Proliferation and committed differentiation into dopamine neurons of neural stem cells induced by the active ingredients of radix astragali

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Neural stem cells (NSCs) are important cellular sources of transplantation therapies for Parkinson's disease. This study aimed to determine the effects of extracts of radix astragali on the proliferation and differentiation into dopamine (DA) neurons in NSCs. NSCs were dealt with astragaloside IV (ASI), astragalus polysaccharide (APS), and astraisoflavan (ASF), the main active ingredients of radix astragali. First, the results from cell-count kit-8 (CCK-8) assay showed that ASI, ASF, and APS had positive effects on the proliferation of NSCs. Next, we also confirmed the effects of ASI, APS, and ASF on BrdU and nestin by immunocytochemistry. Moreover, results from quantitative RT-PCR showed ASI, APS, and ASF could promote the expressions of tyrosine hydroxylase and dopamine transporter mRNA, which are specifically expressed in DA neurons. Simultaneously, sonic hedgehog (*Shh*), orphan nuclear hormone 1 (*Nurr1*), and pituitary homeobox 3 (*Ptx3*) are considered to motivate the formation of DA neurons. Our result showed ASI, APS, and ASF can also promote the expressions of *Shh*, *Nurr1*, and *Ptx3* mRNAs. In conclusion, our study verifies that the active ingredients of radix astragali can promote the proliferation of NSCs and induce NSC differentiation toward DA neurons *in vitro*. These phenomena may occur through upregulation of *Shh*, *Nurr1*, and *Ptx3* in the process of drug treatment. *NeuroReport* 29:577–582 Copyright © 2018 The Author(s). Published by Wolters Kluwer Health, Inc.

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Keywords: astragaloside IV, astragalus polysaccharide, astraisoflavan, differentiation, dopaminergic neurons, neural stem cells, proliferation

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Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative diseases characterized by the progressive loss of midbrain dopamine (DA) neurons in the substantia nigra. Although L-DOPA replacement therapy is currently the main method to treat PD, it cannot increase DA neurons and effect a radical cure. Currently, neural stem cells (NSCs) transplantation is thought to be a promising method to cure PD. NSCs are able to self-renew, migrate, and differentiate into the essential cells, such as neurons, astrocytes, and oligodendrocytes [1,2]. However, how to induce NSCs to differentiate into DA neurons is indeed a challenge.

Chinese medicinal herbs have been shown to have a variety of effects on proliferation and differentiation of NSCs [3,4]. Radix astragali (*Astragalus menbranaceus* Fisch; Huangqi), a Chinese medicinal herb, has been widely used in the therapies for PD in China. Previous studies have confirmed that radix astragali has an effect on the proliferation and differentiation of NSCs into neurons and astrocytes [5–8]. The main active components of radix astragali are astragaloside IV (ASI), astragalus polysaccharide (APS), and astraisoflavan (ASF). Therefore, we hypothesized that ASI, APS, and ASF might also induce differentiation of NSCs to DA neurons, which had not been reported in any studies.

In this study, we further investigated the effects of the main active components of radix astragali on the proliferation and differentiation of NSCs *in vitro*. Cell-count kit-8 (CCK-8) assay and BrdU staining were used to detect the proliferation of NSCs, and qRT-PCR was used to detect the mRNA levels of differentiation factors, tyrosine hydroxylase (TH), dopamine transporter (DAT), sonic hedgehog (*Shh*), orphan nuclear hormone (*Nurr1*), and pituitary homeobox 3 (*Ptx3*), after drug treatment. These findings would be helpful to find a new method for the adjuvant treatment of PD after NSC transplantation.

Materials and methods

Prepare for ASI, ASF, APS, and complete DMEM/F12 medium

ASI (assay standard, No.: B20564), ASF (assay standard, No.: B20563), and APS (assay standard, No.: B20562)

were purchased from Yuanye Biotechnology Company (Shanghai, China), and they were dissolved in complete DMEM/F12 medium (Gibco, New York City, New York, USA), containing 10 ng/ml of basic fibroblast growth factor (Gibco), 20 ng/ml of epidermal growth factor (PeproTech, Rocky Hill, Connecticut, USA), 2% B-27 supplement without vitamin A (Gibco), 1% N2 (Invitrogen, New York City, New York, USA), and penicillin (100 U/ml)/streptomycin (100 ug/ml) (Boster, Wuhan, China).

