



Quercetin Increases Doxorubicin-Induced Apoptosis through Oxidative DNA Damage in KATO III Gastric Cancer Cells

Mingxiang Chen^{1,*}, Chunxiao Duan¹ and Jun Pan^{1,*}

¹ Department of Oncology, Yizheng People's Hospital, Jiangsu Province, China

* **Corresponding author:** Mingxiang Chen, Department of Oncology, Yizheng People's Hospital, Jiangsu Province, China. Email: cmxy8478@163.com; Jun Pan, Department of Oncology, Yizheng People's Hospital, Jiangsu Province, China. Email: panjun1109@126.com

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Abstract

Background: Gastric cancer is the most common gastrointestinal malignancy with an increasing incidence rate worldwide. Finding novel curative and preventive approaches that could target the tumor cells without affecting the normal cells and overcome drug resistance will be tremendously useful.

Objectives: This study aimed to evaluate the effects of quercetin (QUE) in combination with doxorubicin (DOX) on apoptosis and its underlying mechanisms in the KATO III gastric cancer cell line.

Methods: The effects of Que and DOX on cell viability were measured using an MTT assay. Western blot was used for the measurement of γ H2AX protein expression. The expression levels of 8-Hydroxy-2'-deoxyguanosine were evaluated by enzyme-linked immunosorbent assay. The 2, 7-dichlorofluorescein diacetate fluorescence dye was used to detect the formation of reactive oxygen species (ROS). The activities of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glutathione S-transferase) were also assessed. For evaluation of apoptosis, the terminal deoxynucleotidyl transferase dUTP nick end labeling assay was used.

Results: Based on the findings, QUE significantly increased the cytotoxic effects of DOX. Besides, QUE considerably increased the expression levels of γ H2AX. Upon QUE treatment, ROS levels increased, and antioxidant enzyme expression levels markedly decreased. Moreover, QUE treatment resulted in the potentiation of doxorubicin-induced apoptosis in KATO III cells, compared to the cells treated with either QUE or DOX.

Conclusion: Overall, co-administration of QUE and DOX enhances cytotoxicity, increases ROS levels, induces oxidative DNA damage, and decreases cellular antioxidant defense, and thereby might promise a therapeutic regimen in promoting the clinical efficacy of the treatment of patients with gastric cancer.

Keywords: Apoptosis, Doxorubicin, Oxidative DNA damage, Quercetin, ROS

1. Background

Gastric cancer is the most prevalent gastrointestinal malignancy, with more than a million new cases estimated annually worldwide (1). Moreover, since it can only be diagnosed at the advanced stages, its mortality rate is high, making it the third leading cause of cancer-associated deaths (2). Strikingly, gastric cancer is two times more prevalent in males than females (3). The standard treatment for gastric cancer includes a combination of multi-drug chemotherapy, to which a large number of patients respond. In contrast, approximately all patients acquire resistance to chemotherapeutic agents which results in median overall survival of less than one year (4). Furthermore, combination chemotherapies are complicated owing to the diversity in pharmacokinetics, bio-distribution, membrane transport mechanisms, and drug clearance systems (5,6).

Doxorubicin (DOX) is the first-line chemotherapeutic agent, mainly administered to gastric cancer patients, which is slightly effective (7). The DOX toxicity in tumor cells primarily occurs via intercalation with double-strand DNA leading to breakage of strands and repression of both replication

and transcription processes (8). The severe side effects and intrinsic or acquired DOX-resistance are pivotal obstacles that have led to the failure of treatment with mono-administration of DOX (9). Besides, chemotherapeutic drugs overexpress reactive oxygen species (ROS) *in vitro*, which intensifies their genotoxic effects. Activation of ROS-scavenging mechanisms led to the decrease of ROS levels and less DNA damage, resulting in enhanced chemoresistance (10). Hence, promotion of curative methods that potentiate DOX impacts, provide dose mitigation, and preserve normal tissues are crucially required to improve the treatment of gastric cancer patients.

