Research Article

iMedPub Journals
www.imedpub.com

Journal of Biomedical Sciences
ISSN 2254-609X

2020
Vol.9 No.4: 42

The Cytotoxic Effects of Spike Proteins and Hydroxychloroquine

Robert Zheng*

New York University, USA

***Corresponding author:** Robert Zheng

Robzheng3@gmail.com

Robert Zheng, New York University, USA

Citation: Zheng R. (2020) The Cytotoxic Effects of Spike Proteins and Hydroxychloroquine. J Biomedical Sci Vol.9 No.4:42

Abstract

Title: This study shows the effect of Hydroxychloroquine (HCQ), one of the drugs currently under investigation for Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) treatment, on the cytotoxicity caused by spike proteins.

Background: The emergence of the novel pathogenic SARS-coronavirus 2 (SARS-CoV-2) is responsible for a worldwide pandemic. The first step of the viral replication cycle, attachment to the surface of respiratory cells, is mediated by the spike viral protein. It sets off a sequence of reactions that would eventually lead to viral spread and cell death. If we debilitate the spike protein either with a drug like Hydroxychloroquine that's known to mitigate Spike proteins from similar viruses, then the cytotoxic effect of SARS-CoV-2's spike protein (SARS-S-2) is stopped.

Methods and Findings: We utilized multiple 96 well plates and a single 6 well plate with cell cultures of Vero cells to perform various tests. Such as the: MTT Cell Proliferation Assay, LDH Cytotoxicity Assay, Caspase Apoptosis Assay, The Hematoxylin and Eosin (H&E) Staining, and the Trypan-Blue Cell Exclusion Assay, to assess the number of dead cells in each culture and by extension the level of Cytotoxicity. For all 5 tests, the process could be summarized as adding a chemical to the cultures, recording a reaction over a period of time ranging from a few minutes to over 48 hours, and then comparing the results between the groups. These cell cultures were divided into different treatment groups, some were injected and incubated with recombinant SARS-S-2, a certain dilution of HCQ, both, and neither.

The MTT Cell Proliferation Assay uses a student t-test to determine consistent statistical significance in the group of results, unfortunately, the % viability results from the MTT Assay didn't pass the test. The LDH Cytotoxicity Assay also had Cytotoxicity results that didn't pass the 95% confidence interval for the cell cultures with spike proteins in them. The Caspase Apoptosis Assay found that overall there're greater levels of apoptosis caused by spike proteins than HCQ. The H&E staining revealed that the group with HCQ added to the spike protein had a greater area of living cells than the group with only spike proteins added. Specifically, 113.9796% and 106.8545% of the control group's area, compared to a mere 49.7479% on its own, with t-test confidence of over 99%. Unfortunately, the Trypan Blue Assay had the same issue as the MTT and LDH Assay with the failure to break out of statistical significance. Outside of that issue pervading through 3 out of the 5 experiments, the main limitations of this experimental design is the use of Vero cells rather than critical organs in vivo and that the H&E Staining and Trypan Blue Staining experiments were reliant on samples of photos taken on the microscope and not necessarily a reading of the cell culture as a whole.

Conclusion: The general consensus among all the assays, was either inconclusive or in support of the idea that the presence of HCQ mitigates the cytotoxic effect of SARS-S-2; With the added caveat that HCQ on its own was cytotoxic in its own right. In the future, one could follow up on this study with alternate combinations of drugs like Remdesivir or Camostat Mesylater.

Keywords: SARS-CoV-2; Spike Protein; Hydroxychloroquine; Cytotoxicity; LDH Assay; MTT Assay; Trypan Blue Assay; H&E Staining; Caspase Assay

Received: December 10, 2020; **Accepted:** December 14, 2020; **Published:** December 28, 2020

Introduction

The risk of severe COVID-19, if an individual becomes infected, is known to be higher in older individuals and those with underlying health conditions. Understanding the number of individuals at increased risk of severe COVID-19 and how this varies between countries should inform the design of possible strategies to shield or vaccinate those at the highest risk.

Covid-19 impact on humans

The recent coronavirus pandemic crisis is due to viral infection of severe acute respiratory syndrome-related coronavirus-2 (SARS-CoV-2), causing uncontrolled inflammatory conditions in the human lung and other organs. Factors such as age, sex, existing compromising diseases [1], and diabetes [2] are all considered comorbidities, though people of any demographic are susceptible to infection and subsequent death.

