Article published online: 2024-09-30

The Chinese Herbal Formula Weichang'an Reduces the Proliferation of Human Gastric Cancer Cells via Mitochondrial Apoptosis

Weixia Chen[1,2] Yaofei Niu[3] Bin Chen[4] Aiguang Zhao[2]

[1] Department of Oncology, Henan Province Hospital of Traditional Chinese Medicine, Zhengzhou, Henan, China
[2] Department of Oncology, Longhua Hospital Shanghai University of Traditional Chinese Medicine, Shanghai, China
[3] Department of Infection, Henan Provincial People's Hospital; Zhengzhou University People's Hospital, Zhengzhou, Henan, China
[4] Department of Oncology, Putuo Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China

Address for correspondence Aiguang Zhao, PhD, Department of Oncology, Longhua Hospital Shanghai University of Traditional Chinese Medicine, 725 Wanping South Road, Shanghai 200032, China (e-mail: aiguangzhao@qq.com).

CMNP 2024;4:e117–e125.

Abstract

Objective The aim of the study was to investigate the effects of Chinese herbal formula Weichang'an (WCA) on the proliferation and mitochondria-mediated apoptosis of human gastric cancer cells.

Methods Cell Counting Kit-8 (CCK8) was used to evaluate the antiproliferative activity of WCA on MKN45 cells; Giemsa staining was used to investigate cell colony formation; flow cytometry was used to analyze cell cycle, apoptosis rate, and caspases activation; and Hoechst staining was used to analyze the morphology of cell nuclei. The mitochondrial membrane potential was analyzed by both flow cytometric measurements and fluorescence microscopy. Western blot was used to analyze the protein levels of pro-caspase-3, Bcl-2, Bax, and Bcl-X. MKN45 cells were subcutaneously injected into the right forelimb of 16 nude mice to establish the tumor xenograft model, and a total of 12 nude mouse tumor xenograft models were successfully created. The nude mice were divided into the control group and the WCA-treated group. The two groups received normal saline and WCA treatment at 35.49 g/kg by a single daily oral gavage for 21 days. The body weight and tumor size of the nude mice were measured twice a week. The ultrastructural changes of subcutaneous tumors were observed by a transmission electron microscope.

Results WCA can suppress cell proliferation and colony formation. It can also induce changes in the mitochondrial membrane potential and increase the activities of caspases-3, caspases-8, and caspases-9 in MKN45 cells. WCA induced S-phase arrest and apoptosis in MKN45 cells, and decreased the expression levels of antiapoptotic proteins Bcl-2 and Bcl-X in MKN45 cells, while increasing the expression levels of the proapoptotic proteins Bax and pro-caspase-3 compared with the control group. WCA inhibited the growth of a xenografted MKN45 tumor in nude mice, and the protein levels of Bax and pro-caspase-3 were significantly increased, while Bcl-X and Bcl-2 were

Keywords
- gastric cancer
- Chinese herbal formula
- Weichang'an
- mitochondrial apoptosis

received
May 11, 2024
accepted after revision
July 22, 2024

DOI https://doi.org/10.1055/s-0044-1790538.
ISSN 2096-918X.

Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany

reduced compared with the control group. The differences are statistically significant ($p < 0.05$).

Conclusion WCA could suppress the proliferation of gastric cancer cells via mitochondrial apoptosis.

Introduction

Gastric cancer (GC) is the fourth most common malignancy of the digestive tract worldwide, affecting more than 1 million people and causing about 800,000 deaths annually.[1] To date, surgical resection with the assistance of radiotherapy and chemotherapy is the most effective treatment.[2] However, 60% of patients with GC have local recurrence or distant metastasis after radical resection, and the median overall survival (OS) of GC patients is only 7 to 13 months after palliative chemotherapy. Although some expensive targeted molecular therapeutic drugs have been clinically employed to treat advanced GC, their efficacy is still limited and unsatisfactory.[3–5]

Chinese herbal medicine has unique medical advantages and is the most important complementary and alternative therapy in China. Some Chinese herbal formulae have been used in the treatment of cancer and have been found to improve the prognosis after surgical resection. A previous study showed that as an independent prognostic factor, Weichang'an (WCA) can improve the OS of GC patients after radical gastrectomy and chemotherapy from 14 to 20 months.[6] This formula also exhibited a positive effect on the prognosis of GC patients with peritoneal metastasis.[7] It also suppressed colorectal cancer in a mouse model.[8] Further studies indicated that WCA showed similar tumor inhibitory activity to 5-fluorouracil and regulated the expression of cancer-related genes in an animal model using a human GC cell line.[9] These results indicate that the formula WCA is a valuable anticancer agent in complementary medicine. However, even though WCA has been applied clinically in the treatment of GC for more than 30 years, the molecular mechanisms of its anticancer activity are still unclear.