Among anticancer agents, flavonoids are the most studied ones. These compounds can interfere with particular malignancy stages, repress cell growth, and induce programmed cell death in tumor cells (11,12). Quercetin (QUE; with the IUPAC name 3,5,7,3',4'-Penta hydroxyl flavone; C₁₅H₁₀O₇) is one of the most abundant flavonoids present in many vegetables, seeds, and fruits (13). Conflicting findings have been reported in cell cycle arrest by QUE; for instance, it appears that QUE can cause cell cycle arrest at G₀/G₁ in leukemia, S phase in colorectal cancer, or G₂/M phases in breast cancer, and oesophageal

adenocarcinoma cells (14,15).

Aside from the antioxidant activities of QUE, it has an apoptotic effect on neoplasm cells and can hinder tumor expansion (16). Furthermore, QUE has been used in combination with many chemotherapy agents, including DOX and cisplatin (17,18). Due to its lipophilic nature, QUE can traverse the cell membrane and initiate synergistic effects on DOX and other chemotherapeutic agents (19-21).

2. Objectives

The functional mechanism of QUE in combination with DOX during the induction of apoptosis in gastric cancer is mostly unclear. Therefore, this study aimed to scrutinize the effects of QUE in combination with DOX on apoptosis and its underlying mechanisms in KATO III gastric tumor cells.

3. Methods

3.1. Materials and reagents

KATO III cell line was purchased from Sun Yat-sen University Cancer Center (Guangzhou, China) and fetal bovine serum (FBS) was provided by Thermo Fisher Scientific (Waltham, MA). Moreover, RPMI-1640 media, phosphate-buffered saline (PBS), and Trypsin-EDTA were bought from Gibco Life Technologies (Gibco, USA). Antibiotics, dimethyl sulfoxide (DMSO), and trypan blue were purchased from Sigma Aldrich Co., USA. In addition, Bio Basic Co. provided 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Furthermore, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG, ab201734) enzyme-linked immunosorbent assay (ELISA) competitive and genomic DNA isolation were bought from the Abcam Co., Cambridge, UK. Besides, ROS, catalase (CAT), superoxide dismutase (SOD), glutathione (GSH) peroxidase (GPx), GSH s-transferase (GST, E BC K029), and GSH reductase (GR, E-BC-K099) assay kits were purchased from the Elabscience Biotechnology Co. Wuhan, China. Primary monoclonal antibody against γ -H2AX from Boster Biological Technology (USA, Cat. No. #M00241) and β -actin were provided by Santa Cruz Biotech (Cat. No. sc-130300).

3.2. Cell culture

The KATO III cells were seeded in RPMI-1640 (+2 mM L-glutamine) accompanied by 10% FBS, 1% pen/strep at 37 °C with 95% O₂, and 5% CO₂ in the incubator. Cells in the logarithmic phase were collected for functional analysis, and the number of surviving cells was defined by performing the trypan blue dye exclusion test.

3.3. MTT assay

Cell viability was performed using the MTT assay. The KATO III cells were seeded in a 96-well microplate overnight at the density of 2×10^4 cells/200 μ l/well

their growth capability was evaluated. Afterward, the cells were exposed to a serum-containing medium with QUE (0-125 μ M) and DOX (0-1.25 μ mol/L) alone or in combination for 48 h. At the end of the treatment, cells were exposed to 5 mg/mL of MTT (20 μ l) for 4 h. Subsequently, 150 μ l of DMSO was added for solubilization of formazan crystals, followed by persistent shaking for 20 min at 37 °C. Afterward, the absorbance value was detected at 570 nm by ELx808 plate auto-reader.

3.4. Evaluation of 8-Hydroxy-2'-deoxyguanosine levels

For measurement of 8-Hydroxy-2'-deoxyguanosine amounts as a marker of DNA damage, we extracted DNA from KATO III cancer cells by commercial DNA extraction kit. An ELISA kit was employed for the detection of this marker.

