

Effects of Si-Jun-Zi decoction polysaccharides on cell migration and gene expression in wounded rat intestinal epithelial cells

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Si-Jun-Zi decoction (SJZD), a traditional Chinese herbal prescription, has been used clinically for treating patients with disorders of the digestive system. Previous studies indicated that the polysaccharides of SJZD (SJZPS) are the active components contributing towards its pharmacological effects in improving gastrointestinal function and immunity. However, the protective and restitutive effects on intestinal epithelial cells remain unknown. In the present study, SJZPS were first extracted and chemically characterized. Then their stimulatory and restitutive effects on intestinal epithelial cells (IEC-6 cells) were elicited by different *in vitro* models including migration of wounded IEC-6 cells and cell proliferation. Results indicated that SJZPS not only protects the cells against the harmful impairment of indomethacin but also enhances re-epithelialization of a wounded monolayer at an optimal dose of 100 µg/ml at 24 h incubation. To elucidate the modulatory effect of SJZPS on wounded IEC-6 cells at the molecular level, an oligonucleotide microarray was employed to study differential gene expression of SJZPS-treated IEC-6 cells and the candidate genes were validated by RT-PCR. There was increased expression of genes coding for ion channels and transporters, which are critical to cell migration and restoration of wounded intestinal cells, suggesting a possible mechanism for re-epithelialization. In conclusion, our data show for the first time that SJZPS can enhance intestinal restitution and protect against indomethacin-induced damage of intestinal epithelial cells. These findings provide new insight into the mechanism of action of a traditional Chinese herbal prescription, SJZD, in intestinal wound restitution.

Si-Jun-Zi decoction: Polysaccharides: IEC-6 cell: Microarray

The intestinal mucosal epithelium plays an important role in preventing translocation of deleterious substances or organisms present within the lumen of the gut and in preserving normal homeostasis (Dignass & Podolsky, 1995). Impairment of the epithelial surface barrier may facilitate the penetration of harmful factors from the intestinal lumen, leading to acute and chronic inflammatory changes caused by the initiation of a complex network of immune responses. The inability to repair damage to the intestinal mucosal epithelium is a common physiological event that may account for numerous gastrointestinal diseases or disorders, such as infectious diarrhoea, gastrointestinal ulcer and inflammatory bowel disease. Indeed, the intestinal epithelium exerts enormous regenerative capabilities to allow rapid healing of injury by at least two different processes: migration and proliferation. Re-establishment from superficial injury can be achieved initially within minutes to hours by the migration of viable epithelial cells adjacent to or just beneath the injured surface to cover the denuded area, a process termed epithelial restitution or re-epithelialization (Lacy, 1988; Moore *et al.*

1989; McCormack *et al.* 1992). After sealing of the epithelial surface to regain its integrity, epithelial cell proliferation takes place to replenish the decreased cell pool (Dignass & Podolsky, 1995).

Around 50% of the Western population relies on herbal medicines for the treatment or prevention of digestive disorders (Langmead & Rampton, 2001). Si-Jun-Zi decoction (SJZD), a traditional Chinese herbal prescription, is well known for treating disorders of digestive function manifested by poor appetite, indigestion and watery stools or diarrhoea. It also has been used as a tonic supplement for health maintenance. It is formulated from four Chinese herbs: Dangshen (*Codonopsis pilosula* (Franch.) Nannf); Gancao (*Glycyrrhizae uralensis* Fisch.); Baizhu (*Atractylodes macrocephala* Koidz.); Fuling (*Poria cocos* (Schw.) Wolf), of which the polysaccharides are considered the main active components contributing towards its therapeutic effects. *In vivo* studies have shown that SJZD can improve intestinal mucosal immunofunction by counteracting the adverse effect of cyclophosphamide on intestinal mucosal-associated lymphoid

Abbreviations: ASIC1, acid-sensing ion channels, proton-gated cation channels; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *JUND*, JunD proto-oncogene; Kv4, neuronal delayed rectifier K⁺ channel; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBK, phosphorylase kinase catalytic subunit; RCK4, putative potassium channel subunit protein; RIIA, type I regulatory subunit of cAMP-dependent protein kinase; SJZD, Si-Jun-Zi decoction; SJZPS, polysaccharides of SJZD.

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tissues of mice (Liu *et al.* 2000); improve intestinal disturbances to alleviate diarrhoea in rats caused by radiation (Chen & Fu, 1996); and ameliorate intestinal injury in scalded rats and prevent the translocation of intestinal bacteria (Guo *et al.* 2003). As SJZD is used clinically for treating patients with gastrointestinal disorders, some clinical studies have indicated that SJZD is effective in treating chronic gastritis and intestinal metaplasia (Zhong *et al.* 1997; Langmead & Rampton, 2001).

With the advancement of molecular medicine, more evidence-based and scientific support has accumulated to strengthen the scientific foundation and promote the modernization of traditional Chinese medicine. Genome-wide expression monitoring of genes by microarray analysis seems to provide a holistic approach to examine the biochemical effects of Chinese medicines. This functional genomic approach has successfully revealed the possible mechanisms of action for some herbal extracts such as *Ginkgo biloba* (Watanabe *et al.* 2001), *Coptidis* rhizome (Iizuka *et al.* 2003), a preparation of eight different herbs (Bonham *et al.* 2002) and four other Chinese herbal extracts (Sadava *et al.* 2002). Recently, the mechanism of SJZD in human gastric cancer grafted onto nude mice was studied at the gene expression level (Zhao *et al.* 2002). Since migration of intestinal epithelial cells is the principal force behind early restitution of mucosal injury and the mechanism of action of the polysaccharides of SJZD (SJZPS) on intestinal function has not been elucidated, the aim of the present study was to characterize the effects of SJZPS on intestinal epithelial wound healing (restitution) *in vitro*. The non-transformed rat small intestinal epithelial cell line IEC-6 was used in this study because it is a common cell model for demonstrating the process of migration during early mucosal restitution (McCormack *et al.* 1992; Dignass & Podolsky, 1995; Cario *et al.* 2000). The restitutive effect of SJZPS in the wound healing assay and its stimulatory and protective effects against indomethacin-induced damage in a proliferation assay were investigated. Furthermore, to elucidate the molecular mechanism of the modulatory effect of SJZPS on wounded IEC-6 cells, gene expression profiling with an oligonucleotide microarray was employed.

