Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi: 10.1016/S1995-7645(14)60094-8

Effect of spinal cord extracts after spinal cord injury on proliferation of rat embryonic neural stem cells and Notch signal pathway *in vitro*

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ARTICLE INFO

Article history:
Received 10 March 2014
Received in revised form 15 April 2014
Accepted 15 June 2014
Available online 20 July 2014

Keywords:
Neural stem cell
Cell culture
Spinal cord extract
Notch signal pathway

ABSTRACT

Objective: To investigate the effect of the spinal cord extracts (SCE) after spinal cord injuries (SCIs) on the proliferation of rat embryonic neural stem cells (NSCs) and the expressions of mRNA of Notch1 as well as of Hes1 in this process *in vitro*. **Methods:** The experiment was conducted in 4 different mediums: NSCs+PBS (Group A-blank control group), NSCs+SCE with healthy SD rats (Group B-normal control group), NSCs+SCE with SD rats receiving sham-operation treatment (Group C-sham-operation group) and NSCs+ SCE with SCIs rats (Group D- paraplegic group). Proliferative abilities of 4 different groups were analyzed by MTT chromatometry after co-culture for 1, 2, 3, 4 and 5 d, respectively. The expressions of Notch1 and Hes1 mRNA were also detected with RT-PCR after co-culture for 24 and 48 h, respectively. **Results:** After co-culture for 1, 2, 3, 4 and 5 d respectively, the MTT values of group D were significantly higher than those of group A, group B and group C ($P<0.05$). However, there were no significantly differences regarding MTT values between group A, group B and group C after co-culture for 1, 2, 3, 4 and 5 d, respectively ($P>0.05$). Both the expressions of Notch1 and Hes1 mRNA of group D were significantly higher than those of other 3 groups after co-culture for 24 h and 48 h as well ($P<0.05$). But there was no difference oin expressions of Notch1 and Hes1 mRNA among group A, group B and group C after co-culture for 24 h and 48 h ($P>0.05$). There was no difference in expressions of Notch1 and Hes1 mRNA between 24 h and 48 h treatment in group D. **Conclusions:** SCE could promote the proliferation of NSCs. It is demonstrated that the microenvironment of SCI may promote the proliferation of NSCs. Besides, SCE could increase the expression of Notch1 and Hes1 mRNA of NSC. It can be concluded that the Notch signaling pathway activation is one of the mechanisms that locally injured microenvironment contributes to the proliferation of ENSC after SCIs. This process may be performed by up-regulating the expressions of *Notch1* and *Hes1* gene.

1. Introduction

Spinal cord injuries (SCIs) have many causes, but are typically associated with major trauma from motor vehicle accidents, falls, sports injuries and violence. Currently SCIs is characterized by high morbidity, high disability rate, high cost of treatment and mainly young adults[1], which remains to be a global challenge. Primary and secondary damages in the spinal cord lead to neuronal degeneration, necrosis, loss in large quantities and tracts breakage of spinal cord, and demyelination changes is the pathological basis for permanently loss of motor, sensory and sphincter function below the level of damage. Therefore, it is the common interests of global scientists to explore how to supplement the lost neuron and to promote axonal and myelin sheath regeneration for functional recovery.

It has been demonstrated by many researchers that neural stem cell (NSCs), which have the potential for division,

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Foundation project: It is supported by Health Bureau of Luzhou No: 2012-S-40(1/5) and Health Department of Sichuan (120389).
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proliferation and self-renew and the ability for differentiate to all types of nerve cells in central nervous system (CNS) (*eg*. neuron, astrocyte and oligodendroglia cells)[2] have been widely distributed in CNS such as adult mammal spinal cord[3-7]. It has been reported that replenishing lost neuron and gliocyte by activating and promoting endogenous neural stem cells (ENSCs) to proliferate, migrate and differentiate after SCI could enhance recovery of function after SCI. Autotransplantation of ENSCs could prevent many issues arise from exogenous transplant, thus it is more appropriate for clinical use and has broad application prospects[8].

