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Regular paper

Combination of metformin and oxaliplatin inhibits gastric cancer cell proliferation and induces apoptosis

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Background: Gastric cancer is one of the most common cancers worldwide. The disease has a poor prognosis, especially when the tumor becomes inoperable. The present study investigated the potential synergistic effects of oxaliplatin and metformin in gastric cancer cells. Methods: The effect of oxaliplatin and metformin on cell proliferation was assessed with CCK-8 assay in human gastric cancer cell lines SGC7901 and SNU-16, where , the IC50 and (combination index) CI values were determined. RT-PCR and Western blotting were used to determine mRNA and protein expression levels of cell cycle- and apoptosis-related genes. The apoptotic rate was detected with flow cytometry in SGC7901 and SNU-16 cells. Results: The CCK-8 assay showed inhibited proliferation of SGC7901 and SNU-16 cells upon oxaliplatin or metformin treatment and an increase in inhibitory potency when the drugs were administered in combination. Similarly, cell apoptosis was increased in both cell lines in the combination group compared to the metformin and oxaliplatin groups. Both metformin and oxaliplatin reduced Bcl-2 and increased Bax and caspase-3 expression in SGC7901 and SNU-16; and these effects were enhanced when the drugs were used in combination. Conclusion: The combination of metformin and oxaliplatin inhibited proliferation and induced apoptosis in gastric cancer cells. The underlying mechanisms may be related to the suppression of cyclin D1, Bcl-2 and the increase of expression of Bax and caspase-3.

Keywords: oxaliplatin, metformin, apoptosis, proliferation, gastric cancer

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BACKGROUND

Gastric cancer is the fourth most common and the second deadliest cancer worldwide, causing an estimated 800 000 deaths annually (Daniyal *et al.*, 2015). The highest incidence of gastric cancer is in China, South America, and Eastern Europe. The disease has a poor prognosis, especially when the tumor becomes inoperable. The median survival for patients with advanced or metastatic gastric cancer is only 11–14 months (Cunningham *et al.*, 2008; Koizumi *et al.*, 2008; Yamada *et al.*, 2015). Recurrent post-resection gastric cancer and primarily unresectable advanced gastric cancer are treated with systemic chemotherapy. However, a consensus standard chemotherapy regimen has not been established yet. Cisplatin with 5-fluorouracil or epirubicin, together with cisplatin and 5-fluorouracil are widely used (Rivera *et al.*, 2007), but the administration of cisplatin is limited by nephrotoxicity.

Oxaliplatin is a third-generation platinum compound with a better safety profile than cisplatin (Di Francesco *et al.*, 2002). The drug can inhibit DNA replication by cross-linking double-stranded DNA. Oxaliplatin has been used for systemic chemotherapy for advanced gastric cancer in combination with fluorouracil or fluoropyrimidine (Al-Batran *et al.*, 2008; Kang *et al.*, 2009). However, the most effective and safest dose remains unclear, as the therapy often induced thrombocytopenia (Cunningham *et al.*, 2008). Also, as seen with other drugs, cancer cells eventually develop oxaliplatin resistance (Takahashi *et al.*, 2016). Therefore, identification of agents to use in combination with oxaliplatin is of high clinical relevance.

Metformin is commonly used to treat type 2 diabetes; it decreases hyperglycemia by inhibiting liver glucose production. It has been found that metformin improved survival among diabetic patients with head and neck cancer (Franciosi *et al.*, 2013; Noto *et al.*, 2012). Other studies have also demonstrated the anti-tumor activity of metformin with inhibited cell proliferation and induced apoptosis of various cancer cells (Ben Sahra *et al.*, 2008; Brown *et al.*, 2010; Kato *et al.*, 2012; Rego *et al.*, 2015). Recent studies reported that the combination of metformin and traditional chemotherapeutic drugs (e.g., doxorubicin, paclitaxel) could synergize anti-tumor activities (Hanna *et al.*, 2012; Iliopoulos *et al.*, 2011; Zhang *et al.*, 2016).

In the current study, we examined the potential synergistic effects of oxaliplatin and metformin in the gastric cancer cell line SGC7901 and SNU-16. The effect of the combined drugs on cell proliferation and apoptosis was investigated, and molecular mechanisms underlying the anti-tumor activities were also examined.