3.5. Measurement of reactive oxygen species levels

Effects of various treatments on intracellular ROS levels were evaluated by ROS Assay kit. For this purpose, cells were incubated with QUE, DOX, and their combination, washed with PBS at pH 7.4, and then incubated with 10 μ mol/L of 2, 7-dichlorofluorescein diacetate (DCFH-DA) for 40 min at 37 °C in the dark. Intracellular ROS was reacted with DCFH-DA which resulted in a highly fluorescent compound dichlorofluorescein (DCF). The DCF fluorescence intensity was assessed with excitation wavelength at 485 nm and emission wavelength at 525 nm. The data are presented as relative DCF fluorescence (ratio DCF-induced fluorescence/DCF-induced control fluorescence).

3.6. Evaluation of glutathione peroxidase activity

The GPx activity was evaluated based on the protocol. In this assay, GPx catalyzes the reaction between H₂O₂ and GSH to generate H₂O and oxidized GSH (GSSG). The rate of enzymatic reaction can be used for the evaluation of the GPx activity. Hence, the activity of GSH can be determined by calculating the consumption of alleviated GSH levels. Since H₂O₂ and diminished GSH can react without catalysis of GPx, the part of GSH decrement via non-enzymatic reaction should be decreased.

The reaction of GSH with dinitrobenzoic acid can produce 5-thio-dinitrobenzoic acid anion that has a stable golden color. The absorbance value was read at 412 nm using an ELISA plate reader. Enzyme-specific activities were expressed as units/mg of protein. According to previous research, one unit of GPx in 1 mg of protein can catalyze the consumption of 1 μ mol/L GSH with deduction of the effect of non-enzyme reaction at 37 °C for five min.

3.7. Measurement of glutathione s-transferase activity

The GST activity was evaluated using the GST assay kit. In this kit, the activity of GST was measured with regard to the fact that GST catalyzes

the binding of GSH to 1-chlorine-2, 4-dinitrobenzene. It should be noted that the activity of GST is linearly correlated with the change of GSH level. The absorbance value was detected at 412 nm using a plate auto-reader.

3.8. Measurement of Glutathione reductase activity

Glutathione reductase (GR) activity could be detected indirectly by a coupled reaction with GSH. The GSSG is recycled to its diminished state by GR and nicotinamide adenine dinucleotide phosphate (NADPH). Oxidation of NADPH to NADP⁺ is accompanied by a decline in absorbance at 340 nm. Hence, the rate of reduction in absorbance is directly proportional to the GR activity in the sample. Enzyme-specific activities were expressed as units/mg of protein. The amount of enzyme of 1 mmol of NADPH catalyzed by 1 g protein per min is defined as 1 unit.

3.10. Western blotting

After appropriate exposure to drugs, the total cellular proteins were trypsinized and extracted from the cells using radioimmunoprecipitation assay lysis buffer. Afterward, centrifugation (14,000 × g) at 4 °C for 20 min was employed for harvesting the lysates and protein concentration was determined using the Bradford method. In total, 50 µg of protein was separated in a 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane. Afterward, the membranes were blocked with 5% skim milk in Tris-buffered saline, 0.1% Tween 20 (TBST) for one h and incubated with primary antibody against γ -H2AX overnight at 4 °C.

After being washed with TBST, the membranes were incubated with anti-rabbit or anti-mouse IgG-conjugated secondary antibodies for one h. Blots

were visualized by ImageJ software (version 1.42q) was used for Band analysis.

3.11. Statistical analysis

Results are expressed as mean±SD, and a p-value of less than 0.05 was considered statistically significant. All quantitative results were processed by GraphPad Prism software (version 7.04). Kolmogorov-Smirnov and Levene tests were used to evaluate the normality of the results. The groups were compared using the one-way ANOVA post-hoc (Tukey and Dunnett) test.