Materials and methods

Sources of IEC-6 cells and reagents

The intestinal epithelial cell line (IEC-6 cells; ATCC CRL 1592) was purchased from the American Type Culture Collection (Rockville, MD, USA) at passage 14. This IEC-6 cell line originated from intestinal crypt cells, as judged by morphological and immunological criteria. The stock culture was maintained in a T-150 flask in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 µg insulin/ml and 50 µg gentamicin sulphate/ml. The stock culture was incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The cells were sub-cultured once per week at a dilution of 1:20 and the medium was changed three times per week. In the experiments, cells at passage 15–20 were taken up with 0.25% (w/v) trypsin/1 mM-EDTA in Hanks' basic salt solution without Ca and Mg. All chemicals used in the study were purchased from Sigma (St. Louis, MO, USA) unless specified otherwise. DMEM, FBS, PBS, antibiotics, insulin and sodium pyruvate were obtained from Gibco (Invitrogen Life Technologies, Carlsbad, CA, USA).

Extraction and characterization of polysaccharides from Si-Jun-Zi decoction

SJZD was derived from four herbs, Gancao (*G. uralensis* Fisch.), Dangshen (*C. pilosula* (Franch.) Nannf), Baizhu (*A. macrocephala* Koidz.) and Fuling (*P. cocos* (Schw.) Wolf), in a ratio of 1:2:2:2 by weight. SJZD was used for the preparation of SJZPS as described previously (Ou, 1993; Chun *et al.* 2002) with modification. Dried herbs (70 g) were soaked in distilled water for 30 min at room temperature and then extracted twice in boiling distilled water at 1:8 w/v for 4 h. The fractions were combined and concentrated by rotary evaporation. The supernatant was recovered by centrifugation and then dialysed against running water for 2 d. The extract was precipitated with ethanol to a final concentration of 60% (v/v) at 4°C overnight and the precipitate was pelleted by centrifugation. Then, the supernatant was concentrated by rotary evaporation, another ethanol precipitation performed to a final concentration of 80% (v/v) and the precipitate obtained by centrifugation. The pellets from the two ethanol precipitations were combined, dissolved in distilled water and deproteinated by the Sevag method (Staub, 1965). The crude polysaccharide precipitate was extracted by shaking vigorously with five volumes of chloroform–n-butanol (4:1, v/v) for 20 min. The extraction was repeated five more times and the polysaccharides were recovered by ethanol precipitation as stated earlier. Finally, the SJZPS were dissolved in distilled water and sterilized by filtration through a 0.2 µm membrane.

The sugar content of the SJZPS was determined by colorimetry with 3,5-dinitrosalicylic acid (Miller, 1959). Glucose was used as standard. The total protein content of the deproteinated polysaccharides was assayed by the Lowry method using bovine serum albumin as protein standard (Lowry *et al.* 1951). The lipopolysaccharide content of SJZPS was determined by the *Limulus* amoebocyte lysate kinetic assay. The sugar composition of SJZPS was analysed by HPLC after acid hydrolysis. Briefly, polysaccharides (200 mg) were hydrolysed with 2 M-H₂SO₄ at 100°C for 4 h. The depolymerized polysaccharides containing the neutral sugars were neutralized with NaB(OH)₄ to pH 7. The supernatant was collected by centrifugation and concentrated before HPLC analysis using an Alltech Prevail Amino column (0.3 µm, 2.1 mm × 150 mm) and Alltech 2000 ELSD detector (Alltech Associates, Inc., Deerfield, IL, USA). Column temperature was maintained at 25°C. Degassed acetonitrile–distilled water (9:1 v/v) was used as the eluent at a flow rate of 0.3 ml/min.

Wounding assay

The wounding assay was performed as described previously (McCormack *et al.* 1992). Briefly, Matrigel was diluted with cool serum-free medium at a dilution of 1:7 (v/v) and coated onto six-well plates at room temperature for 1 h. Excess diluted Matrigel was discarded. After washing twice with cool serum-free medium, the coated plates were ready for wounding assay. Briefly, IEC-6 cells were seeded at a density of 6.25 × 10⁴ cells in six-well plates and cultured in 10% FBS-supplemented DMEM for 24 h. Then, the cells were washed with PBS and further cultured for another 24 h in serum-free medium. To initiate migration, the cell monolayer was scratched with a razor blade followed by washing with serum-free medium. Fresh serum-free medium containing SJZPS to a final concentration of 50, 100, 200, 400 or 800 µg/ml was then added and recovery of the wounded monolayers due to cell migration towards the denuded area was evaluated at intervals of