METHODS

Cell culture

The human gastric cancer cell line SGC7901 was shared by the Department of Pathology and Pathophysiology, Wuhan University, China. SNU-16 cells were purchased from the Cell Bank of Institute of Biochemistry and Cell Biology (Shanghai, China). The cells were cultured in RPMI-1640 medium (Hyclon, Logan, UT, USA) with 10% fetal bovine serum (Zhejiang Tianhang Biotechnology, China), 100 mg/mL streptomycin and 100 units/mL penicillin (Gibco, Grand Island, NY, USA), at

37°C and 5% CO_2 Metformin and oxaliplatin were purchased from Signa-Aldrich (St. Louis, MO, USA).

Cell proliferation assay

A SGC7901 and SNU-16 cells were seeded plated in a 96-well plate (10000 cells/well) and cultured in the presence or absence of metformin and/or oxaliplatin for 48 hrs. Next, 10 μL CCK-8 solution (CCK-8 cell proliferation test kit, Beyotime Biotechnology, Shanghai, China) was added to each well and the cells were incubated for another 4 hours, after which absorbance at 450 nm was measured using a microplate reader. The rate of growth inhibition was calculated with the formula: (Control – Treatment)/ (Control – Blank) ×100%, and the values of IC_{50} were calculated statistically (how?) .

Combination Index analysis

The Combination Index (CI) of metformin and oxaliplatin was calculated using Chou-Talalay method (Chou, 2006) and CompuSyn software, where. The potency and dose-effect curves for each drug were plotted. The do-effect parameters of each drug (m_1, $(Dm)_1$, m_2, $(Dm)_2$), alone and combined ($M_{1,2}$, $(Dm)_{1,2}$) were calculated, and used to determine the CI value.

Cell apoptosis assay

Cells were seeded at a density of 3×10^5 cells/well in a 6-well culture plate for 24 hrs. Metformin (IC50), oxaliplatin (IC50), and metformin in combination with oxaliplatin were added to respective wells for 48 hrs. Cells were then harvested and prepared for flow cytometry. 5 μL of Annexin V-FITC staining solution was added to the cell suspension (Annexin V-FITC Cell Apoptosis Detection Kit, Tianjin Sungene Biotech Co., Ltd., China). The mixture was gently vortexed and incubated for 10 min at 25°C in the dark. The cells were analyzed by flow cytometry within 1 hr.

Real-time quantitative PCR

Total RNA was extracted from the cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Reverse transcription was conducted using First Strand cDNA Synthesis kit (TOYOBO, Osaka, Japan). Quantitative PCR (qPCR) amplification of the target genes was conducted using SYBR Premix Ex Taq (TAKARA, Shiga, Japan) in StepOne Real-Time PCR. The primers used in the qPCR reactions are listed in Table 1.

Western blotting

Cell lysates were harvested. Proteins were electrophoretically separated in 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. After blocking, the membranes were incubated with antibodies, including GAPDH (Abcam Inc., Cambridge, MA, USA), cyclin D1 (Abcam Inc., Cambridge, MA, USA), caspase-3 (Cell Signaling Technology, MA, USA), Bcl-2 (TDY Biotech Co., Ltd., Beijing, China), and Bax (Beijing Biosynthesis Biotechnology Co., Ltd, China). Immunoreactivity signals were detected using a commercial ECL kit.

Statistical analyses

Quantitative data expressed as mean ± S.D. were compared using ANOVA; quantitative data expressed in percentage (%) were compared using the Chi-square test. $P<0.05$ was considered as significant. The Pearson linear function test was used to examine the correlation between drug concentration and inhibition rate. All statistical analyses were performed with SPSS 17.0 software.

