2.1 Cell Cultures and Samples

Cell cultures were acquired from ATCC. SK-N-SH and CCD-841 CoN cells were cultured in MEM (Minimum essential media), with the addition of 10% Fetal Bovine Serum. The cells were cultured within incubated flasks at 37°C, 5% carbon dioxide concentration and 95% humidity. The media was changed once per week. Upon reaching mass density, the cells are detached with

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trypsin and split evenly into 2 flasks with fresh media. The experimental samples were created by treating the cells with 10 uM spike protein and berberine at concentrations of .1 uM, 1 uM, and 10 uM. The spike protein was obtained from MyBioSource.

2.2 LDH Assay
The Invitrogen LDH Assay Kit was used, following the instructions provided. Two sets of 30 uL SK-N-SH and CCD 841 CoN cell samples were prepared on a 96 well plate, one lysed to detect Maximum (Total) LDH release and the other to detect Spontaneous LDH release. 10 microliters of sterile water were added to the Spontaneous wells, while 10 microliters of 10x lysis buffer were added to the Maximum wells. 30 microliters of reaction substrate mix were added to both groups before being left in the dark for 30 minutes, kept at room temperature. 30 microliters of stop solution are then added. The samples were then analyzed in a spectrophotometer at absorbances 490 and 655.

2.3 MTT Cell Viability Assay
SK-N-SH and CCD 841 CoN cells were detached with 4 mL 38% trypsin and neutralized with additional medium to prevent cell damage. A 96-well plate was then prepared with 5 microliters of cell sample per well. 10 mg of MTT was dissolved in 2 mL phosphate buffered saline (PBS). 10 microliters of MTT solution were then added per well, after which the plate was left in the incubator for 1.5 hours. The resulting formazan crystals were dissolved with 800 microliters of DMSO per well and set aside for 10 minutes to develop the color. The wells were examined at absorbance 595 in the spectrophotometer.

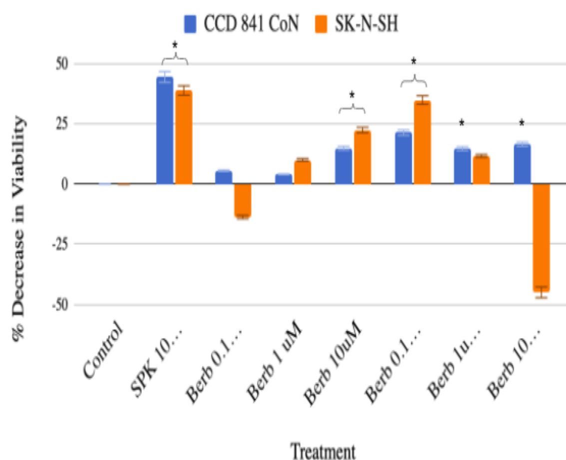
2.4 Enzyme Linked Immunosorbent Assay for TNF-alpha and IL-6
The equipment required for ELISA was obtained from Bolster Biological Technology. A set of standards were prepared with concentrations 0-1000 pg/mL. Then, CCD 841 CoN cell culture samples were centrifuged and stored in a freezer at approximately -20°C. The samples were brought back to room temperature before assaying. 100 uL of each sample were added to the plate, with two replicates per sample. The plate used was precoated with Anti-Human TNF antibodies. The capture antibody used was a biotinylated Anti-Human TNF antibody, and the detection protein used was Avidin-Biotin-Peroxidase Complex. After assay completion, the plates were examined at absorbance 450 in a microplate reader.

2.5 Statistical Analysis
ELISA, MTT, and LDH plates were read with a spectrophotometer. Statistical significance was done using Google Sheets. All experiments were repeated thrice. A two-tailed T-Test was used to evaluate the data, with $p < 0.05$ and $p< 0.01$ as significant.

3.0 Results

3.1 Berberine on Cell Viability of Spike Protein Treated Cells
The effect of berberine alone and with spike protein was assessed using an MTT assay coupled with an LDH assay on both CCD 841 CoN cells and SK-N-SH cells. Firstly, it was observed that berberine alone appeared to be mildly cytotoxic at certain concentrations (~10uM) according to

the MTT assay (**Fig 2A**). It was found that all concentrations of berberine used in the MTT assay significantly increased measured cell viability of CCD 841 CoN cells treated with spike protein as compared to the spike protein control. It was also noted that berberine was capable of increasing measured cell viability in CCD 841 CoN cells used in the LDH assay by more than fourfold at 1 and 10 uM (**Fig 2B**). Regarding the SK-N-SH cells, the MTT assay revealed that berberine alone was effective at increasing cell viability compared to the spike protein control. Of all the concentrations tested, .1uM and 10 uM berberine appeared to be the most significantly effective in increasing cell viability ($p < 0.01$), , with the 10 uM dosage actually promoting cell proliferation ($p < 0.01$) (**Fig 2A**). The LDH assay found that berberine had increased the cell viability of spike protein treated cells, bringing it back to control value (**Fig 2B**). Overall, berberine is capable of alleviating cell viability damage.



