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***Basic Study***

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

Zhu WQ *et al*. Thymoquinone inhibits proliferation in GC

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**Data sharing statement:** Technical appendix, original data, images, and statistical code of this manuscript are available from the corresponding author at dwg@whu.edu.cn. Participants gave informed consent for data sharing.

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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、SGC7901 cells were cultured *in vitro* and treated with thymoquinone (0, 10, 25, 50, 75, 100, 125 μmol/L) for 12 h, 24 h and 36 h, and then the proliferation inhibitory rates were detected by MTT assay. Apoptosis was observed after Hoechst staining. The protein expressions of STAT3, p-STAT3, STAT5, p-STAT5, p-Janus-activated kinase 2 (JAK2), JAK2, p-Src, Src, GAPDH, Lamin-A, survivin, Cyclin D, Bcl-2, Bax, PPAR, Caspase-3,7,9 were detected by Western blot. The cell cycle and apoptosis were determined with flow cytometry. TQ induced dose-dependent apoptotic cell death in HGC27 cells, as measured by Annexin V-FITC/PI analysis and Hoechst 33258.

**RESULTS:** The results showed that TQ inhibits phosphorylation of STAT3, but not STAT5 phosphorylation. TQ-induced down-regulation of STAT3 activation is associated with a reduction in Janus-activated kinase 2 (JAK2) and c-Src activity. TQ also down-regulates the expression of STAT3- regulated genes such as Bcl-2, cyclin D, surviving and vascular endothelial growth factor, and activates caspase-3,7,9. to the *in vitro* results, it has been shown that the treatment of TQ represents a significantly effective antitumor agent in a xenograft tumor mouse model.

**CONCLUSION:** This study provides strong evidence that down-regulation 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 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 STAT3 and its relative pathway *in vitro* and *in vivo*.

Zhu WQ, Wang J, Guo XF, Liu Z, Dong WG. Thymoquinone inhibits proliferation in gastric cancer *via* STAT3 pathway *in vivo* and *in vitro*. *World J Gastroenterol* 2016; In press

**Introduction**

Based on GLOBOCAN estimates, about 14.1 million new cancer cases and 8.2 million deaths occurred in 2012 worldwide[1]. Among them, the incidence and mortality rates of gastric cancer account for the fourth and third place in male patients, respectively, and ranks fifth in female patients[1]. Chemotherapy is commonly used in the treatment of gastric cancer, and the commonly used chemotherapy drugs are 5-fluorouracil, cisplatin and doxorubicin, which have toxic side effects easy to resistance and other defects[2]. Therefore, it is important to search for low toxicity and efficient anti-cancer drugs inhibit gastric cancer development.

Thymoquinone (TQ, also called 2 - Isopropyl -5 - methyl-1,4 - benzo-quinone, C10H12O2), as the active constituents of black cumin (Nigella sativa) seed oil (Figure 1A), was first extracted by EI-Dakhakhany[3]. TQ has been shown to suppress the proliferation, induced apoptosis and drug resistance, and so on in various tumor cells, including colorectal carcinoma, pancreatic carcinoma, breast adenocarcinoma, prostate cancer, and so on[4-9]. A phase I study had reported that in adult patients who had solid tumors or hematological malignancies were treated with TQ, results showed out with 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 gradually attracted the attention of scholars in the world, but the research in tumors of digestive system especially in gastric cancer is rare.

STAT which refers to Signal transducer and activator of transcription have been shown as a fundamental factor in tumor cell survival and proliferation[11]. STAT3, a member of STAT protein family, is a critical molecule of the JAK/STAT signaling pathway, through many signals such as IL-6, TNF-α, and VEGF STAT3 can be activated by a variety of ligands related to these signals[12]. It was reported that in a variety of human malignancies such as gastric, colon, breast and lung, abnormal expression and constitutive activation of STAT3 are involved[12,13]. Once activated, STAT3 would continue to 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 vascular endothelial growth factor (VEGF) expression, which was 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. Previous study had shown that TQ could induce apoptosis and augment 5-FU-induced apoptosis in gastric cancer cells[15]. However, the specific mechanism of TQ in gastric cancer has not been examined.

