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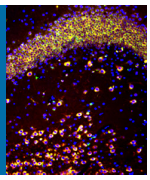

Original article

BDNF administration in skeletal muscle is effective to promote locomotor function improvement in SCT rats

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Abstract

Spinal cord injury is a prevalent disease in surgical clinic with a high morbidity and mortality. Due to its complex pathological process, there is no satisfied strategies for its repair. BDNF, an important member of neurotrophin family, is crucial in supporting survival and differentiation of neurons. However, the concentration of BDNF in CNS is very low, so as to not reach an effective level for the treatment. It therefore needs to develop new strategy to increase BDNF release in injury region. Human simple herpes virus(HSV), a neural invasion virus, has been developed as a vector to carry bio-factors into organism. Here, adult SD rats are divided into sham, SCT, and SCT with HSV-BDNF administrated group. The effect of HSV-BDNF recombinant infection on motor function recovery evaluated by BBB scale was detected, and labeled cells of HSV is observed under fluorescence microscope, as well as the BDNF expression level is evaluated using Western Blot and IHC. As the result, HSV-BDNF recombinant is detected in motor neurons from spinal cord tissue, and the level of BDNF substantially is increased. Finally, motor function recovery of rats in HSV-BDNF group is significantly improved, compared with the SCT group, which confirmed HSV-BDNF releasing in skeleton muscle could be as an effective strategy for the treatment of SCI.

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Introduction

Spinal cord injury (SCI) is a prevalent injury in clinical practice which always induce permanent loss of motor and sensory function under the injury level(Haider T, et al., 2015). Myophagism and depression happened in SCI patients, which caused a severe burden on the life quality of patients(Castro MJ, et al., 1990; Craig A, et al., 2015). According to the pathological process, SCI is divided into two phases: primary injury and secondary injury(Mortazavi MM, et al., 2015). In primary injury, contusion and extrusion usually induce ischemia and reperfusion in tissue. Then inflammation and necrosis cascades were followed in secondary injury, which plays more severe roles in physiological functions loss. Meanwhile, organism has gradual and limited capacity of functions recovery by intrinsic program or rescue themselves by neurotrophins.

Neurotrophins, consisting a family, includes nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) (Hempstead BL, 2015). BDNF is a vital member protein that can support survival and differentiation of neurons, mediated by TrkB receptor(Barde YA, et al., 1982; Chao MV, 2003). As reported, BDNF plays important roles in synaptic function(Lohof AM, et al., 1993), long-term potentiation(Cowansage KK, et al., 2010) and hippocampal function(Lyons WE, et al., 1999). Previously, BDNF is researched as a prominent candidator in neuroprotection, axonal regeneration, and synaptic plasticity in SCI(Ji XC, et al., 2015). Whereas, it's concentration is too low to reach effective level for treatment in central neuron systems.

Human simplex Herpes virus (HSV), a philic-nerve neurotrophic virus, usually leads to significant morbidity and mortality in nervous system in certain clinical settings. Approximately two-third world-wild population has been infected with HSV which causes ocular, facial and genital herpetic diseases in the life(Dasgupta G, et al., 2009). Recently, HSV with elimination side effect has been developed as a prevalent vector for biological factors releasing, especially in nervous system.

Here, we established a technique that HSV could carry BDNF to release in skeletal muscle, and transferred into spinal cord, so as increase motor function improvement after SCI, which is useful for the treatment of SCI in cline.

Materials and methods

Animals and grouping

Total 30 rats, provided by Zhejiang University, are equivalently and randomly divided into three groups- sham, SCT, SCT with HSV-empty-vector, SCT with HSV-BDNF.

All rats are all raised in independent cages for 12 hours light/dark cycle and freely accessible to water and food.

HSV-BDNF recombination preparation

HSV-BDNF with GFP report gene was constructed and validated in previous experiment (Wang SL, et al., 2012).

Spinal cord transection

3.6% chloral hydrate is intraperitoneally used to anesthesia by a dosage of 1mL per 100 g body weight. When rats are unconscious, their back skins are sterilized using iodine after fur shaved and they are placed prone in operating table. Their skins are cut and muscles are separated to expose vertebral plate. Afterwards, vertebral plates are resected at T9-T11 segments to expose spinal cords and spinal cords are transected. The sham group is processed with vertebral plate resection only. Finally, muscle, anadesma and skin are sutured respectively. The wounds are applied with iodine to sterilize to avoid infected. After operation, rats are cared gently three times every day, besides, penicillin is intraperitoneally injected until hematuresis is disappeared.

