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Research Article

The Proliferation Enhancing Effects of Salidroside on Schwann Cells In Vitro

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Derived from *Rhodiola rosea* L., which is a popular plant in Eastern Europe and Asia, salidroside has pharmacological properties including antiviral, anticancer, hepatoprotective, antidiabetic, and antioxidative effects. Recent studies show that salidroside has neurotrophic and neuroprotective effects. However, the effect of salidroside on Schwann cells (SCs) and the underlying mechanisms of the salidroside-induced neurotrophin secretion have seldom been studied. In this study, the effect of salidroside on the survival, proliferation, and gene expression of Schwann cells lineage (RSC96) was studied through the examinations of the cell viability, proliferation, morphology, and expression of neurotrophic factor related genes including BDNF, GDNF, and CDNF at 2, 4, and 6 days, respectively. These results showed that salidroside significantly enhanced survival and proliferation of SCs. The underlying mechanism might involve that salidroside affected SCs growth through the modulation of several neurotrophic factors including BDNF, GDNF, and CDNF. As for the concentration, 0.4 mM, 0.2 mM, and 0.1 mM of salidroside were recommended, especially 0.2 mM. This investigation indicates that salidroside is capable of enhancing SCs survival and function in vitro, which highlights the possibility that salidroside as a drug agent to promote nerve regeneration in cellular nerve scaffold through salidroside-induced neurotrophin secretion in SCs.

1. Introduction

Fractures, hematomas, contusions, and compression always caused peripheral nerve injury characterized by the disruption of myelin sheaths and axons [1, 2]. Recovery without drug treatment is time-consuming and incomplete and sometimes would result in functional impairment and decreased quality of life [3, 4]. In crushed peripheral nerve injury, the basal lamina Schwann cells (SCs) tubes are still intact and regenerated axons can proceed along the original basal lamina tubes towards target organs [5]. One significant feature of SCs is their ability to produce an array of trophic factors such as brain derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), and cerebral dopamine neurotrophic factor (CDNF) that promote the growth of regenerating axons after acute or delayed peripheral nerve injuries [6–8]. SCs also have the ability to dedifferentiate, migrate, and proliferate in endogenous restoration of peripheral nerves [9]. During the repair process, SCs migrate from the periphery into the injury site and produce some signals and cytokines which are crucial for peripheral nerve repair [10]. Therefore, to enhance the ability of SCs to promote

regeneration in peripheral neurons is of significance [11]. Drug therapy is one of the most typical choices to enhance the ability of SCs, which has attracted considerable research interest [12, 13].

Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical diseases [14]. Traditional Chinese herbs are highly recommended because of their constituents, multitarget, and minimum side effects, as concluded from the long history of the clinical application. *Rhodioloside* (syn. salidroside) which has been identified to enhance nerve regeneration is a phenylpropanoid glycoside isolated from *Rhodiola rosea L.*, a popular plant in traditional medicine in Eastern Europe and Asia possessing pharmacological properties including antiviral activities and anticancer, hepatoprotective, antidiabetic, and antioxidative effects [15–19]. Recently, it has been demonstrated that salidroside exerted neurotrophic and neuroprotective effects in numerous studies in vitro [20–22]. Sheng QS reported that salidroside achieved successful nerve regeneration in the rat as evidenced by walking track analysis, electrophysiological assessment, and histological evaluation [23]. However, the effect of salidroside on SCs growth has seldom been studied and whether it is the key component of *Rhodiola rosea L.* that contributes to nerve regeneration is still unknown.

We hypothesized that salidroside promoted survival and proliferation of Schwann cells through the secretion of GDNF, BDNF, and CDNF. In this study, we investigated the effect of salidroside on the survival, proliferation, and gene expression of Schwann cells to explore the underlying mechanism of the salidroside-induced neurotrophin secretion in SCs.

2. Material and Methods

2.1. Preparation of Salidroside. Salidroside was purchased from Chengdu Best Reagent Co., China, and was dissolved in 0.2% DMSO to be prepared as a stock solution with the final concentration of 2 mM. The stock solution was stored at −20°C. The stock solution was diluted with culture medium immediately to various concentrations ranging from 0.0125 mM to 2 mM prior to use.

2.2. Cell Culture. RSC96 Schwann cells were purchased from China Center for Type Culture Collection (CCTCC) and were cultured in Dulbecco's Modified Eagle's medium (DMEM) : F12 = 1 : 1 (Thermo Fisher Beijing, China) supplemented with 10% fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co.) and 1% of penicillin/streptomycin in incubator at 37°C in humidified atmosphere containing 5% CO_2.

2.3. Preliminary Drug Screening and Cell Cytotoxicity Assay. Preliminary screening and cytotoxicity analysis were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) method. For preliminary screening, cells were cultured with various concentrations of salidroside (0.0125 mM, 0.025 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.4 mM, 0.8 mM, 1 mM, and 2 mM) for 3 days, Optimal concentrations of salidroside were chosen for further study based on the results of preliminary screening. For cell cytotoxicity assay, Schwann cells were seeded in 24-well plates at a density of 10,000 cells/well with the addition of salidroside (0, 0.1, 0.2, and 0.4, resp.) or 10 ng/mL nerve growth factor (NGF, Pepro Tech, USA) [24] for 2, 4, and 6 days. Briefly, MTT (0.5 mg/mL) was added to each well and the plates were incubated in the dark at 37°C for 4 h. Then for removal of the solution, dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added for formazan-crystal solubilization. The spectrometric absorbance at 570 nm was read using an enzyme-labeled instrument (Thermo Fisher Scientific, UK). Preliminary screening and cytotoxicity assays were performed in triplicate.

