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Baicalin promotes the viability of Schwann cells *in vitro* by regulating neurotrophic factors

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Abstract. The proliferation and migration of Schwann cells (SCs) are key events in the process of peripheral nerve repair. This is required to promote the growth of SCs and is a challenge during the treatment of peripheral nerve injury. Baicalin is a natural herb-derived flavonoid compound, which has been reported to possess neuroprotective effects on rats with permanent brain ischemia and neuronal differentiation of neural stem cells. The association of baicalin with neuroprotection leads to the suggestion that baicalin may exert effects on the growth of SCs. In the present study, the effects of baicalin on SCs of RSC96 were investigated. RSC96 SCs were treated with various concentrations of baicalin (0, 5, 10 or 20 μM) for 2, 4 and 6 days. Cell attachment, viability and gene expression were monitored via the MTT assay and reverse transcription-quantitative polymerase chain reaction. The gene expression levels of several neurotrophic factors, such as glial cell-derived neurotrophic factor, brain-derived neurotrophic factor and ciliary neurotrophic factor, which are considered important factors in the process of never cell regeneration, were detected. The results indicated that baicalin was able to promote the viability of RSC96 SCs in a dose-dependent manner and the concentration of 20 μM of baicalin exhibited the greatest cell viability and gene expression of the studied neurotrophic factors. The present findings suggested that baicalin likely affects SCs metabolism, through modulating the expression of neurotrophic factors. To conclude, the present study indicates that baicalin may be potential therapeutic agent for treating peripheral nerve regeneration.

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Introduction

Peripheral nerve injuries occur with a high frequency, accounting for up to 3% of all trauma injuries (1,2). In the majority of cases, surgical intervention is necessary due to the self-regenerative capability of nerves; however, this is time-consuming and incomplete, as described in (3), which may cause functional impairment. Although autograft transplantation is the first choice of treatment, the shortage of donor resources and the repercussions of this invasive treatment to the donor present as major limitations (4,5). However, the discovery of an alternative therapy to replace autografts and treat peripheral nerve injury has presented as a challenge.

Among the typical approaches for treating nerve crush injury, Schwann cell (SC)-based therapy is highly recommended (6). SCs, the principle glia in the peripheral nervous system, have an important role in the development, function and regeneration of peripheral nerves (7). Following peripheral nerve injury, SCs aid in phagocytizing the damaged end of the axon and provide physical support to regenerate axons by forming 'Bands of Büngner'. Furthermore, SCs create a suitable axonal growth environment by producing neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF) and neurotrophic factors-3, -4/5 and -6 (NT-3, NT-4/5, and NT-6, respectively) (8-12). However, the slow growth rate of SCs is reported to be one of the major limitations of SC application in regenerative medicine (13). In addition, elevating the proliferation ability of SCs is important for constructing tissue-engineered nerves (6). As a result, researchers have been exploring various promoting agents for SCs proliferation, such as interleukin-1β (14) and tanshinone IIA (15).

Utilizing plant-derived traditional Chinese medicines to treat various types of diseases has a long history in East Asian countries, such as China, Korea and Japan (16). Furthermore, some western medicines are derived from major constituent of traditional Chinese medicine (17) *Scutellaria baicalensis* Georgi (Huangqin in Chinese), a traditional Chinese medicine, has been used to treat inflammation, fever, ulcers and cancer for hundreds of years (18-20) and a recent study has reported that flavonoids from the stems and leaves of *S. baicalensis* Georgi have neuroprotective effects (21). Baicalin, one of the major flavonoid isolated from the root of *S. baicalensis*, has a variety of biological functions, including anti-inflammatory, anti-oxidant and anti-apoptotic activities (22-24). Previous studies have revealed that baicalin had neuroprotective effects on permanent brain ischemia in rats (25) and was able to promote the neuronal differentiation of neural stem cells (26,27). However, little is known on whether baicalin is capable of exerting positive or negative effects on SC proliferation and differentiation. The present study aimed to investigate the effects of different concentrations of baicalin on the viability of RSC96 SCs. The results revealed that baicalin was able to promote the viability of RSC96 SCs at a particular concentration.

