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CLINICAL FEATURE
REVIEW

Advances in the use of chloroquine and hydroxychloroquine for the treatment of COVID-19

JingKang Sun [a,b], YuTing Chen [a,b], XiuDe Fan[b], XiaoYun Wang[b], QunYing Han[b] and ZhengWen Liu[b]

[a]Xi'an Medical University, Xi'an, China; [b]Department of Infectious Diseases, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China

ABSTRACT

Coronavirus Disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is spreading worldwide. Antiviral therapy is the most important treatment for COVID-19. Among the drugs under investigation, anti-malarials, chloroquine (CQ) and hydroxychloroquine (HCQ), are being repurposed as treatment for COVID-19. CQ/HCQ were shown to prevent receptor recognition by coronaviruses, inhibit endosome acidification, which interferes with membrane fusion, and exhibit immunomodulatory activity. These multiple mechanisms may work together to exert a therapeutic effect on COVID-19. A number of *in vitro* studies revealed inhibitory effects of CQ/HCQ on various coronaviruses, including SARS-CoV-2 although conflicting results exist. Several clinical studies showed that CQ/HCQ alone or in combination with a macrolide may alleviate the clinical symptoms of COVID-19, promote viral conversion, and delay disease progression, with less serious adverse effects. However, recent studies indicated that the use of CQ/HCQ, alone or in combination with a macrolide, did not show any favorable effect on patients with COVID-19. Adverse effects, including prolonged QT interval after taking CQ/HCQ, may develop in COVID-19 patients. Therefore, current data are not sufficient enough to support the use of CQ/HCQ as therapies for COVID-19 and increasing caution should be taken about the application of CQ/HCQ in COVID-19 before conclusive findings are obtained by well-designed, multi-center, randomized, controlled studies.

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1. Introduction

Coronavirus disease-19 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is rapidly spreading worldwide, resulting in the third outbreak of coronaviruses in the 21st century. The pandemic of COVID-19 constitutes a serious threat to the whole world [1]. To control the pandemic of COVID-19, effective and easily accessible antiviral drugs and vaccines are urgently needed, in addition to the implementation of epidemiological measures such as strict quarantine. However, until now, no drugs have been demonstrated to be effective against COVID-19. Among the various drugs under investigation are repurposed anti-malarial drugs chloroquine (CQ) and its analog hydroxychloroquine (HCQ), which are among the most used drugs because they are easy to obtain and have a proven favorable safety record at relatively low cost. CQ/HCQ are derivatives of 4-aminoquinoline. They are lipophilic weak bases that quickly pass across cell membranes and accumulate in acidic organelles, such as lysosomes, endoplasmic reticulum and Golgi [2]. CQ/HCQ are used to treat and prevent malaria attacks due to their anti-plasmodium activity and to treat autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) owing to their immunomodulatory activity [3]. In addition, CQ/HCQ display antibacterial, antifungal and antiviral activities [4]. *In vitro* studies have shown that CQ/HCQ possess antiviral activity against RNA viruses, such as HIV [5], rabies virus [6] and polio virus [7] and various DNA viruses as diverse as hepatitis B virus [8] and herpes simplex virus [9]. This article reviews the current status of CQ/HCQ against SARS-CoV-2 and their use in the treatment of COVID-19.

2. Antiviral activity of CQ/HCQ against human coronavirus *in vitro* and *in vivo*

The antiviral activity of CQ against MERS-CoV and HCoV-229E was assessed in the human hepatoma cell line (Huh-7) and found that the 50% effective concentrations (EC_{50}) were 3.0(±1.1) μM and 3.3 (±1.2) μM, the 50% cytotoxic concentrations (CC_{50}) were 58.1(±1.1) μM and >50, and the selectivity indexes (SI; calculated as CC50/EC50) were 19.4 and >15, respectively [10]. CQ could inhibit an early step in the MERS-CoV replication cycle. Addition of CQ to VeroE6 cells 1 h after MERS-CoV infection did not affect virus production. However, when CQ was added 1 h before MERS-CoV infection, 16 μm and 32 μM concentrations of CQ could reduce the virus production of 1-log and 2-log, respectively [10].

In VeroE6 cells, the antiviral activity of CQ/HCQ against SARS-CoV had an EC_{50} of 4.1 (±1.0) μM and 34(±5) μM, CC_{50} of >128 μM and CC_{50} > 100 and SI of >31 and SI>3, respectively [10,11]. SARS-CoV replication was inhibited by 99% at 16 μM CQ 3 days post-infection [12]. The data indicates that CQ has stronger anti-SARS-

CONTACT Zhengwen Liu, liuzhengwen113@xjtu.edu.cn Department of Infectious Diseases, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province 710061, China

CoV activity than HCQ. In addition, CQ has both a prophylactic and a therapeutic advantage. Vincent et al. tested various concentrations of CQ (0.1–10 μM) added 20–24 h prior to SARS-CoV infection and found that 0.1, 1, and 10 μM CQ reduced infectivity by 28%, 53%, and 100%, respectively; when CQ was added immediately after virus adsorption, 0.1–1 μM and 33–100 μM reduced the infection by 50% up to 90–94%; addition of CQ 3 and 5 h after virus adsorption was still significantly effective, yet to achieve equivalent antiviral effect, a higher concentration of CQ was needed [13]. In HRT-18 cells, the antiviral activity of CQ against HCoV-OC43 had an EC_{50} of 0.306 (±0.091) μM, CC_{50} of 419.0 (±192.5) μM, and SI of 1.369 [14].

