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Full Paper

Toosendanin induces apoptosis in human gastric cancer SGC-7901 cells through mitochondrial and death receptor pathways

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Abstract: Toosendanin (TSN) is a triterpenoid derivative which exerts anti-cancer effects on various cancer cell lines. However, it is still not clear whether TSN has similar anti-cancer effects on gastric cancer. In the present study the effects of TSN on human gastric cancer SGC-7901 cells were investigated by trypan blue exclusion, inverted microscopy, confocal microscopy and flow cytometric analysis. The results show that TSN significantly inhibits the proliferation of SGC-7901 cells in a dose- and time-dependent manner. In addition, TSN induces the formation of apoptotic bodies, increases the apoptosis rate and decreases the mitochondrial membrane potential in the SGC-7901 cells, indicating that TSN can induce apoptosis in gastric cancer cells. Further analysis shows that expression of the genes, Bax, cytochrome c and Fas is increased by TSN, whereas Bcl-2 and PARP expression is reduced. Furthermore, the effects of TSN on activation of caspase-3, caspase-8 and caspase-9 indicate that the mitochondrial and death receptor pathways are involved in the TSN-induced apoptosis. These results suggest that TSN may be an effective candidate agent for treating gastric cancer.

Keywords: toosendanin, SGC-7901 cells, apoptosis, gastric cancer

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INTRODUCTION

Gastric cancer is a term that most commonly refers to gastric adenocarcinoma, which represents 90-95% of all malignant gastric neoplasms [1]. The incidence rate varies dramatically, with more than two-thirds of all cases occurring in developing countries, and reports of up to 43% of cases occurring in China, with a geographical variability similar to that of other cancers [2]. Currently, chemotherapy is the main approach to gastric cancer treatment. However, the majority of chemotherapeutic drugs have numerous side effects, limiting their clinical application [3]. It is urgently necessary to identify more effective chemotherapeutic agents with lower toxicity.

Toosendanin (TSN), a triterpenoid extracted from the root bark of *Melia toosendan* [4], has its origin from traditional Chinese medicine and has been used as an insecticide for decades in China [5]. Tang et al. [6] in 2003 reported that TSN induces outgrowth of neuronal processes and apoptosis in PC12 cells. Since then TSN has been found to inhibit tumour cell proliferation and promote tumour cell apoptosis in glioma [7], human ovarian cancer [8], hepatocellular carcinoma [9], colorectal cancer cells [10] and human gastric cancer [11]. The results have suggested that TSN could potentially be used as an agent for treatment of malignant tumours in patients [12].

TSN exhibits anti-cancer effects on various human cancer cells through multiple signalling pathways [7]. For example, it inhibits pancreatic cancer tumour growth via deactivating Akt/mTOR signalling [13] and induces colorectal cancer cells apoptosis through suppression of AKT/GSK-3β/β-catenin pathway [14]. JNK signalling pathway [15] and p38 MAPK pathway [16] are also involved in the anti-tumour effects of TSN in HL-60 cells and human gastric cancer cells. In addition, TSN suppresses oncogenic phenotypes of human gastric carcinoma SGC-7901 cells, partly via miR-200a-mediated downregulation of β-catenin pathway [17]. Our previous study [18] shows that TSN induces apoptosis of human gastric cancer MGC-803 cells by regulating the expressions of Bax and Bcl2 genes. However, the mechanism by which TSN induces apoptosis of human gastric cancer has not been fully understood.

Apoptosis, also called “programmed cell death”, can be induced by mitochondria-mediated pathway or death receptor-mediated apoptosis pathway [18, 19]. Previous studies have revealed that TSN induces apoptosis via the mitochondria-mediated pathway in hepatocellular carcinoma cells and glioma PC12 cells [6, 19, 20]. However, whether TSN induces gastric cancer cell apoptosis via the mitochondria-mediated pathway or death receptor-mediated apoptosis pathway in gastric cancer cells requires further study.

In the present work, to study gastric cancer cells further, another gastric cancer cell line, SGC-7901, is used to investigate 1) the effect of TSN on proliferation of gastric cancer SGC-7901 cells; 2) whether TSN can induce apoptosis in SGC-7901 cells; and 3) the potential mechanism of apoptosis induced by TSN.

MATERIALS AND METHODS

Cell Culture

SGC-7901 cells were purchased from the Tumor Biology Test Center of Beijing and 293T cells were provided by Northeast Normal University (Changchun, China). Both cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Thermo Fisher Scientific Inc., USA) supplemented with 10% foetal bovine serum (Sangon Biotech Co., China), 100 μg/ml

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penicillin sodium (Beyotime Biotech. Inc., China) and 100 U/ml streptomycin (Beyotime Biotech. Inc., China) at 37°C in a humidified atmosphere with 5% CO_2 according to the method described by Fernandez-Araujo et al. [21].

2,5-Diphenyl-2H-Tetrazolium Bromide (MTT) Assay

SGC-7901 and 293T cells growing in the exponential phase were seeded in 96-well plates (1×10^4 cells/well in 100 µl) and cultured overnight at 37°C in a humidified atmosphere containing 5% CO_2. Both cell lines were treated with TSN (98% analysis grade, Nantong Feiyu Biological Technology Co., China) and 5-fluorouracil (5-FU; Shanghai Xudonghaipu Pharmaceutical Co., China), a known anti-cancer drug used as positive control, by adding the drugs to RPMI-1640 medium to obtain the final concentrations of 20, 40, 60, 80 and 100 nmol/l for 24, 48 and 72 hr. Subsequently, the cells were incubated with 0.5 µg/µl MTT (Beyotime Biotech. Inc., China) at 37°C for 4 hr. Absorbance was measured using a multi-well plate reader (Bio-Rad Laboratories Inc., USA) at 570 nm. The cell inhibitory rate (%) was calculated according to the following formula: (1 – absorbance of cells treated with drug / absorbance of untreated control cells) ×100. The IC_{50} value is defined as concentration of drug that reduces cell growth by 50% compared with control treatment.