Inhibition of tumor cell proliferation and induction of apoptosis are common methods in cancer therapy. Generally, apoptosis is an orderly genetically controlled death process that serves an organism to achieve better adaptation to the living environment.[10] However, aberrant regulation of apoptosis plays an important role in the occurrence and development of cancer. Among the elucidated apoptotic pathways, including the death receptor–mediated, mitochondria-mediated, and endoplasmic reticulum–mediated routes, the endogenous mitochondrial pathway is dominant.[11] The Bcl-2 family and the caspases family of proteins, which include both proapoptotic and antiapoptotic factors, are important molecules in the mitochondrial apoptotic pathway.[12,13]

Our previous gene microarray analysis showed that WCA regulated the expression of *Stat3*, *Bcl-2*, *PTBP3*, and other genes in GC cell lines, which may affect the proliferation and apoptosis of tumor cells and play key roles in inhibiting tumor growth.[13,14] Based on these findings, the effects of WCA on the proliferation and mitochondrial apoptosis of the MKN45 GC cell line were analyzed in vivo and in vitro.

Materials

Experimental Animals and Cells

Male BALB/C mice aged 6 to 7 weeks, weighing 18 to 20 g, were purchased from the Cancer Institute Animal Laboratory [Shanghai, China; certification NO. SCXK (Shanghai) 2002-0001]. The mice were housed and fed strictly abiding the specific pathogen-free (SPF) standard. The mice were euthanatize via CO_2 exposure followed by cervical dislocation. All the animal experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the laboratory animal ethics committee of Shanghai University of Traditional Chinese Medicine (Approval number: LHERAW-20003).

The human GC cell line MKN45 was donated by the school of pharmacy of East China University of Science and Technology (Shanghai, China) and cultivated in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), streptomycin (100 U/mL), and penicillin (100 U/mL) at 37° C in a humidified atmosphere comprising 5% CO_2.

Drugs and Reagents

The following drugs and reagents were used in the study: RPMI-1640 medium (Catalog No. 11879020, Gibco, United States); CCK8, Ultrasensitive ECL chemiluminescence detection kit (Catalog Nos. 40203ES60, 36208ES60, Yisheng, China); PI/RNase staining buffer, BD Annexin V: FITC apoptosis detection kit I, JC-1 (Catalog Nos. BDB550825, BDB556547, BDB551302, BD Biosciences, United States); caspases-3 activity kit, caspases-8 activity kit, caspases-9 activity kit (Catalog Nos. K183-100, K188-100, K189-100, BioVision, United States); pro-caspase-3 recombinant rabbit monoclonal antibody (Catalog No. ET16023-26, HuaAn Biotechnology, China); human Bcl-2 polyclonal antibody, Bax polyclonal antibodies, β-actin monoclonal antibody, HRP-conjugated AffiniPure goat anti-mouse IgG (H + L), HRP-conjugated AffiniPure goat anti-rat IgG (H + L; Catalog Nos. 12789-1-AP, 50599-2-Ig, 66009-1-Ig, SA00001-1, SA00001-15, Proteintech, China); and Anti-Bcl-X antibody (Catalog No. ab32370, Abcam, United Kingdom).

Experimental Instruments

The following experimental instruments were used in the study: flow cytometry (FACSVerse, BD, United States); confocal laser scanning microscope (TCS-SP8, Leica, Germany);

and transmission electron microscope (TEM; CM-120, Philips, the Netherlands).

Methods

Preparation of WCA

All the herbs were provided by the pharmacy department of traditional Chinese medicine, Longhua Hospital Shanghai University of Traditional Chinese Medicine. Each herb was identified by Mrs. YanXi (pharmacist of traditional Chinese medicine in the pharmacy department of Longhua Hospital). For decoction preparation, *Radix pseudostellariae* (12 g), *Atractylodes macrocephala* (12 g), *Wolfiporia cocos* (15 g), *Rhizoma Pinelliae* (9 g), *Pericarpium Citri Reticulatae* (9 g), *Sargentodoxa cuneata* (30 g), *Vitis quinquangularis* Rehd (30 g), *Ostrea gigas* Thunberg (30 g), *Gekko japonicus* Dumeril and Bibron (3 g), *Prunella vulgaris* (9 g), and *Flos Pruni mume* (6 g) were decocted twice with eightfold volume of distilled water for 1 h. The decoctions were collected, filtered, combined, lyophilized, and then stored at 4°C. The WCA powder was dissolved in phosphate buffered saline (PBS) and filtrated using a 0.22μm membrane before application. The quality of the prepared WCA decoction was determined as described previously, using high-performance liquid chromatography (HPLC) using a Lichrospher C18 column with linear gradient elution at a flow rate of 1 mL/min at 25°C.[9] The prepared freeze-dried WCA powder was dissolved in RPMI-1640 medium to a final concentration of 0.2 kg/L as reported previously.[15]

Cell Viability and Colony Formation Assay

The antiproliferative activity of WCA in MKN45 cells was evaluated using CCK8. MKN45 cells were seeded in a 96-well plate at a density of 8,000 cells/well and cultured for 24 h. MKN45 cells were treated with different concentrations (0, 0.25, 0.5, 1, and 2 kg/L) of WCA to examine the dose- and time-dependent effects. Cell viability was calculated using the absorbance at 450 nm according to the kit's manual.

For the colony formation assay, 1×10^3 MKN45 cells were treated with different concentrations (0, 0.25, and 0.5 kg/L) of WCA for 14 days under standard culture conditions. The number of colonies with more than 50 cells was counted after staining with Giemsa.

Cell Cycle Assay

After 48 h of treatment with the selected doses of WCA (0.5 and 1 kg/L), the obtained MKN45 cell colonies were fixed with 500μL ice-cold 70% ethanol overnight at 4°C. The cells were subsequently washed twice and stained with 0.5mL staining buffer containing PI/RNase for 15 min at room temperature in the dark and analyzed by flow cytometry.

