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Hydroxychloroquine treatment on SARS-CoV-2 receptor ACE2, TMPRSS2 and NRP1 expression in human primary pterygium and conjunctival cells

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ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the pathogen for coronavirus disease 2019 (COVID-19) pandemic. Its infection depends on the binding of spike protein to the host cell receptor angiotensin-converting enzyme 2 (ACE2), type II transmembrane serine protease (TMPRSS2) and neuropilin-1 (NRP1). Hydroxychloroquine has been applied as one of the COVID-19 treatment strategies. Here we aimed to evaluate hydroxychloroquine treatment on SARS-CoV-2 receptor expression in human primary pterygium and conjunctival cells and its potential influences. Expression of ACE2, TMPRSS2 and NRP1 proteins were found in the epithelial layer of both primary pterygium and conjunctiva tissues as well as in their isolated fibroblasts. High concentration of hydroxychloroquine treatment significantly reduced the viability of both primary pterygium and conjunctival cells. ACE2 protein expression was significantly decreased in both pterygium and conjunctival cells after hydroxychloroquine treatment. Hydroxychloroquine also reduced NRP1 protein expression in conjunctival cells. In contrast, TMPRSS2 protein expression showed slightly increased in conjunctival cells. Notably, ROS production and SOD2 expression was significantly elevated in both pterygium and conjunctival cells after hydroxychloroquine treatment. In summary, this study revealed the reduction of ACE2 and NRP1 expression by hydroxychloroquine in human primary pterygium and conjunctival fibroblasts; yet with the increase in TMPRSS2 expression and oxidative stress and decrease in cell viability. Implementation of hydroxychloroquine for COVID-19 treatment should be carefully considered with its potential side effects and in combination with TMPRSS2 inhibitor.

1. Introduction

Coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Hu et al., 2021), has affected more than 190 million individuals in over 200 countries and regions, imposing a serious threat to the global public health (Wu et al., 2020b). SARS-CoV-2, a member of the coronaviridae family (Lu et al., 2020), infects the target cells through the binding of its spike (S) protein to the host cell receptor angiotensin-converting enzyme 2 (ACE2) (Chan et al., 2020; Hatmal et al., 2020). Moreover,

; COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; ACE2, angiotensin-converting enzyme 2; TMPRSS2, type II transmembrane serine protease; NRP1, neuropilin-1; ROS, reactive oxygen species; SOD2, superoxide dismutase 2; S, spike; CO_2, carbon dioxide; CK13, cytokeratin 13; MUC5, mucin-5; PBS, phosphate buffered saline; RIPA, radio-immunoprecipitation assay; SDS, sodium dodecyl sulphate; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's Modified Eagle Medium; CCK-8, Cell Counting Kit-8; OD, optical density; DAPI, 4′,6-diamidino-2-phenylindole; SD, standard deviation; ANOVA, analysis of variance.

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transmembrane serine protease (TMPRSS2) and neurophilin-1 (NRP1) are recently found to be involved in promoting SARS-CoV-2 invasion and spreading (Cantuti-Castelvetri et al., 2020; Hoffmann et al., 2020). SARS-CoV-2 RNA could be detected in tears and conjunctival secretions of COVID-19 patients (Chen et al., 2020; Zhang et al., 2020). Furthermore, we previously identified the expression of *ACE2* and *TMPRSS2* genes in mouse cornea but inconsistent in human primary pterygium and conjunctival cell lines (Ma et al., 2020). SARS-CoV-2 infection through the ocular surface route should be possible.

Hydroxychloroquine was initially approved to treat the patients with malaria and autoimmune diseases due to its immunodulatory capacity (Martinez et al., 2020). For COVID-19, hydroxychloroquine has been shown effectively reducing the copy number of SARS-CoV-2 (Wang et al., 2020a). It was also shown to disrupt the binding of S protein to ACE2 and prevent virus entry and infection *in vitro* (Vincent et al., 2005). Accordingly, hydroxychloroquine has been applied in COVID-19 treatment due to its potential blocking the ACE2 signaling (Braz et al., 2020; Wang et al., 2020b). However, hydroxychloroquine possesses side effects and induce retinopathy (Ozawa et al., 2021). Whether hydroxychloroquine would be useful to alter SARS-CoV-2 receptor expression in ocular surface cells remains elusive. Herein, this study aimed to determine the protein expression of SARS-CoV-2 receptors, ACE2, TMPRSS2 and NRP1, in human primary pterygium and conjunctival cells after hydroxychloroquine treatment. Its effects on cell viability and oxidative stress were also evaluated.