Morphological Studies

SGC-7901 and 293T cells were cultured on histogrip-coated glass coverslips (Invitrogen Thermo Fisher Scientific Inc., USA) in 6-well plates at a density of 1×10^4 cells/well and treated with 2 ml of TSN (50, 70 and 90 nmol/l) or 2 ml of 5-FU (70 nmol/l) for 48 hr. Subsequently, the medium was discarded and 1 ml phosphate buffered saline (PBS) and 200 µl acridine orange (AO) solution (0.5 µg/µl) were added to the wells and incubated at 25°C for 3-5 min. The cellular morphology was observed and imaged using an inverted microscope (Olympus IX71, Olympus Corporation, Japan). To further observe the morphological changes, the cells were fixed by adding 1 ml methanol to each of the wells of the plates for 20 min. Fixed cells were incubated with 5% bovine serum albumin (BSA) and 0.2% Triton X-100 in PBS for 1 hr, and then 200 µl 4',6-diamidino-2-phenylindole (DAPI) was added to each well. Finally, the fixed cells were imaged using a laser confocal microscope (Leica TCS SP8, Leica Microsystems GmbH, Germany).

Annexin V-FITC/Propidium Iodide (PI) Staining

SGC-7901 cells (5ml; 1×10^4 cells/ml) in the exponential growth phase were cultured separately in cell culture flasks and treated with TSN (5 ml; 50, 70 and 90 nmol/l) and 5-FU (5 ml; 70 nmol/l) for 48 hr. Then the cells were collected and washed twice with cold PBS, followed by adding 500 µl binding buffer. Subsequently, the cells were stained with 5 µl Annexin V-FITC (code:C1062S, Beyotime Biotech. Inc., China) and 5 µl PI (code:C1062S, Beyotime Biotech. Inc., China) for 15 min. according to the reagent instruction manual. Finally, the cells were immediately analysed using a flow cytometer (Beckman Coulter Inc., USA) to examine the apoptosis rate of the cells.

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Mitochondrial Membrane Potential Assay

SGC-7901 cells (5 ml; 1×10^4 cells/ml) in the exponential growth phase were treated with different concentrations of TSN (50, 70 and 90 nmol/l in 5 ml) and 5-FU (70 nmol/l; 5 ml) for 48 hr. The cells were then collected and washed twice with cold PBS. A quantity of 2×10^5 cells was taken in 1 ml of PBS and 0.5 ml JC-1 dye (5 mg/ml) was added, vortexed for 10-15 sec. and incubated for 20 min. in the dark at 37°C. The cells were washed twice with JC-1 dyeing buffer. Subsequently, the mitochondrial membrane potential of the cells was determined using a flow cytometer (Beckman Coulter Inc., USA).

Cell Cycle Assay

SGC-7901 cells (1×10^4 cells/ml; 5 ml) in the exponential growth phase were treated with different concentrations of TSN (50, 70 and 90 nmol/l in 5 ml) and 5-FU (70 nmol/l; 5 ml) for 48 hr. The cells were collected and washed twice with cold PBS, then fixed in 75% ethanol for 18 hr. The cells were then collected, washed twice with cold PBS, re-suspended in 500 µl PI staining solution (code: C1052, Beyotime Biotech. Inc., China) with 5 µl RNase (50 µg/ml) and incubated at 37°C in the dark for 30 min. The cell cycle was analysed using a flow cytometer (Beckman Coulter Inc., USA).

Reverse Transcription-Quantitative Polymerase Chain Reaction

Total RNA was extracted from SGC-7901 cells using TRIzol (Invitrogen; Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions. The total RNA concentration was determined using a NanoDrop 2000C spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific Inc., USA). First-strand cDNA was synthesised from 1 µg of total RNA from each sample using a TransScript One-Step gDNA Removal and cDNA Synthesis Super Mix (Beijing Transgen Biotech Co., China) according to the manufacturer's instructions. SYBR-Green-based qPCR analysis was conducted to determine the mRNA expression levels of *Bax*, *Bcl-2*, *cytochrome-c (Cyt-c)*, *Fas*, *caspase-3*, *caspase-8*, *caspase-9* and *poly(ADP-ribose) polymerase (PARP)* genes. *β-Actin* was used as endogenous control. The primer sequences are listed in Table 1. The data were obtained from three replicates. The mRNA expression levels of the genes were normalised relative to *β-actin* and calculated using the $2^{-\Delta\Delta Cq}$ method [22].

Western Blot Analysis

After treating with TSN or 5-FU, the SGC-7901 cells were collected and washed twice with cold PBS. Total protein was extracted from the cells and separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes using a wet transfer system (Bio-Rad Laboratories Inc., China). The membranes were incubated with diluted primary antibodies (1:500) against *Bax* (cat. no. ab32503, Abcam, UK), *Bcl-2* (cat. no. ab59348), *Cyt-c* (cat. no. ab13575), *Fas* (cat. no. ab15285), *caspase-3* (cat. no. ab13847), *caspase-8* (cat. no. ab25901), *caspase-9* (cat. no. ab32539), *PARP* (cat. no. ab74290) and *GAPDH* (cat. no. ab22555) at 4°C overnight. After blotting with IRDye 800CW secondary antibodies (LI-COR Biosciences), 1:15,000, for 1 hr at 4°C, the bands were visualised and quantified using LI-COR Odyssey infrared

imaging system (LI-COR Biosciences, USA). The expression of *Bax*, *Bcl-2*, *Cyt-c*, *Fas*, *caspase-3*, *caspase-8*, *caspase-9* and *PARP* was normalised to *GAPDH*.

