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ORIGINAL ARTICLE

Chloroquine enhances catalpol's ability to promote apoptosis by inhibiting catalpol's autophagy-promoting effect on gastric cancer

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Summary

Purpose: *Gastric cancer, which is derived from gastric mucosal epithelial cells, is a representative solid tumour, and more than 1 million cases are diagnosed worldwide each year. However, treatment methods and therapeutics for gastric cancer are limited, and further research is needed to develop novel strategies.*

Methods: *In this experiment, we studied the effect of catalpol from the extract of Dihuang from traditional Chinese medicine on gastric cancer cells.*

Results: *The results showed that catalpol led to a dose-dependent reduction in gastric cancer cell proliferation. When the promotion of autophagy by catalpol was inhibited, the proapoptotic effects of catalpol on gastric cancer cells were enhanced. Bax, an apoptosis-related marker, was upregulated in catalpol-treated cells, and its expression was increased in the group treated with catalpol in combination with an inhibitor compared to the group treated with catalpol alone. Opposite results were obtained with BCL-2 inhibition. Flow cytometry showed that apoptosis rates were higher in cells treated with a combination of autophagy inhibitors. Accumulation of reactive oxygen species (ROS) in gastric cancer cells showed the group treated with the combination of catalpol and an inhibitor enhanced ROS production. Transwell assays showed that catalpol plus autophagy inhibitors exerted a stronger inhibitory effect on the migration ability of AGS cells than catalpol alone.*

Conclusions: *In summary, the above results indicate that inhibition of catalpol-induced autophagy could better promote the apoptosis of gastric cancer cells.*

Key words: *gastric cancer, catalpol, chloroquine, autophagy, apoptosis*

Introduction

As a common digestive tract tumour derived from gastric mucosal epithelial cells, gastric cancer has a worldwide mortality rate second only to lung cancer. GC ranks second in cancer mortality, with two-thirds of patients residing in Asian countries, especially China and Japan [1,2]. In the clinic, chemotherapy is the main method used to treat and control gastric cancer and its recurrence. However, multidrug resistance during chemotherapy is a barrier for gastric cancer patients achieving satisfactory prognosis, and autophagy is an important reason for the resistance of tumour cells [1]. The autophagy-related gene Beclin-1 is generally expressed at high levels in normal epithelial cells, but it is usually expressed at lower levels in cancer cells and tissues [3]. Studies in recent years have found that extracts from some medicinal plants can inhibit the growth of tumour cells [4]. Catalpol has antitumour effects, medicinal value, and application prospects for various organs and tissues. Chloroquine (CQ) is a weak base tertiary

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amine that can accumulate in lysosomes, destroy the acidic environment in lysosomes and then inhibit the fusion of autophagosomes and lysosomes during autophagy [5]. Some studies have noted that cell autophagy can both facilitate tumour cell growth [6] and inhibit the growth of cancer cells [7]. Cell autophagy is unstable, and activation of MAPK-activated protein kinase signalling leads to activation of key transcription factors and, in turn, regulates the expression of antiapoptotic genes. During apoptosis of gastric cancer cells, the expression of Bax is obvious [8]. Several studies suggest that Bcl-2 binding to Beclin-1 can lead to autophagy inhibition [9,10]. Therefore, in our study, we inhibited catalpol's ability to promote autophagy by using an autophagy inhibitor in gastric cancer cells. Our results showed that catalpol treatment resulted in more apoptotic gastric cancer cells in the presence of autophagy inhibitors.

Catalpol (Figure 1) is a cyclic allene terpene glycoside that is extracted from the fresh roots of *Rehmannia glutinosa* and has various pharmacological activities, including hypoglycaemic, antitumour and anti-inflammatory effects [11,12]. Catalpol can inhibit gastric cancer cell growth and proliferation *in vitro* and *in vivo* [13]. Unfortunately, it is unclear whether the ability of catalpol to promote autophagy is associated with the inhibition of gastric cancer progression conferred by catalpol.

This is the first study combining an autophagy inhibitor with catalpol to explore the effect of catalpol's autophagy-promoting effect on its ability to inhibit gastric cancer cell growth. We attempted to demonstrate that inhibiting catalpol's ability to promote gastric cancer cell autophagy can enhance catalpol's ability to inhibit gastric cancer cell growth. This work presents a new reference for the application of catalpol as a possible candidate antitumour drug.

