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Original Article

Anti-fibrotic Effects and Mechanism of Shengmai Injection (生脉注射液) on Human Hepatic Stellate Cells LX-2*

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ABSTRACT **Objective**: To investigate the effects of Shengmai Injection (生脉注射液, SMI) on the proliferation, apoptosis and N-myc downstream-regulated gene 2 (NDRG2, a tumour suppressor gene) expression in varying densities of human hepatic stellate cells LX-2. **Methods**: LX-2 cells were cultured *in vitro*. Then, cells were plated in 96-well plates at an approximate density of 2.5×10^4 cells/mL and cultured for 48, 72, 96 or 120 h followed by the application of different concentrations of SMI (0.6, 1.2, 2.4, 4.8 or 6 μL/mL). Cell proliferation was measured after an additional 24 or 48 h using the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The effects of SMI on different cell growth states (cultured for 48, 72, 96, or 120 h) were observed by light microscopy at 24 h after treatment. When the cells reached 80% confluence, apoptosis was detected by flow cytometry after 24 h. Lastly, LX-2 cells were treated with different concentrations of SMI and extracted with protein lysis buffer. The levels of NDRG2 were measured by Western blot. **Results**: When the LX-2 cells grew for 48, 72, 96 and 120 h, 4.8 and 6 μL/mL of SMI significantly inhibited cell proliferation at 24 and 48 h after treatment ($P<0.05$). And 2.4 μL/mL of SMI also inhibited cell proliferation at 24 h after treatment when cell growth for 48 h ($P<0.05$) and at 48 h after treatment when cell growth for 72, 96 and 120 h ($P<0.05$). The NDRG2 expression level in the LX-2 cell was significantly increased when treated with SMI at concentrations of 1.2, 2.4, 4.8 or 6 μL/mL ($P<0.05$). **Conclusions**: The inhibitory effects of SMI on the proliferation of LX-2 cells were related to not only concentration dependent but also cell density. In addition, SMI (2.4, 4.8 and 6 μL/mL) could accelerate apoptosis in LX-2 cells, and the mechanism might be associated with NDRG2 over-expression.

KEYWORDS Shengmai Injection, liver fibrosis, N-myc downstream-regulated gene 2, LX-2 cell, proliferation, apoptosis, Chinese medicine

The liver is one of the most vital organs in humans due to its role in metabolism, nutrition and biotransformation. It is also easily affected by damaging factors such as alcoholism, the hepatitis virus, and CCl_4, which are health hazards and can ultimately result in liver cancer. These damaging factors can induce liver diseases such as hepatitis, liver fibrosis (LF), cirrhosis and hepatocellular carcinoma (HCC).[1] LF is a characteristic of most types of chronic liver disease.[2] A number of factors such as viral infections (hepatitis B and C), alcoholism, autoimmune diseases and non-alcoholic fatty liver disease may cause LF.[3] It has been reported that the activation of hepatic stellate cells (HSCs) is a central link in LF.[4] LF has been reported to be a reversible injury-repair response, and some scholars believe that LF can be repaired by inhibition of activated HSCs.[5]

In Chinese medicine (CM), no definite name exists for the disease, but it has been categorized as "Xiongxie pain" (chest and rib-side pain) according to its clinical manifestations and is thought to be caused by the improper diet, emotional internal injuries or long-term jaundice. CM theory and clinical experience suggest that the pathogenesis of LF involves "qi stagnation, blood

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stasis" and "faint vital qi of the body",[6] and the treatments involve "promoting blood circulation" plus "supplementing vital qi".[7] In recent years, CM has shown great promise in the treatment of LF.[8,9] For example, it has been proved that Huagan Tongluo Recipe (化肝通络方) could dose-dependently reduce liver damage and improve LF, probably through anti-inflammatory mechanisms and the inhibition of HSCs activation.[10] Shengmai Injection (生脉注射液, SMI), extracted from Shengmai Powder (生脉散), has the effects of supplementing qi, nourishing yin, recovering pulse, and stopping abnormal sweating.[11] Previous research indicated that SMI combined with Puerarin Injection could improve fibrosis indexes of cirrhotic patients.[12]

N-myc downstream-regulated gene 2 (NDRG2, GenBank: AF159092), a new member of the NDRG family, involved in cell growth, differentiation, stress and hormonal responses,[13-15] has been reported as a potential tumour suppressor gene.[16] In clinical specimens, HCCs have low or undetectable levels of NDRG2 compared to normal adjacent tissue.[17] The expression of NDRG is decreased during the activation of HSCs. Moreover, over-expression of the NDRG2 gene could inhibit the activation of HSCs, which might be involved in the pathogenesis of LF.[18]

The LX-2 cell line, a stable and sustainable source of human HSCs, has been widely characterized as an optimal cell-based model for studies of LF.[19] This preliminary study explored the effects of SMI on the proliferation, apoptosis and NDRG2 expression in varying densities of LX-2 cells *in vitro*.