The mortality rate is much higher in COVID-19 patients over 60 years old, especially compared to patients under 20 (The rates in question being 20% and >1% respectively) [1]. The demographic of people over 70 is also generally more susceptible to infection not only due to age causing a weaker immune system, but also because of their statistical likelihood to develop compromising diseases like chronic pains or high blood pressure, or chronic respiratory diseases.

In Clark's study, there is a disparity between the mortality rate between males and females, with males' rate being double the females' [6% to 3%]. Though there's no follow-up report to elaborate on whether it stems from a biological cause or a social push to be more reckless.

Sar-CoV-2 virus and cell entry mechanism

Sars-CoV-2 spike protein

SARS-CoV-2 requires the binding of Spike proteins to cell receptors and the priming of Spike proteins via proteases in order to enter the cell. SARS-CoV-2 uses Angiotensin-Converting Enzyme 2 (ACE2) as the receptor for cell entry and follows up with Tran's membrane Protease Serine 2 (TMPRSS2) as the enzyme which primes the spike glycoprotein [3]. Thus, the ACE2 that's normally expressed to protect from a lung injury, is then debilitated and used as a vehicle for infection by the SARS-CoV-2 Spike Protein [4]. The SARS-CoV-2 Spike protein (SARS-S-2) infects the cell using ACE2 receptors or by brute-forcing with the assistance of TMPRSS2 priming. Therefore ACE2 and TMPRSS2 are presented as primary targets for drugs against SARS CoV -2 infection.

ACE2 receptor for virus entry

The in-vitro study by Hoffman showed that SARS-CoV-2 uses cell receptors like ACE2 for host cell entry, shedding it in order to gain access [3]. The SARS-S-2 binds to ACE2 in order to enter the cell membrane. Without those receptors, infection can't spread. And by extension, if those receptors were bound to something else prior, the virus can't bind and shed it, thus spreading becomes impossible without ACE2 being cleaved by TMPRSS2.

TMPRSS2, TACE and ADAM17's role in virus entry into human cells

TMPRSS2 is an enzyme humans naturally produce that also supports the proliferation of SARS-CoV-2. TMPRSS2 assists in cell entry in two ways: cleaving ACE2 [thereby opening the door for viral uptake] and cleaving the Spike protein [priming it for entry] [5]. The priming of the Spike protein is a required part of the Spike protein's process of bonding to ACE2, but if ACE2 is already bonded, TMPRSS2 could skip that step altogether by cleaving the ACE2, allowing the cell membrane to be shredded even when the ACE2 receptor has been bonded prior to the Spike protein.

A Disintegrin and Metalloproteinase 17 (ADAM17) aka Tumor Necrosis Factor (TNF)-Alpha Converting Enzyme (TACE), is a membrane-bound enzyme that cleaves cell surface proteins. Including Cytokines such as TNF-alpha and receptors like ACE2. ADAM17 and TMPRSS2 actually compete with each other in terms of cleaving ACE2, when one enzyme attempts to cleave ACE2, the other can't. When TMPRSS2 cleaves ACE2 it promotes SARS-S-2 entry, but when ADAM17 cleaves ACE2, it offers indirect protection to the organ [6].

SARS-CoV-2 induction of inflammatory response

The progression of SARS-CoV-2 has been followed with: increased concentrations of cytokines, a loss of white blood cells, and lessened expression of IFN-gamma (an immunoregulatory virion that has antitumor properties). Cytokines are normally released as an immune response to infection, but as an inflammatory response, it can be pushed too far and enter the realm of being a cytokine storm, where the concentration does more harm to tissues than good [6].

In a clinical study run by Han, it was found that specifically IL-6 and IL-10 are the cytokines that are released excessively in severe and critical patients [6]. In addition, these two cytokines have a proven direct relationship with critical forms of COVID-19, which allows for the two to be a reliable indicator of predictive severity.

TNF-alpha is a cytokine that normally coordinates a variety of immune responses, but during inflammatory diseases that fester a cytokine storm, it amplifies the inflammation and becomes immune suppressive. Anti-TNF-alpha therapies have been used to treat a variety of inflammatory diseases including COVID-19 because just by reducing TNF-alpha specifically you also reduce the levels of other cytokines which suggests that TNF-alpha is responsible for triggering other cytokines such as IL-6 or IL-8.