Materials and methods

Cell culture. RSC96 SCs were purchased from China Center for Type Culture Collection (Wuhan, China) and cultured in Dulbecco's modified Eagle medium (DMEM)-F12 (1:1; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China) and 1% of penicillin/streptomycin in an incubator at 37˚C with 95% air and 5% CO_2. Baicalin (Chengdu Best-Reagent Chemical, Co., Ltd., Chengdu, China) was dissolved in 0.2% dimethyl sulfoxide (DMSO) and prepared as a stock solution with a final concentration of 100 mM and stored at -20˚C. The stock solution was diluted with culture medium immediately prior to treatment.

Cytotoxicity assay. To determine the level of cytotoxicity of baicalin on RSC96 SCs, cell cytotoxicity was detected with a MTT assay (Gibco; Thermo Fisher Scientific, Inc.) method. RSC96 SCs were seeded in 96-well plates at a density of 1,000 cells/well and the cell viability was determined by using the MTT assay on day 3. Following treatment with various concentrations of baicalin (0 to 1,000 μM where 0 μM was used as a control) for 3 days, 20 μl MTT (5 mg/ml) was added to each well and plates were incubated in the dark at 37˚C for 4 h. Once MTT was removed, cells were treated with 200 μl DMSO (Amresco, LLC, Solon, OH, USA) for crystal solubilization. The spectrometric absorbance at 570 nm was read using Multiskan™ GO microplate spectrophotometer (Thermo Fisher Scientific, Inc., USA).

Measure of cell viability via the MTT assay. RSC96 SCs were seeded in 96-well plates at a density of 1,000 cells/well and the cell viability was determined by using the MTT assay on days 2, 4, and 6. Once cells were treated with various concentration of baicalin (5, 10 and 20 μM), MTT solution (5 mg/ml) was added and the cells were incubated for 4 h at 37˚C. Following removal of the incubation medium, the dark blue formazan crystals formed in the intact cells and all samples were solubilized with 200 μl DMSO. Subsequently, the absorbance was measured at 570 nm on a microplate spectrophotometer (Thermo Fisher Scientific, Inc.).

Measure of cell viability via fluorescein diacetate staining. Live RSC96 SCs were examined using fluorescein diacetate (FDA) on days 2, 4 and 6. A stock solution of FDA (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) was prepared by dissolving 5 mg FDA in 1 ml acetone. Staining solution was prepared by mixing 5 ml PBS with 8 μl FDA stock solution. Once the culture medium was removed, 0.5 ml staining solution was added and the cells were stained in the dark for 5 min. The evaluation of viability was conducted by fluorescent microscopy (magnification, x100, Nikon Corporation. Tokyo, Japan). ImageJ software (version 1.48v; National Institutes of Health, Bethesda, MA, USA) was used for quantitative analysis of the fluorescein diacetate stained cells.

Hematoxylin and eosin staining. RSC96 SCs were grown at $1x10^5$ cells/ml in DMEM/F12 (1:1) with 0, 5, 10 or 20 μM of baicalin for 2, 4, and 6 days on a 24-well plate with a coverslip set at the bottom. Following fixing in 95% ethanol for 20 min, the coverslip contents were washed in PBS twice, immersed in hematoxylin for 2 min, and washed in water for 1 to 3 sec to remove hematoxylin. The coverslip was washed in 1% hydrochloric acid and ethanol for 2 to 3 sec, water for 10 sec, ammonia for 15 sec and running water for 10 sec. Eosin staining was performed for 1 min and the stain was removed by washing with water for 2 sec, 80% ethanol for 2 sec, 95% ethanol for 5 min and 100% ethanol for 10 min. Subsequently, the coverslip was air-dried and mounted with neutral gum for light microscopy analysis. Images of five random fields of the culture were captured (magnification, x100).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from RSC96 SCs using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), following the manufacturer's instructions. cDNA was synthesized from reverse transcribed total RNA using a PrimeScript RT reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd., Dalian, China). Briefly, residual DNA was removed as follows: 10 μl total volume of 2 μl 5x gDNA eraser buffer, 1 μg total RNA, 1 μl gDNA eraser and RNase-free dH_2O at 42˚C for 2 min. For reverse transcription, 20 μl total volume was used with 10 μl of the reaction solution as described, 4 μl 5x PrimeScript buffer 2, 1 μl PrimeScript RT enzyme mix I, 1 μl RT primer mix and 4 μl RNase-free dH_2O. This reaction was performed at 37˚C for 15 min, followed by incubation in an 85˚C water bath for 5 sec. The synthesized cDNA was cooled at 4˚C for 5 min and then stored at -20˚C until real-time quantitative PCR reactions. PCR was performed on Mastercycler® ep realplex 4 system (Eppendorf, Hamburg, Germany) using FastStart Universal SYBR Green Master (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's protocol. Briefly, a total reaction volume of 20 μl was used containing 10 μl SYBR Master Mix, 0.4 μl each primer (0.4 μmol/l), 2 μl cDNA, and 7.6 μl RNase-free dH_2O. The cycling conditions were as follows, for 35 cycles:

Denaturing, at 94˚C for 30 sec, annealing at 54˚C for 30 sec and extension at 72˚C for 30 sec. A final melting curve analysis was performed utilizing conditions of 95˚C for 15 sec, 60˚C for 60 sec, followed by 95˚C for 15 sec. The PCR products for glial cell-derived neurotrophic factor (GDNF), BDNF and ciliary neurotrophic factor (CNTF) were 129, 182 and 191 bp, respectively. The primer sequences are indicated in Table I. All reactions were performed in triplicate. The relative expression levels of mRNA were calculated using the comparative $2^{-\Delta\Delta Cq}$ method (28) and normalized against GAPDH.

Immunohistochemistry. RSC96 SCs were fixed in 95% ethanol for 20 min and washed in PBS twice. Cells were incubated in H_2O_2 (3%) for 10 min to block peroxidase and rinsed using distilled water. Sections were subsequently washed with PBS three times for 2 min. Rabbit anti-rat S100B antibody (1:200; catalogue no. BA0120; Wuhan Boster Biological Technology, Ltd., Wuhan, China) was added and incubated at room temperature for 2 h and subsequently rinsed with PBS, containing 0.05% Tween-20, three times for 2 min. Slides were incubated with peroxidase-conjugated goat anti-rabbit IgG (1:100; catalogue no. SP-9001; Zhongshan Jin Qiao Biotechnology Co., Beijing, China) for 30 min at 37˚C. Following incubation, sections were washed with PBS, containing 0.05% Tween-20, three times for 2 min. Diaminobenzidine was added to visualize primary antibody staining and samples were washed in distilled water. Subsequently, slides were counterstained with hematoxylin for 20 sec, washed once in water, mounted, dried and dehydrated by immersing in 70% ethanol for 10 min, 95% ethanol for 10 min and 100% ethanol for 10 min. Following dehydration, the mounted slides were observed by using a Nikon light microscope at a magnification, x100).

Statistical analysis. Data were statistically analyzed using the SPSS software package, version 17.0 (SPSS, Inc., Chicago, IL, USA). Statistical analysis among multiple samples was performed by one-way analysis of variance followed by post hoc least significant difference (LSD) tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Cytotoxicity of baicalin. The cytotoxicity of baicalin on RSC96 SCs was examined by MTT assay. RSC96 SCs were treated with baicalin at increasing concentrations (0.1 to 1,000 μM). Minimal cytotoxic effects were observed when RSC96 SCs were treated with baicalin for 3 days at doses 0.1, 0.5, 1, 5 or 10 μM (Fig. 1). However, significant cytotoxic effects were observed in cells treated with >50 μM, indicated by the significantly reduce viability exhibited by the SCs (P<0.001 vs. 0 μM; Fig. 1). Therefore, concentrations of 5, 10 or 20 μM of baicalin were selected for subsequent investigations.

Cell viability. The cell viability of RSC96 SCs was explored using the MTT assay in the present study. The viability of SCs was indicated to be time- and dose-dependent (Fig. 2). Furthermore, SCs were more viable when incubated with various concentrations of baicalin (0, 5, 10 or 20 μM) when compared with the control at different time points. Cell viability following treatment of baicalin (20 μM) significantly increased up to ~15% when compared with the control on day 2 (P<0.05; Fig. 2). In all groups of SCs treated with baicalin, 20 μM of baicalin was the optimal concentration that promoted the highest cell viability of RSC96 SCs.

To further investigate the effects of baicalin on RSC96 SC viability, the live viability of RSC96 SCs was analyzed by FDA staining. As shown in Fig. 3, the number of viable cells, which were green in color, increased with time in all groups. In agreement with the MTT analysis, a greater number of viable cells were presented in baicalin-treated groups when compared with the control at different corresponding culture times. These data support the beneficial effect of baicalin on SC survival. In all baicalin groups, the number of viable cells was highest when incubated in medium with 20 μM baicalin (Fig. 4).