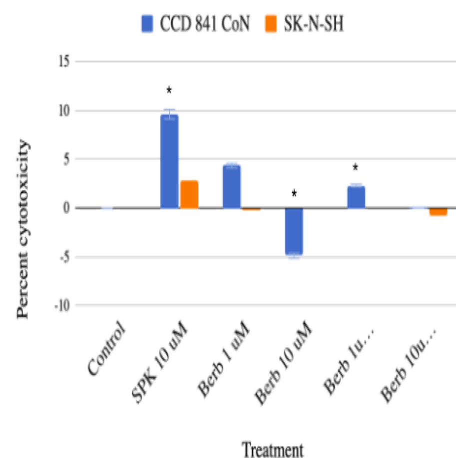
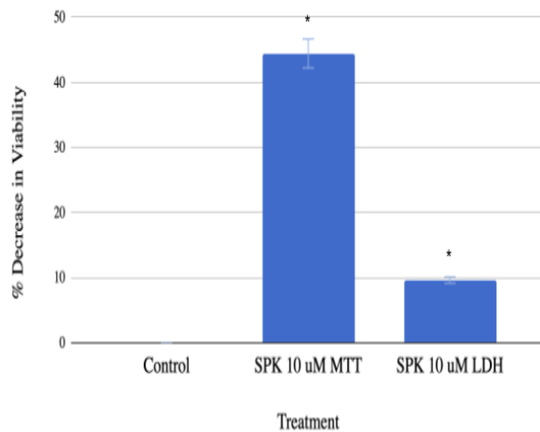


Figure 1. 24-hr MTT and LDH assays were performed to determine berberine's effect on cell viability. Error bars represent +/- 1 SEM, n=5. p<0.05 = *. **A)** MTT assays with CCD 841 CoN and SK-N-SH cells **B)** LDH assay with CCD 841 CoN and SK-N-SH cells

3.2 The Effect of COVID-19 Spike Proteins on Cell Viability.

To assess the effect of spike protein on CCD 841 CoN and SK-N-SH cell viability, an MTT assay was performed alongside an LDH assay. In comparing the results of these assays, it was found that the viability of the cells were affected according to both assays, but to differing extents. The detected decrease in cell viability was greater in the MTT assay than LDH assay. The MTT assay had detected a 38.8% and 44.4% decrease in CCD 841 CoN and SK-N-SH cell viability, respectively, whereas the decrease detected by the LDH assay approximated only around 2.8% and 9.6%. Deducing from these results, it can be concluded that spike protein has a detrimental effect on cellular metabolism without necessarily inducing lysis.



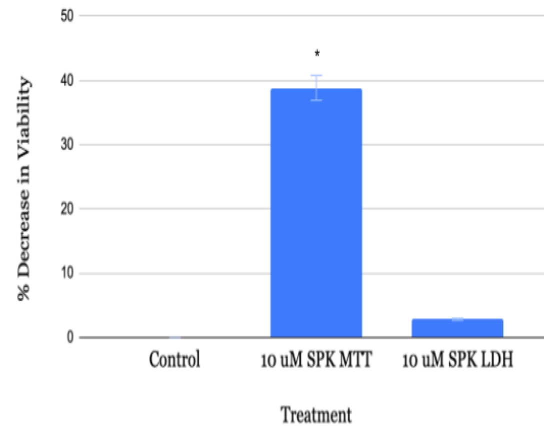
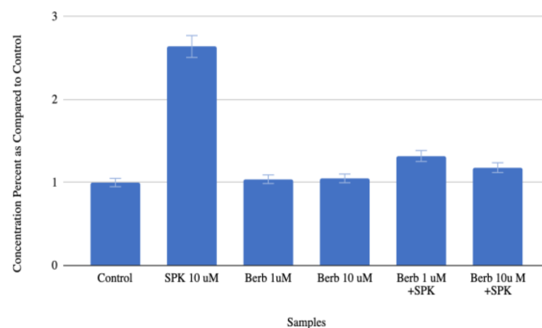


Figure 2. 24-hr MTT and LDH Assays conducted to assess cell viability decrease after treatment of 10uM spike protein. Error bars represent +/- 1 SEM, n=5. p<0.05= *. **A)** Assays with CCD-841 CoN cells. **B)** Assays with SK-N-SH cells.