2. Material and methods

2.1. Human primary pterygium and conjunctival cell culture

The study protocol was approved by the Ethics Committee for Human Medical Research at Joint Shantou International Eye Center of Shantou University and the Chinese University of Hong Kong (approval number: EC20200403(2)-P10), which is in accordance with the tenets of the Declaration of Helsinki. Written informed consent was obtained from all study subjects before inclusion into the study. Human primary pterygium ($n = 3$) and conjunctival ($n = 3$) cell lines have been established and characterized as previously described (Yang et al., 2019a, 2021). Briefly, full-length primary pterygium tissues from the affected eyes as well as conjunctiva tissues from contra-lateral unaffected eyes were freshly collected from pterygium excision surgery with conjunctival autograft. The collected tissues were individually first digested with 50 μg/ml dispase (Gibco®, Rockville, MD) and 100 mM D-sorbitol (Sigma-Aldrich) in Dulbecco's Modified Eagle Medium (DMEM; Gibco®) at 37 °C for 1 h, and further dissociated into single cells by 0.05% trypsin at 37 °C for 5 min with the cell strainer (40 μm, Nunc™). The isolated cells were cultured in advanced DMEM medium supplemented with 5% fetal bovine serum (FBS; Gibco®) and 1x penicillin-streptomycin (Gibco®) in 5% CO_2 incubator at 37 °C. The medium was changed in every 2–3 days. The cells with passage 3 were used in this study. The identity of the isolated cells was confirmed by the immunofluorescence analysis with the markers of conjunctival epithelium (cytokeratin 13, CK13; ab16112, Abcam, Cambridge, the United Kingdom), goblet cell (mucin-5, MUC5; ab3649, Abcam) and fibroblast (vimentin; ab8978, Abcam). Each experiment was performed in 3 primary pterygium and 3 conjunctival cell lines.

2.2. Expression of SARS-CoV-2 receptor protein and in human primary pterygium and conjunctiva tissues and cells

The expression of ACE2, TMPRSS2 and NRP1 in human primary pterygium and conjunctiva tissues and cells were evaluated by immunofluorescence and immunoblotting analyses. For the immunofluorescence analysis, human primary pterygium ($n = 3$) and conjunctiva ($n = 3$) tissues, freshly collected from surgical removal, were fixed with 4% paraformaldehyde in PBS at 4 °C for overnight. After fixation, the tissues were cryoprotected with 10–30% sucrose gradient in PBS. The tissues were sectioned (10 μm) using a cryostat (Leica, Wetzlar, Germany). The tissue sections were blocked and permeabilized with 10% normal goat serum (Biodesign, Saco, ME) and 0.2% Triton® X-100 solution (Sigma-Aldrich, St. Louis, MO) in PBS at room temperature for 1 h, and incubated with rabbit anti-ACE2 (ab272500; Abcam), anti-TMPRSS2 (ab92323; Abcam) or anti-NRP1 antibody (ab81321; Abcam) at 4 °C for overnight, followed by incubating with Alexa Fluor 555-conjugated anti-rabbit IgG antibody (Thermo Fisher Scientific, Waltham, MA) at room temperature for 1 h. The staining without addition of primary antibody was served as negative control. Rat lung tissue sections and human lung epithelial carcinoma A549 cells (catalogue number: CCL-185, American Type Culture Collection, Manassas, VA) were served as positive control. The stained tissue sections were imaged using a confocal microscope (TCS SP5 II, Leica). Co-immunostaining analysis was performed with the cell identity markers to confirm the cell types expressing ACE2, TMPRSS2 and NRP1 proteins. For each tissue sample, 5 sections were imaged.

For the immunoblotting analysis, human primary pterygium ($n = 3$) and conjunctiva ($n = 3$) tissues and cell lines were lysed with the radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) supplemented with 1:100 protease and phosphotase inhibitor cocktail (Thermo Fisher Scientific). Total protein concentrations of the cell lysates were measured by the Protein assay (BioRad, Hercules, CA). Equal amount of total protein (30 μg), after 95 °C denaturation, was resolved in SDS-polyacrylamide gel and electro-transferred to nitrocellulose membranes for probing with primary antibodies against ACE2 (ab108252; Abcam), TMPRSS2 or NRP1 followed by respective horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA). The enhanced chemiluminescence signals (Thermo Fisher Scientific) were detected by the ChemiDocTM XRS^+ system (BioRad). β-actin was used as the housekeeping protein for normalization.

2.3. Hydroxychloroquine treatment

Hydroxychloroqiune (catalog number: H0915; Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich). Pterygium and conjunctival cells were treated with 1, 10, 25, 100 and 500 μM hydroxychloroquine in serum-supplemented advanced DMEM medium for 1–2 days. The cells treated with 0.1% DMSO in advanced DMEM medium were considered as the vehicle control.

2.4. Acute toxic effect of hydroxychloroquine treatment

Acute toxic effect on pterygium and conjunctival cells after hydroxychloroquine treatment was examined by the Cell Counting Kit-8 (CCK-8) assay (Sigma-Aldrich). Briefly, 10,000 cells per well (3 wells for each treatment group) were seeded on the 96-well plates (Corning Life Sciences, Lowell, MA) one day before the start of the treatments, and treated with 1, 10, 25, 100 and 500 μM hydroxychloroquine for 24 h. The cells treated with 0.1% DMSO in advanced DMEM medium were considered as the vehicle control. The CCK-8 assay was performed after 1-day treatment. The CCK-8 signal was measured at the wavelength of 450 nm by a plate reader (Powerwave XS, Bio-Tek Instruments, Winooski, VT). The percentage of cell survival was calculated by the equation of (OD_{450} at Day 1)/(OD_{450} at Day 0) x 100%.