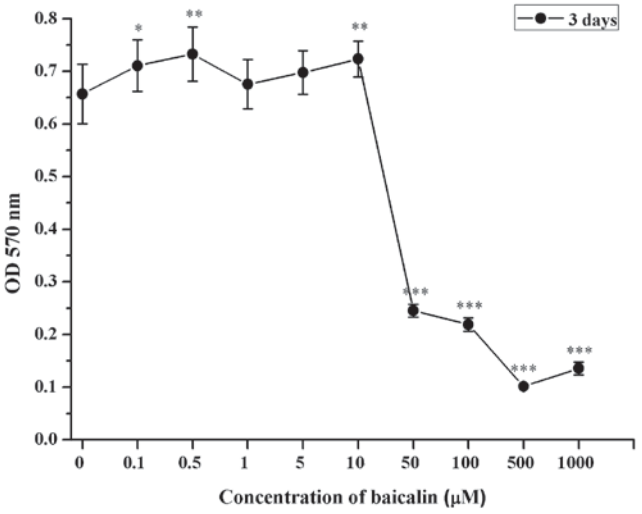

Figure 1. Cytotoxicity analysis of RSC96 Schwann cells treated with different concentrations of baicalin (0 to 1,000 μM) for 3 days (mean ± standard deviation; n=5). *P<0.05; **P<0.01; ***P<0.001 vs. the control group.

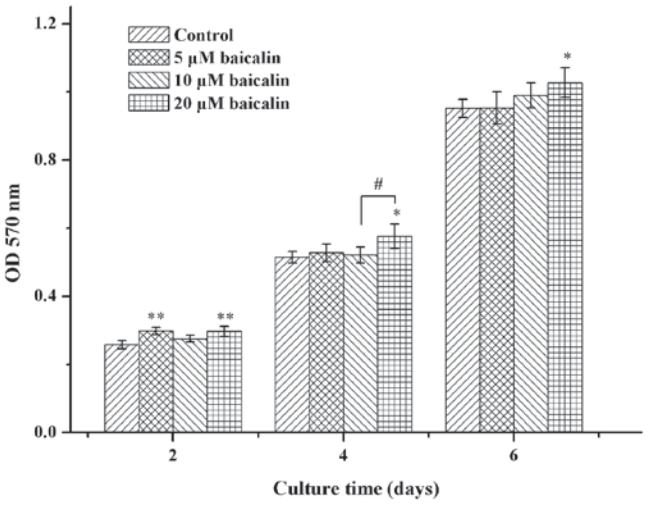

Figure 2. Proliferative effects of baicalin on RSC96 SCs. SCs were incubated with 0 (control), 5, 10 or 20 μM of baicalin for 2, 4 and 6 days. Cell viability was measured by MTT assay. *P<0.05, **P<0.01 vs. the control group. #P<0.05 between the indicated experimental groups. Data are presented as the mean ± standard deviation (n=3). SC, schwann cells.

Table I. Genes and oligonucleotide primers used in PCR analysis.

Gene	Primer sequence (5' to 3')	Length (bp)	Amplicon size (bp)
GDNF	F: AGACCGGATCCGAGGTGC	18	129
	R: TCGAGAAGCCTCTTACCGGC	20	
BDNF	F: TACCTGGATGCCGCAAACAT	20	182
	R: TGGCCTTTTGATACCGGGAC	20	
CNTF	F: ATGGCTTTCGCAGAGCAAAC	20	191
	R: CAACGATCAGTGCTTGCCAC	20	
GAPDH	F: GTCATCATCTCAGCCCCCTC	20	99
	R: GGATGCGTTGCTGACAATCT	20	

PCR, polymerase chain reaction; GDNF, glial cell-derived neurotrophic factor; BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; F, forward primer; R, reverse primer.