NSCs culture and identification

NSCs derived from the mesencephalons of Sprague-Dawley rat embryos at embryonic day 16 were grown in complete DMEM/F12 medium in cell culture flasks in a 37°C incubator with 5% CO_2. The method was as described in the study by Huang *et al.* [9]. In brief, the cells were plated at a density of 1×10^5/ml cells in the medium. Half of the medium was replaced every other day, and passaging was performed every 7 days. For identification *in vitro*, the formed neurospheres were placed in 24-well plates coated with 0.1 mg/ml poly-L-lysine (Sigma, Missouri, USA) and incubated for 24 h, and then fixed with 4% paraformaldehyde for 30 min. The expression of nestin protein, a specific biomarker of NSCs, was determined by immunocytochemistry as described in the study by Huang *et al.* [9]. Anti-nestin antibody produced in rabbit (1 : 100, ab5968; Abcam, Cambridge, UK) was used as primary antibodies, as well as anti-rabbit IgG-Cy3 antibody (1 : 70, No.: BA1032; Boster, Wuhan, China) was used as secondary antibody. DAPI (Boster, Wuhan, China) was applied to label nuclei.

Cell-count kit-8 assays

The cell viability was determined using cell-count kit-8 (CCK-8) (Dojindo, Tokyo, Japan). NSCs were plated into 96-well culture plates coated with poly-L-lysine at an optimal density of 1×10^5 cells/well. Following this, the cells were treated with different concentrations of ASI (including 0, 1, 2, 5, 10, 20, and 50 μg/ml), APS (including 0, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, and 2 μg/ml), and ASF (including 0, 10, 20, 50, 100, 200, 500, and 1000 μg/ml). After 7 days, the medium was gently aspirated, and 5 μl of CCK-8 reagent was added to each well and incubated at 37°C for 2 h. The optical density of each sample was immediately measured in a multiwell spectrophotometer at 450 nm.

Immunocytochemical analysis

The cells were cultured with the corresponding optimum concentrations of ASI, APS, and ASF as determined from CCK-8 method, and then bromodeoxyuridine (BrdU) staining was applied to detect the proliferation of NSCs on days 7 and 14. The cells were pretreatment with 10 μM BrdU (No.: B5002; Sigma) for 5 days. The method was as described in the study by Huang *et al.* [9] and Liu *et al.* [10]. Anti-nestin antibody produced in rabbit and anti-BrdU antibody produced in mouse (1 : 100, No.: B8434; Sigma) were used as primary antibodies, as well as anti-rabbit IgG-Cy3 antibody (1 : 70, No.: BA1032) and anti-mouse IgG-FITC antibody (1 : 50, No.: BA1101) (Boster, Wuhan, China) were used as secondary antibodies. DAPI was applied to label nuclei. Flouorescent images were obtained under a Leica confocal microscope with LAS AF Lite software (QS00IW; Leica, Wetzlar, Germany), and the number of BrdU-positive cells was counted by Image pro plus 6.0 (Media Cybernetics, Maryland, USA).

The differentiation and the mechanism were detected by fluorescence quantitative PCR

To compare the alteration in DA neuronal marker and relative factors of differentiation into DA neurons, total RNA of cells was isolated using Trizol reagent (Invitrogen) and reverse-transcribed to synthesize cDNA with a RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania). The LA PCR Kit (Takara, DaLian, China) was used to detect the expression of *TH*, *DAT*, *Nurr1*, *Ptx3*, and *Shh* mRNAs. The primer sequences are listed in Table 1.