4. Results

4.1. Co-administration of quercetin and doxorubicin could significantly decrease cellular proliferation of KATO III cells

Inhibitory effects of QUE and DOX on cell proliferation of KATO III cells were scrutinized by the MTT assay. As presented in Figures 1A and 1B, different concentrations of QUE (0, 25, 50, 75, 100, and 125 µM) and DOX (0, 0.25, 0.5, 0.75, 1, and 1.25 µM) were used during 48 h. Results revealed the dramatic reduction of cell viability of KATO III cells in a concentration-dependent manner. The IC₅₀ values of QUE and DOX in KATO III cells were 50.37 µM and 0.87 µM, respectively. After combination with Que in a concentration-dependent manner, the IC₅₀ value of DOX was 0.64 which decreased in comparison to DOX alone (P<0.05). In a sense, 50.37 µM of QUE and 0.64 µM of DOX killed 50% of KATO III cell lines after 48 h (Figure 1C).

4.2. Co-administration of quercetin and doxorubicin promotes intracellular reactive oxygen species levels in KATO III cells in gastric cancer

To elucidate whether ROS generation is responsible for QUE and DOX-induced cell death, we

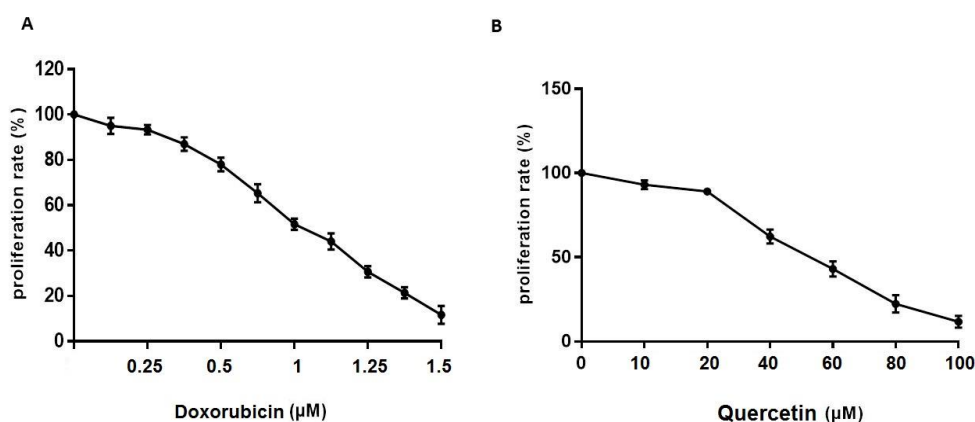


Figure 1. Effects of quercetin and doxorubicin and their combination on cell proliferation. All values are shown as mean±SD.

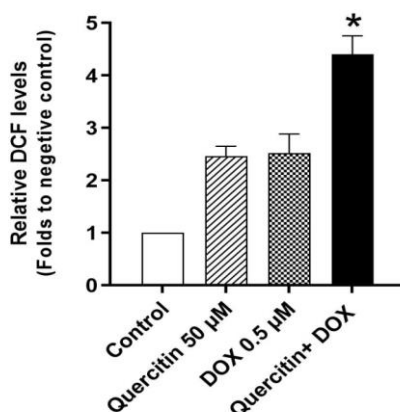


Figure 2. Effects of quercetin and doxorubicin (DOX) and their combination on reactive oxygen species levels. All values are shown as mean±SD. DCF: dichlorofluorescein

examined the effect of their mono-administration and co-administration on the ROS generation using fluorescent probe procedure, DCFH-DA. It was found that QUE and DOX potently elevated the ROS levels, compared to the control group. Moreover, the combination of QUE and DOX had a more powerful impact ($P<0.05$; Figure 2).

