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Chloroquine aggravates the arsenic trioxide (As_2O_3)-induced apoptosis of acute promyelocytic leukemia NB4 cells via inhibiting lysosomal degradation *in vitro*

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Abstract. – OBJECTIVE: Acute promyelocytic leukemia (APL) is a distinct subtype of acute myeloid leukemia (AML), as standing out for its distinguished sensitivity to all-trans retinoic acid and arsenic trioxide (ATO, As_2O_3). The As_2O_3-mediated degradation of PML-RARA (promyelocytic leukemia-retinoic acid receptor-α) oncoprotein via the proteasome pathway appears to be critical for such distinguished sensitivity.

MATERIALS AND METHODS: The present study was to evaluate the influence by chloroquine (CQ), an inhibitor to the release of lysosomal enzymes, on the sensitivity of APL cells to As_2O_3. APL-derived NB4 cell line was treated with As_2O_3 or/and CQ *in vitro*. Then, the cell viability, the induction of apoptosis, and autophagy were examined with MTT assay, with TUNEL staining or with enhanced green fluorescence protein (EGFP)-light Chain 3 (LC3) reporter. The apoptosis- or autophagy-associated proteins were quantified with Western blotting assay.

RESULTS: Our results demonstrated that the As_2O_3 treatment promoted either apoptosis or autophagy in APL NB4 cells and upregulated both apoptosis- and autophagy-associated proteins. However, additional CQ treatment deteriorated the As_2O_3-induced NB4 cell apoptosis, whereas aggravated the As_2O_3-induced accumulation of acidic vesicular organelles (AVOs) and blocked the lysosomal degradation in NB4 cells.

CONCLUSIONS: Chloroquine aggravates the arsenic trioxide-induced apoptosis of APL NB4 cells via inhibiting lysosomal degradation *in vitro*. It implies that chloroquine might be adjuvant to sensitize APL cells to arsenic trioxide.

Key Words:
Acute promyelocytic leukemia, Apoptosis, Arsenic trioxide, Autophagy, Chloroquine.

Introduction

Acute promyelocytic leukemia (APL) is a subtype of acute myeloid leukemia (AML), accounting for approximately 10-15% of all AML cases. APL is characterized by a specific balanced reciprocal translocation t (15;17) (q24.1;q21.2), causing the fusion of PML (promyelocytic leukemia) and RARα (retinoic acid receptor-α) genes. The oncogene PML-RARα could inhibit myeloid differentiation at the promyelocyte stage[1-5]. The APL mortality rate was high in the past, but recently it has dramatically decreased to less than 10%, with the treatment of all-trans retinoic acid (ATRA) and arsenic trioxide (ATO)[6,7]. ATO was used in APL treatment since mid-1990s[8-11]. Arsenic trioxide is used for relapsed APL or the patient with no response to ATRA, further improved the survival rate of patients. Scholars[12-14] have underlined the cellular mechanism of the ATO treatment in APL patients, particularly, a pathway composed of ATR, PML, Chk2, and p53 has been proposed to mediate ATO-induced apoptosis.

The chemical formula for chloroquine (CQ) is a diphosphate salt of N'-(7-chloroquinolin-4-yl)-N, N-diethyl-pentane-1, 4-diamine, is a 4-aminoquinoline class of drug for malaria and autoimmune diseases since 1940s[15]. CQ can enrich in acidic organelles, such as the endosomes, lysosomes and golgi vesicles, and promote the pH of these organelles. Thus, it inhibits the release of lysosomal enzymes and fusion of autophagosomes and lysosomes, finally causing cell death [16]. Due to the function on cell autophagy, CQ has been utilized as adjuvant in various diseases, such as cancers, acute kidney injury, virus infection and

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systemic lupus erythematosus [17-19]. In cancer therapy, the combined treatment of CQ and anti-cancer drugs do better than only anti-cancer drugs treatment in clinical therapeutics. It implies that CQ might ameliorate cancer therapy [20], probably including in the APL therapy, though without such reports. We investigated the apoptosis and autophagy in the APL-derived NB4 cells which were treated with CQ, ATO or CQ plus ATO, especially the combined effect of CQ and ATO. The aim of this research was to explore the effect of chloroquine in APL, which may provide a perspective for further clinical development.

Material and Methods

Cell Culture

The NB4 cell line is a permanent cell line with a low proliferation potential *in vitro*, the cell line was established from a patient with acute promyelocytic leukemia. The NB4 cells were bought from American Type Culture Collection (ATCC, Manassas, VA, USA). The base medium for NB4 cells is RPMI-1640 medium containing 10% (v/v) fetal bovine serum (FBS, GIBCO, Rockville, MD, USA). The cells were incubated at 37°C in a humidified and constant-temperature incubator with 5 % CO_2.