Since TNF-alpha is important in almost all acute inflammatory reactions, being an amplifier of inflammation [7], causing other cytokine levels to rise to undesirable heights, and is immune-suppressive itself at high levels, finding an anti-TNF-alpha solution may ultimately debilitate the harmful effects of COVID-19.

SARS-CoV-2 causes endothelial cell damage (Lung cell damage)

SARS-CoV-2 and conditions that cause endothelial dysfunction such as systemic hypertension, obesity, and diabetes are

comorbidities [7]. In the European Respiratory Journal, two research papers describe the risk of deep vein thrombosis and acute pulmonary embolism in SARS-CoV-2 [8,9]. These descriptions coincide with other documented SARS-CoV-2 comorbidities like thromboembolic disease, systemic vasculitis, endothelial cell apoptosis, and inflammation in various organs. The aforementioned inflammation extends to the SARS-CoV-2 inflammatory response also activating coagulopathy alongside the cytokine storm [10-14]. ACE2 is a part of the Renin-Angiotensin System (RAS), a system that regulates cardiovascular, kidney, lung, and liver systems by maintaining homeostasis of blood pressure, electrolyte balance and inflammatory response. ACE2 acts as a negative regulator in RAS; ACE2 and its interactions with ACE weaken the balance of the system and are thought to be an important contributor to diseases like inflammatory lung disease. When TACE/ADAM17 cleaves ACE2 and ACE, they are released from the epithelial surface and into the airway surface liquid, starting the majority of the endothelial dysfunction.

SARS-CoV-2 causes endothelial cell damage (Lung cell damage)

Did any research show HCQ effect on virus entry into the cell via ACE2 and TMPRSS2/TACE/ADAM17?

There are conflicting opinions and results on what effect Hydroxychloroquine(HCQ) has. On virus entry, in particular, studies may show that Chloroquine (CQ) or HCQ interferes with the glycosylation of ACE2 enzymes and block the space that the Spike protein would use when entering through the membrane surface cell receptors, filling up the ganglioside itself [15]. Or one may find a study that says in the event of TMPRSS2 expression, HCQ will not block virus entry as TMPRSS2 primes the pathway ahead of time [16]. Both studies used Vero cells rather than endothelial cells so pointing to that specific factor to dismiss one argument or the other isn't helpful; all the papers that can be found on the discourse have equally damning factors like study sample size or limited information on certain aspects like receptor interactions or protein comparisons. So all one can do is attempt to run their own experiment and get their own findings.

Aim of research

This study aims to evaluate the potential cytotoxic effect of Spike Proteins alone, the efficacy of Hydroxychloroquine (HCQ) in the treatment of patients with SARS-CoV-2, by using model cell line and discuss possible molecular mechanisms that can be used as targets in the treatment of SARS-CoV-2.

By testing the cytotoxic effect of the Spike Protein, one gains a point of reference. With that reference, the effect of HCQ on a cell culture injected with Spike Protein is comparable. In light of those results, we can reevaluate the validity of other methods of treating SARS-CoV-2, rather than inhibiting the Spike Protein. One could inhibit the Angiotensin-Converting Enzyme (ACE) to maintain stronger homeostasis of ACE2, inhibit TMPRSS2 to prevent viral entry, or inhibit TNF-alpha to prevent the cytokine storm.

Materials and Methods

Cell cultures with cells that expressed both ACE2 and TMPRSS2 receptors were ordered and distributed upon various well plates (multiple sets of 96 well plates, and in one case: 6 well plates). After incubating the cell cultures at 37 degrees Celsius, a variety of in-vitro analysis were then performed: MTT cell proliferation assay, LDH cytotoxicity assay, Trypan blue cell exclusion assay, Hematoxylin pathological staining, and Caspase Apoptosis Assay.

(MTT cell proliferation assay) The TACS MTT Cell Proliferation and Viability Assay were used to measure the proliferation of the cell culture and its level of any apoptosis or necrosis. A section of the 96 well plates had 10 microliters of yellow tetrazolium salt added to them. After the yellow salt breaks down into purple formazan crystals, 80 microliters of dimethyl sulfoxide were added to dissolve the insoluble purple crystals. Immediately, a spectrometer was used on the well plates at a 595-nanometer wavelength to measure the absorbance of the dissolved purple within the cell cultures; the wells would be measured multiple times at intervals of 24 hours. The more purple that was absorbed into the cell culture, the more viable cell growth was in that culture.

