

Basic Study

Thymoquinone inhibits proliferation in gastric cancer *via* the STAT3 pathway *in vivo* and *in vitro*

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Abstract

AIM: To elucidate the mechanism of thymoquinone (TQ)-induced apoptosis in human gastric cancer cells *in vitro* and *in vivo*.

METHODS: HGC27, BGC823, and SGC7901 cells were cultured *in vitro* and treated with TQ (0, 10, 25, 50, 75, 100, 125 $\mu\text{mol/L}$) for 12 h, 24 h, and 36 h, and then the proliferation inhibitory rates were detected by methylthiazole tetrazolium assay. Apoptosis was observed after Hoechst staining. The protein expressions of signal transducer and activator of transcription (STAT)3, p-STAT3, STAT5, p-STAT5, phospho-janus-activated kinase 2 (JAK2), JAK2, p-Src, Src, glyceraldehyde-3-phosphate dehydrogenase, lamin-A, survivin, Cyclin D, Bcl-2, Bax, peroxisome proliferator activated receptor, and caspase-3,7,9 were detected by western blot. Cell cycle and apoptosis were

determined with flow cytometry. TQ induced dose-dependent apoptotic cell death in HGC27 cells was measured by Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) analysis and Hoechst 33258.

RESULTS: TQ inhibited the phosphorylation of STAT3 but not STAT5. TQ-induced downregulation of STAT3 activation was associated with a reduction in JAK2 and c-Src activity. TQ also downregulated the expression of STAT3-regulated genes, such as Bcl-2, cyclin D, survivin, and vascular endothelial growth factor, and activated caspase-3,7,9. Consistent with the *in vitro* results, TQ was significantly effective as an antitumor agent in a xenograft tumor mouse model.

CONCLUSION: This study provides strong evidence that downregulation of the STAT3 signaling pathway mediates TQ-induced apoptosis in gastric cancer.

Key words: Thymoquinone; Gastric cancer; STAT3; Proliferation; Apoptosis

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Core tip: Thymoquinone (TQ) has been demonstrated to exert biological activity in gastric cancer. However, the specific mechanism of TQ in gastric cancer has not been examined. In order to elucidate the mechanism of TQ-induced apoptosis in human gastric cancer cells, we investigated the effects of TQ on signal transducer and activator of transcription (STAT)3 and its related pathway *in vitro* and *in vivo*.

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INTRODUCTION

Based on the Global Burden of Cancer Study (GLOBOCAN) estimates, about 14.1 million new cancer cases and 8.2 million deaths occurred in 2012 worldwide^[1]. Among them, gastric cancer ranks fourth and third for incidence rate and mortality rate, respectively, in male patients and ranks fifth in female patients^[1]. Chemotherapy is commonly used in the treatment of gastric cancer. Commonly used chemotherapy drugs are 5-fluorouracil (5-FU), cisplatin, and doxorubicin, but they are limited by toxic side effects, the development of resistance, and other defects^[2]. Therefore, it is important to search for low toxicity and efficient anti-cancer drugs that inhibit gastric cancer development.

Thymoquinone (TQ, also called 2-isopropyl-5-

methyl-1,4-benzo-quinone, C₁₀H₁₂O₂) is the active constituent of black cumin (*Nigella sativa*) seed oil (Figure 1A) and was first extracted by EI-Dakhkhany^[3]. TQ has been shown to suppress proliferation and induce apoptosis and drug resistance in various tumor cells, including colorectal carcinoma, pancreatic carcinoma, breast adenocarcinoma, prostate cancer, *etc.*^[4-9]. A phase I study reported that in adult patients with solid tumors or hematological malignancies who were treated with TQ, there were no significant systemic toxicities^[10]. It was also reported that the human body could tolerate a dose of 75 to 2600 TQ mg/d. Because of its high efficiency, low toxicity, and natural features in cancer prevention and treatment, TQ has gradually attracted the attention of scholars worldwide, but studies on tumors of the digestive system, especially gastric cancer, are rare.

Signal transducer and activator of transcription (STAT) has been shown to be a fundamental factor in tumor cell survival and proliferation^[11]. STAT3, a member of the STAT protein family, is a critical molecule of the janus-activated kinase (JAK)/STAT signaling pathway through many signals, such as interleukin (IL)-6, tumor necrosis factor (TNF)- α , and vascular endothelial growth factor (VEGF). STAT3 can be activated by a variety of ligands related to these signals^[12]. It was reported in a variety of human malignancies, such as gastric, colon, breast, and lung, that abnormal expression and constitutive activation of STAT3 are involved^[12,13]. Once activated, STAT3 would continue to promote phosphorylation-induced homodimerization, leading to nuclear translocation, DNA binding, and subsequent gene transcription (*e.g.*, Bcl-xL, Bcl-2, survivin, cyclin D, Mcl-1). In addition, STAT3 could also control VEGF expression, which is necessary for angiogenesis and the maintenance of tumor vasculature^[14]. STAT3 has been implicated in the inhibition of immune responses to tumor growth by blocking expression of pro-inflammatory factors. A previous study showed that TQ could induce apoptosis and augment 5-FU-induced apoptosis in gastric cancer cells^[15]. However, specific mechanisms underlying the effects of TQ in gastric cancer have not been examined.

Based on preliminary studies, we explored the specific mechanisms of TQ-induced regulation of proliferation and apoptosis in gastric cancer cells.