MTT Assay and TUNEL Staining Assay

The relative cellular viability of the NB4 cells was determined by MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay (Invitrogen, Carlsbad, CA, USA). The cells were seeded into a 96-well plate before the experiment, and treated the cells with 0, 5, 10 or 20 μM of CQ or ATO for 0, 12, 24 or 48 h. After the treatment, each well was added with 20 μl MTT (5 mg/ml) (Invitrogen, Carlsbad, CA, USA) and cultured for 4 h at 37°C. After that, we removed the supernatant from each well slightly, and 200 μl of dimethyl sulfoxide (DMSO) were added into each well, the plate was shaken for 15 min to dissolve the crystals on a vibrator. Next, the absorbance at 550 nm of each sample was measured by an enzyme-linked immunometric meter (Power Wave XS2, BioTek Instruments, Winooski, VT, USA). The apoptotic cells of each group were detected by the TUNEL assay, with the tdt-mediated DUTP Nick-End Labeling Kit (Roche Diagnostics, Basel, Switzerland) according to the protocol. The nucleic acid was stained into blue by DAPI (4', 6-Diamidino-2-Phenylindole, Dihydrochloride). After staining, the cells were scanned under a laser scanning confocal microscopy (Olympus, Tokyo, Japan) and the TUNEL-stained cells were calculated. The scale bar was indicated as 10 μm in each image, and all the experiments were repeated in triplicate independently.

Western Blot Assay

Expression levels of the apoptosis-related (released cytochrome c (Cyt c), cleaved caspase 3 (C-CASP 3) and lyzed poly ADP-ribose polymerase (L-PARP)) and autophagy-related proteins (LC3A, LC3B, ATG-5 and p62) were detected in the NB4 cells post ATO or CQ treatment by Western blot assay. The NB4 cells were harvested post CQ or ATO treatment, and the cell lysis buffer (Bio-Rad, Hercules, CA, USA) was used to lyses the collected cells. Cell lysis was centrifuged at 12,000 xg for 25 min, and the supernatant was collected. Samples were boiled with 10% SDS (sodium dodecyl sulfate) for 15 min. Protein samples were separated with 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to Hybond-P membranes (Millipore, Billerica, MA, USA). The membrane was blocked with 5% skimmed milk powder overnight at 4°C. The membrane was incubated with the primer mouse-anti-human antibody against released Cyt c, C-CASP 3, L-PARP, LC3A/B, ATG-5, p62 or β-actin (1:1000 diluted) (Abcam, Cambridge, MA, USA) for 1 h at 37°C, and the membrane was washed with TBST. Subsequently, the membrane was incubated with secondary anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. We scanned the membrane by a Smart ChemiTM Lamp Analysis System (Lifescience, Thermo Fisher Scientific, Waltham, MA, USA), and the relative level was quantified according to the band density by Quantity One software (Bio-Rad, Hercules, CA, USA), with β-actin as internal control.

Cell Transfection and Lysosomes Staining

EGFP-LC3 reporter was utilized to trace the formation of autophagy. The EGFP-LC3 reporter was transfected into NB4 cells by Lipofectamine® 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) following to the manufacturer's manual. 24 h later, the green fluorescence of the cells was observed by fluorescence microscope (Olympus, Tokyo, Japan), and counted

the autophagic puncta. The distribution of the lysosomes was detected by LysoTracker™ Deep Red (L12492, Thermo Fisher Scientific, Inc., Waltham, MA, USA) in blank NB4 cells or in the NB4 cells, which were treated with 10 μM CQ, 1 μM ATO, or with 10 μM CQ + 1 μM ATO for 24 h, according to the user's manual. The lysosomes were stained into red. After staining, the cells were observed under confocal microscope, and lysosome dots per cell were quantified using Leica confocal software. The images obtained by confocal microscopy in four replicate experiments. Results were calculated in four independent replicate experiments.

Statistical Analysis

The statistical analysis was performed by GraphPad Prism (GraphPad Software, La Jolla, CA, USA). The difference between two groups was statistically analyzed by Student's *t*-test; to validate ANOVA, Tukey method was used as post-hoc test. Results were averaged for four replicate experiments in each group. Only a *p*-value less than 0.05 was considered statistically significant. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

Results

Influence by CQ On the Cellular Viability and Apoptosis Induction in the APL-Derived NB4 Cells

To investigate the effect of CQ in APL cell apoptosis, we examined the relative cellular viability of the APL-derived NB4 cells. Cells were treated with 0, 5, 10 or 20 μM CQ for 0, 12, 24 or 48 h, by MTT assay. The relative cellular viability was no significant difference among the four groups (treated with 0, 5, 10 or 20 μM CQ) after 0 or 12 h treatment. However, 24 h post treatment, the relative cellular viability of the NB4 cells treated with 20 μM CQ was reduced (20 μM *vs.* 0, 5 or 10 μM CQ) (*$p < 0.05$). With increasing treatment time, the cellular viability was continuously declined in the 20 μM CQ-treated cells (Figure 1 A, **$p < 0.01$). The apoptosis of the APL-derived NB4 cells was performed by TUNEL assay, with nucleic acid as blue and with apoptotic cells as green. As indicated in Figure 1 B, apoptotic cells were significantly increased in the cells treated with 10 μM or 20 μM, compared to the normal group, with concentration dependence. The apoptotic cells were induced by 20 μM CQ than by 0 or 10 μM CQ (Figure 1 C, **$p < 0.01$, ns: no significance). Thus, the apoptosis of the APL-derived cells was enhanced by CQ, and the apoptosis was progressively aggravated with increasing CQ concentration.