Based on preliminary studies, we further explore the specific mechanisms of TQ regulating proliferation and apoptosis in gastric cancer cells.

**Methods and Materials**

***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 DMEM/F12 medium supplemented with heat-inactivated 10% FBS, 1% antibiotics (100 IU penicillin and 100 µg/ml streptomycin) in a humidified incubator at 37 ℃ 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 ℃. Samples were then boiled in loading buffer and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (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 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, GAPDH, Lamin-A, survivin, Cyclin D, Bcl-2, Bax, PPAR, Caspase-3,7,9 (Cell Signaling Technology, United States, 1:1000), overnight at 4 ℃. 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 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) was extracted from the cells for real-time reverse transcription polymerase chain reaction analysis as described previously[16]. The cDNA was used as the template in an SYBR Green Real-Time PCR Master Mixes (Invitrogen, Carlsbad, CA). The PCR reaction was performed with an ABI PRISM 7300 PCR and detection system (Applied Biosystems, Carlsbad, CA). The following primers were used: STAT3 sense 5’- GCCCTTTGGAACGAAGGGTA-3’ and STAT3 antisense 5’- TGGTATTGCTGCAGGTCGTT-3’; GAPDH sense, 5’-TTTGGTATCGTGGAAGGAC-3’, and GAPDH anti-sense, 5’- GTGGAGGAGTGGGTGTCGC-3’. The relative levels of STAT3 messenger RNA (mRNA) transcripts were normalized to the control GAPDH. For relative quantification, the expression levels of *STAT3* gene were calculated based on the method of 2-ΔΔCt[17].

***STAT3-Luciferase reporter gene assay***

Cells were seeded into 12-well plates at a density of 5 × 104 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 1X reporter lysis buffer. After mixing the cell lysates with luciferase substrate (Promega), the luciferase activity was measured by using luminometer (Tecan Trading AG). 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 were expressed as fold transactivation.

***Cell proliferation assay***

Cell proliferation was assessed with MTT (3-(4,5-dimethylthiazolyl-2)-2,5 -diphenyltetrazolium bromide) (Sigma, St. Louis, MO, United States) colorimetric method: The gastric cancer cells were cultured in a 96-well plate in the presence of 0, 25, 50, 75, 100 μmol/L TQ for 12, 24 or 36 h. 20 μl MTT reagent was added into each well and incubated for 4 h at 37 ℃. After the purple precipitate was visible, the cells were added with 150 μl DMSO 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 × 105 cells per well in a six-well plate, which was pretreated with 25 μmol/L, 50 μmol/L, 75 μmol/L TQ for 24 h. The cells were subsequently fixed, washed three times with PBS, and stained with Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, United States) 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).

***Annexin V staining for apoptosis***

Gastric cancer cells were cultured in the six-well plates at 1 × 106 cells/well in DMEM medium supplemented with 0.5% FBS, different concentration of TQ as described above. Cells were washed twice with cold PBS and centrifuged, and then incubated with 5 ml of Annexin V-FITC and 10 ml of PI at room temperature for 5 min in the dark. Flow cytometric analysis was performed with a FACSCalibur using the CellQuest software (BDIS). 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 NIH 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 lightand temperature-controlled environment and 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 × 106) 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, equalvolume 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 days. 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 before the beginning of 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 then, tissue sections (4 μm) were prepared. The 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 × magnification and analyzed using light microscopy (CarlZeiss, Thornwood, NY, United States).