Table 1. Primer sequences for genes

Gene and primer name	Primer sequence	Product size
Bcl-2_F1	atgtgtgtggagagcgtcaac	180
Bcl-2_R1	agacagccaggagaaatcaac	
Bax_F1	aagctgagcgagtgtctcaag	178
Bax_R1	caaagtagaaaagggcgacaac	
Fas_F1	tgatgtggaacacagcaagg	107
Fas_R1	ggctgtggtgactcttagtgata	
Cyt c_F1	ctgggtgacgagtgaaactg	104
Cyt c_R1	tgagcacaacaggaactgga	
Caspase-3_F1	ggaacgaacggacctgtg	135
Caspase-3_R1	gcctccactggtatcttctg	
Caspase-8_F1	ccagaagtacacgcagtcc	327
Caspase-8_R1	cgatagcccaaggaagtg	
Caspase-9_F1	ccagatgctgtcccatacc	228
Caspase-9_R1	attggcgaccctgagaag	
PARP_F1	cgatcttggacagagttgctgatg	400
PARP_R1	acagctttgcttcgtccttgttc	
β-Actin_F1	agcgagcatcccccaaagtt	205
β-Actin_R1	gggcacgaaggctcatcatt	

Statistical Analysis

All experiments were replicated thrice and all data are presented as mean ± standard error of the mean (SEM). Statistical comparisons were conducted using a two-tailed unpaired Student's t-test, Kolmogorov-Smirnov test, or one-way analysis of variance (ANOVA). All analyses were performed using SPSS 16.0 software (SPSS Inc., USA). Statistically significant difference was set at $P<0.05$.

RESULTS AND DISCUSSION

Effects of TSN on Proliferation of SGC-7901 Cells

TSN is a triterpenoid derivative extracted from the bark of *Melia toosendan* Sieb. et Zucc [4] and was previously reported to inhibit proliferation and induce apoptosis of liver cancer cells, glioma cell lines and colorectal cancer cells [7, 19, 23, 24]. Similarly, in the present study TSN is shown to inhibit the proliferation of human gastric cancer SGC-7901 cells. When the cells are treated with TSN, the inhibitory rate significantly increases, both with TSN concentration and over

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time (Figures 1A-C), indicating that the effect of TSN on SGC-7901 cells is dose- and time-dependent. As shown in Figures 1 D-F, the inhibitory rates of 5-FU against SGC-7901 cells are similar to those of TSN at different concentrations throughout the experiment, and the IC_{50} value of TSN (70.008 nmol/l) is similar to that of 5-FU (73.689 nmol/l), an established cancer therapeutic. Notably, in 293T cells, the normal control cell line, the effects of TSN on the inhibitory rate are negligible at all doses and time points (Figures 1 A-C). These results suggest that TSN may be an effective candidate agent for treating human gastric cancer.

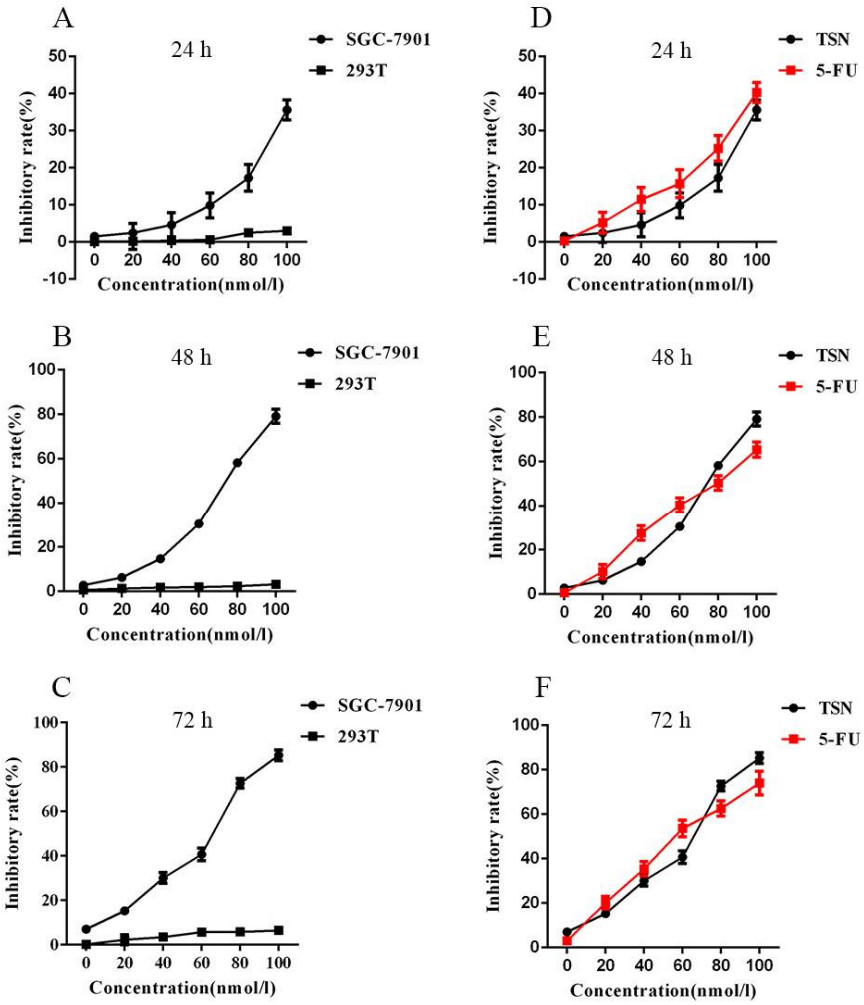

Figure 1. (A–C) Effects of TSN on SGC-7901 and 293T cells at 24 hr, 48 hr and 72 hr; (D–F) Effects of TSN and 5-FU on SGC-7901 cells at 24 hr, 48 hr and 72 hr

