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Title: Lipid emulsion inhibits the cardiac toxicity caused by chloroquine via inhibition of reactive oxygen species production

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Running title: Chloroquine toxicity and lipid emulsion

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Lipid emulsion inhibits the cardiac toxicity caused by chloroquine via inhibition of reactive oxygen species production

Running title: Chloroquine toxicity and lipid emulsion
Abstract

Background: Lipid emulsion (LE) is effective in treating intractable cardiac depression induced by the toxicity of highly lipid-soluble drugs including local anesthetics. However, the effect of LE on chloroquine-evoked cardiac toxicity remains unclear. This study aimed to examine the effect of Lipofundin MCT/LCT, an LE, on the cardiotoxicity caused by chloroquine in H9c2 rat cardiomyoblasts and elucidate the underlying cellular mechanism.

Methods: The effects of chloroquine (1 × 10⁻⁴ M), LE, and the reactive oxygen species (ROS) scavengers mitotempo and N-acetyl-L-cysteine (NAC), alone or combined, on cell viability and migration, apoptosis, ROS production, calcium levels, mitochondrial membrane potential, and adenosine triphosphate (ATP) were examined. Additionally, the effects of LE on the activities of catalase (CAT), malondialdehyde (MDA), and superoxide dismutase (SOD) induced by chloroquine were assessed.

Results: Pretreatment with LE, mitotempo, or NAC reversed the reduction in cell migration and viability, mitochondrial membrane potential, and ATP levels evoked by chloroquine, and inhibited the increase in cleaved caspase-3, ROS, and calcium concentration induced by chloroquine. LE inhibited the increase in Bax expression, TUNEL-positive cells, MDA activity, and late apoptosis, and reversed the reduction in SOD and CAT activity induced by chloroquine. Chloroquine did not significantly affect cleaved caspase-8 expression, and LE did not significantly affect chloroquine concentration.

Conclusion: Collectively, these results suggest that LE with 50% long-chain and 50% medium-chain fatty acids inhibits the cardiotoxicity and late apoptosis induced by chloroquine toxicity via the intrinsic mitochondrial apoptotic pathway, which is associated with direct inhibition of ROS production.
Keywords: Chloroquine; Lipid emulsion; Cardiotoxicity; Apoptosis; Mitochondria; Reactive oxygen species.
Introduction

Lipid emulsion (LE) has primarily been used for parenteral nutrition and as a solvent for propofol and etomidate, but is now also used for the treatment of cardiovascular collapse caused by systemic toxicity of local anesthetics [1]. In addition, it has been reported that LE used as an adjuvant drug can treat intractable cardiovascular collapse induced by a toxic dose of non-local anesthetic drugs with high lipid solubility, which include calcium channel blocker, beta-blocker, antidepressant, and antipsychotic drugs [2]. Chloroquine and hydroxychloroquine are used to treat malaria, systemic lupus erythematosus, and rheumatoid arthritis, and they produce a variety of side-effects that include hypotension, ventricular arrhythmia, QT prolongation, QRS widening, and shock [3,4,5]. The toxic side-effects of these drugs are due to the blockade of cardiac sodium and potassium channels [3,4,5]. Hypotension and cardiac depression induced by chloroquine and hydroxychloroquine toxicity are treated with the following supportive measures: fluid resuscitation, vasopressors (epinephrine, phenylephrine, and norepinephrine), sodium bicarbonate, 3% sodium chloride, dextrose, and activated charcoal [4]. However, in some cases of chloroquine and hydroxychloroquine toxicity that were unresponsive to supportive treatments, LE was reported to be effective in treating chloroquine- and hydroxychloroquine-induced toxicity [6-9]. Chloroquine and hydroxychloroquine exhibit the following pharmacological properties, which are similar to those of the local anesthetic bupivacaine: First, bupivacaine (log P = 3.41) and chloroquine (log P = 4.63) are highly lipid-soluble, which may contribute to LE-mediated absorption of highly lipid-soluble drugs [1]. Second, chloroquine and hydroxychloroquine inhibit cardiac sodium and potassium channels, leading to QRS widening, QT prolongation, and cardiac depression, which are similar to the symptoms of cardiac toxicity due to bupivacaine toxicity [10,11]. Third, in a rat model of pressure overload hypertrophy, Chaanine et al. showed that chloroquine-induced cardiac toxicity was mediated by impaired mitochondrial antioxidant function and enhanced oxidative stress [12]. Furthermore, chloroquine-
induced ototoxicity is mediated by reactive oxygen species (ROS) production in glial cells of the ear [13]. Similar to previous reports which demonstrated that chloroquine toxicity is mediated by oxidative stress and ROS [12,13], bupivacaine-induced cardiac toxicity is also mediated by ROS production, and is inhibited by Intralipid-induced reduction of ROS levels [14,15]. However, the effect of LE on chloroquine-induced cardiac toxicity remains unknown. Recently, chloroquine and hydroxychloroquine were reported to attenuate severe acute respiratory syndrome coronavirus 2 in *in vitro* experiments [16-19]. However, a meta-analysis of further clinical studies reported that treatment with chloroquine and hydroxychloroquine, which were used as alternative drugs to treat patients with coronavirus disease 2019, resulted in cardiac toxicity, including ventricular tachycardia, QT prolongation, torsade de pointes, and cardiac arrest due to the inhibition of sodium and potassium channels in the heart [20,21]. Since bupivacaine and chloroquine share some pharmacological properties, we tested the biological hypothesis that LE inhibits the cardiac toxicity caused by chloroquine via inhibition of ROS production or LE-mediated sequestration of highly lipid-soluble chloroquine [4,10,12-15]. The aim of this *in vitro* study was to examine the effect of Lipofundin MCT/LCT, an LE, on the cardiac toxicity evoked by chloroquine in H9c2 rat cardiomyoblasts and clarify the associated cellular mechanism.
Materials and Methods

Cell culture

The American Type Culture Collection supplied the H9c2 cardiomyoblasts (#CRL-1446, Rockville, MD, USA). H9c2 cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) (#11995, Gibco, Life Technologies, Grand Island, NY, USA) with 10% fetal bovine serum (#16000, Gibco) and 1% penicillin/streptomycin (#15140122, Gibco) at 37°C in a 5% CO2 environment, as reported previously [22]. Prior to drug treatment, the cells were preincubated for 16 h in DMEM without serum.