(LDH cytotoxicity assay) The LDH Cytotoxicity Assay was used to quantify cellular cytotoxicity. In a similar concept as the MTT assay, the cytotoxicity would be determined by adding an indicator that changes color depending on the level of cytotoxicity. This assay differed from MTT as it utilized 3 sets of wells: the experimental well where 10 milliliters of a substrate is added and changes color based on the level of cytotoxicity, one well to be untouched by the substrate, and one well to be filled with only deceased cells in order to determine the total possible LDH value. The second and third wells would be used as a point of comparison after measuring the color absorbance using a spectrophotometer at 490 nanometers. The third well was filled with a Lysis buffer which broke down cells and left in the incubator. After killing the cells for 45 minutes 30 mL of the substrate was added. After both the first and the third wells had substrate added, they were left at room temperature in darkness for approximately 30 minutes before adding 30 mL of stop solution to stop the color change. That's when the spectrophotometer was used on all three wells. With the results from the spectrophotometer, the % Cytotoxicity was determined using the following formula: (First well - Second well) / (Third well - Second well)]*100.

(Trypan blue cell exclusion assay) The Trypan Blue Viability Assay was used to measure the number of dead cells. Trypan Blue dyes cells with membrane leakage, a symptom of a dead cell; after taking photos under a microscope, one can count the number of blue dead cells. In this instance, the 6 well plates were used since they were large enough to fit under the platform of a microscope. A solution was created out of 0.2 gm Trypan Blue and 99.8 ml distilled water and added drops of that solution onto the well plates. After the solution slowly dyed the cells, the cells

were observed under a microscope and took photos using the program ImageJ. In ImageJ, the photos of the dyed cells could be edited to be desaturated, more contrasted, without background and noise. By applying those edits the photos would only be left with the simple shapes of the dyed cells which were counted and recorded.

(Hematoxylin Pathological Staining) The Hematoxylin and Eosin Staining were applied to calculate the proportion of dead to living cells within the cell cultures. Rather than dyeing the cells blue and taking a count of each dead cell, one would use ImageJ to analyze the volume of specific hues within the photos in order to determine the proportion of dead and living cells. After staining and taking pictures, ImageJ is opened, the color space is edited, a threshold color is set as red to denote all the colored cells as red, and then go to the analyze menu to measure the area of the red in the photo.

(Caspase Apoptosis Assay) The Caspase Colorimetric Apoptosis Assay was used to measure the level of apoptosis in cell cultures. The assay is based on the reactions of three chemicals: a Lysis Buffer, an Assay Buffer, and a Substrate Solution. Through the course of their chemical reactions, there will be an optical change that can be detected by a spectrophotometer at 405 nanometers. The absorbance of this optical change indicates the presence and activity of Caspase, which is an enzyme that incites apoptosis.

The Assay takes place in 6 well plates after procuring 10^7 cells and suspending them in a medium using a vortex apparatus. In the case of any attached cells, they were manually removed from the culture plate and suspended alongside the rest of the cells. This would be followed by centrifugation at 600xg for five minutes, which would necessitate another check for attached cells. After re-suspending the cells again, the centrifugation and suspension would be repeated one last time before adding 50 microliters of the Lysis Buffer. After one fifth and final vortex suspending, the cells would be lysed by freezing and thawing them four times.

It's after the final thaw when the Caspase Assay Buffer is added into each well alongside 5 microliters of chromophore p-NitroAniline (pNA) Substrate Solution. When the solution is being mixed, the pNA Substrate Solution breaks down in response to the Caspase Assay Buffer and the presence of Caspase in lysed cells, a chromogenic is released and produces a color change that can be measured at 405 nanometers. Utilizing out the spectrophotometer, the wells were measured every 30 minutes. Between each measuring, the well plates would be covered and incubated at 37 degrees Celsius. The 30 minutes intervals of measuring would continue until the measured output significantly changed, in this case, it was 120 minutes. The change between absorbance before and after the 120 minutes would then be compared between treatment groups.

All of this was to test if SARS-CoV-2 recombinant spike protein can cause cell death or prevent cell replication, alongside whether any other chemicals (such as HCQ) would be able to undo the cell death caused by the spike protein.

Results

LDH cytotoxicity assay

When comparing the % cytotoxicity derived from testing the released and total LDH **Figure 2.**, HQ 1 uM was the highest by a large margin at 6.807%, followed by HQ 10 uM at 4.4% and trailed behind SPK and SPK+HQ 10 uM having 1.99% and 0.5275% respectively. What's most surprising is SPK+HQ 1 uM actually having a negative % cytotoxicity(-1.7%), and looking at the t-tests only SPK+HQ 10 uM is above the 0.05 value maximum (holding 0.892 t-test result) that determined statistical significance **Figure 1**.