2.4. Cell Viability Assay. Live/dead cells were examined by fluorescein diacetate (FDA; Sigma-Aldrich Co., USA) and propidium iodide (PI; Sigma-Aldrich Co., USA) for 2, 4, and 6 days. Staining solution was prepared by mixing 5 mL PBS (phosphate buffer saline) with 8 μL FDA (5 mg/mL) and 50 μL PI (2 mg/mL). After the culture medium was removed, staining solution was added to the cell culture dish and incubated for 5 min in the dark, and then the cells were visualized with a laser scanning confocal microscope (Nikon, Japan).

2.5. Cell Morphological Analysis. RSC96 cells were treated with salidroside of 0, 0.1, 0.2, and 0.4 mM or NGF, respectively, for 2, 4, and 6 days and then fixed in 95% ethanol for 30 min. After washing with PBS for three times, cells were stained using hematoxylin and eosin kit (HE, JianCheng Biotech, China). Cells were then mounted in neutral gummi for light microscopy analysis. Images were photographed by a microscope (Zeiss Corporation, German). Three images (×100) randomly from either the middle or the border of each well were taken and the stained cell nuclei in the images were counted. Each experiment was performed three times in parallel.

2.6. Immunohistochemical Analysis. RSC96 SCs were fixed in 95% ethanol for 30 min. The cells were incubated in H_2O_2 (3%) for 10 min to block peroxidase and rinsed by distilled water. And then nonspecific binding was blocked by normal goat serum for 10 min at room temperature. Rabbit anti-rat IgG antibody (1 : 100) to S100β (Boster, China) which is an astrocyte marker was added and incubated at room temperature for 2 hours and then rinsed with PBS 3 times for 2 min each time. Slides were then incubated with HRP Polymer-anti-Rabbit IgG for 30 min at room temperature. Diaminobenzidine (DAB) was added to visualize primary antibody staining and washed in distilled water. After the slides had been counterstained with hematoxylin for 20 seconds, they were washed once in water, dehydrated, and mounted. The mounted slides were observed by using a microscope (Zeiss Corporation, German).

2.7. Real-Time Quantitative RT-PCR. The expressions of GDNF, BDNF, and CDNF were analyzed by qRT-PCR. Total RNA was extracted from RSC96 SCs using Trizol reagent

Table 1: Genes and oligonucleotide primers used in PCR analysis.

Gene	Primer sequence (5′ to 3′)	Length (bp)	Amplicon size (bp)
GDNF	F: CGGACGGGACTCTAAGATGA	20	109
	R: CGCTTCGAGAAGCCTCTTAC	20	
BDNF	F: GTGGTTACCTGACTGGGCTC	20	195
	R: ACAGGGGATTCAGTGGGACT	20	
CDNF	F: CGAGGGCTGACTGTGAAGTA	20	182
	R: GGTGGCCGAGTCTTTGGTAG	20	
β-actin	F: CCCATCTATGAGGGTTACGC	20	150
	R: TTTAATGTCACGCACGATTTC	21	

PCR: polymerase chain reaction; GDNF: glial cell-derived neurotrophic factor. BDNF: brain-derived neurotrophic factor; CDNF: cerebral dopamine neurotrophic factor; F: forward primer; R: reverse primer.

following the manufacturer's instructions, and cDNA was synthesized from the total RNA using a PrimeScript RT reagent kit with gDNA Eraser (Takara, Dalian). For reverse transcription polymerase chain reaction (RT-PCR), the PCR reaction consisted of 35 cycles of denaturing at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s. The PCR products for GDNF, BDNF, and CDNF were 109, 195, and 182 bp, respectively. The primers were shown in Table 1. For real-time quantitative RT-PCR, the procedures were performed using FastStart Universal SYBR Green Master (Roche, US) on a Master cycle reprealplex 4 system (Eppendorf, German). All reactions were run in triplicate. The relative expression of mRNAs was calculated using the comparative $2^{-\Delta\Delta Ct}$ method and normalized against β-actin (Table 1).

2.8. Western Blot Assay. Cell proteins were extracted using RIPA Lysis Buffer (Beyotime, China) and PMSF (Beyotime, China) after 6 days. The protein concentration was determined by the BCA assay reagent. 60 μg proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to a PVDF membrane (Millipore, USA), and incubated overnight at 4°C with the following antibodies: anti-BDNF, anti-CDNF, anti-GDNF, and anti-β-actin (Boster company, Wuhan, China). These membranes were incubated with Alexa Fluor 790 dye-conjugated secondary antibodies (Invitrogen, USA) for 1 h at room temperature. Immunoreactive bands were detected by an Odyssey Infrared Imaging System (LI-COR) according to the manufacturer's instructions.