8, 16 and 24 h. The area covered by the migrating cells was measured with an inverted phase-contrast microscope (model TMS; Nikon Corporation, Tokyo, Japan) and the images analysed using Motic Images Plus 2.0 software (Motic Instruments Inc., Richmond, Canada). The time-dependent effect of SJZPS on wounded IEC-6 cells expressed as migration rate compared with the medium as control:

$$\text{Migration rate} = [(\text{Treatment}_{\text{area}} - \text{Control}_{\text{area}}) / \text{Control}_{\text{area}}] \times 100 \%$$

Determination of IEC-6 cell proliferation

The dose effect of SJZPS on IEC-6 cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Mosmann (1983) with modification. Briefly, IEC-6 cells were seeded at a density of 1×10^4 cells/well in 96-well plates and cultured in DMEM supplemented with 10% FBS for 24 h. Then, cells were washed with PBS and cultured for a further 24 h in serum-free medium. The cultures were then supplemented with serum-free medium containing sterilized SJZPS at a final concentration of 50, 100, 200, 400 or 800 $\mu\text{g/ml}$ and subsequently incubated at 37°C for 24 h. Two sets of positive control experiments were performed in parallel: one using 20 ng epithelial growth factor/ml and the other 10% FBS alone. Cell proliferation was measured using the Cell Proliferation Kit I (Roche Diagnostics Corp., Indianapolis, IN, USA) according to the manufacturer's instructions. The colour development from the tetrazolium compound, which is directly proportional to the number of viable cells in culture, was measured spectrophotometrically at 550 nm using an ELISA plate reader (Sunrise; Tecan Instruments, Maennedorf, Switzerland).

To examine the protective effect of SJZPS against indomethacin-induced damage of IEC-6 cells, indomethacin (40 μM) and various concentrations of SJZPS (50, 100 and 200 $\mu\text{g/ml}$) were co-incubated with IEC-6 cells that had been starved for 24 h in serum-free medium. After further incubation for 24 h, the extent of cell proliferation was determined using the Cell Proliferation Kit I (Roche Diagnostics Corp.). All data are expressed as percentage relative viability: Relative viability (%) = $(\text{OD}_{\text{experiment}} / \text{OD}_{\text{control}}) \times 100 \%$, where OD is optical density.

Gene expression profiling analysis

IEC-6 cells (6.25×10^4 cells) were seeded in 10 cm Matrigel-coated Petri dishes and incubated in DMEM supplemented with 10% FBS in a humidified atmosphere of 95% air and 5% CO_2 . After 72 h, the cells became confluent, the medium was replaced by serum-free DMEM and incubation was continued for a further 24 h. Confluent cells were wounded vertically, horizontally and diagonally using a multi-channel pipette with attached yellow tips (eight channels). The distance between each line of denuded area was about 0.9 cm. Cells were washed with PBS. SJZPS, at a final concentration of 100 $\mu\text{g/ml}$, was added and the cells were incubated for another 24 h. A control was given the same wounding but without SJZPS treatment. The same batch of IEC-6 cells at passage 18 was used for three independent sets of microarray experiments.

Total cellular RNA was isolated from the control and SJZPS-treated IEC-6 cells using the Atlas™ Glass Total RNA Isolation Kit (Clontech, Palo Alto, CA, USA) according to the

manufacturer's instructions. The quantity and quality of RNA were determined on the basis of spectrometric measurements (260 and 280 nm) and electrophoresis in 1.0% Tris–boric acid–EDTA agarose gels, respectively.

cDNA probes for microarray analysis were prepared using the Atlas™ PowerScript™ Fluorescent Labeling Kit (Clontech). Briefly, 8 μg total RNA isolated from control and SJZPS-treated cells were primed separately with 2 μl CDS (cDNA synthesis) primer mix and 1 μl cDNA synthesis control, incubated at 70°C for 5 min in a thermal cycler (PTC-100®; MJ Research, Inc., Watertown, MA, USA) and cooled to 48°C. Then, a master mix containing 4 μl 5× first-strand buffer, 2 μl 10× dNTP, 2 μl dithiothreitol (100 mM), 1 μl deionized water and 1 μl PowerScript Reverse Transcriptase was mixed with the primed RNA and incubated at 48°C for 1 h. The reaction was stopped by heating at 70°C for 5 min. Then, the tubes were cooled to 37°C and 0.2 μl RNase H (10 U/ μl) was added, followed by an additional incubation at 37°C for 15 min. The reaction was then stopped by adding 0.5 μl 0.5 M-EDTA, pH 8.0 and the transcribed cDNA was purified by QuickClean™ Purification Resin (Clontech). Newly synthesized cDNA was precipitated with sodium acetate–absolute ethanol at –20°C for 1 h and collected by centrifugation at 12 000 rpm for 20 min. cDNA pellets were washed once with 70% ethanol and re-suspended in 10 μl 2× fluorescent labelling buffer (Clontech). Then the control and SJZPS-treated cDNAs were mixed with 0.5 μl coupling reaction control oligos (Clontech) and subsequently coupled with 10 μl 5 mM-Cy5 and Cy3 reactive dyes (Amersham Pharmacia, Piscataway, USA, NJ), respectively, at 24°C for 1 h. The labelled probes were precipitated and collected by the same procedure as mentioned earlier. Finally, the pellets were dissolved in 100 μl sterile water (Clontech). Unincorporated reactive dye and other impurities were removed on a Microcon YM-30 column (Millipore, Billerica, MA, USA).