MATERIALS AND METHODS

Cell culture and reagents

Three human gastric cancer cells (HGC27, BGC823, and SGC7901) were obtained from Key Laboratory of Hubei Province for Digestive System Disease (Wuhan University). The cells were maintained in continuous exponential growth in Dulbecco's Modified Eagle's Medium (DMEM)/F12 medium supplemented with heat-inactivated 10% fetal bovine serum (FBS) and 1% antibiotics (100 IU penicillin and 100 μ g/mL

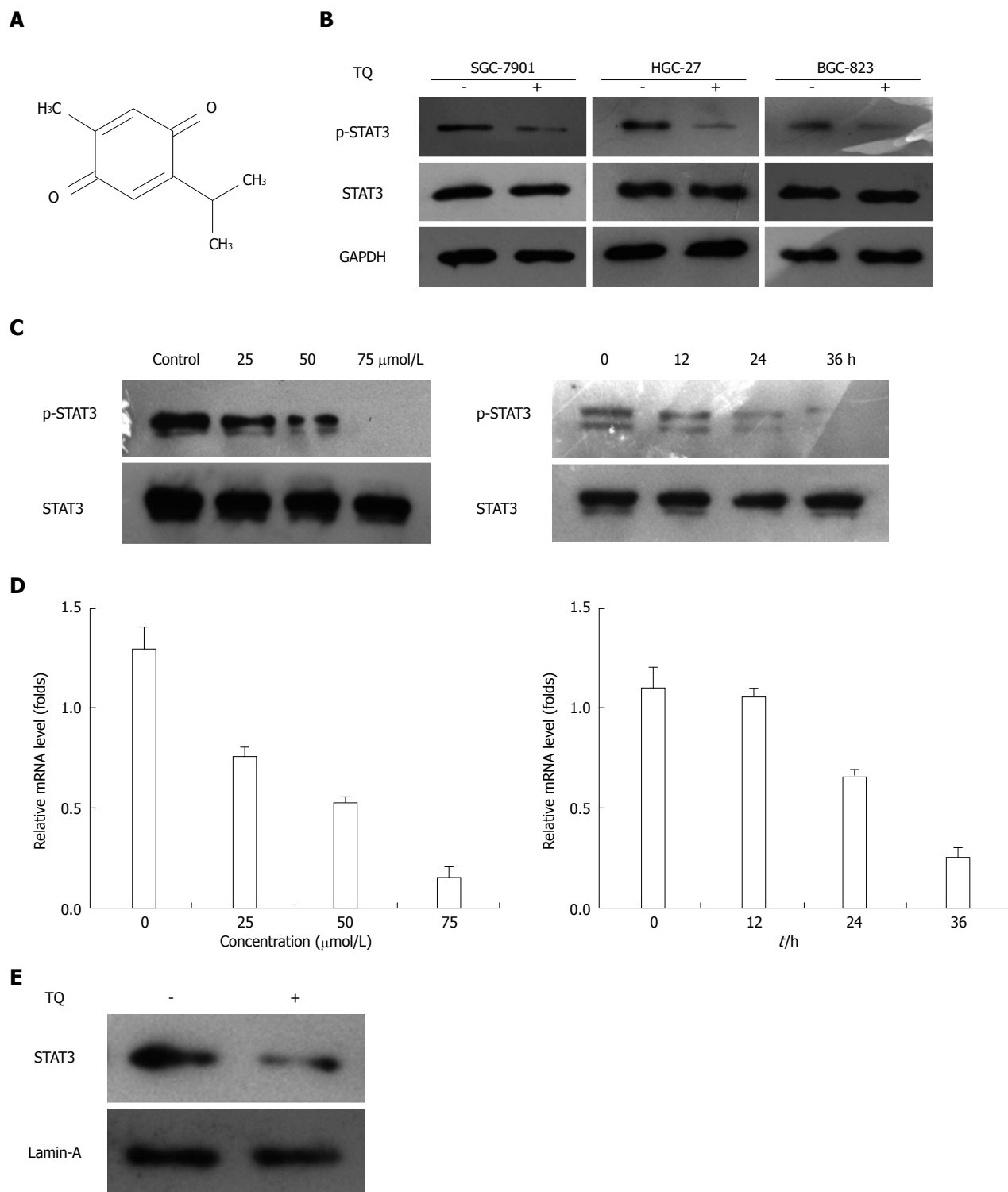


Figure 1 Thymoquinone suppresses constitutive STAT3 activation in three gastric cancer cell lines. A: Human gastric cancer cells (HGC27, BGC823, and SGC7901) were incubated with 50 μmol/L thymoquinone (TQ) for 24 h, and western blotting was performed as described previously; B: The same blots were stripped and reprobed with the GAPDH antibody to verify equal protein loading; C: TQ suppressed phospho-STAT3 levels in a concentration- and time-dependent manner. HGC27 cells (1 × 10⁶/mL) were treated with the indicated concentrations of TQ for 24 h, and western blotting was performed (left). HGC27 cells (1 × 10⁶/mL) were treated with 50 μmol/L TQ for the indicated times, and western blotting was performed (right). The same blots were stripped and reprobed with the STAT3 antibody to verify equal protein loading; D: TQ suppressed STAT3 gene levels in a concentration- and time-dependent manner. HGC27 cells (1 × 10⁶/mL) were treated with the indicated concentrations of TQ for 24 h, and real-time RT-PCR was performed (left). HGC27 cells (1 × 10⁶/mL) were treated with 50 μmol/L TQ for the indicated times, and real-time RT-PCR was performed (right); E: TQ suppressed STAT3 nuclear translocation. HGC27 cells (1 × 10⁶/mL) cells were treated with or without 50 μmol/L TQ for 24h, and nuclear extracts were assessed for the detection nuclear accumulation of STAT3. Lamin-A was used as a nuclear extract marker. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; RT-PCR: Reverse transcription polymerase chain reaction; STAT: Signal transducer and activator of transcription.

streptomycin) in a humidified incubator at 37 °C and 5% CO₂.