4.3 Co-administration of quercetin and doxorubicin promotes DNA damage in KATO III cells in gastric cancer

To ascertain the effects of QUE, DOX, and their combination on DNA damage in KATO III cells, we surveyed 8-OXO-dG levels as a valid biomarker of DNA oxidation. Our results showed that QUE and DOX increased the 8-OXO-dG levels ($P<0.05$; Figure 3A). Notably, QUE and DOX in combination exerted a more potent effect on increasing the DNA damage ($P<0.05$; Figure 3A). Furthermore,

the expression levels of γ H2AX that played a substantial role in DNA damage response were also evaluated in this study. The findings demonstrated that QUE and DOX mono-administration resulted in a dramatic increment in γ H2AX levels in both groups ($P<0.05$; Figure 3B and C). Additionally, co-administration of QUE and DOX increased γ H2AX levels more drastically ($P<0.05$; Figure 3B and C).

4.4 Co-administration of quercetin and doxorubicin tackled the activities of the antioxidant enzymes in KATO III cells in gastric cancer

We assessed the activity of antioxidant enzymes, such as CAT, SOD, GST, GR, and GPx. As shown in Figure 4, the collected data indicated that the co-administration of QUE and DOX dramatically repressed the above-mentioned enzymatic activities, compared to the mono-administration ($P<0.05$).

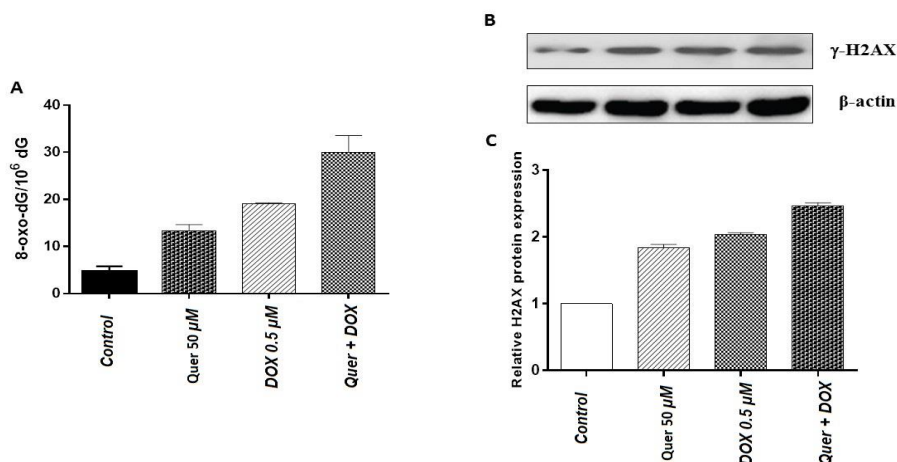


Figure 3. Effects of quercetin (Quer) and doxorubicin (DOX) and their combination on DNA damage. A) Levels of 8-oxo-dG in stomach DNA in the studied groups, B) protein levels of γ -H2AX. β -actin was applied as the loading control, C) quantitative interpretation of γ -H2AX. All values are shown as mean±SD.

