Effect of the serum of rats treated with Suo Quan pill on embryonic stem cells

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Abstract: Suo Quan pill(SQP), a well-known and classical traditional Chinese medicine compound, consists of three traditional Chinese medicine: *Alpinia oxyphylla Miq., Lindera aggregata (Sims) Kosterm., Dioscorea opposite.* Its effect was summarized as supplementing kidney- yang and shrinkaging urination. This study evaluated the effects of the serum of rats treated with Suo Quan pill on embryonic stem cells(ES cells). Cell proliferation was detected by MTT assay. Cell cycle and apoptosis of ES cells were evaluated with flow cytometry. Nanog mRNA expression was verified by fluorescence quantitative PCR and Nanog protein in ES cells was determined by Western blot. The serum of SQP-treated rats not only promoted ES cells proliferation and Nanog expression in ES cells, but also inhibited H_20_2 stimulated cell apoptosis. Furthermore, the serum of rats containing SQP affected the cell cycle distribution of ES cells, reducing the percentage of cells in G0/G1phase and increasing the percentage of cells in G2/M phase, increasing the proliferation index of ES cells. These results illustrate that the enhanced effect of SQP on ES cells proliferation is in part due to the increased expression of Nanog in ES cells, the accelerated cell cycle period and the inhibited apoptosis of ES cells.

Keywords: Suo Quan pill, embryonic stem cells, serologic pharmacological method, Chinese medicine.

INTRODUCTION

Suo Quan pill (SQP) is a classical Chinese medicine prescription from TCM ancient book “Furen Liangfang”. According to the record, SQP has the efficacy of supplementing kidney-Yang, reducing urine, and is mainly used to treat frequent urination, urinary incontinence and urorrhagia with good clinical effect (Li and Chen, 1993). Based on TCM theory and clinical experience, diseases such as frequent urination, urorrhagia and urinary incontinence are attributed to deficiency of kidney-yang. So such diseases can be cured through supplementing kidney-Yang (Zhang *et al*., 2013).

Kidney is an important fig. in TCM theory. Kidney theory of TCM, especially the connotation of the Kidney Essence as the root of the life has inherent connection with ES cells (Zhang *et al*., 2006). Kidney Essence has a close relationship with human reproduction, growth, development and aging (Hu *et al*., 2008). ES cells derived from inner cell mass of mammalian blastocysts grow rapidly and unlimitedly while maintaining pluripotency, the ability to differentiate into all types of cells (Evans and Kaufman, 1981; Martin, 1981). These properties of ES cells are main tained by symmetrical self-renewal, producing two identical stem cell daughters upon cell division (Burdon *et al*., 2002). ES cells was very similar with kidney essence in source or function, it can explain most of the function of kidney essence (Zhang *et al*., 2006). Thus, the essence in kidney has inherent connection with ES cells.

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Nanog is a recently discovered homeodomain transcription factor that directs propagation of undifferentiated ES cells. Nanog mRNA is present in pluripotent mouse and human cell lines, and is absent from differentiated cells (Chen *et al*., 2006). Gain-of-function and loss-of-function studies suggest that Nanog not only plays a crucial role in the maintenance of pluripotency of murine embryos and ES cells but also blocks the differentiation of ICM and ES cells into endoderm (Chambers *et al*., 2003; Mitsui *et al*., 2003; Yoshida-Koide *et al*., 2004; Hamazaki *et al*., 2004).

Previous research has shown that SQP can obviously decrease the urination of the kidney-yang deficiency model rats with polyuria. On account of the inherent connection between Kidney Essence and ES cells, the current research is in order to investigate the effects of SQP as well as the method of supplementing kidney-Yang on ES cells by the serologic pharmacological test.

MATERIALS AND METHODS

Materials

SQP consists of three TCM drugs: *Alpinia oxyphylla Miq., Lindera aggregata (Sims) Kosterm., Dioscorea opposite.* according to a ratio of 1:1:1. SQP (lot No: 100302) were purchased from Hunan Hansen Pharmaceutical Co. Ltd. (Hunan, China). Jin Gui Shen Qi Pill (JGSQP, Tong Ren Tang Technologies Co., Ltd, Beijing, China, Lot. 0030377); 2-Mercaptoethanol, L-Glutamine were purchased from invitrogen (Carlsbad, CA, USA); LiF was purchased from millipore (Billerica, MA, USA);