Cell viability assay

The Cell Counting Kit 8 (CCK-8) (#CK04-13, Dojindo Molecular Technologies, Kumamoto, Japan) was used to determine cell viability in accordance with the manufacturer's procedure, as reported previously [22]. Briefly, H9c2 cells were plated in 24-well dishes at $2 \times 10^4$ cells per well and treated for 24 h with various doses of chloroquine ($10^{-6}$, $10^{-5}$, $10^{-4}$ and $10^{-3}$ M). To investigate the effect of LE (Lipofundin MCT/LCT) or Intralipid on the decreased cell viability caused by toxic levels of chloroquine, cells were treated with chloroquine ($10^{-4}$ M) alone for 24 h; LE or Intralipid (0.1, 0.2, 0.5, 0.75, and 1%) for 1 h, followed by chloroquine ($10^{-4}$ M) for 24 h; or LE or Intralipid (0.1, 0.2, 0.5, 0.75, and 1%) alone for 25 h [23]. In addition, to examine how the ROS scavenger N-acetyl-L-cysteine (NAC) and mitochondrial ROS scavenger mitotempo affect the reduced cell viability produced by toxic amounts of chloroquine, cells were treated with chloroquine ($10^{-4}$ M) alone for 24 h; LE (0.75%) or ROS scavenger ($10^{-4}$ M NAC or $10^{-5}$ M mitotempo) for 1 h, followed by chloroquine ($10^{-4}$ M) for 24 h; and LE (0.75%) or ROS scavenger ($10^{-4}$ M NAC or $10^{-5}$ M mitotempo) for 25 h. After the treatment, the cells were incubated with 500 μl of DMEM including 10% CCK-8 solution for 3 h under 5% CO2 at 37°C. The absorbance at 405 nm was determined.
using a VersaMax® microplate reader (Molecular Devices, Sunnyvale, CA, USA).

**Wound healing assay**

A wound healing assay was performed to observe the effects of LE and ROS scavenger on cell migration after chloroquine treatment of H9c2 cells, as described previously [24]. H9c2 cells were seeded in 100 mm culture dishes (at a density of $1 \times 10^6$ cells) and incubated in a CO$_2$ (5% at 37°C) incubator. Cells were grown to 90–100% confluency. After scratching the cells with a pipette tip (200 μl yellow tip) and washing them with phosphate-buffered saline (PBS), culture medium was added to the culture dishes, and cell images were obtained using a microscope (Nikon Eclipse Ti2; Nikon Co., Tokyo, Japan). Cells were then treated with chloroquine (10$^{-4}$ M) alone for 18 h; LE (Lipofundin MCT/LCT, 0.75%) or ROS scavenger (10$^{-4}$ M NAC or 10$^{-5}$ M mitotempo) for 1 h, followed by chloroquine (10$^{-4}$ M) for 18 h; and LE (0.75%) or ROS scavenger (10$^{-4}$ M NAC or 10$^{-5}$ M mitotempo) alone for 19 h. A light microscope fitted with camera (Nikon Eclipse Ti2) was then used to capture images of the cells.

**Western blot analysis**

Analysis of cleaved caspase-3, Bax, and cleaved caspase-8 protein levels in H9c2 cells was performed using western blotting, as reported previously [22]. For the evaluation of cleaved caspase-3 expression, cells were treated with chloroquine (10$^{-4}$ M) alone for 12 h; LE (Lipofundin MCT/LCT, 0.75%) or ROS scavenger (10$^{-4}$ M NAC or 10$^{-5}$ M mitotempo) for 1 h, followed by chloroquine (10$^{-4}$ M) for 12 h; and LE (0.75%) or ROS scavenger (10$^{-4}$ M NAC or 10$^{-5}$ M mitotempo) alone for 13 h. For the evaluation of Bax expression, cells were treated with chloroquine (10$^{-4}$ M) for 4 h; LE (0.75%) for 1 h, followed by chloroquine (10$^{-4}$ M) for 4 h; or LE (0.75%) alone for 5 h. For the evaluation of cleaved caspase-8 expression, cells were treated with chloroquine (10$^{-4}$ M) for
2 h; LE (0.75%) for 1 h, followed by chloroquine (10^{-4} M) for 2 h; or LE (0.75%) alone for 3 h. After treatment, the cells were harvested in radio-immunoprecipitation assay lysis buffer (#9806, Cell Signaling Technology, Beverly, MA, USA) supplemented with protease and phosphatase inhibitor cocktails (#78440, Thermo Fisher Scientific, Rockfield, IL, USA). Protein amounts were quantified using a bicinchoninic acid protein assay reagent kit (#23227, Thermo Fisher Scientific). After 10 minutes of heating at 100°C, the protein samples were fractionated via 10–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride membranes (#IPVH00010, Millipore, Bedford, MA, USA). After transfer, membranes were blocked in 5% skim milk or 5% bovine serum albumin in Tris-buffered saline containing 0.5% Tween-20 (TBST) for 1 h at room temperature (22–27°C), followed by incubation with primary antibodies (anti-cleaved caspase-3 [1: 1000], anti-cleaved caspase-8 [1: 1000], anti-Bax [1:1000], and anti-β-actin [1: 10000] antibody) for 16 h at 4°C. The membranes were rinsed with TBST, and incubated with horseradish peroxidase–conjugated goat anti-rabbit or anti-mouse secondary antibody (1: 5000) for 1 h at room temperature (22–27°C). The proteins were visualized using the Westernbright™ ECL western blotting Detection Kit (#K-12045-D50, Advansta, Menlo Park, CA, USA) and ECL images were obtained using a ChemiDoc™ Touch Imaging System (Bio-Rad Laboratories Inc., Berkely, CA, USA). The density of the bands was assessed using ImageJ software (version 1.45s; National Institutes of Health, Bethesda, MD, USA).

**Terminal deoxynucleotidyl transferase dUTP nick end labeling assay**

Apoptotic cells were identified by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. A TUNEL kit (#11684795910, Roche Applied Science, Indianapolis, IN, USA) was used following the manufacturer’s directions as previously reported [22]. H9c2 cells were
seeded on coverslips in 6-well dishes and treated with chloroquine \((10^{-4} \text{ M})\) alone for 12 h; LE (Lipofundin MCT/LCT, 0.75%) for 1 h, followed by chloroquine \((10^{-4} \text{ M})\) for 12 h; or LE (0.75%) alone for 13 h. Cells were then counterstained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescence images were captured on a fluorescence microscope (Nikon Eclipse Ti2). The number of TUNEL-positive cells was determined as a percentage of DAPI-stained cells in each field of view.

