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*Original Article*

# Chloroquine sensitizes hepatocellular carcinoma cells to chemotherapy via blocking autophagy and promoting mitochondrial dysfunction

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**Abstract:** Cisplatin/cisplatin-based combination chemotherapy is the main therapy strategy against hepatocellular carcinoma. However, the cisplatin efficiency is dimmed by the development of drug resistance. Numerous clinical trials are now revealing the promising role of chloroquine, an autophagy inhibitor, as a novel antitumor drug. In the present study, we investigated the regulation by chloroquine on the autophagy and on the sensitivity of hepatocellular carcinoma cells in vitro. Reverse transcription quantitative-polymerase chain reaction (RT-qPCR), western blotting assay, confocal microscopy and flow cytometry (FCM) were used to analyze the autophagy induction by cisplatin in hepatocellular carcinoma HepG2 cells, to examine the chloroquine-mediated autophagy inhibition on the cisplatin-induced apoptosis in HepG2 cells, and to explore the possible involvement of mitochondrial dysfunction in such process. Our results found the autophagy induction by cisplatin in HepG2 cells, basing on such results as increased induction of autophagic vesicles and upregulated conversion of A subunit (LC3-A) to B (LC3-B) subunit of microtubule-associated protein 1 light chain 3. Flow cytometry analysis results demonstrated that the cisplatin-induced apoptosis was aggravated by chloroquine. In addition, the mitochondrial function was downregulated by cisplatin and was deteriorated by chloroquine in HepG2 cells; the mitochondrial membrane potential (MMP) downregulation, the accumulation of reactive oxygen species (ROS) and the mitochondrial superoxide were markedly higher in the chloroquine/cisplatin-treated HepG2 cells than in the cisplatin-treated cells. In conclusion, we concluded that chloroquine sensitized the chemotherapy efficiency of cisplatin against hepatocellular carcinoma HepG2 cells, probably via blocking autophagy and via deteriorating the mitochondrial dysfunction. Chloroquine might be a potential adjuvant agent for overcoming chemotherapy resistance in hepatocellular carcinoma.

**Keywords:** Autophagy, chloroquine, chemotherapy, hepatocellular carcinoma cells, mitochondrial dysfunction

## Introduction

Hepatocellular carcinoma (HCC) is on the top list of incurable malignancies [1, 2] with a high mortality [3], due to the limitation to therapeutic approaches. Surgical resection is the main eradication option for most HCC patients [4], with only approximately 15% of HCC cases surgically-removed [2]. Moreover, HCC is resistant to most conventional chemotherapeutic agents [5, 6]. Therefore, it is urgent to identify molecular mechanisms underlining the HCC chemoresistance or to develop more effective HCC treatment strategies.

In recent years, multiple biomarkers have been recognized to contribute to the HCC chemoresisitance [5-7], including deregulated microRNAs [5], up- or down-regulated protein markers [6, 8]. Various types of molecular mechanisms are also implies involving in the HCC chemoresistance, such as epithelial mesenchymal transition [7], autophagy [9-11]. Autophagy is a survival mechanism in response to adverse conditions, such as starvation, growth factor deprivation, hypoxia, damaging stimuli, and therapeutic agents [12, 13]. Particularly, autophagy is activated in multiple types of tumors [14-16]. Accumulating evidence demonstrates that

autophagy is activated to promote survival in HCC cells under different stress conditions, including chemotherapy [9]. Thus, autophagy might be a promising target of the amelioration of the HCC chemoresistance.

In the present study, we investigated the regulation by chloroquine on the autophagy and on the sensitivity of hepatocellular carcinoma cells in vitro, via analyzing the autophagy induction by cisplatin in hepatocellular carcinoma HepG2 cells, via examining the chloroquine-mediated autophagy inhibition on the cisplatin-induced apoptosis in HepG2 cells, and via exploring the possible involvement of mitochondrial dysfunction in such process. Our results indicated that chloroquine sensitized the chemotherapy efficiency of cisplatin against hepatocellular carcinoma HepG2 cells, implying it might be a potential adjuvant agent for overcoming chemotherapy resistance in hepatocellular carcinoma.

## Materials and methods

### *Reagents, cell culture and treatment*

HCC HepG2 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and was cultured at 37°C in a humidified atmosphere under 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Pittsburgh, PA, USA) supplemented with 10% fetal bovine serum (FBS; Sijiqing, Hangzhou, China) (or 2% FBS for cell maintaining) and with 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). Cisplatin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in DMEM for short time use. For the cisplatin treatment, 85%-confluent HepG2 cells were updated with % FBS-supplemented DMEM, with various concentration of cisplatin for 12, 24 or 48 hours.