Apoptosis Assay and Nuclear Morphology Analysis

The apoptotic rates were measured using the BD Annexin V: FITC apoptosis detection kit I via flow cytometry. MKN45 cells treated with different concentrations of WCA (0.5 and 1 kg/L) were collected and incubated with 5μL FITC annexin V and 5 μL PI according to the kit's manual.

For nuclear morphology analysis, cells were trypsinized and then incubated in six-well plates (1×10^4 cells/well) with a cover slip for 24 h. MKN45 cells were then treated with different concentrations of WCA (0.5 and 1 kg/L). After 48 h, the cells were fixed with 4% paraformaldehyde, washed twice with PBS buffer, and stained with Hoechst 33258. Finally, the slides were mounted and images were obtained using a confocal laser scanning microscope.

Mitochondrial Membrane Potential Assay

After 48 h of treatment with different concentrations of WCA (0.5 and 1 kg/L), the MKN45 cells were incubated with 0.5 mL of JC-1 working solution at 37°C for 20 min. The mitochondrial membrane potential was then analyzed by both flow cytometric and fluorescence microscopy according to a previous report.[16]

Caspases Activation Assay

The activity of caspases-3, caspases-8, and caspases-9 were measured using the caspases-3, caspases-8, and caspases-9 activity kit. After 48 h of treatment with different concentrations of WCA (0.5 and 1 kg/L), MKN45 cells were collected and incubated with 1μL FITC-IETD-FMK (caspases-3), 1μL FITC-IETD-FMK (caspases-8), and 1μL FITC-IETD-FMK (caspases-9), respectively, in assay buffer at 37°C for 1 h. The cells were then suspended in 300μL of wash buffer for flow cytometric analysis.

Western Blot Analysis

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at a constant voltage of 120 V for 90 min and then transferred onto a polyvinylidene fluoride (PVDF) membrane as described previously.[17] The obtained PVDF membranes were blocked and incubated with antibodies against pro-caspase-3 (1:1,000), Bcl-2 (1:2,000), Bax (1:2,000), Bcl-X (1:1,000), and β-actin (1:10,000) at 4°C overnight. After that, the membranes were incubated with the secondary antibody (1:2,000) and developed using chemiluminescence.

Nude Mouse Xenograft Tumor Model

MKN45 cells (2×10^6 cells per animal) were subcutaneously injected into the right forelimb of 16 nude mice to establish the tumor xenograft model. When the tumor volume of MKN45 cells reached about 100 mm^3, the nude mouse tumor xenograft model was successfully established. In this study, a total of 12 nude mouse tumor xenograft models were successfully created. The nude mice were divided into the control group and the WCA-treated group. The two groups received normal saline and WCA treatment at 35.49 g/kg by a single daily oral gavage for 21 days. The body weight and tumor size of the nude mice were measured twice a week. Tumor volumes were determined as follows: tumor volume (mm^3) = 0.52 × length (mm) × width (mm^2).

Transmission Electron Microscope Analysis

The ultrastructural changes of subcutaneous tumors derived from cultured gastric carcinoma cells after WCA intervention

were observed by TEM. The tumor tissues were fixed with 0.25 g/L glutaraldehyde and 0.1 g/L osmium acid, dehydrated in a series of ethanol/acetone solutions, and embedded in Epon. Ultrathin sections were examined under a tacna1 TEM.

Statistical Analysis

All data were processed using SPSS 20.0 statistical software (IBM Corp., United States) and presented as means ± standard deviations. Single factor analysis of variance (one-way ANOVA) was used to analyze the experimental results. Normality was investigated using the Van der Waerden test. The homogeneity of the variance was analyzed using Levene's test. Multiple comparisons were performed using the least significant difference (*LSD*) and Student–Newman–Keuls *(SNK)* tests. The data obtained from animal experiments were analyzed by independent *t*-tests after the normality test. Differences with $p < 0.05$ were considered statistically significant.

Results

WCA Potently Inhibited GC Growth in Vivo

To determine whether WCA could inhibit GC in vivo, we established an MKN45 cell–induced xenograft model in nude mice. Even though the body weight of the nude mice was not significantly affected, the average weights and volumes of MKN45 xenograft tumors from the WCA-treated mice were significantly reduced compared with the control group ($p < 0.01$), suggesting that the growth of the MKN45 cell tumors was significantly inhibited by the treatment with WCA (►**Fig. 1A**). The fact that WCA induced apoptosis and had an antiproliferative effect in vivo was further observed by transmission electron microscopy analysis, as shown in ►**Fig. 1B**. Cancer cells in the control group showed active proliferation and double nucleoli, while WCA treatment decreased the nuclear/cytoplasmic ratio of tumor cells, tumor cells swelling, and chromatin condensation. At the same time, apoptotic bodies and some necrotic tumor cells with irregular distribution, unclear boundaries, and swelling of cytoplasm were also observed. Additionally, western blot analysis of tumor tissues suggested that the protein expression levels of Bax and pro-caspase-3 were significantly upregulated in the WCA group ($p < 0.01$), while those of Bcl-2 and Bcl-X were significantly repressed compared with the control group ($p < 0.05$; ►**Fig. 1C**).