**Annexin V-fluorescein isothiocyanate/propidium iodide (Annexin V-FITC/PI) staining**

Annexin V-FITC/PI staining was used to assess early/late apoptosis using flow cytometry with a FITC Annexin V Apoptosis Detection Kit (V13242, Invitrogen-Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s protocol, as described previously [22]. Briefly, cells were inoculated in 6-well dishes at \(1 \times 10^5\) cells per well and subsequently treated with chloroquine \((10^{-4} \text{ M})\) alone for 24 h; LE (Lipofundin MCT/LCT, 0.75%) for 1 h, followed by chloroquine \((10^{-4} \text{ M})\) for 24 h; or LE (0.75%) alone for 25 h. The cells were collected after treatment, rinsed two times in PBS, and resuspended with 1× binding buffer. All cells were incubated with annexin V-FITC and a PI staining solution \((100 \mu\text{g/mL})\) in the dark at room temperature \((22–27^\circ\text{C})\) for 15 min. Binding buffer \((1\times)\) was added to each tube, and cells were examined with a BD LSRRFortessa X-20 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) to determine the proportion of apoptotic cells in the cell population. The BD FACSDiva™ software (version 6.0; BD Biosciences) was used to analyze the data.

**Measurement of intracellular levels of ROS**

Intracellular ROS production during chloroquine treatment was analyzed using 2', 7'-dichlorofluorescin diacetate (H\(_2\)DCFDA), as described previously [15]. The cells were seeded in 6-well dishes (at a density of \(1 \times 10^5\) cells per well) containing cover glasses and incubated in a CO\(_2\)
incubator overnight. The cells were treated with chloroquine (10⁻⁴ M) alone for 1 h; LE (Lipofundin MCT/LCT, 0.75%) or ROS scavenger (10⁻⁴ M NAC or 10⁻⁵ M mitotempo) for 1 h, followed by chloroquine (10⁻⁴ M) for 1 h; and LE (0.75%) or ROS scavenger (10⁻⁴ M NAC or 10⁻⁵ M mitotempo) alone for 2 h. Cells were treated with 10 µM H₂DCFDA for 30 min at 37°C before being washed with PBS. The mounting solution (#S3023, DAKO fluorescent mounting medium, Dako North America, Inc., Carpinteria, CA, USA) was added drop-wise, and the cell-bound cover glass was placed on the slide. Then, images of the cells were obtained using a fluorescence microscope (Nikon Eclipse Ti2) and ROS generation was analyzed.

Mitochondrial membrane potential measurement

The mitochondrial membrane potential was measured using the JC-1 mitochondrial membrane potential detection kit (#30001, Biotium Inc. Hayward, CA, USA), as previously described [15]. Cells were seeded in 6-well dishes containing cover glasses at a density of 1 × 10⁵ cells per well. After incubation, the cells were treated with chloroquine (10⁻⁴ M) alone for 6 h; LE (Lipofundin MCT/LCT, 0.75%) or ROS scavenger (10⁻⁴ M NAC or 10⁻⁵ M mitotempo) for 1 h, followed by chloroquine (10⁻⁴ M) for 6 h; or LE (0.75%) or ROS scavenger (10⁻⁴ M NAC or 10⁻⁵ M mitotempo) alone for 7 h. The cells were washed with PBS and then, 1× JC-1 dye reagent working solution was added to the cells for 15 min, and the cells were washed with PBS. The mitochondrial membrane potential was analyzed using a fluorescence microscope (Nikon Eclipse Ti2) and calculated as the fluorescence intensity of the JC-1 red/green ratio and normalized to the control.

Measurement of intracellular calcium concentration

The change in intracellular calcium levels [Ca²⁺]i was assessed using a confocal laser microscope (IX70 Fluoview, Olympus, Tokyo, Japan), as described previously [15]. H9c2 cells were cultured...
in a confocal cell culture dish (SPL, Pocheon, Korea) and incubated with 2.5 μM Fluo-4 AM (#F14217, Invitrogen-Life Technologies) in Hank’s Balanced Salt Solution (HBSS, #14175, Gibco) for 30 min. Subsequently, cells were washed twice with PBS and after replacing the HBSS, the cells were treated with chloroquine (10⁻⁴ M) alone and LE (Lipofundin MCT/LCT, 0.75%) or ROS scavenger (10⁻⁴ M NAC or 10⁻⁵ M mitotempo), followed by chloroquine (10⁻⁴ M). Ca²⁺ levels were monitored every 2.5 seconds at excitation and emission wavelengths of 485 and 520 nm, respectively. Ca²⁺ levels were analyzed in scanned images and calculated as drug-treated fluorescence intensity (F) divided by baseline fluorescence intensity (F₀) before drug treatment. The net change in calcium ions was indicated as (Fₘₐₓ-F₀)/F₀, where Fₘₐₓ represents the maximum calcium level corresponding to the fluorescence intensity after chloroquine (10⁻⁴ M) treatment in the presence or absence of LE or ROS scavenger pretreatment. Changes in Ca²⁺ levels were measured for 7 min.

Measurement of malondialdehyde, superoxide dismutase, and catalase activity

The levels of malondialdehyde (MDA, #ab118970), superoxide dismutase (SOD, #ab65354), and catalase (CAT, #ab83464) were measured with MDA, SOD, and CAT assay kits (Abcam, Cambridge, MA, USA), respectively, according to the manufacturers’ protocols, as described previously [25]. To measure SOD, MDA, and CAT activity, H9c2 cells were cultured in 100 mm culture dishes at a density of 2 × 10⁶, 1 × 10⁶, and 1 × 10⁶ cells, respectively. Cells were treated with chloroquine (10⁻⁴ M) alone for 18 h, LE (Lipofundin MCT/LCT, 0.75%) for 1 h followed by chloroquine (10⁻⁴ M) for 18 h, or LE (0.75%) alone for 19 h. The samples collected for MDA activity measurement were incubated with MDA lysis buffer, homogenized, and centrifuged to collect the supernatant. Samples were incubated with thiobarbituric acid reagent at 95°C for 60 min. After allowing the samples to cool to room temperature (22-27°C) on ice, the analysis was performed with a VersaMax® microplate reader. The MDA concentration was calculated using the formula provided with the assay kit. The
harvested cells for SOD activity measurement were lysed in lysis buffer and centrifuged to collect the supernatant. SOD activity was measured using water-soluble tetrazolium salts working solution, dilution buffer, and enzyme working solution. SOD activity was measured at 450 nm using a VersaMax® microplate reader. The SOD level was calculated using the formula described in the assay kit. Samples for CAT activity measurement were lysed in CAT assay buffer and the supernatant was collected. For the CAT reaction, H2O2 was added to the samples and incubated at 25°C, followed by the addition of stop solution. The prepared Developer Mix was mixed with the samples and incubated at 25°C. CAT activity was measured at 570 nm using a VersaMax® microplate reader, and calculated using the formula provided with the assay kit.