Western blot analysis

Protein expression levels were assessed by western blot analysis. Briefly, cells were collected, washed twice, and lysed with lysis buffer. Cell lysates were kept on ice for 30 min and centrifuged at 4 °C. Samples were then boiled in loading buffer and separated by 10% SDS-PAGE. After electrophoresis, protein was transferred onto a nitrocellulose membrane (Millipore, Bedford, MA, United States), which was incubated with blocking solution [10% non-fat dry milk in Tris-buffered saline with tween (TBST) containing 0.05% Tween-20] for 2 h and immunoblotted with primary antibodies, including STAT3, p-STAT3, STAT5, p-STAT5, p-JAK2, JAK2, p-Src, Src, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Lamin-A, survivin, Cyclin D, Bcl-2, Bax, peroxisome proliferator activated receptor, caspase-3,7,9 (Cell Signaling Technology, Beverly, MA, United States, 1:1000), overnight at 4 °C. After washing with TBST, the membranes were incubated with anti-rabbit (or anti-mouse) immunoglobulin conjugated to horseradish peroxidase secondary antibody (Promega, Madison, WI, United States, 1:500) for 2 h at room temperature, developed using the enhanced chemiluminescence (ECL) western blotting kit (Millipore, Bedford, MA, United States), and then exposed to Kodak X-ray Film. Protein band intensities were determined densitometrically using the video imaging CMIASWIN system (BioRad, Hercules, CA, United States).

Real-time reverse transcription polymerase chain reaction

Total RNA and complementary DNA (cDNA) were extracted from the cells for real-time reverse transcription polymerase chain reaction (RT-PCR) analysis as described previously^[16]. The cDNA was used as the template in an SYBR Green Real-Time PCR Master Mix (Invitrogen, Carlsbad, CA, United States). The PCR reaction was performed with an ABI PRISM 7300 PCR and detection system (Applied Biosystems, Carlsbad, CA, United States). The following primers were used: STAT3 sense 5'-GCCCTTTGGAACGAAGGGTA-3' and STAT3 antisense 5'-TGGTATTGCTGCAGGTCGTT-3'; GAPDH sense, 5'-TTTGGTATCGTGGGAAGGAC-3'; and GAPDH antisense, 5'-GTGGAGGAGTGGGTGTCGC-3'. The relative levels of STAT3 mRNA transcripts were normalized to the control GAPDH. For relative quantification, the expression levels of the STAT3 gene were calculated based on the method of $2^{-\Delta\Delta Ct}$ ^[17].

STAT3-Luciferase reporter gene assay

Cells were seeded into 12-well plates at a density of 5×10^4 cells/well prior to transfection. Cells were transfected with p-STAT3-TA-luc (Clontech, Palo Alto, CA, United States) or control vector using Genefectin transfection

reagent (Genetrone Biotech, Seoul, South Korea). After 24 h of transfection, cells were treated with TQ for an additional 24 h, and cell lysis was carried out with $1 \times$ reporter lysis buffer. After mixing the cell lysates with luciferase substrate (Promega), luciferase activity was measured using a luminometer (Tecan Trading AG, Shanghai, China). The β -galactosidase assay was carried out according to the supplier's instructions (Promega Enzyme Assay System) for normalizing the luciferase activity, and the results are expressed as fold transactivation.

Cell proliferation assay

Cell proliferation was assessed with 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) (Sigma St. Louis, MO, United States) colorimetric method. Gastric cancer cells were cultured in a 96-well plate in the presence of 0, 25, 50, 75, or 100 μ mol/L TQ for 12, 24, or 36 h. MTT reagent (20 μ L) was added into each well and incubated for 4 h at 37 °C. After the purple precipitate was visible, the cells were added with 150 μ L dimethylsulfoxide and incubated at room temperature in the dark for 2 h. The absorbance was recorded at 490 nm. For each experiment, the total procedure was repeated three times.

Hoechst 33258 staining for apoptotic cells

Gastric cancer cells in exponential growth were placed at a final concentration of 5×10^5 cells per well in a six-well plate, which was pretreated with 25 μ mol/L, 50 μ mol/L, or 75 μ mol/L TQ for 24 h. The cells were subsequently fixed, washed three times with phosphate buffered saline (PBS), and stained with Hoechst 33258 (Sigma-Aldrich) according to the manufacturer's protocol. Apoptotic features were assessed by analyzing chromatin condensation and by staining the fragments under an inverted fluorescent microscope (Olympus, Center Valley, PA, United States).

Annexin V staining for apoptosis

Gastric cancer cells were cultured in six-well plates at 1×10^6 cells/well in DMEM medium supplemented with 0.5% FBS and different concentrations of TQ as described above. Cells were washed twice with cold PBS and centrifuged, and then incubated with 5 mL of Annexin V-fluorescein isothiocyanate (FITC) and 10 mL of propidium iodide (PI) at room temperature for 5 min in the dark. Flow cytometric analysis was performed with a FACSCalibur using the CellQuest software (BDIS, San Jose, CA, United States). For each experiment, the total procedure was repeated three times.

Xenograft tumor model

Female BALB/c athymic nude mice (4-wk-old) were obtained from the Center of Experimental Animals of Wuhan University, and all procedures were performed in compliance with the National Institutes of Health

Guide for the Care and Use of Laboratory Animals. Nude mice were divided into four groups, and mice were housed (four animals per cage) in standard mouse plexiglass cages in a light and temperature-controlled environment. Mice were provided with food and water *ad libitum*. Gastric cancer cells were harvested from subconfluent cultures, washed in serum-free medium, and resuspended in PBS. Cells (5×10^6) resuspended in 0.2 mL PBS were subcutaneously inoculated into the lower right flank of nude mice. When the developing tumors reached 100-150 mm³ in size, mice were randomly assigned to the following treatment groups ($n = 5$): (1) untreated control, equal volume physiologic saline; (2) 10 mg/kg TQ; (3) 20 mg/kg TQ; and (4) 30 mg/kg TQ. All therapies were administered three times per week *via* intraperitoneal injection. Next, the mice were weighed, and the size of each tumor and its central necrotic area was monitored using calipers every 3 d. Following the last dose of TQ, all mice were sacrificed on day 30. During the autopsy procedure, the tumor was neatly excised and weighed. One part of the tissue was fixed in formalin, and another part was frozen in liquid nitrogen. Ethical approval was obtained prior to the start of the experiments.

TdT-mediated dUTP-biotin nick end-labeling assay

For histological examination, tumor tissues were fixed in 10% buffered formalin and embedded in paraffin, and tissue sections (4 μ m) were prepared. The TdT-mediated dUTP-biotin nick end-labeling (TUNEL) assay for apoptosis was conducted using an apoptosis detection kit (Roche Diagnostics, Branchburg, NJ, United States) according to the manufacturer's instructions. Positive cells were counted as the number of TUNEL-labeled cells per 100 epithelial cancer cells in 10 fields of the most affected tumor areas with 400 \times magnification and analyzed using light microscopy (Carl Zeiss, Thornwood, NY, United States).