Effects of TSN on Morphology of SGC-7901 Cells

Morphological changes were observed in TSN-treated SGC-7901 cells compared with control treatments (Figure 2). AO and DAPI staining show that the cell volume shrinks, the shape of the nucleus is rippled or creased, chromatin is released, and vacuoles or apoptotic bodies are formed following TSM treatment (Figures 2 C-D). The number and shape of 293T cells are not markedly altered following treatment with 70 nmol/l TSN compared with control group (Figure 3). The 5-FU-treated 293T cells exhibit shrinkage and deformation, with an increased number of floating

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cells in the culture medium, reduced number of adherent cells and increased apoptosis (Figure 3). The results show that TSN treatment alters the morphology of SGC-7901 cells but does not affect the morphology of the 293T cells.

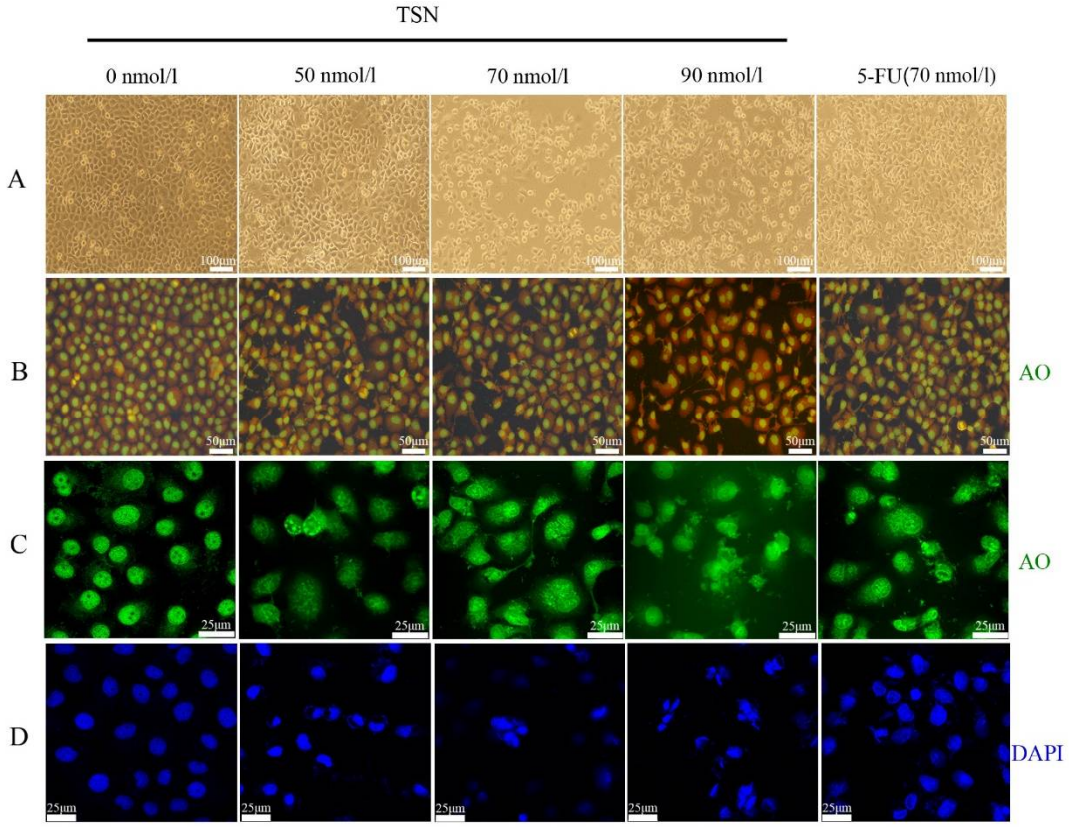

Figure 2. Morphological changes of SGC-7901 cells treated with TSN and 5-FU: (A) by light microscope (×100); (B) by fluorescence microscope (×200); (C) stained with AO (×726); (D) stained with DAPI (×752)

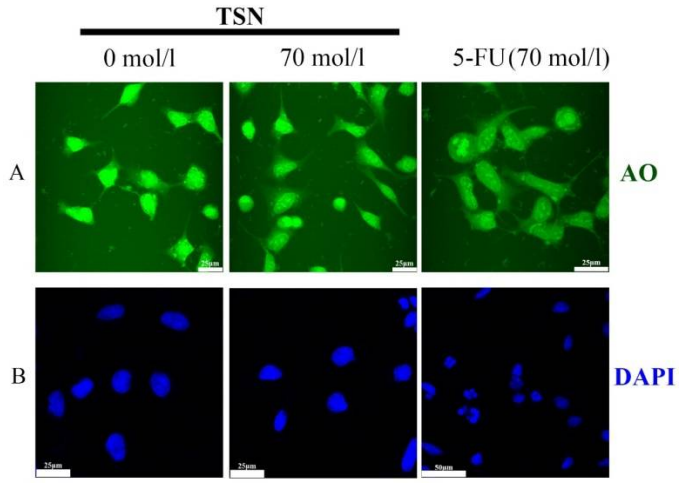

Figure 3. 293T cells treated with TSN and 5-FU for 48 hr. Cells were stained using (A) AO (×726) and (B) DAPI (×752)