**Adenosine triphosphate measurement**

Intracellular adenosine triphosphate (ATP) levels in chloroquine-treated H9c2 cells were measured using an ATP detection kit (#ab83355, Abcam) according to protocol of the manufacturer, as described previously [26]. H9c2 cells were seeded and incubated in a serum-free medium in a 37°C CO2 incubator. Cells were treated with chloroquine (10^{-4} M) alone for 18 h and LE (Lipofundin MCT/LCT, 0.75%) or ROS scavenger (10^{-4} M NAC or 10^{-5} M mitotempo) for 1 h, followed by chloroquine (10^{-4} M) for 18 h. Cells were lysed with ATP assay buffer, and centrifuged (13,000 \times g) at 4°C for 5 min to collect the supernatant and transferred to a new tube. The prepared ATP reaction mixture was added to the sample and standard, mixed, and incubated at room temperature (22–27°C) for 30 min in the dark. ATP levels were measured using a VersaMax® microplate reader at 570 nm, and a standard curve was created with authentic ATP for quantitative analysis.

**Effect of LE on chloroquine concentration in the distilled water**

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Chloroquine ($10^{-4}$ M) was emulsified with LE (Lipofundin MCT/LCT, 0.75%) in distilled water, as described earlier [15]. The sample emulsion was centrifuged at $18,500 \times g$ for 30 min to estimate the release of chloroquine from the samples. The concentration of chloroquine in the aqueous phase was analyzed using ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF MS; Waters, Milford, MA, USA). The aqueous layer was injected into an Acquity UPLC BEH C$_{18}$ column (100 × 2.1 mm, 1.7 m; Waters) equilibrated with water/acetonitrile (99:1), including 0.1% formic acid (FA), and dissolved in a linear gradient (1–100%) of acetonitrile, including 0.1% FA, for 5 min at a flow rate of 0.35 mL/min. Q-TOF MS in positive electrospray ionization mode was used to evaluate the eluted chloroquine. The optimal MS operating conditions were set as follows: The voltages of capillary and sampling cones were set to 3 V and 30 kV, respectively; the source and desolvation temperatures were 400 and 100°C, respectively; and the desolvation flow was 800 L/h. The eluted chloroquine was detected by multiple reaction monitoring mode with precursor and product ions of m/z 409.14 and 294.08, respectively. To assure the reproducibility and accuracy of all analyses, a lock spray was performed with leucine-enkephalin ([M + H] = 556.2771) used as the lock mass. All mass results were gathered and evaluated using the UIFI 1.8.2 (Waters).

Materials

The purest chemical substances are commercially available. Lipofundin MCT/LCT 20% was donated by B. Braun Melsungen AG (Melsungen, Germany). Intralipid 20% was obtained from Fresenius Kabi AB (Upsala, Sweden). Chloroquine (#PHR1258), anti-β-actin antibody (#A5441), NAC (#A9165), mitotempo (#SML0737), DAPI (#D9542), and H$_2$DCFDA (#287810) were purchased from Sigma-Aldrich (St Louis, MO, USA). The anti-cleaved caspase-8 antibody was purchased from Novus Biologicals (#NB100-56116, Littleton, CO, USA). Anti-cleaved caspase-3 (#9661) and anti-Bax (#2772) antibodies were obtained from Cell Signaling Technology. All drugs

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were dissolved in distilled water.

**Statistical analysis**

Normality tests were performed using a Kolmogorov-Smirnov or Shapiro-Wilk test. The primary end points were the effects of LE, Intralipid, and ROS inhibitors on cell viability and migration, TUNEL-positive cells, apoptosis, cleaved caspase-3, cleaved caspase-8, and Bax expression, which is induced by chloroquine. The effects of LE, Intralipid, chloroquine, and ROS inhibitors, alone or in combination, on cell viability and migration, TUNEL-positive cells, apoptosis, cleaved caspase-3, cleaved caspase-8, Bax expression, mitochondrial membrane potential, intracellular calcium level, MDA, SOD and CAT activity, and ATP were analyzed using a one-way analysis of variance, followed by Bonferroni’s test (Prism, Version 5.0; GraphPad Software, San Diego, CA, USA). The effects of LE and chloroquine, alone or in combination, on mitochondrial membrane potential were analyzed using the Kruskal-Wallis test, followed by Dunn’s multiple comparison test. The Kruskal-Wallis test, followed by Dunn’s multiple comparison test was used to analyze the effects of LE, chloroquine, and ROS inhibitors, alone or in combination, on ROS production. An unpaired Student’s *t*-test was used to analyze the effect of LE on the chloroquine concentration. The number of independent experiments was determined using the resource equation method and based on previous similar experiments [25,27]. Results with *P* < 0.05 were considered statistically significant.
Results

Effects of LE and ROS scavenger on the decreased cell viability and migration induced by a toxic dose of chloroquine

Treatment with chloroquine (10^{-4} and 10^{-3} M) reduced cell viability ($P < 0.001$ versus the control, 95% confidence interval [CI]: 66.78 to 70.86 and 97.61 to 101.7 at 10^{-4} and 10^{-3} M, respectively, Fig. 1A). Lipofundin MCT/LCT (0.1 to 1%), which is made up of 50% long-chain triglycerides and 50% medium-chain triglycerides, reversed the decrease in cell viability induced by chloroquine (10^{-4} M) (0.1 to 1%: $P < 0.001$ versus chloroquine alone, 95% CI: -10.0 to -2.1, -15.1 to -7.2, -25.0 to -17.0, -34.2 to -26.8, and -30.8 to -23.0 at 0.1, 0.2, 0.5, 0.75, and 1%, respectively, Fig. 1B). In contrast, Intralipid (0.2 to 1%), which contains only 100% long-chain triglyceride, only slightly reversed the chloroquine (10^{-4} M)-induced reduction in cell viability (0.2%: $P = 0.012$ versus chloroquine alone, 0.5 to 1%: $P < 0.001$ versus control, 95% CI: -8.2 to -0.6, -11.3 to -3.7, -15.1 to -7.5, and -17.5 to -9.9 at 0.2, 0.5, 0.75, and 1%, respectively, supplementary Fig. 1). Lipofundin MCT/LCT (0.2, 0.5, and 0.75%) alone slightly improved cell viability (0.2, 0.5 and 0.75%: $P < 0.001$ versus control, 95% CI: -10.2 to -2.3, -12.2 to -4.3, and -11.4 to -3.5 at 0.2, 0.5, and 0.75%, respectively, Fig. 1B), but Intralipid (0.1, 0.2, 0.75, and 1%) alone reduced cell viability (0.75%: $P = 0.006$ versus control, 0.1, 0.2, and 1%: $P < 0.001$ versus control, 95% CI: 3.7 to 11.4, 2.3 to 10.0, 1.0 to 8.6, and 2.6 to 10.2 at 0.1, 0.2, 0.75, and 1%, respectively, supplementary Fig. 1). The ROS scavenger NAC (10^{-4} M) and mitochondrial ROS scavenger mitotempo (10^{-5} M) reversed the reduction in cell viability evoked by chloroquine (10^{-4} M) ($P < 0.001$ versus chloroquine alone; 95% CI: -19.7 to -14.0, and -10.9 to -5.2 at mitotempo and NAC, respectively, Fig. 1C). In addition, chloroquine treatment (10^{-4} M) reduced cell migration ($P < 0.001$ versus the control; 95% CI: -64.7 to -50.0, Fig. 2A). However, pretreatment with LE (0.75%) increased cell migration compared with chloroquine alone ($P < 0.001$; 95% CI: 9.2 to 23.9, Fig. 2A). Additionally, pretreatment with the
ROS scavenger NAC (10⁻⁴ M) or mitotempo (10⁻⁵ M) increased cell migration compared to chloroquine alone (P < 0.001; 95% CI: 20.3 to 36.8, and 16.4 to 32.9 at NAC and mitotempo, respectively, Fig. 2B).