Studies have shown that mediating ENSCs proliferation and differentiation through Notch signaling pathway mainly depends on the exposure microenvironment[9,10]. Based on the speculated interactions between Notch signaling pathway and local microenvironment after SCI as an external mediating signal to regulate ENSCs proliferation, the regulating mechanism of ENSCs proliferation after SCI can provide theoretical basis for accelerating the repair of injury of spinal cord by microenvironment intervention and the promotion of ENSCs proliferation. We used SCE to simulate the microenvironment after SCI to explore the effects of different SCEs on NSCs proliferation and Notch signaling pathway.

2. Materials and methods

2.1. Experimental animals

12 estrus female adult SD rats (body weight range 220-250 g, clean level) and 4 healthy adult male SD rats (body weight range 220-250 g, clean level) were provided by the Animal Center of Luzhou Medical College, Sichuan Province. Male and female SD rats were caged together at the ratio of 1:3. The next morning in the same cage white vaginal plug was observed. 12 h later, it was recorded as pregnancy Day 1. Pregnant SD rats were fed until Day 15 of gestation, when these SD rats were executed to obtain the embryonic rat neural stem cells.

2.2. Experimental reagents & instruments

MTT assay kit was purchased from Shanghai Biyuntian Biological Co., Ltd. Total RNA purification kit was obtained from Tiangen Biotech(Beijing)Co., Ltd. RT-PCR kit was the product of Chengdu Furuike Biotechnology Co., Ltd. Other reagents were analytically pure (A.R.). Inverted phase contrast microscope was from Leica Camera AG, Germany; full spectrum spectrophotometer was from Thermo Electron, USA. PCR amplifier and digital gel image analysis system was from Bio-Rad Laboratories, Inc. All surgical instruments used during the study were provided by the animal center of Luzhou Medical College, Sichuan Province.

2.3. Assay

2.3.1. Cell culture

Pregnant SD rats with 15 days of gestation were killed by cervical vertebra dislocation and placed in 75% ethanol for 10 min. Then these rats were fasten to the super clean bench in supine position, T shape incision was used to open the abdominal cavity and fetal rats were taken out from V shape uterus appearing as a "string of beads". These fetal rats were placed into pre-heated water bath (until 37 ℃) with 0.01 M phosphate buffer solution pH 7.2 (PBS) for 10 min; head skins were separated from unshaped rat skull under dissecting microscope and fetal brains were taken out to the second sterile Petri dishes with pre-heated 0.01 M PBS (pH 7.2) for 10 min. Surface blood was cleared; pia mater was dissected from choroid plexus tissues under dissecting microscope and was rinsed. The tissues were transferred to the third dish as mentioned above, the tissues were cut to pieces of about 1 mm^3 each and pipettes were used to transfer tissues to 5 mL round-bottom polypropylene tubes. Single cell suspensions from the tissues was prepared, live cells density was counted using trypan blue exclusion method and cells were inoculated in the flasks at the density of 1×10^6/mL. Cells were cultivated in incubator of 37 ℃, 5% CO_2 and saturated humidity. The culture medium was replaced during the cell growth based on the density, nutrient consumption rate and by-product formation rate was determination every 2 to 3 d. The cell growth status was observed under inverted phase contrast microscope and cells were subcultured every 5 d.