2.5. Expression of SARS-CoV-2 receptor and oxidative stress marker proteins in human primary pterygium and conjunctival cells after hydroxychloroquine treatment

The expressions of SARS-CoV-2 receptors (ACE2, TMPRSS2 and NRP1) as well as oxidative stress marker (SOD2) proteins in human primary pterygium and conjunctival cells after hydroxychloroquine treatment were evaluated by the immunoblotting analysis. Briefly, 2 ×

10^5 cells per dish were seeded on the 60-mm dishes (Corning Life Sciences) one day before the start of the treatments and treated with 10 μM hydroxychloroquine for 2 days. After the 2-day treatment, the treated cells were lysed with the RIPA buffer (Sigma-Aldrich) supplemented with 1:100 protease and phosphotase inhibitor cocktail (Thermo Fisher Scientific). Total protein concentrations of the cell lysates were measured by the Protein assay (BioRad). Equal amount of total protein (30 μg), after 95 °C denaturation, was resolved in SDS-polyacrylamide gel and electro-transferred to nitrocellulose membranes for probing with primary antibodies against ACE2, TMPRSS2, NRP1 or SOD2 (#13141; Cell Signaling Technology) followed by respective horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology). The enhanced chemiluminescence signals (Thermo Fisher Scientific) were detected by the ChemiDocTM XRS$^+$ system (BioRad). β-actin was used as the housekeeping protein for normalization.

2.6. Reactive oxygen species level in human primary pterygium and conjunctival cells after hydroxychloroquine treatment

Intracellular reactive oxygen species (ROS) level was determined using the CellROX™ Orange Reagent (Invitrogen, Waltham, MA) according to the manufacturer's instructions. Briefly, 2×10^4 cells per well (3 wells for each treatment group) were seeded in a 24-well plate one day before the start of the treatments and treated with 10 μM hydroxychloroquine for 2 days. The cells treated with 0.1% DMSO in advanced DMEM medium were considered as the vehicle control. After the 2-day treatment, the medium was replaced by the CellROX™ Orange Reagent at a final concentration of 5 μM and incubated at 37 °C for 30 min. After PBS washes, the cells were incubated with DAPI nuclear counterstain at room temperature for 30 min. The stained cells were imaged under a confocal microscope (TCS SP5 II, Leica). The corrected total cell fluorescence of each cells was measured using the ImageJ software (version 1.47; NIH, Bethesda, MD). For each cell lines, 100 cells were used in the measurement.

2.7. Statistical analysis

The experimental data was presented as mean ± standard deviation (SD). Mean of the 3 tissues or cell lines was compared by one-way analysis of variance (ANOVA) with post-hoc LSD test for multiple testing correction. All statistical analyses were performed by the commercially available software (IBM SPSS Statistics 21; SPSS Inc., Chicago, IL). Significance was defined as $p < 0.05$.

3. Results

3.1. Expression of ACE2, TMPRSS2 and NRP1 in human primary pterygium and conjunctival tissues and cells

Immunoblotting analysis demonstrated that ACE2, TMPRSS2 and NRP1 proteins were all found to be expressed in human primary pterygium and conjunctiva tissues (Fig. 1A) as well as in human lung epithelial carcinoma A549 cells (Supplementary Fig. 1A). Similar patterns of ACE2, TMPRSS2 and NRP1 protein expression were also found in the isolated primary pterygium and conjunctival cells (Fig. 1B). Strong immunofluorescence signals of ACE2, TMPPRSS2 and NRP1 were all found in A549 cells and rat lung tissue (Supplementary Figs. 1B and 1C). In contrast, strong signal of TMPRSS2 and moderate signal of NRP1 were observed in the epithelium and stroma of primary pterygium and conjunctiva tissues, and comparatively weaker ACE2 signal was only detected in the stroma of conjunctiva as well as the stroma and epithelium of primary pterygium tissues (Fig. 1C and D). Co-staining analysis demonstrated that ACE2, TMPRSS2 and NRP1 signals were co-localized in the isolated primary pterygium and conjunctival cells expressing vimentin (Fig. 2A and B), which did not express CK13 and MUC5 (Supplementary Figs. 2A and 2B). Collectively, our results validated the expression of SARS-CoV-2 receptors in human primary pterygium and conjunctiva tissues as well as their isolated fibroblasts, suggesting their susceptibility for SARS-CoV-2 infection.