CQ Aggravates Apoptosis in the NB4 Cells Post Arsenic Trioxide Treatment

APL is sensitive to all-trans retinoic acid and arsenic trioxide[9]. We examined the effect of ATO in NB4 cells, with MTT and TUNEL assay, by treating NB4 cells with 0, 1 or 2 μM ATO for 0, 12, 24 or 48 h. To investigate the combined effect of CQ and ATO, we extra treated the cells with 1 μM ATO + 10 μM CQ for 0, 12, 24 or 48 h. As shown in Figure 2 A, the relative cellular viability was gradually declined in the NB4 cells which were treated with 1, 2 ATO (*$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$). Moreover, additional 10 μM CQ aggravated the ATO-induced (1 μM) viability reduction for 24 or 48 h (**$p < 0.01$). In addition, the images in Figure 2 B and 2 C showed that the apoptotic cells were gradually increased in the NB4 cells treated with 1 μM and 2 μM ATO (**$p < 0.01$ or ***$p < 0.001$). More apoptotic cells were induced by 1 μM ATO + 10 μM CQ than 1 μM ATO (**$p < 0.01$). Therefore, the combined treatment of CQ and ATO was more effective.

The Expression Level of Apoptosis-Related Proteins in the ATO- or/and CQ-Treated NB4 Cells

The apoptotic cells were increased of the NB4 cells post the ATO or CQ treatment. To further verify the apoptosis in the ATO- or/and CQ-treated NB4 cells, we detected the expression level of the apoptosis-related proteins by Western blot assay, such as released Cyt c, C-CASP 3 and L-PARP. The representative image of Western blotting assay was shown in Figure 3 A, the protein bands of the three apoptosis-related proteins were deeper in the group of CQ-treated, ATO-treated and CQ+ATO-treated cells to some degree than the control group. In addition, the relative protein level of released Cyt c, C-CASP 3 and L-PARP was represented in Figure 3 B-D, based on the data of WB assay, with β-actin as internal control. The CQ treatment and ATO treatment increased the expression level of released cyt c, C-CASP 3 and L-PARP in the NB4 cells (*$p < 0.05$). However, the protein level of released cyt c, C-CASP 3 and L-PARP were mark-