2.9. Statistical Analysis. Date were presented as the means ± SD. The data were analyzed using the SPSS17.0 statistical package (Chicago, USA). Statistical significance was determined using Student's t-test for data comparisons. The level of significance was set to $P < 0.05$.

3. Results

3.1. Preliminary Drug Screening and Cell Cytotoxicity Assay. For preliminary screening, Schwann cells were cultured and treated with salidroside in increasing concentrations (0.0125 to 2 mM) compared to the control group (0 mM). As shown in Figure 1, no or very low cytotoxic effect was observed when

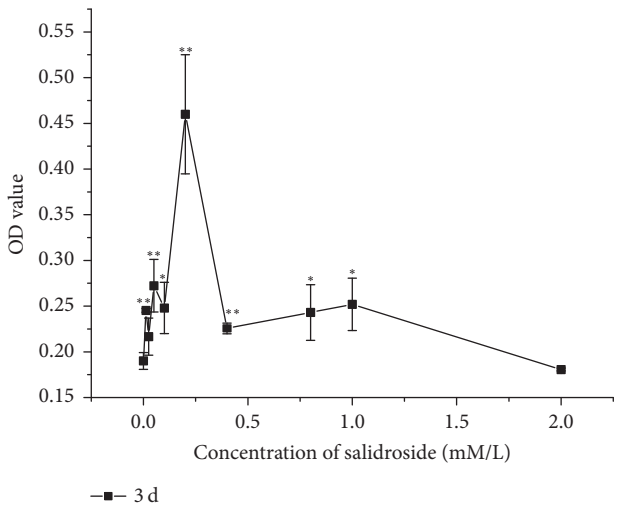

Figure 1: Preliminary drug screening analysis of SCs treated with different concentrations of salidroside after 3 days (mean ± SD, $n = 4$). Notes: $^{*}P < 0.05$, $^{**}P < 0.01$, versus control group.

SCs were treated with the salidroside ranged from 0.0125 to 1 mM. The concentrations of 0.1, 0.2, and 0.4 mM within which a peak value was observed were chosen for further investigation.

To determine the effects of salidroside on RSC96 growth, MTT was used to analyze cell proliferation of SCs in five groups. As shown in Figure 2, the proliferation of SCs is both time- and dose-dependent. Comparatively, SCs grew faster when incubated with various concentration of salidroside than control at different time point. Cell viability after treatment of salidroside of 0.1, 0.2, and 0.4 mM increased compared with control. We did not observe a significant difference between the 0.2 mM of salidroside and NGF group at 2 days and 4 days. Among the three concentrations, 0.2 mM of salidroside was the optimal concentration which stimulated the proliferation of RSC96 SCs.

3.2. Cell Viability. Live-dead viability of RSC96 SCs was analyzed by FDA/PI staining. As shown in Figure 3, viable cells which were green in color increased with time in all groups. In agreement with the MTT analysis, more viable

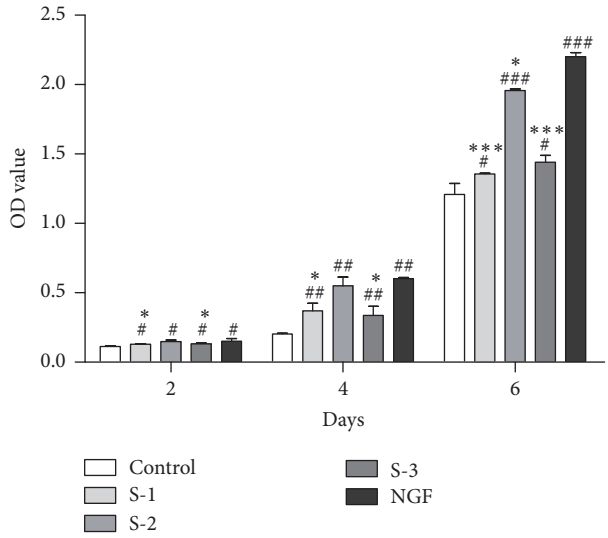

FIGURE 2: Proliferative effects of salidroside on RSC96 SCs. SCs were incubated with different doses of salidroside (control: 0 mM, S-1: 0.1 mM, S-2: 0.2 mM, S-3: 0.4 mM) or NGF for 2, 4, and 6 days. Cell proliferation was measured by MTT assay. Data of each bar are shown as the mean of three independent experiments ± SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with the control; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the positive control (NGF).