Methods

Cell culture

The human gastric cancer cell line AGS (the Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) was used for *in vitro* experiments.

Cell viability assay

The effects of catalpol on AGS cell viability were tested using CCK-8 assay. In brief, AGS cells (10^3 cells/well) were seeded in 96-well plates (Corning, USA) and maintained in complete culture medium which consisted of 10% fetal bovine serum (FBS) and 90% f-12 medium). The next day, AGS cells were subjected to different treatments as described in each experiment. Next, the CCK-8 analysis kit (Beyotime Biotechnology Company, China) was used to measure cell viability at 570 nm.

Cell migration assays

Transwell assay: AGS cells (10^3 cells/well) were placed in the upper chamber coated with a 24-well (pore size: 8 μm) membrane filter (EMD Millipore, USA). AGS cells were maintained in serum-free medium (F-12 medium only,without FBS and received different drug treatments. Cells in the bottom chamber were counted using a high-power microscope after 4% paraformaldehyde fixation and 0.1% crystal violet staining (5 min).

Scratch test

A thin scratch was made in a 24-well plate seeded with AGS cells. Scratch healing was observed under a high-power microscope. Then, in serum-free medium (F-12 medium only, FBS was added, and the relative treatment drug was added according to each group. After 24 h of incubation, the cells were washed three times with phosphate buffered saline (PBS). Images were taken, and the reduction ratio of the scratch area of each group was analysed with ImageJ software.

Immunoblotting analysis

AGS cell lysates were centrifuged at 15,000×g for 15 min. The supernatants were assayed for purity using a bicinchoninic acid kit following the manufacturer's instructions (Thermo Fisher Scientific, USA). Protein extracts (15 μg) underwent 10% SDS-PAGE separation and membrane transfer (EMD Millipore). Immunoblots were analysed by incubating with primary antibodies, secondary antibodies, and electro-chemi-luminescence (ECL) reagent (Beyotime Biotechnology Company, China). The primary antibodies (dilution: 1:2000, Beyotime Biotechnology Company; Proteintech) included LC3B, ATG12, Beclin-1, Bcl-2, Bax and GAPDH.

Hoechst 33342 analysis

AGS cells treated with drugs of different groups for 24 h were harvested in a 6-well plate and washed 3 times with ice-cold PBS, followed by staining with 0.5 ml Hoechst 33342 solution (Beyotime Biotechnology Company) in the dark (37°C, 5 min), and then, the cells were observed using an inverted fluorescence microscope (Nikon, Japan).

Flow cytometry analysis

Apoptosis was calculated using an Annexin V-amino actinomycin (7-Amino-actinomycin D) apoptosis detection kit (BD Pharmingen, San Jose, CA, USA). AGS cells were

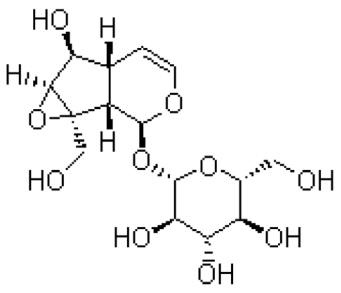

Figure 1. The chemical structure of catalpol.