MTT cell proliferation assay

When looking at the MTT viability values all of the treatment groups have a higher % viability than control, with SPK and HQ 10 uM being both the highest two(at 116.1% and 108.28%) **Figure 4.** and the two with the t-test values low enough to qualify for statistical significance(them being 0.140 and 0.368) **Figure 3**. The other groups may be a bit higher than the control group but have a t-test value too high to prove statistical significance.

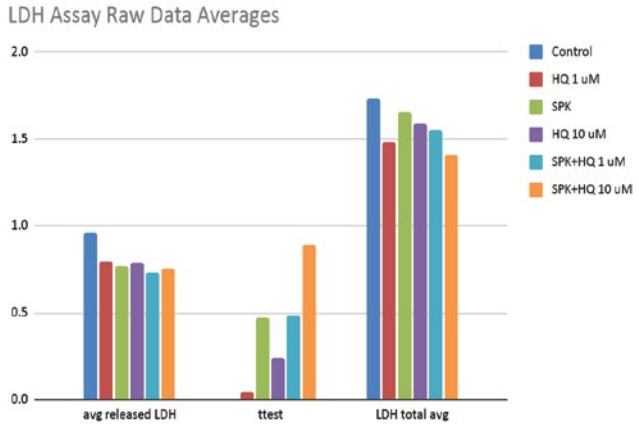

Figure 1 LDH Assay X axis: Measurement Group Y axis: Micrograms per mililiterMicrograms per mililiter.

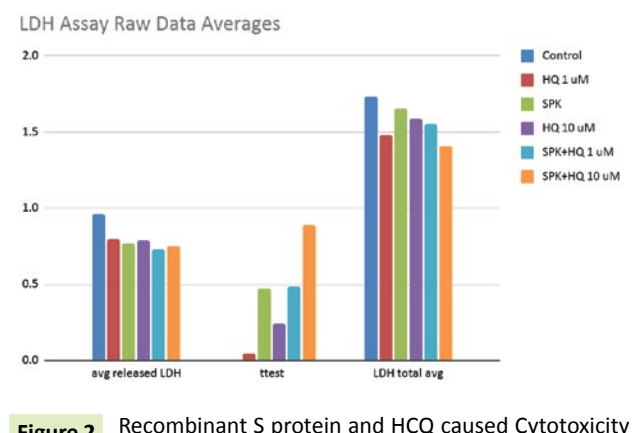

Figure 2 Recombinant S protein and HCQ caused Cytotoxicity X axis:(handled by legend) Y axis: % Cytotoxicity.

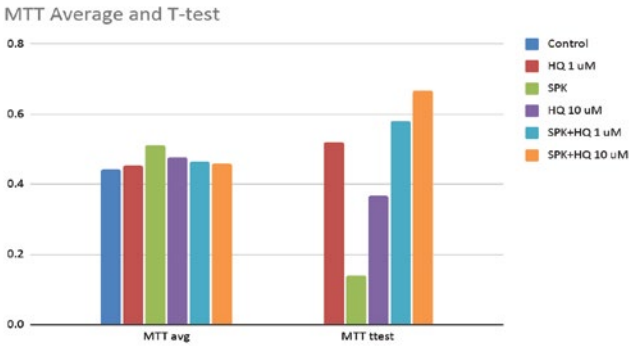

Figure 3 MTT Average and T Test X axis: Measurement Group Y axis: Micrograms per Mililiter.

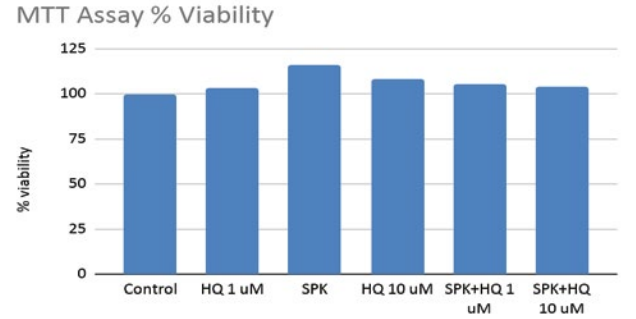

Figure 4 MTT % Viability X axis: Treatment Group Y axis:% Viability.