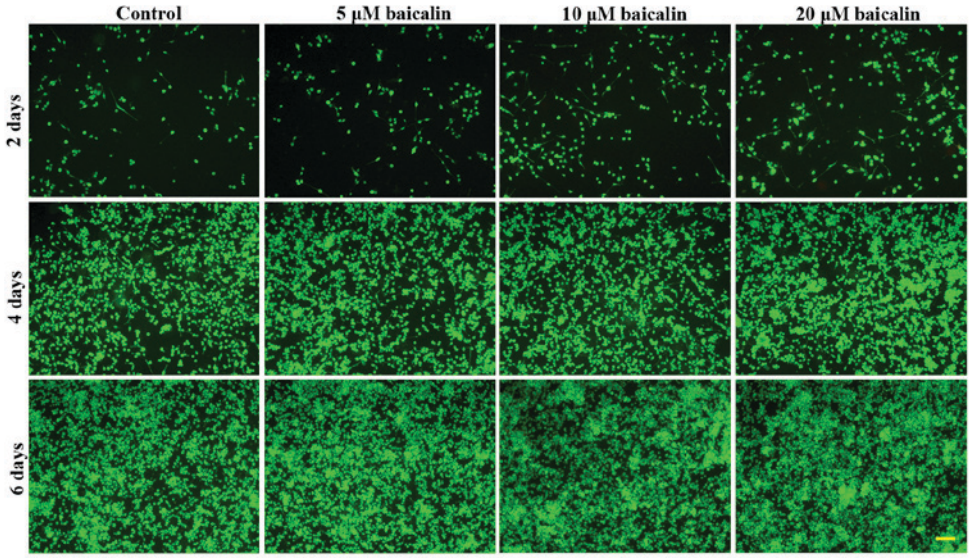

Figure 3. Cell viability was measured by fluorescein diacetate staining under a light microscope. RSC96 Schwann cells were incubated with 0 (control), 5, 10 or 20 μM of baicalin for 2, 4 and 6 days (magnification x100; scale bar, 200 μm).

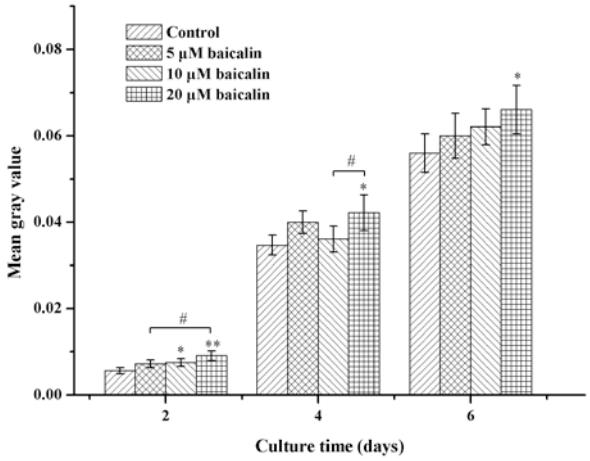

Figure 4. Quantitative analysis of the fluorescein diacetate stained cells, derived from ImageJ software. The mean gray value was obtained by integrated density divided by the area. *P<0.05, **P<0.01 vs. the control group. $^{\#}$P<0.05 between the indicated experimental groups.

Cell morphology. Hematoxylin and eosin staining was used to observe RSC96 SC morphology. Dendrites, the typical component of nerve cells, were clearly observed under the microscope following 2 days of culture; however, over time, the number of cells with dendrites decreased whereas the number of rounded cells increased. As showed in Fig. 5, the SCs grew slower in control when compared with the groups treated with baicalin at 2, 4 and 6 days. Furthermore, among the three concentrations, the present data suggests that 20 μM of baicalin stimulated cell proliferation the most prominently.

Gene expression. The effect of 0, 5, 10 or 20 μM of baicalin on RSC96 SCs was further investigated by detecting the gene expression of the important neurotrophic factors, GDNF, BDNF and CNTF. The expression levels of these genes were examined at 2, 4 and 6 days. Gene expression levels of GDNF, BDNF and CNTF were markedly increased in all baicalin-treated RSC96 SCs and significantly increased in RSC96 SCs treated with 20 μM baicalin when compared with the control (P<0.01),