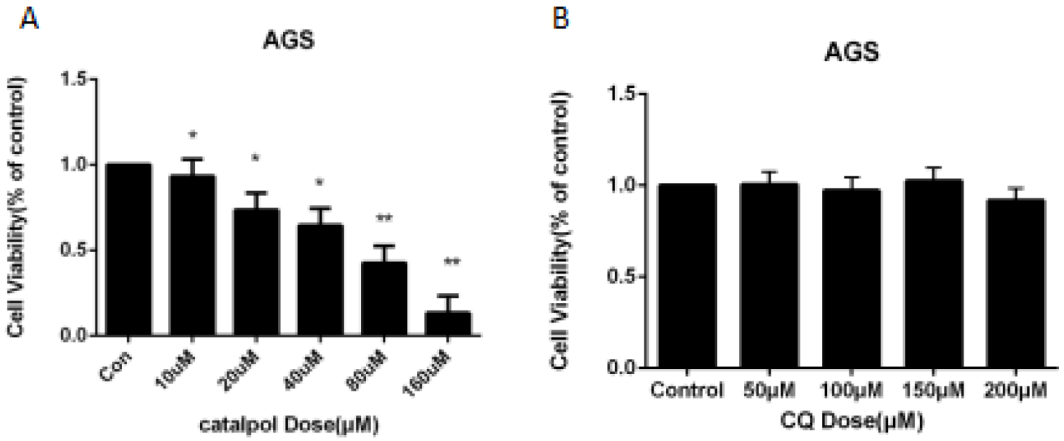

Figure 2. Effects of catalpol and the autophagy inhibitor CQ on AGS cell viability. **A:** A CCK-8 assay was used to analyse the viability of AGS cells in the presence of catalpol at various concentrations for 24 h. **B:** A CCK-8 assay was used to analyse the viability of AGS cells in the presence of CQ at various concentrations for 24 h. *p<0.05, **p<0.01 (mean±SEM; n=3).