Cy5 (control) and Cy3 (SJZPS-treated) labelled probes were combined and mixed with GlassHyb hybridization solution (Clontech) in the ratio suggested by the company. Finally, the probe mixture was hybridized at 50°C overnight to the Atlas™ Glass Rat 1.0 Microarray (Clontech), which includes 1081 rat genes (80-mer oligo array), nine housekeeping genes, negative and positive controls. Thereafter, the microarrays were washed once in GlassHyb washing solution (Clontech), twice in GlassHyb washing solution diluted with 1× standard saline citrate and once in GlassHyb washing solution diluted with 0.1× standard saline citrate; each washing was for 10 min at room temperature. The hybridized arrays were spun dry prior to scanning.

Hybridized arrays were scanned using the ScanArray 5000 confocal laser scanner (Packard BioChip/GSI Lumonics, Billerica, MA, USA). To get the best-fit image, laser power and PMT (photomultiplier tube) voltage were adjusted manually to minimize background and saturated spots. Cy5 and Cy3 signal intensities of each spot were obtained using QuantArray Analysis Software (Packard BioChip/GSI Lumonics). Comparisons were made between the normal (Cy5) and SJZPS-treated IEC-6 cells (Cy3) from the same microarray. The signal intensities of each spot were corrected by subtracting local background signals. Then the Cy3 and Cy5 signal intensities of each spot were further normalized by taking the total averaged signal intensities across all spots in the microarray to be equal for both channels (Hegde *et al.* 2000). To eliminate any unreliable data, spots were screened by first setting a threshold of minimum acceptable signal

(background plus two standard derivations) to eliminate expression ratios of extremely high or low spots due to an undetectable signal in either channel and, second, performing visual inspection to eliminate any irregular spots. The genuine spots were subjected to calculation for the fold change (Cy3: Cy5). Differential gene expression was considered up- or down-regulated if the averaged ratio (Cy3: Cy5) between the two conditions was > 1.5 or < -1.5 , respectively.

Validation of candidate genes by semi-quantitative RT-PCR

Semi-quantitative RT-PCR was used to verify microarray data of the selected genes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used to compare and monitor the efficiency of cDNA synthesis between different samples. The primers were as follows: GAPDH sense primer AAG ATT GTC AGC AAT GCA TCC, antisense primer ACA GTC TTC TGA GTG GCA GTG A; phosphorylase kinase catalytic subunit (PBK) sense primer TCA CAG AAA CCA GCC CTT TAC C, antisense primer CAG GCG GTG ATG GCA AGG; JunD proto-oncogene (*JUND*) sense primer ACC CTC AAA AGC CAG AAC ACC, antisense primer CGC TCG GGC TCA ATA CGC; putative potassium channel subunit protein (RCK4) sense primer ATG CTG AAG CCC GAG AGA GAC, antisense primer TCG TGT GCT GTT ATC TGT GTG C; type I regulatory subunit of cAMP-dependent protein kinase (RIIA) sense primer TGG TGA AAT TGC CCT GCT GAT G, antisense primer ACT GCT GGA TGT TTC GCT TGA G; neuronal delayed rectifier K⁺ channel (Kv4) sense primer TTC ACA CTG CCA CCC TCT TCC, antisense primer TGG TCA CAC TGG AGC TAC ACA C. RT-PCR was carried out according to the manufacturer's instructions. Briefly, cDNA templates for RT-PCR were synthesized from 1 µg total RNA using the ThermoScript™ RT-PCR system (Invitrogen) and oligo-dT primer. PCR mixtures (final volume 50 µl) contained 100 ng cDNA template, 0.2 µM of each primer, 0.2 mM of each dNTP, 1 × *Taq* buffer (Pharmacia), 1.5 mM-MgCl₂ and 1 U *Taq* DNA polymerase (Pharmacia). The reactions went through a touchdown cycle of 94°C for 5 min; then fifteen cycles at 94°C for 1 min, 65°C for 1 min (minus 1°C per cycle) and 72°C for 1 min; then fifteen to twenty cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min; and a final extension at 72°C for 10 min. Reactions were performed in a PTC-200® thermal cycler (MJ Research Inc.). PCR products were analysed by electrophoresis in 1.5% agarose gel and Kodak 1D Image Analysis Software (Rochester, NY, USA) was used for the densitometric analysis. Due to variations in abundance of the different mRNA in the cell, the PCR cycles (Tsien & Tsien, 1990; McCormack *et al.* 1998) and different titrations of the RT reaction (equal to 100 ng, 500 ng and 1 µg total RNA) were performed in duplicate to ensure that the PCR reaction was in the linear range.

Statistical analysis

Data are expressed as mean and standard deviation. Significance of differences among groups (e.g. among different concentrations of SJZPS and the control) were tested by one-way ANOVA. Dunnett's *post hoc* procedure was used for *post hoc* testing; Dunnett's test compares the mean of each treatment group (e.g. SJZPS-treated cells) with that of a designated control. The effect of both incubation time intervals (time-dependent effect) and SJZPS concentration (dose-dependent effect) on cell migration was analysed

by two-way ANOVA, followed by a multiple *post hoc* test using least significant difference. Differences were considered significant if $P < 0.05$. SPSS 10.0 software (SPSS Inc., Chicago, IL, USA) was used to perform all calculations.

Results

Extraction and characterization of polysaccharides from Si-Jun-Zi decoction

The total polysaccharides from SJZD were successfully extracted and chemically characterized. As shown in Table 1, SJZPS were composed of 51.82% total sugar and 37.5% protein; no lipopolysaccharides were detected. In terms of sugars, the composition of SJZPS was glucose (44.61%), galactose (33.96%), arabinose (10.85%), rhamnose (5.26%), xylose (4.84%), mannose (0.46%) and fructose (0.02%) as determined by HPLC analysis. These results reveal that glucose and galactose are the main components of SJZPS.