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Effects of TSN on SGC-7901 Cell Cycle

There are two possible reasons for the inhibitory effects of TSN on SGC-7901 cells: cell proliferation might be blocked, or there is mass mortality of the cells, or both. To explore this, we examined the cell cycle of SGC-7901 cells using flow cytometry. The results show that the number of SGC-7901 cells in the G1 phase significantly increases at 48 hr after treatment with 50 and 70 nmol/l TSN, while the numbers of cells in the S and G2 phases are reduced (Figure 4), indicating that the cell cycle is arrested in the G1 phase, thus reducing cell proliferation. Moreover, compared to 0 hr (untreated with TSN), TSN treatment decreases the number of SGC-7901 cells (Figure 1), indicating that TSN increases cell death. These results show that TSN inhibits cell proliferation by blocking the cell cycle as well as increasing the death of the SGC-7901 cells.

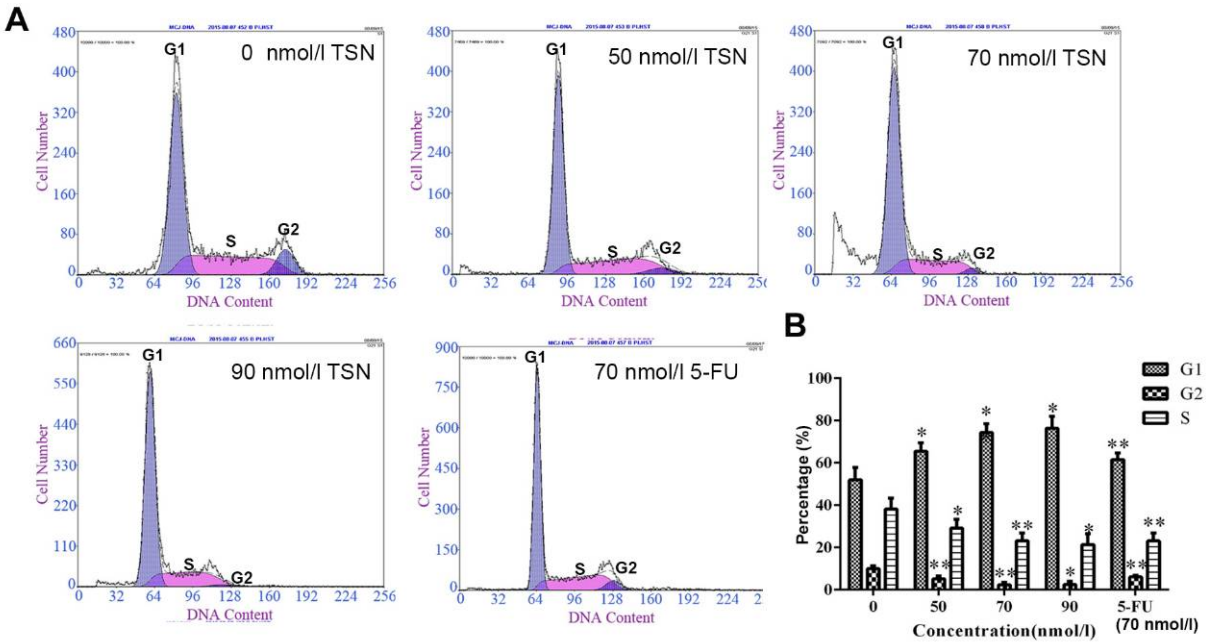

Figure 4. Effects of TSN on cell cycle in SGC-7901 cells: (A) cell cycle DNA content and (B) statistical diagram of cell proportion at each stage of cell cycle; * P<0.05, ** P<0.01 vs control

Effects of TSN on Apoptosis of SGC-7901 Cells

In addition to killing cells directly, apoptosis is one of the mechanisms that can be generated by drugs to induce cell death in malignant tumours [25]. In the present study (Figures 2C-D) the formation of apoptotic bodies, which are a typical characteristic of apoptosis [25], is observed in SGC-7901 cells at 48 hr after treatment with 50 and 70 nmol/l TSN, indicating that apoptosis occurs in the SGC-7901 cells treated with TSN. To confirm this, the early apoptosis rate of SGC-7901 cells is measured using flow cytometry with Annexin V-FITC/PI staining, and the results show that early apoptosis is induced by TSN in a dose-dependent manner (Figure 5A). The apoptosis rate is 29.32% in the TSN-treated (70 nmol/l) cells; in contrast, this rate is 3.43% in the untreated cells and 21.82% in the 5-FU-treated (70 nmol/l) cells (Figure 5B).

A decline in the mitochondrial membrane potential ($\Delta\Psi$) is a marked event during early cell apoptosis [26]. Therefore, changes in the mitochondrial membrane potential of SGC-7901 cells are analysed at 48 hr after treatment with TSN and 5-FU (Figure 5C). Compared to the control (0

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nmol/l), 70 and 90 nmol/l TSN significantly decrease the mitochondrial membrane potential by 68.07% and 83.54% respectively (Figure 5D). The mitochondrial membrane potential in SGC-7901 cells treated with 70 and 90 nmol/l TSN is significantly lower than that with 70 nmol/l 5-FU. These results strongly indicate that TSN induces apoptosis in SGC-7901 cells. A similar phenomenon was previously observed in MGC-803 cells [18], indicating that TSN can induce apoptosis in gastric cancer cells, potentially reducing tumour growth.

