Induction of Angiogenesis and Neurogenesis by Serum from Rats Treated with Shunaoxin Dropping Pills

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Abstract: **Objective** Shunaoxin Dropping Pills (SDPs), a Chinese patent medicine, has been used widely in China for the treatment of headache, amnesia, and insomnia. The aim of the present study is to observe the effect of SDPs on inducing angiogenesis and neurogenesis *in vitro*. **Methods** The present testing system using the serum obtained from animals ig treated with SDPs and a co-culture system *in vitro* was used to investigate if SDPs promotes brain microvascular endothelial cells (BMECs) tube formation and neural differentiation of neural stem/progenitor cells (NSPCs), which plays important roles in angiogenesis and neurogenesis. **Results** The SDPs serum sampled from rats ig treated with SDPs for 3 d dose-dependently promoted the tube like structure formation of cultured BMECs, and enhanced the fraction of MAP-2 positive cells of NSPCs, which co-cultured with the BMECs and astrocyte. In addition, there was no significant change in the percentage of glial fibrillary acidic protein positive cells. **Conclusion** Our results show that SDPs serum can induce neural differentiation and BMECs tube formation *in vitro*.

Key words: angiogenesis; brain microvascular endothelial cell; neural stem/progenitor cells; neurogenesis; Shunaoxin Dropping Pills

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Introduction

Nowadays, numerous people were affected by central nervous system (CNS) disorder every year. When brain cells are damaged, headache, insomnia, depression, and memory deficits will be caused. Due to complexity of CNS disease, there is increasing evidence that Chinese materia medica (CMM) can represent a better strategy against brain injury based on its combination therapies (Feigin, 2007; Kim, 2005). CMM can not only benefit brain blood vessels and nourish brain functions, but also stimulate the body's own healing mechanisms, such as promoting angiogenesis and neurogenesis when brain cells are damaged.

Currently, the drugs responsible for angiogenesis and neurogenesis have been the subject of intense investigation because identification of effects that regulate the behavior of neural stem/progenitor cells (NSPCs) and brainmicrovascular endothelial cells (BMECs) may allow for their eventual manipulation in the CNS therapies for injury or disease. Stem cell expansion and differentiation were regulated *in vivo* by environmental factors encountered in the stemcell niche (Teng *et al*, 2008). BMEC and astrocytes were the main component of the microenvironment (Shen *et al*, 2004; Song, Stevens, and Gage, 2002). Neural progenitor cells lay in close proximity to endothelial cells, bursts of angiogenesis occurred at the same time as neurogenesis, and endothelial cells secreted soluble factors, which stimulated NSPCs proliferation and neurogenesis (Shen *et al*, 2004; Palmer, Willhoite, and Gage, 2000). These findings indicated that in the CNS, angiogenesis and neurogenesis are closely linked in theadult brain. But

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when the drugs under investigation were added directly to the medium, the effects of herbal medicines might be lost. Their pharmacological effects could not be adequately evaluated by conventional *in vitro* methods using direct addition of extracts to cultured cells because of the complex chemical nature of herbal medicines. By utilizing serum obtained following drug administration, *in vitro* experimental results may accurately reflect those observed *in vivo* (Umeda, Amagaya, and Ogihara, 1988; Iwama, Amagaya, and Ogihara, 1987).

Shunaoxin Dropping Pills (SDPs), a Chinese patent medicinal mixture, have been used widely in China for the treatment of headache, amnesia, and insomnia. SDPs is composed of two herbal drugs, *Angelica Sinensis Radix* and *Szechwan Lovage Rhizome*, containing some known chemical constituents (ligustilide, ferulic acid, and ligustrazine, *etc*). Their cerebral protection and angiogenesis actions have been investigated by many researchers (Cheng *et al*, 2008; Kim *et al*, 2007; Zhu *et al*, 2009; Mamiya, Kise, and Morikawa, 2008; Wan *et al*, 2008). However, the effect of SDPs on CNS disorder has not been fully studied. Therefore, to address the possible beneficial effects of SDPs against CNS disease, in the present study, SDPs was orally given to rats and their serum was then studied as the drug by its direct addition to the medium of BMECs, to explore angiogenesis potentials of SDPs, and on the other hand, we co-cultured NSPCs, BMECs, and astrocytes to explore the neurogenesis effects of SDPs *in vitro*.