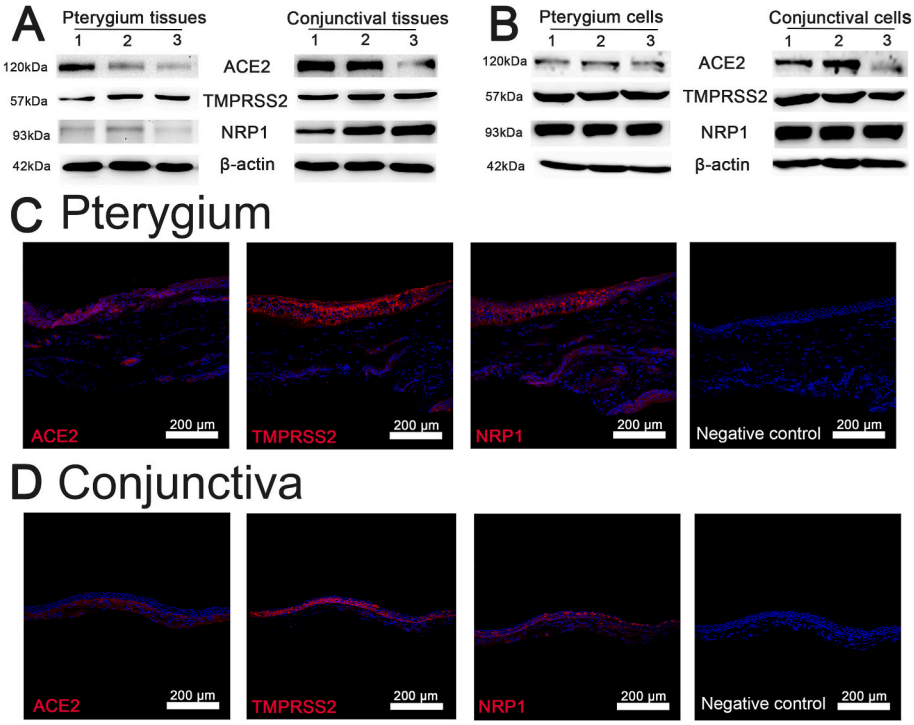

Fig. 1. Expression of SARS-CoV-2 receptor in human primary pterygium and conjunctival tissues and cells. (A–B) Immunoblotting analysis on ACE2, TMPRSS2 and NRP1 protein expression in human primary pterygium and conjunctival (A) tissue and (B) cells. β-actin was used as the housekeeping protein for normalization. (C–D) Immunofluorescence analysis of ACE2, TMPRSS2 and NRP1 protein expression (red) in human (C) primary pterygium and (D) conjunctival tissues. DAPI (blue) as nucleus counter-stain. Negative control was the sections without addition of primary antibodies. Scale bar: 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

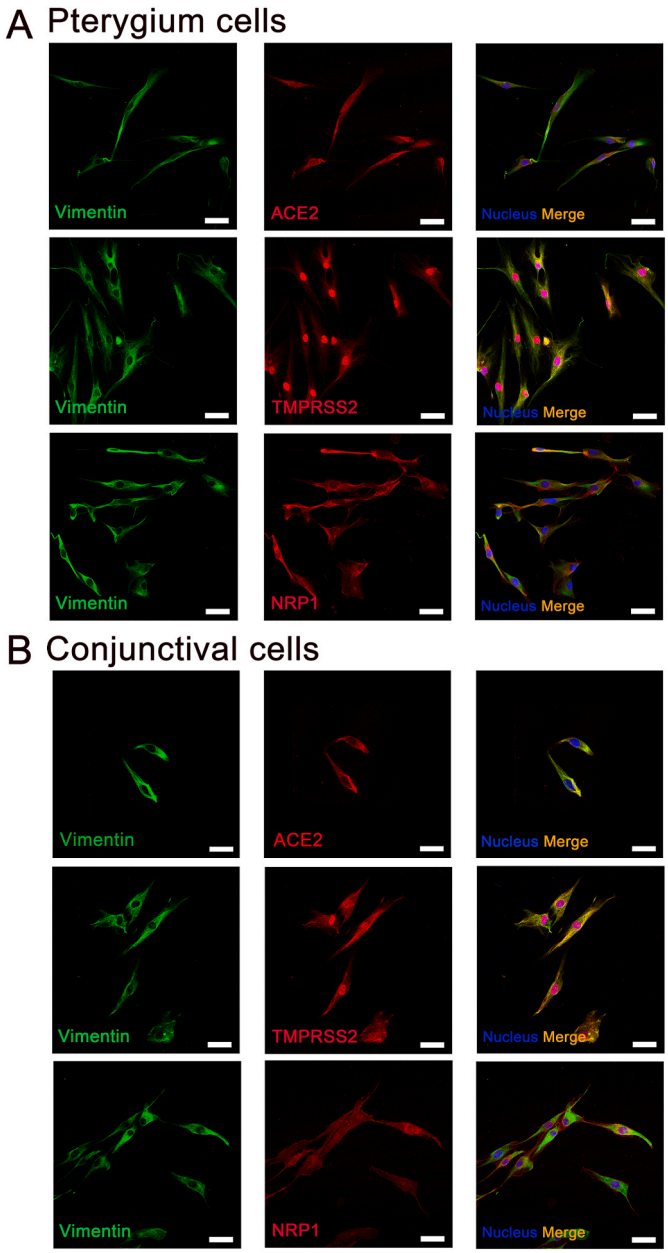

Fig. 2. Immunofluorescence analysis of ACE2, TMPRSS2 and NRP1 expression in isolated human primary pterygium and conjunctival cells. Co-staining of ACE2, TMPRSS2 and NRP1 protein (red) with fibroblast marker (vimentin; green) in isolated (A) primary pterygium and (B) conjunctival cells. DAPI (blue) as nucleus counter-stain. Scale bar: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Hydroxychloroquine treatment on human primary pterygium and conjunctival cell viability