Statistical analysis

Results were analyzed by SPSS version 17.0 (SPSS, Inc., Chicago, IL, United States). All data are presented as the mean \pm standard deviation (SD). One-way analysis of variance (ANOVA, multiple comparisons) and *t*-tests (two groups comparisons) were performed accordingly. $P < 0.05$ was considered to be statistically significant.

RESULTS

TQ inhibited constitutive STAT3 phosphorylation and nuclear translocation in gastric cancer cells

Since HGC27, BGC823, and SGC7901 cells were shown to express constitutive STAT3 activation, we determined whether TQ could inhibit this activation in these cells. Based on western blot results, the protein level of p-STAT3 was significantly reduced in all three

types of cancer cells treated with TQ compared to cells not treated with TQ (Figure 1B). These findings suggest that TQ inhibited STAT3 activation in all these cells in a concentration- and time-dependent manner, with maximum inhibition occurring at 75 μ mol/L (Figure 1C). Although TQ did not affect protein levels of STAT3 (Figure 1B, lower panel), it did suppress the mRNA expression of STAT3 in a concentration- and time-dependent manner, as demonstrated by real-time RT-PCR (Figure 1D). Because the active dimer of STAT3 is capable of translocating to the nucleus and inducing transcription of specific target genes^[18], we determined whether TQ suppresses the nuclear translocation of STAT3. Western blot clearly demonstrated that TQ blocked the translocation of STAT3 into the nucleus in HGC27 cells (Figure 1E). The phosphorylation of STAT5 and the expression of STAT5 were not affected by TQ (Figure 2A and B), indicating that TQ specifically inhibits STAT3 tyrosine phosphorylation.

TQ suppressed constitutive activation of other proteins

Since JAK2 and c-Src kinase are two of the main kinases involved in the activation of STAT3^[19,20], we examined the effect of TQ on the expression and phosphorylation of these two proteins. As shown in Figure 2C, JAK2 was constitutively active in HGC27 cells, and pretreatment with TQ clearly suppressed its phosphorylation in a time-dependent manner. TQ treatment did not affect total JAK2 levels. In addition, we found that TQ suppressed the constitutive phosphorylation of c-Src kinase in a time-dependent manner (Figure 2D), while the levels of total c-Src kinase remained unchanged under the same conditions (Figure 2D, lower panel).

TQ suppresses STAT3-dependent reporter gene expression

STAT3 is constitutively active in gastric cancer and plays a critical role in cell proliferation through transcriptional activation of pro-survival genes^[21]. We found that TQ reduced STAT3 reporter gene activity in HGC27 cells transiently transfected with STAT3-luc vector (Figure 3A). Moreover, TQ attenuated the protein expression of STAT3 target gene products, such as survivin, cyclin-D, VEGF, and Bcl-2 and increased the expression levels of Bax (Figure 3B and C).

TQ activated caspase activity and induced PARP cleavage

Next, we investigated whether suppression of constitutively active STAT3 in HGC27 cells by TQ caused cell apoptosis. In HGC27 cells, TQ activated procaspase-3,7,9 in a concentration-dependent manner (Figure 3D). Activation of downstream procaspase-3 led to the cleavage of a 116 kDa poly(ADP-ribose)-polymerase (PARP) protein into an 85 kDa fragment (Figure 3C). These results clearly suggested that TQ induces caspase-3-dependent apoptosis in HGC27 cells.

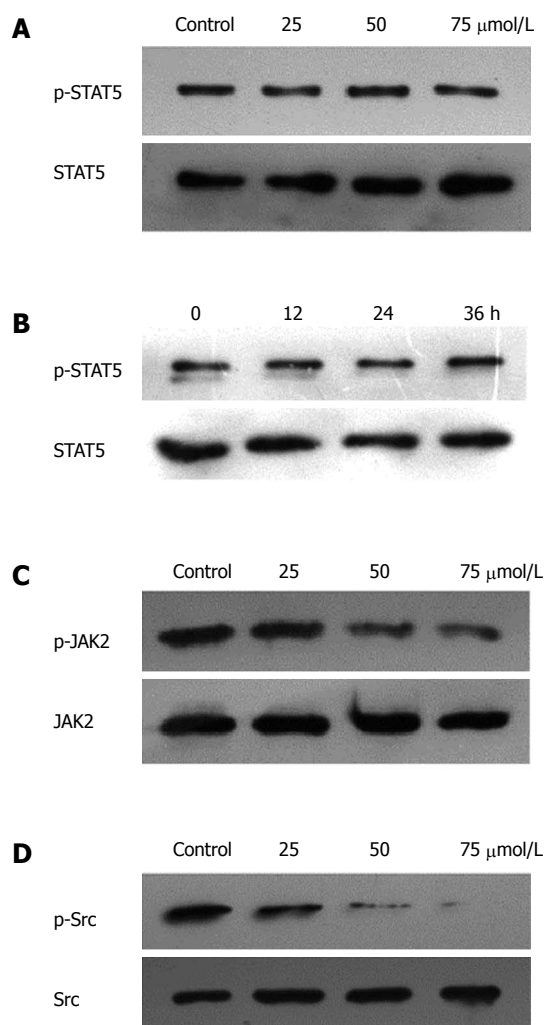


Figure 2 Thymoquinone suppresses phospho-JAK2 and phospho-Src levels, but not STAT5 levels. A: HGC27 cells (1×10^6 /mL) were treated with the indicated concentrations of thymoquinone (TQ) for 24 h, and western blotting was performed; B: HGC27 cells (1×10^6 /mL) were treated with 50 μ mol/L TQ for the indicated times, and western blotting was performed. The same blots were stripped and reprobbed with the STAT5 antibody to verify equal protein loading; C: TQ suppressed phospho-JAK2 levels in a concentration-dependent manner. HGC27 cells (1×10^6 /mL) were treated with the indicated concentrations of TQ for 24 h, and Western blotting was performed. The same blots were stripped and reprobbed with the JAK2 antibody to verify equal protein loading; D: TQ suppressed phospho-Src levels in a concentration-dependent manner. HGC27 cells (1×10^6 /mL) were treated with the indicated concentrations of TQ for 24 h, and Western blotting was performed. The same blots were stripped and reprobbed with the Src antibody to verify equal protein loading. JAK: Janus-activated kinase.