Effect of polysaccharides from Si-Jun-Zi decoction on cell migration of wounded IEC-6 cells

A cell migration assay was used to mimic the early stage of intestinal epithelial restitution *in vitro*, in which the migration rate of IEC-6 cells after wounding was determined (Table 2). In addition, the dose effect of SJZPS and the incubation time for re-epithelialization of wounded IEC-6 cells were evaluated. Concerning the time effect, significant re-epithelialization of the wounded IEC-6 cells was observed 24 h after the addition of medium as control or SJZPS. However, the migration rate in the control group was much lower than that in the SJZPS-treated groups. Concerning the dose effect, SJZPS at a final concentration of 100 µg/ml was the optimal dose for stimulation of epithelial cell restitution in terms of cell migration towards the denuded area *in vitro*. Stimulation of epithelial restitution was increased by 38.45, 102.39 and 132.74% at 8, 16 and 24 h, respectively, compared with the control.

Protective effect of polysaccharides from Si-Jun-Zi decoction on IEC-6 cells

We examined the relative viability of IEC-6 cells after treatment with various concentrations of SJZPS ranging from 50 to 800 µg/ml in the proliferation assay (Fig. 1). The results show that SJZPS significantly enhanced the growth of IEC-6 cells by more than 40% at the optimal dose of 100–200 µg/ml. Indomethacin, a common non-steroidal anti-inflammatory

Table 1. The composition and sugar content analysis of the polysaccharides of Si-Jun-Zi decoction

Total sugar (wt %)	51.82
Protein (wt %)	37.50
Lipopolysaccharides	ND
Sugar components (mol %)	
Glucose	44.61
Galactose	33.96
Arabinose	10.85
Rhamnose	5.26
Xylose	4.84
Mannose	0.46
Fructose	0.02

ND, not detected.

Table 2. The restitutive effect of the polysaccharides of Si-Jun-Zi decoction (SJZPS) on the migration of IEC-6 cells upon injury. Various concentrations of SJZPS were used to treat the wounded IEC-6 cells and the migration area for each group of SJZPS-treated cells was monitored at 8, 16 and 24 h. Values are means with their standard deviation from three separate experiments. Migration rate at each time interval is expressed as a percentage of the control

SJZPS concentration ($\mu\text{g/ml}$)	Migration area (mm^2) at different intervals								
	8 h			16 h			24 h		
	Mean	SD	Migration rate (%)	Mean	SD	Migration rate (%)	Mean	SD	Migration rate (%)
Control	141.9	16.5	–	223.1	25.0	–	708.6	148.5	–
50	136.6	23.2	3.8	250.3	11.6	12.2	890.1*	130.5	25.7
100	196.5	33.6	38.5	451.5*	111.8	102.4	1649.2***	307.1	132.7
200	172.1	44.9	21.3	321.2	35.2	44.0	1293.0***	116.8	82.5
400	174.6	8.9	23.1	350.2	62.0	57.0	1485.0***	171.5	109.6
800	204.6***	72.2	44.2	443.4	162.3	98.8	1266.5***	237.6	78.7

Least significant difference test between means (two-way ANOVA): * $P < 0.01$, *** $P < 0.001$ with regard to control values.

drug that causes inhibition of re-epithelialization, was used to study the protective effect of SJZPS on IEC-6 cells (Fig. 2). Indomethacin alone induced nearly 40% inhibition of cell growth in comparison with the control. However, addition of SJZPS at a concentration of 100 $\mu\text{g/ml}$ produced 30% more viable IEC-6 cells than the indomethacin treatment.

Gene expression profiling of IEC-6 cells upon induction with polysaccharides from Si-Jun-Zi decoction

To explore the differential gene expression upon induction with SJZPS, DNA microarray analysis was performed. Wounded IEC-6 cells treated with 100 μg SJZPS/ml for 24 h were subjected to the Atlas Glass Rat 1.0 Microarray, which included nine housekeeping genes, negative and positive controls and a total of 1081 rat genes. Three sets of microarray hybridization experiments were performed, and the genes up- and down-regulated in response to SJZPS induction are shown in Table 3. These genes could be classified into different categories, mainly related to ion channels such as voltage-gated ion channels, gradient-driven transporters, calcium-modulating ion channel and calcium-binding proteins, transcription factors, intracellular

kinase members and G-proteins. In particular, six genes, those coding for *JUND*, phosphorylase B kinase, protein kinase type 1, K^+ voltage-gated channel, K^+ inwardly-rectifying channel and K^+ channel protein CDRK, were considered as up-regulated ($\text{Cy3:Cy5} > 1.5$). Five genes, those coding for γ -aminobutyric acid transporter, solute carrier family 6 (serotonin transport), neural visinin-like Ca^{2+} -binding protein type 2 and 3, and Ras-related GTP-binding protein 4b, were down-regulated ($\text{Cy3:Cy5} < 0.6$) (Table 3). Besides, three additional genes, those coding for K^+ channel 1, acid-sensing ion channels, proton-gated cation channels (ASIC1) and metabotropic glutamate receptor 2, with expression ratio of 1.4-fold were also selected and considered as marginal candidate genes. The expression levels of K^+ channel 1 and ASIC1 proton-gated cation channels were confirmed by RT-PCR.