Trypan blue cell exclusion assay

The difference in Trypan Blue average dead cell count at 4x **Figure 5.** magnification are all statistically insignificant, at 10x **Figure 6**. magnification all except for HQ 100 uM have significant difference however the lowered sample size that comes with greater magnification also implies statistical insignificance. That makes it easy to dismiss the trend of HQ 10 uM and HQ 100 uM increasing or stagnating the number of dead cells at 10x magnification (6 and 5 dead cells on average) alongside SPK as opposed to the groups with only HQ (2 and 3 dead cells) or only SPK (5 dead cells on average).

Hematoxylin pathological staining

There's a significant drop between the control group and the group treated with Spike proteins dropping down to 49.7479% of the control group's area, adding credence to the hypothesis of spike proteins being poisonous on their own without the property of allowing viral entry.

Following that line of thought, the Statistically significant Rise that comes with adding HQ 100 uM or HQ 10 uM raising it up to 113.9796% and 106.8545% of the control group's area, also implies that Hydroxychloroquine is fully capable of mitigating the toxic effects of Spike proteins on their own **Figure 7**.

Caspase apoptosis assay

The trend of the pigment absorption increasing over time is to be expected during the Caspase Apoptosis assay as the color is meant to change after mixing in the lysate and substrate. The differences lie in the severity of the absorption change, greater change denoting greater amounts of dead cells. The lower concentration of Hydroxychloroquine causes a visibly lower change in absorption compared to the higher concentration, yet

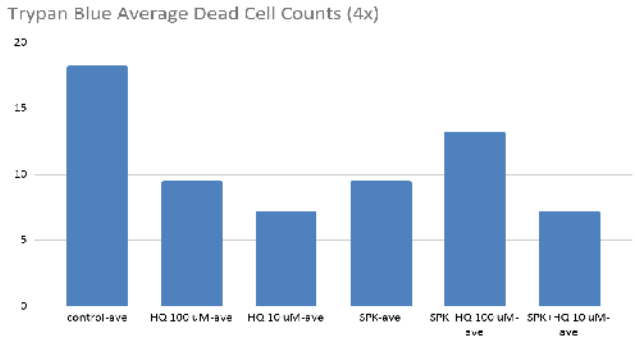

Figure 5 Trypan Blue (4x) X axis: Treatment Group Y axis: # of Dead Cells.

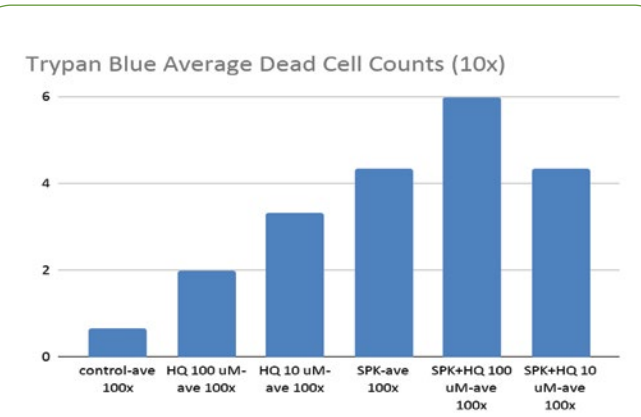

Figure 6 Trypan Blue (10x) X axis: Treatment Group Y axis: #of Dead Cells.

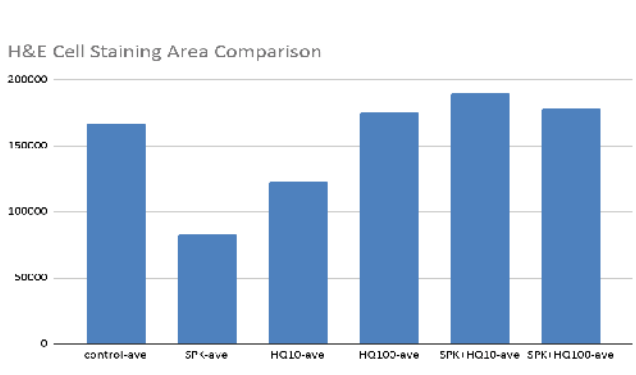

Figure 7 H&E Cell Staining X axis: Treatment Group Y axis: Square Micrometers.