3.3 Berberine's Influence on TNF-alpha and IL-6 in Spike-Protein Treated CCD 841 CoN Cells.

TNF-alpha and IL-6 are inflammatory cytokines commonly detected during COVID-19 infection. In order to quantify the anti-inflammatory effect of berberine on spike protein treated CCD-841 cells, an ELISA assay was performed, targeting the aforementioned cytokines. Spike-protein treated cells were treated with berberine at 1µM and 10µM concentrations. The resulting values were compared to the spike protein control group to detect the possible inhibitory effects of Berberine. The spike protein control had elevated cytokine concentration in comparison to the untreated control, resulting in a 138% increase in the cells tested for IL-6 and a 164% increase in the cells tested for TNF-alpha. It was found that the concentration of the inflammatory cytokines in spike protein and berberine treated cells was substantially lower than that of the spike protein control, bringing the concentration down close to that of the untreated cells. Within these cells, IL-6 experienced a decrease of 46.6% and 51.7% for dosages 1µM and 10µM (compared to the spike protein control), respectively; TNF-alpha experienced a decrease of 50% and 55.3% for the same dosages, respectively. Berberine alone, however, doesn't appear to have a significant impact on the expression of TNF-alpha and IL-6.



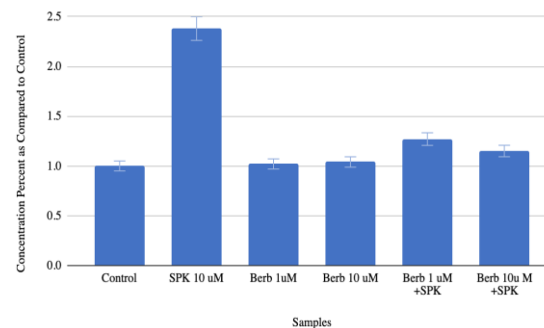


Figure 3. 24-hr ELISA assay conducted with CCD 841 CoN cells. Error bars represent +/- 1 SEM, n=5. **A)** TNF-Alpha is visibly upregulated by the presence of spike protein, and is significantly downregulated by the addition of berberine. **B)** IL-6 is also upregulated by the presence of spike protein and subsequently downregulated by the presence of berberine. Neither of the berberine controls have significant effects.

4.0 Discussion

The objective of this study was to assess the efficacy of berberine as part of a possible treatment for COVID-19. The results indicate that the spike protein of COVID-19 alone was sufficient to trigger significant effects within the cells. The presence of berberine is capable of mitigating the damage to cell viability caused by COVID-19 spike proteins in both CCD 841 CoN and SK-N-SH cells. Furthermore, berberine also appears to alleviate expression of inflammatory cytokines in CCD 841 CoN cells.

The cell lines SK-N-SH (neuroblastoma) and CCD 841 CoN (colon) were chosen primarily due to the fact that COVID-19 has been shown to affect intestinal and nerve tissue. Furthermore, both tissues feature ACE2, which would allow for significant binding of spike protein and uptake by cells. Mice neuroblastoma cells have previously been implemented in studies centering around the effect of ACE2 mediation on oxidative stress (Xia et al., 2011). Given the fact that mice are commonly used in the modeling of neurological disease (Hafezparast et al., 2002), it would be reasonable to assume a structural likeness between human and mice neurons. Thus, SK-N-SH was determined as a viable cell line for the experiment. Furthermore, it has been previously reported that the colon is a targeted area for COVID-19 infection (Zhang et al., 2020) with detectable ACE2 expression, thus also being suitable for this experiment.

The spike protein's effects were measured via the viability tests, coupled with the ELISA. Though the COVID-19 virus itself has been confirmed to be capable of cellular damage, the results suggest that the spike protein of the virus alone is able to interfere with cellular functions. It was observed that the protein caused a significant decrease in the cell viability while also elevating the expression of inflammatory cytokines. This appears to be consistent with previous experiments that observe the use of the spike proteins alone in cells. A previous experiment was done with a focus on the same cytokines used in this experiment, using spike protein from

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SARS-CoV. In both instances, spike protein appeared to significantly upregulate the production of the inflammatory cytokines (Wang et al., 2007). In addition, viability of the cells was primarily determined through MTT and LDH assays. The results of these two assays displayed a large disparity, with the MTT assay detecting higher amounts of damage than LDH. The MTT assay measures cell metabolic activity, whereas the LDH assay measures the damage to the cellular membrane via concentrations of lactate dehydrogenase. Concluding from these results, it can be inferred that the spike protein primarily causes damage contained within the cell and doesn't necessarily induce cell lysis.