**Effects of LE and ROS scavenger on apoptosis induced by a toxic dose of chloroquine**

The cleavage of caspase-3 (a signaling molecule downstream of the common pathway associated with both intrinsic and extrinsic apoptotic cell death) is a well-known marker of the apoptotic cell death process [28]. Treatment with chloroquine (10⁻⁴ M) induced cleaved caspase-3 expression (P < 0.001 versus control; 95% CI: -4.94 to -3.96, Fig. 3A), whereas LE (0.75%), ROS scavenger NAC (10⁻⁴ M), and mitotempo (10⁻⁵ M) treatment inhibited the chloroquine (10⁻⁴ M)-induced cleaved caspase-3 expression (P < 0.001 versus chloroquine alone; 95% CI: 4.14 to 5.15, 1.09 to 2.07, and 1.2 to 2.17 at LE, mitotempo, and NAC, respectively, Fig. 3A), leading to inhibition of apoptotic cell death. Chloroquine (10⁻⁴ M) produced an increase in the expression of the intrinsic proapoptotic protein Bax (P < 0.001 versus control; 95% CI: -1.043 to -0.4667, Fig 3B), which is associated with mitochondrial stress [28]. LE treatment (0.75%) reduced the increase in Bax expression induced by chloroquine (10⁻⁴ M) (P < 0.001 versus chloroquine alone; 95% CI: 0.23 to 0.81, Fig. 3B), suggesting that it reduces mitochondrial stress. However, chloroquine treatment (10⁻⁴ M) did not significantly affect the expression of the extrinsic proapoptotic protein cleaved caspase-8, which is associated with apoptotic cell death activated via cell death receptors [28], and LE did not significantly affect the expression of cleaved caspase-8 induced by chloroquine (10⁻⁴ M) (Fig. 3C). Chloroquine (10⁻⁴ M) treatment induced TUNEL-positive cells (P < 0.001 versus the control; 95% CI: -32.6 to -27.7, Fig. 4A), suggesting the presence of DNA fragmentation due to apoptosis, whereas LE (0.75%) treatment inhibited the chloroquine (10⁻⁴ M)-induced increase in TUNEL-positive cells (P < 0.001 versus chloroquine alone; 95% CI: 23.5 to 28.3, Fig. 4A), suggesting
inhibition of apoptosis. Furthermore, chloroquine (10^{-4} \text{ M}) treatment increased both early and late stages of apoptotic cell death (early apoptosis: \( P = 0.005 \) versus control, late apoptosis: \( P < 0.001 \) versus control; 95% CI: -9.53 to -1.97 and -19.3 to -14 at early and late apoptosis, respectively, Fig. 4B). Chloroquine (10^{-4} \text{ M}) increased necrosis (\( P = 0.034, 95\% \text{ CI: } -8.7 \text{ to } -0.2 \), Fig. 4B), however, LE (0.75%) treatment inhibited the late stage of apoptotic cell death evoked by chloroquine (10^{-4} \text{ M}) (\( P < 0.001 \) versus chloroquine alone; 95% CI: 8.1 to 13.4, Fig. 4B).

**Effects of LE and ROS scavengers on physiological changes induced by toxic dose of chloroquine**

Chloroquine treatment (10^{-4} \text{ M}) increased ROS levels (\( P < 0.001 \) versus control, Fig. 5A), whereas pretreatment with LE (0.75%), the ROS scavenger NAC (10^{-4} \text{ M}), and mitotempo (10^{-5} \text{ M}) inhibited the ROS production evoked by chloroquine (10^{-4} \text{ M}) (\( P < 0.001 \) versus chloroquine alone; Fig. 5A). Chloroquine treatment (10^{-4} \text{ M}) reduced the mitochondrial membrane potential (\( P < 0.001 \) versus control; Fig. 5B), suggesting that it promotes mitochondrial dysfunction. However, treatment with LE (0.75%), NAC (10^{-4} \text{ M}), and mitotempo (10^{-5} \text{ M}) reversed the reduction in mitochondrial membrane potential evoked by chloroquine (10^{-4} \text{ M}) (\( P < 0.001 \) versus chloroquine alone; 95% CI: -0.608 to -0.384, and -0.592 to -0.368 at NAC, and mitotempo, respectively, Fig 5B), indicating the attenuation of mitochondrial dysfunction. Chloroquine treatment (10^{-4} \text{ M}) increased the intracellular calcium levels (Fig. 6A), suggesting that calcium regulation is impaired by this drug. However, treatment with LE (0.75%), the ROS scavenger NAC (10^{-4} \text{ M}), and mitotempo (10^{-5} \text{ M}) reduced the calcium levels increased by chloroquine (10^{-4} \text{ M}) (\( P < 0.001 \) vs. chloroquine alone; 95% CI: 0.50 to 1.34, 0.47 to 1.30, and 0.81 to 1.64 at LE, NAC, and mitotempo, respectively, Fig. 6A), suggesting that they reduce the mitochondrial ROS-induced increase in calcium. Chloroquine treatment (10^{-4} \text{ M}) increased the levels of MDA, an indicator of oxidative stress (\( P < 0.001 \) versus
control; 95% CI: -120 to -71, Fig. 6B), whereas LE treatment (0.75%) inhibited the increased MDA activity evoked by chloroquine (10^{-4} M) ($P < 0.001$ versus chloroquine alone; 95% CI: 55 to 104, Fig. 6B), suggesting an inhibition of increased oxidative stress. Chloroquine treatment (10^{-4} M) reduced the activities of SOD and CAT (SOD and CAT: $P < 0.001$ versus control; 95% CI: 29.5 to 41.8 and 3.7 to 4.7 for SOD and CAT, respectively, Fig. 6C and D), which are antioxidant enzymes, suggesting a relative increase in oxidative stress. However, LE (0.75%) reversed the reduction in the activities of SOD and CAT induced by chloroquine (10^{-4} M) ($P < 0.001$ versus chloroquine alone; 95% CI: -21.9 to -9.5 and -2.3 to -1.3 for SOD and CAT, respectively, Fig. 6C), suggesting that it promotes relatively decreased oxidative stress.