Confirmation of microarray data by RT-PCR

Among the up-regulated genes found in the microarray study, the following were chosen for confirmation by semi-quantitative RT-PCR: *PBK* (accession no. M73808); *JUND* (accession no. D26307); *RCK4* (accession no. X16002); *RIIA* (accession no. M17086); and *Kv4* (accession no. M68880). Because of the variations in abundance of mRNA of different genes in the cell, the

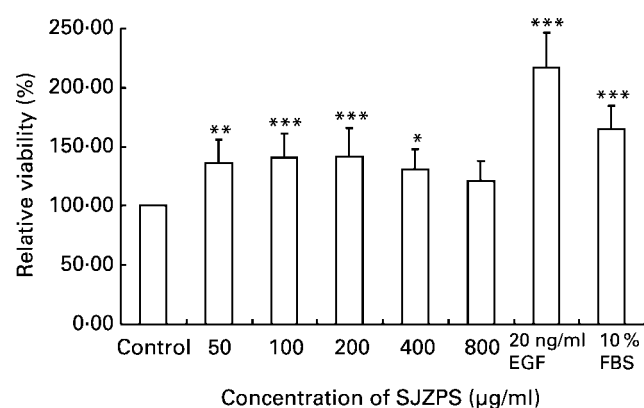


Fig. 1. Dose effect of the polysaccharides of Si-Jun-Zi decoction (SJZPS) on proliferation of IEC-6 cells. 'Control' represents the medium as control; positive controls are 20 ng epithelial growth factor (EGF)/ml and 10% fetal bovine serum (FBS). Values are means and standard deviation shown by vertical bars for three separate experiments. Statistical analysis was performed by one-way factorial ANOVA ($P < 0.0001$) with Dunnett's *post hoc* test. Significant difference v. control medium: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. SJZPS significantly enhances the growth of IEC-6 cells by more than 40% at the optimal dose of 100–200 $\mu\text{g/ml}$.

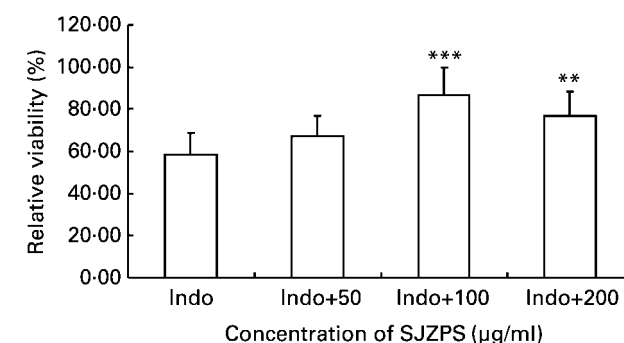


Fig. 2. Mucosal restitution effect of the polysaccharides of Si-Jun-Zi decoction (SJZPS) on indomethacin (Indo)-treated IEC-6 cells. Values are means and standard deviation shown by vertical bars for three separate experiments. Statistical analysis was performed by one-way factorial ANOVA ($P < 0.0001$) with Dunnett's *post hoc* test. Significant difference v. indomethacin treatment: ** $P < 0.01$, *** $P < 0.001$. SJZPS at the concentration of 100 $\mu\text{g/ml}$ shows the best restorative effect on IEC-6 cells, resulting in approximately 86% relative viability.

Table 3. IEC-6 cells seeded in a Matrigel-coated Petri dish were incubated in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum in a humidified atmosphere with 95% air and 5% CO₂ for 72 h, followed by 24 h serum-free incubation. Confluent cells were mechanically wounded with the tip of a multi-channel pipette. Cells were washed with PBS and incubated with 100 µg/ml polysaccharides of Si-Jun-Zi decoction (SJZPS) for 24 h. Total RNA was extracted, reverse-transcribed into cDNA labelled with Cy5 (for control) and Cy3 (for treatment) and hybridized to the Altas™ Glass Rat 1.0 Microarray (Clontech, Palo Alto, CA, USA). The average gene expression ratio of control medium and SJZPS-treated IEC-6 cells from three independent microarray experiments was calculated. Genes with an expression ratio >1.5 or <0.6 were selected as candidate genes. '#' indicates that the gene is a marginal candidate. Genes marked with '*' had their expression level confirmed by RT-PCR

Genes expressed differentially in wounded IEC-6 cells in response to SJZPS induction	GenBank accession no.	Cy3: Cy5
Transcription factors		
<i>JunD proto-oncogene*</i>	D26307	1.7
Intracellular kinase network members		
<i>Phosphorylase B kinase catalytic subunit*</i>	M73808	1.8
<i>Protein kinase, cAMP-dependent, regulatory, type 1*</i>	M17086	1.5
Voltage-gated ion channels		
<i>Potassium voltage-gated channel, shaker-related subfamily, member 4*</i>	X16002	1.5
<i>Potassium inwardly-rectifying channel, subfamily J, member 5</i>	L35771	1.5
<i>Potassium channel protein CDRK</i>	M77482	1.5
<i>Potassium channel 1*†</i>	M68880	1.4
Gradient-driven transporters		
<i>ASIC1 proton-gated cation channel †</i>	U94403	1.4
<i>GABA transporter</i>	U28927	0.6
<i>Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4 (5-hydroxytryptamine (serotonin) transporter)</i>	M79450	0.4
Calcium-modulated ion channels		
<i>Metabotropic glutamate receptor 2 (GRM2; MGLUR2)†</i>	M92075	1.4
Calcium-binding proteins		
<i>Neural visinin-like Ca²⁺-binding protein type 2</i>	D13125	0.6
<i>Neural visinin-like Ca²⁺-binding protein type 3</i>	D13126	0.5
G-protein		
<i>Ras-related GTP-binding protein 4b</i>	X78605	0.5

GABA, γ -aminobutyric acid.

*Genes had their expression level confirmed by RT-PCR.

†Gene is a marginal candidate.