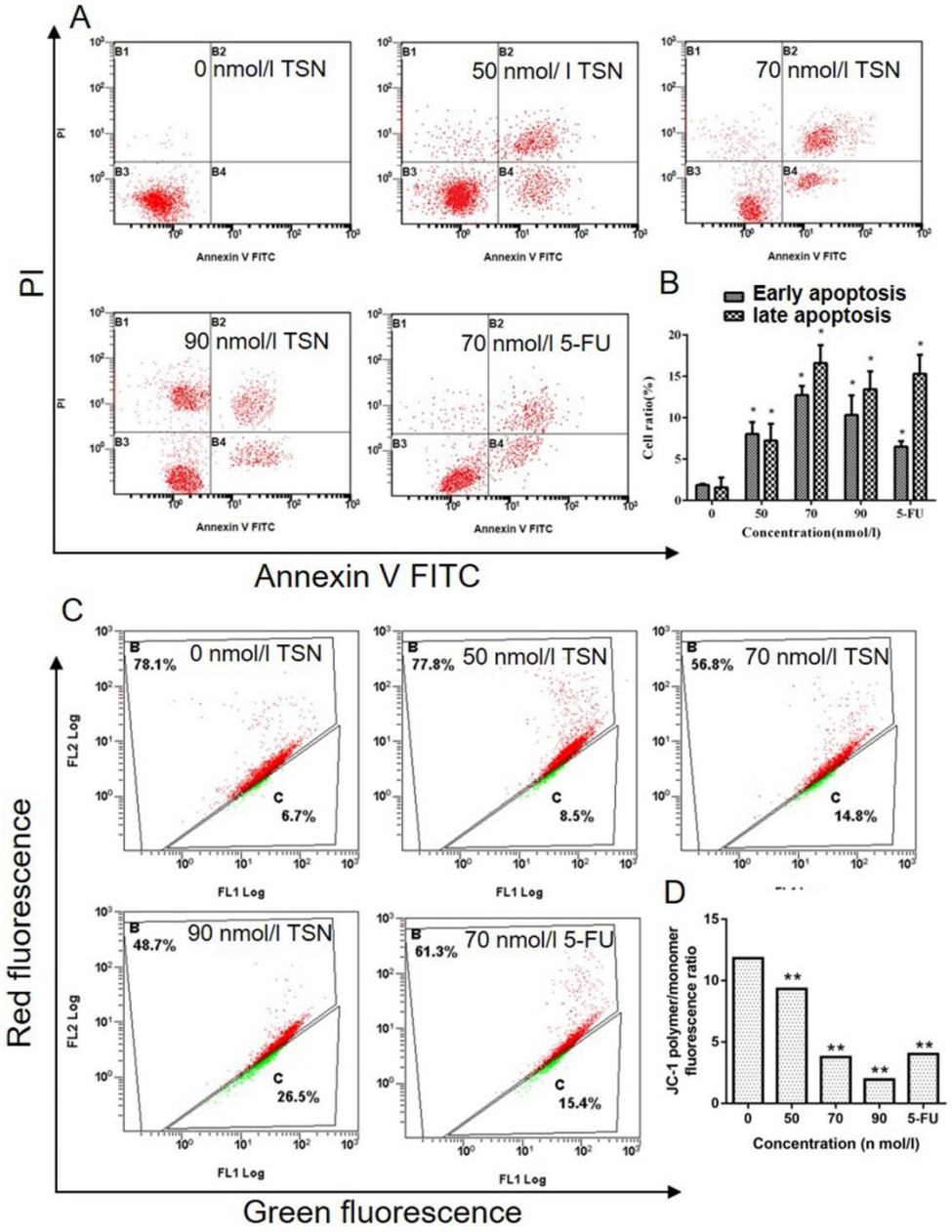

Figure 5. Effects of TSN on cell apoptosis and mitochondrial potential in SGC-7901 cells: (A-B) SGC-7901 cells are treated with TSN (50, 70 and 90 nmol/l) and 5-FU (70 nmol/l) for 48 hr. Apoptosis is analysed using Annexin V-FITC/PI; (C-D) Analysis of mitochondrial membrane potential. Red fluouescence represents JC-1 polymer, green fluorescence represents JC-1 monomer. * $P<0.05$, ** $P<0.01$ vs control

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Effects of TSN on Expression of Bax, Bcl-2, Cyt-c, Fas, Caspase-3, Caspase-8, Caspase-9 and PARP