RESULTS

Inhibition of proliferation in cells treated with metformin and oxaliplatin

The proliferation assay results showed that treatment with metformin or oxaliplatin alone could inhibit cell proliferation within 48 hours (Fig. 1). The rates of inhibition of proliferation by metformin were: 26% (at 12.5 mM), 39% (at 25 mM), 50% (at 50mM) and 61% (at 100nM) for SGC-7901, and 25% (at 12.5 mM), 41% (at 25 mM), 50% (at 50 mM) and 59% (at 100 mM) for SNU-1. The inhibitory effect increased with the increase of drug concentration ($r=0.710$, $P<0.05$). Similar correlation was observed in cells treated with oxaliplatin ($r=0.708$, $P<0.05$). The proliferation inhibition rates of oxaliplatin were: 35% (at 12.5 ug/ml), 43% (at 25 ug/ml), 60% (at 50 ug/ml) and 72% (at 100 ug/ml) for SGC-7901 and 34% (at 12.5 ug/ml), 43% (at 25 ug/ml), 61% (at 50 ug/ml) and 71% (at 100 ug/ml) for SNU-16. The proliferation inhibition rates were higher for oxaliplatin than for the same doses of metformin (all $P<0.05$). The proliferation inhibition rates by both drugs administered simultaneously were higher than for each of them separately in both SGC-7901 and SNU-16 cells. The IC_{50} values based on proliferation assay for were 46 mM in SGC-7901 and 44 mM in SNU-16. for metformin and

Table 1. Sequences of primers used for real-time qPCR

Primer name		Primer sequence (5′– 3′)	Product length (bp)
H-GAPDH	Forward	GGTCGGAGTCAACGGATTTG	218
	Reverse	GGAAGATGGTGATGGGATTTC	
H-cyclin D1	Forward	TCGTGGCCTCTAAGATGAAGG	246
	Reverse	CACAGAGGGCAACGAAGGTC	
H-Bax	Forward	TGTCGCCCTTTTCTACTTTGC	164
	Reverse	GAGGCTTGAGGAGTCTCACCC	
H-Bcl-2	Forward	ACATCGCCCTGTGGATGACT	160
	Reverse	AGGGCCAAACTGAGCAGAGTC	
H-Caspase-3	Forward	GAACTGGACTGTGGCATTGAGAC	164
	Reverse	GCACAAAGCGACTGGATGAAC	

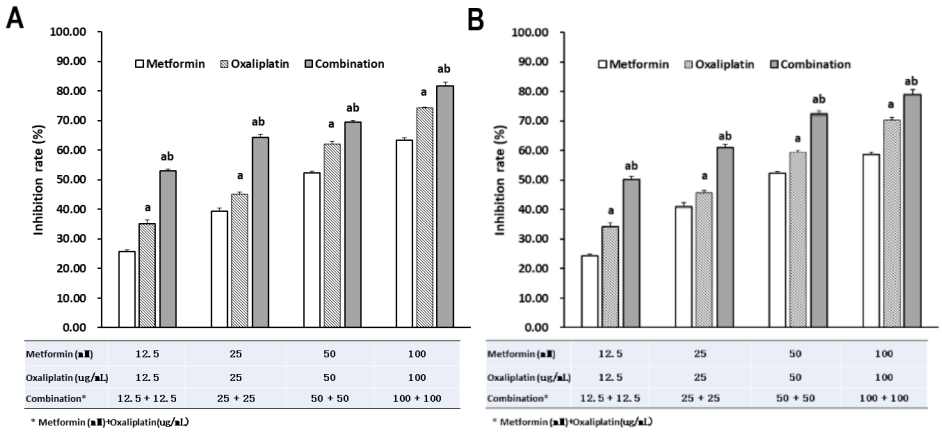

Figure 1. Metformin and oxaliplatin inhibit the proliferation of gastric cancer cells in a dose-dependent and synergistic manner.. All samples were exposed to drugs for 48 hours.
(**A**) Cell viability of SGC-7901 cells, (**B**) Cell viability of SNU-16 cells. Letters a and b in the graphs indicate statistical significance of the following comparisons: a. comparison to metformin group at each concentration, $P<0.05$; b. comparison to oxaliplatin group at each concentration, $P<0.05$)

30 μg/mL in SGC-7901 and 28 μg/mL in and SNU-16 for oxaliplatin (in subsequent experiments, these concentrations were used to treat the cells). The combination index (CI) values of metformin and oxaliplatin were 0.67 in SGC-7901 and 0.81 in SNU-16.