METHODS

Drugs and Reagents

SMI (lot No.13070121), composed of *Panax Ginseng* (0.1 g/mL), *Radix Ophiopogonis* (0.312 g/mL), and *Fructus Schisandrae Chinensis* (0.156 g/mL), was produced by Yibin Pharmaceutical Co. (Sichuan, China). The 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco (Ohio, USA). Antibodies directed against NDRG2 were purchased from Abcam (Cambridge, UK). The antibodies directed against β-actin, phenylmethylsulfonyl fluoride (PMSF), sodium fluoride, sodium chloride, and sodium phosphate were purchased from the Beyotime Institute of Biotechnology (Xi'an, China). The horseradish peroxidise (HRP)-conjugated secondary antibodies were purchased from Boster (Wuhan, China).

Cell Culture

LX-2 cells were obtained from the Cell Engineering Research Center of The Fourth Military Medical University (China). LX-2 cells were cultured at 37 ℃ in 5% CO_2 using high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL). Cultures were placed in a humidified atmosphere of 5% CO_2 at 37 ℃, and the medium was changed once per day. When the cell density reached 80%–90%, the cells were treated with 0.25% trypsin for approximately 2–3 min and passaged.

Drug Concentration

The extracellular fluid in a 50-kg person who is 170 cm in height accounts for 20% of their weight, i.e., 10 L. In humans, the administration and dose of SMI is 60 mL once per day. Therefore, the drug concentration in the body is 0.6 μL/mL. The following concentration gradients for SMI were used: 0.6, 1.2, 2.4, 4.8 and 6 μL/mL.

MTT Proliferation Assay

The inhibitory effect of SMI on growth was evaluated in LX-2 cells using an MTT assay. LX-2 cells were seeded at a density of 2.5×10^4 cells/mL in 96-well plates in the growth medium (100 μL) after centrifugation at 1,000 r/min for 5 min and then cultured for 48, 72, 96 or 120 h. Then, the medium was changed, and SMI was added to each well at a final concentration of 0.6, 1.2, 2.4, 4.8 or 6 μL/mL. Four replicates plus a negative control were performed for each concentration. After SMI treatment for 24 or 48 h, 20 μL of 5 mg/mL MTT was added and incubated at 37 ℃ for 4 h. Then, the formazan crystals were dissolved, and 180 μL of dimethyl sulfoxide (DMSO) was added. After shaking the 96-well plate at room temperature for 10 min, the absorbance (OD) of the plate was measured at a wavelength of 490 nm. The proliferation inhibition rate (%)= (1–absorbance of the experimental group/absorbance of the control group) × 100%. Each treatment was performed in triplicate.

Observation of Cell Growth by Light Microscopy

LX-2 cells in the logarithmic growth phase were centrifuged at 1,000 r/min for 5 min, and a total of 100 μL of cell suspension containing approximately 2.5×10^4 cells/mL was plated in 96-well plates and cultured for 48, 72, 96 or 120 h. Different concentrations

of the SMI (0.6, 1.2, 2.4, 4.8 or 6 μL/mL) were applied at the 4 time points listed above. Four replicates plus a negative control were performed for each concentration. Twenty-four hours after applying SMI, the medium was changed, and the cell state was observed using a light microscopy (DMI4000B, Leica, Germany).

Apoptosis Measurement by Flow Cytometry

The apoptosis of LX-2 cells was detected by flow cytometry. Propidium iodide (PI)-annexin-V staining was performed using an Annexin-V-FLUOS staining kit (Roche Diabetes Care GmbH, Swizerland) according to the manufacturer's instructions. Briefly, LX-2 cells were seeded onto 6-well plates (5×10^6 cells/mL) and treated with SMI at different concentrations (0.6, 1.2, 2.4, 4.8 or 6 μL/mL). After 24 h, the LX-2 cells were digested with 0.25% trypsin containing 0.02% ethylene diamine tetraacetic acid (EDTA), harvested, and washed twice with cold phosphate buffer solution (PBS). Then, apoptosis was measured using a FACSAria flow cytometer (Becton Dickinson Co., USA) following the manufacturer's instructions.