### *Quantitative analysis of autophagic vesicles with GFP-LC3 reporter*

Green fluorescence protein (GFP)-LC3B reporter was utilized to quantify the induction of autophagic vesicles in HepG2 cells. In brief, GFP-LC3B reporter plasmid was transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) into the HepG2 cells, which were cultured to approximately 85% confluence. 6 hours post translation and incubation (at 37°C in a humidified atmosphere under 5% $CO_2$), HepG2 cells were updated with fresh DMEM medium (2% FBS), containing 0, 5 or 10 μM cisplatin for another incubation for 24 hours. The autophagic vesicles were presented as GFP-positive puncta in HepG2 cells and counted under fluorescence microscopy.

### *Western blot analysis of autophagy-associated biomarkers in HepG2 cells*

HepG2 cells post treatment were collected with a cell scratch and were washed with ice-cold 1× phosphorylated buffer solution (PBS) via centrifuging with 800×g at 4°C. The precipitated cells were then used to isolate the cytosol and mitochondria proteins with a Mitochondria/Cytosol Fractionation Kit (Abcam, Cambridge, UK) according to the kit's manual. Each protein sample was supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland) and was quantified for protein concentration using the BCA protein assay reagent (Thermo Scientific, Rockford, USA). Cytosol proteins were separated with 12% gradient SDS-PAGE gel, were transferred to a nitrocellulose (NC) membrane (Millipore, Bedford, MA, USA), and then were blocked with 2% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) at °C overnight. For the specific blotting for LC3-A/B, autophagy-related gene (ATG) 7, the NC membrane was respectively inoculated with rabbit polyclonal antibody against LC3-A/B (1:500, Cat. Ab128025, Abcam, Cambridge, UK), against ATG 7 (1:800, Cat. D12B11, Cell Signaling Technology Inc., Danvers, MA, USA), or against β-actin (as control) (1:1000, Cat. Ab8227, Abcam, Cambridge, UK) at 4°C for two hours. Then the membrane was incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (1:600, Jackson ImmunoResearch, West Grove, PA, USA) at room temperature (26°C) for another one hour, and was finally incubated with Enhanced chemiluminescence (ECL) kit (Thermo Scientific, Rockford, IL, USA) according to the product's guidance. Four-time washing for the membrane with 1× PBS adding Tween 20 (PBST) was performed before each incubation. The specific binding was visualized with UVP BioSpectrum 500 imaging system (Upland, CA, USA) and was presented as a relative level to β-actin.

### *MTT (Methyl Thiazolyl Tetrazolium) assay*

Cellular viability of the treated HepG2 cells was assayed by the MTT Cell Viability Assay Kit

(Abnova, Walnut, CA, USA). In brief, HepG2 cells in 96-well plates with 85% confluence were treated with 0, 5 or 10 μM cisplatin or (and) with 0, 15 or 40 μM chloroquine for 24 hours. Then cell wells were added with MTT solution for an incubation at 37°C for two hours. Post the reaction termination with dimethyl sulfoxide, the absorbance was on aspectrophotometer (Crystaleye, Olympus, Tokyo, Japan) at 570 nm.

*Apoptosis examination*

Apoptosis in HepG2 cells was examined with both Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate NickEnd Labeling (TUNEL) Assay and FACScan flow analysis. For the FACScan flow analysis, HepG2 cells post treatment were digested with 0.25% EDTA-free trypsin and were immediately washed with ice-cold PBS for two time. Then cells were resuspended in 500 μL binding buffer containing 1 μL annexin-V-phycoerythrin (PI) for an incubation at room temperature (26°C) for 15 minutes. Finally, the apoptosis analysis was performed in a FACScan flow cytometer (Bio-Rad, Hercules, CA, USA) according to the guidance of the manufacturer's recommendations, and the data analysis was performed with FlowJosoftware (Tree Star, Ashland, OR, USA). The TUNEL staining was performed using the TUNEL Kit (Roche, Indianapolis, IN, USA) under the guidance of the manufacturer's protocol. In brief, HepG2 cells post treatment was incubated with TUNEL reaction mixture for at room temperature (26°C) for one hour and then was stained with DAPI for nucleus. Each sample was randomly captured for five non-overlapping fields at 200× magnification with a light microscope (Nikon Corporation, Tokyo, Japan).

