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Book Review

Safety considerations of chloroquine in the treatment of patients with diabetes and COVID-19

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ABSTRACT

Patients with underlying diseases and coronavirus disease 2019 (COVID-19) are at increased risk of death. Using the recommended anti-COVID-19 drug, chloroquine phosphate (CQ), to treat patients with severe cases and type 2 diabetes (T2D) could potentially cause harm. We aimed to understand the safety of CQ in patients with T2D by administrating the recommended dose (63 mg/kg twice daily for 7 days) and a high dose (126 mg/kg twice daily for 7 days) of CQ in T2D rats. We found that CQ increased the total mortality of the T2D rats from 27.3% to 72.7% in the recommended and high-dose groups during the whole period. CQ also induced hematotoxicity of T2D rats in the high-dose group; the hepatic enzymes in T2D rats were significantly elevated. CQ also changed the electrocardiograms, prolonged the QTc intervals, and produced urinary leukocytes and proteins in the T2D rats. Histopathological observations revealed that CQ caused severe damage to the rats' heart, jejunum, liver, kidneys, spleen, and retinas. Furthermore, CQ significantly decreased the serum IL-1β and IL-6 levels. In conclusion, the CQ dosage and regimen used to treat COVID-19 induced adverse effects in diabetic rats, suggesting the need to reevaluate the effective dose of CQ in humans.

1. Introduction

Coronavirus disease 2019 (COVID-19), caused by a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, has resulted in a worldwide pandemic and elicited serious global public health concerns. From December 2019 to March 31, 2022, more than 485 243 022 worldwide COVID-19 cases were identified, of which more than 6 137 553 patients died [1]. Patients with COVID-19 were more likely to have comorbidities, such as cardiovascular disease, causing severe symptoms and worsening clinical outcomes [2]. Many early studies have indicated that cardiovascular disease and diabetes were the most common comorbidities in patients with COVID-19 at high risk of adverse effects and in-hospital death [3,4]. Clinical reports have shown that hypertension (15%–30%), diabetes mellitus (19%), and coronary heart disease (2.5%–15%) were the most common comorbid diseases associated with COVID-19 [5–7]. Among these comorbidities, diabetes mellitus was one of the most critical risks of mortality in patients with COVID-19 due to its roles in the dysregulation of immune and inflammatory responses and increasing SARS-CoV-2 replication [8,9]. If patients with diabetes were infected by SARS-CoV-2, they could experience increased cardiovascular events, a weakened immune response, and increased all-cause mortality [3,10]. Therefore, treatment of patients with COVID-19 has been highly complicated and involved administering drugs, such as camostat mesylate, protease inhibitors, RNA-dependent RNA polymerase inhibitors, and corticosteroids, which could worsen hyperglycemia [3].

Repurposing old drugs to treat SARS-CoV-2 infection was a quick way to develop an effective tool against the COVID-19 pandemic. Among which, chloroquine (CQ) and its derivative, hydroxychloroquine (HCQ), were suggested as potential therapeutics for COVID-19 [11]. On February 18, 2020, the National Health Commission (NHC) of China issued the recommendation to use CQ for the treatment of COVID-19 at a

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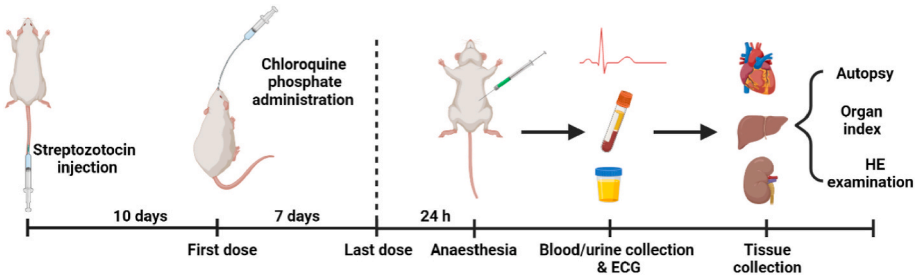

Fig. 1. Schematic diagram of animal experiments.

dose of 500 mg BID (*bis in die*, twice daily) for 10 days [12,13]; however, this regimen was quickly reduced due to safety concerns [14]. Similarly, the United States Food and Drug Administration (FDA) released an Emergency Usage Authorization (EUA) for CQ and HCQ to treat COVID-19 but subsequently withdrew this authorization [15]. Many clinical trials were approved worldwide to test the efficacy and safety of CQ and HCQ on patients with COVID-19 generating controversial results [16,17]. In the early stages, clinical trials in China and France demonstrated that CQ and HCQ effectively repressed SARS-CoV-2 infection in small sample sizes [18,19]. Soon after, large, randomized clinical trials of CQ and HCQ in several countries eliminated the CQ and HCQ recommendation for treating patients with COVID-19 due to lack of efficacy and risk of adverse events and mortality [20–23]. Moreover, using CQ and HCQ as postexposure prophylaxis or for the treatment of COVID-19 in randomized and controlled clinical trials also failed to prove its efficacy [24,25]. Furthermore, in vitro studies confirmed that HCQ did not inhibit SARS-CoV-2 infection in human lung cells [26]. However, in an extensive analysis of patients with COVID-19 admitted to the hospital in Belgium, HCQ was independently associated with a lower in-hospital mortality rate [27]. Considering the COVID-19 pandemic was worldwide, the efficacy and safety of CQ and HCQ therapy have been at the center of debates.

Currently, little is known about the possible reasons for the controversial CQ and HCQ findings during the COVID-19 pandemic. From an applied toxicology perspective, the dosage regimen of CQ and HCQ and the concomitant disease of enrolled patients in clinical trials both contributed to the outcome. Furthermore, the high incidence rate of diabetes mellitus predisposed patients with COVID-19 to severe outcomes and increased risk of death [2,3]. In addition, there was concern over the potential for interactions between antiviral drugs and diabetes. Moreover, SARS-CoV-2 itself induces multiple organ injuries, affecting the safety of using experimental anti-COVID-19 drugs in confirmed patients [28,29]. Thus, we hypothesized that diabetes mellitus could contribute to the mixed clinical outcomes of CQ and HCQ against COVID-19 disease.

Although long-term use of CQ has an acceptable safety profile as antimalarial and anti-rheumatoid arthritis therapeutics, potential safety concerns such as QT prolongation, ventricular tachycardia, and retinopathy resulting from high dosages or prolonged use should be given more attention [30,31]. In this study, we aimed to evaluate the safety of CQ by using it in a COVID-19 therapy regimen in patients with diabetes. We also elucidated the underlying reason for the CQ controversy from a toxicology perspective. Our findings could help determine the potential causes for the approval and then halt using CQ and HCQ in the battle against COVID-19.

2. Materials and methods

2.1. Reagents and drugs

We obtained nicotinamide (Sigma-Aldrich, 72340), streptozotocin (Aladdin, S110910, Shanghai, China), chloroquine phosphate (Aladdin, C129284, Shanghai, China), citric acid/sodium citrate buffer (pH = 4.5, Yuanye Bio-Technology, R22384, Shanghai, China), diethyl ether (Sinopharm Chemical Reagent, 10009318, AR, Shanghai, China), 4% paraformaldehyde (Biosharp, BL539A, Hefei, China), and 0.9% (w/v) sodium chloride (Guojing Pharmaceutical Co., Ltd, Lishui, Zhejiang, China). Chloroquine phosphate was prepared as it was used, and streptozotocin solution was be freshly prepared for each injection.