***Statistical analysis***

Results were analyzed by SPSS version 17.0 (SPSS, Inc., Chicago, IL, United States) and All data are presented as the mean ± SD. One-way 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 translocatin in gastric cancer cells***

Since HGC27, BGC823 and SGC7901 cells have been shown to express constitutive STAT3 activation, whether TQ could inhibit this activation in these cells were detected. According to the results of the western blot, the protein levels of p-STAT3 were significantly lower in three types of cancer cells which were treated with TQ than in the cells treated without TQ (Figure 1B), suggesting the STAT3 activation was inhibited by TQ in all these cells in a concentration- and time-dependent manner, with maximum inhibition occurring at 75 μmol/L (Figure 1C). Although TQ showed no effect on the expression of STAT3 proteins (Figure 1B, lower panel), the expression levels of STAT3 protein-encoding gene were suppressed by TQ also in a concentration- and time-dependent manner as demonstrated by Real-time 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 demonstrates that TQ blocked the translocation of STAT3 into the nucleus in HGC27 cells (Figure 1E). However, the phosphorylation of STAT5 as well as th eexpression of STAT5 was not affected by TQ (Figure 2A and B).

***TQ suppresses constitutive STAT3 phosphorylation and nuclear translocation***

Human gastric cancer cells are well-known to express constitutively active STAT3. Whether TQ can modulate the constitutive STAT3 activation in these cells was investigated. As shown in Figure 1C (upper panel), TQ suppressed the constitutive activation of STAT3 in a concentration- and time-dependent manner, with complete inhibition occurring at 75 μmol/L. TQ had no effect on the expression of STAT3 proteins (Figure 1C, lower panel). Besides, TQ also inhibits the levels of STAT3 gene in a concentration- and time-dependent manner, as demonstrated by Real-time 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 demonstrates that TQ blocked the translocation of STAT3 into the nucleus in HGC27 cells (Figure 1E). We also determined the effect of TQ on STAT5 activation. As shown in Figure 2A and B, TQ did not inhibit STAT5 phosphorylation or the expression of STAT3 protein (Figure 2A and B). The results indicate that TQ specifically inhibits STAT3 tyrosine phosphorylation.

***TQ suppressed constitutive activation of other proteins***

Since JAK2 and c-Src kinase were two of the main kinases involved in the activation of STAT3[19,20], we also examined the effect of TQ on the expression and phosphorylation of those two proteins. As shown in Figure 2C, JAK2 was constitutively active in HGC27 cells and the pretreatment with TQ clearly suppressed this phosphorylation in a time-dependent manner. However, JAK2 was remained at the same level with no change. At the same time, we found that TQ suppressed the constitutive phosphorylation of c-Src kinase in a time-dependent manner as well (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***

Since STAT3 was 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 the STAT3 reporter gene activity in HGC27 cells which were 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, Bcl-2, and increased the expression levels of Bax (Figure 3B, C).

***TQ activated caspase activity and induced PARP cleavage***

Whether suppression of constitutively active STAT3 in HGC27 cells by TQ caused cell apoptosis was also investigated. In HGC27 cells treated with TQ, there was a concentration-dependent activation of pro-caspase-3, 7, 9 (Figure 3D). Activation of downstream procaspase-3 led to the cleavage of a 116 kDa PARP protein into an 85 kDa fragment (Figure 3C). These results clearly suggested that TQ induces caspase-3-dependent apoptosis in HGC27 cells.

***TQ inhibited proliferation and induces apoptosis***

According to the results of MTT assay, the cell viabilities were reduced in the present of TQ (25, 50 or 75 μmol/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 were also used in treated cells, which showing that apoptotic bodies containing nuclear fragments were generated in apoptosis cells (Figure 5B).

***Anti-tumor effects in vivo***

According to the analysis of apoptosis induction in gastric cancer cell *in vitro*, the antitumor effect of TQ was in a dose-dependent manner. 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 in contrast to the control group mice (*P <* 0.05). (Figure 6A, B). Tumor tissues isolated from the xenograft mice of four groups were processed for TUNEL assay, 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 the isolated from control group mice. In addition, we further measured the levels of STAT3 in tumor tissue by western blot, and the results showed TQ could also inhibit STAT3 phosphorylation *in vivo* (Figure 6E).