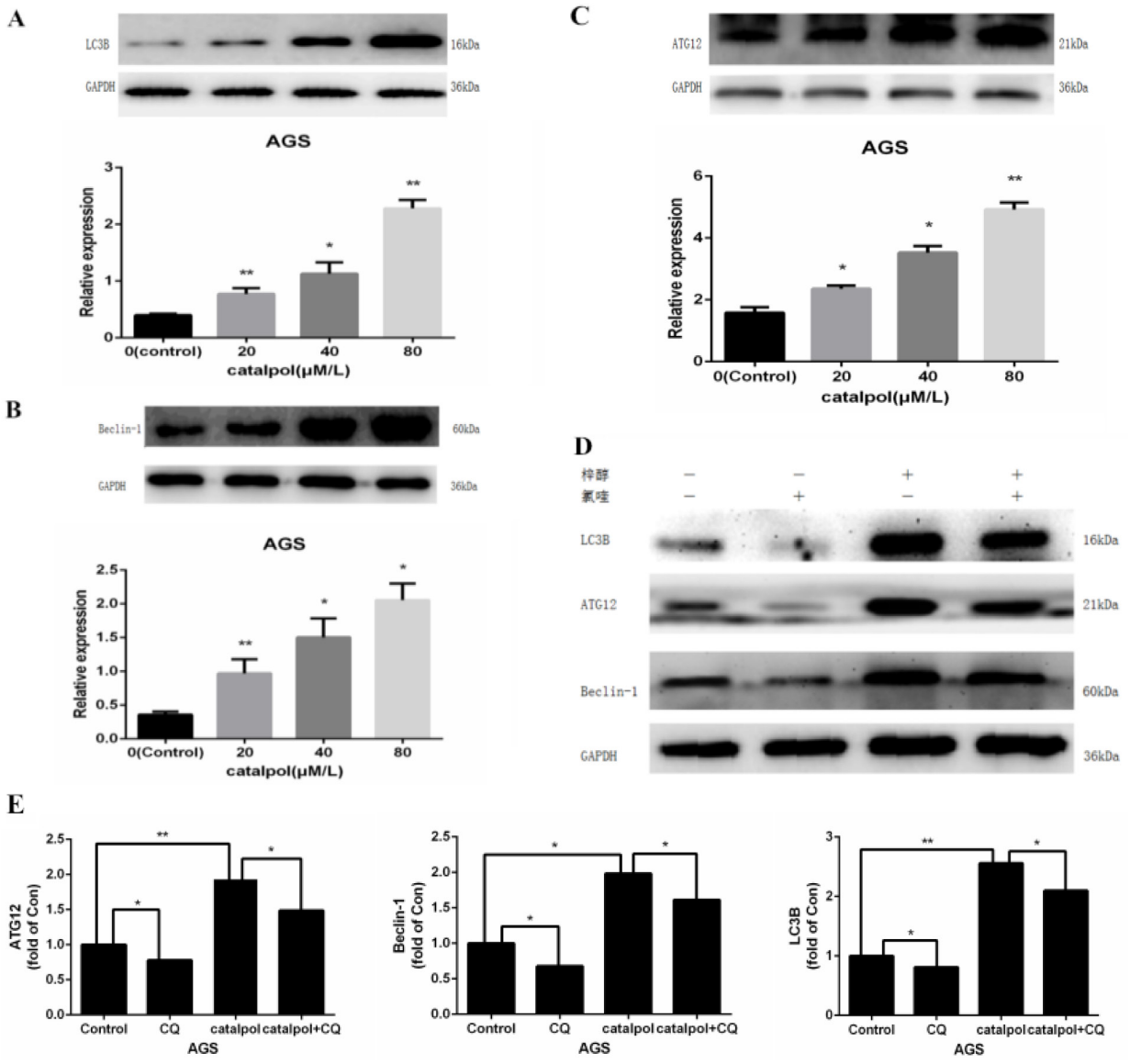

Figure 3. Catalpol promotes autophagy in AGS cells, whereas CQ inhibits this effect. **A-C:** Catalpol promotes the expression of the autophagy-related proteins LC3B **(A)**, Beclin-1 **(B)**, and ATG12 **(C)**. **D,E:** CQ inhibits catalpol-induced autophagy in AGS cells. *p<0.05 and **p<0.01 (mean±SEM; n=3).

harvested from a 6-well plate and washed 3 times with ice-cold PBS. Following a 15-min incubation with Annexin V plus 7-AAD in the dark, flow cytometry (BD Biosciences, San Jose, USA) was employed to gate apoptotic cells.

Reactive oxygen species (ROS) determination

An active oxygen analysis kit (Beyotime Biotechnology Company) was used to determine ROS generation. AGS cells were seeded in a 6-well plate (5×10^5/ml) and then stained with 10 μM DCFH-DA. Then, stained cells were washed 3 times with serum-free DMEM and detected by fluorescence microscopy (Nikon, Japan) to quantify ROS generation.

Statistics

The results are shown as mean ± standard deviation of the mean (SEM). SPSS 25.0 software was used for statistical analyses and variance tests. A p value less than 0.05 indicated significant difference.

Results

Catalpol constrains AGS cell viability, while the autophagy inhibitor CQ does not

Initially, whether catalpol affects AGS cell viability was assessed by CCK-8. The results revealed a dose-dependent reduction in AGS cell viability 24 h after catalpol treatment ($p<0.01$, Figure 2A). Treatment of AGS gastric cancer cells with the autophagy inhibitor CQ for 24 h had no effect on cell viability ($p=0.681$, Figure 2B). The above results show that catalpol can reduce the viability of AGS gastric cancer cells in a dose-dependent manner and that the autophagy inhibitor CQ has no significant effect on viability.

Autophagy inhibitor CQ inhibits catalpol promotion of autophagy in AGS cells

To investigate the role of catalpol in mediating autophagy in AGS cells, the autophagy-related proteins LC3B, Beclin-1, and ATG12 in AGS cells following treatment with different concentrations of catalpol were determined by immunoblotting analysis. The results showed that catalpol triggered a dose-dependent increase in LC3B, Beclin-1, and ATG12 in AGS cells (Figures 3A, 3B and 3C). Next, we chose catalpol at a concentration of 80 μM and CQ at a concentration of 100 μM to treat AGS cells alone or in combination. The immunoblotting analysis results showed increased LC3B, Beclin-1 and ATG12 protein expression upon catalpol treatment and decreased LC3B, Beclin-1 and ATG12 protein expression upon CQ treatment. In contrast to the control group, the catalpol combined with CQ group presented increased LC3B, Beclin-1, and ATG12 protein expression. In contrast to the control group, the catalpol group presented decreased protein expres-