An *in vivo* study found that CQ could exert anti-HCoV-OC43 activity transplacentally or via maternal milk. The data from mouse models showed that 98.6% of the pups survived when pregnant mice were treated with 15 mg/kg of CQ, and survival rates decreased in a dose-dependent manner, with 88% and 13% survival when treated with 5 mg/kg and 1 mg/kg CQ, respectively [14]. The survival rate of newborn mice via maternal milk was 69.0% with 15 mg/kg of CQ [14]. In another mouse study, CQ strongly attenuated HCoV-OC43 replication in the brain and prevented the infection from spreading to the spinal cord [15]. The above studies confirmed that CQ/HCQ have a broad-spectrum anti-HCoV activity *in vitro* and *in vivo*.

3. Antiviral activity of CQ/HCQ against SARS-CoV-2 *in vitro*

In VeroE6 cells, the EC_{50}, CC_{50} and SI of CQ against SARS-CoV-2 were 1.13 μM, >100 and >88.50, respectively. CQ functioned at the entry, and post-entry stages of SARS-CoV-2 infected cells [16]. In the same cell line, at different multiplicities of infection (MOIs, 0.01, 0.02, 0.2, and 0.8) of SARS-CoV-2, the EC_{50} for CQ (2.71, 3.81, 7.14, and 7.36 μM) was slightly lower than that of HCQ (4.51, 4.06, 17.31, and 12.96 μM). Consequently, the SI of CQ (100.81, 71.71, 38.26, and 37.12) was slightly higher than that of HCQ (55.32, 61.45, 14.41, 19.25) [17]. These results indicate that the anti-SARS-CoV-2 activity of CQ seems to be more potent than HCQ *in vitro*. However, another *in vitro* cell experiment showed that after SARS-CoV-2 infection of VeroE6 cells, the EC_{50} values for CQ were 23.90 μM and 5.47 μM, and EC_{50} values for HCQ were 6.14 μM and 0.72 μM, at 24 and 48 h, respectively; When administered prior to SARS-CoV-2 infection of VeroE6 cells, EC_{50} values for CQ were >100 μM and 18.01 μM, and the EC_{50} values for HCQ were 6.14 μM and 0.72 μM, at 24 and 48 h, respectively [18]. These results showed that the anti-SARS-CoV-2 activity of CQ was worse than HCQ *in vitro*. The conflicting results of these two studies may be related to different cell culture methods and experimental conditions. In short, these *in vitro* studies show that CQ/HCQ have strong anti-SARS-CoV-2 activity.

4. Mechanisms of CQ/HCQ in treating COVID-19

4.1. Antiviral activity

4.1.1. Hindrance of receptor recognition process

The S protein of SARS-CoV-2 is cleaved by host proteases into two subunits, S1 and S2 [19]. The S1 subunit binds to the host cell surface receptor angiotensin-converting enzyme 2 (ACE2) for virus attachment, and the S2 subunit fuses the virus and the host cell membrane [19]. The investigation of the effect of CQ on ACE2 in VeroE6 cells showed that effective anti-SARS-CoV-2 concentrations of CQ had no significant effect on the synthesis and glycosylation of S protein on the surface of SARS-CoV, and although it had no significant effect on the cell surface expression of ACE2, CQ could destroy the glycosylation at the terminal glycosylation site of ACE2 [13]. Therefore, the mechanism of anti-CoV activity of CQ/HCQ may be at least partly related to the impairment of terminal glycosylation of ACE2, which may result in reduced binding affinities between ACE2 and SARS CoV S protein, thereby blocking receptor recognition (Figure 1).

In addition to protein membrane receptors, infection of host cells by HCoVs also relies on sialic acid-containing glycoproteins and gangliosides, which are used by a broad range of viruses as receptors, such as influenza [20] and HCoVs including SARS-CoV [21] and HCoV-OC43 [13,22,23]. A recent molecular structure analysis showed that SARS-CoV-2 not only uses ACE2 as a receptor, but also recognizes highly conserved gangliosides on the host cell surface through sialic acid [24,25]. CQ/HCQ binds sialic acids and gangliosides with high affinity, which can prevent the attachment of SARSCoV-2 S protein to gangliosides [25]. CQ had inhibitory effect on quinone reductase 2 (QR2) involved in the biosynthesis of sialic acids [26,27]. Hence, the mechanism of anti-CoV activity of CQ/HCQ may also be related to hindering the recognition process of sialic acid and ganglioside (Figure 1).