2.3.2. Preparation of spinal cord extracts (SCE)

15 healthy adult female SD rats (body weight of 220-250 g) were randomized into sham- operation group (n=5), surgically paralysed group (n=5), control group (n=5) after acclimation for 2 weeks. Weight-drop (WD) method was adopted to establish adult SD rat model of SCI. 5 days after rats modeling, the rats were anesthetized and fixed in situ, the skin and muscle were incised to expose T8-T10 spinal cord. Spinal nerve roots outside of the spinal cord were separated using self-made hook, segments T8-T10 (110-120 mg per specimen) were taken out and immediately placed in 4 ℃ sterile PBS (alkaline phosphat buffer solution without Ca^{2+} and Mg^{2+}). Envelope and part nerve roots were trimmed and washed off the surface blood. Specimen was transferred into 2 mL grinder and added with 1 m 4 ℃ sterile PBS. They were homogenized and centrifuged at 3 000 rpm for 10 min. The supernatant was filtered using 0.22 μm syringe filters twice and froze at -20 ℃ for storage (1 mL each tube). Same procedures were used for rats in the sham-

operation group and control group.

2.3.3. Methods for evaluating the effect of SCE on NSCs growth curve

Cell density of single cell suspension was adjusted to 1×10^6/mL, added with 500 μL/well in a 24 well microplate and divided into 4 groups. In group A 25 μL PBS (0.01mol/L, PH7.2) was added into each well; in group B, added with 25 μL spinal cord extracts from normal controls; in group C, added with 25 μL spinal cord extracts from sham operation group; in group D, added with 25 μL spinal cord extracts from SCI group. The viable cells from each of the three wells in 4 groups were counted every 24 h. The average values of each three wells were recorded. The growth curves were drawn with time as abscissa and cell population as ordinate.

2.3.4. MTT assay for detecting the influences of different SCEs on NSCs proliferation

Experimental groups and culture conditions were the same as mentioned above and inoculation cell volume was 100 μL. The added volumes of PBS or different spinal cord extracts were 5 μL/well and each group had 9 duplicate wells. Cultures were incubated at a constant temperature of 37 ℃, 5% CO_2 incubator gas conditions. The viability of NSCs were detected by MTT at 24, 48, 72, 96 and 120 h.

2.3.5. RT-PCR method for Notch 1 and Hes1 mRNA detection

Logarithmic growth phase NSCs were seeded in 6-well plate at the density of 1×10^6/mL with the volume of 2 mL in each well and each group had 6 duplicate wells. Experimental groups and culture conditions were the same as mentioned before and the added volumes of PBS or different spinal cord extracts were 100 μL/well. Cultures were incubated at a constant temperature of 37 ℃, 5% CO_2 incubator gas conditions. According to the total RNA kit instruction manual, total RNA was extracted from NSCs at 24 and 48 h, respectively.

All the primers were synthesized and qualified by Sangon Biotech (Shanghai) Co.,Ltd. Notch1 gene primer: target fragment length 119 bp Upstream primer: 5′-CCAGTACAACCCGCTAAGGC-3′Downstream primer: 5′-GGGACAAGGTATTGGTGGAGA-3′Hes1 gene primer:target fragment length 267 bp pstream primer: 5′-GCGCCGGGCAAGAATAAATG-3′ ownstream primer: 5′-TCGGTGTTAACGCCCTCACAC-3′GAPDH gene primer:target fragment length 450 bp pstream primer: 5′-ACCACAGTCCATGCCATCAC-3′Downstream primer: 5′-TCCACCACCCTGTTGCTGTA-3′.

The total RNA was used as template and RT-PCR cycle was initiated. In accordance with the RT-PCR kit instruction, detailed reactions were as following: the reaction program was devised for 35 cycles, each with 30 s at 94 ℃ denature, 30 s at 37 ℃ annealing (annealing temperature varies with the primers: 58 ℃, 56 ℃and 59 ℃ for Notch1, Hes1 and GAPDH respectively) and a final extension at 72 ℃ for 30 s. PCR reaction products were stored at -20 ℃ until analyzed by agarose gelelectrophoresis.

2.4 Statistical method

SPSS 11.5 was used for statistical analysis. P value less than 0.05 was considered as statistical significance. All data were expressed as mean±standard error (mean±sd). One-way ANOVA was used to compare the means of four matched groups. SNK method was used to compare the differences between groups.