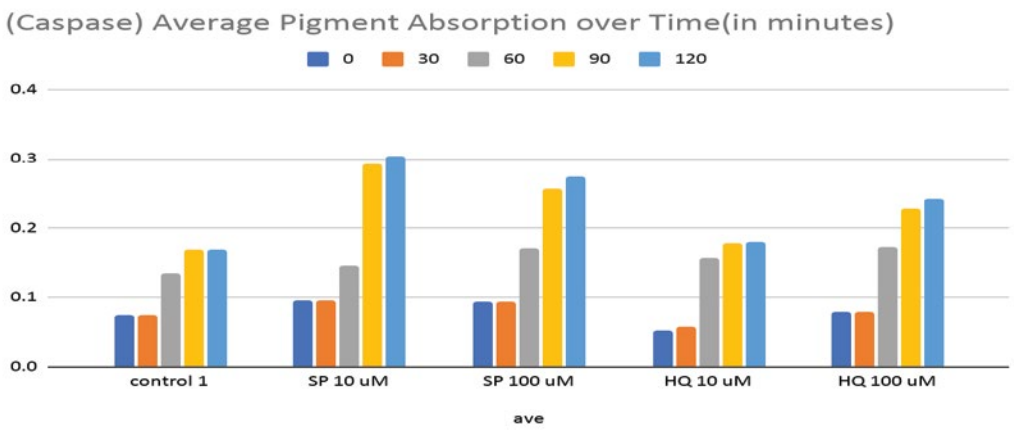

Figure 8 Caspace Assay X axis: Time in Minutes Y axis: Micrograms per mililiter.

is still larger than the control group, which coincides with the consensus that HCQ is toxic. There's a less obvious difference in severity between the Spike Protein groups. While both groups have higher absorption readings on the spectrometer, the lower concentration reaches a higher peak of 0.304 Absorption Change as opposed to the higher concentration (SP 100 uM) only reaches 0.275 Absorption Change after 120 minutes. However, the roles are reversed at the 60-minute mark, where SP 100 uM is at 0.171 while SP 10 uM is at 0.1465 Absorption Change, implying that the unusually high peak is an outlier and that the higher concentration(SP 100 uM) is trending more consistently **Figure 8**.

Discussion

The Trypan Blue Dying and Caspase Apoptosis Assay data largely coincides with the community consensus on the effects of HCQ and Spike proteins. Though SARS-S-2 appears to neutralize HQ's cytotoxicity as shown in the LDH and MTT assay, it begs the question as to whether it also neutralizes HQ's effectiveness at either suppressing or hindering SARS-CoV-2. Though conceptually that shouldn't be the case given that SARS-S-2 would be present while SARS-CoV-2 is proliferating in an infected patient, it's the presence of a virus to hinder that neutralizes the cytotoxicity, rather than the lack of virus-hindering-capacity in the chemical.

Limitations

The use of Vero cells rather than lung cells calls into question whether the cytotoxicity results would be the same in the critical organs. This study is done *in vitro*, which has been reliably shown to have different results in vivo.

While previous studies showed that SARS-COV spike protein can induce human cell apoptosis [17-19], currently there is very limited research on SARS-CoV-2 spike protein's cytotoxic effect on human cells.

There are multiple proteins capable of binding with cell surfaces. As such, it would be naive to rely only on HCQ to inhibit virus entry without taking into consideration TMPRSS2, or taking a look at targeting the spike protein rather than the cell membrane in order to see if that method has less complication.

Many of the conclusions made about the SPK reducing the loss in LDH is made from the statistical significance rather than actually decreased readings in LDH. MTT readings were unusual with it being full of outliers and impossibilities. Trypan blue only shows the number of dead cells and not the proportion or ratio of dead to alive cells. The Caspase Apoptosis Assay only took an average of two trials.

Conclusion

Our study establishes that Hydroxychloroquine (HCQ) reduces the cytotoxic effect in the presence of Spike proteins. We found lessened apoptosis or cell death in the presence of HCQ treatment. Though this was only in comparison to cultures with spike protein, on its HCQ had cytotoxic effects on its own. Thus, treatment with HCQ shouldn't be taken lightly. In conclusion, our evaluation of HCQ after experimenting with its effects on Spike Proteins supports its use as an antiviral agent for the treatment of SARS-CoV-2 in humans.

Acknowledgment

Dr. Zhu for helping me obtain the equipment and overlooking the experiments.

Conflicts of Interest

The authors have no conflicts of interest.

Funding

This research was self-financed. All Equipment either being borrowed by Dr. Zhu or ordered off the internet.