4.1.2. Interference of the membrane fusion process

CoVs are enveloped RNA viruses, and their cell entry processes involve a principal route of receptor-mediated endocytosis [28]. Membrane fusion takes place in the endosomal compartment after endocytosis, which needs additional triggers such as pH acidification or proteolytic activation [29]. Multiple cellular proteases, such as trypsin, furin, proprotein convertase (PC) family, cathepsins, transmembrane protease/serine (TMPRSS) proteases and elastase, are involved in S protein activation, which can induce membrane fusion [30]. Among them, cathepsin L, with anoptimal pH of 3.0 to 6.5, is most commonly associated with activation of a variety of CoV S proteins [30], such as SARS-CoV [19], MERS-CoV [31], HCoV-229E [32], and mouse hepatitis virus 2 (MHV-2) [33]. A recent study found that SARS-CoV-2 enters 293/hACE2 cells mainly through endocytosis, in which cathepsin L is critical for priming of SARS-CoV-2 S protein [24]. A study investigated the detailed mechanism of action of CQ/HCQ in inhibiting SARS-CoV-2 entry, and co-localization of SARS-CoV-2 with early endosomes (EEs) or endolysosomes (ELs) in VeroE6 cells, and the results showed that CQ/HCQ hampered the transport of SARS-CoV-2 from EEs to ELs, indicating that CQ/HCQ might inhibit endosomal maturation [17]. These studies revealed that the mechanism of anti-CoV activity of CQ/HCQ may involve the inhibition of the endosome acidification process, which might inactivate lysosomal proteases, thus interfering with the fusion of virus and host membranes [34,35] (Figure 1).

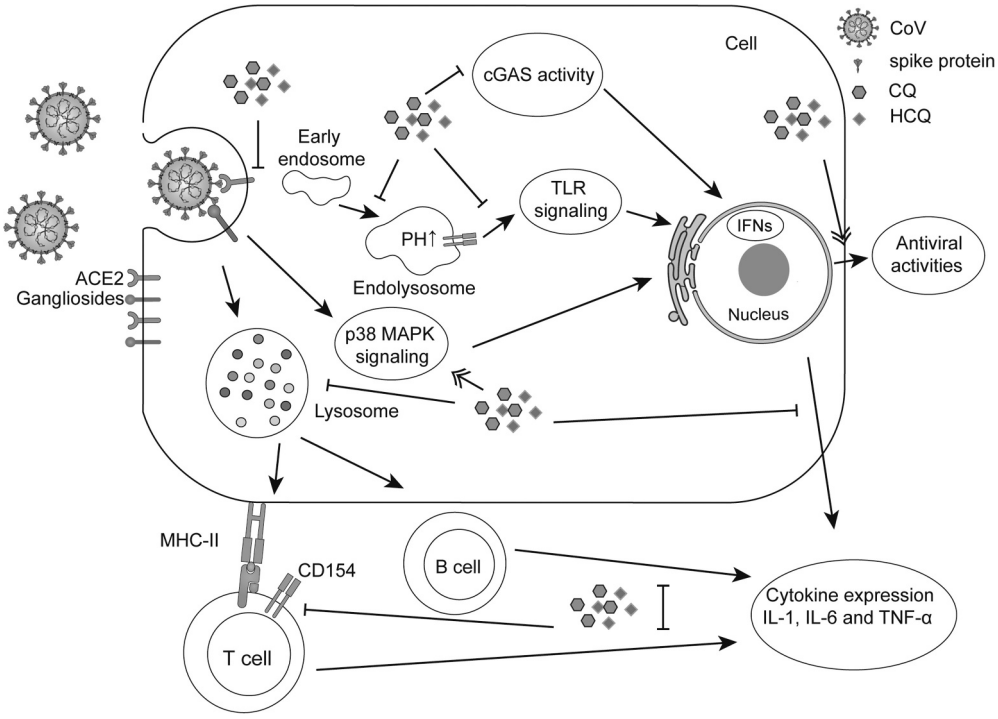

Figure 1. Schematic representation of the possible mechanisms of CQ/HCQ against CoVs replication and modulating immune response. CQ/HCQ may synergistically exert antiviral and immunomodulatory effects on COVID-19 through multiple mechanisms including hindering the receptor recognition process by influencing the affinity of ACE2 and S protein, and the affinity for sialic acid and ganglioside; inhibiting the membrane fusion process by suppressing endolysosome acidification; suppressing the p38 activation and affecting host defense machinery, and preventing MHC class II expression (block expression of CD154 on the surface of CD4 + T cell) and TLR signaling and reducing the production of cytokines through inhibiting the activation of T cells and B cells.

ACE2, angiotensin-converting enzyme 2; COVID-19, coronavirus disease 2019; CQ, chloroquine; HCQ, hydroxychloroquine; CoVs, coronaviruses; MAPK, mitogen-activated protein kinase; MHC-II, major histocompatibility complex class II; TLR, toll-like receptor; cGAS, cyclic GMP-AMP synthase; IFN, interferon; IL, interleukin; TNF-α, tumor necrosis factor-α.

4.1.3. Effects on cell signaling pathway and host defense machinery

The mitogen-activated protein kinase (MAPK) pathway transmits signals from the cell surface to the nucleus involved in the infection of CoVs such as MHV [36] and SARS-CoV [37]. CQ could inhibit HCoV-229E replication in human embryonic lung epithelial cells (L132) through suppressing the activation of p38 MAPK [38]. Moreover, HCQ could markedly induce the production of cellular reactive oxygen species (ROS), which play an important role in the activation of innate immunity [39]. HCQ also could trigger the host defense mechanism through the mitochondrial antiviral signaling (MAVS) pathway, resulting in anti-dengue virus activity [39]. Therefore, CQ/HCQ may also exert their antiviral activity by suppressing the activation of p38 MAPK pathway and affecting the host defense machinery (Figure 1).