PD0325901,CHIR-99021 were obtained from cayman (Lot : 0426978-53 , 0438531-4, USA); MTT was from Sigma Chemical Co. (USA); Cell cycle kit was obtained from Beijing 4A Biotech Co., Ltd (Lot. 4AC291211, Beijing, China); Annexin V-FITC Apoptosis Detection Kit was from eBioscience (Lot.59033000, Frankfurt, Germany); Trizol (Lot: 604,Merck,USA); RT reagent Kit, SYBR Premix ExTaq II (Lot: 00074722 and 00117666, Fermentas, Lithuania); the primers for nanog and GAPDH were synthesised by Sangon Biotech, Co., Ltd (Shanghai, China); Nanog Antibody for Western blot was provided by eBioscience (Lot: E04352-1630,San Diego, USA).

Preparation of drug-containing serum

Adult Male SD rats weighing 180-220g were purchased from the Laboratory animal center of Guangzhou University of Chinese Medicine, Guangzhou, PR China. The animal experiment was conducted according to the management regulations of experimental animals of Guangzhou University of Chinese Medicine. 15 rats were used to prepare for the drug-containing serum and they were divided into five groups.

Normal group was treated with intragastrical administration of saline. JGSQP group was treated with intragastrical administration of 2.16g/kg of JGSQP, and three SQP groups were treated with intragastrical administration of 0.59, 1.17, 2.34g/kg of SQP for 7 days, respectively. Blood samples were obtained from abdominal aorta. The serum was obtained by centrifugation of blood and then stored at−20□ for use after filtration (Zhang *et al*., 2007).

Cell culture

Strain 129 Mouse Embryonic Stem (mES) Cell was obtained from the Stem Cells and Tissue Engineering Research Center (Sun yat-sen university, China). All mES cells were cultured on 0.1% gelatin-coated dishes using LIF-containing medium (Ying *et al*. 2008). 2i media was prepared by addition of final concentrations of 3μM CHIR99021 and 1μM PD0325901 to growth media. 129 mES cells were passaged every 48h using 0.25% of trypsin to obtain single cell suspension after harvesting by centrifugation.

Experimental procedures

mES cells were inoculated into 6 hole cell culture plate by density of 1×10^4 cells/ml for 24h incubation, and then were incubated in the serum-free medium for 6h. After that, the cells were divided into five groups, and cultured for 24h in fluid medium containing serum of rats (1/9, v/v, serum/ medium) treated with different drugs. The experiment conducted in accordance with the following different methods.

Assessment of proliferation of mES cells by the MTT assay

After stimulating cells with different serum containing drug for 24h, 100ul of 1.0mg/ml MTT in DMEM medium was added into each hole. Then, mES cells were incubated for further 4h, 100ul of DMSO was added into each hole to dissolve formazan crystal (Mosmann, 1983). At last, absorbance was measured with ELISA microplate reader at 492 nm.

Observation of cycle of mES cells

Cycle distribution of mES cells was detected by flow cytometry. After stimulating mES cells with different drugs for 24h, single cell suspension was got by 0.25% of trypsin. Then the cells were adjusted to a density of 10^6cells/ml, fixed with 4ml 95% cold ethanol at 4°C for 24h, and finally dyed with 4ml PI solution (Zhang *et al*., 2009). These treated cells were measured with FACS Canto Ⅱ flow cytometer, and the index of cell proliferation was obtained based on the following formula.

Proliferation index (%)= (G2/M +S)/(G0/ G1+S+ G2/M)×100%.

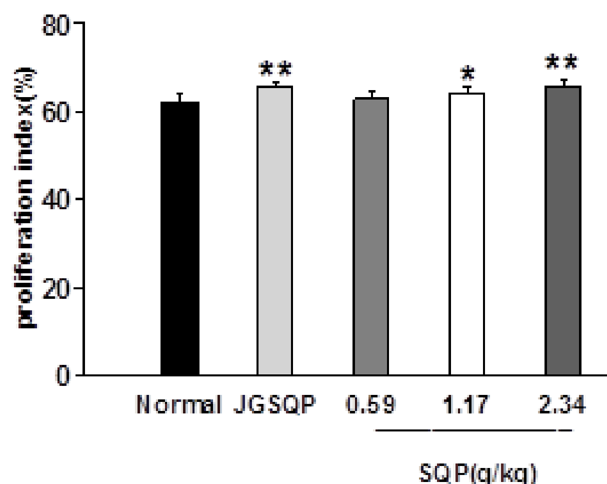
Detection of early apoptosis of H_20_2-sitmulated mES cells

Apoptosis of mES cells was measured with flow cytometry. After adding with different serum containing drug for 24 h, the cells were induced by hydrogen peroxide (H_20_2) in accordance with the procedure of Liu (Liu *et al*., 2012).The harvested cells were washed with PBS, and resuspended in the binding buffer. After adding annexin V-FITC/PI solution into the cell suspension, the treated samples were stored in a dark place for 10 minutes to stain the damaged DNA in the apoptotic cells. The early apoptosis rate of mES cells was analyzed by FACS Canto Ⅱ flow cytometer.