WCA Suppressed the Proliferation and Colony Formation of GC Cells

The antiproliferative effects of WCA on MKN45 cells were evaluated using the CCK8 assay. As shown in ►**Fig. 2A**, the proliferation of MKN45 cells was significantly inhibited by the treatment with different concentrations of WCA, with a half-maximal inhibitory concentration of 1.014 kg/L at 48 h. To further observe the long-term effect of WCA on the proliferation of GC cells, MKN45 cells were treated with 0, 0.25, and 0.5 kg/L WCA and cultivated for 14 days. The colony formation of MKN45 cells was reduced by about 60% by the treatment with 0.25 kg/L WCA ($p < 0.001$), and more than 90% colony formation of GC cells was inhibited by a high dose of WCA (0.5 kg/L) compared with the control group ($p < 0.001$; ►**Fig. 2B**). These results suggest that WCA indeed has an antiproliferative effect on GC cells in vitro at high doses.

WCA Induced S-Phase Arrest and Apoptosis in GC Cells

To further investigate the mechanism by which WCA inhibited the growth of GC cells, the cell cycle distribution and apoptosis of MKN45 cells treated with 0.5 and 1 kg/L WCA were analyzed by flow cytometry. As shown in ►**Fig. 3A**, treatment with 0.5 and 1 kg/L of WCA resulted in a significant increase of MKN45 cells in the S phase compared with the control group ($p < 0.01$), indicating that the GC cells underwent S-phase blockage. Flow cytometry showed the proportion of apoptosis of GC cells increased with the increase in concentration of WCA intervention compared with the control group ($p < 0.01$; ►**Fig. 3B**). After 48 h of treatment with WCA on MKN45, Hoechst 33258 staining revealed that the nuclei of the control group were normal and blue in color, while the WCA intervention group cells showed the chromatin of condensation, with nuclei that were dense, thick, or small, and the formation of apoptotic bodies (►**Fig. 3C**). This finding supports the induction of cancer cell apoptosis by WCA in vitro.

WCA Decreased Mitochondrial Membrane Potential in GC Cells

The decrease in mitochondrial membrane potential is an important event in the early stages of apoptosis. The change in mitochondrial membrane potential after the GC cells were treated with WCA was also analyzed. Compared with the control group, the ratio of green to red fluorescence intensity was increased in the WCA-treated cells at all studied doses (0.5 and 1 kg/L) in a dose-dependent manner ($p < 0.01$; ►**Fig. 4**). These results indicate that WCA can reduce the mitochondrial membrane potential and thereby induce mitochondrial dysfunction.

WCA Regulated the Activity of Caspases and Expression of Proteins Associated with Mitochondrial Apoptosis in GC Cells

Apoptosis is mediated by activation of the specific proteases named caspases. Caspases-3, also called death protease, is the most important terminal shearing enzyme in apoptosis. Caspases-8 and caspases-9 are upstream proteins of apoptotic signaling pathways that can self-activate and initiate downstream caspases-3, acting on specific substrates, altering cell morphology, and leading to apoptosis.[17] Activity analysis showed that the WCA treatment significantly increased the activities of caspases-3, caspases-8, and caspases-9 in the MKN45 cells compared with the control group ($p < 0.01$; ►**Fig. 5A–C**). To further elucidate the details of how WCA induced GC apoptosis, the expression levels of apoptosis-associated proteins were analyzed by western blot. After the WCA treatment, the expression level of pro-caspase-3 was increased compared with the control group, demonstrating the induction of apoptosis by WCA in GC cells.