Berberine was included as both a control and a treatment in the viability assays, meant to determine its effect alone and on spike protein treated cells. Firstly, it can be noted that berberine used alone has mild cytotoxic properties. The highest dose (10 uM) appears to cause the highest decrease in SK-N SH cell viability, as measured by the MTT assay. The cytotoxicity appears to increase with the dosage amount. The values for CCD 841 CoN cells were inconsistent, however. From this, it is also reasonable to assume that the effect of berberine is dosage dependent, as the cytotoxicity percentage varies with the dosages. Similarly, the migitative effect berberine exerts on the spike protein treated cells is also dose dependent. 0.1 uM berberine had the least migitative effect on SK-N-SH cells, whereas 10 uM berberine had the highest. CCD 841 CoN cells also followed in the dose-dependent difference between These discoveries are in line with previous studies done with berberine concerning cell viability, where various dosages of berberine were assessed for potential effects on cell apoptosis, proliferation cycle, etc. (Gu et al., 2015). Regarding the actual effect of the berberine itself on the treated cells, the results showed a considerable increase in cell viability as compared to the spike protein control. This suggests that, with reference to cytotoxicity levels, berberine can be used as a possible treatment to restore cell viability.

Inflammatory cytokines were measured using an ELISA assay, targeting specifically the proteins TNF-alpha and IL-6. Interestingly, the graphs for these two proteins are remarkably similar, indicating that the same treatments produced similar concentrations for both cytokines. A possible explanation for this phenomenon is the regulation of the NF-kB pathway via TNF-alpha. The pathway is responsible for inducing the production of both cytokines, while TNF-alpha serves to trigger the process in a sort of positive loop (Tanabe et al., 2010). Berberine alone did not cause significant differences in cytokine levels when compared to the blank control, but appeared to inflict a dose-dependent suppression of the elevated levels of the cytokines. This is consistent with previous experiments assessing the inhibitory effect of berberine on the expression of TNF-alpha in vascular endothelial cells (Liu et al., 2015). By inhibiting the production of TNF-alpha, berberine also may serve to inhibit the NF-kB pathway, which further inhibits IL-6 production. Inferring from this information, it can be reasonably suspected that the berberine is capable of manipulating the NF-kB pathway via TNF-alpha suppression. Consequently, berberine is also viable as an anti-inflammatory treatment for the uncontrolled inflammation induced by COVID-19. It can also be noted, after examining both viability results and ELISA results, that the (Fig 2A, Fig 3A and 3B) charts correspond to each other. The results compared showed that the rise in cytokine concentration was reflected in the viability essays by a

decrease in cell viability, though the extent of change in the two assays differed. Concluding from these two results, it can be concluded that inflammation was successfully triggered and is negatively correlated with cell viability.

5.0 Future Research

Due to lack of time, ELISA tests for the inflammatory proteins weren't performed on the SK-N-SH cells. This may be a point of future research to inspect, as berberine is expected to have an effect on the expression of these cytokines. These effects, however, may vary depending on the cell types—as observed in the cell viability assays. In addition, though it can be inferred that the effect berberine has on the production of inflammatory cytokines is through the NF-kB pathway, the tests performed here hadn't accounted for extraneous variables that could be attributed to different cellular workings. Possible methods of investigating the NF-kB pathway directly include gene reporter assays and western blots. This is definitely a point of future interest that could be viable to explore. There is also the question of the effect of berberine on a different type of cells (such as immune cells, which are directly involved in the inflammation process itself) since there are visible differences between the results of the CCD 841 CoN and SK-N-SH cells. There is also the option of testing berberine with various other structures of the virus.

6.0 Conclusion

This purpose of this study was to examine the effect that berberine can have on spike protein treated cells, thus testing the possible use of berberine as a treatment for COVID-19. Spike protein was determined to have an detrimental impact on cell viability despite being isolated from the main body of the virus. Berberine's behavior is dose-dependent, determined by the difference in both cytotoxicity and cytokine values of the various samples. Berberine is capable of significantly mitigating cytotoxicity and inflammatory cytokine release at specific dosages. Overall, berberine appears to be beneficial as a possible treatment to the COVID-19 spike protein treated cells. Coupled together, these results could be further studied in future research focused on the role of berberine as a possible medical treatment.

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