Statistical analysis

All quantitative data are expressed as mean ± SD. Differences between each group were assessed by one-way analysis of variance. P value less than 0.05 was considered statistically significant. All data were analyzed using SPSS 21.0 (SPSS Inc., Chicago, USA).

Results

Primary culture and identification of NSCs

The isolated cells formed floating neurospheres after culturing for 3 days (Fig. 1a1), and when adhered for 24 h, the branches were noted (Fig. 1a2). The result from immunocytochemistry showed positive immunoreactivity to nestin, a marker of NSCs (Fig. 1a3), and Tuj-1, a marker of neurons (Fig. 1a4), which indicated the cells were NSCs and expressed genes to promote differentiation into neurons.

ASI, APS, and ASF promoted the proliferation of NSCs

The results from CCK-8 showed that the cell viability of ASI group in 20 μg/ml (424 ± 108%) and 5 μg/ml (391 ± 71%), APS group in 0.02 μg/ml (198 ± 36%) and 0.05 μg/ml (188 ± 45%), and ASF group in 10 μg/ml (145 ± 7%), 20 μg/ml (167 ± 14%), and 50 μg/ml (155 ± 11%) was increased compared with the control ($P < 0.05$) (Fig. 1b), suggesting ASI, APS, and ASF were able to promote proliferation of NSCs.

Next, NSCs were treated with 2 μg/ml of ASI, 0.02 μg/ml of APS, and 20 μg/ml of ASF, and then BrdU and nestin staining were used to detect the proliferation viability of NSCs after drug treatment. On days 7 and 14, the ratios of BrdU-positive cells in the ASI (82.4 ± 6.1 and

Table 1 Oligonucleotide sequences of primers used for qPCR

Targets	Forward primer	Reverse primer
TH	CTCCTCCTTGTCTCGGGCTGTA	GTCAGACACCCGACGCACAGAG
DAT	GGCGTTGGGTTTGGAGTGCTGA	TAGATGATGAAGATGAGCCCAGG
Nurrl	TGGTTCGCACGGACAGTTT	ATAGAGCCAGTCAGGAGATCGTA
Ptx3	TGCAGAGCTGGGTCGCTCG	TCCACTTGGTGCCATAGGAAAA
Shh	CTACTTGGGTTCTGAATGGAATGCT	GAACGGCTGACGCCCATCTTA
RN18s	AACGTCTGCCCTATCAACTTTC	CTGCTGCCTTCCTTGGATGTG

DAT, dopamine transporter; Nurr1, orphan nuclear hormone 1; Ptx3, pituitary homeobox 3; Shh, sonic hedgehog; TH, tyrosine hydroxylase.

84.0 ± 3.4%), APS (75.8 ± 7.2% and 80.6 ± 3.4%), and ASF (85.0 ± 6.4 and 96.2 ± 2.3%) groups were all increased compared with the control (61.8 ± 9.2 and 65.6 ± 5.6%) ($P < 0.05$), especially in ASF group on day 14 ($P < 0.05$) (Fig. 1d1), which suggests NSCs might have a high sensitivity to ASF. The expression of nestin was significantly decreased on the seventh day (60.5 ± 10%) compared with the control (77.9 ± 6%) ($P < 0.05$), whereas other groups had no obviously difference compared with the control group ($P > 0.05$) (Fig. 1d2).

ASI, APS, and ASF promoted the differentiation of NSCs to DA by up-regulating the expressions of TH, DAT, Nurr1, Shh, and Ptx3