Materials and methods

Reagents and chemicals

SDPs were provided by Tianjin Zhongxin Pharmaceutical Group Co., Ltd., (Tianjin, China). Male Wistar rats (200 ± 20) g (Certificate No. SCXX 2006-0009, Vital River Laboratory Animal Technology Co., Ltd.,) were used in this study. All experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. SDPs at a dose of 90.72 mg/(kg·d) were ig administered to rats. After SDPs treatment for 3 d, the abdominal aorta blood samples were collected. The serum was collected and put into 56 ℃ water bath for half an hour to inactivate complement. Then the serum was steriled by 0.22 μmol/L filter and used in the following study. Serum sample prepared from rats treated with the same volume water was used as control serum.

Endothelial cell growth factor (ECGF) was obtained from Roche Molecular Biochemicals (Mannheim, Germany). Matrigel was obtained from Becton Dickinson (Bedford, USA), rabbit polyclonal antibody against micro-tubule-associated protein 2 (MAP-2) and mouse monoclonal antibody against glial fibrillary acidic protein (GFAP) were purchased from Millipore (Billerica USA), anti-human related factor VIII antibody, goat anti-rabbit IgG linked to FITC, and goat anti-mouse IgG linked to Cy3 were from Zhongshan Biotech Co. (Beijing, China).

Culture of rat BMECs

This procedure was performed essentially as described previously (Calabria *et al*, 2006). Brains were removed from male rats (80－120 g). The cortices were digested with 1.0 mg/mL type 2 collagenase for 1 h at 37 ℃. Afterwards, the enzyme solution was centrifuged at 1000 × *g* for 10 min at 4 ℃. The pellet was re-suspended in a 20% bovine serum albumin and DMEM solution and centrifuged for 20 min at 1000 × *g*. Then, the pellet was further digested in 1 mg/mL collagenase/dispase for 1 h at 37 ℃. The digested microvessels were seeded on 2% gelatin and PBS solution coated plastic culture plates. The medium is made from DMEM supplemented with ECGF, heparin, FBS, and antibiotics. Confluent first passage of endothelial cells was used for our experiments.

In vitro tube like structure (TLS) formation assay

Endothelial tube formation was assessed using Matrigel as described previously with minor modifications (Sengupta *et al*, 2004). Matrigel (diluted to 1 mg/mL) was added to 12-well plates with a total volume of 300 μL in each well and allowed to polymerize for 1 h at 37 ℃. BMECs were plated on the Matrigel at a density of 5×10^4 cells per well for 12-well plates, in a final volume of 1 mL DMEM medium with various concentrations of SDPs serum (5%, 10%, and 15%). Cells plated in DMEM plus 5%, 10%, and 15% control serum served as the control, respectively. Endothelial tube formation on Matrigel after 48 h was assessed and photographed by inverted phase contrast microscopy.

Neural differentiation study in an *in vitro* model of co-culture system

Cortical NSPCs was prepared from pregnant Wistar rat embryos on day 14 according to a protocol

detailed previously (Davis and Temple, 1994). Briefly, rat embryonic cerebral cortices were dissected, mechanically triturated in cold D-Hank's balanced salt solution. The dissociated cells were cultured in T75 culture flasks in the serum-free medium containing B27 neural supplement (Gibco-Invitrogen), 20 ng/mL EGF, and bFGF. After 6 d *in vitro*, the proliferating cells formed the neurospheres, which were suspended in the medium. Subsequently, the neurospheres were passaged by treatment with accutase about 5 min at 37 ℃ until they were gently dissociated, and then subcultured as single cells in a new T75 culture flask at a density of 10 000 cells/cm^2 in the fresh culture medium. The procedure of subculture was repeated again to achieve the purified cortical NSPCs and proliferate neurospheres.