**Effects of LE and ROS scavengers on ATP levels decreased by toxic dose of chloroquine**

Additionally, chloroquine treatment (10^{-4} M) reduced ATP levels ($P < 0.001$ versus the control; 95% CI: 61.16 to 84.39, Fig. 7). However, treatment with LE (0.75%), the ROS scavenger mitotempo (10^{-5} M), and NAC (10^{-4} M) reversed the reduction in ATP levels evoked by chloroquine (10^{-4} M) ($P < 0.001$ versus chloroquine alone; 95% CI: -44.93 to -22.70, -59.71 to -37.47, and -37.87 to -15.63 for LE, NAC, and mitotempo, respectively, Fig. 7).

**Effect of LE on chloroquine concentration**

LE (0.75%) did not significantly alter chloroquine concentration (10^{-4} M) in distilled water ($P = 0.0572$, and Fig. 8), suggesting that LE does not absorb chloroquine.
Discussion

This study demonstrates that LE inhibits the cardiac toxicity caused by chloroquine toxicity, and that this is due to the direct inhibition of ROS production. The major results of this study are as follows: 1) treatment with LE and the ROS scavengers NAC and mitotempo reversed the reduction in cell viability and migration and increase in cleaved caspase-3 expression evoked by chloroquine; 2) LE treatment inhibited Bax expression and the late apoptosis induced by chloroquine; 3) treatment with LE and ROS scavengers reversed ROS production, increased calcium levels, and reduced ATP levels and mitochondrial membrane depolarization caused by chloroquine.

Intralipid reverses the reduction in cell viability induced by verapamil, amlodipine, and bupivacaine, which are highly lipid soluble (log P >2) [14,15,22,29,30]. In addition, NAC attenuates the bupivacaine-induced reduction in cell viability [15]. In line with earlier reports, LE, NAC, and the mitochondrial ROS scavenger mitotempo reversed the reduction in cell viability evoked by toxic levels of highly lipid-soluble chloroquine, which suggests that the ROS production induced by chloroquine contributes to a reduced cell viability [14,15,23]. In accordance with the results of the cell viability study, chloroquine treatment reduced cell migration, whereas LE, NAC, and mitotempo treatment reversed the reduction in cell migration induced by chloroquine. Intralipid inhibits cleaved caspase-3 expression in rat cardiomyoblasts exposed to a toxic dose of bupivacaine, suggesting that Intralipid inhibits apoptotic cell death [31]. Similar to this report, LE, NAC, and mitotempo inhibited the cleaved caspase-3 expression induced by toxic concentrations of chloroquine (Fig. 3A) [31]. In addition, LE inhibited the Bax expression induced by toxic concentrations of chloroquine (Fig. 3B); however, a toxic dose of chloroquine did not significantly change cleaved caspase-8 expression (Fig. 3C). When combined, these results suggest that LE inhibits chloroquine-evoked apoptosis via the intrinsic mitochondrial apoptotic pathway, which is mediated by the inhibition of ROS production. Compared to Lipofundin MCT/LCT, Intralipid displays greater
inhibition of the decrease in cell viability induced by a toxic dose of bupivacaine [31]. However, in
the current study, Lipofundin MCT/LCT substantially reversed the decrease in cell viability due to a
toxic dose of chloroquine in comparison to Intralipid. The differences in these results may be due
to specific physicochemical interactions between the fatty acids (100% long-chain alone or 50% long-
chain plus 50% medium-chain) and the offending drugs (bupivacaine or chloroquine), as well as the
different concentrations of Intralipid and Lipofundin MCT/LCT used. The Lipofundin MCT/LCT-
mediated increased reversal of reduced cell viability caused by chloroquine may be associated with
medium-chain triglycerides contained in Lipofundin MCT/LCT, compared to Intralipid (which is
comprised of 100% long-chain fatty acid alone). In addition, this difference may be ascribed to the
differences in cell viability evoked by Lipofundin MCT/LCT or Intralipid alone (increased cell
viability evoked by Lipofundin MCT/LCT alone versus decreased cell viability evoked by Intralipid
alone; Fig. 1B versus supplementary Fig. 1). Further studies should examine which type of medium-
chain triglyceride present in Lipofundin MCT/LCT is the main contributor to the reversal of reduced
cell viability.