Investigation of Nanog mRNA expression in mES cells

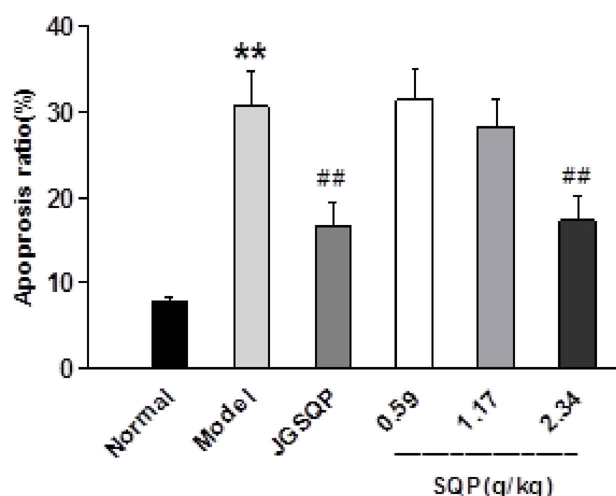
The total RNA from mES cells were extracted by Trizol and the purity of the RNA was assessed by the ratio of A260/A280. First-strand cDNA was produced by adding 2.5μg total RNA, 5×Reaction Buffer, 4μl; Oligo (dt) 18 Primer, 1μl; *RevertAid* ^{TM}*M-M*uLV *Reverse* Transcripase, 1μl; 10mMdNTP Mix, 2μl; Ribolcok ^{TM}RNase inhibitor , 1μl and DEPC-treated water were used to reach a reaction volume of 12μl. The conditions of RT were as follows: 1h at 42°C and 70°C for 5 min.

All the primers were set spanned an intron and the information of primer were performed in Table 1. The PCR reaction of components were combined in a master mix composed of SYBR Green Realtime PCR Master mix, 12.5μl; PCR Forward Primer, 0.75μl; PCR Reverse Primer, 0.75μl; nuclease-free Water, 8μl; cDNA, 3μl. The real-time quantitative PCR used ABI7500 (Applied Biosystems, USA). All the real-time QPCR experimentation were conducted strictly according to the rules of the MIQE.



Note: *: compared with normal group, P<0.05; **: compared with normal group, P<0.01.

Fig. 1: Bar graphs showing the value of the proliferation index of mES cells.

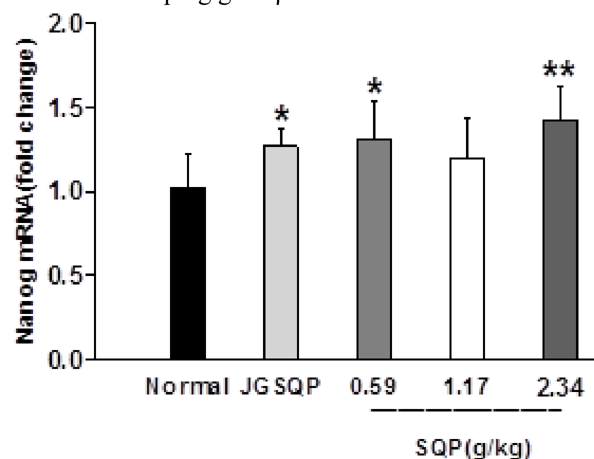


Note: **: compared with normal group, P<0.01; ##: compared with model group, P<0.01.

Fig. 2: Effect of the drug-containing serum of SQP on early apoptosis of mES cells.