To establish an *in vitro* co-culture system, astrocyte (AC) and BMECs were co-cultured according to Millicell insert as described by Perrière *et al* (2005). Millicell inserts were coated on the upper side with 200 μL of gelatin. Primary cultures of astrocytes were prepared from the cerebral cortex of newborn rats. Three days before BMECs passaging, 8×10^4 cells/cm^2 astrocytes were seeded in the bottom of 12-multiwell inserts for further co-culture with BMECs. The BMECs suspension was seeded on the upper side of the inserts in 0.5 mL of medium with a concentration of 4×10^5 cells/mL.

For neural differentiation experiments, to differentiate the clones, NSPCs were seeded on glass coverslips in the bottom of 12 well plates, which were pre-coated with poly-*L*-lysine. The millicells which have a mono-confluence of BMECs and AC were placed above on the neural stem cells. The cells were co-cultured for 3 d. bFGF was then removed and SDPs serum (1% or 2%) was subsequently added in the upper side of the millipore insert. As control, equal volume of serum was added.

Immunocytochemistry

For immunocytochemical characterization, cultured cells were fixed in ice-cold 4% paraformaldehyde. After fixing, cells were incubated with primary antibodies for overnight at 4 ℃. For characterization the primary culture cells and double-labeling of ACs and neuron of the differentiated NSCs. The primary antibodies including mouse anti-GFAP (1:500), rabbit anti-factor VIII polyclonal antibody (1:100), and rabbit anti-MAP-2 (1:500) were used in this study, and FITC-conjugated goat anti-mouse IgG (1:100), Cy3-conjugated goat anti-rabbit IgG (1:1000), and rabbit anti-goat IgG (1:1000) were used as secondary antibodies to visualize the signal by reacting with cells for 60 min at room temperature.

Data analysis

Results are expressed as $\bar{x} \pm s$. Data were analyzed by One-way ANOVA followed by *post hoc* Dunnett's test for pair-wise comparison. $P < 0.05$ was considered to be significant.

Results

Characterization of BMECs

To determine the endothelial origin of the cells, microscopy studies including immunofluorescence characterization were performed. As shown in Fig. 1, we were able to isolate the capillary fraction (Fig. 1A) from the cerebral cortical tissue. Endothelial cells in culture exhibited classic cobblestone or spindle-shaped morphology. Based on morphology, 95% of the populations were endothelial cells (Fig. 1B and C). As presented in Fig. 1D, immunostaining staining for factor VIII related antigen, a marker of endothelial cells, presented diffusely in the cytoplasm of > 98% of the primary cultures.

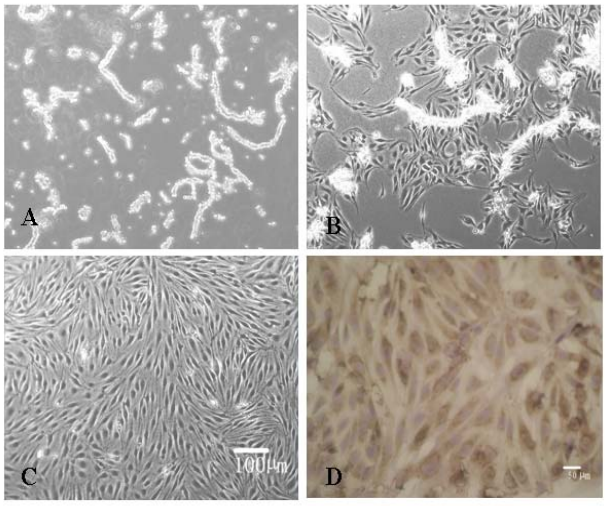

Fig. 1 Photomicrographs of rat BMECs

A: Rat brain microvessel, obtained subsequent to brain tissue disruption and collagenase/dispase digestion

B: After 2 d in culture, BMECs migrate out of isolated microvessels

C: BMECs form confluent monolayer demonstrating cobblestone and spindle-shaped morphology after 4 d culture

D: BMECs cultured on gelatin-coated slides stain positiely with antibody to factor VIII related antigen

Effects on *in vitro* TLS formation

To investigate the effects of SDPs on angiogenesis, *in vitro* tube formation assay was performed using BMECs in a 12-well plate coating with Matrigel. A significant increase in capillary like tube formation was detected when endothelial cells were incubated with the SDPs serum, compared with the endothelial cells incubated with control serum. As shown in Fig. 2, TLS was not observed in the controls when BMECs were plated on the matrigel (Fig. 2A, B, and C). However, in the presence of SDPs serum, cells were aligned and formed a capillary-like network within 48 h, and the TLS was significantly increased with the serum increased (Fig. 2D, E, and F). These findings at least partly indicated that SDPs increased angiogenesis.