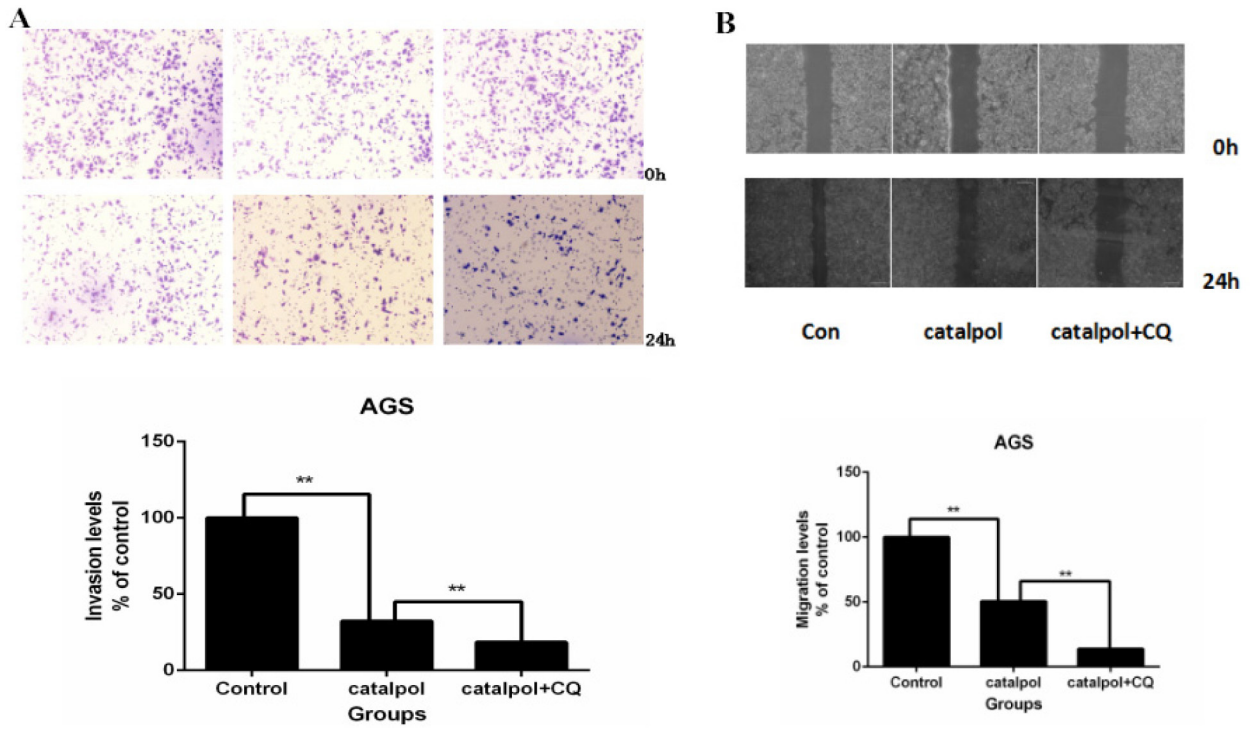

Figure 4. Inhibiting catalpol-induced autophagy suppresses AGS cell migration. **A:** AGS cells were assayed to evaluate migration ability by Transwell analysis in the presence of 80 μM catalpol and/or 100 μM CQ. **B:** AGS cells were assayed to evaluate the migration ability by scratch test analysis in the presence of 80 μM catalpol and/or 100 μM CQ. *p<0.05 and **p<0.01 (mean±SEM; n=3).

sion (Figures 3D, 3E). These results suggest that catalpol could trigger autophagy in AGS cells, whereas CQ inhibits the autophagy caused by catalpol.

Inhibiting catalpol-induced autophagy suppresses AGS cell migration

Transwell migration analysis showed that treating AGS gastric cancer cells with catalpol in combination with an autophagy inhibitor reduced the migration of gastric cancer cells ($p<0.01$, Figure 4A). Next, we used scratch experiments to verify the results ($p<0.01$, Figure 4B). The above findings indicate that the inhibiting catalpol's autophagy effect on AGS gastric cancer cells can better inhibit AGS gastric cancer cell migration.

Inhibiting catalpol-induced autophagy facilitates AGS cell apoptosis

Apoptosis has received attention as a mechanism of cell death in tumour cells [14,15]. Accord-