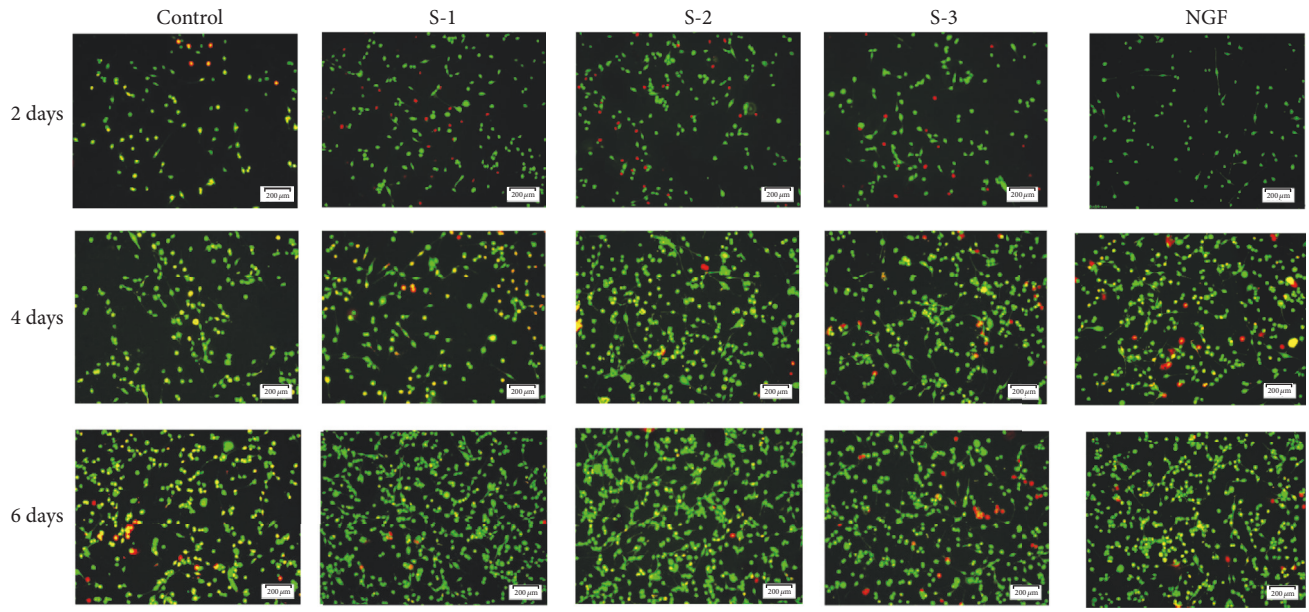

FIGURE 3: Cell viability was measured by FDA/PI staining under microscope. RSC96 SCs were incubated with different doses of salidroside (control: 0 mM, S-1: 0.1 mM, S-2: 0.2 mM, S-3: 0.4 mM) or NGF for 2, 4, and 6 days. Viable cells were green in color and dead cells were red (original magnification ×40).

cells and less dead red cells were presented in salidroside groups than the control at different culture time. Cell imaging also strongly supported the beneficial effect of salidroside on SCs survival. In all the salidroside groups, the amount of live cells was the highest when incubated in medium with 0.2 mM salidroside.

3.3. Cell Morphology. HE staining was used to observe the RSC96 SCs morphology (Figure 4(a)). Spindle-shaped bi- or tripolar SCs could be clearly observed under the microscope at early days and cells increased to form clusters with time elapsed. For each specimen, the mean number of SCs in 3 fields of view from different areas of the slide was counted as showed in Figure 4(b). The SCs grew slower in control than in groups treated with salidroside at 2, 4, and 6 days supported by quantitative data. We did not observe a significant difference between the 0.2 mM of salidroside and NGF group at 2, 4, and 6 days. Among the three concentrations, salidroside at