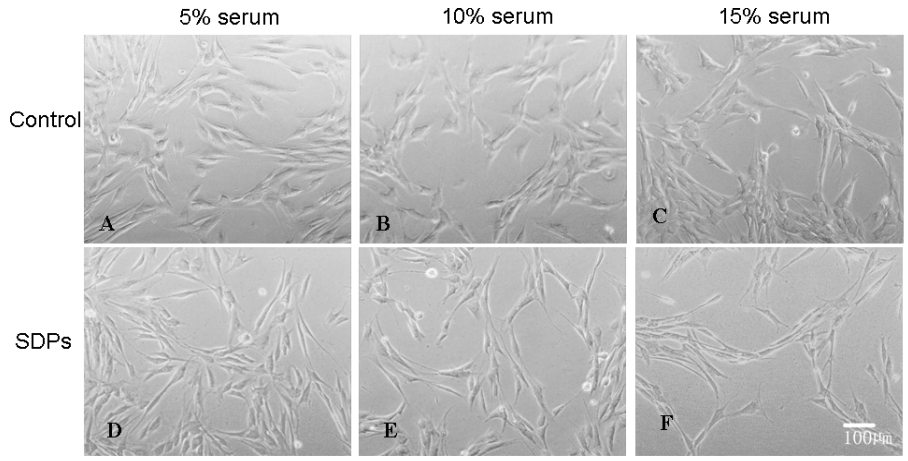

Fig. 2 SDPs serum promotes BMECs tube formation on matrigel matrix *in vitro*

BMECs were seeded onto Matrigel coated 12 well plates for 48 h, control serum (A, B, and C) or SDPs (D, E, and F) serum at different concentrations (5%, 10%, and 15%) were added. Light microscope photographs showed that SDPs serum promoted capillary-like tube formation of BMECs, with a tendency of increase in a dose-dependent manner. This experiment is representative of three similar experiments

Effects on neural differentiation in an *in vitro* co-culture system

For the differentiation assay, double-labeling of astrocytes and neuron assay was performed. Immunostained cells were visualized under the fluorescence microscope (Lica DF300, Germany). Six fields of view per section immunostained with different antibodies were digitized. The number of MAP-2$^+$, GFAP$^+$ cells and the total number of DAPI (Sigma) cells were counted and the percentage of each cell type was determined. Within 3 d *in vitro*, neurospheres under the co-culture system with neuronal morphology appeared in greater numbers. After fixing the spheres and double-immunostaining for MAP-2 and GFAP, cells exhibited a significant increase in the number of MAP-2 (neuronal marker)-positive cells in media containing 2% SDPs serum compared with the corresponding numbers from the control (Fig. 3), Quantitative analyses of MAP-2/GFAP double-labeled cells with neuronal or astrocytes morphology showed a two-fold increase ($P < 0.05$) in neuronal numbers in SDPs serum treated spheres (Fig. 3D), but no significant changes in numbers of astrocytes.

Discussion

The development of brain protective, vascular, and even neural regenerative drugs is essential for the treatment of CNS disease. An overall research of brain injury therapies would be carried out, whose goal is not only to salvage acutely threatened brain tissue but also to promote repair and restoration of function. This study has focused on the evaluation of the effects of SDPs on TLS formation and neural differentiation *in vitro*, and some important steps in the onset of angiogenesis and neurogenesis. The greatest challenge when the pharmacological mechanisms of complex medicines to be assessed *in vitro* is how to accurately reflect the effects observed *in vivo*. One of the major difficulties encountered is the complex chemical nature of herbal medicines, and another difficulty is the unknown metabolism of their components. The results obtained from *in vitro* experiments using serum from animals ig treated with herb medicine are in accord with those obtained from the *in vivo* experiments (Chung, Maruyama,