*Determination of mitochondrial membrane potential with JC-1 staining*

Mitochondrial membrane potential in HepG2 cells were examined using JC-1 (5,5',6,6'-tetrachloro-1,1',3,3' tetraethyl benzimidazolyl carbocyanine iodide) (Life Technologies, UK) as described previously [17]. In brief, cells post treatment were washed with ice-cold PBS via a centrifugation (800×g) and then were stained with JC-1 reagent (10 μM) for 30 minutes at 37°C in dark, and the fluorescence was measured using a fluorescence spectrophotometer (Fa 2000, Hitachi, Tokyo, Japan) with the Ex/Em of 488/529 nm to measure monomers (green) and with the Ex/Em 488/590 nm to measure J-aggregates (red). The JC-1 staining was observed under fluorescent microscope (Olympus, USA) with TRITC filter at 200× magnification.

*Measurement of mitochondrial superoxide and reactive oxygen species*

The mitochondrial superoxide levels were detected using MitoSOX™ Red mitochondrial superoxide indicator (Invitrogen Molecular Probes, Carlsbad, CA, USA), which is a fluorogenic dye (Ex/Em=510/580 nm) for highly selective detection of superoxide in the mitochondria of cells. Cells post various treatment were incubated with 5 μM MitoSOX™ Red at 37°C for 20 min, and then the MitoSOX™ Red fluorescence were detected After the removal and washing of cells. Intracellular level of ROS was quantified by the oxidation of the ROS-sensitive fluorophore 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate (Invitrogen, Carlsbad, CA, USA) according to the reagent manual. In brief, confluent HepG2 cells in six-well plates ($5\times10^5$/well) were loaded with 5 μM probe, 5-chloromethyl-2,7-dichlorodihydrofluorescein diacetate in Hanks' balanced salt solution and were incubated at 37°C and 5% $CO_2$ for 30 min. And then the cells were rinsed with HBSS, post the removal of probe. 2,7-dichlorofluo-rescein (DCF) fluorescence was measured by using a luminescence spectrometer with the excitation source at 488 nm and emission at 530 nm.

*Statistical analysis*

All experiments were carried out in triplicate, and all results are expressed as mean ± SEM of at least three independent experiments. Statistical significance was determined by analysis of variance and subsequently applying the Student's t test ($P<0.05$).

## Results

*Cisplatin induces autophagy in HCC HepG2 cells*

Specific acidicvesicular organelles (AVOs), also known as autophagosomes, develop in cytosol [18] in autophagy. AVOs are observable by the staining for the LC3B on AVO membrane, and present as green punctain cytosol under fluo-