2.2. Animals

Fifty healthy Sprague-Dawley 7-week-old male rats (specifically pathogen-free, SPF, 200–250 g) were purchased from Charles River (Beijing Vital River Laboratory Animal Technology Co., Ltd.). All rats were reared in the laboratory animal center of Nanjing Agricultural University, which had a barrier system of constant temperature (24 °C ± 1 °C) and constant humidity (55% ± 5%) in light/dark (12 h/12 h) controlled housing. Groups of three or four rats were kept in each squirrel cage to guarantee enough activity space. Standard rodent chow diet and clean water were available to the rats throughout the entire study except when fasting was needed. All protocols used in the animal experiments were approved by the Committee on Animal Welfare and Ethics of Nanjing Agricultural University (No. PZ2020112) according to the regulations on the administration of laboratory animals in China.

2.3. Establishment of the type II diabetic (T2D) rat model

According to a previous report, the T2D rat model was established with minor alterations [32]. In brief, 9-week-old rats (250 g ± 20 g) were weighed accurately to 1 g and subsequently fasted for 8 h with normal drinking water before streptozotocin administration. Nicotinamide was injected intraperitoneally at a dose of 230 mg/kg to protect partial β-cell function from the diabetogenic effect of streptozotocin. Within 15 min of the nicotinamide injection, streptozotocin solution (dissolved in 50 mM sodium citrate buffer) was injected at a 65 mg/kg dose via the caudal vein. We ensured that the streptozotocin solution was injected within 5 min of dissolution. The control rats were administered an equal volume of sodium citrate buffer via intravenous injection. All the rats were then returned to the cages and were provided free access to standard food and clean water. Next, on experimental day 10, the non-fasting blood glucose levels of all the rats' tail vein blood were measured using Accu-Chek Active Blood Glucose Meter (Roche, Shanghai, China). When the non-fasting blood glucose concentration was higher than 16.7 mmol/L, the rat was labeled as diabetic for the following safety evaluation of CQ.

2.4. The CQ dosage regimen in T2D rats

The above established T2D rats were randomized into three groups: 0 mg/kg CQ (given an equal volume of saline, 11 rats), 63 mg/kg CQ (equivalent to clinical dosage, 11 rats), and 126 mg/kg CQ (equivalent to double the recommended dosage, 11 rats). These doses of CQ used in rats were equivalent to the human dose by calculation using the following formula [33]: Animal equivalent dose (AED; mg/kg) = Human dose (HED; mg/kg) × Km ratio, where Km ratio = (Human Km/Animal

Km), Km is estimated by dividing the average body weight (kg) of the species to its body surface area (m^2). The remaining 6 healthy rats were used as negative controls and an equal volume of saline water was administered during the entire experiment. The CQ solution and saline water were administrated intragastrically every 12 h for 7 consecutive days. Regular food and clean water were provided for all the rats during the CQ administration period. The schematic diagram of the animal experiment is shown in Fig. 1.

2.5. Clinical symptom observation

During the establishment of the T2D rat model and CQ administration period, the clinical signs of all rats including physical appearance, behavior, response to external stimuli, diet, water intake, respiratory rate, feces, and urine status, were observed twice daily. Clinical observations were carried out by individually checking the rats outside their home cage at approximately the same time of day. Any abnormal signs concerning their nature, severity and onset time of the observed conditions were carefully recorded. Additionally, after CQ administration, eye reactions, such as hyperemia, edema, hemorrhage, inflammation, and secretion, were observed twice daily. The rats' food consumption and water intake in each group were recorded daily before CQ administration. The bodyweights of the rats were measured before the administration of CQ in the morning, every two days. The postprandial blood glucose levels of the rats' tail vein blood were monitored every three days.

2.6. Electrocardiogram (ECG) monitoring

Electrocardiograms were monitored according to a previous report [34] to explore the effect of CQ on the rat hearts on day 8. In brief, three rats from each group were anesthetized by diethyl ether inhalation. When the anesthesia reached the appropriate level, the electrocardiogram signals were continuously recorded for at least 10 min using the TaiMeng biological signal collecting system (Chengdu, China) with limb leads. After that, the changes in the rat cardiac cycle were analyzed by calculating the PR, QRS, and QT intervals.

2.7. Sample collection and pretreatment

At the end of the CQ administration period on day 8, samples from the rats such as plasma, serum, urine, and organic tissues were collected for further biological analysis. All the rats were fasted for 12 h and given normal clean water until they were euthanized. Urine collection was carried out using a metabolic cage, providing an easy way to get clean urine from the rats. After urine collection, the rats were euthanized by cutting the jugular vein, and 200 μL of blood per rat was collected in an EDTA anticoagulation tube, with the rest of the blood collected in a non-anticoagulant tube to obtain serum. The blood used for the hematological assay was kept in a 4 °C refrigerator and analyzed within 4 h. Additionally, the rat serum was obtained by 1000×*g* centrifugation for 10 min at 4 °C. The rat serum was then placed at −20 °C until the biochemical analysis and cytokine detection. The organic rat tissues were collected with minimum artificial damage to observe the effect of CQ on histopathological changes. The tissues were from the heart, liver, spleen, lung, kidney, duodenum, jejunum, ileum, cecum, colon, rectum, bladder, skeletal muscle, pancreas, stomach, thymus, testis, and eyeballs in triplicate from each group. The tissues of suitable sizes were immediately soaked in 4% paraformaldehyde solution, and the eyeballs were placed in Davidson's fixative solution for 24 h, followed by histopathological examination as described below.

2.8. Hematological examination

The blood was analyzed using an automatic blood-counter system (Mindray, Shenzhen, China) to determine the hematological changes of diabetic rats induced by different doses of CQ. The tested blood parameters were red blood cell (RBC), hematocrit (HCT), mean corpuscular volume (MCV), hemoglobin (HGB), mean hemoglobin (MCH), mean hemoglobin concentration (MCHC), red blood cell distribution width (RDW-SD), white blood cell (WBC), lymphocyte count (LYM), monocyte (MONO), neutrophils (NEUT), eosinophils (EO), basophils (BASO), platelet (PLT), and mean platelet volume (MPV). The blood of each group was measured at less in quadruplicate within 4 h of the sample collection.

2.9. Serum biochemical analysis

The rat serum of rats was used to detect biochemical indexes including glucose (GLU), total protein (TP), albumin (ALB), globulin (GLB), total bile acid (TBA), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (T-BIL), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine kinase (CK), creatinine (Cr), uric acid (UA), total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and amylase (AMY). The rat serum was analyzed at less in triplicate for each group using an automatic biochemical analyzer (Beckman Coulter AU5800, Brea, CA, USA) within 2 days of the sample collection.

2.10. Urinary analysis

In addition, to understand the effect of CQ on the urine of rats, the urinary examination was carried out using a urine analyzer (Mindray, Shenzhen, China). The urinary parameters included specific gravity, pH, leukocytes, nitrite, urobilinogen, protein, occult blood, ketone bodies, bilirubin, glucose, and vitamin C, which were analyzed within 1 h after urine collection. The urine samples were collected at less in triplicate of each group.

2.11. Serum inflammatory cytokine assay

To further explore the effect of CQ on the rat serum cytokine levels, an enzyme-linked immunosorbent assay (ELISA) was performed using ELISA kits according to the manufacturer's instructions (MultiSciences Biotech., Hangzhou, China). These cytokines included TNF-α, IFN-γ, IL-1β, IL-4, IL-6, and IL-10. Briefly, the standard curves of the tested cytokines were developed by double diluting the standard solutions. Then, the levels of the tested cytokines were measured by enzyme coated plate soaking, serum loading, antibody loading, horseradish peroxidase-labeled streptavidin incubating, chromogenic substrate tetramethylbenzidine loading, and stop buffer addition. Finally, the optical density values of each well were determined using a multifunctional microplate reader (BioTek Synergy H1, Winooski, United States) at 450 nm and 630 nm as reference wavelengths.