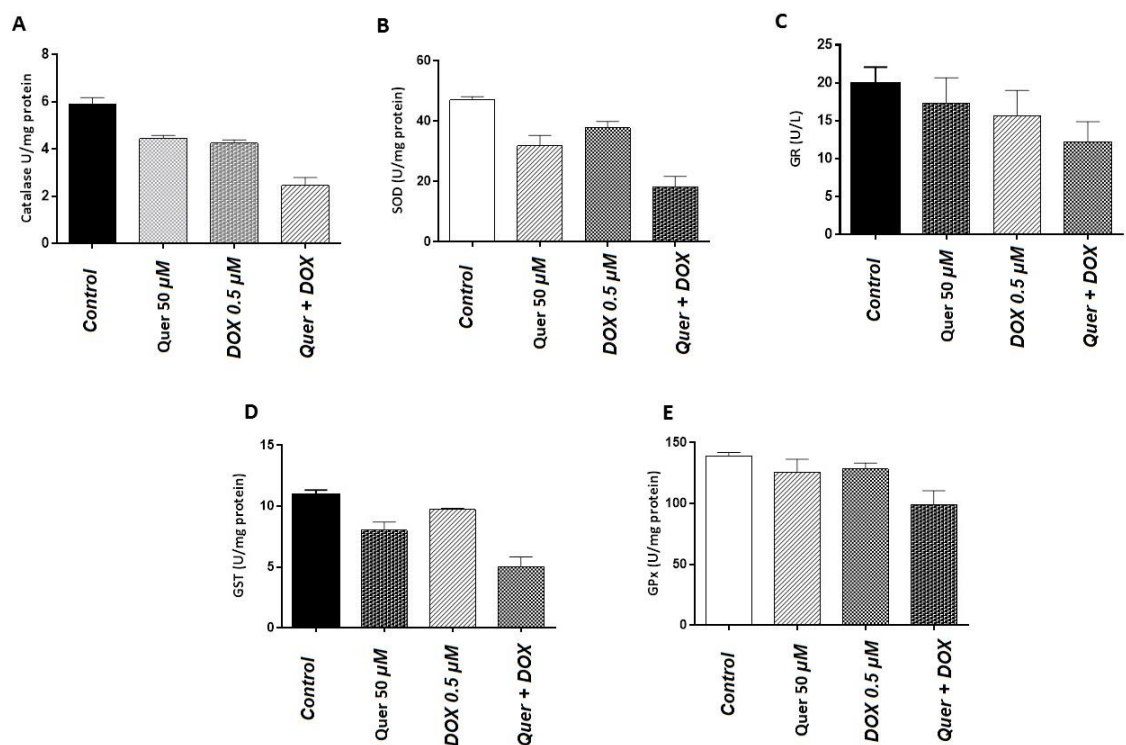


Figure 4. Effects of quercetin (Quer) and doxorubicin (DOX) and their combination on the activity levels of antioxidant enzymes including (A) catalase, (B) SOD, (C) GR, (D) GST, and (E) GPx. Results are shown as mean±SD. SOD: superoxide dismutase, GR: glutathione reductase, GPx: glutathione peroxidase, and GST: glutathione S-transferase.

5. Discussion

Findings of the present study implied three significant results that allow us to better understand the effects of co-administration of QUE and DOX on the increase in apoptosis through oxidative DNA damage in the KATO III gastric cancer cell line. Firstly, it was found that QUE could significantly diminish the IC₅₀ value of DOX in the KATO III cells, compared to its mono-administration.

Secondly, it was indicated that the co-administration of QUE and DOX had a more potent effect on apoptosis induction. Thirdly, this co-administration dramatically enhanced the 8-OXO-dG and ROS levels and also diminished antioxidant enzyme levels in comparison with mono-administration. These valuable findings highlighted that the co-administration of QUE and DOX exacerbated *in vitro* apoptosis in the gastric cells.

Although DOX is a potent anticancer agent, its clinical utility is restricted due to its toxicity to healthy tissues. Besides, drug-resistance development is another limitation in the employment of this agent in the eradication of tumor cells (22). Therefore, expansion of the novel, effective, and safe agents with lesser cytotoxicity and side effects are required for tumor treatment (23). It has been well documented that QUE has

extensive therapeutic effects, including anti-infective and antioxidant activities. Moreover, apart from its potent effects, QUE induced little or no toxicity in healthy tissues (8).

Based on the results of previous studies, QUE induced toxicity in many human cancer cell lines with differing sensitivities (15,24,25). Furthermore, it has been revealed that QUE affects several cancers by intensifying the DOX activity. Co-administration of QUE and DOX is highly effective against several tumor types, including human lymphoid, myeloid leukemia, and breast cancer cell lines, since it causes repression of cell growth and invasion through the repression of P-glycoprotein (17,21). Herein, the collected data indicated that the combination of QUE and DOX could markedly diminish the IC₅₀ value of DOX in the KATO III cells, compared to the mono-administration of DOX.