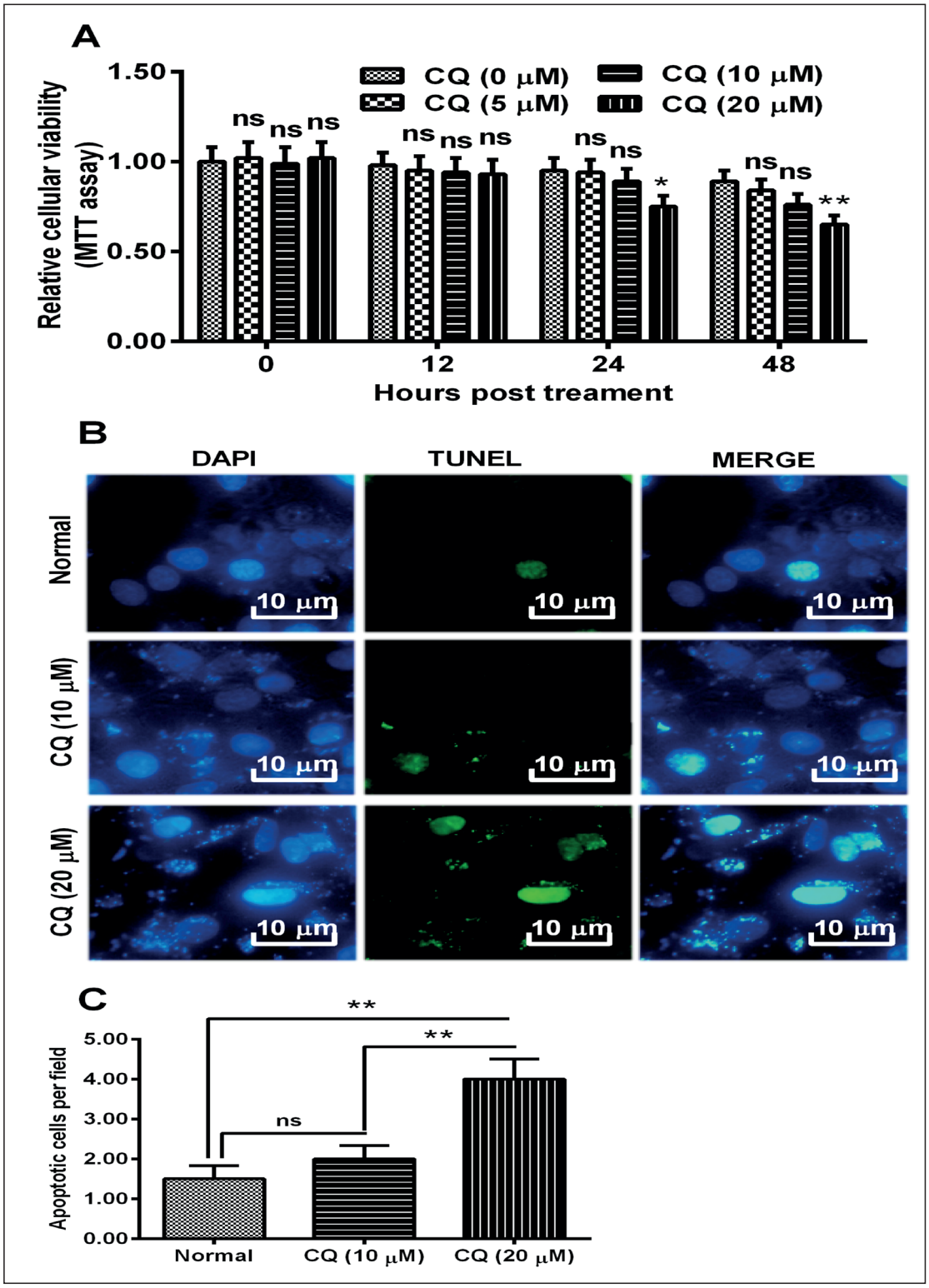

Figure 1. TUNEL staining for the apoptosis in the APL-derived NB4 cells, which were treated with various concentration of chloroquine (CQ). A: MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay for the viability of APL-derived NB4 cells, which were treated with 0, 5, 10 or 20 μM chloroquine (CQ) for 0, 12, 24 or 48 h; ***B,*** TUNEL staining for the NB4 cells, which were treated with 0, 10 or 20 μM CQ; C: Counting of TUNEL-stained (apoptotic) cells, post CQ treatment. Scale bar was indicated as 10 μm in each image. Experiments were repeated in triplicate independently. *$p < 0.05$, **$p < 0.01$, ns: no significance.