3. Results

3.1. Rat embryonic NSCs growth status

Primary NSCs isolated from embryonic rat brain were small, characteristically round and regular in shape and has bright color observed under microscope. 24 h after NSCs cultivation, suspended single cells division or proliferation could be observed (Figure 1A); on the third day, a couple or dozens of cell aggregates resembling botryoid or sphere had transparent appearance and high refractivity under inverted phase contrast microscope (Figure 1B). On the fifth day, aggregation of large amount of cells to form cells spherical aggregates were observed. Around the spherical aggregates, there was transparent appearance and they had high refractive index, while the refractivity began to decrease in the center of the spherical aggregates. By this time, single cell suspension could be easily prepared (Figure 1C). On the eighth day, the number of neurospheres in the culture medium decreased gradually, mutually fused or the volume of single neurosphere continued to increase (Figure 1D).

Table 1

Cell numbers of NSC of each groups at deferent time point (n=3).

Group	1 d	2 d	3 d	4 d	5 d	6 d	7 d	8 d
Blank control group	0.663±0.031	0.920±0.026	1.767±0.025	2.363±0.050	2.697±0.015	2.877±0.015	3.043±0.061	3.087±0.015
Normal spinal cord group	0.667±0.031	0.907±0.032	1.783±0.031	2.280±0.046	2.707±0.021	2.850±0.020	3.067±0.045	3.097±0.006
Sham-operation group	0.677±0.153	0.873±0.021	1.780±0.020	2.313±0.055	2.683±0.012	2.893±0.021	3.007±0.015	3.073±0.015
SCI group	0.797±0.153*	1.213±0.025*	2.003±0.055*	2.563±0.031*	2.903±0.025*	3.130±0.095*	3.287±0.015*	3.333±0.015*

*Compared with other groups, P<0.05.

Table 2
MTT values of NSC of each groups at deferent time point (n=10).

Group	1 d	2 d	3 d	4 d	5 d
Blank control group	0.132±0.025	0.186±0.019	0.238±0.029	0.287±0.024	0.322±0.018
Normal spinal cord group	0.142±0.033	0.181±0.032	0.245±0.031	0.281±0.034	0.309±0.027
Shame-operation group	0.149±0.030	0.186±0.031	0.228±0.027	0.297±0.020	0.315±0.017
SCI group	0.194±0.161*	0.238±0.035*	0.291±0.331*	0.353±0.0274	0.383±0.017*

*Compared with other groups, P<0.05.

Table 3
Expression of mRNA in each group after cocultured for 24 h (n=3).

Group	Notch1	Hes1
Blank control group	0.994±0.024	0.923±0.036
Normal spinal cord group	0.988±0.032	0.976±0.034
Shame-operation group	0.934±0.053	0.916±0.062
SCI group	1.150±0.036*	1.235±0.103*

*Compared with other groups, P<0.05.

A large amount of cloned neurospheres were obtained by TrypLTM Express digestion and mechanical isolation, with appropriate size and good shape (Figure 1E). Neurospheres adhered to the surface and the interconnection between them to form networks (Figure 1F).

3.2. SCE effects on NSCs growth curve

On day 1, 2, 3, 4, 5, 6, 7 and 8, cells numbers of NSCs from all groups were determined (Table 1). According to the statistical analysis, cells numbers of group D at all the observed time points were significantly higher than those in group A, B and C (P<0.05), while the difference in cells numbers at different time points among group A, B and C was not statistical significant (P>0.05).

3.3. NSCs proliferation ability by MTT

On day 1, 2, 3, 4 and 5, MTT values of NSCs in all groups were determined (Table 2). According to the statistical analysis, all the MTT values in group D were significantly higher than those in group A, B and C (P<0.05), while there was no difference among group A, B and C (P>0.05).