PCR cycles (Tsien & Tsien, 1990; McCormack *et al.* 1998) and titration of the RT reaction (equal to 100 ng, 500 ng and 1 µg total RNA) were performed in duplicate to ensure that PCR was in the linear range (data not shown). The relative expression ratios of selected genes were normalized with regard to ribosomal gene *S29* (Fig. 3). The results indicate that the expression patterns of the selected genes as determined by DNA microarray and RT-PCR were highly comparable.

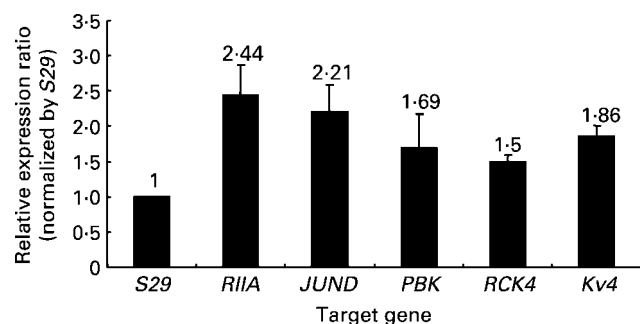


Fig. 3. Confirmation of microarray data by semi-quantitative RT-PCR. cDNA was synthesized from 1 µg RNA in duplicate. The expression ratio for type I regulatory subunit of cAMP-dependent protein kinase (RIIA) was observed at 12 cycles; *JunD proto-oncogene* (*JUND*) and *phosphorylase kinase catalytic subunit* (*PBK*) were observed at 25 cycles; *putative potassium channel subunit protein* (*RCK4*) and *neuronal delayed rectifier K⁺ channel* (*Kv4*) were observed at 35 cycles. Expression of all target genes was normalized by that of the gene coding for ribosomal protein *S29* (*S29*). All genes were found to be up-regulated and the expression patterns were comparable to the microarray data.

Discussion

The use of herbal medicinal decoctions to modulate body homeostasis is a common practice among the Chinese. Si-Jun-Zi decoction, a traditional Chinese herbal prescription formulated by four Chinese herbs (Dangshen, Gancao, Baizhu and Fuling) has been used to treat disorders of the digestive system with symptoms such as poor appetite, indigestion and diarrhoea. Previous studies showed that many of its active constituents, in particular the polysaccharides, shared anti-tumour and immunostimulatory effects (Haranaka *et al.* 1985; Lin, 1988; Xu *et al.* 1994; Liu *et al.* 2000; Chang, 2002; Kiyohara *et al.* 2002). However, the protective effect of the polysaccharides of SJZD on the gastrointestinal epithelium had not been studied previously.

To elicit the gastrointestinal restitution effect of SJZPS, we performed several lines of bioassay. First, the proliferative effect of SJZPS on IEC-6 cells was demonstrated by MTT assay (Fig. 1). It can be inferred from the observations that replacing lost cells through cell proliferation can enhance restitution of damaged epithelial cells. Second, SJZD was found to protect IEC-6 cells from indomethacin-induced injury. Indomethacin, a cyclooxygenase inhibitor, is the most potent of the non-steroidal anti-inflammatory drugs and can cause gastroduodenal mucosal injury, ulceration and delay ulcer healing (Larkai *et al.* 1987). Previous studies showed that the ulcerogenic action of indomethacin could be attributed to the increase of intestinal permeability (Kimura *et al.* 1998), influx of neutrophils (Bjarnason *et al.* 1993) and prostaglandin depletion (Ding *et al.* 1998). Indomethacin has a

pronounced effect on human intestinal mucosal injury (Li *et al.* 1999; McCormack *et al.* 1999), whereas SJZPS can restore the recovery of IEC-6 cells after treatment with indomethacin. This suggests that SJZPS can exert a certain repairing ability in the early phase of mucosal restitution. Third, the gastrointestinal restitution effect of SJZPS was further demonstrated by the *in vitro* migration assay (McCormack *et al.* 1993). Accordingly, replacement of lost cells or tissues is partly achieved by cell migration (Tarnawski *et al.* 1995). In the current study, the confluent monolayer of IEC-6 cells was scratched with a razor blade and the cells were allowed to migrate towards the denuded area. The results indicated that SJZPS could enhance migration of the IEC-6 cells; the most effective dosage was 100 $\mu\text{g/ml}$.

To gain further insight into the modulating effect of SJZPS during intestinal epithelial restitution, DNA microarray technology was applied to decipher the biological pathways involved. Among the 1081 genes profiled in the microarray experiments, the candidate genes that were significantly up-regulated coded for voltage-gated ion channel, voltage-gated K^+ channel, inwardly-rectifying K^+ channel, K^+ channel protein CDRK (Circumvallate Papilla Delayed Rectifier K^+ channel), K^+ channel 1 and gradient-driven transporter (proton-gated cation channel, ASIC1). Previous reports have shown that K^+ channels play a very important role in the migration of intestinal epithelial cells through the modulation of membrane potential and Ca^{2+} influx (Wang *et al.* 2000; Rao *et al.* 2002). In addition, Ca^{2+} is a well-known secondary messenger in many cellular activities and biochemical pathways. Alteration of cytosolic Ca^{2+} concentration would determine the response of cells to the surrounding environment, sequential signal transduction linked with the surface receptors and hence the final behaviours of the cells. Basically, in the present study, the expression of K^+ channel genes due to the effect of SJZPS acting on wounded IEC-6 cells correlated positively with the cell migration assay.