2.12. Organ index and histopathological examination

The rats' organs (hearts, livers, spleen, lungs, kidneys, testis, and thymuses) were weighed, and the organ indexes were calculated in triplicate. Herein, organ index = organ weight (g) * 100/body weight (g). In addition, all the obtained organs from each group were used for histopathological examination. Briefly, the fixed tissues were cut into square sections (5 mm × 5 mm) and dehydrated in a series of alcohol solutions (50%–100%) for 30 min at each concentration, followed by xylene twice for transparency. The tissue blocks were then embedded into paraffin for at least 2 h. Next, these paraffin blocks were cut into thin slices of about 3 μm using a slicer (Leica, Germany). The slices were subsequently transferred to glass slides for drying and dewaxing. These prepared tissue slices were stained using hematoxylin and eosin (H&E) and appropriate washing to remove the free dyes. The stained tissue slices were sealed by a cover glass with dropwise neutral balsam. The

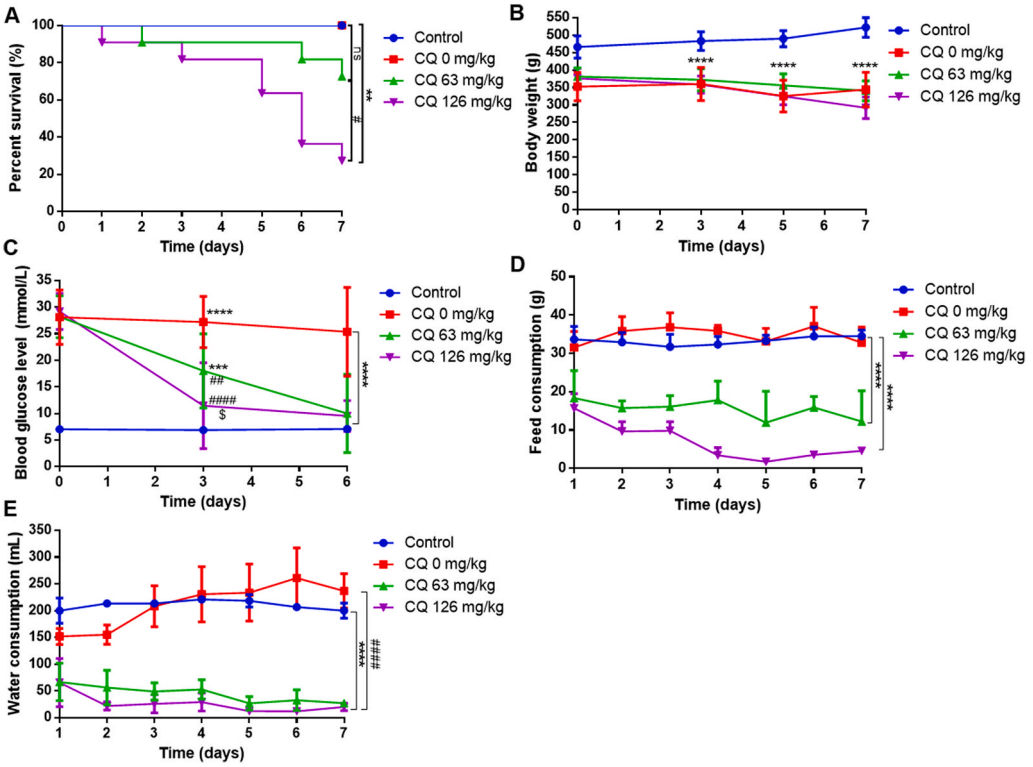

Fig. 2. CQ increases the mortality of T2D rats. Healthy and diabetic rats orally administrated saline and CQ at two doses (63 mg/kg or 126 mg/kg) twice daily for seven consecutive days. (A) Survival rate of rats (n = 6-11). "ns" is short for no significance; **, $P<0.01$, compared to healthy and diabetic rats; #, $P<0.05$, compared to CQ (126 mg/kg) group; The significance between different survival curves was carried out by performing log-rank (Mantel-Cox) test. (B) Body weight of all rats were weighed on 0 d, 3 d, 5 d and 7 d post-drug administration. ****, $P < 0.0001$, compared to healthy and diabetic rats. (C) Blood glucose of all rats were monitored on 3 d and 6 d after CQ administration. ***, $P < 0.001$ and ****, $P < 0.0001$ compared to healthy rats; ##, $P < 0.01$ and ####, $P < 0.0001$ compared to diabetic rats; $, $P < 0.05$, compared to 63 mg/kg CQ-treated diabetic rats. (D) Feed consumption of rats was recorded in each day. ****, $P < 0.0001$, compared to healthy and diabetic rats on day 7. (E) Water consumption of rats was recorded every day. Data are shown as mean ± SD, n = 3–11. ****, $P < 0.0001$, compared to healthy rats on day 7; ####, $P < 0.0001$, compared to diabetic rats on day 7.

histopathological changes of each tissue slice were observed and photographed under a light microscope equipped with a digital camera (Leica, Buffalo Grove, United States).

2.13. Statistical analysis

The data are shown as mean ± standard deviation (SD). The data were analyzed using SPSS 20.0 version (IBM, Chicago, IL, USA) and tested by performing a two-way analysis of variance (ANOVA), followed by the least-significant difference (LSD) test. The statistical differences were set when $P < 0.05$.

3. Results

3.1. CQ increases the mortality rate of T2D rats

First, the clinical signs and survival rate of the diabetic rats were observed during CQ administration. The diabetic rats displayed symptoms of depression, decreased activity, disordered hair, and exhibited slow responses to external stimuli three days post-administration of the approved dose of CQ (Table S1). These clinical symptoms were also observed in the high-dose CQ-treated diabetic rats, that exhibited more severe symptoms, such as slow reaction, emaciation, and standing dorsal hair (Table S1). As the time increased after CQ administration, most diabetic rats grew weak and experienced sudden death, while the diabetic rats without CQ administration experienced weight loss only. Starting on the fifth day after CQ treatment, even using the recommended dose, the diabetic rats began to die. At the end of the CQ administration period, the survival rate of the CQ-treated diabetic rats decreased to 72.7% (approved dose of CQ) and 27.3% (high dose of CQ, $P < 0.01$) (Fig. 2A). However, all the diabetic rats receiving saline survived throughout the entire animal experiment. In addition, compared to the healthy control group rats, the bodyweight of the diabetic rats all decreased significantly among the control group ($P < 0.0001$), approved-CQ dose group ($P < 0.0001$), and high-dose CQ group ($P < 0.0001$) (Fig. 2B). Moreover, the doses of CQ (63 mg/kg and 126 mg/kg) decreased the diabetic rats' blood glucose levels on day 3 ($P < 0.01$) and day 6 ($P < 0.0001$) (Fig. 2C), and these rats showed decreased food and water intake on day 7 ($P < 0.0001$), compared to the diabetic and

Table 1
Hematological analysis of rats on day 7 post-administration of CQ.