4.2. Inhibitory effect on T cell activation and cytokine production

CQ/HCQ regulate the release of various pro-inflammatory factors, which are important immunomodulators. Intracellular alkalinization by CQ/HCQ inhibits lysosomal activity, preventing antigen processing, major histocompatibility complex (MHC) class II expression and immune activation [40]. This process can inhibit T cell activation and block expression of CD154 on the surface of CD4 + T cells [41]. CQ also reduces cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor-α (TNF-α) produced by T cells and B cells [42]. At the same time, changes of endosomal pH can interfere with Toll-like receptor (TLR) signaling, such as TLR7 and TLR9 processing, inhibiting the activation and production of cytokines [43]. CQ/HCQ also weaken the cyclic GMP-AMP (cGAMP) synthase (cGAS) activity by inhibiting cytosolic DNA, thereby reducing type I interferon production [44]. *In vitro*, CQ/HCQ can also inhibit phospholipase A2, altering the metabolism of arachidonic acid, and reducing the production of prostaglandins [45]. Some clinical studies have found that high concentrations of cytokines and pro-inflammatory factors such as IL-6 and IL-10 are elevated in the plasma of critically ill patients infected with SARS-CoV-2 [46,47], suggesting that cytokine release syndrome (CRS) is associated with disease severity. In the aspect of immune response, HCQ/CQ therefore are likely to inhibit CRS, delaying the progression of COVID-19 (Figure 1).

5. Clinical efficacy of CQ/HCQ in the treatment of COVID-19

Only two published clinical reports have studied the efficacy of CQ in COVID-19 patients (Table 1). One study used CQ to treat more than 100 patients with COVID-19 and claimed that CQ was superior to the control group in suppressing the deterioration of pneumonia, improving lung imaging, promoting viral conversion and shortening the course of disease. Serious adverse effects were not observed in these patients

Table 1. Outcomes and advantage/limitation of chloroquine (CQ)/hydroxychloroquine (HCQ) clinical studies for COVID-19.

Author (Reference)	Study design	Patients	Treatment	Outcomes	Advantage	Limitations
Gao et al [48].	Rough and simple description	Not mentioned	CQ (No specific dosage was mentioned)	CQ was superior to control in suppressing pneumonia deterioration, improving lung imaging, promoting viral conversion and shortening disease course.	Not significant.	No study design and the specific number of patients and controls were provided and thus the result appears to be unconvincing.
Borba et al [49].	Randomized controlled trial	High-dose CQ group: n = 40; Low-dose CQ group:n = 41	high-dose CQ (600 mg/2 times/day, for 10 day); low-dose CQ (450 mg/2 times/day for 5 days, double dose on 1st day)	The mortality rate in the high-dose group was more than double that in low-dose group	Double-blind study; 2 dosages of CQ for the first time in severe COVID-19	Small sample size; single-center design; Lack of a placebo control; Lack of exclusion criteria based on the QTc interval at baseline
Chen et al. [50]	Randomized trial	HCQ group: n = 31; control group: n = 30	HCQ (200 mg/2 times/day for 5 days)	HCQ group have small improvement in body temperature and cough compered with control group	Randomized trial.	Small sample size; Single-center design; Small improvement in temperature and cough.
Mahévas et al [53].	Comparative study	HCQ group: n = 84; control group: n = 97	HCQ 600 mg/day for 7 days	Compared with control group, a reduction of admissions to ICU or death 7 days after hospital admission was not observed in HCQ group.	Relatively larger sample size in HCQ treatment and control groups.	Nonrandomized design; In propensity score model, four possible important prognostic variables were unbalanced.
Tang et al [54].	Open label, randomized controlled trial	HCQ group: n = 70; control group: n = 80.	HCQ 1200 mg daily for 3 days, 800 mg daily for 2 weeks (mild to moderate disease)/ 3 weeks(severe disease)	HCQ did not show additional benefits of vial elimination in patients with mild to moderate COVID-19.	Randomized controlled study.	Lack of a placebo control group; Design introduces the possibility of biased investigator determined assessment and unbalanced dosage adjustment; Randomization of sequential envelopes may be biased. The antiviral efficacy of HCQ was not assessed at an earlier stage; Most patients are mild to severe, and the effect of HCQ on disease progression or regression could not be provided. The trial terminated early due to the difficulty to recruit enough patients. Some secondary endpoints could not be analyzed by the cutoff date; Viral RNA specimens are mostly from the upper respiratory tract rather than bronchoalveolar lavage fluid, which may cause false negative results.
Geleris et al [55].	Observational study	HCQ group: n = 811; no-HCQ group: n = 565	HCQ (600 mg/2 times on the first day, then 400 mg once a day for 4 days)	No correlation between the HCQ use and significant higher or lower risk of intubation or death was observed.	Large sample size; Minimization of the unmeasured confusion and error through multivariable Cox model with inverse probability weighting according to the propensity score.	Single-center design; missing of some variables; Potential for inaccuracies in the electronic health records.
Yu et al [56].	Retrospective study	HCQ group: n = 48; no-HCQ group: n = 502	HCQ (200 mg/2 times/day, for 7 to 10 days)	The fatalities of HCQ group was significantly lower than no-HCQ group. HCQ treatment was related to significantly reduced mortality in critically ill COVID-19 patients and greatly lowered IL-6 level.	Mortality was used as a measure of outcome and the study included critically ill patients.	Retrospective design of the study and the number of HCQ group patients was small.
Gautret et al. [57]	Open label, nonrandom cohort study	HCQ group: n = 12; HCQ + azithromycin group: n = 6; control group: n = 12	HCQ (200 mg/3 times/day for 10 days)	HCQ alone or in combination with azithromycin could effectively eliminate nasopharyngeal virus in 3–5 days.	Nasopharyngeal virus determination was used as main endpoint.	Small sample size; Six patients dropped out due to critical illness or intolerance to the drug; Lack of clinical outcomes; Limited follow-up results.