Western Blot Analysis

Western blot was used to evaluate the expression of protein in LX-2 cells. Cells were treated with SMI (0.6, 1.2, 2.4, 4.8 or 6 μL/mL for 24 h, washed twice in ice-cold PBS, lysed in 100 μL radio-immunoprecipitation assay (RIPA) buffer containing 1% PMSF and 2% protease inhibitor on ice for 20 min, centrifuged at 12,000 r/min for 15 min at 4 ℃, and then the supernatant was collected. The amount of protein in the supernatant was measured using a bicinchoninic acid assay (BCA). The proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking for 1 h with 5% milk, the membranes were incubated with anti-NDRG2 and anti-β-actin antibodies (1:1000) at 4 ℃ overnight. After washing 3 times with Tris-buffered saline containing 0.1% Tween 20 (TBST), the cells were then incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. After washing 3 times with TBST, the protein bands were detected using ChemiDOTMXRS and analysed with Quantity One software (v4.6.7, Bio-rad, California, USA).

Statistical Analysis

All values were presented as mean ± standard deviation ($\bar{x} \pm s$). Statistical analyses of differences between two groups were tested by Student's *t*-test (SPSS Inc., Chicago, IL, USA). Statistical comparisons between multiple groups used one-way analysis of variance (ANOVA) or one-way repeated ANOVA (followed by LSD post-hoc test). $P<0.05$ was considered statistically significant.

RESULTS

MTT Assay

When the cells grew for 48, 72, 96 and 120 h, high concentrations (4.8 and 6 μL/mL) of SMI significantly inhibited cell proliferation in a concentration- and time-dependent manner at 24 or 48 h after treatment ($P<0.05$). A moderate concentration (2.4 μL/mL) of SMI also inhibited cell proliferation at 24 h after treatment when cell growth for 48 h ($P<0.05$) and at 48 h after treatment when cell growth for 72, 96 and 120 h ($P<0.05$, Figure 1).

Cell Growth State

As shown in Figure 2, when the cells grew for 48 h, each concentration of SMI (0.6, 1.2, 2.4, 4.8 or 6 μL/mL) inhibited cell proliferation at 24 h, and pyknosis, condensation and cell volume were rapidly decreased. When the cells grew for 96 and 120 h, obvious cell apoptosis morphology was observed only at high SMI concentrations (4.8 and 6 μL/mL).

Apoptosis Assay

As shown in Figure 3, the lower-left quadrant (B3) represents living cells, the upper-left quadrant

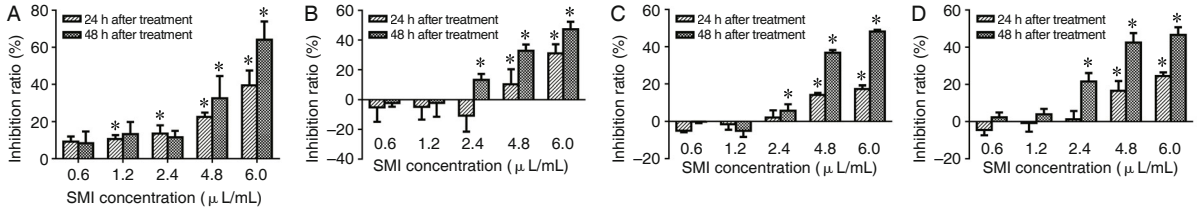

Figure 1. Effect of Different Concentration of SMI on Proliferation of LX-2 Cells

Notes: SMI: Shengmai Injection. A–D: Cell growth for 48, 72, 96 and 120 h, respectively. *$P<0.05$ *vs*. the last concentration at the same time point