TH and *DAT* are markers of DA neurons. In our study, the expressions of *TH* and *DAT* mRNA were measured by qPCR. On the seventh day, the expressions of *TH* and *DAT* mRNA were significantly increased after treating with ASI (33.5 ± 0.4 and 34.6 ± 0.4%, respectively) and ASF (34.8 ± 0.1 and 34.7 ± 0.1%, respectively) compared with the control group (28.5 ± 0.3 and 28.7 ± 0.2%, respectively) ($P < 0.05$). Moreover, *DAT* mRNA was increased after treating with APS (30.5 ± 0.5%) ($P < 0.05$). On the 14th day, the expression of *TH* mRNA was obviously increased after treating with ASI (32.2 ± 0.1%), APS (32.5 ± 0.1%), and ASF (31.2 ± 0.1%) compared with the control group (29.6 ± 0.2%) ($P < 0.05$), whereas the expression of *DAT* mRNA was significant increased after treating with ASI (35.2 ± 0.1%), APS (34.9 ± 0.1%), and ASF (34.7 ± 0.1%) compared with the control group (28.6 ± 0.4%) ($P < 0.05$) (Fig. 2a and b). These results suggested ASI, APS, and ASF might be able to induce differentiation of NSCs to DA neurons.

Moreover, we found that the expressions of *Nurr1*, *Ptx3*, and *Shh* mRNA were also increased in ASI, APS, and ASF groups on days 7 and 14 ($P < 0.05$). However, there was no difference in the expression of Shh between the ASF group and control on the seventh day ($P > 0.05$). The genes had no apparent change over time in our study (Fig. 2c–e and Table 2). In summary, these results revealed that ASI, APS, and ASF might be able to induce differentiation of NSCs to DA by up-regulating the expression of *Nurr1*, *Ptx3*, and *Shh*.

Discussion

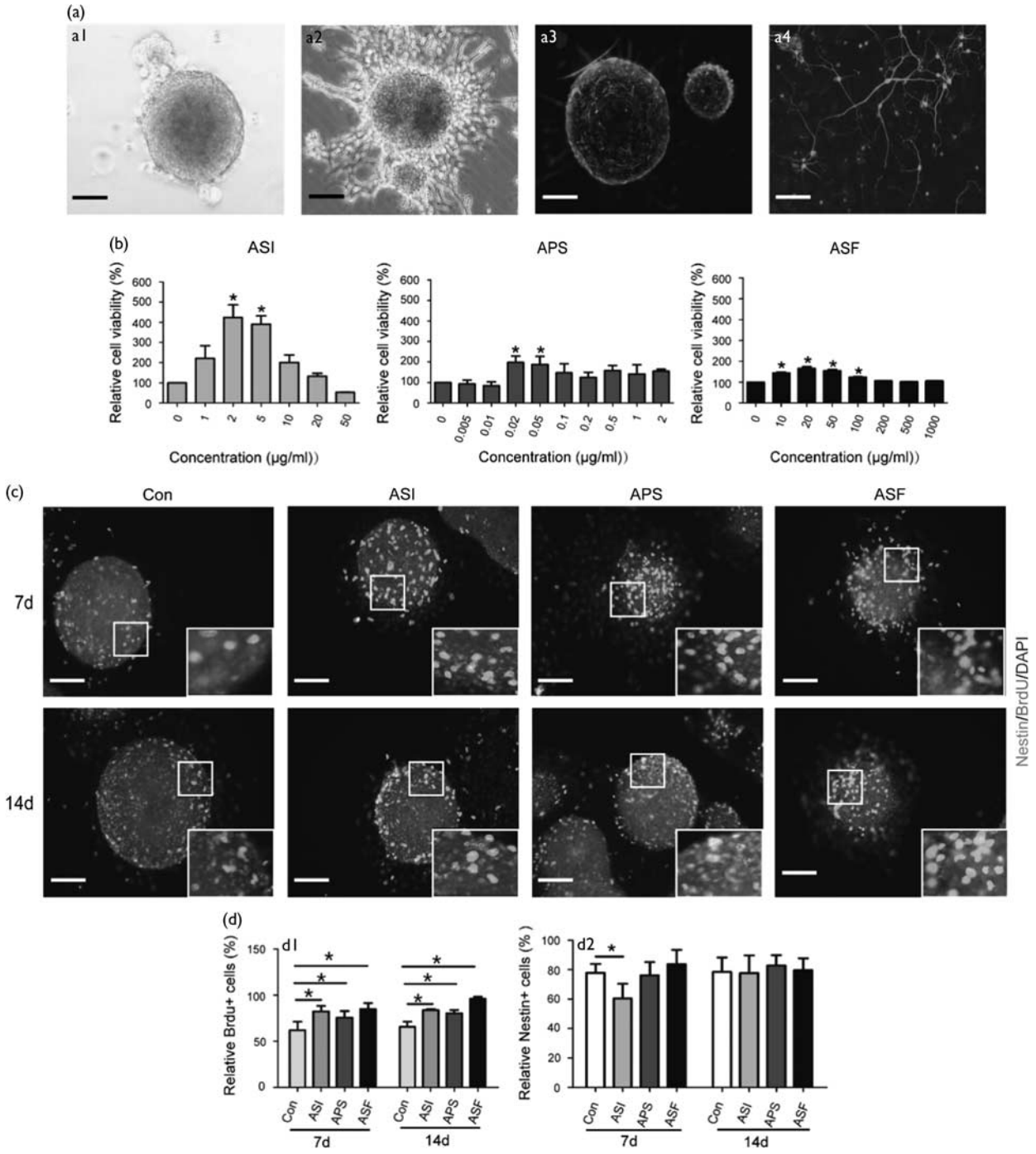
To cure PD, an increasing number of studies focus on the drugs that can influence the survival, regeneration, and differentiation of NSCs, and the traditional Chinese medicine is a valuable candidate. Radix astragali has been widely used to postpone the development of PD in clinical treatment [11,12]. Therefore, we hypothesized that radix astragali might induce the neurogenesis of DA neurons. Related studies have reported that R. astragali could promote the proliferation and differentiation of NSCs; however, the potential to differentiate into DA has not been reported.