(*Continued*)

Table 1. (Continued).

Author (Reference)	Study design	Patients	Treatment	Outcomes	Advantage	Limitations
Gautret et al [58].	Observational study	HCQ group: n = 80; no control	HCQ, 200 mg/3 times/day for 10 days combined with azithromycin 500 mg on the 1st day, 250 mg/day afterward for 5 days	The nasopharynx viral load in most patients received HCQ decreased rapidly.	Observation of nasopharynx viral load.	Observational study design and no control group; No clinical outcomes were analyzed. Possible confounding factors were not adjusted.
Magagnoli et al [59].	Retrospective study	HCQ group: n = 97 HCQ + azithromycin: n = 113 No HCQ group: n = 158	Not specified.	The use of HCQ, either with or without azithromycin, didn't reduce the risk of mechanical ventilation in patients hospitalized with COVID-19; Patients treated with HCQ alone was associated with increased overall mortality.	The study data comes from a comprehensive electronic medical record; Strictly defined covariates and outcomes; Using propensity scores adjustment for a large number of relevant confounders to make results more persuasive.	Retrospective nature of the study; The subjects included only men and most of them were black; Despite adjustments to many possible confounding factors, there may still be undiscovered factors.
Rosenberg et al [60]	Retrospective multicenter cohort study	HCQ group: n = 271; HCQ + azithromycin group: n = 735 control group: n = 221	HCQ:200 mg/400 mg/ 600 mg/other/unknown, frequency: once a day/ twice a day/other/ unknown azithromycin:200 mg/ 250 mg/400 mg/500 mg/ other/unknown, methods:Oral/IV/ unknown, frequency: only once/ once a day/twice a day/ other/unknown	Treatment with HCQ, azithromycin, or both, compared with neither treatment, was not significantly associated with differences in in-hospital mortality.	This study include a large, random sample from 25 metropolitan New York hospitals. The sample was drawn early in the epidemic to include patients with long, complicated, and ongoing hospital stays; In-hospital mortality was used as primary outcome.	Retrospective study design; There may be missing information; Mortality was limited to in-hospital death; There may be potential confounders. The dosing in the doses and frequencies of HCQ and azithromycin varied greatly. The confidence intervals for some of the findings are wide.
Mehraet al [61]	Multinational real-world analysis	Teatment groups: n = 14,888 Control group: n = 81,144	Mean daily dose: CQ 765 mg, (SD 308); HCQ 596 mg(126); CQ with a macrolide 790 mg(320); HCQ with a macrolide 597 mg(128). mean duration:CQ 6.6 days(2.4); HCQ 4.2 days(1.9);CQ with a macrolide 6.8 days(2.5); HCQ with a macrolide 4.3 days (2.0)	A benefit of HCQ or CQ, when used alone or with a macrolide, on in-hospital outcomes for COVID-19 was not observed.	Large multinational real-world data and large number of study populations.	There may be potential confounders; It did not measure QT intervals and stratify the arrhythmia pattern; It did not determine whether the increased risk of death in-hospital and use of drug treatment regimens were directly related to cardiovascular risk; It did not observe the risk of the drug dose-response analysis.

[48]. However, this report did not provide any details about the study design and patient data, thus it is difficult to evaluate the validity. Recently, a parallel, double-blind, randomized, phase IIB clinical trial was performed in Brazil [49]. In this study, 81 severe COVID-19 patients were randomly divided into two groups: 41 patients received high-dose CQ (600 mg/2 times/day for 10 day) and 40 patients received low-dose CQ (450 mg/2 times on day 1 and then 450 mg/1 time/day for 4 days). The 13-day mortality rate in the high-dose group was more than double that in low-dose group (39.0% *vs*. 16.0%). The high-dosage group exhibited more instance of corrected QT (QTc) interval prolongation (>500 milliseconds (ms); 7 of 37 [18.9%]) than the low-dosage group (4 of 36 [11.1%]). These findings suggest that the higher CQ dosage should not be recommended for critically ill patients with COVID-19 [49].