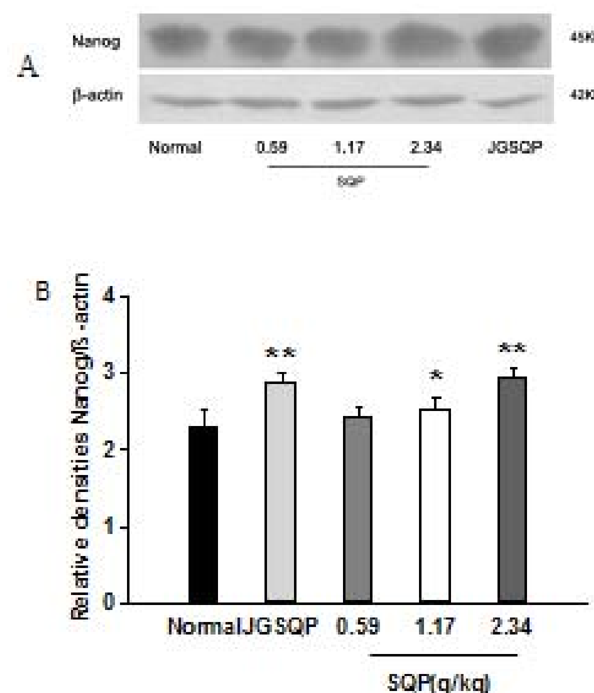
Investigation of Nanog protein expression in mES cells

Western blot analysis were used to measure the protein level of Nanog in mES cells in all experimental groups. The total protein extracts were separated by SDS-PAGE and then subsequently transferred to a PVDF membrane. The membrane were rinsed in PBS and then immersed for 24h in blocking PBS buffer containing 5% skimmed milk. The blots were incubated in a 1:1000 dilution of the primary anti- Nanog monoclonal antibody at 37□ for 1h. After further washing, the membrane was incubated with biotinylated goat anti-mouse IgG (1:6,000) in PBS containing 5% skim milk at 37°C for 1h. Then the membrane reacted with mixture of equal volume ECL reagent A and B. Then the specific protein bands were catched by X-ray film, scanned and quantitated in relation to the housekeeping gene β-actin.



Note: *: compared with normal group, P<0.05; **: compared with normal group, P<0.01.

Fig. 3: Effect of drug-containing serum of SQP on Nanog mRNA expression.



Note: *: compared with normal group, P<0.05; **: compared with normal group, P<0.01.

Fig. 4: Effect of drug-containing serum of SQP on Nanog protein expression.

STATSTICAL ANALYSIS

The experiment data were expressed as mean ±SD. Statistical analysis were conducted using the SPSS 18.0 software package. Comparisons among groups were calculated with ANOVA variance analysis and p value less than 0.05 was considered of statistical significant.

RESULTS

Effect of drug-containing serum of SQP on the proliferation of mES cells

As seen from table 2, cultivation of the cells with the serum of rats which were intragastrical administration with 1.17 and 2.34g/kg of SQP promoted the proliferation of mES cells ($P<0.05$ or $P<0.01$). Compared with the normal group the growth rate were 11.6% and 14.0% respectively. The experiment proved that drug-containing serum of SQP significantly promotes proliferation of mES cells *in vitro*.

Effect of drug-containing serum of SQP on the cycle of mES cells

As indicated in Table 3, cultivation of the cells with the serum of rats which were intragastrical administration with 1.17 and 2.34 g/kg of SQP reduced the percentage of cells in G0/G1phase and increased the percentage of cells in G2/M phase ($P<0.05$ or $P<0.01$). fig. 1 revealed that the serum of rats treated with 1.17 and 2.34 g/kg of SQP increased the proliferation index of mES cells ($P<0.05$ or $P<0.01$). The experiment showed that drug-containing serum of SQP could promote the cell cycle to enhance proliferation of mES cells.

Effect of drug-containing serum of SQP on early apoptosis of mES cells

As shown in fig. 2, early apoptotic ratio of mES cells was very obvious after exposing them to H_20_2 for 4h ($P< 0.01$ vs. normal group). However, cultivation of the cells with the serum of rats which were intragastrical administration with 2.34g/kg of SQP reduced early apoptotic ratio of mES cells ($P<0.01$ vs. model group), showing that the drug-containing serum of SQP could diminish early apoptosis of mES cells.

Effect of drug-containing serum of SQP on Nanog mRNA expression

The result in fig. 3 showed that cultivation of the cells with the serum of rats which were intragastrical administration with 0.59 and 2.34 g/kg of SQP enhanced the expression of Nanog mRNA, indicating that the drug-containing serum of SQP could enhance Nanog mRNA expression.

Effect of drug-containing serum of SQP on Nanog protein expression

As seen from fig. 4, cultivation of the cells with the serum of rats which were intragastrical administration with 1.17 and 2.34 g/kg of SQP enhanced the expression of Nanog protein, indicating that the drug-containing serum of SQP could enhance Nanog protein expression.