The dosages of hydroxychloroquine in clinical treatment vary from different diseases (Catteau et al., 2020; Liu et al., 2019). To elucidate the acute toxic effect of hydroxychloroquine on human primary pterygium and conjunctival cells, we first analyzed their cell viability after hydroxychloroquine treatment. The CCK8 assay demonstrated that, upon 25–500 μM hydroxychloroquine treatment, the survival of pterygium cells was significantly and dose-dependently reduced by 64.16%–98.99% as compared to the vehicle (0.1% DMSO)-treated cells ($p < 0.001$; Fig. 3A). Treatment of 1 (93.50 ± 13.64%, $p = 0.583$) and 10 μM hydroxychloroquine (90.37 ± 17.34%, $p = 0.435$) did not significantly influence the survival of pterygium cells as compared to the vehicle control group (100.87 ± 12.97%). Similarly, the survival of conjunctival cells was significantly and dose-dependently decreased by 71.64%–98.50% as compared to the vehicle-treated cells ($p < 0.001$; Fig. 3B). Conjunctival cells treated with 1 (90.21 ± 17.17%, $p = 0.515$) and 10 μM hydroxychloroquine (93.53 ± 19.83%, $p = 0.758$) showed no statistically significant differences as compared to the vehicle control group (96.50 ± 13.75%). Our results suggest that exposure of ≥25 μM hydroxychloroquine would reduce both primary pterygium and conjunctival cell viability. To examine the subsequent influences, 10 μM hydroxychloroquine treatment was selected for further investigations.

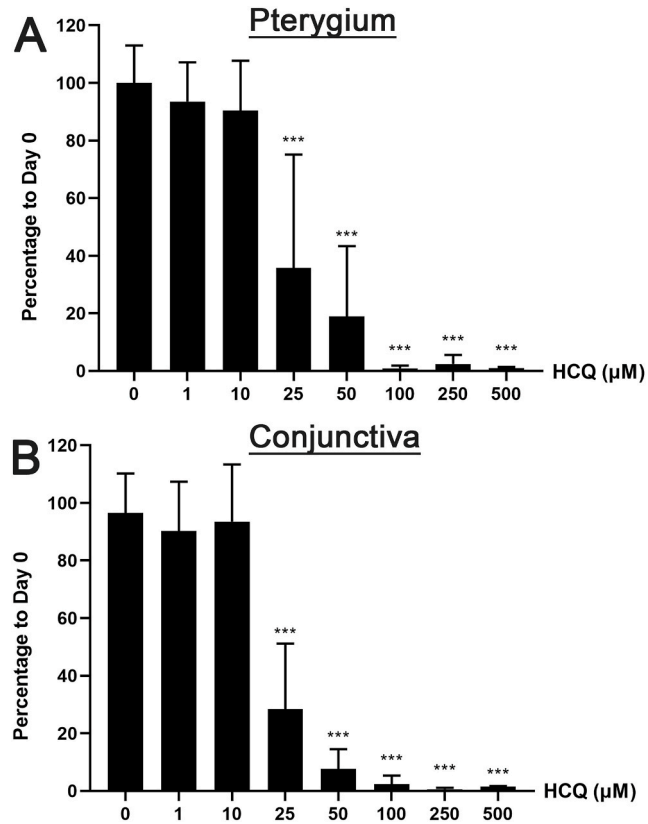

Fig. 3. Survival analysis on human primary pterygium and conjunctival cells after hydroxychloroquine treatment. (A) Pterygium and (B) conjunctival cells were treated with 1–500 μM hydroxychloroquine (HCQ) in serum-supplemented medium for 24 h and compared to the vehicle control group (0.1% DMSO). Cell survival was assessed by the CCK-8 assay after 1-day treatment. The data was presented as the mean of 3 pterygium or conjunctival cell lines ±standard deviation. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3.3. Hydroxychloroquine treatment on SARS-CoV-2 receptor protein expression in human primary pterygium and conjunctival cells

Hydroxychloroquine has been applied to treat patient with COVID-19 for its capacity of interfering the connection of SARS-CoV-2 and ACE2 (McKee et al., 2020). We aimed to evaluate the protein expression of SARS-CoV-2 receptors in human primary pterygium and conjunctival cells after hydroxychloroquine treatment. Immunoblotting analysis showed that the expression of ACE2 protein was significantly decreased by 24% in pterygium cells ($p < 0.001$) and 28% in conjunctival cells ($p = 0.008$) after 2-day 10 μM hydroxychloroquine treatment as compared to the vehicle-treated cells (Fig. 4A and B). Moreover, the expression of NRP1 protein was also found to be significantly reduced by 18% in conjunctival cells after 2-day 10 μM hydroxychloroquine treatment as compared to the vehicle control group ($p < 0.001$; Fig. 4A and C). In