Indexes	Control	Type 2 diabetic rats		
		CQ (0 mg/kg)	CQ (63 mg/kg)	CQ (126 mg/kg)
RBC	7.15 ± 3.01	6.33 ± 3.59	8.29 ± 3.59	11.43 ± 1.21*#
HCT	40.85 ± 17.17	35.42 ± 19.74	45.34 ± 18.62	63.43 ± 9.19*#
MCV (fL)	57.37 ± 1.89	56.28 ± 2.05	55.64 ± 3.16	56.30 ± 2.84
HGB (g/L)	130.09 ± 58.31	122.00 ± 69.59	150.25 ± 63.58	208.00 ± 24.10*#
MCH (pg)	18.04 ± 1.57	19.09 ± 0.77	18.33 ± 0.74	18.55 ± 0.52
MCHC (g/L)	314.36 ± 24.84	339.30 ± 12.74	329.75 ± 7.74	329.50 ± 11.96
RDW-SD (%)	27.97 ± 2.19	26.22 ± 1.18	25.76 ± 1.04	29.88 ± 2.74
WBC ($10^{\wedge}9$/L)	6.82 ± 3.96	7.54 ± 4.50	9.08 ± 5.24	16.28 ± 4.83**##$
LYM ($10^{\wedge}9$/L)	5.13 ± 3.14	4.78 ± 2.83	5.24 ± 3.32	2.27 ± 2.74
MONO	0.17 ± 0.11	0.22 ± 0.15	0.19 ± 0.09	0.38 ± 0.28
NEUT ($10^{\wedge}9$/L)	1.28 ± 0.73	2.29 ± 1.68	3.37 ± 2.05	12.76 ± 5.99**##$$
EO	0.08 ± 0.05	0.09 ± 0.08	0.04 ± 0.03	0.04 ± 0.03
BASO	0.15 ± 0.12	0.17 ± 0.12	0.25 ± 0.17	0.84 ± 0.22**##$$
PLT (10^9/L)	513.64 ± 328.12	196.30 ± 189.62*	579.00 ± 428.78	784.00 ± 274.11
MPV (fL)	7.45 ± 0.41	7.29 ± 0.57	6.83 ± 0.41	7.23 ± 0.22

Note: *, $P < 0.05$, compared with control group; #, $P < 0.05$, compared with CQ (0 mg/kg) group; $, $P < 0.05$, compared with CQ (63 mg/kg) group; **, $P < 0.01$, compared with control group; ##, $P < 0.01$, compared with CQ (0 mg/kg) group; $$, $P < 0.01$, compared with CQ (63 mg/kg) group.

Table 2
Effect of CQ on serum biochemical indices of rats.

Indexes	Control	Type 2 diabetic rats		
		CQ (0 mg/kg)	CQ (63 mg/kg)	CQ (126 mg/kg)
TP (g/L)	64.81 ± 3.71	59.50 ± 6.32	62.33 ± 4.25	56.93 ± 5.37
ALB (g/L)	30.69 ± 1.07	27.92 ± 3.32	27.50 ± 1.17	24.37 ± 1.40
GLB (g/L)	34.12 ± 3.11	31.58 ± 3.55	34.83 ± 3.52	32.57 ± 4.04
TBA (μmol/L)	11.82 ± 5.33	87.56 ± 100.28*	43.80 ± 59.11	20.93 ± 27.34
ALT (U/L)	48.82 ± 13.83	82.70 ± 33.85	139.38 ± 45.47**$^{\#}$	253.33 ± 124.50**$^{\#\#\$\$}$
AST (U/L)	160.64 ± 44.55	231.20 ± 54.68	957.50 ± 1001.20**$^{\#\#}$	952.33 ± 710.43*
AST/ALT	3.33 ± 0.53	3.01 ± 0.68	4.28 ± 1.78$^{\#}$	7.68 ± 1.36**$^{\#\#\$\$}$
T-BIL (μmol/L)	<0.7	<0.7	<0.7	<0.7
ALP (U/L)	209.64 ± 31.04	224.50 ± 85.85	257.50 ± 91.73	279.33 ± 107.08
LDH (U/L)	1585.55 ± 808.17	3127.1 ± 663.39**	2839 ± 1267.97**	3361.33 ± 1044.77**
CK (U/L)	1621 ± 929.72	4793.70 ± 3352.77**	4748.25 ± 2805.42*	6439.33 ± 3474.54**
Cr (U/L)	25.09 ± 5.32	20.9 ± 3.87	25.00 ± 3.89	27.00 ± 13.86
Urea (mmol/L)	4.78 ± 0.60	10.54 ± 4.34**	6.94 ± 2.83$^{\#}$	9.26 ± 6.81
UA (μmol/L)	156.45 ± 115.50	122.1 ± 30.38	145.25 ± 66.38	150.67 ± 48.01
TC (mmol/L)	1.82 ± 0.35	1.63 ± 0.34	2.21 ± 0.49*$^{\#\#}$	1.52 ± 0.66$^{\$}$
TG (mmol/L)	1.23 ± 0.49	0.76 ± 0.29	1.08 ± 0.82	0.54 ± 0.15
HDL-C (mmol/L)	1.11 ± 0.21	1.10 ± 0.31	1.20 ± 0.22	0.77 ± 0.43
LDL-C (mmol/L)	0.38 ± 0.07	0.38 ± 0.09	0.45 ± 0.09	0.37 ± 0.15
AMY (U/L)	2463 ± 314.29	1829.00 ± 669.69**	1775.38 ± 395.25**	1027.00 ± 133.29**$^{\#\$}$

Note: *, $P < 0.05$, compared with control group; $^{\#}$, $P < 0.05$, compared with CQ (0 mg/kg) group; $^{\$}$, $P < 0.05$, compared with CQ (63 mg/kg) group; **, $P < 0.01$, compared with control group; $^{\#\#}$, $P < 0.01$, compared with CQ (0 mg/kg) group; $^{\$\$}$, $P < 0.01$, compared with CQ (63 mg/kg) group.

healthy rats administered saline (Fig. 2D and E).

3.2. CQ alters T2D rat hematological and biochemical blood indices

As shown in Table 1, compared with healthy rats and diabetic rats, high-dose CQ (126 mg/kg) administration significantly increased the level of red blood cells (RBC) ($P < 0.05$), hematocrit (HCT) ($P < 0.05$), hemoglobin (HGB) ($P < 0.05$), white blood cells (WBC) ($P < 0.01$), neutrophils (NEUT) ($P < 0.01$), and basophils (BASO) ($P < 0.01$). In contrast, the diabetic rats given the recommended dose of CQ showed no significant hematological effects ($P > 0.05$). Compared with healthy rats, diabetic rats without administration of CQ exhibited significantly reduced levels of platelets (PLT) ($P < 0.05$).

As shown in Table 2, the recommended and high doses of CQ significantly elevated the levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and the ratio of ALT/AST compared to the healthy rats and diabetic rats ($P < 0.05$ and $P < 0.01$, respectively). Furthermore, the diabetic rats and the CQ-treated diabetic rats (both doses) all had significantly increased levels of lactate dehydrogenase (LDH) and creatine kinase (CK). The urea levels of T2D rats were significantly higher than those of the healthy rats ($P < 0.01$); however, CQ effectively decreased urea to normal levels. The amylase (AMY) levels were significantly reduced in the diabetic rats and CQ-treated diabetic rats ($P < 0.01$).

3.3. CQ induces abnormal electrocardiogram (ECG) of T2D rats

As shown in Table 3, high-dose CQ significantly prolonged the QRS interval of the T2D rats ($P < 0.01$). Of note, the diabetic rats and CQ-treated diabetic rats showed a significant increase in QT intervals ($P < 0.01$) compared to healthy rats. Compared to the diabetic rats, the recommended-dose and high-dose CQ groups had significantly prolonged QT intervals ($P < 0.01$). Compared to the recommended dose of CQ, the high dose of CQ induced more severe QT prolongation in the diabetic rats ($P < 0.01$). In addition, compared to the healthy and diabetic rats, the recommended dose (63 mg/kg) of CQ treatment decreased the heart rate (HR) of the diabetic rats ($P < 0.05$). The relevant electrocardiographs of rats are presented in Fig. 3 and show CQ markedly changed the peak profile, especially QT-interval prolongation.