DISCUSSION

For a long time the pharmacology study for Chinese traditional medicine often uses crude drugs directly in the *in vitro* assays (Chen *et al*., 2003; Nishida and Satoh, 2003). However, physical and chemical characters of crude drugs (pH, osmotic pressure, impurities etc.) are bound to affect validity of experimental results (Meng *et al*., 1999). Moreover, compositions of traditional Chinese drugs are generally complicated, and a number of compositions do not produce effects on the body until they undergo a series of biotransformation after absorption from gastrointestinal tract. Many researchers believe that Serum Pharmacology is more appropriate for Chinese traditional medicine (Wang *et al*., 2005).

According to TCM theory, polyuria is closely related to the function of Kidney, that is, warming kidney-yang and holding excessive urination can effectively cure the disease. Our previous research has shown that SQP can evidently decrease the urination of several kidney-yang deficiency models of rats with polyuria (Cao *et al*., 2014; Li *et al*., 2011).

ES cells were very similar with Kidney Essence in source or function, it can explain most of the function of Kidney Essence (Zhang *et al*., 2006). Thus, Kidney Essence has inherent connection with ES cells. In this study, we used ES cells as the model of Kidney Essence at the cellular level.

According to the warming kidney-yang property of SQP, and embryonic stem cells have the characteristics of the Kidney Essence. Our main research objective is to study the effect of SQP on proliferation, cell cycle, apoptosis of ES cells. The result showed that the drug-containing serum of SQP could promote ES cells proliferation, interfere with the cycle of ES cells, decrease the percentage of cells in G0/G1phase and increase the percentage of cells in G2/M phase, increase the proliferation index of ES cells, and inhibit H_2O_2 stimulated cell apoptosis. It also indicated that SQP could strengthen the vitality of ES cells by means of supplementing kidney-yang. The results, in return, proved that SQP may cure kidney deficiency syndrome such as frequent urination, urorrhagia and urinary incontinence through cultivating and raising the role of Kidney Essence.

ES cells have two definitive properties, self-renewal and pluripotency, which make them very attractive in clinic. Nanog, a homeodomain-containing transcription factor, was found at 2003 and specially expressed in pluripotency cells. Nanog plays important roles in ES cells pluripotency, early differentiation and self-renewal (Ji and Jiang, 2011). Therefore, we studied the effect of drug-containing serum of SQP on the expression of Nanog mRNA and protein in ES cells. The result suggested that the enhanced effect of SQP on ES cells proliferation is partially attributed to increased Nanog expression in ES

Table 1: Sequences of the primers for RT-PCR.

Genes	Sense primers (5ʹ to 3ʹ)	Antisense primers (5ʹ to 3ʹ)	Product Size (bp)
Nanog	CTGCTACTGAGATGCTCTGCAC	AGCTTTTGTTTGGGACTGGTAG	106
GAPDH	GGTGAAGGTCGGTGTGAACG	CTCGCTCCTGGAAGATGGTG	233

Table 2: Effect of drug-containing serum of SQP on the proliferation of mES cells.

Group	OD value	Growth rate (%)
Normal	0.43±0.03	
JGSQP	0.50±0.03**	16.3
SQP 0.59g/kg	0.45±0.04	4.7
SQP 1.17g/kg	0.48±0.02*	11.6
SQP 2.34g/kg	0.49±0.03**	14.0

Table 3: Effect of drug-containing serum of SQP on the cycle of mES cells.

Group	G_0/ G_1 (%)	S (%)	G_2/M (%)
Normal	37.68±2.02	54.73±1.61	7.60±0.80
JGSQP	34.19±0.85**	56.07±0.86	9.74±0.84**
SQP 0.59g/kg	37.18±1.67	54.31±1.35	8.50±0.91
SQP 1.17g/kg	35.63±1.28*	55.70±1.17	8.67±0.84
SQP 2.34g/kg	34.29±1.64**	56.29±1.59	9.42±1.77**

Note: *: compared with normal group, P<0.05; **: compared with normal group, P<0.01.

cells, accelerated cell cycle period and inhibited apoptosis of ES cells.

CONCLUSION

In conclusion, these experiments indicate that SQP has the capacity not only to accelerate ES cells proliferation, which is partially attributed to improvement of Nanog expression in ES cells and interference with the cell cycle of ES cells, but also depress ES cells apoptosis. All the above provide further evidences for better understanding effects and mechanisms of SQP. Results of our present studies also reflect Kidney theory of TCM is associated with ES cells from an aspect.

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