References

1 Clark A, Jit M, Warren-Gash C, Guthrie B, Wang H, et al. (2020) Global, regional, and national estimates of the population at increased risk of severe COVID-19 due to underlying health conditions in 2020: a modeling study. Lancet Glob Health 8: e1003-e1017.

2 Orioli L, Hermans MP, Thissen JP, Maiter D, Vandeleene B, et al. (2020) COVID-19 in diabetic patients: Related risks and specifics of management. Ann Endocrinol 81: 101-109.

3 Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, et al. (2020) SARS-CoV-2 Cell entry depends on ace2 and tmprss2 and is blocked by a clinically proven protease inhibitor. Cell 181: 271-280. e8.

4 Lukassen S, Chua RL, Trefzer T, Kahn NC, Schneider MA, et al. (2020) SARS-CoV-2 receptor ACE2 and TMPRSS2 are primarily expressed in bronchial transient secretory cells. EMBO J 39: e105114.

5 Heurich A, Hofmann-Winkler H, Gierer S, Liepold T, Jahn O, et al. (2014) TMPRSS2 and ADAM17 cleave ACE2 differentially and only proteolysis by TMPRSS2 augments entry driven by the severe acute respiratory syndrome coronavirus spike protein. J Virol 88: 1293-1307.

6 Xiao L, Sakagami H, Miwa N (2020) ACE2: The key Molecule for Understanding the Pathophysiology of Severe and Critical Conditions of COVID-19: Demon or Angel?. Viruses 12: 491.

7 Huertas A, Montani D, Savale L, Pichon J, Tu L, et al. (2020) Endothelial cell dysfunction: a major player in SARS-CoV-2 infection (COVID-19)?. Eur Respir J 56: 2001634.

8 Criel M, Falter M, Jaeken J, Kerrebroeck MV, Lefere I, et al. (2020) Venous thromboembolism in SARS-CoV-2 patients: only a problem in ventilated ICU patients, or is there more to it?. Eur Respir J 56: 2001201.

9 Bompard F, Monnier H, Saab I, Tordjman M, Abdoul H, et al. (2020) Pulmonary embolism in patients with COVID-19 pneumonia. Eur Respir J 56: 2001365.

10 Dolhnikoff M, Duarte-Neto AN, de Almeida Monteiro RA, Ferraz da Silva LF, de Oliveira Ep, et al. (2020) Pathological evidence of pulmonary thrombotic phenomena in severe COVID-19. J Thromb Haemost 18: 1517-1519.

11 Danzi GB, Loffi M, Galeazzi G, Gherbesi E (2020) Acute pulmonary embolism and COVID-19 pneumonia: a random association?. Eur Heart J 41: 1858.

12 Poissy J, Goutay J, Caplan M, Parmentier E, Duburcq T, et al. (2020) Pulmonary embolism in COVID-19 patients: awareness of an increased prevalence. Circulation 142: 184-186.

13 Ullah W, Saeed R, Sarwar U, Patel R, Fischman DL (2020) COVID-19 complicated by acute pulmonary embolism and right-sided heart failure. JACC Case Rep 2: 1379-1382.

14 Ruiz FA, Lea CR, Oldfield E (2004) Human platelet dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and unicellular eukaryotes. J Biol Chem 279: 44250-44257.

15 Fantini J, Di Scala C, Chahinian H, Yahi N (2020) Structural and molecular modeling studies reveal a new mechanism of action of chloroquine and hydroxychloroquine against SARS-CoV-2 infection. Int J Antimicrob Agents 55: 105960.

16 Hoffmann M, Mösbauer K, Hofmann-Winkler H, Kaul A, Kleine-Weber H, et al. (2020) Chloroquine does not inhibit infection of human lung cells with SARS-CoV-2. Nature 585: 588-590.

17 Han H, Ma Q, Li C, Liu R, Zhao L, et al. (2020) Profiling serum cytokines in COVID-19 patients reveals IL-6 and IL-10 are disease severity predictors. Emerg Microbes Infect 9: 1123-1130.

18 Feldmann M, Maini RN, Woody JN, Holgate ST, Winter G, et al. (2020) Trials of anti-tumor necrosis factor therapy for COVID-19 are urgently needed. Lancet 395: 1407-1409.

19 Jia Hongpeng (2016) Pulmonary Angiotensin-Converting Enzyme 2 (ACE2) and Inflammatory Lung Disease. Shock 46: 239-248.