3.4. Effect of CQ on T2D rat urine

As shown in Table 4, compared to healthy rats in the control group, the diabetic rats had high nitrite and glucose urine levels. In addition, the diabetic rat urine levels of leukocytes, nitrite, and protein increased markedly after CQ administration for seven days. Of note, the CQ treatment decreased the glucose levels of the diabetic rat urine, which was in agreement with the altered CQ-treated diabetic rats' blood glucose levels. Other CQ-treated diabetic rat urine parameters showed no distinct changes (Table 4).

3.5. CQ increases organ indexes and induces histopathological damages

Next, the CQ-treated rat organ index changes are shown in Table 5. They indicate the diabetic rats and CQ-treated diabetic rats had significantly increased organ indexes for the livers, lungs, and testis ($P < 0.05$ or $P < 0.01$) compared to those of the healthy rats. Similarly, 63 mg/kg CQ administration induced a significant increase in the spleen index ($P < 0.05$) compared with the diabetic rats. In contrast, the thymus indices of the diabetic and CQ-treated diabetic rats significantly decreased ($P < 0.05$ and $P < 0.01$, respectively) compared to the control group. However, there was no significant difference between the diabetic rats and

Table 3
Electrocardiogram changes of T2D rats induced by CQ.

Parameter	Control	Type 2 diabetes + CQ (0 mg/kg)	Type 2 diabetes + CQ (63 mg/kg)	Type 2 diabetes + CQ (126 mg/kg)
PR (ms)	48.87 ± 1.68	47.82 ± 2.80	52.08 ± 1.55	51.47 ± 5.17
QRS (ms)	24.13 ± 1.61	28.05 ± 6.19	29.21 ± 3.48	33.05 ± 3.00**
QT (ms)	40.47 ± 2.29	51.97 ± 1.21**	62.45 ± 8.30**$^{\#\#}$	73.48 ± 6.66**$^{\#\#\$\$}$
HR (bpm)	422.40 ± 5.37	405.60 ± 15.06	355.20 ± 52.20*$^{\#}$	380.40 ± 49.40

Note: HR is an abbreviation for 'heart rate'. *, $P < 0.05$, **, $P < 0.01$, compared with control group; $^{\#\#}$, $P < 0.01$, compared with CQ (0 mg/kg) group; $^{\$\$}$, $P < 0.01$, compared with CQ (63 mg/kg) group.

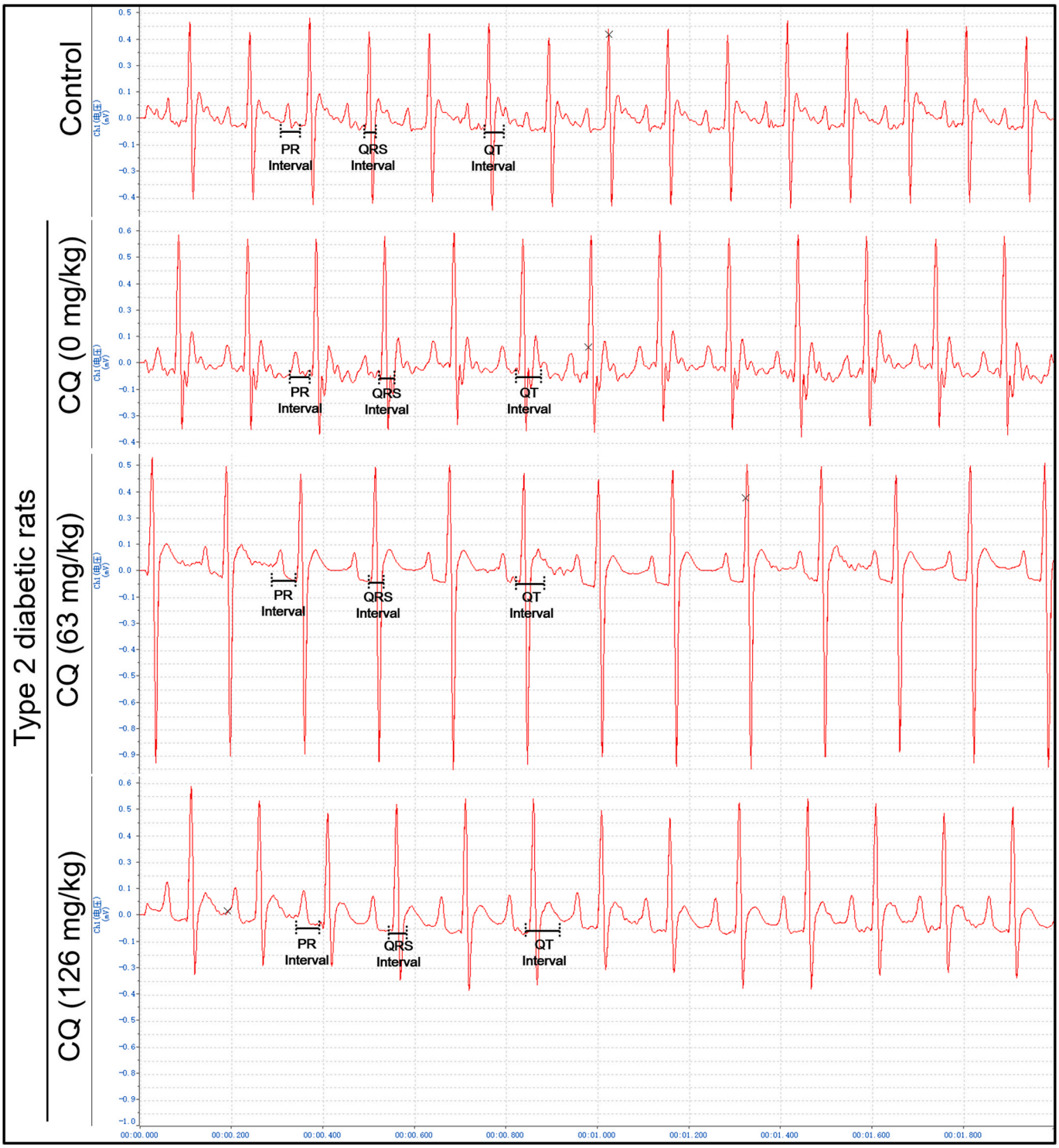

Fig. 3. Effect of CQ on electrocardiogram of T2D rats. The electrocardiograms (ECG) of rats were recorded after CQ exposure for 7 days. Three rats in each group were taken to ECG determination under general anesthesia. The ECG signals were continuously recorded for at least 10 min by using biological signal collecting system. The cardiac cycles of CQ-treated rats were marked with abnormal intervals such as PR, QRS and QT interval in the representative ECG photos.

CQ-treated diabetic rats ($P > 0.05$). Moreover, during the CQ administration period, the diabetic rat heart index did not significantly change ($P > 0.05$).