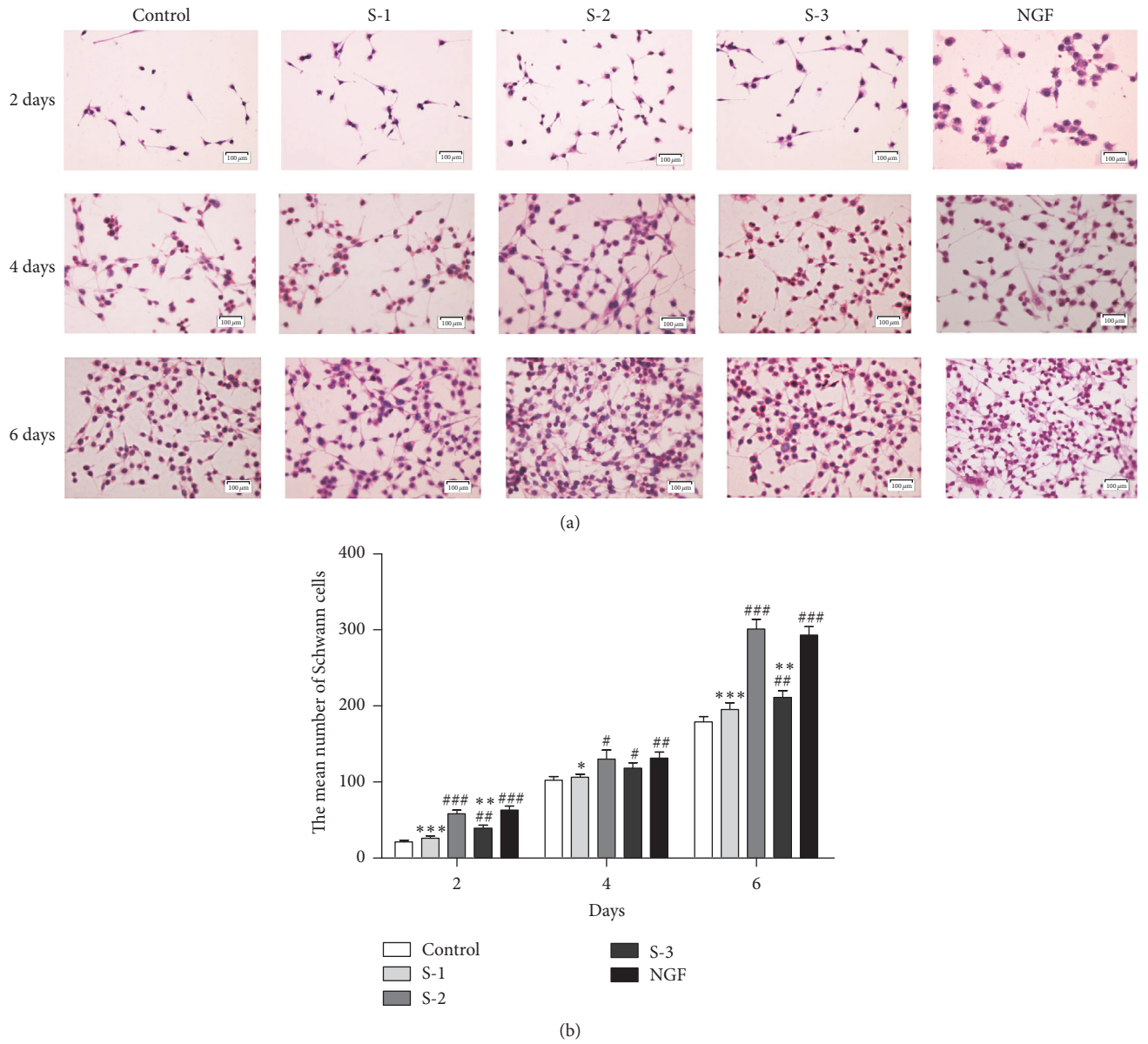

Figure 4: (a) Hematoxylin-eosin staining images showing the morphology of SCs cultured in vitro with different doses of salidroside (control: 0 mM, S-1: 0.1 mM, S-2: 0.2 mM, S-3: 0.4 mM) or NGF for 2, 4, and 6 days. Spindle-shaped bi- or tripolar SCs could be clearly observed (original magnification ×100). (b) Quantitative data of the mean number of SCs. Data of each bar are shown as the mean of three independent experiments ± SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with the control; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the positive control (NGF).

the concentration of 0.2 mM stimulated the cell proliferation the most prominently.

3.4. Immunohistochemical Analysis. The production of S100 was evaluated by immunohistochemical assay after treatment for 2, 4, and 6 days. As shown in Figure 5(a), SCs showed positive cytoplasmic staining for S-100 in which the cell bodies were brown with the round or oval blue nucleus. The RSC96 SCs grew faster treated with salidroside than the control supported by quantitative data as shown in Figure 5(b). Among the three concentrations, salidroside at the concentration of 0.2 mM stimulated the cell proliferation the most prominently as NGF.

3.5. Gene Expression. The effect of various concentration of salidroside on RSC96 SCs was further investigated by detecting gene expression of several important neurotrophic factors, such as GDNF, BDNF, and CDNF. The expression of these genes was examined at 2, 4, and 6 days. As shown in Figure 6(a), the gene expression of GDNF, BDNF, and CDNF in experiment groups was obviously higher than control, which indicated that salidroside stimulated the transcription