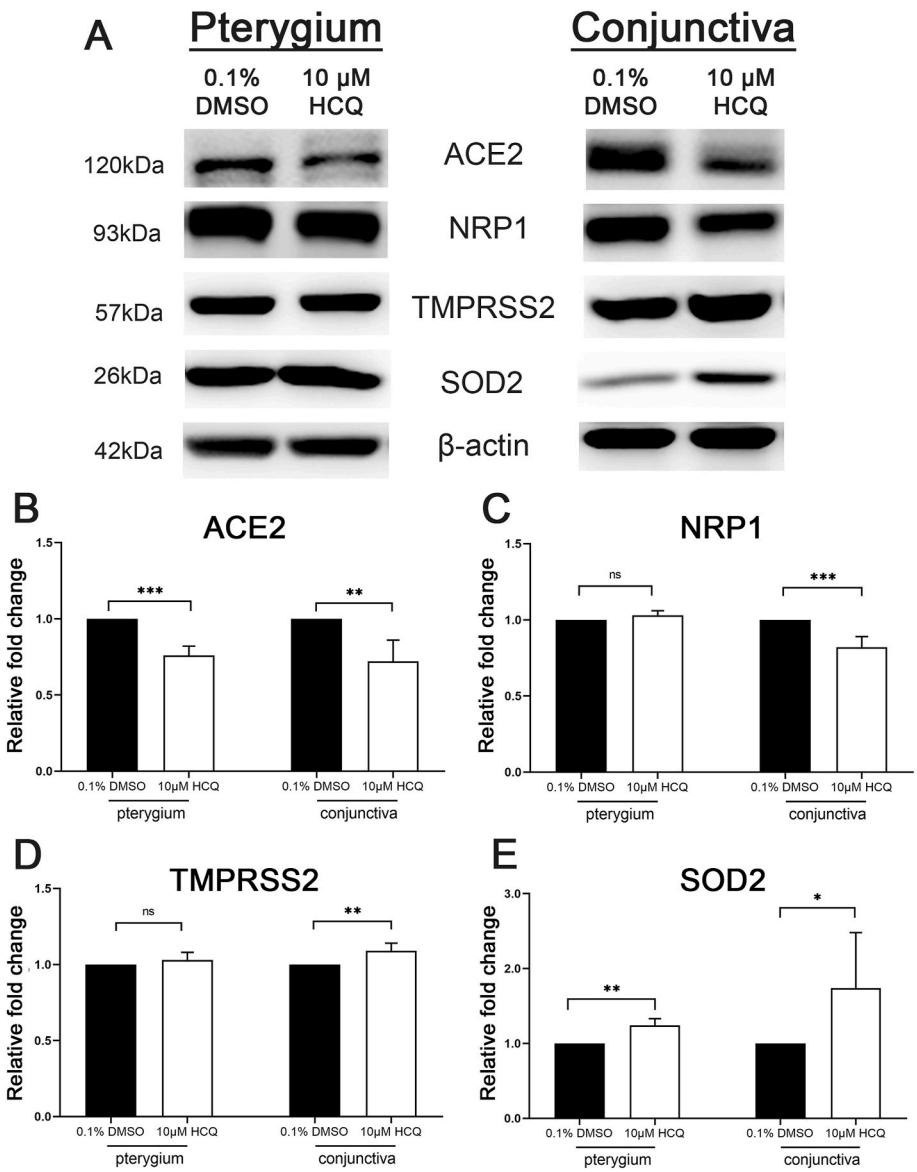

Fig. 4. Expression of SARS-CoV-2 receptor and oxidative stress marker proteins in human primary pterygium and conjunctival cells after hydroxychloroquine treatment. (A) Immunoblotting analysis of pterygium and conjunctival cells treated with 10 μM hydroxychloroquine (HCQ) in serum-supplemented medium for 2 days and compared to the vehicle control group (0.1% DMSO). Relative expression of (B) ACE2, (C) TMPRSS2, (D) NRP1 and (E) SOD2 as compared to the vehicle (0.1% DMSO)-treated cells. β-actin was used as the housekeeping protein for normalization. The data was presented as the mean of 3 pterygium or conjunctival cell lines ±standard deviation. ns: not significant; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

contrast, the expression of TMPRSS2 protein was slightly but significantly increased by 9% in conjunctival cells after hydroxychloroquine treatment compared to the vehicle-treated cells ($p = 0.004$; Fig. 4A and D). Our results indicated that non-lethal dosage of hydroxychloroquine treatment could reduce the expression of ACE2 and NRP1 in conjunctival cells, suggesting its potential to reduce SARS-CoV-2 infection.

3.4. Hydroxychloroquine induced oxidative stress in human primary pterygium and conjunctival cells

Hydroxychloroquine has been reported to induce oxidative DNA damage in mouse embryonic fibroblasts and human hepatocellular carcinoma cells (Besaratinia et al., 2021; Chen et al., 2021). We aimed to evaluate the oxidative stress status of pterygium and conjunctival cells after hydroxychloroquine treatment by determining the expression of SOD2 (an oxidative stress marker (Yang et al., 2019b); and the cellular ROS levels. Immunoblotting analysis demonstrated that the expression of SOD2 protein was significantly upregulated by 24% in pterygium cells ($p = 0.002$) and 74% in conjunctival cells ($p = 0.049$) after 2-day 10 μM hydroxychloroquine treatment as compared to the vehicle-treated cells (Fig. 4A and E). In addition, CellROX™ Orange oxidative stress analysis illustrated that the ROS level in hydroxychloroquine-treated pterygium cells (21.67 ± 5.47) was 2.53-fold higher than that in the vehicle-treated cells (8.55 ± 3.00, $p = 0.006$; Fig. 5A and C). Similarly, the ROS level in hydroxychloroquine-treated conjunctival cells (20.84 ± 6.42) was 4.79-fold higher than that in the vehicle-treated cells (8.55 ± 3.00, $p = 0.003$; Fig. 5B and C). Collectively, our results confirmed that hydroxychloroquine treatment could induce cellular oxidative stress in human primary pterygium and conjunctival cells.