3.4. Notch1 and Hes1mRNA detection by RT-PCR

According to our statistical analysis, co-cultivation of rat SCE and NSCs about 24 and 48 h later, the expression quantities of Notch 1 and Hes 1 in group D was significantly higher than those in group A, B and C (P<0.05), while there was no difference inexpression quantities of Notch 1 orHes 1 genes among group A, B and C (P>0.05). Statistical analysis between 24 hours and 48 h expression quantities indicated that there was no significant difference between them, although the values of 48 h detection were higher than the 24 h values (P>0.05) (Figure 2&3, Table 3&4).

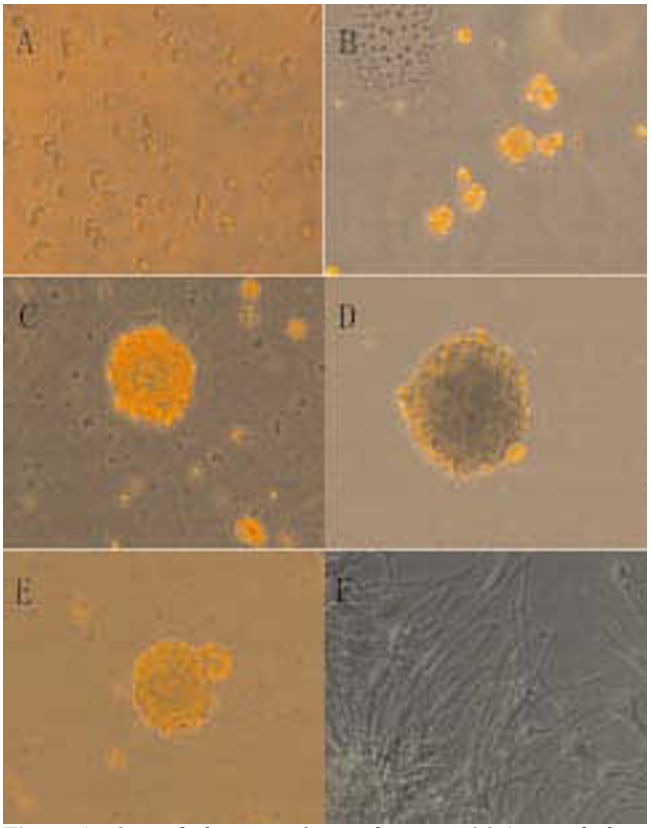

Figure 1. General character of rat embryonic NSC (inverted phase contrast microscope) primary culture 24 hours (×100) , culture 3 d (×100), 5 d (×100), 8 d (×100), to fifth generation culture 5 d (×100) and 5 d after differentiation (×200) (Figure A-F).

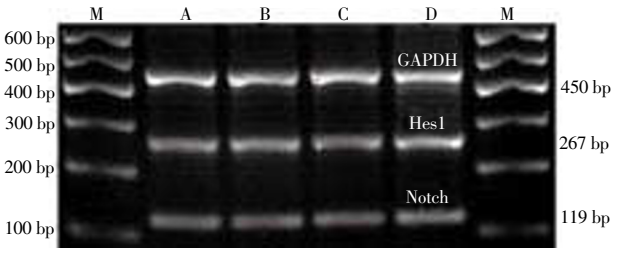

Figure 2. Electrophoresis of PCR products of mRNA in each group after coculture for 24 h.

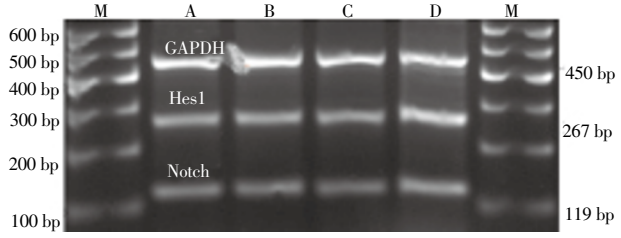

Figure 3. Electrophoresis of PCR products of mRNA in each group after coculture for 48 h.

Table 4
Expression of mRNA in each group after cocultured for 24 h (n=3).