We further evaluated the histopathological changes of the CQ-treated rats for seven days. The results showed that several organic rat tissues were damaged to varying degrees. These organs and tissues include the heart, jejunum, liver, kidney, retina, and spleen. In detail, compared with healthy and diabetic rats, the approved dose of CQ induced vacuolar degeneration and lipid deposition of the heart (Fig. 4). In contrast, a high dose of CQ caused more severe myocardial damage such as focal necrosis, myocardial fiber swelling, and inflammatory cell infiltration (Fig. 4). In addition, the CQ-treated T2D rat jejunum were severely damaged in a dose-dependent manner; the histopathologic structure changes included necrosis and shedding of intestinal villus, disruption of intestinal crypt cells structure, and attenuation of the serosal layer (Fig. 4). However, other intestinal segments of the CQ-

Table 4
Urinalysis of rats on day 7 post-administration of CQ.

Test Items		Control	Type 2 diabetic rats		
			CQ (0 mg/kg)	CQ (63 mg/kg)	CQ (126 mg/kg)
Quantitative measures [a]					
Specific gravity		1.020 ± 0.005	1.022 ± 0.003	1.030 ± 0.000	1.027 ± 0.006
pH		6.800 ± 0.447	6.200 ± 1.037	5.7 ± 0.570	7.000 ± 1.500
Qualitative measures [b]					
Leukocyte	Negative	5	3	1	0
	Trace	0	2	3	3
	1+	0	0	1	0
Nitrite	Negative	5	2	1	1
	1+	0	3	4	2
Urobilinogen	Negative	5	5	5	3
Protein	Negative	3	3	0	0
	Trace	2	2	0	0
	2+	0	0	2	1
	3+	0	0	3	2
Occult blood	Negative	5	4	4	2
	1+	0	0	1	1
	3+	0	1	0	0
Ketone body	Negative	5	5	3	3
	Trace	0	0	1	0
	1+	0	0	1	0
Bilirubin	Negative	5	5	5	3
	1+	0	0	0	0
Glucose	Negative	5	0	4	2
	Trace	0	0	1	1
	2+	0	1	0	0
	3+	0	4	0	0
Vitamine C	Negative	5	5	4	3
	2+	0	0	1	0

[a] Values are represented as mean ± standard deviation.
[b] Number of rats under the same qualitative item.

Table 5
Organ indexes of rats on day 7 post-administration of CQ.

Organs	Control	Type 2 diabetic rats		
		CQ (0 mg/kg)	CQ (63 mg/kg)	CQ (126 mg/kg)
Heart (g/100g)	0.36 ± 0.03	0.42 ± 0.09	0.38 ± 0.03	0.41 ± 0.02
Liver (g/100g)	3.01 ± 0.20	3.61 ± 0.43**	3.47 ± 0.35*	3.53 ± 0.09
Spleen (g/100g)	0.19 ± 0.03	0.17 ± 0.03	0.21 ± 0.03#	0.18 ± 0.03
Lung (g/100g)	0.40 ± 0.07	0.54 ± 0.08**	0.50 ± 0.06*	0.57 ± 0.01**
Kidney (g/100g)	0.68 ± 0.05	0.95 ± 0.21**	0.82 ± 0.10	0.86 ± 0.03
Testis (g/100g)	0.77 ± 0.19	0.96 ± 0.16*	0.98 ± 0.13*	1.02 ± 0.05
Thymus (g/100g)	0.16 ± 0.06	0.11 ± 0.04*	0.11 ± 0.05*	0.035 ± 0.05**

Note: *, $P < 0.05$, compared with rats of control group; **, $P < 0.01$, compared with control group.

treated T2D rats had no apparent lesions compared with healthy rats (Supplemental Fig. S1). For the liver, CQ aggravated the cytoplasm vacuolization and granular degeneration induced by STZ pretreatment (Fig. 4) compared with healthy rats. Furthermore, the approved dose of CQ induced T2D rat glomerular cell swelling, and the high dose of CQ caused renal tubules necrosis, extreme vacuolization, and the loss of cellular integrity (Fig. 4). However, we observed focal vacuolar degeneration of the renal tubular epithelial cells in T2D rats with no CQ (Fig. 4). For the eyes of T2D rats, the approved dose of CQ triggered congestion of the retinal optic nerve layer vessels (Fig. 4), and the high dose of CQ induced hemorrhage in the inner granular layer of the retina (Fig. 4). CQ treatment caused diffuse vacuolization of macrophages in the medullary regions of the T2D spleen. However, this phenomenon was not observed in the healthy rat spleen (Fig. 4). STZ-induced rat pancreas injury during T2D model development was alleviated by CQ treatment in a dose-dependent manner, including reduced vacuolar degeneration (Supplemental Fig. S1). However, other organs and tissues showed no apparent pathologic injury. These organs were the lungs, cecum, colon, rectum, thymus, testis, bladder, stomach, and local tissues of the eye (Supplemental Fig. S1).

3.6. CQ inhibits serum IL-1β and IL-6 of T2D rats

Next, we analyzed the T2D rat serum cytokines after CQ administration for seven days. As shown in Fig. 5, compared with healthy and diabetic rats receiving saline, the high dose of CQ significantly decreased the levels of IL-1β and IL-6 ($P < 0.05$). However, the other four inflammatory cytokines were not significantly changed in T2D rats ($P > 0.05$). Moreover, after the treatment of the approved and high doses of CQ, the concentrations of these serum cytokines in T2D rats did not significantly change ($P > 0.05$).

4. Discussion

Because CQ and hydroxychloroquine HCQ have been reported as effective against COVID-19, determining their efficacy and safety has been controversial due to the varying therapeutic regimens with great difference in the enrolled patient populations in clinical trials [35–38]. The specific reasons for the controversial efficacy and safety of CQ and HCQ used against COVID-19 are unclear. Based on the T2D rat model, the current study found that the clinically high dose of CQ and the complicating disease, diabetes, caused treatment failure during the COVID-19 pandemic. First, CQ administration with an approved dosage (63 mg/kg, 7 d) increased the T2D rat mortality rate, suggesting serious toxic effects of CQ. This finding is also confirmed by an international collaborative meta-analysis of randomized clinical trials that demonstrated HCQ was associated with increased mortality in COVID-19 patients, and CQ had no effect on COVID-19 [21]. Similarly, data from a randomized, controlled, open-label trial demonstrated that COVID-19 patients in the HCQ group had a higher incidence of invasive mechanical ventilation or death [22]. However, there are no other relevant analyses on the relationship between using of HCQ or CQ and the risk of death for patients with COVID-19 and comorbidities. The mortality of patients with COVID-19 and comorbidities, such as diabetes, was 7.9% versus 0.9% in patients with no comorbidities [5]. Thus, using experimental CQ and HCQ against COVID-19 in patients with comorbidities could have severe consequences due to the difficulty of distinguishing whether COVID-19 or the use of the improper drug caused the adverse outcomes. Given the varied CQ and HCQ regimens and COVID-19 patient population in previous clinical trials, scientists have called for dosage optimization in each unique patient population [11,39]. Moreover, a small dose of HCQ may achieve an antiviral activity in vivo due to its long elimination half-life [40]. A limitation of the current study is that we did not set a low dose of CQ to understand further the safety range of CQ in diabetic rats, which could provide information for clinical dose setting. Additionally, regarding the narrow range of CQ against viral infections, low-dose CQ may be less critical for translation to clinical antiviral treatment, even if a low dose of CQ is safe for humans. Based on the findings in this study, we propose that dose-optimization based on clinical pharmacokinetic models was critical to repurpose CQ for patients with diabetes and COVID-19.