4. Discussion

Results of this study show that: (1) human primary pterygium and conjucntival tissues and fibroblasts express SARS-CoV-2 receptors ACE2, TMPRSS2 and NRP1; (2) high concentration (≥25 μM) of hydroxychloroquine could reduce primary pterygium and conjunctival cell viability; (3) hydroxychloroquine could significantly reduce the expression of ACE2 and NRP1 in conjunctival cells, but slightly increase the expression of TMPRSS2; (4) hydroxychloroquine could significantly increase cellular ROS levels and SOD2 expression in primary pterygium and conjunctival cells after hydroxychloroquine treatment. Collectively, our results indicated that low concentration (≤10 μM) of

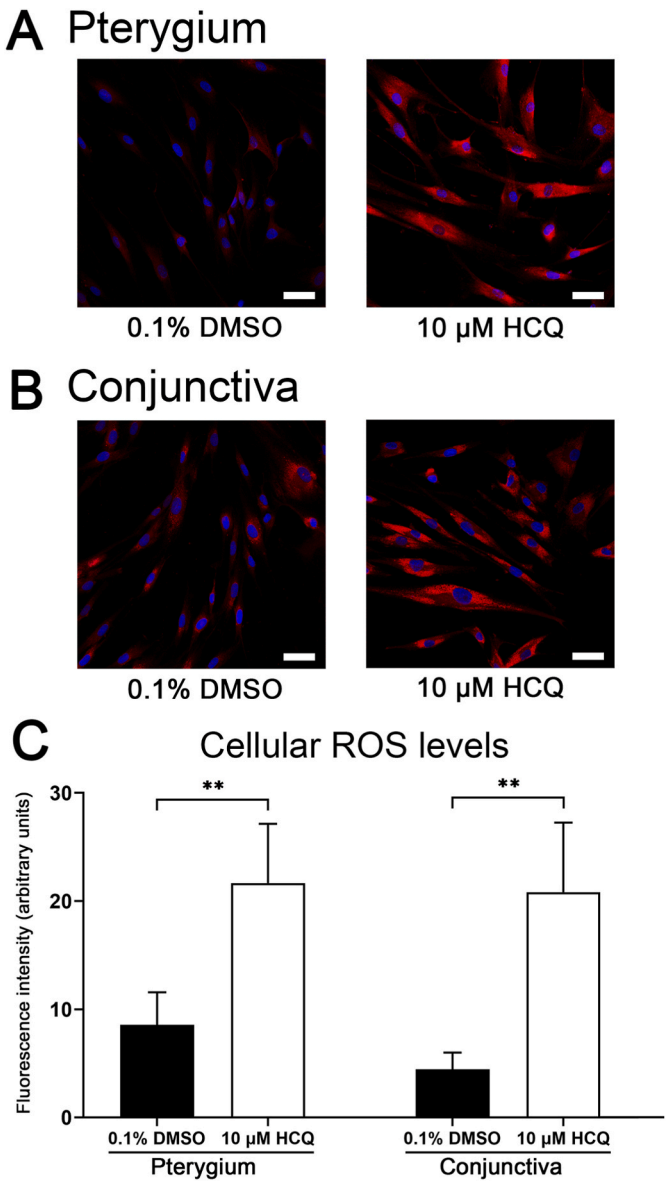

Fig. 5. Reactive oxygen species level in human primary pterygium and conjunctival cells after hydroxychloroquine treatment. CellROX™ Orange-stained (A) pterygium and (B) conjunctival cells treated with 10 μM hydroxychloroquine (HCQ) or vehicle (0.1% DMSO) in serum-supplemented medium for 2 days. Scale bar: 50 μm. (C) Quantification of fluorescence intensity in CellROX™ Orange-stained pterygium and conjunctival cells. The data was presented as the mean of 3 pterygium or conjunctival cell lines ±standard deviation. $^{**}p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

hydroxychloroquine could reduce SARS-CoV-2 receptor ACE2 expression but inducing oxidative stress in conjunctival fibroblasts without influencing cell viability.

SARS-CoV-2 shares 85% identity to the genome of a bat SARS-like coronavirus (bat-SL-CoVZC45, MG772933.1) (Zhu et al., 2020). Both SARS-CoV and SARS-CoV-2 invade the host cells primarily via binding to the host receptor ACE2 (Wu et al., 2020a; Zhou et al., 2020), which is expressed in multiple human cell types and organ systems and plays a critical role in blood pressure regulation (Sun et al., 2021). In addition, TMPRSS2 was reported to be responsible for S protein priming, which is also necessary for ACE2 entry (Bestle et al., 2020). TMPRSS2 inhibitor could block SARS-CoV-2 infection in lung cells (Hoffmann et al., 2020). Recently, NRP1 was identified to be another host factor for SARS-CoV-2 infection, which is abundantly expressed in the respiratory and olfactory (Daly et al., 2020). The fragment of S protein could directly interact with NRP1. Co-expressing with ACE2 and TMPRSS2, NRP1 could promote the infection and spreading of SARS-CoV-2 (Cantuti-Castelvetri et al., 2020). Understanding the expression of SARS-CoV-2 receptors ACE2, TMPRSS2 and NRP1 should be able to predict the possibility of SARS-CoV-2 entry and infection in human system.