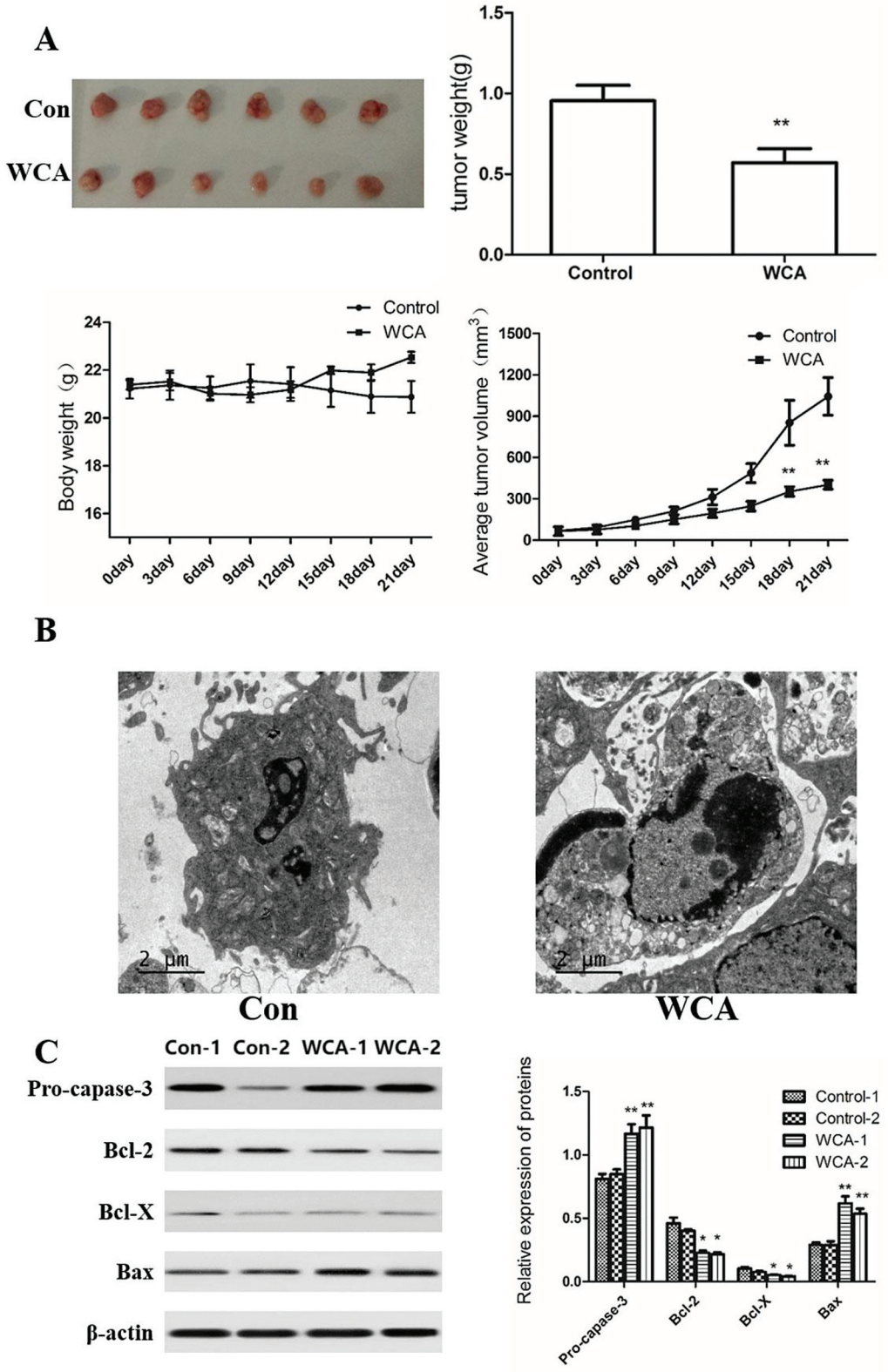

Fig. 1 WCA inhibited tumor growth in the MKN45 xenograft model. (**A**) The effect of WCA treatment on the mass and tumor mass and volume of MKN45 xenograft model mice ($n = 6$). (**B**) Transmission electron microscopy analysis of cancer cell morphology ($n = 6$). (**C**) WCA regulates the expression of proteins associated with mitochondria-mediated apoptosis ($n = 3$). Compared with the control group, $^{*}p < 0.05$ and $^{**}p < 0.01$. "Con" represents the control group.

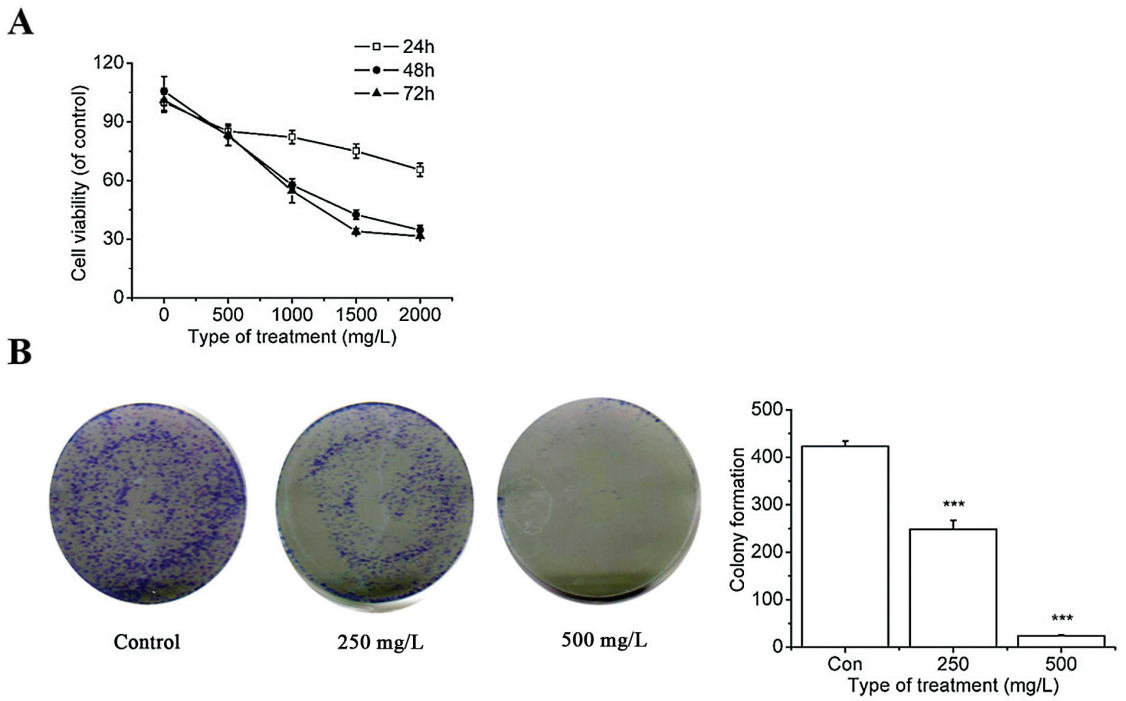

Fig. 2 WCA significantly inhibited cell proliferation in vitro. (**A**) The viability of MKN45 cells was measured using the CCK8 assay. (**B**) Clonogenicity was measured using the colony formation assay. Compared with the control group, $^{***}p < 0.001$. "Con" represents the control group.