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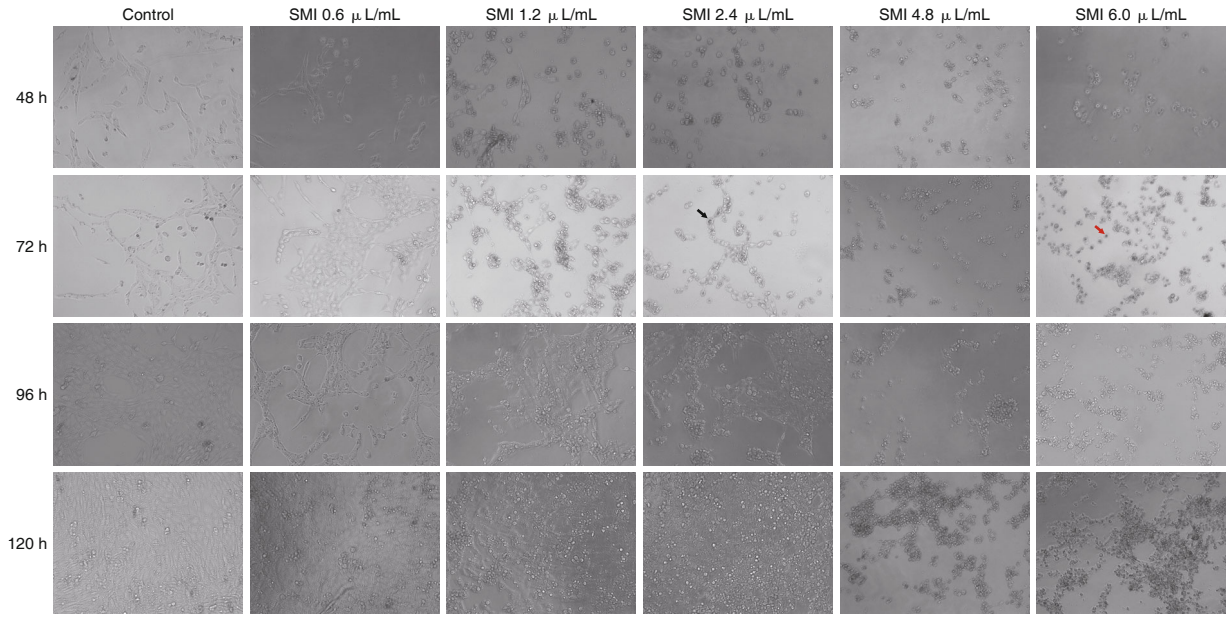

Figure 2. LX-2 Cell Growth State after Treatment with SMI for 24 h under Light Microscopy (×20)

Notes: SMI: Shengmai Injection. LX-2 cells were found in different degrees of cell shrinkage, some nucleus pycnosis (red arrow) and chromatin condensation (black arrow)

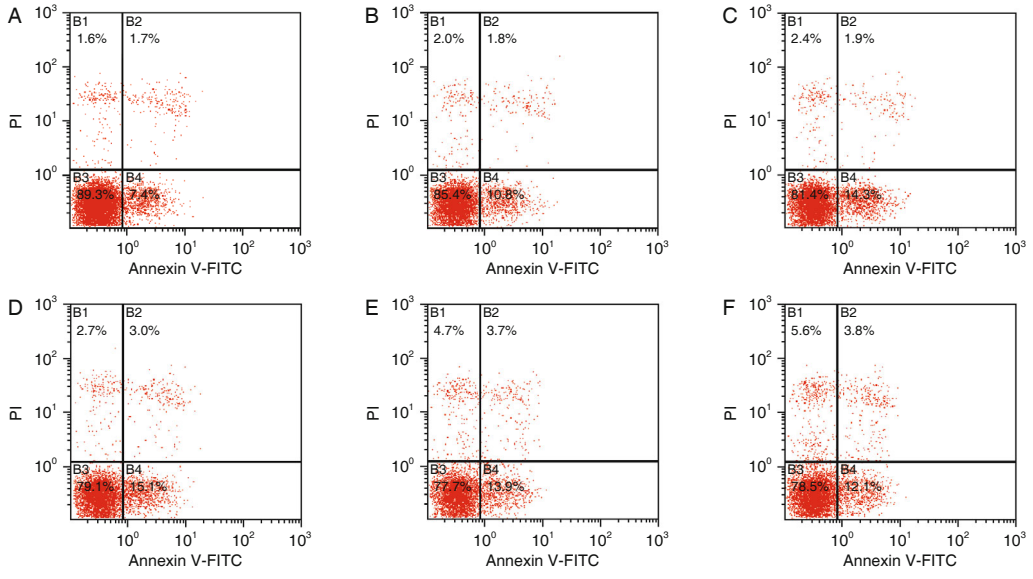

Figure 3. Apoptosis of LX-2 Cells Treated with SMI for 24 h

Notes: SMI: Shengmai Injection; PI: propidium iodide. A: negative control group; B–F: 0.6, 1.2, 2.4, 4.8, 6 μL/mL of SMI, respectively

(B1) represents dead cells, the upper-right quadrant (B2) contains late apoptotic cells, and the lower-right quadrant (B4) indicates early apoptotic cells. Respective percentages of these cells arising from apoptosis and necrosis (B2+B4+B1) after treatment with SMI (0.6, 1.2, 2.4 and 4.8 μL/mL) for 24 h were 14.6%, 18.6%, 20.8% and 22.3%, respectively. SMI induced apoptosis in a concentration-dependent manner in LX-2 cells at 24 h.

Western Blot

The level of NDRG2 was greatly increased by SMI (1.2, 2.4, 4.8 or 6 μL/mL for 24 h, $P<0.05$), while the level of β-actin was unaffected (Figure 4).