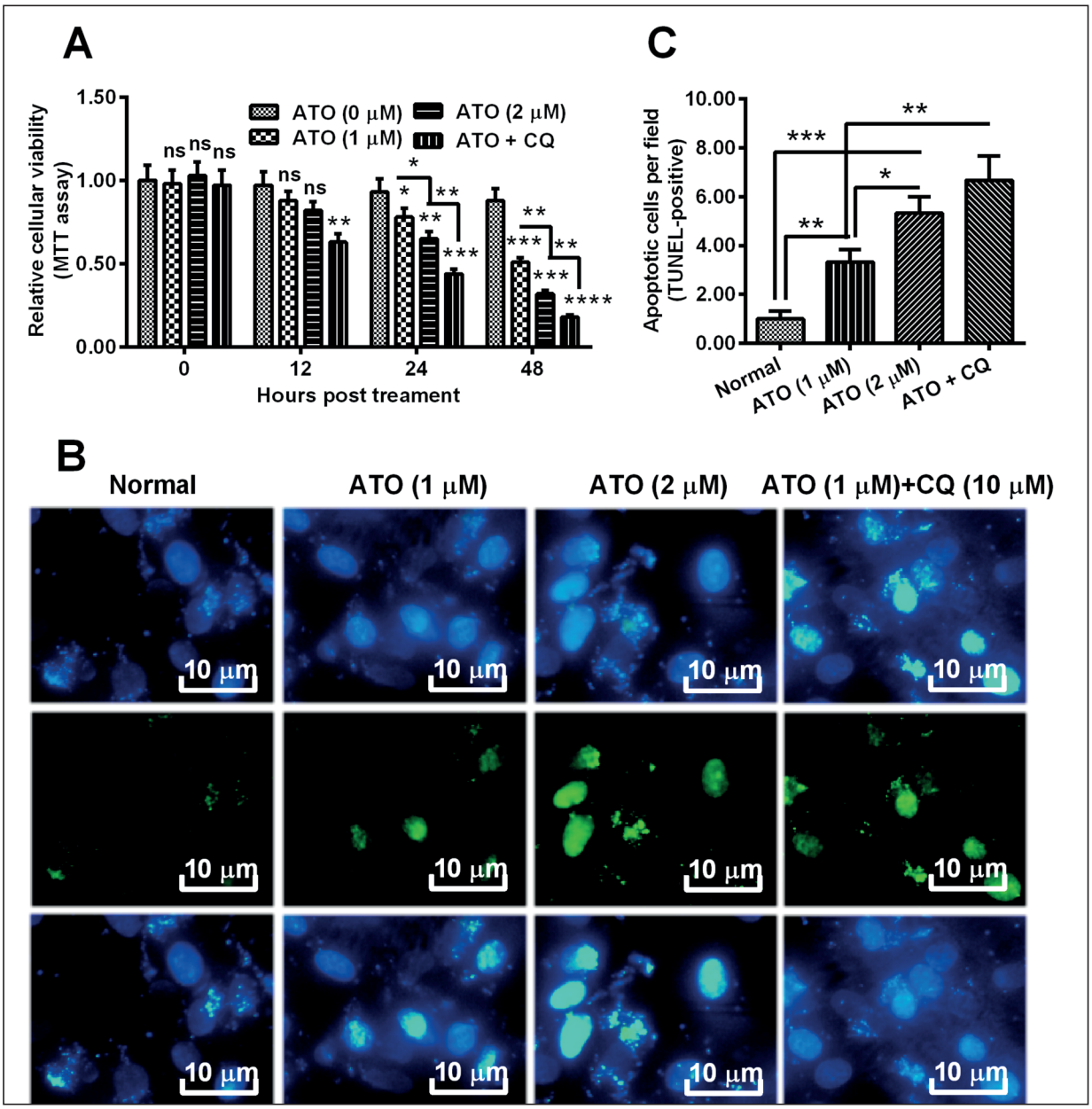

Figure 2. TUNEL staining for the apoptosis in the NB4 cells, which were treated with Arsenic trioxide (ATO). ***A,*** MTT assay for the viability of APL-derived NB4 cells, which were treated with 0, 1 or 2 μM ATO or with 1 μM ATO + 10 μM CQ for 0, 12, 24 or 48 h; ***B,*** TUNEL staining for the NB4 cells, which were treated with 0, 1 or 2 μM ATO or with 1 μM ATO + 10 μM CQ for 24 h; ***C,*** Counting of TUNEL-stained (apoptotic) cells, post the CQ or (and) ATO treatment. Scale bar was indicated as 10 μm in each image. Experiments were repeated in triplicate independently. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ or ****$p < 0.0001$, ns: no significance.

edly increased in the cells treated with both CQ and ATO (**$p < 0.01$, ***$p < 0.001$). All these data implied that combined treatment of CQ and ATO could positively regulate the expression of released cyt c, C-CASP 3 and L-PARP on protein level in NB4 cells.

Autophagy Induction In The ATO- or/and CQ-Treated NB4 Cells

Based on the above findings, ATO and CQ could enhance the apoptosis of NB4 cells. This work made further efforts to investigate the autophagy in the ATO- or/and CQ-treated NB4 cells.

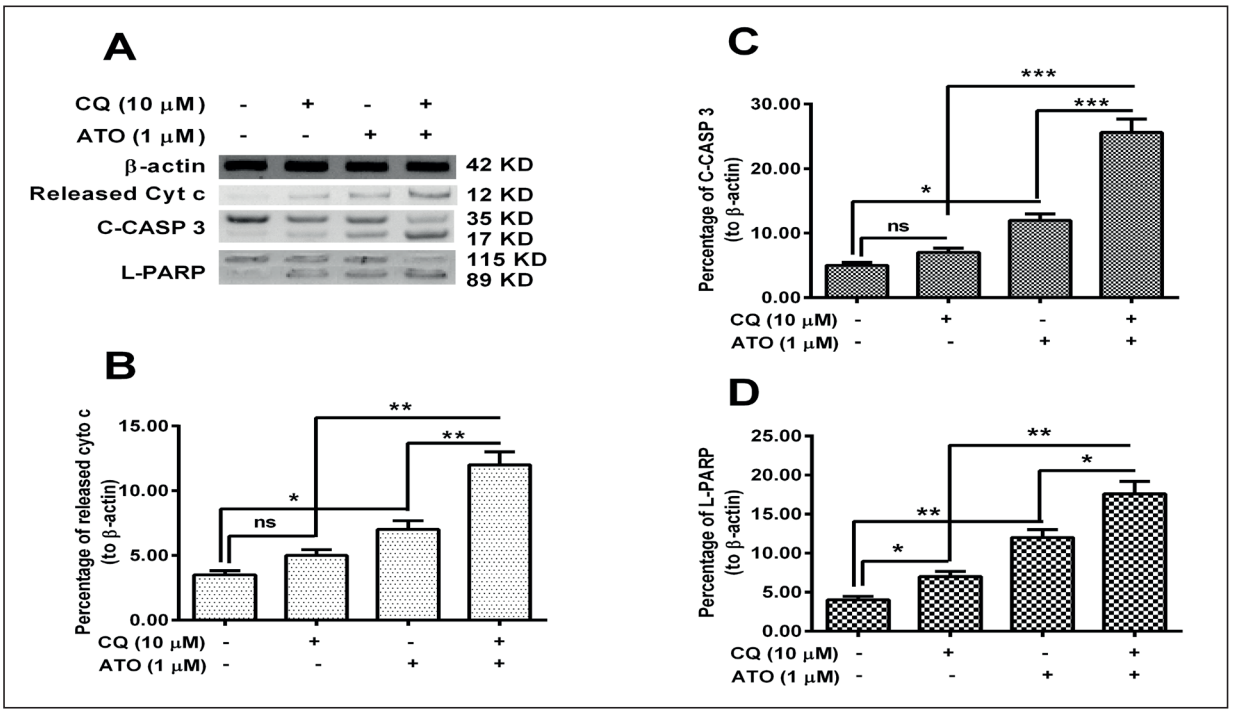

Figure 3. Western blot analyses of apoptosis-related proteins in the ATO- or/and CQ-treated NB4 cells. ***A,*** Western blotting assay for released cytochrome c (released cyt c), cleaved caspase 3 (C-CASP 3) and lyzed poly ADP-ribose polymerase (L-PARP) in the ATO- (1 μM) or (and) CQ-treated (10 μM) NB4 cells for 24 h, with β-actin as internal control; B-D: Relative levels of released cyt c ***(B)***, C-CASP 3 ***(C)*** or L-PARP ***(D)*** to β-actin in the ATO- (1 μM) or (and) CQ-treated (10 μM) NB4 cells. Experiments were repeated in triplicate independently. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ or ****$p < 0.0001$, ns: no significance.