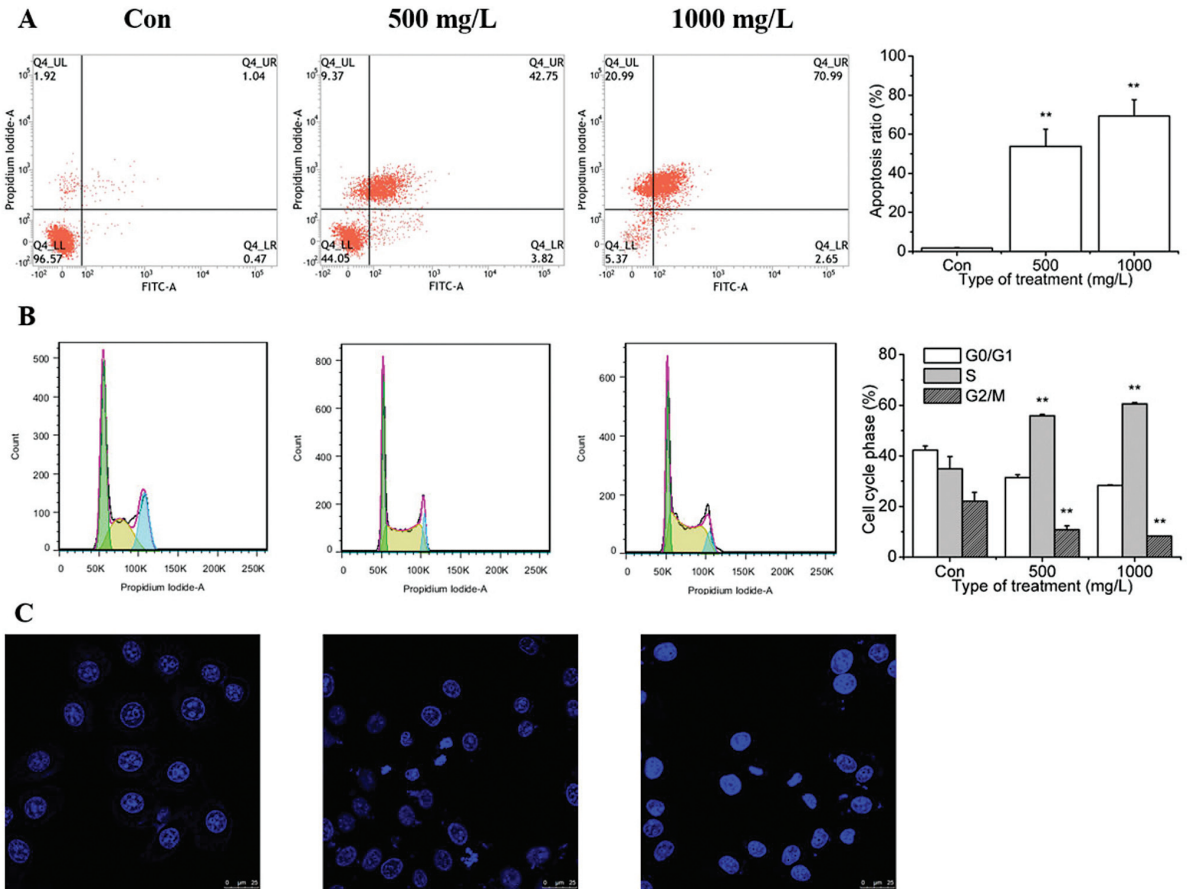

Fig. 3 WCA induced S-phase arrest and apoptosis in GC cells in vitro. (**A**) WCA can induce apoptosis in GC cells. (**B**) WCA led to a significant cell cycle arrest at the S phase. (**C**) Hoechst 33258 staining of the nuclei of MKN45 cells treated with WCA for 48 h. Compared with the control group, $^{**}p < 0.01$. "Con" represents the control group.