**Discussion**

As the second most common cause of cancer-related death in the world, gastric cancer was reported to present particularly high incidence and mortality rates in eastern Asia, and its survival rate was substantially lower than those of patients with other types of cancers[22].

Thymoquinone (TQ), the oil active constituent of these seeds, exhibited inhibitory effects on 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]. In addtion, TQ was proved to induce apoptosis and augment 5-FU-induced apoptosis in gastric cancer cells[15]. In this study, we found that TQ inhibits phosphorylation of STAT3 in three gastric cancer cells. TQ also inhibits the levels of *STAT3* gene in a concentration- and time-dependent manner, as demonstrated by Real-time PCR.

The phosphorylation was required for the activation of STAT3, which would result 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. The results showed that TQ could inhibit JAK2 and Src phosphorylation, supposing that the suppression of STAT3 phosphorylation at Tyr705 was due to the inhibition of JAK2 and Src activity. Interestingly, the inhibition of tyrosine phosphorylation induced by TQ was only observed at STAT3 rather than at STAT5. These results suggested 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) and anti-apoptotic gene products (Bcl-2, survivin), and angiogenic gene product (VEGF) in HGC27 cells. Activation of STAT3 signalling 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 down-regulation of the expression of Bcl-2, Bcl-xL and survivin was likely to be linked with TQ’s ability to induce apoptosis in HGC27 cells as evidenced by cleavage of PARP. In addition, we also observed that TQ could activate caspase-3, 7, 9 activities in a concentration-dependent manner. The results of MTT assay showed TQ could inhibit cell viability in a concentration- and time-dependent manner. TQ induced dose-dependent apoptotic cell death in HGC27 cells, as estimated by Annexin V-FITC/PI analysis and Hoechst33258.

Besides the results focused on the *in vitro* culture cells, we further analyzed the effect of TQ on xenograft tumor *in vivo*. The results showed that the treatment of TQ presented obvious antitumor effects in a xenograft tumor mouse model. The therapeutic effect has been demonstrated by TUNEL analysis, which displayed obvious cell death in tumor mass *via* apoptosis. Moreover, we also detected the STAT3 protein in tumor tissue and found that the levels of p-STAT3 in 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. TQ may be a candidate for a cancer Chemopreventive or chemotherapeutic agent.

**Comments**

***Background***

Thymoquinone (TQ), has demonstrated to exert biological activity in gastric cancer. However, the specific mechanism of TQ in gastric cancer is still need to be examined. So in order to elucidate the mechanism of TQ-induced apoptosis in human gastric cancer cells, we investigated the effects of TQ about STAT3 and its relative pathway *in vitro* and *in vivo*.

***Research frontiers***

In 2014, a study written by Kundu J has stated that in human colon cancer HCT116 cells, TQ induces apoptosis through inactivation of STAT3 by blocking JAK2- and Src-mediated phosphorylation of EGF receptor tyrosine kinase. Which is similar with our study, JAK2- and Src- got our attention as well.

***Innovations and breakthroughs***

Previous study had shown that TQ could induce apoptosis in gastric cancer cells. However, the specific mechanism of TQ in gastric cancer has not been examined. Our study demonstrated that in gastric tumor cells, TQ inhibited proliferation and induced apoptosis though down-regulation of the STAT3 signaling pathway. Which means TQ may be a Chemopreventive or chemotherapeutic candidate for gastic cancer.

***Applications***

In the further research, as we know that STAT3 is the main signaling pathway when TQ inhibits proliferation of gastic cancer, TQ can be used in chinical safely, however, if TQ still can be treated in gastric cancer with drug resistance may not been studied yet. It is a new direction the authors can go further.

***Terminology***

Thymoquinone (TQ, also called 2 - Isopropyl -5 - methyl-1,4 - benzo-quinone, C10H12O2), as the active constituents of black cumin (Nigella sativa) seed oil (Figure 1A), was first extracted by EI-Dakhakhany. STAT refers to Signal transducer and activator of transcription. VEGF refers to vascular endothelial growth factor.