TQ inhibited proliferation and induces apoptosis

According to the results of the MTT assay, cell viability was reduced in the presence of TQ (25, 50 or 75 μ mol/L) in a time- and concentration-dependent manner (Figure 4). Annexin V staining of cells treated with the indicated concentrations of TQ showed that the compound induced apoptosis in a concentration-dependent manner (Figure 5A). In addition, Hoechst 33258 staining was performed in TQ treated cells and showed that apoptotic bodies containing nuclear fragments were generated in apoptotic cells (Figure 5B).

Anti-tumor effects in vivo

Based on the induction of apoptosis in gastric cancer cell *in vitro*, the antitumor effect of TQ was dose-dependent. In a xenograft tumor mouse model, the treatments of intraperitoneally administered injections of TQ for 30 d led to significant decreases in tumor weight and size compared to the control group mice ($P < 0.05$). (Figure 6A and B). Tumor tissues isolated from the xenograft mice of the four groups were processed for TUNEL assay, and the results showed (Figure 6C and D) that the tumors derived from high doses of TQ treated mice exhibited a markedly higher count of apoptotic bodies compared with that from control group mice. In addition, we measured the levels of STAT3 in tumor tissue by western blot and found that TQ inhibited STAT3 phosphorylation *in vivo* (Figure 6E).

DISCUSSION

As the second most common cause of cancer-related death in the world, gastric cancer has been reported to have particularly high incidence and mortality rates in eastern Asia. Its survival rate is substantially lower than that of patients with other types of cancers^[22].

TQ, the active constituent of black cumin seed oil, was shown to inhibit the proliferation of various types of tumor cells, including lung carcinoma, breast adenocarcinoma, pancreatic cancer, colorectal cancer, acute lymphoblastic leukemia, and prostate cancer^[23,24]. It has also been shown to enhance detoxification and inhibit benzo(a)pyrene (BP)-induced fore-stomach tumors in a female Swiss albino mouse model^[25]. TQ was also induced apoptosis and augmented 5-FU-induced apoptosis in gastric cancer cells^[15]. In this study, we found that TQ inhibited phosphorylation of STAT3 in three gastric cancer cells. TQ also inhibited the expression of the *STAT3* gene in a concentration- and time-dependent manner, as demonstrated by real-time RT-PCR.

Phosphorylation of STAT3 is required for its activation and results in dimerization, nuclear translocation, DNA binding, and transcriptional activation of target genes^[26]. To investigate the mechanism of TQ-induced STAT3 inhibition in HGC27 cells, we analyzed the activation of upstream protein kinases, such as JAK and Src. We found that TQ could inhibit JAK2 and Src phosphorylation, and presumably the suppression of STAT3 phosphorylation at Tyr705 was due to the inhibition of JAK2 and Src activity. Interestingly, TQ-induced inhibition of tyrosine phosphorylation was only observed for STAT3, not STAT5. These results suggest that TQ specifically inhibited phosphorylation of STAT3 at Tyr705.

We also observed that TQ suppressed the expression of several STAT3-regulated genes, including proliferative (cyclin D1), anti-apoptotic (Bcl-2, survivin), and angiogenic (VEGF) gene products in HGC27 cells. Activation of