In this study, we first confirmed that ASI, ASF, and APS had significant effects on proliferation of NSCs *in vitro*. In ASI and ASF groups, with increasing concentration, proliferation gradually increased and then decreased. Next, we also found that BrdU-positive cells were increased after drug intervention, indicating ASI, ASF, and APS were able to promote NSCs proliferation, especially ASF on the 14th day. Meanwhile, ASI could reduce the expression of nestin, which might be related to the increased differentiation in the process of cells culture.

Moreover, our study showed ASI, ASF, and APS could promote the expression of *TH* and *DAT* at the mRNA level. TH, produced by DA neurons in central nervous system, is the enzyme responsible for catalyzing the conversion of the amino acid L-tyrosine to L-3,4-dihyroxyphenylalanine, which is a precursor for dopamine. Then *TH* is transmitted to the synaptic destinations by the axons and produce dopamine [13,14]. DAT is a membrane-spanning protein that pumps the neurotransmitter dopamine out of the synaptic cleft back into cytosol, and is highly expressed in the substantia nigra pars compacta and located in cell bodies, axonal, and dendritic plasma membranes [15,16].

Nonetheless, how do these drugs regulate the differentiation of NSCs? *Nurr1* plays a key role in the maintenance of the DA system of the brain [17], and the mutation in this gene leads to disorders of DA dysfunction, including PD. *Nurr1* induces DA gene expression in NPCs, and some co-activators, such as Ptx3, can potentiate Nurr1-induced DA gene transcription [18]. Ptx3 gene, a transcription factor belonging to the *RIEG/PITX* homeobox family, can regulate terminal