To further investigate the effects of metformin and oxaliplatin on cell proliferation, we examined the levels of mRNA and protein of the cell cycle regulator cyclin D1 (Fig. 2) using the IC_{50} concentrations. The separate treatments of metformin or oxaliplatin resulted in a reduced expression of cyclin D1 mRNA compared to the control group (1.1 *vs* 0.55, $P<0.05$ for metformin; 1.1 *vs* 0.6, $P<0.05$ for oxaliplatin in SGC-7901 cells; 0.59 *vs* 0.37, $P<0.05$ for metformin; 0.59 *vs* 0.29, $P<0.05$ for oxaliplatin in SNU-16 cells), and this reduction was enhanced when the drugs were used in combination (1.1 *vs* 0.28, $P<0.05$ in SGC-7901 and 0.59 *vs* 0.09, $P<0.05$ in SNU-16). The change in cyclin D1 protein levels was similar to change in mRNA levels. The lowest expression of cyclin D1 was observed under the combination treatment (0.6 *vs* 0.08, $P<0.05$ in SGC-7901; and 1.14 *vs* 0.3, $P<0.05$, in SNU-16).

Promotion of apoptosis in cells treated with metformin and oxaliplatin

Promoting cell apoptosis is a crucial activity of chemotherapy drugs. We evaluated apoptosis in the cells treated with metformin, oxaliplatin or the combination of the

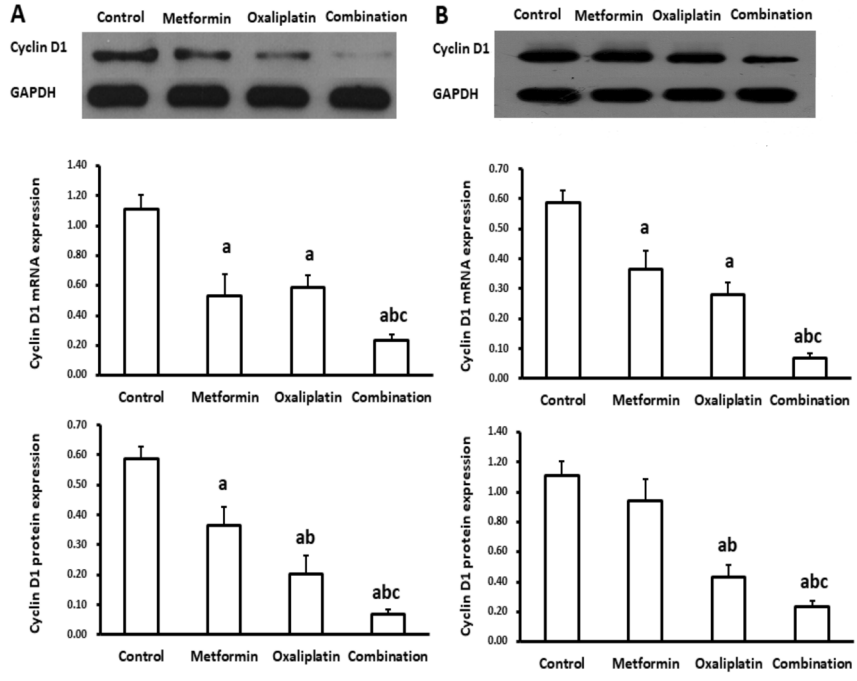

Figure 2. Metformin and oxaliplatin reduce cyclin D1 expression level in a synergistic manner.
All samples were exposed to IC_{50} concentration of the drugs for 48 hours. (**A**) Cyclin D1 expression level in SGC-7901, (**B**) Cyclin D1 expression level in SNU-16. Letters a, b, c in the graphs indicate statistical significance of the following comparisons: a. comparison to the control group, $P<0.05$; b. comparison to the metformin group, $P<0.05$; c. Comparison to the oxaliplatin group, $P<0.05$.