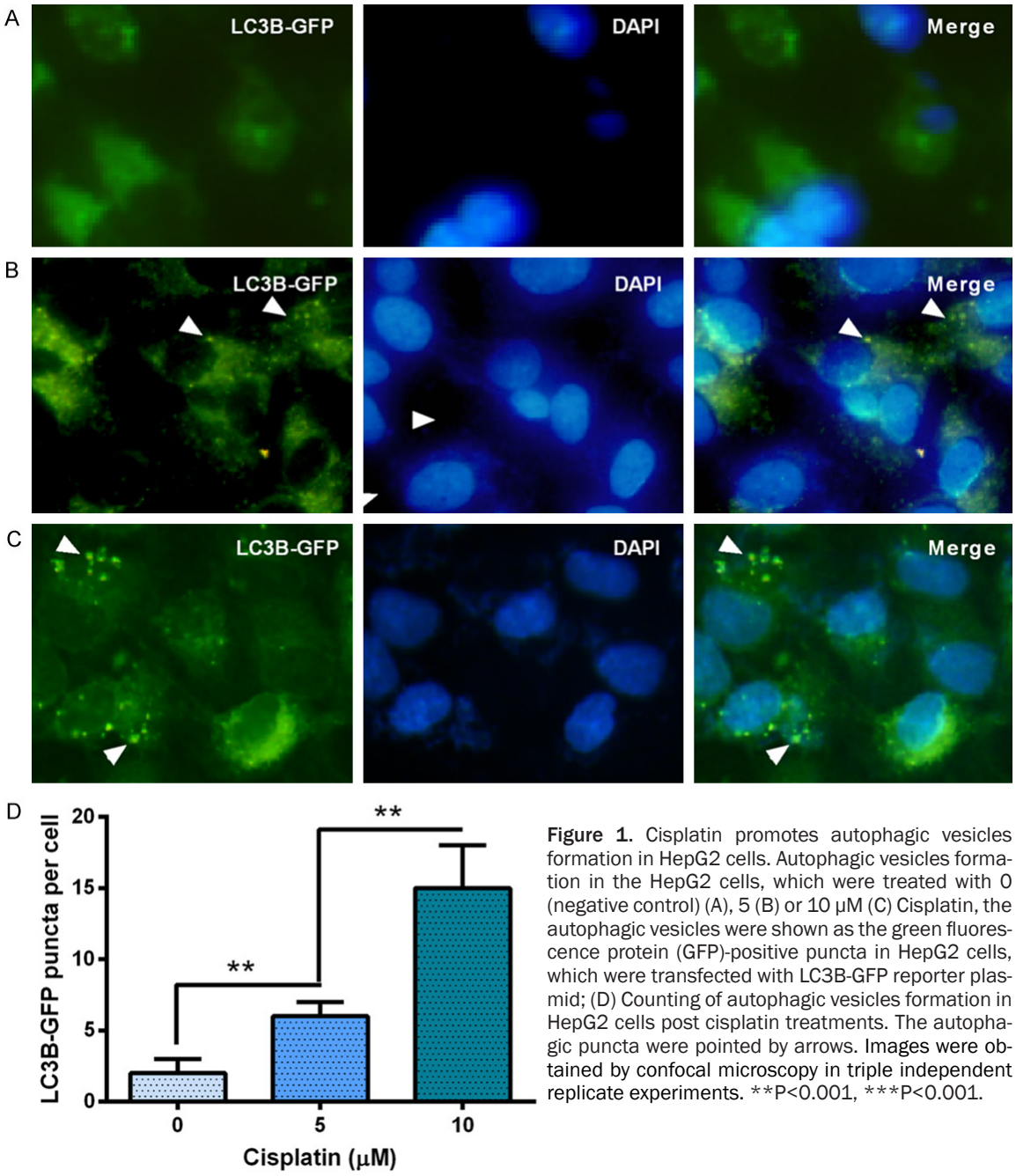


**Figure 1.** Cisplatin promotes autophagic vesicles formation in HepG2 cells. Autophagic vesicles formation in the HepG2 cells, which were treated with 0 (negative control) (A), 5 (B) or 10 μM (C) Cisplatin, the autophagic vesicles were shown as the green fluorescence protein (GFP)-positive puncta in HepG2 cells, which were transfected with LC3B-GFP reporter plasmid; (D) Counting of autophagic vesicles formation in HepG2 cells post cisplatin treatments. The autophagic puncta were pointed by arrows. Images were obtained by confocal microscopy in triple independent replicate experiments. **P<0.001, ***P<0.001.

rescence microscope by the transfection with GFP-LC3B reporter. To examine a possible autophagy induction in the cisplatin-treated HCC HepG2 cells, we observe AVOs in HepG2 cells, which were treated with 5 or 10 μM cisplatin and were transfected with GFP-LC3B reporter plasmid. Results demonstrated that the GFP-positive puncta were significantly more in the HepG2 cells post the treatment with 5 or 10 μM cisplatin, than in the blank HepG2 cells (**Figure 1A-D**). The high conversion of microtubule-associated protein 1A/1B-light chain 3 (LC3) A to LC3B is another autophagy-specific phenomenon [19]. Both the conversion of LC3A to LC3B and the expression of *Autophagy Related (ATG) Gene 7* were then examined to validate the autophagy induction by cisplatin in HepG2 cells. Western blotting results (**Figure 2A**) demonstrated that there were higher levels of LC3B/LC3A ratio (**Figure 2B**) and Atg7 expression