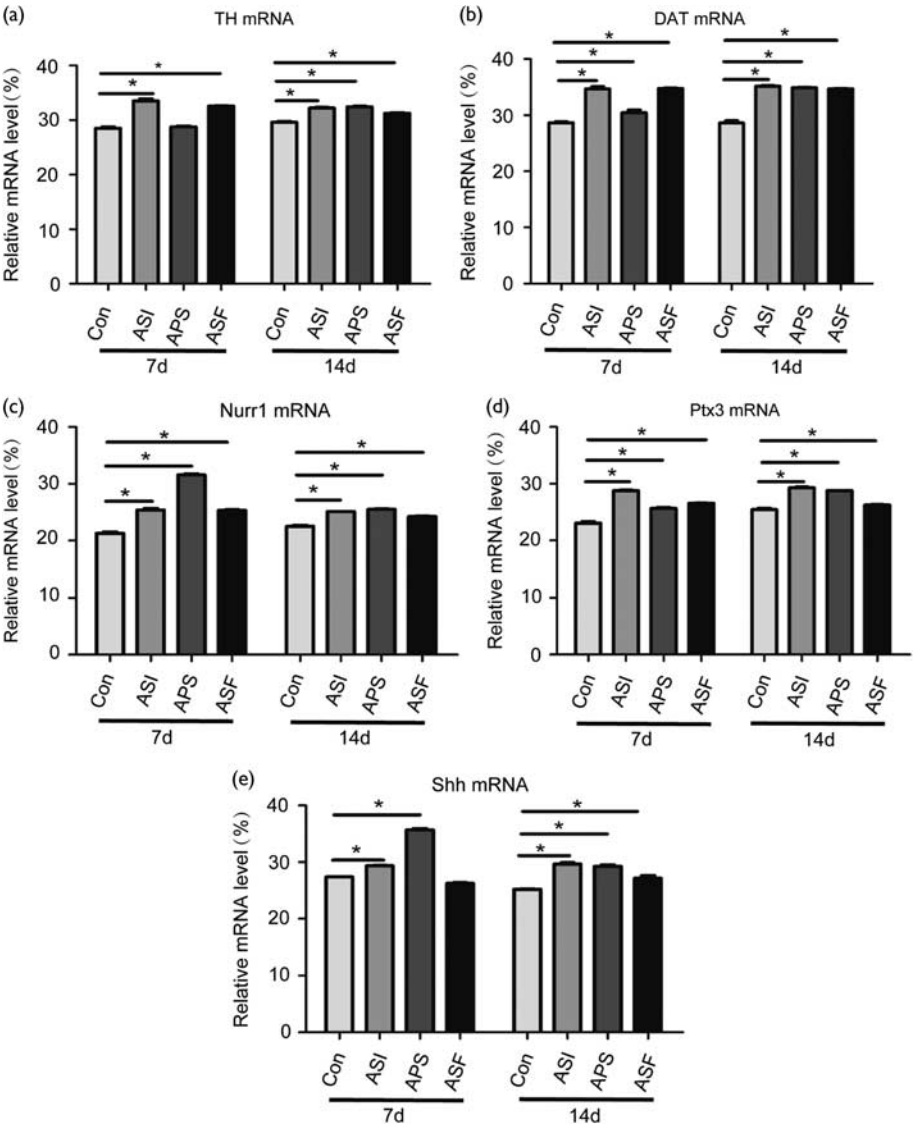
Fig. 1



(a) The primary neural stem cells (NSCs) cultured for 3 days (a1); The NSCs branched after adhering for 24 h (a2); Immunostaining of NSCs with nestin (a3); and Tuj-1 (a4). Scale bar: (a1–a3) 200 μm, (a4) 100 μm. (b) The optical density of NSCs treated with differentiation of ASI, APS, and ASF as measured by CCK-8 assay. (c) Expression of BrdU and nestin after treating with ASI, APS, and ASF; DAPI was used to counterstain nuclei. Scale bar: 200 μm. (d) Quantitative analysis of relative $BrdU^+$ cells (d1) and $Nestin^+$ cells (d2). Data was as mean ± SEM; $N = 3–4$/group. $^*P < 0.05$. APS, astragalus polysaccharide; ASF, astraisoflavan; ASI, astragaloside IV.

differentiation of mesencephalic DA neurons in the substantia nigra compacta of brain [19]. It has been reported that overexpression of *Nurr1* and *Ptx3* genes could promote the differentiation of NSCs into DA neurons and induce the expression of TH in the brains of PD models [20]. Additionally, Shh, which has an

Fig. 2



The expression of relative mRNA by quantitative RT-PCR. The mRNA levels were semiquantified, and normalized with *RN18s* as internal control. Data were represented with GraphPad Prism 5.02 (GraphPad Software Inc., California, USA). Data were as mean ± SEM; $N = 3$/group. *$P < 0.05$ compared with the control group. APS, astragalus polysaccharide; ASF, astraisoflavan; ASI, astragaloside IV; *DAT*, dopamine transporter; *Nurr1*, orphan nuclear hormone 1; *Ptx3*, pituitary homeobox 3; *Shh*, sonic hedgehog; *TH*, tyrosine hydroxylase.