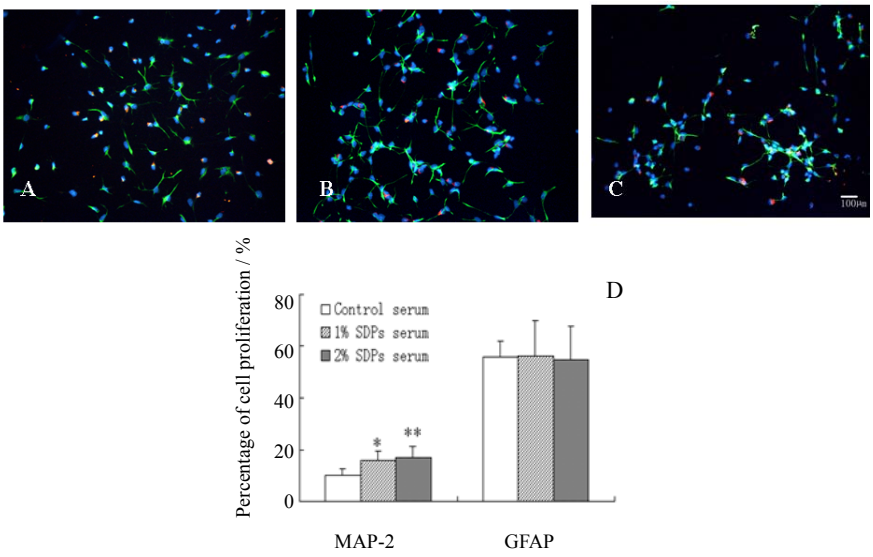

Fig. 3 SDPs serum induces neuronal differentiation in NSPCs

Neurospheres were cultured for 3 d in the co-culture system, cells were double-immunostained with MAP-2 and GFAP antibodies

A: NSCPs were incubated with 2% control serum

B and C: NSCPs were incubated with 1% and 2% SDPs serum

D: Percentage of MAP-2 or GFAP positive cells were counted as described in the method, the values of represent $\bar{x} \pm s$ (n = 6) from three independent experiments

and Tani, 2004). We used the serum from rats ig treated with SDPs in the present study. It was worth noting that SDPs serum, which may contain some small active components and metabolites, promoted the TLS formation of BMECs and enhanced neural differentiation of NSPCs. And further chemical studies on endogenous serum components and organic in SDPs serum are ongoing.

Therapeutic angiogenesis was critical to cerebral ischemia (Beck and Plate, 2009). Angiogenesis required a coordinated and sequential involvement of a number of cellular events, such as cell migration and tube formation and angiogenic agents that affected any step of the process of angiogenesis may exert a promotive effect on angiogenesis. Our present data provided a clear evidence that the SDPs serum exerted a promotive effect on angiogenesis through promoting BMECs TLS formation. Recent evidence suggested that ferulic acid, one of the most important active components of SDPs, exhibited a augmenting effect on angiogenesis through functional modulation of endothelial cells (Lin *et al*, 2009). Our data were in agreement with the conclusion above. Although the mechanism of the promotive effect on angiogenesis remained to be explored in detail, SDPs might exert the cerebral protection through beneficial modulation of the brain vascular system and the promotion of angiogenesis.

Neurogenesis is a multistep process including proliferation, migration, fate determination, and neuronal maturation of NSPCs (Fallon *et al*, 2000). Among these processes, one of critical steps during NSPCs differentiation was the decision to generate neuronal or glial cells. We intended to assess whether treatment with the SDPs serum would increase the percentage of neuron differentiation across an *in vitro* co-culture system. In our study, the differentiation of NSPCs was determined by double-labeling immuno-cytochemistry analysis. We observed here that when NSPCs were treated for 3 d with 2% serum in the co-culture system, the neuron production was clearly increased. However, the percentage of the GFAP differentiation was not modified compared with the control.

In conclusion, we have demonstrated that SDPs exerts potential effects on the angiogenic and neurogenesis capacity. The effect of SDPs on CNS disease at least involved in facilitating TLS formation of BMECs and enhancing neural differentiation of NSPCs. Thus, SDPs may ultimately impact on vessel growth and contribute to the neurogenesis. The present findings expand our knowledge on pharmacological

actions of SDPs on CNS disorder.

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