Several trials evaluated the efficacy of HCQ for the treatment of COVID-19 (Table 1). In a randomized clinical trial from Wuhan about HCQ treatment of mild COVID-19 [50], 31 out of 62 patients received HCQ (200 mg/2 times/day for 5 days). The results showed that the temperature recovery time in the HCQ group was improved compared with the control group (average days, 2.2 *vs*. 3.2); the cough relief time was shorter in the HCQ group than the control group (average days, 2.4 vs. 3.1); and the improvement rate of pneumonia in the HCQ group was higher than the control group (80.6% *vs*. 54.8%). However, only 48% of patients (15/31) in HCQ group and 71% of patients (22/31) in control group had cough at baseline and the duration of cough was not described. Improvement of symptoms were small and the trial was terminated prematurely. These factors and the low sample size compromise the reliability of the results of this study. Importantly, evaluation of HCQ in the COVID-19 pandemic areas have shown that HCQ can help patients with mild symptoms, and may potentially reduce transmission in areas lacking isolation facilities [51,52]. However, in areas with strict isolation standards, the use of HCQ to reduce transmission or for treatment of mild COVID-19 cases may not be beneficial in risk-benefit analysis [51,52]. Nevertheless, there is an urgent need for drugs and therapeutics in severe cases, which require randomized controlled trials. A study from four French tertiary care centers included 181 patients hospitalized for COVID-19 and requiring oxygen (2 L/min): 84 patients received HCQ (600 mg/day) within 48 hours of admission (HCQ group) and 97 did not (no-HCQ group) [53]. The results showed that the patients transferred to the ICU or died within 7 days and developed ARDS within 7 days had no significant differences between the HCQ group and no-HCQ group. Eight patients in the HCQ group (9.5%) discontinued HCQ due to electrocardiogram alterations. These results do not support the use of HCQ for treating hospitalized COVID-19-related hypoxic pneumonia patients. It is worth noting that in the study's propensity score model, four possible important prognostic variables were unbalanced and a center effect was not considered, which all can cause bias for study results. In a multicenter, randomized, parallel trial about HCQ in patients with mainly mild to moderate COVID-19 [54], 80 patients received 'standard care' and 70 patients received HCQ (1200 mg daily for 3 days, and then 800 mg daily for 2 weeks [mild to moderate disease] or 3 weeks [severe disease]). The results showed that the 28-days negative conversion probability in 'standard care' + HCQ group was 85.4%, similar to the 'standard care' group (81.2%). HCQ did not show additional benefits of viral elimination in patients with mild to moderate COVID-19. However, the study could not evaluate the antiviral effect of HCQ at early stages of disease, which is a critical period of antiviral treatment. In addition, viral RNA specimens were mostly from the upper respiratory tract rather than bronchoalveolar lavage fluid, which may cause false negative results. Due to the small number of severe patients, this study could not provide evidence regarding the effect of HCQ on the disease progression or regression. In another large observational study involving 1376 cases of COVID-19 from New York, 811 patients received HCQ (600 mg/2 times on the first day, then 400 mg once a day for 4 days) within 24 or 48 hours of admission and 565 did not [55]. This study found no correlation between HCQ use and significantly higher or lower risk of intubation or death. However, in this study, even after the propensity score-matching, the diseases in patients receiving HCQ were more severe at baseline than those in the patients not receiving. Notably, according to another recent study [56], low dose of HCQ reduced fatality of critically ill patients with COVID-19 without apparent toxicity. This retrospective study included 550 patients who need mechanical ventilation, of which 48 received HCQ treatment (200 mg/2 times/day for 7 to 10 days) and 502 did not. The fatalities of the HCQ group was significantly lower than no-HCQ group (18.8% vs 47.4%, $P < 0.05$), and the inflammatory cytokine IL-6 in the HCQ group decreased significantly from 22.2 (8.3 to 118.9 pg/mL) at the beginning of treatment to 5.2 (3.0 to 23.4 pg/mL) at the end of treatment. The authors deemed that the anti-inflammatory effect of low-dose HCQ and the activity of inhibiting viral replication may have important significance in critically ill patients with COVID-19. Yet, this study is flawed due to its retrospective nature and the small number of HCQ treated patients included. In short, some initial studies have shown that HCQ appears to have a curative effect on patients with mild COVID-19, but subsequent studies indicate that HCQ had no significant benefit in COVID-19 patients with viral conversion and the risk of intubation or death. Although some recent studies show that low-dose HCQ could potentially reduce the mortality of severe COVID-19 patients, there are other studies showing that the HCQ use had no effect on risk of intubation or death.

There are also several reports that investigated the efficacy of CQ or HCQ in combination with a macrolide in the treatment of COVID-19 (Table 1). In an open nonrandom clinical trial conducted in France [57], of the 36 participants, 20 patients were given HCQ (200 mg/3 times) with 6 receiving added azithromycin, and 16 controls. The results showed that compared with the control group, HCQ alone or in combination with azithromycin could effectively eliminate nasopharyngeal virus in 3–5 days. On the 6th day after treatment, the virus clearance rates of HCQ combined with azithromycin, HCQ alone and controls were 100%, 57.1% and 12.5%, respectively ($P < 0.001$). This study indicated that the combined