Table 2 The expression of Nurr1, Ptx3, and Shh mRNAs (%)

Groups	Control	ASI	APS	ASF
Nurr1				
7 day	21.3 ± 0.2	25.4 ± 0.2*	31.6 ± 0.2*	25.3 ± 0.1*
14 day	22.5 ± 0.2	25.1 ± 0.1*	25.5 ± 0.1*	24.3 ± 0.0*
Ptx3				
7 day	23.1 ± 0.3	28.8 ± 0.1*	25.7 ± 0.2*	26.6 ± 0.0*
14 day	25.5 ± 0.2	29.3 ± 0.1*	28.8 ± 0.0*	26.2 ± 0.1*
Shh				
7 day	27.4 ± 0.0	29.4 ± 0.1*	35.7 ± 0.2*	26.3 ± 0.1
14 day	25.2 ± 0.1	29.6 ± 0.3*	29.3 ± 0.2*	27.15 ± 0.5*

*$P < 0.05$, significant.
APS, astragalus polysaccharide; ASF, astraisoflavan; ASI, astragaloside IV; *Nurr1*, orphan nuclear hormone 1; *Ptx3*, pituitary homeobox 3; *Shh*, sonic hedgehog.

important role in the generation of a neural model, is a mitogenic signal that induces differentiation of various cells, as well as the proliferation and differentiation of NPCs in the embryonic and postnatal periods [21,22]. To further verify the mechanisms of NSCs differentiation induced by ASI, ASF, and APS, we tested the expression of *Nurr1*, *Ptx3*, and *Shh* in mRNA levels. We found that *Shh*, *Nurr1*, and *Ptx3* mRNAs were significantly enhanced after treatment of ASI, ASF, and APS, except Shh mRNA in ASF group on the seventh day. This may suggest that ASF might affect the differentiation into DA neurons by up-regulating *Nurr1* and *Ptx3*. Additionally, in the APS

group, the expressions of *Nurr1*, *Ptx3*, and *Shh* mRNA were all elevated on day 7. Our study suggested that ASI, ASF, and APS have the abilities to enhance NSCs to differentiate to DA neurons partially by up-regulating the expression of *Nurr1*, *Ptx3*, and *Shh*.

Conclusion

Our study confirmed the components of radix astragali had significant effects on proliferation and differentiation of NSCs. Notably, we found the active ingredients of radix astragali could induce the differentiation of NSCs to DA neurons possibly though upregulation of *Shh*, *Nurr1*, and *Ptx3*.

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Authors contributions: Han Gao and Lianrong Dou contributed equally toward performing the experiment and analyzing the data, and Liang Shan participated in writing the paper. The experiment was directed by Yan Sun and conceived and designed by Wentao Li.

Conflicts of interest

There are no conflicts of interest.