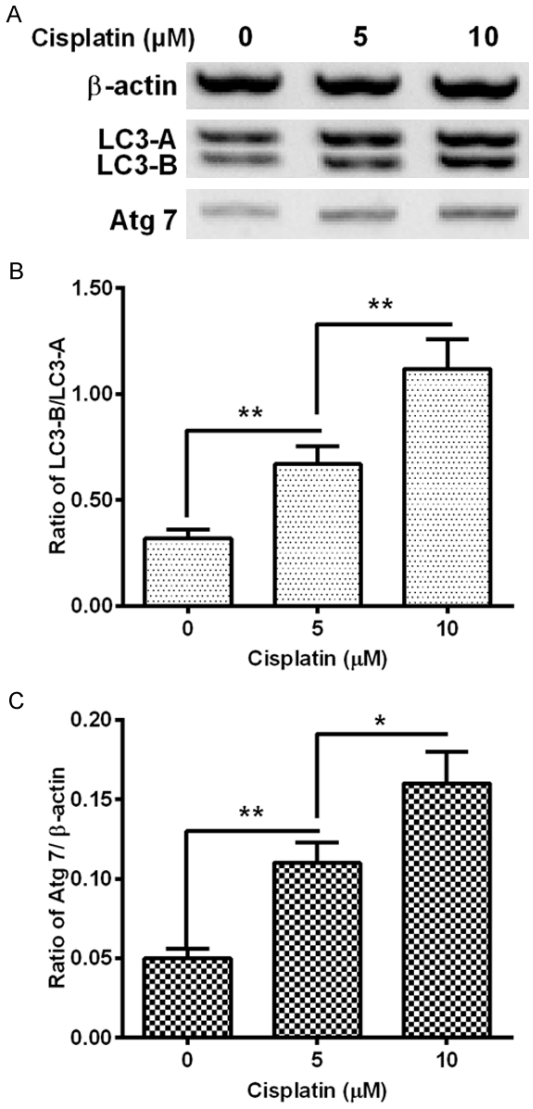


**Figure 2.** Cisplatin promotes the autophagy-associated molecules in HepG2 cells. (A-C) Western blotting analysis (A) of the conversion of LC3A to LC3B (B) and the relative level of autophagy-associated gene 7 (Atg7) to β-actin (C) in the HepG2 cells, which were treated with 0, 5 or 10 μM Cisplatin. All experiments were repeated independently in triplicate. Statistical significance was shown as *P<0.05, **P<0.01, ***P<0.001.

(**Figure 2C**) in the cisplatin-treated HepG2 cells, dose-dependently ($P<0.05$ or $P<0.01$). Taken together, autophagy was induced by cisplatin treatment in HCC HepG2 cells.

*Chloroquine enhances the cisplatin-induced apoptosis in HCC HepG2 cells*

Chloroquine has recently been found to be potential as an adjuvant agent against various types of cancers [20-22], particularly to be promising to overcome chemoresistance [23, 24]. In order to further analyze whether the significantly-induced autophagy exerts a regulation on the treatment effect of cisplatin in HepG2 cells, we evaluated the influence of autophagy inhibitor, chloroquine, on the cisplatin-treated HepG2 cells. **Figure 3A** demonstrated that cisplatin markedly aggravated the viability reduction of HepG2 cells 24 hours post the cisplatin treatment (5 or 10 μM). Either 15 or 40 Mm chloroquine markedly reduced the cellular viability of HepG2 cells than only cisplatin treatment (either 5 or 10 μM) ($P<0.05$, $P<0.01$ or $P<0.001$); and there was a dose-dependence of such reduction ($P<0.05$).

We examined the apoptosis induction in the HepG2 cells, post treatments with cisplatin or (and) chloroquine. As demonstrated in **Figure 3B**, either 5 or 10 μM cisplatin induced significant amount of apoptotic HepG2 cells ($P<0.05$ or $P<0.01$), dose-dependently ($P<0.05$ for 5 vs 10 μM). Moreover, additional chloroquine treatment (15 or 40 μM) markedly aggravated the cisplatin-mediated apoptosis induction (10 μM) ($P<0.01$ or $P<0.001$), also dose-dependently ($P<0.05$ for 15 vs 40 μM). To re-confirm the regulation by chloroquine on the cisplatin-mediated apoptosis induction in HepG2 cells, we performed TUNEL assay in cisplatin-treated HepG2 cells, with or without chloroquine treatment. As indicated in **Figure 3C**, 10 μM cisplatin promoted TUNEL-positivity of HepG2 cells (Second column vs first column), and more TUNEL-positive HepG2 cells were found in the cisplatin (10 μM)/chloroquine (15 μM) group (Third column vs second column). Quantitative analysis confirmed the significant difference of the apoptosis induction between the third and second column, or between the second and first column ($P<0.001$ or $P<0.01$, **Figure 3D**). Taken together, chloroquine enhances the cisplatin-induced apoptosis in HCC HepG2 cells.