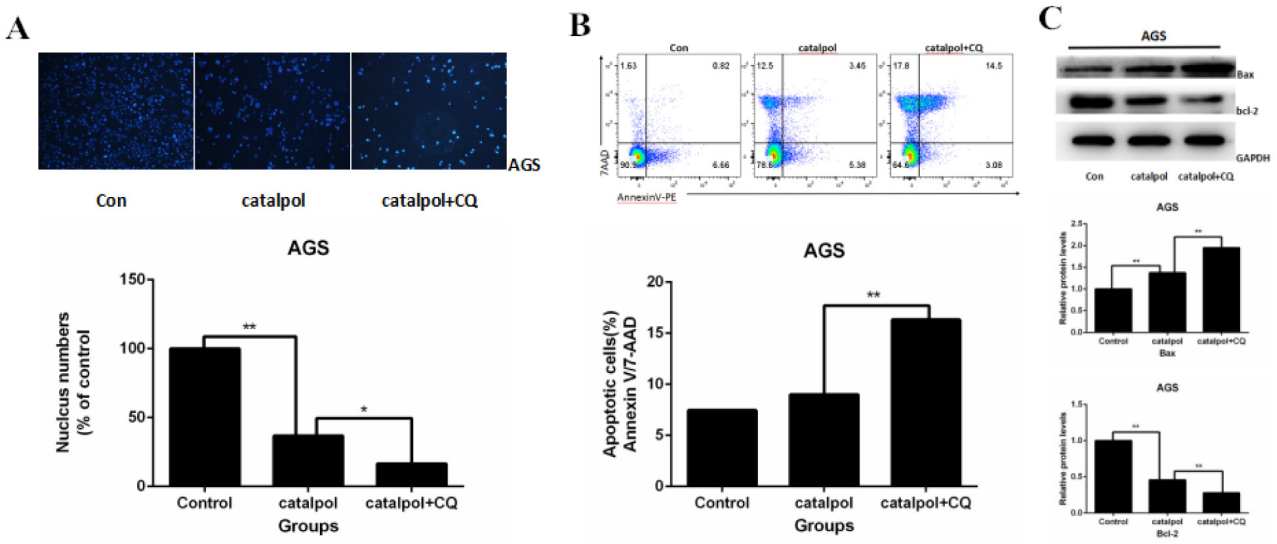

Figure 5. The effect of catalpol plus CQ on AGS cell apoptosis was detected by Hoechst 33342 and flow cytometric analysis. **A:** Quantification of Hoechst 33342-stained nuclei (blue). **B:** Apoptotic AGS cells were examined by flow cytometric analysis. **C:** Immunoblots of Bax and Bcl-2 proteins and their quantification. *p<0.05 and **p<0.01 (mean±SEM; n=3).

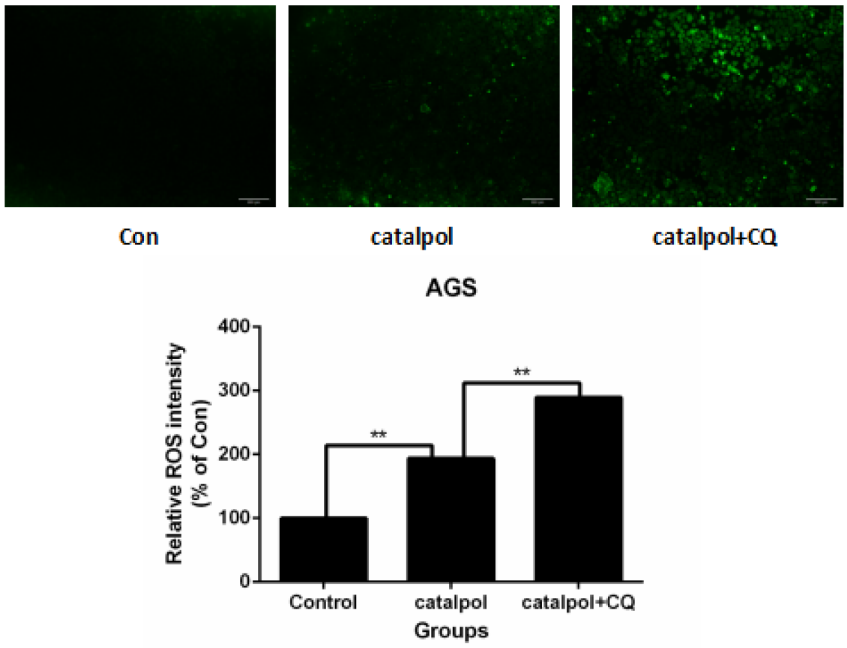

Figure 6. Effects of catalpol plus CQ on ROS production in AGS cells. AGS cells were assayed to measure ROS production in the presence of 80 μM catalpol and/or 100 μM CQ. **p<0.01 (mean±SEM; n=3).

ingly, we studied the role of catalpol in regulating the apoptosis of AGS cells. Once AGS cells were treated with 80 μM catalpol and 100 μM CQ alone or in combination for 24 h, Hoechst 33342 staining was performed. The results showed that inhibition of catalpol-induced AGS autophagy could increase the apoptosis of AGS cells ($p<0.05$, Figure 5A). As depicted in Figure 5B, the results of flow cytometric analysis revealed increased apoptosis and death rates after inhibiting catalpol's autophagy effect on AGS cells ($p<0.01$). In addition, we further analysed the effects of different treatments on the apoptosis markers of AGS gastric cancer cells by immunoblotting analysis. The results showed that catalpol enhanced the Bax expression level ($p<0.05$), and the Bax expression level was higher upon treatment with CQ plus catalpol, which reduced Bcl-2 expression. After combination treatment with CQ, protein expression was further reduced. The above results show that catalpol can induce apoptosis, and after inhibiting autophagy, catalpol-induced apoptosis was enhanced in AGS cells, causing cell death.