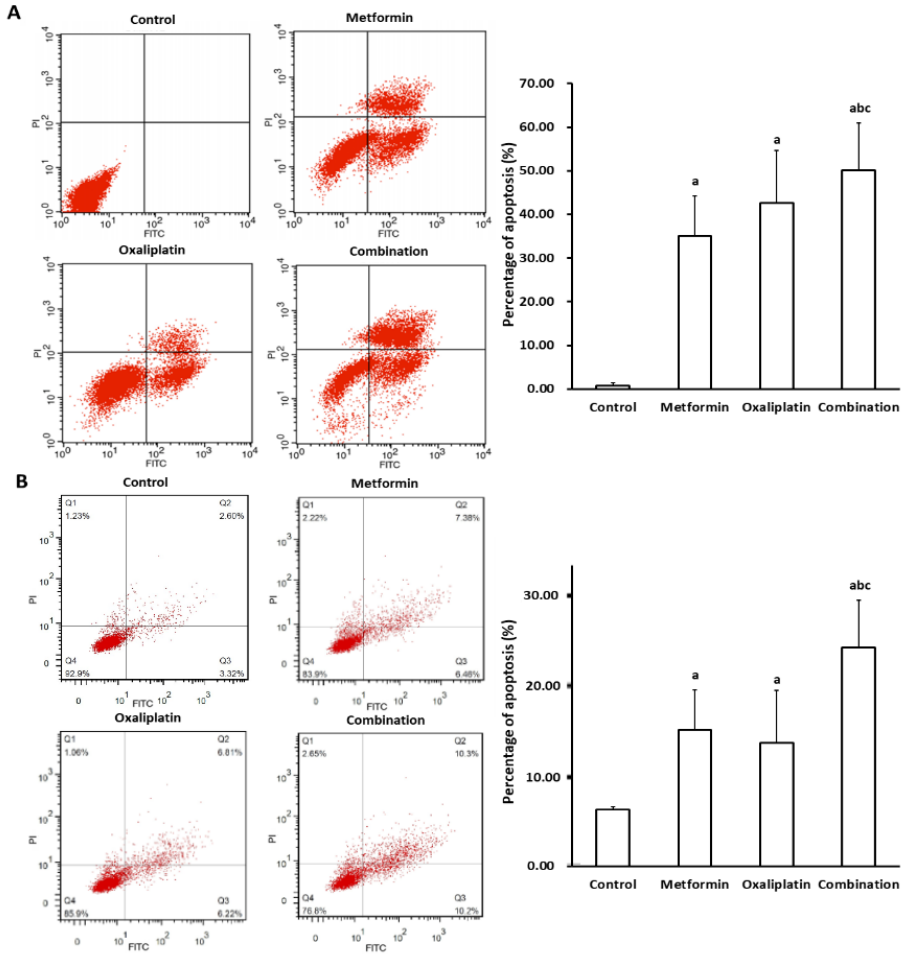

Figure 3. Metformin and oxaliplatin induce apoptosis in a synergistic manner.
All of the samples were exposed to IC_{50} concentration of the drugs for 48 hours. (**A**) apoptosis in SGC-7901, (**B**) apoptosis in SNU-16. Letters a, b, c in the graphs indicate statistical significance of the following comparisons: a. comparison to the control group, $P<0.05$; b. comparison to the metformin group, $P<0.05$; c. comparison to the oxaliplatin group, $P<0.05$.

drugs using flow cytometry. (Fig. 3). Both in SGC7901 and SNU-16 cells, the proportion of apoptotic cells was significantly increased after the treatment compared to the control (0.1 *vs* 0.35, for metformin 0.1 *vs* 0.4 for oxaliplatin, both $P<0.05$, in SGC-7901; 0.07 *vs* 0.15 for metformin, 0.07 *vs* 0.14 for oxaliplatin, both $P<0.05$, in SNU-16). This increase was the highest when the drugs were used in a combination (0.35 *vs* 0.5, 0.4 *vs* 0.5, both $P<0.05$, for SGC-7901; 0.15 *vs* 0.25, 0.14 *vs* 0.025, both $P<0.05$, for SNU-16).