References

1 Lim DA, Alvarez-Buylla A. An adult neural stem cells stake their ground. *Trends Neurosci* 2014; **37**:563–571.
2 Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 1992; **255**:1707–1710.
3 Song X. Pharmacological study of single herb and its active ingredient on cerebral ischemia in 2009. *Chin J Med Guide* 2010; **12**:1589–1590.
4 Si YC, Li Q, Xie CE, Niu X, Xia XH, Yu CY. Chinese herbs and their active ingredients for activating xue (blood) promote the proliferation and differentiation of neural stem cells and mesenchymal stem cells. *Chin Med* 2014; **9**:13.
5 Liu J, Yao Z, Qin M, Chen X, Jian-fang C. Effect of simple astragali, floscarthami, salviam iltiorrhiza injection on differentiation of neural stem cells. *Acta Acad Med Militaris Tertiae* 2006; **28**:1470–1472.
6 Qi C, Liu Y, Tian Y, Zhang J, Chen X, Zhang P, *et al.* Effects of tetramethylpyrazine on cell proliferation in dentate gyrus in rat brain after cerebral ischemia-reperfusion injury. *J Xi'an Jiaotong Univ (Med Sci)* 2007; **28**:1671–1675.
7 Wang C, Zhang Y, Feng Y, Zhuang P, Wang J, Liu H, Wang L. Effect of astragaloside IV on neurogenesis in adult hippocampus of rats after transient forebrain ischemia. *Chin Tradit Herbal Drugs* 2009; **40**:754–758.
8 Zhang W, Li Y, Zhong Z, Wang S, Chen L. Effect of Salidroside on neural stem cell. *Heilongjiang Med Pharmacy* 2005; **28**:3–4.
9 Huang F, Yin Z, Wu D, Hao J. Effects of controlled release of brain-derived neurotrophic factor from collagen gel on rat neural stem cells. *NeuroReport* 2013; **24**:101–107.
10 Liu D, Wang Z, Zhan J, Zhang Q, Wang J, Zhang Q, *et al.* Hydrogen sulfide promotes proliferation and neuronal differentiation of neural stem cells and protects hypoxia-induced decrease in hippocampal neurogenesis. *Pharmacol Biochem Behav* 2014; **116**:55–63.
11 Liu Y, Li RK. Clinical observation on the treatment of Parkinson's disease with zhichan decoction. *Shanxi Zhongyi* 2000; **18**:16–17.
12 Shi H, Song J, Yang X. Zhichan decoction induces differentiation of dopaminergic neurons in Parkinson's disease rats after neural stem cell transplantation. *Neural Regen Res* 2014; **9**:931–936.
13 Alcaro A, Huber R, Panksepp J. Behavioral functions of the mesolimbic dopaminergic system: an affective neuroethological perspective. *Brain Res Rev* 2007; **56**:283–321.
14 Nagatsu T. Tyrosine hydroxylase: human isoforms, structure and regulation in physiology and pathology. *Essays Biochem* 1995; **30**:15–35.
15 Ledley FD, Di Lella AG, Kwok SC, Woo SL. Homology between phenylalanine and tyrosine hydroxylases reveals common structural and functional domains. *Biochemistry* 1985; **24**:3389–3394.
16 Nirenberg MJ, Vaughan RA, Uhl GR, Kuhar MJ, Pickel VM. The dopamine transporter is localized to dendritic and axonal plasma membranes of nigrostriatal dopaminergic neurons. *J Neurosci* 1996; **16**:436–447.
17 Sacchetti P, Carpentier R, Segard P, Olive-Cren C, Lefebvre P. Multiple signaling pathways regulate the transcriptional activity of the orphan nuclear receptor NURR1. *Nucleic Acids Res* 2006; **34**:5515–5527.
18 Yi SH, He XB, Rhee YH, Park CH, Takizawa T, Nakashima K, Lee SH. Foxa2 acts as a co-activator potentiating expression of the Nurr1-induced DA phenotype via epigenetic regulation. *Development* 2014; **141**:761–772.
19 Smidt MP, Smits SM, Burbach JP. Molecular mechanisms underlying midbrain dopamine neuron development and function. *Eur J Pharmacol* 2003; **480**:75–88.
20 Messmer K, Remington MP, Skidmore F, Fishman PS. Induction of tyrosine hydroxylase expression by the transcription factor Pitx3. *Int J Dev Neurosci* 2007; **25**:29–37.
21 Han YG, Spassky N, Romaguera-Ros M, Garcia-Verdugo JM, Aguilar A, Schneider-Maunoury S, *et al.* Hedgehog signaling and primary cilia are required for the formation of adult neural stem cells. *Nat Neurosci* 2008; **11**:277–284.
22 Jessell TM. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat Rev Genet* 2000; **1**:20–29.