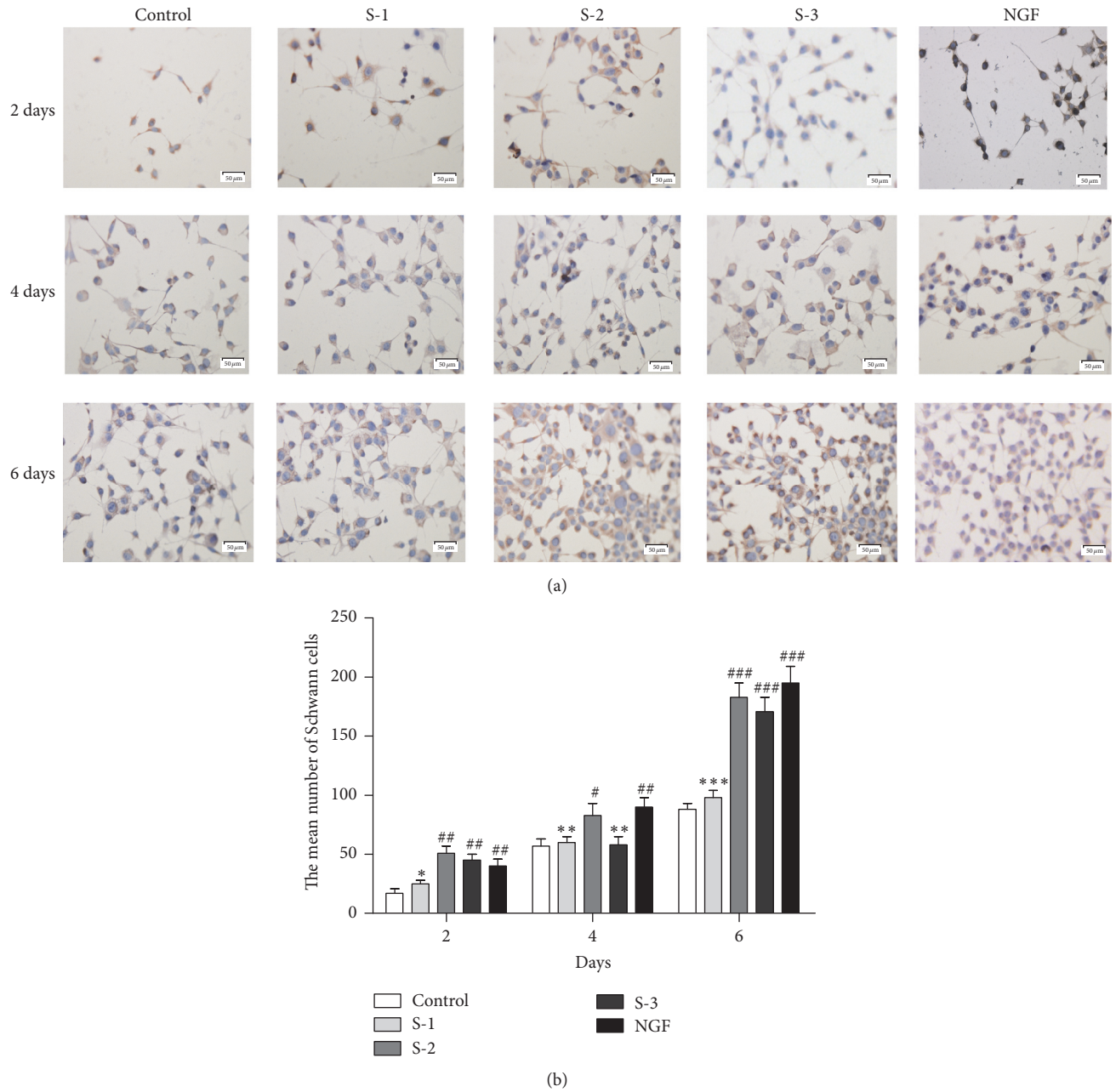

Figure 5: (a) Immunohistochemical staining images revealed the presence of S100. SCs cultured in vitro with different doses of salidroside (control: 0 mM, S-1: 0.1 mM, S-2: 0.2 mM, S-3: 0.4 mM) or NGF for 2, 4, and 6 days. SCs showed positive cytoplasmic staining for S-100 in which the cell bodies were brown with the round or oval blue nucleus (original magnification ×200). (b) Quantitative data of the mean number of SCs. Data of each bar are shown as the mean of three independent experiments ± SD. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with the control; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the positive control (NGF).

of GDNF, BDNF, and CDNF genes. In addition, the figure suggested that 0.2 mM salidroside achieved the best performance on SCs.

3.6. Western Blot Assay. As shown in Figure 6(b), the expression of BDNF, CDNF, and GDNF proteins was examined after 6 days. S-2 and NGF groups expressed higher level compared with other groups which agree with the results of RT-PCR.

4. Discussion

Previous studies had shown that Schwann cells derived from the neural crest through intermediate SC precursors could encircle the axon to form a myelin sheath to contribute to the promotion of axonal regeneration [24]. In the therapy of peripheral nerve injury, the acceleration of the proliferation of nerve cells is of significance because the slow axonal growth