DISCUSSION

LF is a public health problem, and its pathogenesis is complex. It is caused by inappropriate tissue repair via connective tissue deposition, e.g., metabolic imbalance or synthesis over-degradation in the liver extracellular matrix (ECM).[20] ECM is formed by the complex network of proteins and sugars surrounding cells in all solid tissues, including

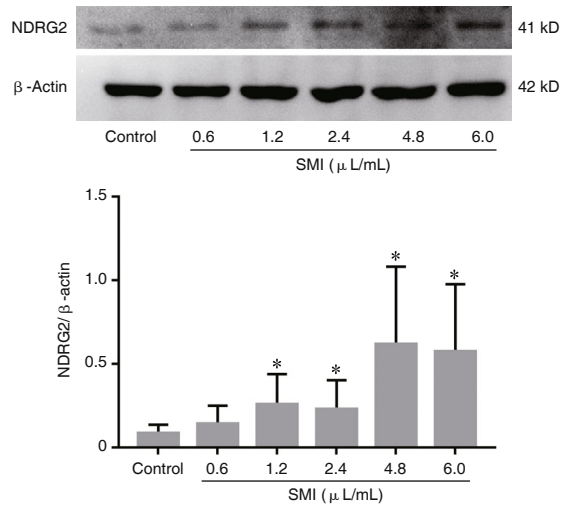

Figure 4. NDRG2 and β-Actin Expressions in LX-2 Cells Treated with SMI for 24 h

Notes: SMI: Shengmai Injection; NDRG2: N-myc downstream-regulated gene 2. *P<0.05 *vs*. control group

collagen types Ⅰ, Ⅲ and Ⅳ, fibronectin, elastin, laminin, and proteoglycans.[21] HSCs are the main source of ECM. The activation of HSCs plays an important role in the occurrence and progression of LF. In addition, hepatocyte apoptosis and/or necro-apoptosis, sustained hepatic inflammation, activation of Kupffer cells and recruitment of monocytes/macrophages are key contributors to the LF process.[22] Studies have shown that NDRG2 can mediate liver regeneration by regulating the cell cycle and apoptosis.[23] It also plays a critical role in HCCs by inhibiting ECM-based, Rho-driven tumour cell invasion and migration.[17]

SMI is composed of *Panax Ginseng*, *Radix Ophiopogonis* and *Fructus Schisandrae Chinensis*. Modern pharmacological research reveals that *Panax Ginseng* can enhance superoxide dismutase activity, scavenge oxygen free radicals, inhibit lipid peroxidation, stabilize and protect lysosomal membranes and enhance the body's immunity.[24] *Radix Ophiopogonis* has the ability to dilate the blood vessels, improve microcirculation and anoxaemia.[25] *Fructus Schisandrae Chinensis* can improve liver detoxification functions, promote liver regeneration and protect liver cell membranes.[26] SMI could not only inhibit the LX-2 cells proliferation, but also mitigate myocardial ischaemia and reperfusion injury in rats.[27] What is more, SMI could provide protection against cerebral ischemia/reperfusion injury and appeared to be more effective in ameliorating quality of life and reducing adverse reactions of chemotherapy in patients with non-small cell lung cancer.[28,29]

In this study, we found that when the LX-2 cells grew for 48, 72, 96 and 120 h, 4.8 or 6 μL/mL of SMI inhibited cell proliferation at 24 and 48 h after treatment (P<0.05), and 2.4 μL/mL of SMI also inhibited cell proliferation at 24 h after treatment when cell growth for 48 h (P<0.05) and at 48 h after treatment when cell growth for 72, 96 and 120 h (P<0.05), indicating that the drug concentration and different cell densities must be considered. In addition, we examined the apoptosis rate of LX-2 cells using flow cytometry at 24 h after SMI treatment. It was found that SMI could lead to LX-2 cell apoptosis. Finally, to determine whether NDRG2 was involved in the apoptosis caused by SMI, we detected the expression of NDRG2 by Western blot and found that SMI could increase the expression of NDRG2, which might act as an anti-hepatic fibrotic mechanism.

In conclusion, this study confirmed that SMI inhibited LX-2 cell proliferation and induced its apoptosis. Over-expression of NDRG2 might be involved in the process. Further study of this mechanism could be beneficial to clinical treatment.

Conflict of Interest

The authors claimed no potential conflicts of interest relevant to this article.

Author Contributions

Zhang Y, Ma LT, Zheng J and Li J designed and performed the study and wrote the manuscript. Qiao Y, Liu JY, Wang J, Ren QY, Liu Y and Hu JT performed the study. All of the authors read and approved the manuscript.

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