Intracellular ROS, which are mostly produced in mitochondria, induce disturbances in respiratory
chain function, leading to further ROS generation, a reduced membrane potential of mitochondria,
and reduced ATP production [28]. Eventually, ROS in the mitochondria, which are produced
endogenously or exogenously, induce p53 and c-Jun N-terminal kinase activation, leading to the
activation of proapoptotic Bcl-2 proteins (Bad, Bax, Puma, Noxa, and Bak) [28]. In addition, ROS
induces cardiolipin oxidation, leading to cytochrome c release and mitochondrial membrane
depolarization [28]. Intralipid, NAC and mitotempo have been shown to reduce ROS production
and mitochondrial membrane depolarization induced by a toxic dose of bupivacaine [14,15].
Similar to previous reports involving bupivacaine, LE and the ROS scavengers NAC and mitotempo
inhibited the ROS production and mitochondrial membrane depolarization induced by chloroquine in
rat cardiomyoblasts [12-15,25]. Combined with these results, it suggests that the LE-mediated attenuation of mitochondrial membrane depolarization caused by chloroquine is mediated by a decrease in ROS production. In agreement with the LE-evoked reduction in ROS produced by chloroquine, LE reversed the chloroquine-induced increase in the activity of MDA, which is produced via lipid peroxidation by free radicals [32]. However, LE reversed the chloroquine-induced reduction of activities of the antioxidant enzymes SOD and CAT. Taken together, the LE-mediated reduction of ROS produced by chloroquine mainly contributes to the attenuation of cardiotoxicity induced by a toxic level of chloroquine. ATP treatment nearly reversed the decreased myocardial contractility caused by bupivacaine [33]. Bupivacaine has been shown to attenuate cardiac mitochondrial ATP synthesis [34,35], and Intralipid contributes to the attenuation of bupivacaine-induced cardiotoxicity by stimulating fatty acid oxidation, which is associated with ATP production [36]. Taken together, these previous studies suggest that fatty acid oxidation and subsequently ATP production mediated by Intralipid attenuates bupivacaine-induced cardiotoxicity [33-36]. Similar to these reports regarding bupivacaine-induced cardiotoxicity [33-36], chloroquine (10^{-4} M)-evoked ROS production reduces ATP production, whereas LE, NAC, and mitotempo treatment reversed the reduction in ATP production induced by a toxic level of chloroquine (10^{-4} M) via the inhibition of ROS production [14,15, 33-36]. Further studies are needed to investigate detailed upstream and downstream (for example: mitochondrial cytochrome c release) cellular signaling pathways associated with the chloroquine-induced ROS production in cardiomyoblasts. Mitochondria are important for maintaining calcium homeostasis [28]. ROS production in the mitochondria disrupts calcium homeostasis in the intracellular compartment, leading to increased intracellular calcium levels [37]. Intralipid was shown to increase calcium retention capacity and subsequently inhibited mitochondrial permeability transition pore opening in bupivacaine-induced cardiotoxicity, suggesting that Intralipid provides better control of calcium overload [36]. Intralipid, NAC, and mitotempo
reduce the calcium level increased by toxic dose of bupivacaine [15]. Comparable to these reports [15,36], our results indicated that LE, NAC, and mitotempo inhibited the increase in intracellular calcium levels induced by chloroquine treatment (10^{-4} \text{ M}). When combined with these results, it suggests that LE-mediated inhibition of ROS production by chloroquine contributes to the inhibition of increased intracellular calcium levels caused by the toxic concentration of chloroquine.

The widely accepted underlying mechanism of LE resuscitation for drug toxicity caused by drugs with high lipid solubility is an indirect “lipid shuttle,” in which the lipid compartment of LE absorbs lipid-soluble drugs (for example: bupivacaine; log P of bupivacaine = 3.41) from the heart and brain, and then LEs containing lipid-soluble drugs are delivered to the muscle, adipose tissue, and liver for storage and detoxification [38]. In addition, Intralipid (0.25, 1 and 2%) has been shown to decrease bupivacaine concentration [39]. As the lipid solubility of chloroquine (log P = 4.63) is higher than that of bupivacaine (log P = 3.41), high-performance liquid chromatography was carried out to examine whether the lipid compartment of LE absorbed highly lipid-soluble chloroquine. However, as LE (0.75%) did not significantly reduce chloroquine (10^{-4} \text{ M}) concentration, we surmise that the LE-evoked reduction in ROS generation induced by chloroquine is due to the direct inhibition of chloroquine-induced ROS production by LE. The direct underlying mechanisms of LE resuscitation include inotropic effects, reversal of mitochondrial dysfunction, attenuation of ischemic reperfusion injury, and inhibition of nitric oxide release [1]. These observations indicate that the underlying mechanism associated with the LE-evoked reversal of cardiotoxicity induced by chloroquine toxicity is associated with the reversal of mitochondrial dysfunction via the inhibition of ROS production [1].

This study has some limitations. First, the current study did not examine the effect of LE on the cardiac dysfunction caused by a toxic dose of chloroquine in an \textit{in vivo} model. High chloroquine concentrations of 1 and 3 \times 10^{-5} \text{ M} have been shown to decrease left ventricular pressure and heart rate, respectively, in rats [40]. Furthermore (and similar to our results in the current study), Blignaut
et al. also demonstrated that a high dose of chloroquine \((1 \times 10^{-4} \text{ M})\) decreased the cell viability of isolated ventricular myocytes \([40]\). However, further investigation will be needed to confirm these findings \textit{in vivo}. Second, LE was administered prior to treatment with a toxic dose of chloroquine in this study, whereas LE is clinically administered after the cardiovascular collapse evoked by a toxic dose of chloroquine. Third, the H9c2 rat cardiomyoblast cell line was used in this experiment, but primary cultured cardiomyocytes are more clinically relevant. Despite these limitations, considering that chloroquine and hydroxychloroquine have similar pharmacological and toxicological properties, this study suggests that LE treatment may be helpful to treat critical patients with cardiac toxicity due to chloroquine or hydroxychloroquine toxicity, who need emergency anesthesia and intensive care. Furthermore, LE may be effective in treating the cardiotoxicity observed after chloroquine and hydroxychloroquine treatment in patients suffering from coronavirus disease 2019 \([20,21]\). LE was reported to be effective in alleviating cardiovascular depression caused by a toxic dose of highly lipid soluble drugs that inhibit cardiac sodium or potassium channels, which include amitriptyline, bupropion, lamotrigine, and flecainide \([10,41,42]\). Thus, LE may be a valuable adjuvant drug in the treatment of patients with intractable cardiovascular depression caused by toxicity of the afore-mentioned drugs in the operating room or intensive care unit. In addition, further studies are needed to examine the effect of LE alone on the cardiovascular system and elucidate the details of the underlying mechanisms.