Inhibiting catalpol-induced autophagy caused ROS production in AGS cells

The production of ROS can induce apoptosis and lead to cell death, which may be a strategy for inhibiting cancer progression [16]. After treating AGS cells for 24 h with different drug combinations, the results of ROS generation showed that catalpol combined with CQ promoted the ability of AGS gastric cancer cells to produce ROS (Figure 6A) ($p<0.01$).

Discussion

Gastric cancer is a frequently occurring malignant tumour [17], with increasing recurrence and mortality rates worldwide but treatment strategies are limited. Tumour cells can spread from the stomach to other organs, such as the lung, liver, and bone [18]. Especially in China, analysis of the clinical experience show that most patients present an advanced stage disease at their first diagnosis, and their 5-year survival rate is relatively low. Therefore, the treatment of gastric cancer requires more novel and effective drugs and strategies. Catalpol has many pharmacological capabilities, and its inhibitory effect on gastric cancer has also been confirmed [13]. In addition, other researchers have also discovered the effectiveness of catalpol in inhibiting other tumours. For example, catalpol can inhibit the proliferation, invasion, and migration of liver cancer cells [19], non-small cell lung cancer cells [20], and osteosarcoma [21]. However, these studies did not note a relationship between catalpol and the autophagy of cancer cells. Ke et al [22] demonstrated that Tenovin-6 plus the autophagy inhibitor CQ led to a cytotoxic effect by inducing accumulation of LC3B-II and by triggering cell cycle arrest and inducing apoptosis. In our experiments, catalpol promoted autophagy in gastric cancer cells in a dose-dependent manner, and autophagy inhibitors inhibited this effect. When used in combination with an autophagy inhibitor, catalpol enhances gastric cancer cell apoptosis, enhances ROS production, and inhibits cancer cell migration. Therefore, the results show that by inhibiting the autophagy of catalpol, its anticancer ability can be enhanced, which is a new method of drug combination.

Apoptosis represents one of the physiological mechanisms of programmed cell death and is characterized by cell shrinkage, chromosomal condensation, fragmented nuclei, and DNA fragmentation [23,24]. We demonstrated that catalpol combined with autophagy inhibitors had stronger inhibition of the anti-apoptotic protein Bcl-2 than catalpol alone, and the proapoptotic protein Bax was increased. Flow cytometry also confirmed that the proportion of apoptosis of gastric cancer cells after catalpol combined with autophagy inhibitors was greater than that of the catalpol monotherapy group, indicating that catalpol's ability to promote gastric cancer cell apoptosis was enhanced by inhibiting catalpol's autophagy.

We found through Transwell and scratch experiments that gastric cancer cells treated with increasing doses of autophagy inhibitors had lower migration ability than those treated with catalpol alone, suggesting that inhibiting catalpol's autophagic effect could enhance its ability to inhibit gastric cancer migration.

Studies have found that the production of ROS can damage tumour cell proteins, lipids, and DNA [25]. In our experiments, we found that the amount of ROS released was increased upon treatment with catalpol plus the autophagy inhibitor CQ compared with catalpol alone. Therefore, we believe that inhibiting autophagy with catalpol can increase the amount of ROS produced and thus inhibit gastric cancer cell growth.

Our results provide evidence that catalpol can reduce the proliferation of gastric cancer cells *in vitro*, promote gastric cancer cell apoptosis and inhibit gastric cancer cell migration. When autophagy inhibitors inhibit catalpol, their ability to inhibit the tumourigenic properties of gastric cancer cells is enhanced. The above results indicate that the combination of catalpol with an

autophagy inhibitor increases the ability of catalpol to inhibit gastric cancer cell growth. Our experiments provide a novel avenue for catalpol to treat gastric cancer.

Conclusion

Inhibition of catalpol's autophagy effect in gastric cancer cells can enhance catalpol's inhibitory effect on gastric cancer cells.

Acknowledgements

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Conflict of interests

The authors declare no conflict of interests.

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