Apoptosis in mammalian cells is mainly mediated through the extrinsic (death receptor) and intrinsic (mitochondrial) pathways [27-30]. Multiple genes and gene families, including *Fas* and *Fas ligand* (*FasL*), *Bcl-2* family (*Bax* and *Bak*), *caspase* family (*caspase-3*, *caspase-8* and *caspase-9*) and *Cyt-c*, are involved in this process [31-33]. Fas binds to FasL and activates the death receptor pathway where caspase-3 and caspase-8 promote apoptosis. *Bcl-2*, *caspase-3*, *Cyt-c* and *Bax* genes are involved in the mitochondrial-mediated apoptosis pathway [34]. Bax and Bcl-2 induce the release of Cyt-c, and the release of Cyt-c from the mitochondria into the cytoplasm can activate caspase-3 and caspase-9, both of which can trigger the mitochondrial apoptosis pathway [23, 35]. Previous studies have suggested that TSN induces apoptosis in human Ewing's sarcoma cells [36], ovarian cancer [8] and hepatocellular carcinoma [19] through the mitochondrial apoptotic pathway. In the present study, to study the mechanism by which TSN induces apoptosis of human gastric cancer SGC7901, the mRNA expression levels of *Bax*, *Bcl-2*, *Cyt-c*, *Fas*, *caspase-3*, *caspase-8*, *caspase-9* and *PARP* are examined using RT-qPCR analysis, and the data are evaluated using the $2^{-\Delta\Delta Cq}$ method. The results indicate that TSN significantly increases the mRNA levels of *Bax*, *Cyt-c*, *Fas*, *caspase-3*, *caspase-8*, *caspase-9* and *PARP*, and decreases *Bcl-2* mRNA expression (Figure 6). The highest mRNA expression of *Bax*, *Cyt-c*, *Fas*, *caspase-3*, *caspase-8*, *caspase-9* and *PARP*, and the lowest level of *Bcl-2* are induced by 70 nmol/l TSN. The effects of 5-FU on RNA expression levels are similar to those of TSN.

The changes in protein expression level are similar to changes in mRNA expression level (Figure 7). TSN treatment increases the protein expression of *Bax*, *Cyt-c* and *Fas* in a dose-dependent manner in SGC-7901 cells, and the highest expression is observed following treatment with 70 nmol/l TSN ($P<0.05$). The expression levels of pro-caspase 3, pro-caspase 8, pro-caspase 9, Bcl-2 and PARP decrease, and the lowest expression is produced by treatment with 70 nmol/l TSN ($P<0.05$). The effects of TNS on protein expression are greater than those of 5-FU at the same concentration (70 nmol/l).

Furthermore, the relative expression ratio of *Bax/Bcl-2* is increased by TSN, which further confirms its effects on apoptosis [14, 37]. Additionally, the mitochondrial membrane potential decreases (Figures 5C-D) while the expression of Cyt C, Caspase9 and Caspase3 increases (Figure 7). These results indicate that TSN can induce apoptosis in SGC-7901 cells through a mitochondria-mediated pathway, as observed in human gastric cancer MGC-803 cells [18]. Furthermore, the expression of Fas and Caspase8 also increases (Figure 7), indicating that the death receptor-mediated pathway is also involved in the apoptosis of SGC-7901 cells.

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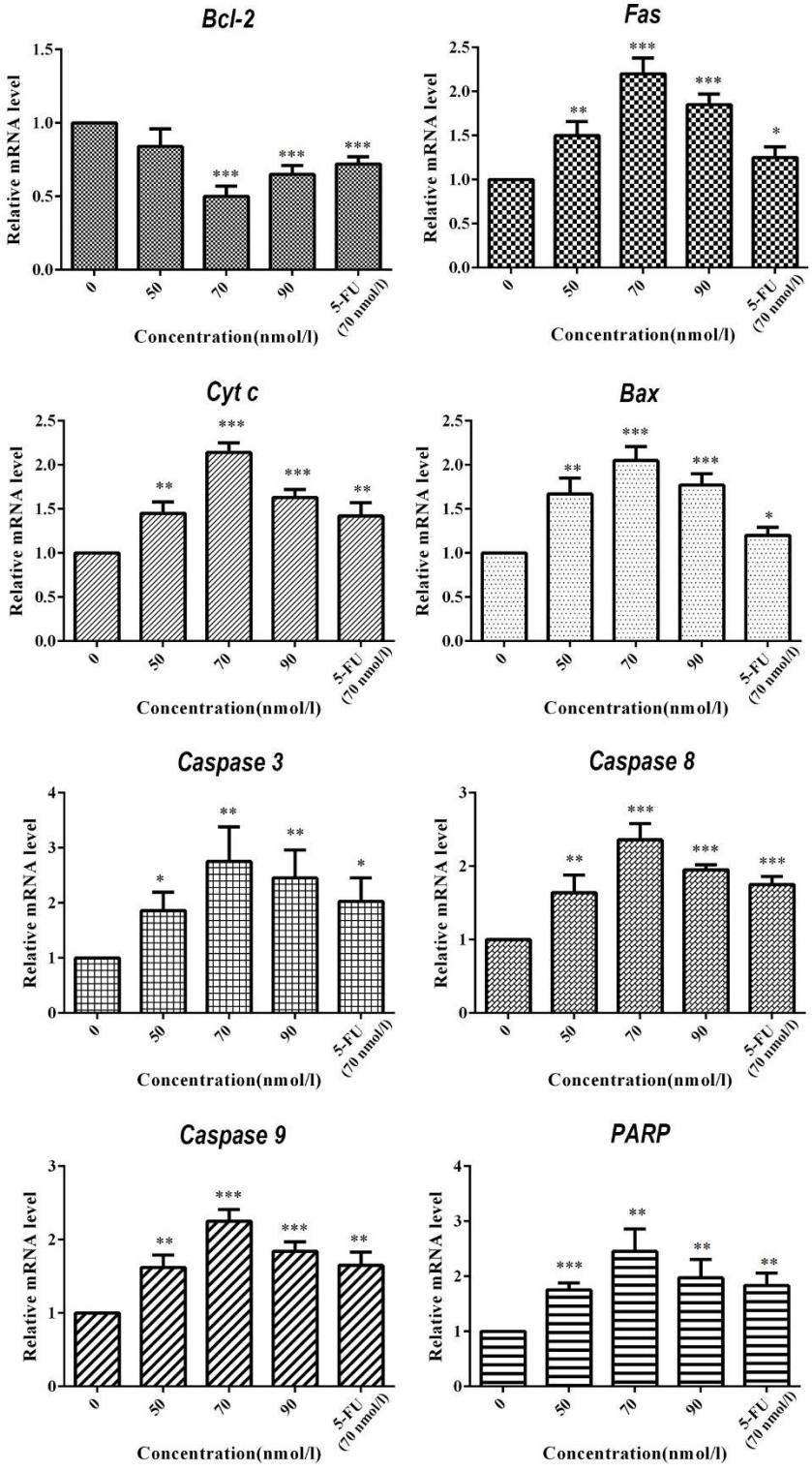