The EGFP-LC3 reporter was utilized to make the autophagic puncta visible. The images were depicted in Figure 4 A, and the GFP-positive autophagic puncta were calculated in Figure 4 B. The autophagic puncta were much more in the cells treated with 10 μM CQ and 1 μM ATO than the control group (*$p < 0.05$, **$p < 0.01$, Figure 4 B, with 10 μM Rapamycin as positive control). However, compared to the groups of the 10 μM CQ-treated cells and 1 μM ATO-treated cells, the autophagic puncta in the cells treated with both 10 μM CQ + 1 μM ATO, were significantly increased (**$p < 0.01$). Besides, the expression level of the autophagy-related makers, such like the LC3B/LC3A, ATG-5 and p62, were also examined by Western blot assay. ATO and CQ treatment increased the expression of LC3B, ATG-5 and p62 on protein level (*$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$, Figure 4 C and 4 D). In addition, the positive regulation was most significant in the cells treated with both ATO and CQ. All the data showed that the ATO and CQ could promote the autophagic vesicle formation, especially the combination of CQ and ATO.

CQ Inhibited The Lysosomal Degradation in NB4 Cells, With or Without ATO Treatment

Previous analysis revealed that CQ treatment deteriorated the As_2O_3-induced NB4 cell apoptosis, whereas aggravated the As_2O_3-induced accumulation of acidic vesicular organelles. To further investigate the mechanism, the lysosomes level in the ATO- or/and CQ-treated NB4 cells was detected by lysotracker Red kit, with lysosomes stained into red. Figure 5 A demonstrated the lysosomes staining in each group. The lysosome dots per cell were quantified in Figure 5 B, which illustrated that CQ treatment reduced the lysosomes in the NB4 cells; in contrast, the lysosome dots were much higher in the ATO-treated NB4 cells to the control group (*$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$). Whereas, the extra CQ treatment in the ATO-treated NB4 cells down-regulated the level of lysosomes significantly, compared to the cells treated with ATO only (**$p < 0.01$). The activity of cathepsin B was also detected to verify the change of lysosomes. The change of the cathepsin B activity