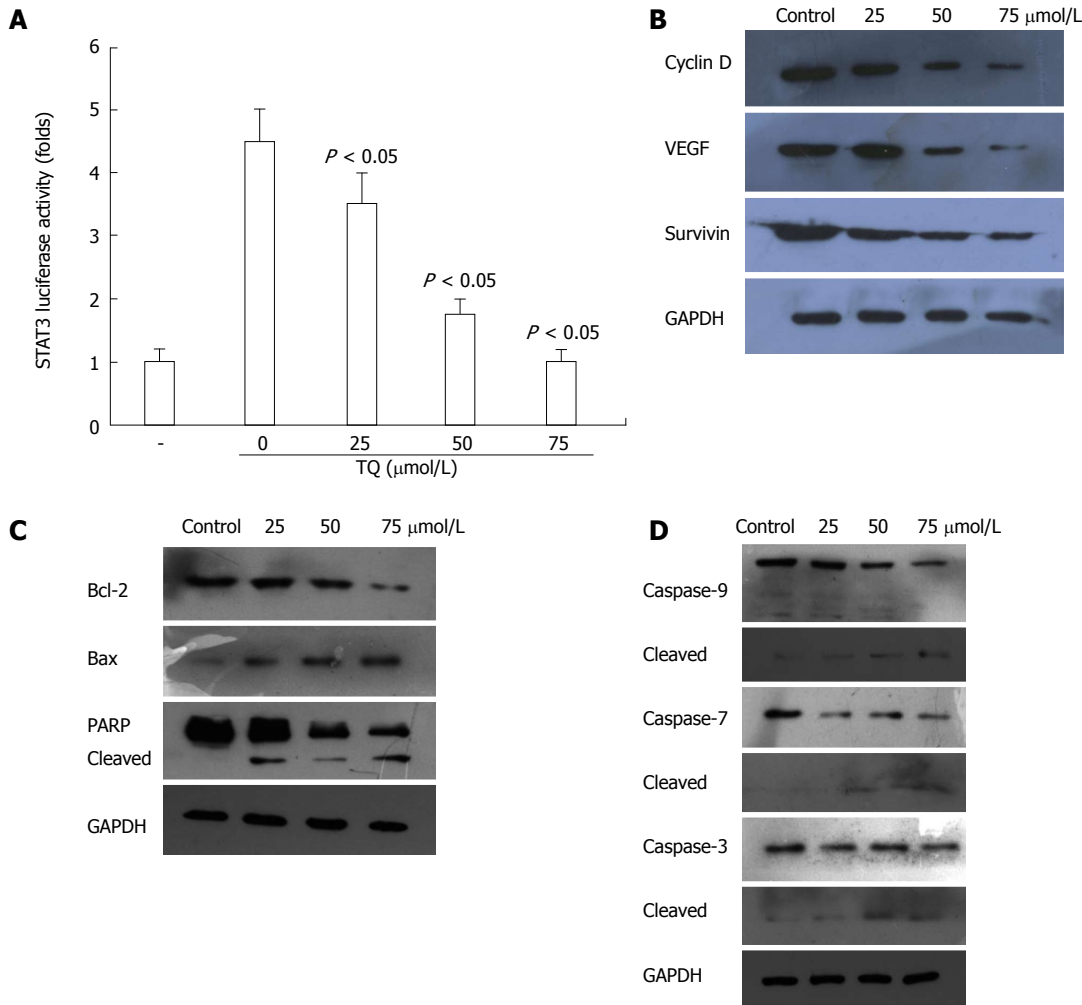


Figure 3 Thymoquinone suppresses STAT3-dependent reporter gene expression and activates caspase activity. A: Thymoquinone (TQ) suppressed STAT3 transcriptional activity. HGC27 cells (1×10^6 /mL) were transfected with STAT3-luciferase (STAT3-Luc) plasmid, incubated for 24 h, and treated with 25, 50, and 75 μ mol/L TQ for 24 h. Whole-cell extracts were then prepared and analyzed for luciferase activity. The results shown are representative of three independent experiments; B: TQ inhibited the expression of Cyclin D, survivin, and VEGF. HGC27 cells (1×10^9 /mL) were treated with the indicated concentrations of TQ for 24 h, and western blotting was performed. The same blots were stripped and reprobbed with the GAPDH antibody to verify equal protein loading; C: HGC27 cells (1×10^6 /mL) were treated with the indicated concentrations of TQ for 24 h, and whole-cell extracts were prepared, separated by SDS-PAGE, and subjected to western blot against Bcl-2, Bax, and PARP antibody. The same blot was stripped and reprobbed with GAPDH antibody to show equal protein loading; (D) TQ activates caspase activity. HGC27 cells (1×10^6 /mL) were treated with the indicated concentrations of TQ for 24 h, and western blotting was performed. The results shown are representative of three independent experiments. PARP: Poly(ADP-ribose)polymerase

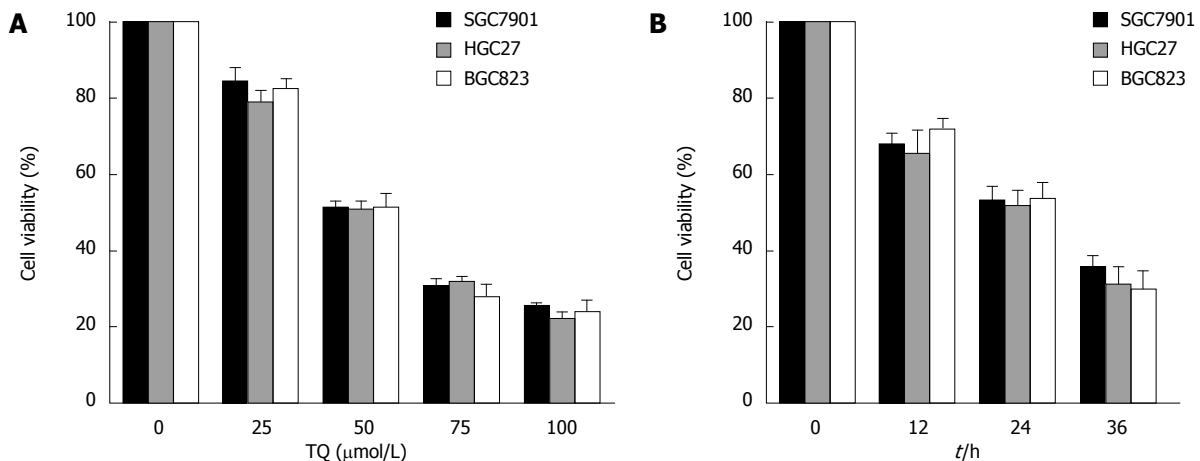


Figure 4 Thymoquinone inhibits proliferation in gastric cancer cells. A: Gastric cancer cells were treated with 25 μ mol/L, 50 μ mol/L, or 100 μ mol/L TQ for 24 h, after which an MTT assay was performed; B: Cells were treated with 50 μ mol/L TQ for 12, 24, or 36 h, and an MTT assay was performed. TQ: Thymoquinone.