Figure 6. Effects of TSN and 5-FU on mRNA expression of *Bax*, *Bcl-2*, *Cyt-c*, *Fas*, *caspase-3*, *caspase-8*, *caspase-9 and PARP*. Data are presented as mean ± SEM of three independent experiments.

* P<0.05, ** P<0.01, *** P<0.001 vs control (0 nmol/l)

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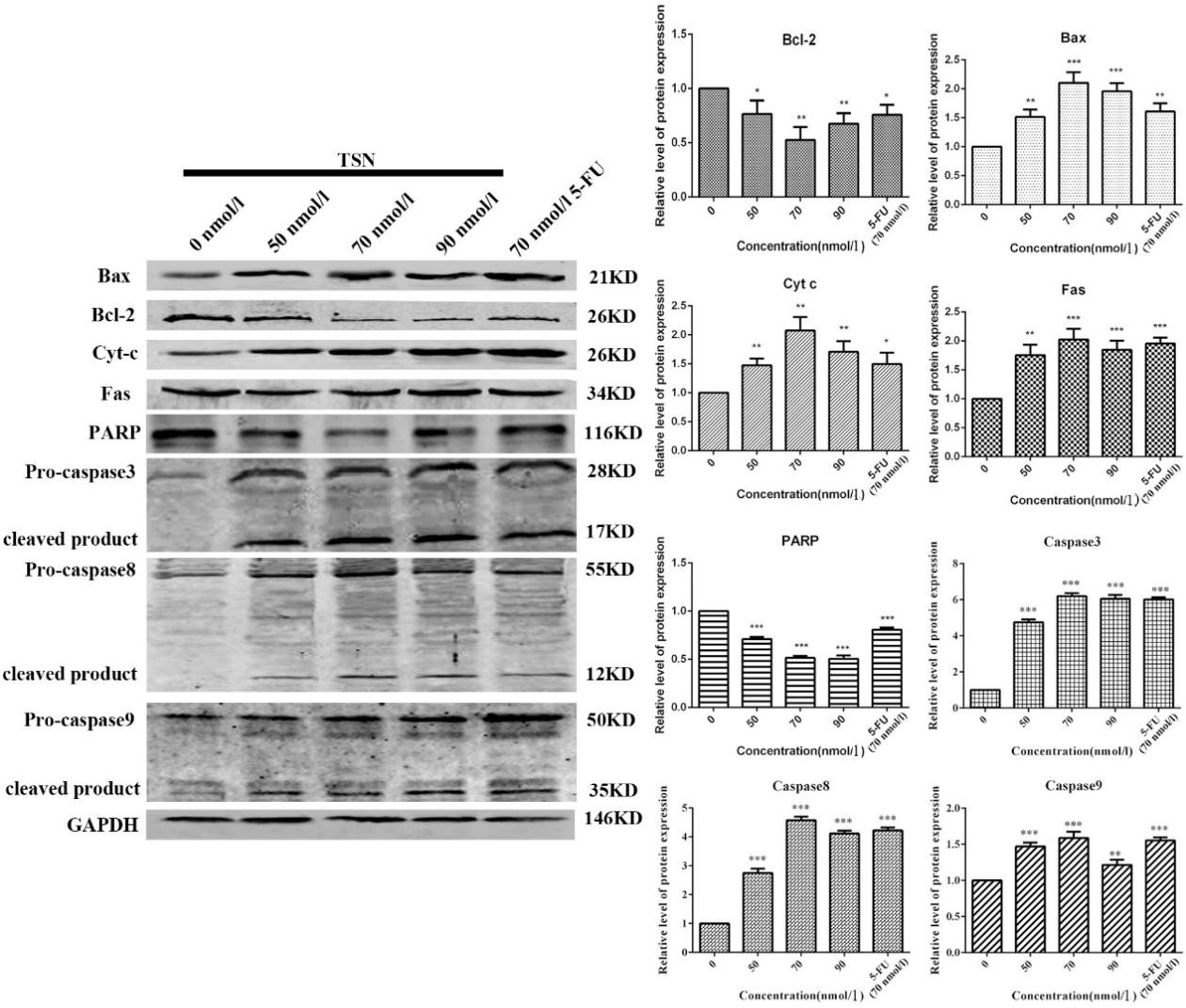

Figure 7. Expressions of apoptosis-related proteins in SGC-7901 cells treated with TSN or 5-FU. Data are presented as mean ± SEM of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs control (0 nmol/l)

CONCLUSIONS

The present study has demonstrated that TSN inhibits proliferation and induces apoptosis in SGC-7901 cells. The mitochondria-mediated and death receptor-mediated pathways are both involved in the induction of apoptosis caused by TSN. These results enrich our understanding of the anti-cancer effects of TSN on human gastric cancer cells and suggest that TSN may be an effective candidate agent for treating human gastric cancer.

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