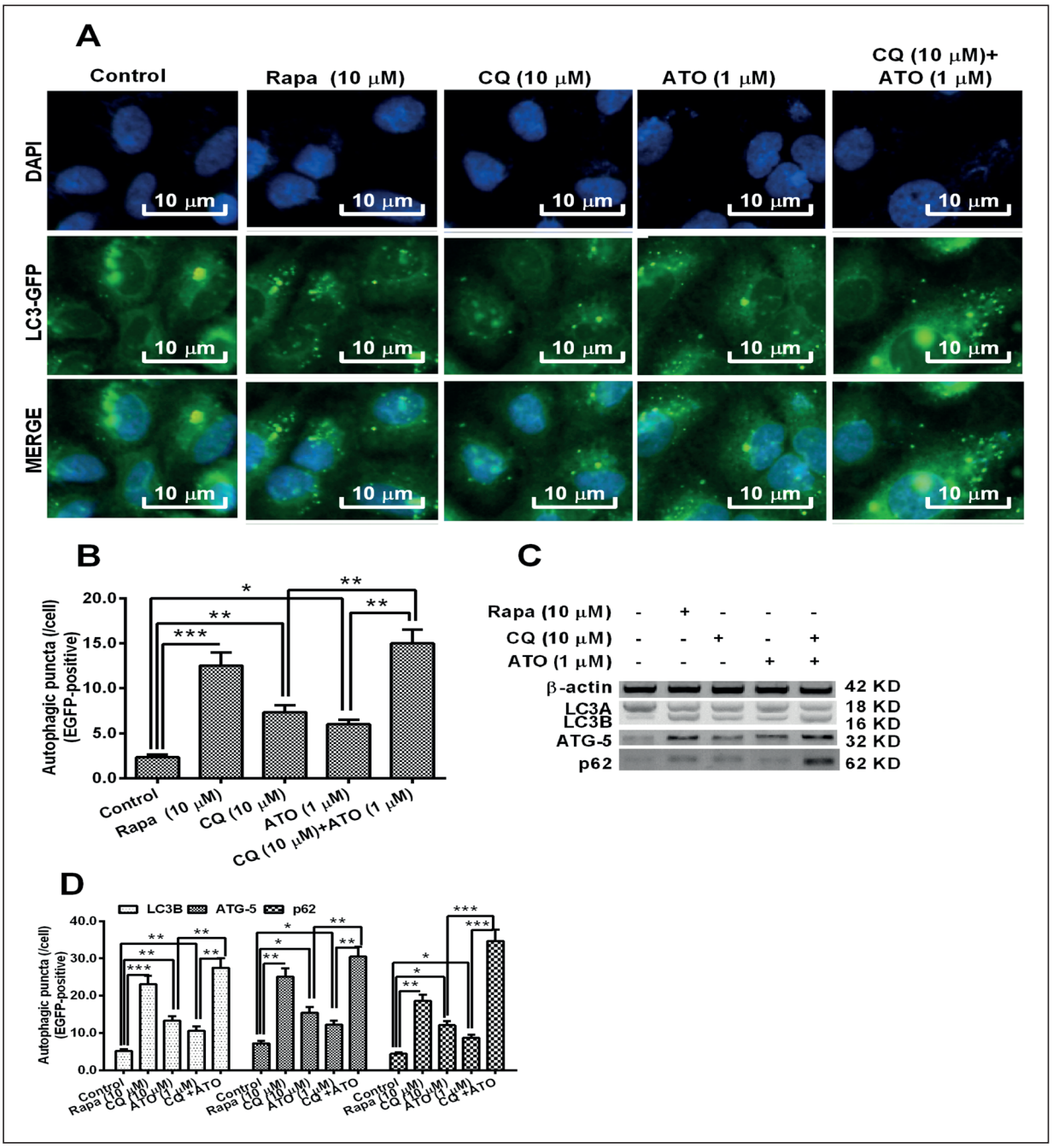

Figure 4. Autophagy induction in the ATO- or/and CQ-treated NB4 cells. ***A,*** Autophagic puncta were visualized post the transfection (24 h) with EGFP-LC3 reporter in blank NB4 cells or the NB4 cells, which were treated with 10 μM Rapamycin (as positive control), 10 μM CQ, 1 μM ATO, or with 10 μM CQ + 1 μM ATO for 24 h; ***B,*** Counting of the GFP-positive autophagic Puncta in the NB4 cells, post CQ or (and) ATO treatment; Scale bar was indicated as 10 μm in each image. ***C,*** and ***D,*** Western blot analysis ***(C)*** and quantification ***(D)*** of autophagy-related genes (ATGs) in the CQ- or ATO-treated NB4 cells. Results were averaged from four independent replicate experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ns: no significance.

was similar to the image results, CQ decreased the cathepsin B activity, ATO treatment increased the cathepsin B activity on the contrary, and the CQ down-regulated the ATO-induced cathepsin B activity as well (Figure 5 C, **$p < 0.01$, ***$p < 0.001$). Thus, CQ blocked the lysosomal degradation in NB4 cells with or without ATO treatment.