The activities of the voltage-gated K^+ channels that regulate membrane potential determine the cytosolic free Ca^{2+} concentration by regulating Ca^{2+} influx. IEC-6 cells are a type of non-excitable cell that does not express L-type voltage-dependent Ca^{2+} channels (Himmel *et al.* 1993; Nilius *et al.* 1997). Thus the cytosolic free Ca^{2+} concentration is regulated by the influx of extracellular Ca^{2+} through passive Ca^{2+} leakage, receptor-operated Ca^{2+} channels, stored-operated Ca^{2+} channels and the Ca^{2+} released from intracellular organelles such as endoplasmic and sarcoplasmic reticulum (Quaroni *et al.* 1979; Tsien & Tsien, 1990; Himmel *et al.* 1993; Putney & Bird, 1993; Bilato *et al.* 1995; Pauly *et al.* 1995; Nilius *et al.* 1997; Moore *et al.* 1998). Moreover, the magnitude of Ca^{2+} influx has been found to depend on both the transmembrane Ca^{2+} gradient and the membrane potential (Fleischmann *et al.* 1993; Bilato *et al.* 1995). Previous studies have also indicated that the membrane potential of eukaryotic cells is basically controlled by the number and functional ability of membrane K^+ channels (Fleischmann *et al.* 1993; Bilato *et al.* 1995). When the gene expression of K^+ channels is being induced, the sequential increase of available K^+ channels and their functional ability result in membrane hyperpolarization. Since membrane potential is the major driving force controlling Ca^{2+} influx, this tendency increases the magnitude of Ca^{2+} influx into the cells and finally contributes to the elevation of cytosolic Ca^{2+} concentration. Raising cytosolic free Ca^{2+} concentration stimulates cell contraction and migration through the initiation and participation of

cytoskeleton reorganization, such as activation of the Ca^{2+} /calmodulin (Pauly *et al.* 1995) and Ca^{2+} /RhoA (Rao *et al.* 2001) signalling pathways, mediation of cadherin-catenin-associated interaction (Guo *et al.* 2003) and the Ras superfamily of small GTP (guanosine triphosphate)-binding proteins.

Another candidate gene also up-regulated by the action of SJZPS was that coding for acid-sensing ion channels, proton-gated cation channels (ASIC1). This gene is a member of the ASIC subfamily (Waldmann & Lazdunski, 1998) that shares certain identity (approximately 20–25%) with the subunit of the epithelial Na channel with similar topological domains and intracellular termini. Both of its subunits (ASIC1 α and ASIC1 β) were shown to associate with the formation of Na^+ -selective ion channels (Fyfe *et al.* 1998), but with different permeability for Ca^{2+} (Chen *et al.* 1998). ASIC might provide certain means for controlling the Ca^{2+} entry pathway as well as modulating neuronal activity (Waldmann & Lazdunski, 1998). Furthermore, ASIC was found to participate in the formation of mechanosensitive ion channels that were implicated to be associated with the cytoskeleton (Price *et al.* 2000).

Surprisingly, a proto-oncogene (*JUND*) was found to be over-expressed in the SJZPS-induced IEC-6 cell system. JunD is a member of the Jun family, which consists of three members (c-jun, JunB and JunD) and they are all cellular transcription factors. They occupy a central role in cellular signal transduction and regulation of proliferation (Lamph *et al.* 1988) through their binding to specific DNA sequences as homodimers or heterodimers with Fos proteins, forming a complex called activator protein-1 (Curran & Vogt, 1992). Previous studies showed that c-jun, JunB and JunD mRNA levels were elevated several times after vascular injury and such focal expression indicates the consequences of the initiation of DNA synthesis and migration after vascular injury. Increasing evidence has confirmed that c-jun and JunB are involved in the initiation of cell cycle and proliferation. Conversely, the role of JunD in cell proliferation is still unclear. Some data showed that JunD could slow down cell proliferation (Hirai *et al.* 1989; Ryder *et al.* 1989; Li *et al.* 2002) while some reports still proposed that it could induce cell proliferation. There are two processes involved in the achievement of mucosal restitution: the rapid removal of damaged cells by sloughing followed by the migration of adjacent cells and the replacement of lost cells through cell proliferation. The former occurs within 1 h while the latter process may begin around 16 h after injury (Silen, 1987). So the over-expression of JunD can be explained in the sense that it transiently slowed down the proliferation of IEC-6 cells, while the cells were actively migrating under the influence of SJZPS by modulating the K channels and cytosolic Ca^{2+} concentration.

In the present study, we used IEC-6 cells as a cellular model to elicit the protective and restitution effects of SJZPS on gastrointestinal lining. Our results indicate that SJZPS not only exerts a stimulatory effect on the proliferation of IEC-6 cells, but also restores the recovery of IEC-6 cells after treatment with indomethacin. In addition, we also found that SJZPS can increase the cell migration rate after wounding. In applying the DNA microarray technology to study differential gene expression of SJZPS-induced IEC-6 cells, we discovered increased expression of a group of genes that are related to K and ion channels. The microarray data were confirmed by RT-PCR.

In research on traditional Chinese medicine formulations, the pharmacological actions of polysaccharides are usually neglected

probably owing to the complexity of their chemical nature. However, with the introduction of DNA microarray technologies into the study of Chinese medicine, it is possible to correlate the effects of the herbal compound at the gene level (Watanabe *et al.* 2001; Bonham *et al.* 2002; Sadava *et al.* 2002; Iizuka *et al.* 2003). SJZD is a classic Chinese herbal formula with tonic properties on the gastrointestinal system. In this work we have demonstrated that polysaccharides of SJZD can significantly enhance the *in vitro* migration and proliferation of IEC-6 cells. These data provide further evidence to support the therapeutic value of SJZD in traditional Chinese medicine formulations.

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