Western blotting and qPCR analyses were performed to investigate the effects of drugs on Bax, Bcl-2, and caspase-3 (Fig. 4). Bcl-2 mRNA expression was significantly reduced upon the addition of metformin or oxaliplatin, compared to the control (1.1 *vs* 0.6 for metformin, 1.1 *vs* 0.7 for oxaliplatin, both $P<0.05$, in SGC-7901 cells; 0.07 *vs* 0.15 for metformin, 0.07 *vs* 0.14 for oxaliplatin, both $P<0.05$, in SNU-16 cells). The results were similar in the protein expression of Bcl-2 (0.6 *vs* 0.35 for metformin, 0.6 *vs* 0.35 for oxaliplatin, both $P<0.05$, in SGC-7901; 1.1 *vs* 0.45 for metformin, 1.1 *vs* 0.7 for oxaliplatin, both $P<0.05$, in SNU-16). The expression of Bax and caspase-3 was significantly increased compared to the control. These effects were more prominent where the drugs were used in combination.

DISCUSSION

Oxaliplatin is a platin analog widely used in gastrointestinal malignancies, but has been reported with moderate anti-tumor activity due to low accumulation in tumor tissues *in vivo*. The drug displays side effects when used alone (Zeng *et al.*, 2016). Major side effects of oxaliplatin include gastrointestinal toxicity, neurotoxicity, and thrombocytopenia (Erdem *et al.*, 2016). Reducing the side effects of therapy may improve the overall prognosis. The combination of drugs may contribute to achieving this goal (Florou *et al.*, 2013).

Metformin, a drug commonly used to treat type 2 diabetes, was also found to have anti-tumor activities (Kato *et al.*, 2012; Rego *et al.*, 2015). Studies showed that metformin could down-regulate the expression of G1 phase proteins, such as cyclin D1, CDK4, and CDK6, and reduce phosphorylation of Rb protein, resulting in G0/G1 phase arrest (Cantrell *et al.*, 2010; Kato *et al.*, 2012). Metformin could also reduce the phosphorylation of EGFR and IGF-1 receptors in gastric cancer cells *in vitro* and *in vivo* (Kato *et al.*, 2012). In addition, metformin has been associated with promoting apoptosis by increasing caspase-3 activation, but only at high concentrations (Cantrell *et al.*, 2010; Guimaraes *et al.*, 2016). Indeed, our