application of azithromycin and HCQ appears to have a synergistic effect. However, the trial design and the results were unreliable, as six patients in the HCQ group discontinued treatment early due to critical illness or intolerance to the drugs and were excluded from the analysis. The assessment of efficacy was based on viral load rather than a clinical endpoint. An observational study in 80 COVID-19 patients evaluated the efficacy of HCQ (200 mg/3 times/day for 10 days) in combination with azithromycin (500 mg on the first day, 250 mg/day afterward for 5 days) and showed that all patients' clinical symptoms were improved, except for one patient aged over 86 years who died due to critical illness [58]. The nasopharynx viral load in most patients decreased rapidly, and the negative rates of viral nucleic acid conversion on days 7 and 8 were about 83% and 93%, respectively. About 97.5% of patients had negative virus culture in respiratory specimens on the fifth day. However, this study had no control group, thus the results were difficult to interpret [58]. Some recent studies have yielded different results about the efficacy of HCQ combined with azithromycin. A retrospective study including 368 patients (97 patients received HCQ, 113 patients received HCQ + azithromycin and 158 patients received no HCQ) from USA [59] showed that the rates of ventilation in the HCQ, HCQ+azithromycin and no HCQ groups had no significant differences. Unfortunately, theHCQ group (but not in the HCQ+azithromycin group) had a higher risk of death from any case than the no HCQ group. This study showed no evidence that the use of HCQ, either with or without azithromycin, reduced the risk of mechanical ventilation in patients hospitalized with COVID-19. Noticeably, in patients treated with HCQ alone, an association with increased overall mortality was observed [59]. In this study, the subjects included were only men and most of them were black, which may affect the generality of the results. In addition, the patients who received HCQ or azithromycin were more severe, which may also affect the results. In a retrospective multicenter cohort study of a random sample of COVID-19 patients from 25 hospitals in New York [60], totaling 1438 patients, 735 received HCQ and azithromycin, 271 received HCQ alone, 211 received azithromycin alone and 221 received neither drug (HCQ or azithromycin). The results showed that the hospital mortality rate of patients receiving HCQ + azithromycin was 25.7%, HCQ alone was 19.9%, azithromycin alone was 10.0% and neither drug was 12.7%. In adjusted Cox proportional hazards models, compared with patients receiving neither drug, there were no significant differences in hospital mortality rate for patients receiving HC + azithromycin, HCQ alone, or azithromycin alone. In this study, the sample size is large and includes patients with long-term, complex and ongoing hospitalization. However, the mortality rate of this study was limited to in-hospital deaths, and patients discharged during the study period were considered alive, which may underestimate the morality rate. Recently, a multinational registry analysis about HCQ or CQ with or without second-generation macrolides (especial azithromycin and clarithromycin) for treatment of COVID-19 was reported [61]. A total of 96,032 patients were included in this study. Of these, 1868 received CQ, 3783 received CQ with a macrolide, 3016 received HCQ and 6221 received HCQ with a macrolide and 8114 patients as control group. After controlling various confounding factors related to disease, when compared with the mortality in the control group (9.3%), CQ group was 16.4%, CQ with a macrolide group was 22.2%, HCQ group was 18.0% and HCQ group with a macrolide was 23.8%; each group was associated with an increased risk of hospital mortality independently. Apart from this, compared with the control group (0.3%), CQ group (4.3%), CQ with a macrolide group (6.5%), HCQ group (6.1%) and HCQ with a macrolide group (8.1%) were independently associated with a risk for ventricular arrhythmia during hospitalization [61]. This study showed that CQ or HCQ (used alone or combination with a macrolide) was associated with an increased hazard for in-hospital death and an increased risk of ventricular arrhythmias. This study included a large number of patients, but it is not a randomized clinical trial. In short, some small studies have shown that HCQ combined with azithromycin could quickly and effectively eliminate viruses, but the design of these studies was flawed in many aspects, making the results unconvincing. Several subsequent studies have shown that the combination of HCQ or CQ and macrolides (azithromycin or clarithromycin) has no obvious correlation with a reduced risk for mechanical ventilation, and may even increase the risk of arrhythmia and in-hospital mortality.

In summary, although CQ/HCQ appeared to exhibit a favorable effect on COVID-19 patients in some initial studies of small numbers of patients, the most recent studies with larger sample sizes revealed that CQ/HCQ exhibited no significant improvement of disease but even an increased overall mortality in COVID-19 patients. The studies on the combination of HCQ or CQ and macrolides (azithromycin or clarithromycin) also showed conflicting findings. Therefore, caution should be taken regarding the use of CQ/HCQ treatment in COVID-19 due to the uncertainty of efficacy, the potential adverse effects and the various defects in the studies. According to the Chinese Clinical Trial Registry (ChiCTR) (http://www.chictr.org.cn/index.aspx) and the International Clinical Trials Registry Platform (ICTRP) (https://www.who.int/ictrp/en/), currently, there are more than 200 ongoing clinical trials for CQ/HCQ. Current findings suggest that CQ/HCQ alone or in combination with macrolides should not be recommended for widespread use in COVID-19 (except in clinical trials). Results from these ongoing prospective, randomized, controlled studies are required before these drugs are recommended for the treatment of COVID-19.

6. Safety

CQ/HCQ are basic medications for malaria with a long history of reliable safety records [3,62]. However, the therapeutic window of CQ is narrow, and the toxic dose is 3 times higher than the therapeutic dose [55,63]. The most common adverse effects of taking CQ are gastrointestinal discomforts, such as nausea, vomiting, diarrhea, and anorexia. These symptoms are mild and can be controlled by reducing the dose [3]. However, long-term and high-dose CQ intake can cause irreversible damage to the ear, cardiovascular system, and blood system, such as neurological deafness, conduction disorder cardiomyopathy, and leukopenia, though these adverse effects are very rare [3]. Compared to CQ, HCQ has fewer adverse effects,