We previously reported inconsistent mRNA expression of *ACE2* gene in human primary pterygium and conjunctival cells (Ma et al., 2020). There are discrepancy in ACE2 expression among different studies. No ACE2 signal was found in conjunctiva tissue from human without SARS-CoV-2 infection (Lange et al., 2020). On the contrary, strong or weak expression of ACE2 was observed in the epithelium layer of conjunctiva (Mencucci et al., 2021). In this study, weak ACE2 protein expression signal in the stroma of conjunctiva as well as the stroma and epithelium of primary pterygium tissues (Fig. 1). Moreover, the ACE2 signal was co-localized with vimentin, the marker of fibroblasts, in the isolated primary pterygium and conjunctival cells (Fig. 2), further confirming the observation of ACE2 expression in the stromal layer of primary pterygium and conjunctival tissues. Besides, our previous study also reported that lack of mRNA expression of *TMPRSS2* genes in human primary conjunctival cells in RT-PCR analysis, but weak expression in SYBR green PCR analysis (Ma et al., 2020). Instead, this study demonstrated strong expression of TMPRSS2 protein in human conjunctival tissues and cells (Fig. 1). The inconsistent results among our previous gene expression analysis as well as different studies could be related to the ACE2 antibody applied and the conjunctival samples from the study subjects as well as genetic and epigenetic controls on ACE2 and TMPRSS2 protein expression regulation. However, considering the necessity of both ACE2 and TMPRSS2 for SARS-CoV-2 infection, the stromal expression of ACE2 and strong epithelial and weak stromal expression of TMPRSS2 in human conjunctival tissues (Fig. 1D) further indicated that conjunctiva could less likely be susceptible for SARS-CoV-2 infection. Nevertheless, we, for the first time, identified NRP1 protein expression in human primary pterygium and conjunctival epithelial cells.

COVID-19 pandemic is still ongoing in the world (Khan et al., 2020). It is warranted to develop effective therapeutic treatments against COVID-19. One possible strategy raised to reduce SARS-CoV-2 entry by interfering its connection with the host receptors. Hydroxychloroquine possesses anti-myriad and immunomodulatory capacities, and it has been commonly used for malaria and autoimmune diseases, including rheumatoid arthritis (Lim et al., 2009; Martinez et al., 2020). Moreover, hydroxychloroquine has been shown to interact with ACE2 terminal glycosylation *in vitro*, disrupting the connection between ACE2 and SARS-CoV-2 (Biot et al., 2006; Brufsky, 2020). The patients with COVID-19, receiving combined treatment of hydroxychloroquine and azithromycin, showed significant reduction in viral carriage (Gautret et al., 2020). Low dose of hydroxychloroquine has been reported effectively reducing the mortality rate of COVID-19 hospitalized patients as compared to those only with supportive care (Catteau et al., 2020). Yet, COVID-19 patients received 800 mg loading dose of hydroxychloroquine and 400 mg daily for 9 days did not show significant differences in viral clearance and symptom resolution as compared to the placebo group (Reis et al., 2021). Besides, post-exposure therapy with hydroxychloroquine also did not limit the SARS-CoV-2 infection in healthy persons (Mitja et al., 2021). In this study, we further provided a possible mechanism for hydroxychloroquine against COVID-19 that the expressions of ACE2 and NRP1 proteins were significantly downregulated in human conjunctival cells after hydroxychloroquine treatment but TMPRSS2 protein expression showed slightly increase (Fig. 4A–D). As hydroxychloroquine could not block SARS-CoV-2 entry mediated by TMPRSS2 (Ou et al., 2021), the net effect of hydroxychloroquine on viral infectivity still needs to be further evaluated in future studies. Combination of hydroxychloroquine with TMPRSS2 inhibitor could be considered to prevent SARS-CoV-2 infection than

hydroxychloroquine alone (Ou et al., 2021).

Hydroxychloroquine would induce cellular oxidative stress (Besaratinia et al., 2021; Chen et al., 2021). We confirmed that hydroxychloroquine could increase cellular ROS level (Fig. 5) and upregulate the expression of SOD2 protein in both human primary pterygium and conjunctival cells (Fig. 4E), the marker of oxidative stress (Yang et al., 2019b). Apart from oxidative stress induction, we demonstrated that high dose (≥25 μM) of hydroxychloroquine could reduce the viability of human primary pterygium and conjunctival cells (Fig. 3), which is coherent to the results from other studies (Yang et al., 2020). Besides, application of hydroxychloroquine could have potential to induce hydroxychloroquine retinopathy (Ozawa et al., 2021; Yusuf et al., 2017). Whether hydroxychloroquine would be suitable for COVID-19 treatment is still controversial (Bakadia et al., 2021; Guastalegname and Vallone, 2020).

In summary, this study revealed that hydroxychloroquine treatment could reduce the expression of SARS-CoV2 receptors ACE2 and NRP1 proteins in human conjunctival fibroblasts; yet, it could induce oxidative stress and reduce cell viability. Implementation of hydroxychloroquine as COVID-19 treatment should be carefully considered with its action and potential side effects.

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Author contributions

V.J. and T.K.N. conception and design. T.K.N. financial support. D. M., V.J. and T.K.N. provision of study materials. Y.Y., Y.X., X.L.Y., J.J.L. and S.L.C. collection and/or assembly of data. Y.Y., Y.X. and T.K.N. data analysis and interpretation. Y.Y., V.J. and T.K.N. manuscript writing.

Declaration of competing interest

The authors declare that they have no potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exer.2021.108864.

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