Although the acceptable safety of CQ and HCQ are well-known long-term, when repurposing CQ/HCQ to treat patients with diabetes and COVID-19 patients, a more comprehensive risk evaluation may be needed [35]. In the T2D rat model, the approved dose of CQ induced QT interval prolongation, CK elevation, and histopathological damage to

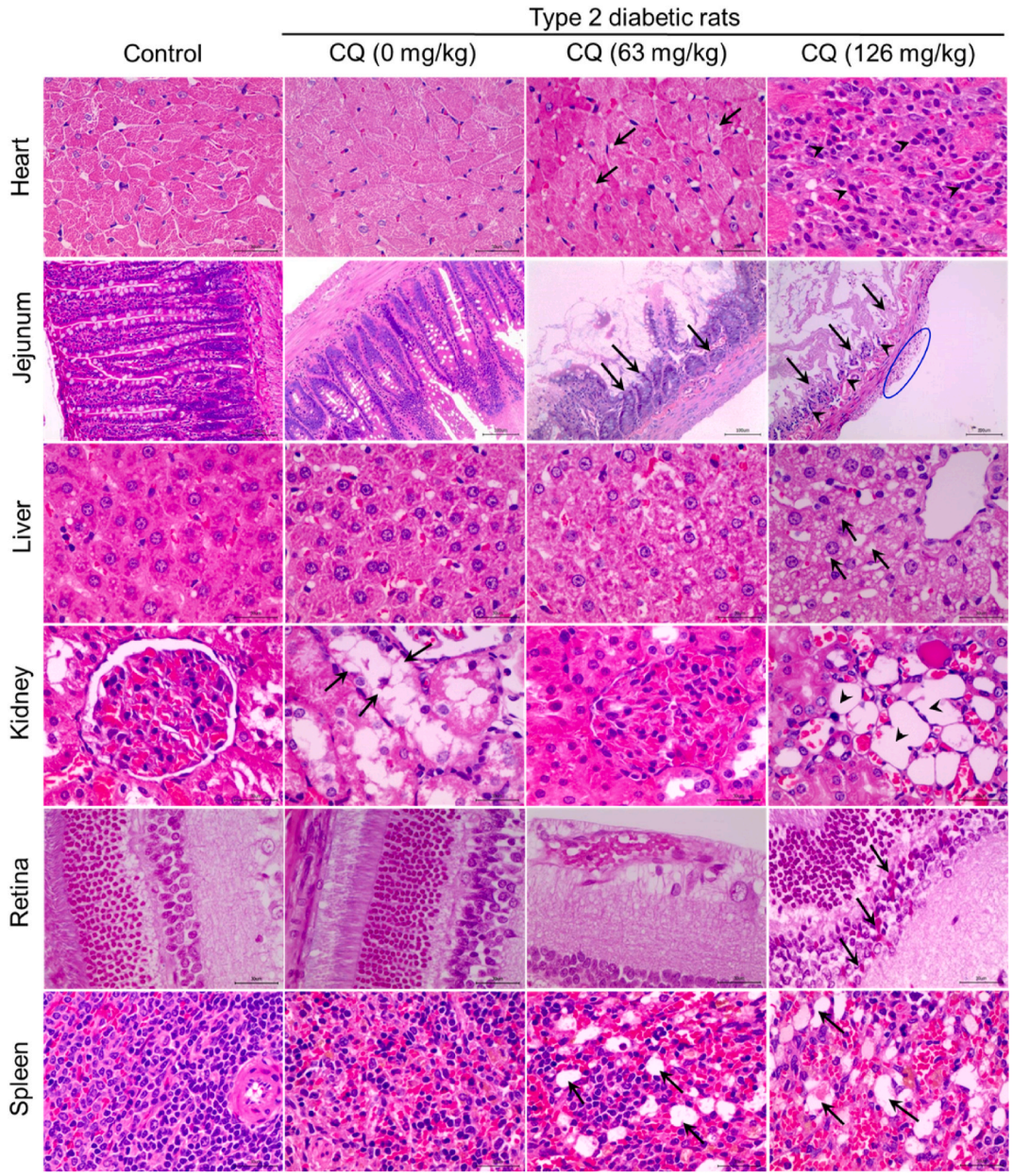

Fig. 4. CQ induces pathological rat tissue damage. Hematoxylin and eosin staining were carried out after tissues of rats were collected. There were various of severely histopathological alterations in several organic tissues, including heart (lipid deposition, black arrows; focal necrosis and lots of inflammatory cell infiltration, arrow heads), jejunum (necrosis and shedding of jejunal villi, black arrows; destruction of intestinal recess, arrow heads, disruption of serosal layer, blue ellipse), liver (incompact cytoplasm; steatosis and vacuolation, black arrows), kidney (vacuolar degeneration of renal tubular epithelial cells, black arrows; necrosis of renal tubular epithelial cells, arrow heads), retina (punctual hemorrhage in the inner granular layer, black arrows) and spleen (the diffuse vacuolization of macrophages, black arrows). Bars = 30 μm or 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the heart, suggesting potential cardiotoxicity occurred when using CQ in the setting of diabetes. In several clinical trials of CQ and HCQ against COVID-19, prolonged QT intervals have been frequently recorded [41,42]. Compared to CQ, HCQ had lower toxicity but also had adverse effects, such as QT interval prolongation in patients with severe COVID-19 [43]. Although a high dose of CQ is needed for treating coronavirus infection, most of the CQ dose regimens used in COVID-19 clinical trials were not likely to induce severe cardiotoxicity [44]; however, the ECG changes and the potential risk of cardiovascular abnormalities should be routinely monitored. Most hospitalized patients with COVID-19 had comorbidities, such as type 2 diabetes, and drug combinations, such as CQ-azithromycin/- propranolol, or other drug-drug interactions could aggravate cardiovascular disease risk of CQ and HCQ [45]. Therefore, a comprehensive understanding of the cardiovascular complications of patients with COVID-19 may help make more precise therapeutic decisions when using CQ.

In addition to lethal outcomes of CQ and HCQ, several adverse events (AEs) have been extensively reported in treating malaria, rheumatoid arthritis, and systemic lupus erythematosus [46]. Among these AEs, gastrointestinal toxicity, hepatotoxicity, nephrotoxicity, and retinal toxicity of CQ in T2D rats were found in a previous study. A higher risk of gastrointestinal AEs was found ($P < 0.01$) in patients with COVID-19 treated by CQ or HCQ [46]. Similar to CQ, the application of HCQ during prophylaxis and treatment of COVID-19 was caused some gastrointestinal toxicity [24,25,47]. In this study, diabetic rats developed more serious jejunum damage and a noticeable decrease in bodyweight and