***Peer-review***

This article analyzed from the mechanism of the role of the TQ, which is a more accurate guidance for clinical administration, with practical research significance.

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**Figure 1 thymoquinone suppresses constitutive STAT3 activation in three gastric cancer cells.** A:Human gastric cancer cells (HGC27, BGC823 and SGC7901) were incubated with 50 mol/L thymoquinone (TQ) for 24 h, after which 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 suppresses 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, after which Western blotting was performed (left). HGC27 cells (1 × 106/ml) were treated with the 50 μmol/L TQ for the indicated times, after which Western blotting was performed (right). The same blots were stripped and reprobed with the STAT3 antibody to verify equal protein loading; D: TQ suppresses 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, after which Real-time PCR was performed (left). HGC27 cells (1 × 106/ml) were treated with the 50 μmol/L TQ for the indicated times, after which Real-time PCR was performed (right); E: TQ suppresses STAT3 nuclear translocation. HGC27 cells (1 × 106/ml) cells were treated with or without 50 μmol/L TQ for 24h, after which nuclear extracts was assessed for the detection nuclear accumulation of STAT3, Lamin-A was used as a nuclear extract marker.

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**Figure 2 thymoquinone suppresses phosphor-JAK2 and phospho-Src levels, but not STAT5 levels.** A: HGC27 cells (1 × 106/ml) were treated with the indicated concentrations of thymoquinone **(**TQ) for 24 h, after which Western blotting was performed; B: HGC27 cells (1 × 106/ml) were treated with the 50 μmol/L TQ for the indicated times, after which Western blotting was performed. The same blots were stripped and reprobed with the STAT5 antibody to verify equal protein loading; C: TQ suppresses phospho-JAK2 levels in a concentration -dependent manner. HGC27 cells (1 × 106/ml) were treated with the indicated concentrations of TQ for 24 h, after which Western blotting was performed. The same blots were stripped and reprobed with the JAK2 antibody to verify equal protein loading; D: TQ suppresses phospho-Src levels in a concentration -dependent manner. HGC27 cells (1 × 106/ml) were treated with the indicated concentrations of TQ for 24 h, after which Western blotting was performed. The same blots were stripped and reprobed with the Src antibody to verify equal protein loading.

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*P* < 0.05

*P* < 0.05

*P* < 0.05

**Figure 3** **thymoquinone suppresses STAT3-dependent reporter gene expression and activates caspase activity.** A: thymoquinone **(**TQ) suppresses STAT3 transcriptional activity. HGC27 cells (1 × 106/ml) were transfected with STAT3-luciferase (STAT3-Luc) plasmid, incubated for 24 h and treated with 25, 50 and 75 μmol/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 inhibits the expression of Cyclin D, surviving and VEGF. HGC27 cells (1 × 106/ml) were treated with the indicated concentrations of TQ for 24 h, after which Western blotting was performed. The same blots were stripped and reprobed with the GAPDH antibody to verify equal protein loading; C: HGC27 cells (1 × 106/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, PARP antibody. The same blot was stripped and reprobed with GAPDH antibody to show equal protein loading; (D) TQ activates caspase activity. HGC27 cells (1 × 106/ml) were treated with the indicated concentrations of TQ for 24 h, after which Western blotting was performed. The results shown are representative of three independent experiments.

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

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**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 ± 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, × 200); D: Quantitative analysis of apoptotic cells is represented as the mean ± SD from three independent experiments. c*P* < 0.05, *vs* control and 25 μmol/L TQ. TQ: thymoquinone.

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*P* < 0.05

*P* < 0.05

*P* < 0.05

**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 μmol/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 ± 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 mL. Original magnification: 400 ×; 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 reprobed with the GAPDH antibody to verify equal protein loading. TQ: thymoquinone.