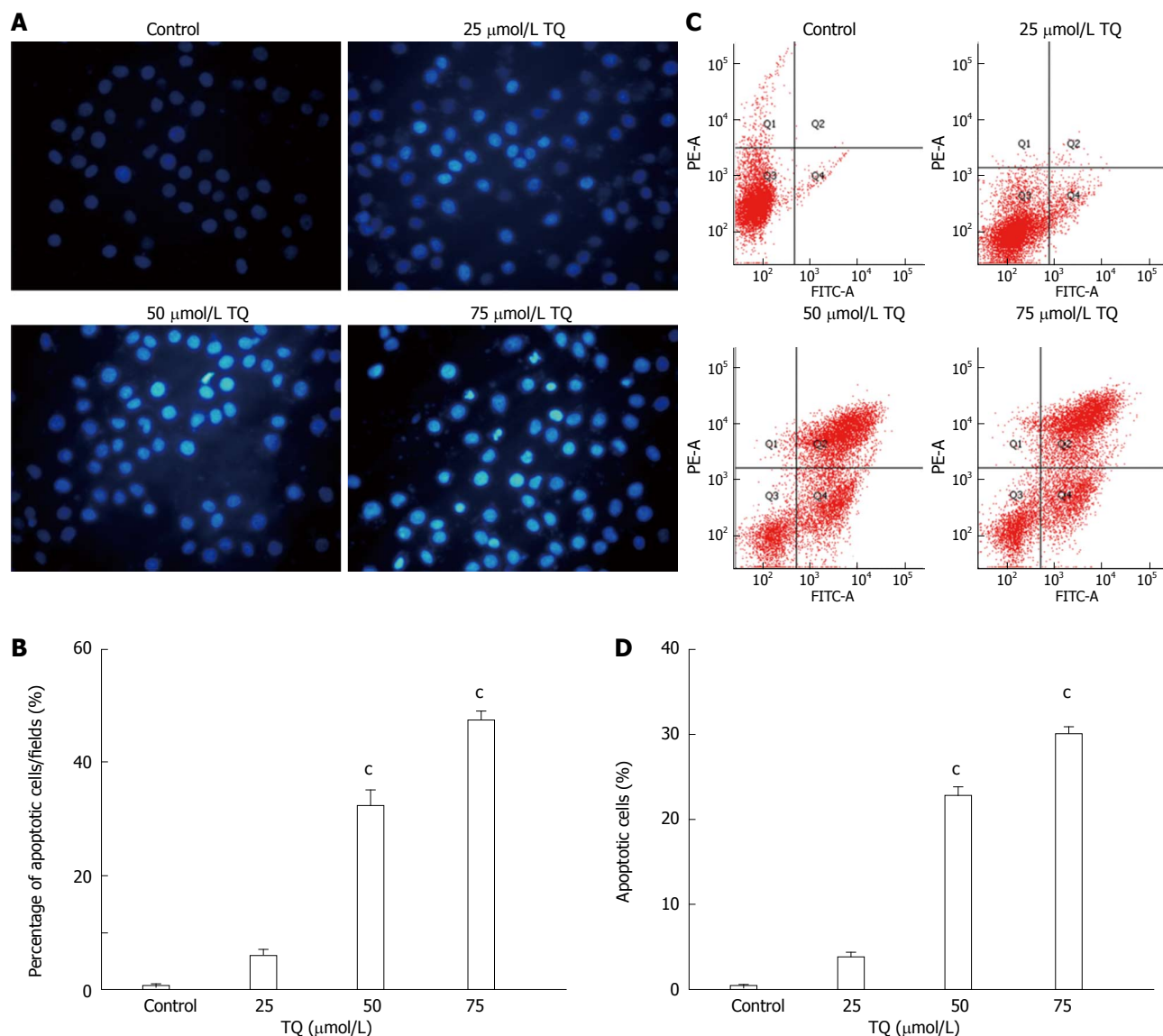


Figure 5 Thymoquinone induces apoptosis in HGC27 cells. A: Detection of apoptosis *via* Annexin V/PI staining (X-axis: annexin V; Y-axis: PI). The proportion of non-apoptotic cells (Q3), early apoptotic cells (Q4), late apoptotic/necrotic cells (Q2), and cell debris or death cell (Q1); B: Data shown are mean \pm SD from three independent experiments; C: Apoptosis was assessed using Hoechst 33258, and apoptotic features were assessed by observing chromatin condensation and fragment staining (original magnification, \times 200); D: Quantitative analysis of apoptotic cells is represented as the mean \pm SD from three independent experiments. ^c*P* < 0.05, vs control and 25 μ mol/L TQ. PI: Propidium iodide; TQ: Thymoquinone.

STAT3 signaling was thought to induce survivin gene expression and confer resistance to apoptosis in human breast cancer cells^[27]. It was reported that blocking cell death by Bcl-2 and Bcl-xL could be induced by a variety of chemotherapeutic agents, in parallel with an increase in chemoresistance^[28,29]. Thus, we considered that the downregulation of the expression of Bcl-2, Bcl-xL, and survivin was likely to be linked with the ability of TQ to induce apoptosis in HGC27 cells, as evidenced by cleavage of PARP. In addition, we observed that TQ could activate caspase-3,7,9 activities in a concentration-dependent manner. The MTT assay showed that TQ could inhibit cell viability in a concentration- and time-dependent manner. TQ dose-dependently induced apoptotic cell death in HGC27

cells, as estimated by Annexin V-FITC/PI analysis and Hoechst33258.

In addition to these *in vitro* studies, we further analyzed the effect of TQ on xenograft tumor *in vivo*. We found that TQ treatment exhibited obvious antitumor effects in a xenograft tumor mouse model. The therapeutic effect was demonstrated by TUNEL analysis, which displayed obvious cell death in the tumor masses *via* apoptosis. Moreover, STAT3 protein was measured in tumor tissue, and we found that the levels of p-STAT3 in the tumor tissue were significantly decreased after TQ treatment.

In conclusion, the present study demonstrated that TQ inhibited proliferation and induced apoptosis *via* down-regulation of the STAT3 signaling pathway.