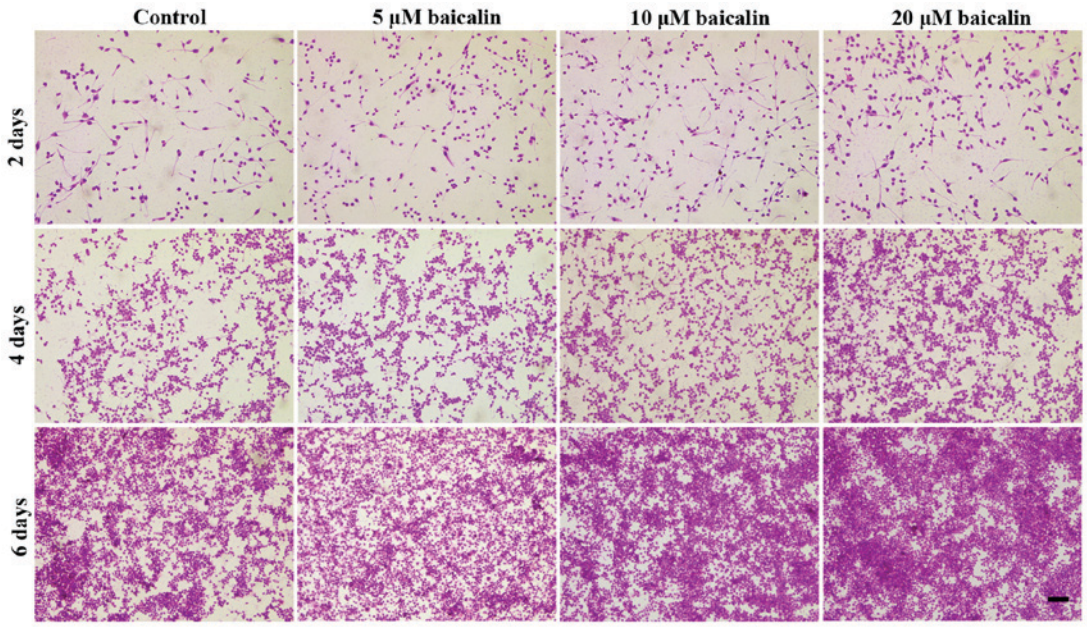

Figure 5. Hematoxylin-eosin staining images showing the morphology of RSC96 SCs cultured *in vitro* with 0 (control), 5, 10 or 20 μM of baicalin for 2, 4 and 6 days (magnification x100; scale bar, 200 μm).