*Chloroquine deteriorates the cisplatin-induced mitochondrial dysfunction in HepG2 cells*

Mitochondrial dysfunction has been indicated by several studies to be one of main pathways in the cisplatin-induced apoptosis [24-28]. To explore whether mitochondrial dysfunction was implicated in the chloroquine-mediated aggravation to cisplatin-induced apoptosis in HepG2

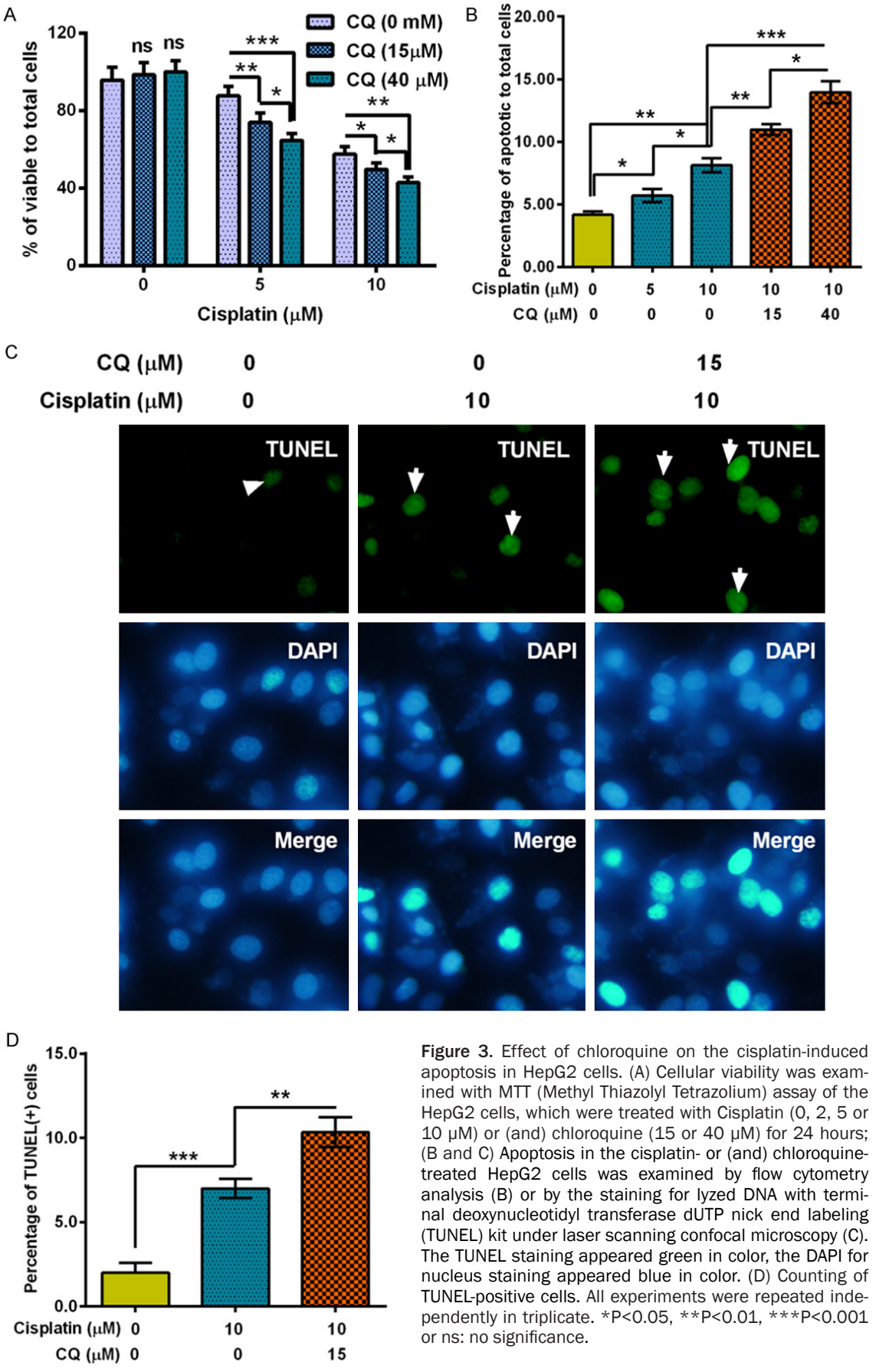


**Figure 3.** Effect of chloroquine on the cisplatin-induced apoptosis in HepG2 cells. (A) Cellular viability was examined with MTT (Methyl Thiazolyl Tetrazolium) assay of the HepG2 cells, which were treated with Cisplatin (0, 2, 5 or 10 μM) or (and) chloroquine (15 or 40 μM) for 24 hours; (B and C) Apoptosis in the cisplatin- or (and) chloroquine-treated HepG2 cells was examined by flow cytometry analysis (B) or by the staining for lyzed DNA with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit under laser scanning confocal microscopy (C). The TUNEL staining appeared green in color, the DAPI for nucleus staining appeared blue in color. (D) Counting of TUNEL-positive cells. All experiments were repeated independently in triplicate. *P<0.05, **P<0.01, ***P<0.001 or ns: no significance.