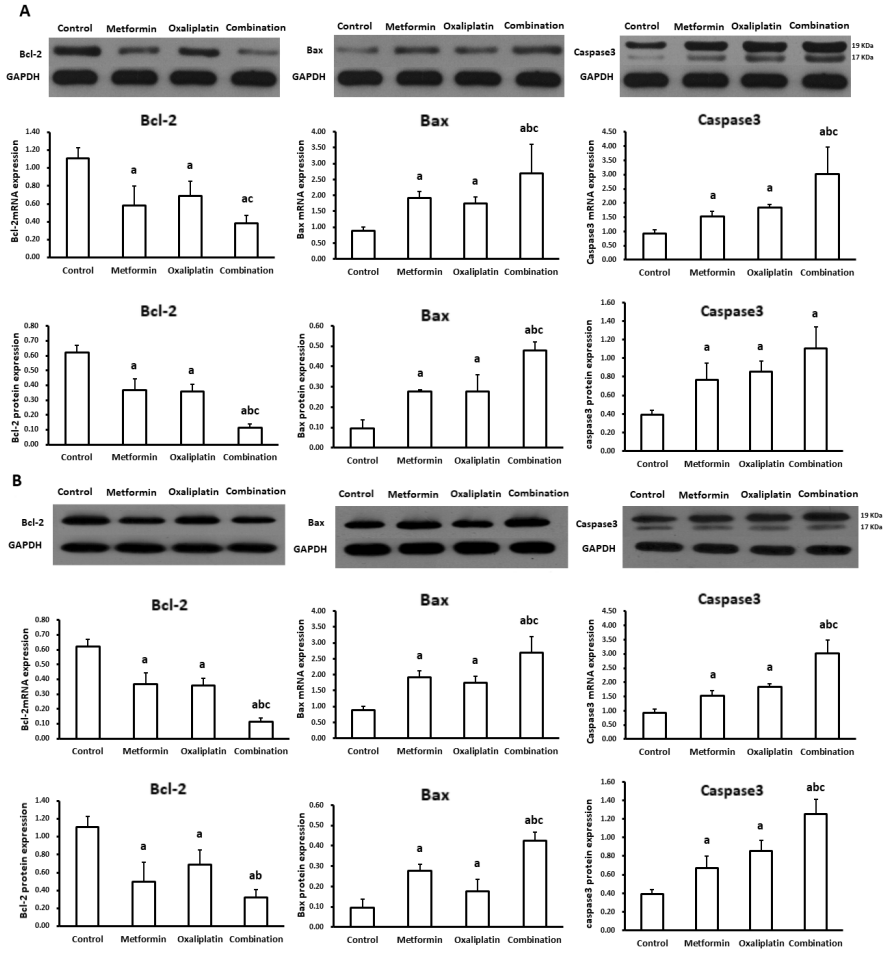

Figure 4. Metformin and oxaliplatin increase the expression level of apoptotic genes in a synergistic manner.
All of the samples were exposed to IC_{50} concentration of the drugs for 48 hours. (**A**) the expression level of Bcl-2, Bax and caspase-3 in SGC-7901 cells; (**B**) the expression level of Bcl-2, Bax and caspase 3 in SNU-16 cells. Letters a, b, c in the graphs indicate statistical significance of the following comparisons: a. comparison to the control group, $P<0.05$; b. comparison to the metformin group, $P<0.05$; c. comparison to the oxaliplatin group, $P<0.05$).

study also found that metformin had only a moderate effect on apoptosis compared to oxaliplatin. Furthermore, the treatment with metformin and oxaliplatin, separately or combined, could inhibit the proliferation of gastric cancer cells dose-dependently. Theoretically, the combined use of the two drugs may increase anti-tumor activity. The previous study by Liu and others (Liu *et al.*, 2020; Cantrell *et al.*, 2010; Guimaraes *et al.*, 2016) concluded that insulin-induced oxaliplatin resistance can be reversed by metformin-mediated AMPK activation in colon cancer patients with diabetes. Huang and others (Huang *et al.*, 2018; Cantrell, *et al.*, 2010; Guimaraes, *et al.*, 2016) observed synergistic cytotoxic effect and cell growth inhibition in DLD-1 cells treated with oxaliplatin combined with metformin. However, there was no study demonstrating anti-tumor activity of the combined use of the two drugs We hypothesize that metformin may increase the sensitivity of gastric cancer cells to oxaliplatin by inhibiting their proliferation. Combining these two drugs in gastric cancer treatment could allow for reducing the dose of oxaliplatin and thus reduce its side effects.

In this study, the results of RT-qPCR and Western blotting showed that both oxaliplatin and metformin, separately and in combination, reduced the expression of cyclin D1. Cyclin D1 is relevant to the abnormal proliferation and prognosis of tumor cells (Alao 2007). The Western blotting results also showed that the expression of anti-apoptotic protein Bcl-1 was reduced, and the pro-apoptotic protein Bax and caspase-3 were increased. The changes in Bcl-1, Bax and caspase-3 levels were especially prominent in the cells treated with metformin and oxaliplatin together. Moreover, the correlation between Bcl-1, Bax and caspase-3 levels and gastric cancer growth was shown in previous studies (Alao 2007). Overall, our results suggest that the metformin-oxaliplatin combination treatment could enhanceG0/G1 phase arrest through regulating cyclin D1 and the regulation of apoptosis.

Although we successfully demonstrated that the combination of metformin and oxaliplatin inhibited the proliferation and promoted apoptosis of gastric cancer cells, there are limitations to this study, as only *in vitro* experiments were performed. The specific molecular mechanism of synergistic effects of oxaliplatin and metformin need to be clarified with the further investigation involving *in vivo* study, and more evidence of the synergistic effects need to be examined under the clinical setting.

The hypothesis that combined use of the two drugs can reduce side effect cannot be proved by *in vitro* study. The future *in vivo* and clinical studies are needed to fully evaluate the clinical use of metformin in gastric cancer.

CONCLUSION

The combination of metformin and oxaliplatin may be a novel therapy for gastric cancer acting via inhibiting cell proliferation and inducing apoptosis.

Declarations

Ethics approval and consent to participate. The research protocol was approved by the Ethics Committee of the Wuhan No.4 Hospital.

Consent for publication. Not applicable.

Competing interests. All authors declare no conflicts of interest.

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