In conclusion, LE with fatty acids, which contain 50% long-chain and 50% medium-chain, inhibited the late apoptosis and cardiotoxicity caused by chloroquine toxicity, which is mediated by the direct reduction of ROS production in the mitochondrial intrinsic apoptotic pathway of rat cardiomyoblasts. LE as adjuvant drug with supportive treatments may be effective in treating intractable cardiac toxicity induced by chloroquine.
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Figure 1.  (A) Effect of chloroquine (CQ) (N = 3) on the viability of H9c2 rat cardiomyoblasts. (B) Effect of lipid emulsion (LE) (Lipofundin MCT/LCT, N = 6) on the reduced cell viability induced by CQ (10^{-4} M) in H9c2 cells. (C) Effect of LE and reactive oxygen species scavenger (10^{-4} M N-acetyl-L-cysteine [NAC] or 10^{-5} M mitotempo; N = 6) on the reduced cell viability induced by CQ (10^{-4} M) in H9c2 cells. Data are shown as mean ± SD. The number N denotes the number of independent experiments. *P = 0.028, and ***P < 0.001 versus control. †††P < 0.001 versus CQ alone.
Figure 2. Effects of chloroquine (CQ) (10^{-4} M), lipid emulsion (LE) (0.75% Lipofundin MCT/LCT; A), and reactive oxygen species scavenger (10^{-4} M N-acetyl-L-cysteine [NAC] or 10^{-5} M mitotempo; B), alone or in combination, on scratch wound healing in rat cardiomyoblasts. The edge of the wound is shown with a solid line. The edge of migrated cells is shown with a dotted line. The pictures were captured immediately (0 h) and 19 h following the creation of the scratch wound. Scale bar: 100 µm. Cell migration, which indicates the change in wound area prior to and following drug treatment, is expressed as a percentage of wound area at 0 h after creation of scratch wound. Data (N = 4) are shown as mean ± SD. The number N denotes the number of independent experiments. *P = 0.026 and ***P < 0.001 versus control. †††P < 0.001 versus CQ alone.
Figure 3.  (A) Effects of chloroquine (CQ) (10⁻⁴ M), lipid emulsion (LE) (0.75% Lipofundin MCT/LCT), and reactive oxygen species scavenger (10⁻⁵ M mitotempo and 10⁻⁴ M N-acetylcysteine [NAC]), alone or combined, on the expression of cleaved caspase-3 in H9c2 cells.  B and C: Effects of LE (0.75%) and CQ (10⁻⁴ M), alone or combined, on the expression of Bax (B) and cleaved caspase-8 (C) in H9c2 cells.  Data (N = 4) are shown as mean ± SD.  The number N denotes the number of independent experiments.  **P = 0.002 and ***P < 0.001 versus control.  †††P < 0.001 versus CQ alone.
Figure 4. (A) Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of H9c2 rat cardiomyoblasts treated with chloroquine (CQ) (10\(^{-4}\) M) or the lipid emulsion (LE) (0.75% Lipofundin MCT/LCT) alone or LE, followed by CQ. Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI). Scale bar: 100 µm. Data (N = 3) are shown as mean ± SD. N indicates the number of independent experiments. ***\(P < 0.001\) versus control. †††\(P < 0.001\) versus CQ alone. (B) Effects of LE (0.75%) on the CQ (10\(^{-4}\) M)-evoked apoptosis of H9c2 cells. Annexin V-fluorescein isothiocyanate-propidium iodide staining, followed by flow cytometric analysis. Plot showing live cells, necrosis, early- and late-stage apoptosis following treatment. Data (N = 3) are shown as mean ± SD. The number N denotes the number of independent experiments. *\(P = 0.034\), **\(P = 0.005\), and ***\(P < 0.001\) versus control. †††\(P < 0.001\) versus CQ alone.
Figure 5. (A) Effects of chloroquine (CQ) \(10^{-4}\) M, lipid emulsion (LE) \(0.75\%\) Lipofundin MCT/LCT, and reactive oxygen species (ROS) scavenger \(10^{-4}\) M N-acetylcysteine [NAC] and \(10^{-5}\) M mitotempo), alone or in combination, on ROS production of H9c2 rat cardiomyoblasts. Cellular ROS levels were measured via flow cytometry after staining with the fluorescent dye 2', 7'-dichlorofluorescin diacetate (H2DCFDA). FI: fluorescence intensity. Scale bar: 100 µm. Data \((N = 3)\) are shown as median ± interquartile range (25 to 75%) and expressed as relative value of control. The number N denotes the number of independent experiments. \(* *** P < 0.001\) versus control. \(††† P < 0.001\) versus CQ alone. (B) Effects of LE \(0.75\%\), NAC \(10^{-4}\) M, and mitotempo \(10^{-5}\) M on the CQ \(10^{-4}\) M-induced changes in the membrane potential of mitochondria in the H9c2 rat cardiomyoblasts. Green and red indicate JC-1 monomers and JC-1 aggregates, respectively. Scale bar: 100 µm. Data \((N = 3, \text{left})\) are shown as median ± interquartile range (25 to 75%) and
expressed as relative value of control. Data (N = 3, right) are shown as mean ± SD and expressed as relative value of control. The number N denotes the number of independent experiments. FI: fluorescence intensity. $*P = 0.049$ and $**P < 0.001$ versus control. $†††P < 0.001$ versus CQ alone.

Figure 6. (A) Effects of lipid emulsion (LE) (0.75% Lipofundin MCT/LCT), reactive oxygen species scavenger N-acetyl-L-cysteine (NAC) (10$^{-4}$ M), and mitotempo (10$^{-5}$ M) on the chloroquine (CQ) (10$^{-4}$ M)-evoked intracellular calcium levels in H9c2 rat cardiomyoblasts. Data (N = 3) are shown as mean ± SD. The number N denotes the number of independent experiments. $***P < 0.001$ versus CQ alone. B, C, and D: Effects of CQ (10$^{-4}$ M) and LE (0.75%), alone or in combination, on malondialdehyde (MDA) (B, N = 6), sulfoxide dismutase (SOD) (C, N = 5), and catalase (CAT) (D, N = 5) activities of H9c2 rat cardiomyoblasts. Data are shown as mean ± SD.
**P = 0.009 and ***P < 0.001 versus control. †††P < 0.001 versus CQ alone.

Figure 7. Effects of chloroquine (CQ) (10⁻⁴ M), lipid emulsion (LE) (0.75% Lipofundin MCT/LCT) and reactive oxygen species scavenger (10⁻⁴ M N-acetylcysteinen [NAC] and 10⁻⁵ M mitotempo), alone or combined, on adenosine triphosphate (ATP) levels of H9c2 rat cardiomyoblasts. Data (N = 5) are shown as mean ± SD. ***P < 0.001 versus control. †††P < 0.001 versus CQ alone.
Figure 8. Effect of lipid emulsion (LE) (0.75% Lipofundin MCT/LCT) on chloroquine (CQ) concentration in distilled water. After emulsification with 0.75% Lipofundin MCT/LCT and CQ (10^{-4} M) in the distilled water, the released CQ from the emulsified sample was analyzed using ultraperformance liquid chromatography-quadrupole time-of-flight mass spectrometry (Q-TOF MS). The eluted CQ was analyzed using Q-TOF MS with a positive electrospray ionization and multiple reactions monitoring mode. Data are shown as mean ± SD. Experiment was repeated six times.
Supplementary Figure 1. Effect of Intralipid (IL) on the reduced viability in H9c2 rat cardiomyoblasts induced by chloroquine (CQ) (10^{-4} M). Data (N = 4) are shown as mean ± SD. The number N denotes the number of independent experiments. **P = 0.006 and ***P < 0.001 versus control. †P = 0.012 and †††P < 0.001 versus CQ alone.