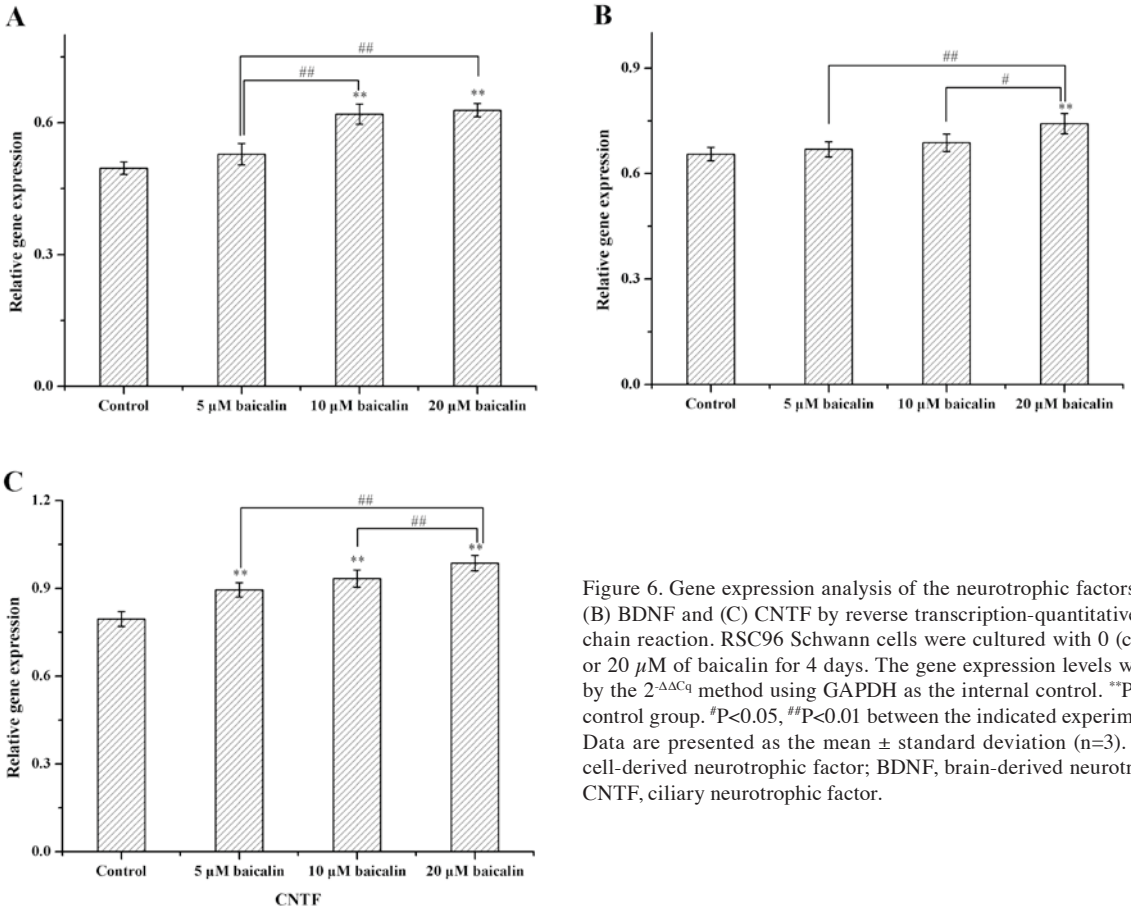

Figure 6. Gene expression analysis of the neurotrophic factors, (A) GDNF, (B) BDNF and (C) CNTF by reverse transcription-quantitative polymerase chain reaction. RSC96 Schwann cells were cultured with 0 (control), 5, 10 or 20 μM of baicalin for 4 days. The gene expression levels were analyzed by the $2^{-\Delta\Delta Cq}$ method using GAPDH as the internal control. **P<0.01 vs. the control group. $^{\#}$P<0.05, $^{\#\#}$P<0.01 between the indicated experimental groups. Data are presented as the mean ± standard deviation (n=3). GDNF, glial cell-derived neurotrophic factor; BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor.

which indicated that baicalin may stimulate the transcription of GDNF, BDNF and CNTF genes (Fig. 6). In addition, the present data suggested that SCs treated with 20 μM baicalin exhibited the highest gene expression levels of GDNF, BDNF and CNTF genes.

Expression of S100β. Expression of S100β was detected by immunohistochemical staining. RSC96 SCs were treated with 0, 5, 10 or 20 μM of baicalin at different time points. As indicated in Fig. 7, the expression of S100β was upregulated when the concentration of baicalin increased and treatment

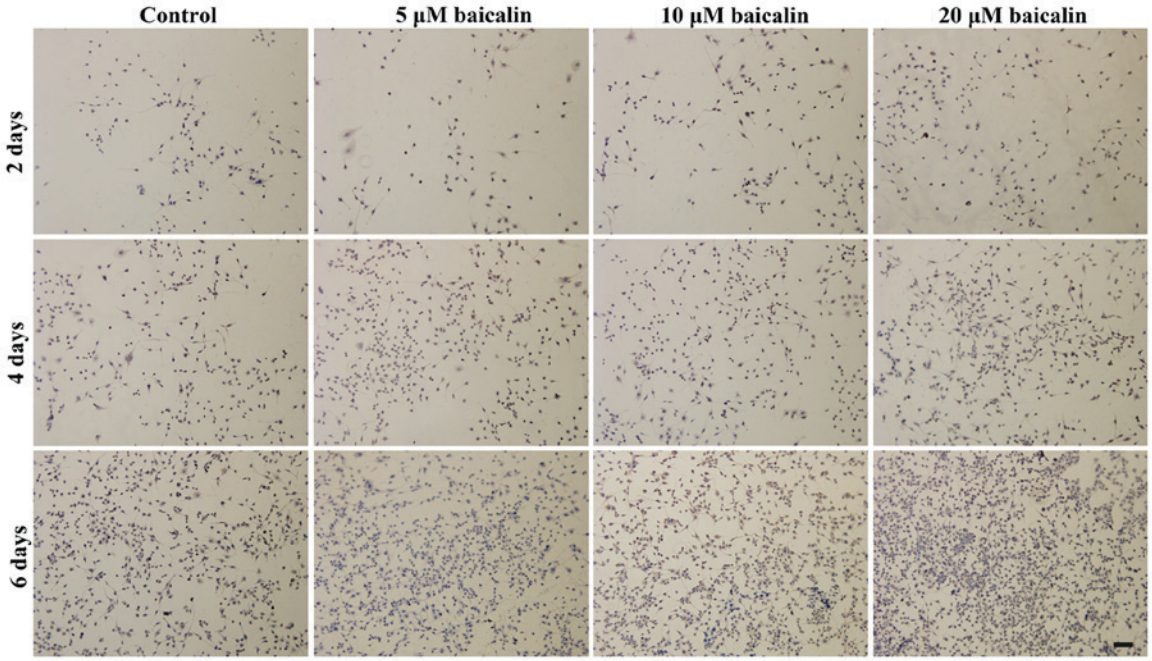

Figure 7. Immunohistochemical staining images revealed the presence of S100β. RSC96 Schwann cells were cultured *in vitro* with 0 (control), 5, 10 or 20 μM of baicalin for 2, 4 and 6 days (magnification x100; scale bar, 200 μm).

with 20 μM of baicalin resulted in the highest expression of S100β in SCs.