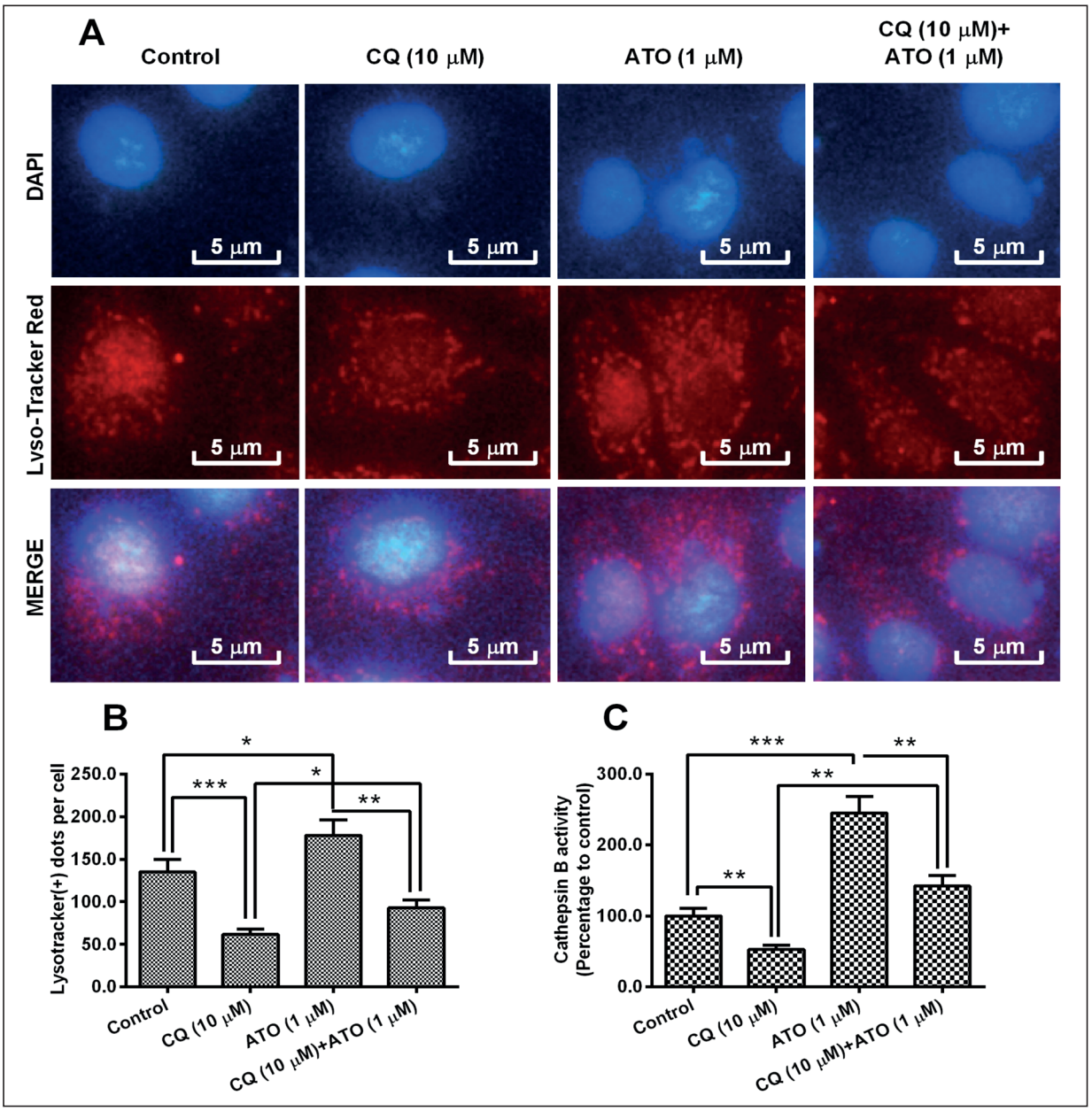

Figure 5. Lysosome inhibition by CQ in NB4 cells, with or without ATO treatment. **A**, and ***B,*** Lysosomes were stained LysoTracker Red, an indicator of acidic intracellular compartments, in blank NB4 cells, or in the NB4 cells, which were treated with 10 μM CQ, 1 μM ATO, or with 10 μM CQ + 1 μM ATO for 24 h; Lysosome dots were were observed under confocal microscope ***(A)*** and lysosome dots per cell were quantified using Leica confocal software ***(B)***. Scale bar was indicated as 5 μm in each image. ***C,*** Proteolytic activity of cathepsin B in the CQ- or (and) ATO-treated (for 24 h) NB4 cells were quantified with a fluorescence-based assay kit. Results were averaged from four independent replicate experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Discussion

The therapy of APL is an exciting achievement in the modern medicine. At present the curative ratio is exceeding 90% with the molecularly targeted therapy with ATRA and ATO[12,21]. We examined the influence by CQ on the sensitivity of APL cells to ATO. It is widely accepted that CQ is an inhibitor of autophagy[22]. In the present study, There was no significant regulation on cellular viability in NB4 cells with 5 or 10 μM CQ, within 48 h. However, the relative cellular viability was

declined and the apoptotic cells were increased, when NB4 cells were treated with 20 μM CQ. Thus, we selected 10 μM as a safe concentration to examine CQ-posed influence on the sensitivity of NB4 cells to ATO. Results showed that relative cellular viability was decreased and the apoptotic cells were up-regulated in the ATO- or/and CQ-treated NB4 cells. The apoptosis induction in the cells treated with 1 μM ATO + 10 μM CQ was most significant. Moreover, the Western blot assay was performed to examine the expression level of the apoptosis-related protein (released cyt c, C-CASP 3, L-PARP), the findings depicted that these three apoptosis-related protein were positively regulated in the ATO- or/and CQ-treated NB4 cells, especially the cells treated with ATO + CQ. Therefore, additional CQ treatment deteriorated the As_2O_3-induced NB4 cell apoptosis. It also has been reported that CQ could inhibit the autophagy process by blocking the release of lysosomal enzymes and the fusion of autophagosomes and lysosomes. To further investigate the mechanism of the influence by chloroquine CQ in APL-derived NB4 cells, which were treated, with ATO, the autophagy induction was explored in the ATO- or/and CQ-treated NB4 cells. The EGFP-LC3 reporter was transfected into the NB4 cells to trace the autophagic puncta. The acidic vesicular organelles were accumulated in the NB4 cells treated with ATO, CQ or ATO+CQ; in addition, the combined treatment of ATO and CQ remarkably inducted the autophagy in the NB4 cells, compared to the positive control (treated with 10 μM Rapamycin). The detection of the autophagy-related protein level showed the similar results, the expression level of LC3A, LC3B, ATG-5 and p62 were significantly up-regulated in the experimental groups. Therefore, extra CQ treatment promoted the accumulation of the acidic vesicular organelle. The fusion of the autophagosome and lysosomes was the key step to the autophagy process[23,24]. It was found that the autophagosome was increased in the experimental groups, consequently, the lysotracker Red staining was used to detect the distribution of the lysosomes. Lysosome dots were lower in the cells treated with CQ than the control group, so CQ inhibited the formation of lysosomes. However, the treatment with ATO up-regulated the lysosome dots in NB4 cells. Interestingly, additional CQ treatment significantly declined the ATO-induced lysosomes. The degradation of autophagy is dependent on lysosome[25]. Nevertheless, CQ inhibited the release of lysosomal enzymes and the fusion of autophagosomes and lysosomes, and the substance contained in the acidic vesicular organelles could not be degraded and finally caused the cell death.

Conclusions

We observed that chloroquine aggravates the arsenic trioxide-induced apoptosis of APL NB4 cells by inhibiting lysosomal degradation *in vitro*. It implies that chloroquine might be adjuvant to sensitize APL cells to As_2O_3.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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