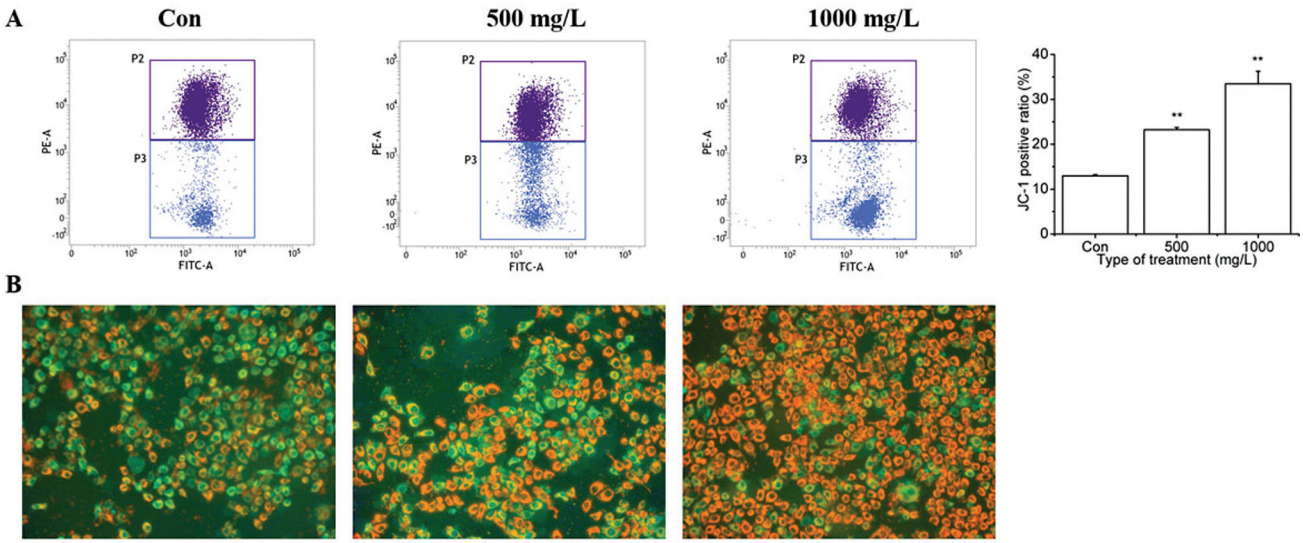

Fig. 4 WCA decreased the mitochondrial membrane potential in MKN45 cells. (**A**) Flow cytometry evaluation of changes in mitochondrial membrane potential of GC cells. (**B**) Fluorescence microscopy analysis of changes in mitochondrial membrane potential of GC cells. Compared with the control group, $^{**}p < 0.01$. "Con" represents the control group.

Interestingly, we found that WCA decreased the expression levels of antiapoptotic proteins Bcl-2 and Bcl-X while increasing the expression levels of the proapoptotic proteins Bax and pro-caspase-3 compared with the control group ($p < 0.01$; ►**Fig. 5D**). Considering the close association between these proteins and mitochondria, we speculated that WCA induces apoptosis via mitochondrial disruption by changing the expression levels of the apoptosis-associated proteins Bcl-2 and Bax.

Discussion

GC is one of the most common malignancies of the digestive tract, representing a highly heterogeneous group of malignant tumors.[18] Although surgical resection is the first choice of treatment, about 50% of patients with advanced GC have tumors that cannot be resected. Therefore, the combined application of chemotherapy and traditional Chinese medicine has garnered much attention in the treatment of GC. It is also generally well tolerated and no herb-related adverse events were observed in our previous clinical studies.[6,7] In this study, the effects of WCA on the proliferation and apoptosis of human MKN45 GC cells were analyzed in further detail.

Inhibition of proliferation and induction of apoptosis have been accepted as effective strategies in cancer treatment. Our results indicated that WCA significantly inhibited the proliferation of MKN45 GC cells in a dose-dependent manner. Altered cell cycle regulation is an important feature of cancer cells and arresting the cell cycle at certain checkpoints may result in apoptosis. Therefore, blocking the S to G1/M phase progression may contribute to the antiproliferative activity of WCA. In addition to arresting the cell cycle at the S phase, the exposure of MKN45 GC cells to WCA also induced significant apoptosis. Mitochondria are the core organelles of apoptosis, and mitochondrial membrane potential is critical to their function.[19] A decreased mitochondrial membrane potential will increase the membrane permeability and then activate caspases-9, leading to activation of the death effector caspases-3 and the initiation of apoptosis.[20] Our study revealed a dose-dependent depolarization of the mitochondrial membrane potential and an increase in mitochondrial membrane permeability after treatment with WCA. These results indicate that the endogenous mitochondrial pathway is responsible for WCA-induced apoptosis.

Generally, the mitochondrial pathway is regulated by members of the Bcl-2 family, such as Bcl-2, Bcl-XL, Bax, and caspases family proteins. The WCA treatment led to an increase in the Bax/Bcl-2 ratio, which can stimulate the release of cytochrome c from the mitochondria into the cytosol, leading to the activation of caspases-3. Measurements of the caspases cascade further revealed that WCA significantly increased the activity of caspases-3, caspases-8, and caspases-9 in MKN45 cells. These results suggest that WCA can activate the mitochondrial apoptotic signaling pathway and the caspases cascade in vitro, leading to apoptosis of human GC cells.

Finally, we established a nude mouse xenograft model of GC to explore the effect of WCA on solid tumors and validate the mechanism. WCA significantly inhibited the proliferation of tumor cells and decreased the tumor weight of the mice. Commonly, inhibition of tumor proliferation in vivo has been considered to be a consequence of multiple factors, including cell cycle arrest, apoptosis, and necrosis.[21] The tumor cells from mice treated with WCA also showed typical hallmarks of apoptosis. In addition, WCA treatment downregulated the expression of Bcl-2 and Bcl-X and upregulated that of Bax and pro-caspase-3. These results indicate that WCA may have an effect on mitochondrial function in human GC cells and may activate the mitochondrial apoptotic pathway in vivo. In addition to this direct inhibition of tumor proliferation by