Discussion

The present study focused on the effect of baicalin on RSC96 SCs *in vitro*. The present findings indicated that baicalin significantly enhanced the viability of SCs. In addition, the expression of GDNF, BDNF and CNTF was significantly upregulated in the presence of 20 μM baicalin. These findings revealed that baicalin is capable of enhancing SCs survival and function *in vitro*. This may corroborate that baicalin is a key component that is able to contribute to nerve repair by *S. baicalensis* (29). Moreover, the present study highlights the possibility of promoting nerve regeneration in cellular nerve grafts through baicalin-induced neurotrophin secretion in SCs.

Acceleration of the proliferation of nerve cells is important due to the slow axonal growth that is the cause of poor functional recovery, which may lead to prolonged denervation of end organs, raising the specter of permanent paralysis (30). In the present study, baicalin exhibited an effect in a dose-dependent manner on the viability of RSC96 SCs, whereby at the concentration of 20 μM, SCs exhibited the highest viability, as evidenced by cell viability assay and histological evaluation. S100, which is a SC marker (31), was elevated when SCs received baicalin treatment, as demonstrated by the increased protein expression levels of S100 in baicalin-treated cells when compared with the control, via immunohistochemical examination. Natural substrates, such as traditional medicinal herbs, are well-known for their relatively minor adverse effects (32). Extracts from *S. baicalensis* are considered to exhibit low cytotoxicity (33) and have neuroprotective properties (34). As one of the active components, baicalin has been reported to promote neuroprotective effects in rats (25,35), which is in agreement with the findings of the present study.

Nerve growth factor and several neurotrophic factors have been reported to elicit stimulatory effects on specific neuronal populations (36,37). They affect several vital aspects of regeneration, including axon growth, SC function and myelination (38). GDNF, BDNF and CNTF are several important neurotrophic factors that are important in the process of nerve cell regeneration (39). A previous study indicated that CNTF is able to enhance myelin formation and myelinate regenerating axons in the course of regrowth (40,41). Furthermore, it has been suggested that BDNF is a necessary component for axon regeneration (42) and a small peptide mimetic of BDNF was demonstrated to promote peripheral myelination (43). Moreover, GDNF has been indicated to be beneficial to peripheral nerve regeneration and functional recovery in multiple experimental nerve injury models (44,45). In addition, a recent study on autograft-based repair revealed that BDNF, GDNF and nerve growth factor showed considerable promise as these factors enhanced modality-specific axon regeneration in autografts (46). In the present study, when RSC96 SCs were incubated with 20 μM baicalin, the gene expression levels of BDNF, CNTF and GDNF were significantly elevated, as determined by RT-qPCR. These findings suggest that baicalin likely promotes SCs viability and proliferation by stimulating neurotrophic factors, such as CNTF and GDNF.

S100 is associated with cell proliferation and differentiation (47). In the S100 protein family, S100B has been reported to be a potentially important factor contributing to neuronal development (48) and differentiation. A previous study has indicated that S100A4 is capable of stimulating neuronal differentiation in cultures of rat hippocampal neurons (49). In the present study, S100 protein expression levels were elevated by baicalin-treatment, as demonstrated by immunohistochemical examination. These findings suggest that baicalin may stimulate SC viability and differentiation via upregulation of S100.

The present results showed that the different concentrations of baicalin (5 to 20 μM) affected the viability of RSC96 SCs, with 20 μM having a significant effect. Among the chosen concentrations, treatment with 20 μM of baicalin indicated the optimal cell viability and stimulated the most secretion of S100 in RSC96 SCs.

In conclusion, the present study corroborated that baicalin has a regulative effect on the viability of RSC96 SCs. Furthermore, the present findings suggest baicalin likely affects SC metabolism by modulating the expression of several neurotrophic factors, such as BDNF, GDNF and CDNF. To conclude, the present study suggests that baicalin may be a promising therapeutic agent for peripheral nerve regeneration.

Acknowledgements

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