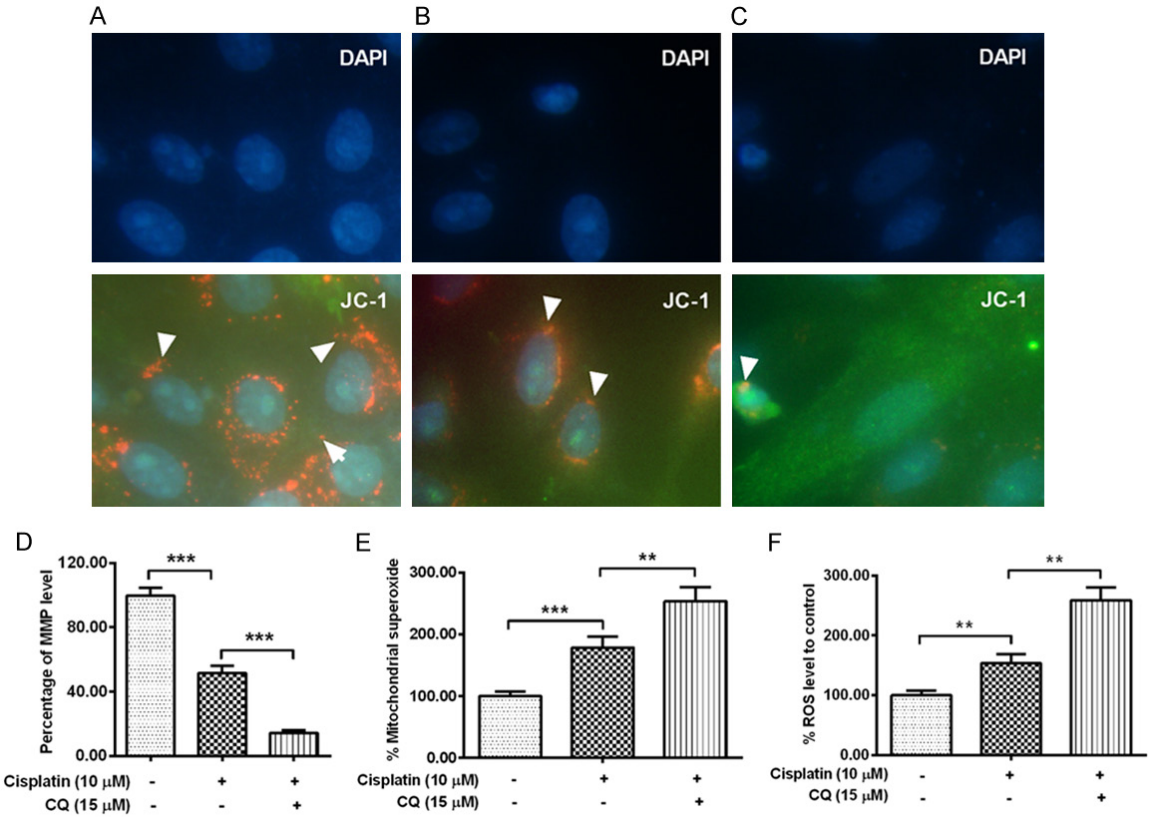


**Figure 4.** Chloroquine deteriorates the cisplatin-induced mitochondrial dysfunction in HepG2 cells. HepG2 cells were treated with Cisplatin (0 or 10 μM) or chloroquine (0 or 15 μM) for 24 hours, then the mitochondrial membrane potential (MMP) was examined with JC-1 staining (A: Blank HepG2 cells, B: HepG2 cells treated with 10 μM cisplatin; C: HepG2 cells treated with 10 μM cisplatin and with 15 μM chloroquine) and was presented as percentage level to which in Blank HepG2 cells (D), the mitochondrial superoxide was examined with MitoSOX™ Red mitochondrial superoxide indicator (E), the mitochondrial reactive oxygen species (ROS) was examined with the ROS-sensitive fluorophore 5-(and-6)-chloromethyl-2,7-dichlorodi-hydrofluorescein diacetate (F). All experiments were repeated independently in triplicate. **P<0.01 or ***P<0.001.