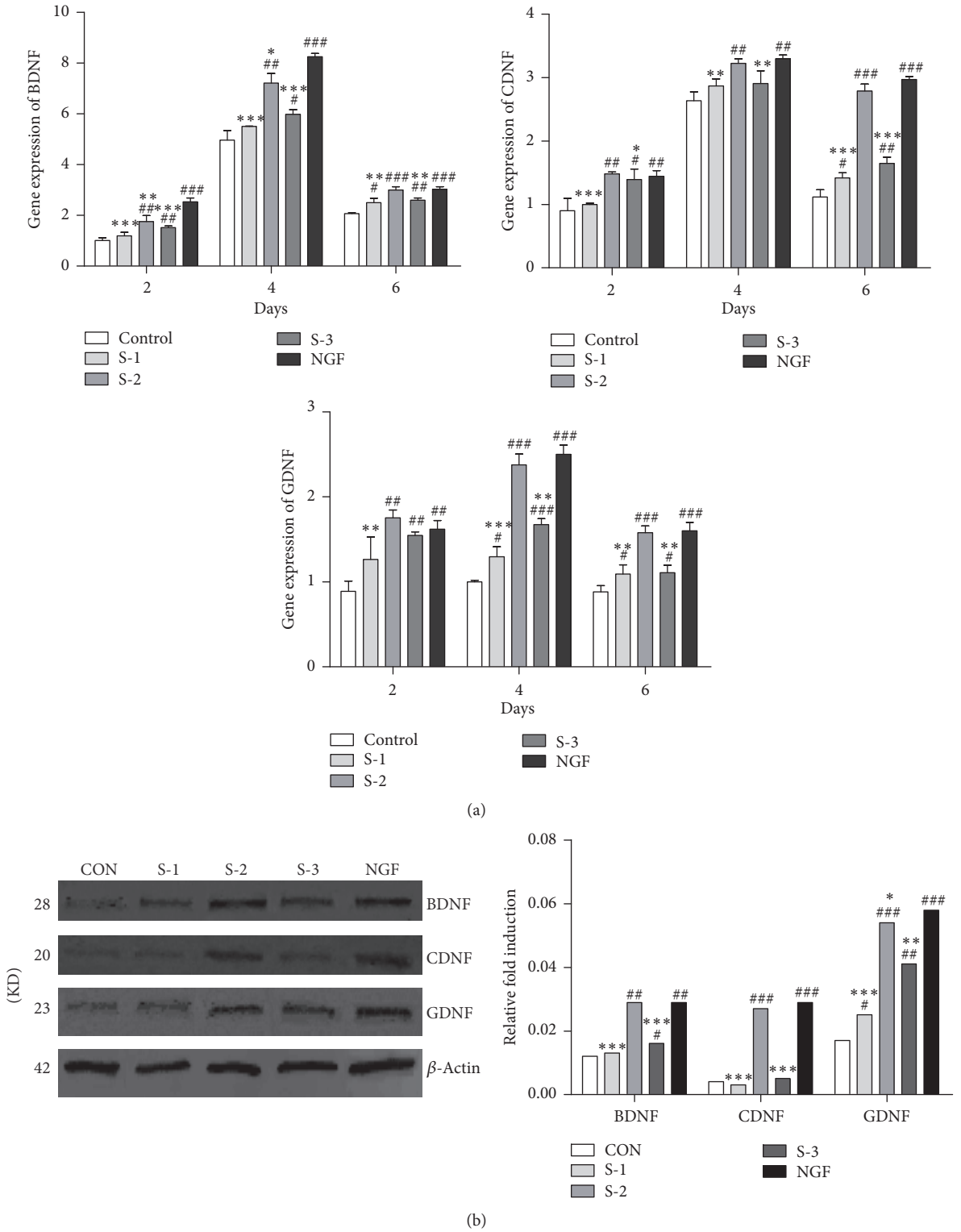

Figure 6: (a) Gene expression analysis of three important neurotrophic factors (BDNF, CDNF, and GDNF) by qRT-PCR. The RSC96 SCs were cultured with different doses of salidroside (control: 0 mM, S-1: 0.1 mM, S-2: 0.2 mM, S-3: 0.4 mM) or NGF for 2, 4, and 6 days. The gene expression levels were analyzed by the $2^{-\Delta\Delta Ct}$ method using GAPDH as the internal control. (b) Western Blot assay of BDNF, CDNF, and GDNF proteins and quantification of the proteins expression after 6 days. The data represent the mean of three independent experiments ± SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with the control; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the positive control (NGF).