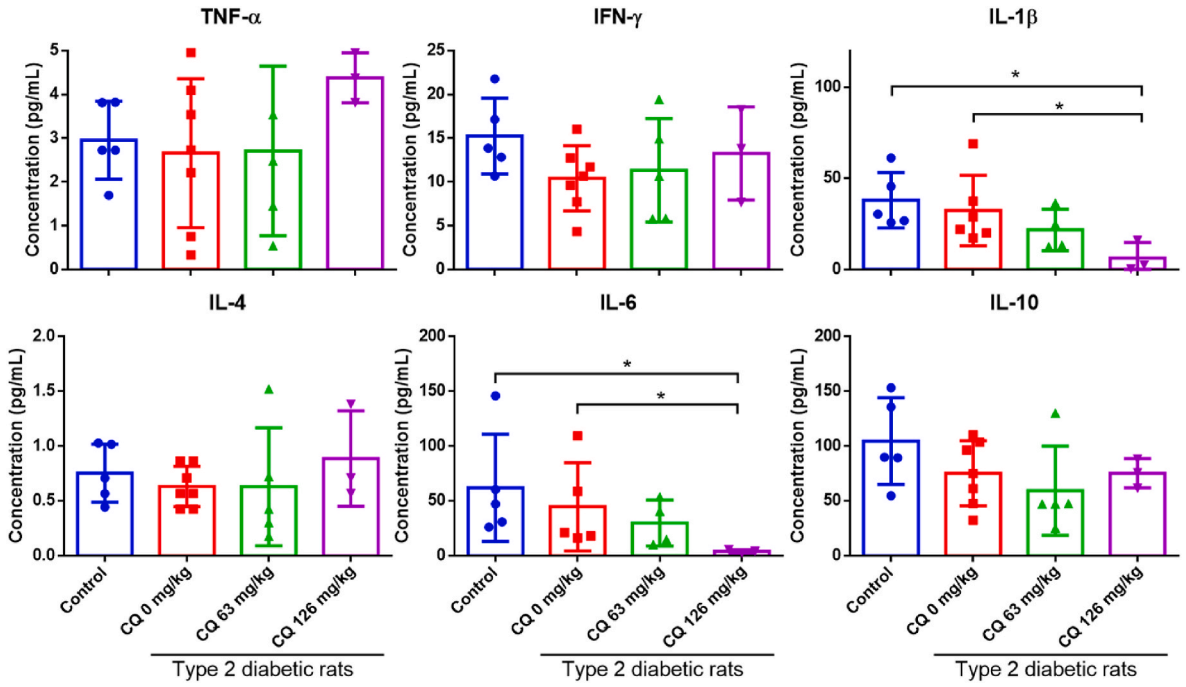

Fig. 5. Effect of CQ on serum inflammatory cytokines of rats. After CQ administration for seven days, serum of healthy and diabetic rats was collected for analyzing the levels of several inflammatory cytokines, including TNF-α, IFN-γ, IL-1β, IL-4, IL-6 and IL-10. The commercial ELISA kits were used to determine the concentrations of cytokines. In each group, the number of samples in each treatment was at least in triplicate. *, $P < 0.05$.

food consumption after CQ administration for 3 days. Therefore, caution against gastrointestinal toxicity should be taken early when repurposing CQ. Moreover, CQ induced ocular toxicity in T2D rats, exhibiting varying degrees of hemorrhagic effusion and retinal injury. Retinopathy incidence was a severe complication and one of the major dose-limited toxicities of CQ or HCQ usage [48]. This also occurred in patients with COVID-19 with blurred vision [49]. Based on the high prevalence (up to 8%) of retinopathy with long-term HCQ use [50], the American Academy of Ophthalmology recommended the limited dose of CQ should be < 2.3 mg/kg using actual body weight, and the dose of HCQ should be $<$ 5.0 mg/kg [51]. The risk of retinopathy has increased in the elderly; therefore, once the patients in clinical trials experience vision problems, the use of CQ or HCQ must be halted due to retinal damage that can persist long-term.

Furthermore, CQ-induced hepatotoxicity was observed in diabetic rats, although this was rare in treating malaria, lupus, or rheumatism [30]. However, as CQ and HCQ accumulate in the liver, acute liver injury may occur. During the COVID-19 pandemic, a case report of hepatotoxicity associated with HCQ administration occurred in Brazil [52]. HCQ was also reported to induce severe liver injury when used for treatment of porphyria cutanea tarda at doses used to treat systemic lupus erythematosus [53]. Such unusual hepatotoxicity of HCQ was associated with using a higher dose, drug-drug interactions, and the health condition of the involved patients. Considering the combination use of antivirals, antimicrobials, and vasoactive drugs in severe COVID-19 cases, the high risk of drug-related hepatotoxicity should not be neglected. Similarly, the nephrotoxicity of CQ should also be considered due to the high rate of kidney dysfunction in hospitalized patients with COVID-19 [54,55]. Kidney insufficiency reduces the excretion and elimination rate of CQ/HCQ, resulting in dose-dependent kidney injuries. During the CQ and HCQ clinical trials against COVID-19, nephrotoxicity in hospitalized patients with COVID-19 was reported [20]. Thus, patients with COVID-19 and diabetes should be monitored for the risk of renal failure with compassionate use of CQ and HCQ. In addition, CQ causes other side effects in patients with COVID-19, including neurological, psychological, dermatic, and respiratory problems [49].

Besides the above-mentioned toxic risks of CQ, some potential benefits were found in this study. First, it was surprising that CQ significantly decreased the blood and urinary glucose levels of the diabetic rats, which has already been demonstrated in previous reports [56–59]. Because HCQ has been approved for treating diabetes in India [60], it is critical to investigate the effect of HCQ on patients with COVID-19 who already took HCQ to control glycemia. In addition, given that CQ and HCQ lower blood glucose, close monitoring of blood glucose and timely reduction of other antidiabetic drugs or insulin in diabetic patients with COVID-19 are essential to avoid hypoglycemia [61]. Second, CQ inhibited the production of proinflammatory cytokines, including IL-1β and IL-6 [62]. Given that the cytokine storms commonly occur in severe COVID-19 patients, the anti-inflammatory properties of CQ and HCQ may provide potential benefits in the treatment of COVID-19 [45]. In clinical practice, a nonpeer-reviewed study suggested that HCQ (200 mg twice per day) for 7–10 days in critically ill patients with COVID-19 significantly decreased mortality and IL-6 levels [63]. However, the anti-inflammation properties of CQ and HCQ for patients with COVID-19 need more randomized clinical trial evidence soon.

Safe using of antiviral drugs was more difficult for elderly patients with underlying diseases like diabetes during the COVID-19 pandemic. Even with proof of the failure of HCQ against COVID-19 officially declared based on the findings of the international Solidarity Trial [64], CQ is still used in China and other countries. Our present study partly answered why this promising drug failed to treat SARS-CoV-2 infection in large population randomized clinical trials due to its narrow safety range especially for diabetic patients. Moreover, our findings also call attention to reevaluate the obtained clinical data of chloroquine on COVID-19 by excluding the data from patients with severe basic disease such as diabetes. On the other hand, the prophylaxis role of CQ and HCQ in patients with COVID-19 has been tested in appropriate trials [65]. Hence, the use of CQ/HCQ during this pandemic remains a questionable topic for the scientific community. Ideal dosing regimens should be evaluated to protect infected patients and avoid unwarranted overdosing to guarantee the safety of CQ and HCQ.

5. Conclusion

Patients with diabetes and COVID-19 can quickly become severely ill. Off-label or compassionate use of CQ to treat COVID-19 in the clinic may induce severe toxicities such as cardiac arrhythmia, gastrointestinal adverse effects, and ocular toxicity, making the situation even more difficult for patients with severe symptoms. A small dose and early application of CQ in diabetic patients combined with COVID-19 may be adequate for antiviral, antidiabetic action, and immunoregulatory function. Furthermore, to ensure the safety of the experimental CQ in vulnerable patients with COVID-19, medical staff should pay close attention to any potential adverse reactions and discontinue the medication as early as possible, that is before the occurrence of intolerable side effects.

CRediT authorship contribution statement

Xiuge Gao: Conceptualization, Investigation, Writing - Original Draft, Funding acquisition. Xian Jing: Methodology, Investigation, Writing - Original Draft. Junqi Wang: Software, Investigation, Resources. Yuling Zheng: Investigation, Validation, Visualization. Yawei Qiu: Methodology, Resources, Writing - Review & Editing. Hui Ji: Resources, Data Curation. Lin Peng: Software, Visualization. Shanxiang Jiang: Project administration, Writing - Review & Editing. Wenda Wu: Formal analysis, Conceptualization, Writing - Review & Editing. Dawei Guo: Conceptualization, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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