cells, we then analyzed the mitochondrial function in the cisplatin or (and) chloroquine-treated HepG2 cells. Firstly, the mitochondrial membrane potential (MMP) was determined with JC-1 staining. **Figure 4A** demonstrated that compared with normal HepG2 cells (**Figure 4A**), there were less JC-1(+) HepG2 cells in the cisplatin group (10 μM) (**Figure 4B**); and much less JC-1(+) HepG2 cells were found in the cisplatin (10 μM)/chloroquine (15 μM) group (**Figure 4C**), with statistical significance ($P<0.001$ respectively, **Figure 4D**). Secondly, the mitochondrial superoxide generation was also measured with MitoSOX, a live-cell-permeable and mitochondrial localizing superoxide indicator. As shown in **Figure 4E**, significantly higher level of in the cisplatin-treated cells ($P<0.001$), and the highest superoxide level was found in the cisplatin (10 μM)/chloroquine (15 μM) group ($P<0.01$, cisplatin/chloroquine vs cisplatin). Additionally, we measured ROS production in the three groups of HepG2 cells. **Figure 4F** demonstrated that there was second higher level of ROS production in the cisplatin-treated cells ($P<0.01$), and the highest level of ROS production in the cisplatin/chloroquine-treated cells ($P<0.01$). Therefore, chloroquine deteriorates the cisplatin-induced mitochondrial dysfunction in HepG2 cells.

## Discussion

Chemotherapy resistance is the leading obstacle against HCC eradication [5, 6]. Multiple biomarkers/molecular pathways have been identified to mediate or involve in the HCC chemo-resistance [5-7], such as Caspase-3-diated apoptosis [29], fibroblast growth factor 19 (FGF19)/FGF receptor 4 signaling [30], choline kinase α [31]. In particular, autophagy is activated in multiple types of tumors [14-16], and is activated to promote survival in HCC

cells under different stress conditions, including chemotherapy [9]. Thus, autophagy might be a promising target of the amelioration of the HCC chemoresistance. In the present study, we identified the autophagy induction by cisplatin in HCC HepG2 cells, increased induction of autophagic vesicles and upregulated conversion of LC3-A) to LC3-B. Moreover, the cisplatin-induced apoptosis was aggravated by chloroquine, a well-known autophagy inhibitor. Thus, our study confirms the involvement of autophagy in HCC chemoresistance.

Mitochondrial damage is one of main pathways in the cisplatin-induced apoptosis [24-28]. Autophagy is another result following mitochondrial damage, under such conditions as hypoxia, oxidative stress or chemotherapy [32, 33]. We hypothesized that mitochondrial dysfunction would be implicated in the chloroquine-mediated aggravation to cisplatin-induced apoptosis in HepG2 cells. The hypothesis was confirmed by such results as that the cisplatin-downregulated mitochondrial function was deteriorated by chloroquine in HepG2 cells; MMP reduction, ROS accumulation and the mitochondrial superoxide were markedly higher in the chloroquine/cisplatin-treated HepG2 cells than in the cisplatin-treated cells. Accumulating studies have found that chloroquine decreases mitochondrial quality in primary neurons [34], in breast cancer cells [35], in cholangiocarcinoma cells [36]. In this study, we firstly found the deterioration by chloroquine to the cisplatin-induced mitochondrial damage. Therefore, we conclude that chloroquine sensitizes hepatocellular carcinoma cells to cisplatin via blocking autophagy and by damaging mitochondrial function.

However, there are still lots detailed signaling pathways need to be uncovered in future. Cisplatin induced both apoptosis and autophagy in HCC HepG2 cells; it is not clear whether the cisplatin-induced autophagy is a feedback protection mechanism against the cisplatin-induced apoptosis or is an independent autophagic death pathway. On the other side, it is unclear which of the two pathways is the main one underlining the chloroquine-mediated HCC sensitization to cisplatin.

In conclusion, we concluded that chloroquine sensitized the chemotherapy efficiency of cisplatin against hepatocellular carcinoma HepG2 cells, probably via blocking autophagy and via deteriorating the mitochondrial dysfunction. Chloroquine might be a potential adjuvant agent for overcoming chemotherapy resistance in hepatocellular carcinoma.

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## Disclosure of conflict of interest

None.

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