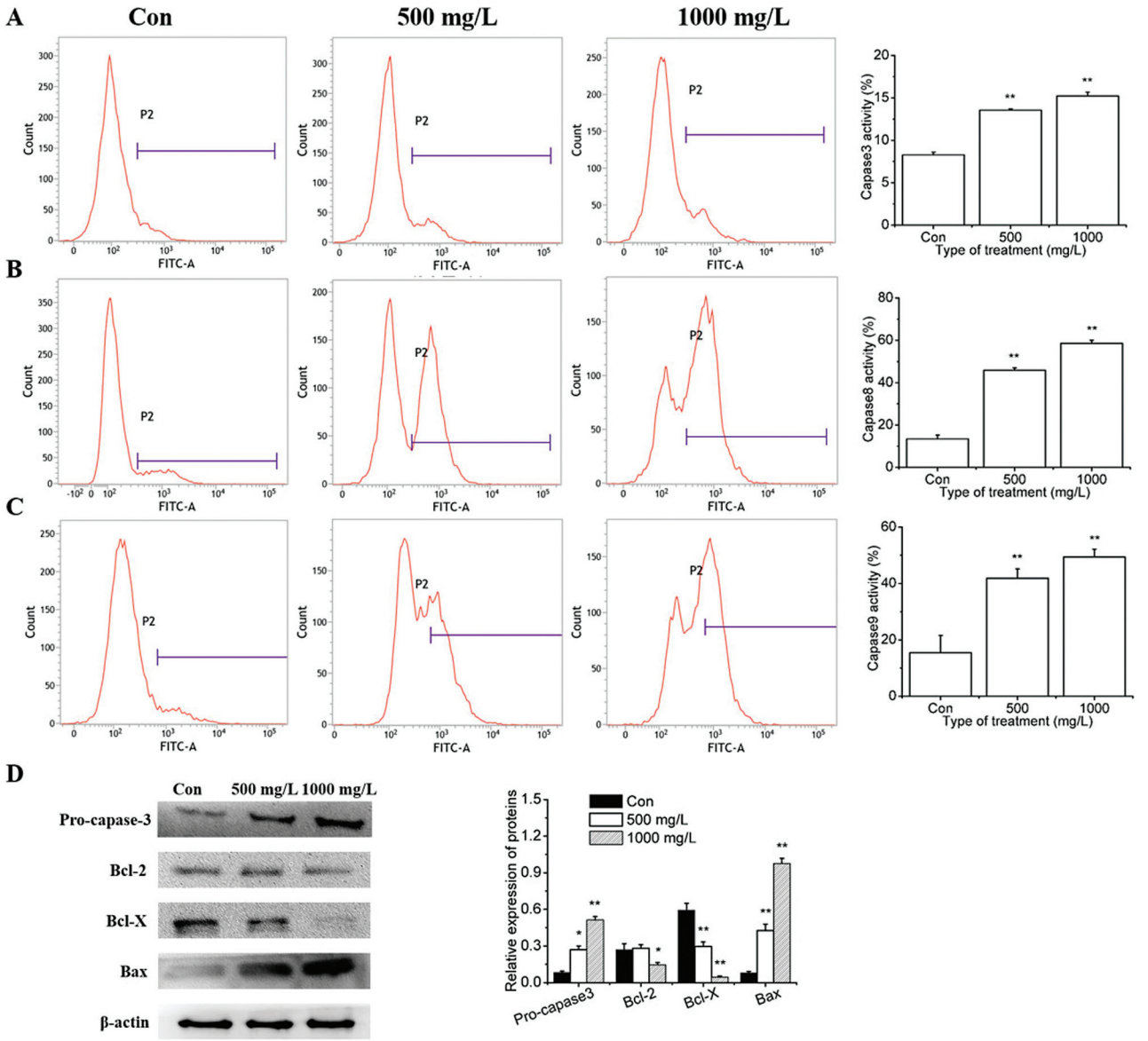

Fig. 5 WCA regulates caspases activity and the expression of proteins related to mitochondria-mediated apoptosis. (**A–C**) The effect of WCA on inducing caspases activity. **(D)** The effect of WCA on the expression of mitochondrial related apoptotic proteins. Compared with the control group, $^{*}p < 0.05$ and $^{**}p < 0.01$. "Con" represents the control group.

WCA in the mouse model, the drug-induced activation of the immune system may also contribute to the anticancer function of WCA. This phenomenon has been observed in a study of a photocarcinogenesis mouse model in which chemical peeling reduced tumor formation in both the treated and nontreated areas.[22] Therefore, the antitumor function of WCA in the mouse model may rely on these multiple biological functions.

Conclusion

WCA can inhibit the proliferation of human GC cells, arrest the cell cycle at the S phase, and induce apoptosis through the caspases-dependent mitochondrial pathway. These results may be helpful for further application and better understanding of the potential mechanism of the anticancer activity of WCA.

Authors' Contribution

Weixia Chen: contributed to conceptualization, methodology, and writing the original draft of the manuscript. **Bin Chen** and **Aiguang Zhao**: contributed to the investigation, funding acquisition, and writing, review, and editing of the manuscript. **Yaofei Niu** contributed to supervision of the study and data curation.

Funding

This study was funded by the Three-Year Plan of Action for the Development of Traditional Chinese Medicine in Shanghai (ZY3-CCCX-3-2003), State Administration of Traditional Chinese Medicine (TCM) Clinical Research Base of China (JDZX2015068), the Key and Promotion Special Topics of Henan (202102310164), and Henan

Province Traditional Chinese Medicine Scientific Research Special Project (2022ZY2031).

Conflict of Interest

The authors declare no conflict of interest.

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