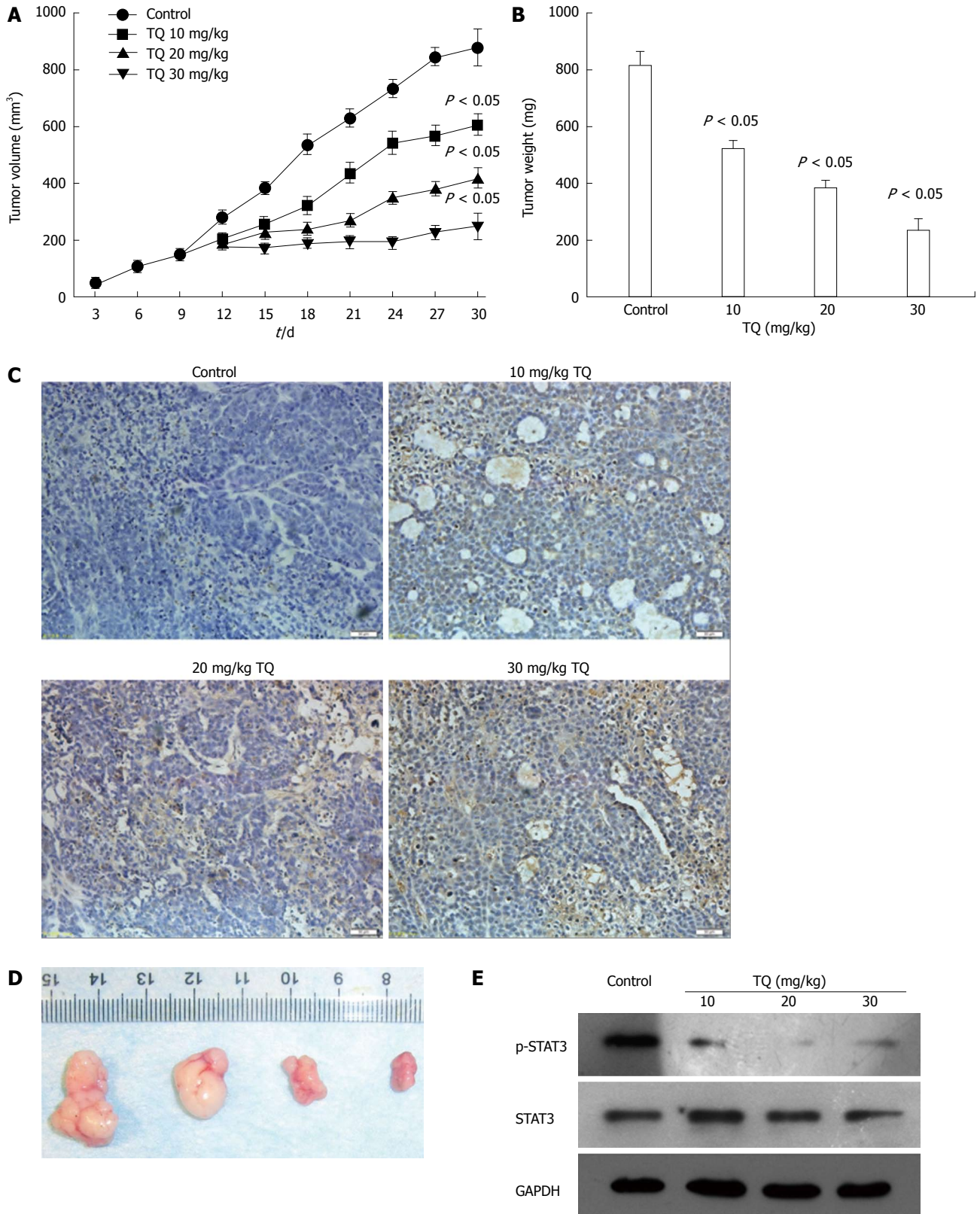


Figure 6 Thymoquinone inhibits tumor growth in a gastric mouse xenograft model. A: Tumor volumes of the xenograft tumors derived from 0, 25, 50, or 75 $\mu\text{mol/L}$ TQ group. Each time point represents the mean tumor volume for each group; B: Tumor weight was obtained at the end of the experiment. Error bars represent the standard error of the mean \pm SD; C: Detection of apoptotic cells in tumor tissue by TUNEL assay. Control: equal-volume physiologic saline; Treatment group: different doses of TQ: 10 mg/kg TQ; 20 mg/kg TQ; 30 mg/kg TQ; all therapies were administered three times per week *via* intraperitoneal injection. The brown color indicating apoptotic signals is shown by the arrows. Scale bar represents 50 μm . Original magnification: 400 \times ; D: Photographs of tumors from control and TQ treatment groups. From left to right: control group, 10 mg/kg TQ; 20 mg/kg TQ; 30 mg/kg TQ group; E: TQ suppresses phosphor-STAT3 *in vivo*. The tumor tissue extracts were prepared, after which Western blotting was performed. The same blots were stripped and reprobbed with the GAPDH antibody to verify equal protein loading. TQ: Thymoquinone; TUNEL: TdT-mediated dUTP-biotin nick end-labeling.

TQ may be a promising candidate as a cancer chemopreventive or chemotherapeutic agent.

COMMENTS

Background

Thymoquinone (TQ) has been demonstrated to exert biological activity in gastric cancer. However, the specific mechanism by which TQ affects gastric cancer remains to be examined. In order to elucidate the mechanism of TQ-induced apoptosis in human gastric cancer cells, we investigated the effects of TQ on signal transducer and activator of transcription (STAT)3 activation and its relative pathway both *in vitro* and *in vivo*.

Research frontiers

In 2014, Kundu *et al* found in human colon cancer HCT116 cells that TQ induced apoptosis through inactivation of STAT3 by blocking janus-activated kinase (JAK)2- and Src-mediated phosphorylation of endothelial growth factor (EGF) receptor tyrosine kinase. This finding is similar with the authors' study, thus they examined JAK2- and Src as well.

Innovations and breakthroughs

It was previously shown that TQ could induce apoptosis in gastric cancer cells. However, the specific mechanism of TQ in gastric cancer had not been examined. Our study demonstrated in gastric tumor cells that TQ inhibited proliferation and induced apoptosis through downregulation of the STAT3 signaling pathway. These findings suggest that TQ may be a potential chemopreventive or chemotherapeutic candidate for gastric cancer.

Applications

Since STAT3 is one of the main signaling pathways involved in TQ-mediated inhibition of gastric cancer, TQ may be used safely clinically. However, it remains to be determined if TQ will be useful in gastric cancer with drug resistance. In the future, this will be examined.

Terminology

Thymoquinone (TQ, also called 2-isopropyl-5-methyl-1,4-benzo-quinone, C₁₀H₁₂O₂) is the active constituent of black cummin (*Nigella sativa*) seed oil (Figure 1A) and was first extracted by El-Dakhkhany. STAT refers to signal transducer and activator of transcription. VEGF refers to vascular endothelial growth factor.

Peer-review

In this article, the authors analyzed the mechanism underlying the effect of TQ in the treatment of gastric cancer. This study has practical research significance, providing important information to guide the clinical administration of TQ in the future.

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