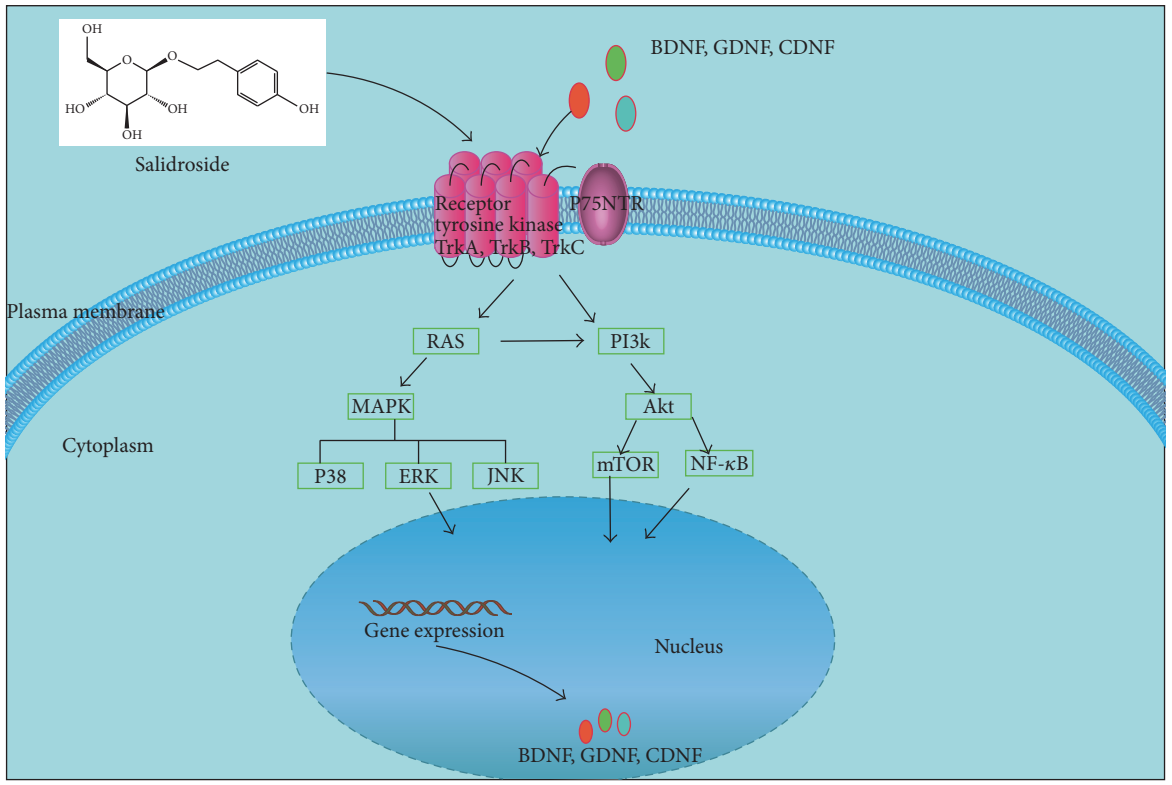

Figure 7: Schematic diagram of the mechanism of action of salidroside in nerve regeneration. Salidroside affects SCs by regulation of neurotrophic factors through PI3K/Akt and RAS-MAP kinase signaling pathways.

is the cause of poor functional recovery which can lead to prolonged denervation of end organs, raising the specter of permanent paralysis [25]. Drug therapy is one of the most typical choices to enhance the survival and proliferation of SCs to promote regeneration in peripheral nerve injury. Natural substrates such as traditional medicinal herbs are well known for the relatively minor adverse effects [26–28]. As one of the active components, salidroside was reported to be biocompatible and had a protective effect on endothelial cells, neuroblastoma cells, and so forth [26, 29]. Salidroside has been shown to be neuroprotective in many studies in vitro, which raises the possibility of using salidroside as a neuroprotective agent after nerve injuries [23, 30, 31]. For example, Sheng QS reported that salidroside achieved functionally successful nerve regeneration in a rat sciatic nerve model [23].

In the present study, salidroside had an effect in a dose-dependent manner on the proliferation of Schwann cells of RSC96, as evidenced by MTT analysis, cell viability assay, histological analysis, and immunohistochemical analysis. We found that salidroside significantly enhanced survival and proliferation of SCs at the concentration of 0.2 mM. Also, the expression of BDNF, GDNF, and CDNF was significantly upregulated. These findings indicated that the underlying mechanism of the salidroside-induced neurotrophin secretion on SCs may be through the modulation of several neurotrophic factors including BDNF, GDNF, and CDNF, which was helpful for the utilization of drug therapy such as salidroside in the therapy of peripheral nerve injury with tissue engineering strategy.

Upregulated expression of neurotrophic factors including BDNF, GDNF, and CDNF was verified in this study [32]. These factors secreted by SCs are crucial in nerve regeneration, myelination, and neuronal survival, which have been studied to characterize their individual effect on nerve regeneration. The transplantation of SCs with overexpressed BDNF promoted axonal regrowth across the transection site in the thoracic cord and increased auditory neuronal survival in the deaf guinea pig [33, 34]. Zhang et al. reported that GDNF significantly increased the number of myelin sheaths produced by SCs and enhanced the proliferation of SCs already in contact with axons [35]. As another neurotrophic factor, CDNF induces a strong promyelinating effect, which is capable of signaling oligodendrocytes to survive, differentiate, or grow [36, 37]. The upregulated synthesis of BDNF, GDNF, and CDNF by treatment of salidroside might hold promising potential for the protection of cell viability. Binding to a small subset of receptors, including the tyrosine kinases TrkA, TrkB, and TrkC as well as neurotrophic receptor p75 (p75NTR), these neurotrophic factors are coupled to the PI3K/Akt pathway and the RAS-MAP kinase signaling through phosphorylation of ERK1 and ERK2 [38]. It has been reported that salidroside exerts neuroprotective effect by regulation of PI3K/Akt and RAS-MAP kinase signaling pathways [39]. Similar drugs, such as the anti-Parkinson drug rasagiline (Azilect) and the neurotransmitter noradrenaline, were reported to regulate neurotrophic factors through PI3K/Akt [40, 41]. Thus, the underlying mechanism may be that salidroside affects SCs by regulation of neurotrophic factors through PI3K/Akt and RAS-MAP kinase signaling pathways (Figure 7).

5. Conclusion

This study corroborated that salidroside had a regulative effect on SCs proliferation and growth. The underlying mechanism that salidroside affects SCs metabolism might be through the modulation of expression of several neurotrophic factors such like BDNF, GDNF, and CDNF. This suggests that salidroside has the potential to be a neuroprotective agent for nerve injury repair through enhancing the survival and proliferation of Schwann cells transplanted in nerve scaffold. However, we have to realize that the neuroprotective effect of salidroside in vivo still needs to be investigated in further studies.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Hui Liu, Peizhen Lv, and Li Zheng conceived the study and participated in its design and coordination. Hui Liu, Peizhen Lv, Huayu Wu, Kun Zhang, and Fuben Xu performed the experiments. Hui Liu and Peizhen Lv drafted the main manuscript and performed the statistical analysis. Li Zheng and Jinmin Zhao corrected the original draft. All authors read and approved the final manuscript. Hui Liu and Peizhen Lv contributed equally to this work.

Acknowledgments

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