The ROS is a pivotal regulator of cellular signal transduction; hence, disturbance of the balance between the producing and cleansing ability of ROS might contribute to cell damage. In a sense, ROS can convert lipids or proteins in the cells into oxidized forms or transform nucleic acids into mutated forms (26). According to previous studies, ROS exert dual function in the cellular process in a concentration-dependent manner since in moderate levels they play a role in cancer onset and development through inflammation, DNA mutation, and cellular damage, while in higher levels they act

as an anticancer agent by induction of apoptosis (27). Hence, this has led to the conclusion that ROS modulators could be employed as primary cancer impediments to improve treatment plans (10).

The ROS attack guanine in the DNA and transform into 8-OXO-dG that can bind to T rather than C; hence, the increased level of 8-OXO-dG is primarily considered hallmark mutagenesis resulting in oxidative stress (OS) (28). Since 8-OXO-dG is regarded as a suitable biomarker to assess DNA damage *in vitro* (29), this factor was evaluated in response to co-administration in this study. As mentioned earlier, we found that 8-OXO-dG elevated in line with ROS overproduction.

Based on the results of previous studies, anticancer properties of DOX, at least in part, mediated through the induction of oxidative DNA damage, which is in line with those of the present study. Findings of the present study indicated that QUE, combined with DOX, exerts more substantial DNA damage in KATO III cells, which subsequently brings about potent apoptosis. In another aspect, Ramasamy et al. demonstrated that the co-administration of QUE and DOX using pH-sensitive polypeptide-based nanocarriers enabled the QUE to dramatically enhance the cytotoxic impression of DOX and additionally induce apoptosis in the tumor cells (30).

Following the intercalation of DOX into the DNA, the structural changes impede a convenient interaction of topoisomerase II with DNA, ultimately impairing ataxia telangiectasia mutated activation resulting in γ H2AX formation (23,31). Phosphorylation of histone H2AX on Ser-139 (γ H2AX) is a sensitive biomarker of DNA injury and DNA damage response, especially the stimulation of DNA double-strand breaks. In this regard, the Western blot analysis of γ H2AX revealed that exposure of cells to Que or DOX led to an increase in γ H2AX in KATO III cells, which was reflective of the findings of the present and previous studies (32,33). Moreover, co-administration of QUE and DOX results in a significant enhancement of γ H2AX levels.

Importantly, QUE, combined with DOX, leads to remarkable alleviation in antioxidant levels, including SOD, catalase, GPx, GR, and GSH S-transferase. Indeed, decreasing intracellular GSH enhances ROS and improves chemotherapy sensitivity. It has been widely accepted that several chemotherapeutic agents and adjuvant therapies cause a decrease in antioxidant capacity and blockage of antioxidant defense, resulting in cell death (34).

Furthermore, our results supported this notion that programmed cell death is the consequence of ROS overproduction and antioxidant defense inhibition, which is considered one of the essential mechanisms in QUE anticancer activity (35).

Furthermore, Staedler et al (21). found that DOX, when combined with QUE in the breast cancer cell lines, synergistically repressed cell growth and induced apoptosis via decrement S-transferase of GR GSH (36). Indeed, over-produced antioxidant enzymes led to the improvement of the tumor cell and prevention of DNA damage, OS, and repression of apoptosis; these conditions can result in resistance to chemotherapies (37).

6. Conclusion

Based on the findings, QUE could induce cytotoxicity through various methods. Moreover, recent findings have revealed that QUE, a DNA intercalator, increases the cytotoxicity of doxorubicin-induced apoptosis via oxidative DNA damage in KATO III gastric cancer cells. Consequently, our data imply that QUE is a hopeful candidate for the treatment of gastric cancer with apoptosis induction.

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Footnotes

Author's contributions: M.C performed the experiments and supervision. C.D analyzed the data. J.P designed experiments and co-wrote the paper.

Conflicts of Interest: The authors have no conflict of interest to declare.

Ethical Approval: This study was approved by Yizheng People's Hospital, Jiangsu Province, China.

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Informed Consent: Informed consent was not necessary for this *in vitro* study.

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