Group	Notch1	Hes1
Blank control group	0.914±0.002	0.876±0.015
Normal spinal cord group	0.905±0.023	0.874±0.012
Shame-operation group	0.878±0.176	0.900±0.031
SCI group	1.226±0.035*	1.347±0.054*

*Compared with other groups, P<0.05.

4. Discussion

In our study, the SCE which simulated SCIs environment *in vitro* were co-cultured with embryonic rat NSCs which undergone amplification, purification and identification. Several hours after co-culture, the number of embryonic rat NSCs and their proliferation ability were significantly higher than those of blank control group, normal SCE group and sham-operation group (P<0.05). It is demonstrated that SCE from SCI group could promote embryonic rat NSCs proliferation *in vitro*, which is correspondence with the results of *in vivo* studies from Laural and Meletis *et al*[11-13]. It can be concluded that, SCE from injured rats could reflect the local microenvironment after SCIs to some extent which may promote NSCs proliferation.

Currently, secondary reactions after SCIs lead to the release of several inflammatory mediators and growth factors, which constitute the complicate microenvironment after SCIs. Prior studies have shown that SCIs contribute to the up-regulation of several inflammatory mediators like a (TNF-α), IL-6, IL-1a, IL-1b[14]. In addition to participating in immunoreactions, these inflammatory mediators together with other growth factors released after SCIs may play their role in the proliferation, differentiation and variability[15-22].

Besides the above mentioned inflammatory mediators and growth factors, the self-sustaining and proliferation of NSCs is also influenced by the signaling pathway mediating the interactions between adjacent cells, *i.e.* the regulation of Notch signaling pathway which have been confirmed in several studies, as well as its role in NSCs differentiation[23,24]. In our study, the Notch 1 and Hes1 mRNA expression levels of NSCs after co-culture of embryonic rat NSCs and SCIs for about 24 and 48 h *in vitro* were significantly higher than those of blank control group, normal SCE group and sham-operation group (P<0.05), which shows the spinal cord extraction from SCIs group may up- regulate the Notch 1 and Hes1 mRNA expression levels of embryonic rat NSCs suggesting that Notch signaling pathway involved in the regulation of SCE after SCI on NSCs. Researchers have stated that after the activation of Notch signaling pathway, the inhibition of Ngn1 on JAK/STAT signaling pathway was eliminated by the inhibition of down-stream Ngn1 gene expression from up-regulated Hes gene, thus the proliferation of NSCs may be promoted. These studies have revealed that the loss of Notch signal correlates with the decrease in the number of NSCs and vice versa[28]. It has been confirmed that, inhibition of NSCs neurogenesis and promotion their proliferation by Notch signaling pathway were mainly depended on the microenvironment NSCs have exposed to[9,10]. Previous studies have verified that the Notch signaling pathway could be activated after central nervous system injury (*eg.* SCIs) which correlates with the proliferation of injured NSCs *in vivo*[29,30]. Combined with our research results *in vitro*, we speculated that one of the mechanisms of locally injured microenvironment promoting ENSC proliferation after SCI may be activation of Notch signaling pathway to participate in the regulation of ENSC proliferation, which mainly depends on the up-regulation of Notch 1 and Hes1 gene expression.

In conclusion, we demonstrate that SCE after SCI could reflect the locally injured microenvironment to some extent and simulate SCI microenvironment *in vitro*, which may promote the proliferation of NSCs and up regulate Notch 1 and Hes1 mRNA expression levels of NSCs. However, the SCE from normal rats or from sham-operation group rats have no effect on NSCs proliferation and Notch 1 and Hes 1mRNA expression level. One of the mechanisms that locally injured microenvironment after SCI may promote ENSC proliferation can be contribute to the activation of Notch signaling pathway involving the regulation of ENSC proliferation which principally rely on the up regulation of Notch 1 and Hes 1 expression.

Conflict of interest statement

We declare that we have no conflict of interest.

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