which may be related to its lower toxicity. In animal models, HCQ was about 40% less toxic than CQ [64]. Reportedly, only overdoses (average daily dose > 5.0 mg/kg) and long-time (more than 5 years) ingestion of HCQ can cause retinopathy [65]. CQ/HCQ have similar pharmacokinetic characteristics, fast absorption in the gastrointestinal tract, fast excretion in liver and kidney, and a long half-life (40–50 days) [3,66]. Therefore, liver and kidney dysfunction may aggravate adverse effects. High-dose CQ (600 mg/2 times/day for 10 day) is associated with increased QTc interval prolongation in critically ill patients with COVID-19 [49] and should not be recommended. In the initial trial from France, of the 84 patients receiving HCQ treatment, 8 patients discontinued HCQ owing to ECG modifications within 4 days. Among them, 7 patients had a prolonged QTc interval more than 60 ms, and one patient developed a first-degree atrioventricular block within 2 days [53]. In a randomized clinical trial from Wuhan, 2 out of 31 patients receiving HCQ treatment had minor adverse effects (headache and rash) [50]. Prolonged QT interval after taking HCQ may also develop in ICU COVID-19 patients [67]. A recent observation showed that patients who received HCQ for the treatment of pneumonia associated with COVID-19 were at high risk of QTc prolongation (19%), and concurrent use of azithromycin was associated with greater changes in QTc (21%) [68]. Another observation in COVID-19 patients admitted to ICU showed that QTc intervals increased in 93% of patients receiving HCQ with or without azithromycin, prolonged QTc was observed in 36% of patients after a duration of the treatment for 2 to 5 days, and 6 of 18 (33%) patients treated with HCQ and azithromycin and 1 of 22 (5%) of those treated with HCQ alone developed an increase in QTc of 500 ms or greater [69]. The use of CQ, CQ with a macrolide, HCQ and HCQ with a macrolide were all found to be independently associated with increased risk for ventricular arrhythmia in hospitalized COVID-19 patients [61]. Therefore, clinicians should carefully weigh risks and benefits if considering CQ/HCQ with or without a macrolide. When CQ/HCQ are used, electrocardiogram examination should be routinely performed before taking the medicine, with close monitoring of QTc and concomitant medication usage. CQ/HCQ should be more cautiously used in patients with existing heart disease, and the use of QT interval prolonging drugs, such as antiarrhythmic drugs, antihistamines, and moxifloxacin should be avoided. In addition, close attention should be paid to symptoms after taking drugs and the drugs should be stopped in time if there are intolerable adverse reactions. In addition, CQ is extremely dangerous for patients with glucose 6-phosphate dehydrogenase (G6PD) deficiency because of the possible induction of hemolytic anemia [70]. Therefore, more caution should be given for patients with G6PD deficiency when CQ is considered for the treatment and the best way should be to detect G6PD deficiency before the use of CQ.

7. Discussion

At present, the COVID-19 pandemic is continuing worldwide. It is still an urgent need to find effective therapies and vaccines for treatment and prevention. CQ/HCQ have diverse biological activities, and their mechanisms against CoVs including SARS-CoV-2 are not yet fully clarified. Current studies show that CQ/HCQ can prevent receptor recognition by CoVs, inhibit endosome acidification, which interferes membrane fusion, and exhibit immunomodulatory activity. These multiple mechanisms may work together to exert a therapeutic effect on COVID-19. A number of *in vitro* studies have revealed that CQ/HCQ have inhibitory effects on various CoVs, including SARS-CoV [12,13], MERS-CoV [10] and SARS-CoV-2 [16–18]. However, conflicting results also exist on the *in vitro* activity of CQ/HCQ against SARS-CoV-2 [17,18]. Several clinical studies have shown that CQ/HCQ may alleviate the clinical symptoms of COVID-19, promote viral conversion, and delay the progression of the disease, with less serious adverse effects [48,50,57,58,]. However, previous studies showed that CQ had anti-Ebola virus activity in cell culture, but it had conflicting results in animal models [71,72]. In addition, CQ has shown beneficial results against chikungunya virus *in vitro*, but in animal models it aggravates the infection and lacks therapeutic effect [73]. More importantly, in recent studies the use of HCQ did not show any favorable effect on patients with COVID-19 and high-dose CQ treatment of severe COVID-19 patients may even increase the risks of mortality and QTc interval prolongation [49,55]. In addition, the optimal daily dose and duration of treatment course are not yet clear. One study suggested that the dose of HCQ should be 400 mg/2 times for 1 day, 200 mg/2 times/day for 4 days based on the physiological pharmacokinetic model [18]. A prospective study of HCQ on COVID-19 patients (13 cases) admitted to the ICU in France showed that the first daily dose of 800 mg/1 time for 1 day, and 200 mg/2 times/day for 7 days was recommended to maintain the HCQ treatment level (1–2 mg/l) based on physiologically pharmacokinetic (PBPK) models for COVID-19 patients in ICU [67]. Whether the dosage of CQ or HCQ should be varied according disease severity is also unclear. A rodent study showed that CQ could exert anti-HCoV-OC43 activity transplacentally or by way of maternal milk [14]. However, in humans, the efficacy of CQ in the prevention and treatment of SARS-CoV-2 infection to both the mother and the child remains to be investigated. Clinical trials in France showed that HCQ combined with azithromycin could enhance the virus clearance [50], but the subsequent reports did not support this combination [59,61]. Furthermore, CQ/HCQ alone or in combination with a macrolide induced high rate of adverse effects, especially prolonged QTc, in the use for COVID-19 treatment [61,68,69]. Therefore, current data are not sufficient enough to support the routine use of CQ/HCQ as therapies for COVID-19 and increasing caution should be taken for the application of CQ/HCQ, alone or in combination with other drugs, in COVID-19 before the conclusive findings are obtained by well-designed, multicenter, randomized, controlled studies.

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ORCID

JingKang Sun http://orcid.org/0000-0002-5530-1527
YuTing Chen http://orcid.org/0000-0003-0448-4711

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