HSV-BDNF recombination injection

Total 7.5 μl HSV-BDNF solution is injected into hind limb gastrocnemius muscle at three points with 2.5 ul for each, by microinjector accompany with micro pump. In detail, the skin in local hind limb is sterilized and subsequently fur is shaved. Then, HSV-BDNF solution is injected into skeletal muscles of hind limbs at the speed of 2.5 μl/min. When solution injection is finished, microinjector is maintained for 2 min and finally evacuated. The empty-vector is injected with equivalent volume HSV-vector.

Motor behavior assessment

Rats in each group are processed with motor behavior assessment by three double-blind researchers at 3, 7, 14, 28 days after HSV interjection, refered by BBB scores.

Sample harvest

Tissues of muscles and spinal cords are harvested at 7 dpo and 28 dpo, respectively. Briefly, Skeletal muscles of hind limb and spinal cords are harvested and stored at -80°Cfor PCR and western blot at 7 dpo, followed by normal saline solution perfusion. At 28 dpo, rats are perfused with 4% paraformaldehyde solution, which is pre-cold at 4°C. And after that, spinal cords and skeletal muscles are stored in 4% paraformaldehyde solution at 4°C.

Morphology observation

Spinal cords and muscles are sliced into 10 μm thickness in a freezing microtome (Leica CM1900, Germany), respectively. Subsequently, sections are washed 3 times in PBS for 5 minutes each time, and then blocked in 5 % goat serum/ PBS with Triton for 30 min at 37 °C. After incubated with primary antibodies BDNF (rabbit, 1:100, bs-0248R, Bioss) and Neun (mouse, Chemicon, 1:500) overnight at 4 °C in 2 % serum, sections are washed 3 times in PBS to remove unbound primary antibody, then incubated with secondary antibodies (goat anti-rabbit, Alexa 594, ZSGB-BIO, 1:200; goat-anti mouse, Alexa 488, invitrogen, 1:100) for 1 h at 37 °C. After that, sections are washed 3 more times in PBST (PBS, with 1 % Tween-20) and covered with DAPI/fluorescence quenching agent (Beyotime). Slides were viewed using a Leica fluorescence microscope (Leica. DMIRB, Germany) coupled with a computer assisted video camera.

Western blot

Protein of spinal cords and muscles are extracted by RIPA buffer in burnisher and then the lysate is centrifuged at 15,000 rpm for 15 min to collect supernatants. Subsequently, concentration from extracted protein is quantitative using bicinchoninic acid (BCA) protein assay reagent, according to which, total 50 mg protein was loaded into 4–12% polyacrylamide gels to separate BDNF based on the marker indicator, and then transferred onto a polyvinylidene difluoride (PVDF) membrane. Next, the PVDF membranes are washed with TBST for 3 times with 5 min for each time at room temperature and then incubated overnight at 4°C with primary antibodies of BDNF (1:1000, Santa Cruz, Rabbit) and Neun (1:500, Santa Cruz, Rabbit). β-actin (1:1000,Abcam, USA) is used as control. Then the blots are washed three times in TBST for three times with 5 min each, and next incubated with second antibody (1:5000, goat anti-rabbit IgG; ZSGB-BIO, Beijing, China) for two hours. Afterwards, ECL Western blot detection kit (Amersham Pharmacia Biotech, Buckinghamshire, England) is used to image the immunoblot. Densitometry analysis was performed by ImageJ software.

Statistically analysis

SPSS 17.0 version is used to analysis collected data statistically. One-Way ANOVA method is applied to analysis difference between each group, and all the results are showed as mean roup, $P<0.05$ indicates there is statistical difference between two group. And $P<0.01$ indicates there is significantly statistical difference.

Results

HSV-BDNF tracing in skeletal muscle and spinal cord

To trace the HSV-BDNF in skeletal muscle and spinal cord, GFP-positive signals are detected in muscle cells and spinal cord under fluorescence microscope. In spinal cord, GFP-positive signals are seen in motor neurons, which are located in ventral horn. Interestingly, these cells expressed NeuN marker simultaneously, confirming their neuronal character. In skeletal muscle, there is no positive signal of GFP could be detected in this observation (Fig 1).

Expression level of BDNF in ventral horn

Compared with sham group, BDNF level in SCI rats are slightly increased. However, in HSV-BDNF group rats, the expression level of BDNF is upregulated greatly about more that in control. (Fig 2)

BDNF administration increase repair of motor function

In SCI rats, BBB scale is significantly lower than in the

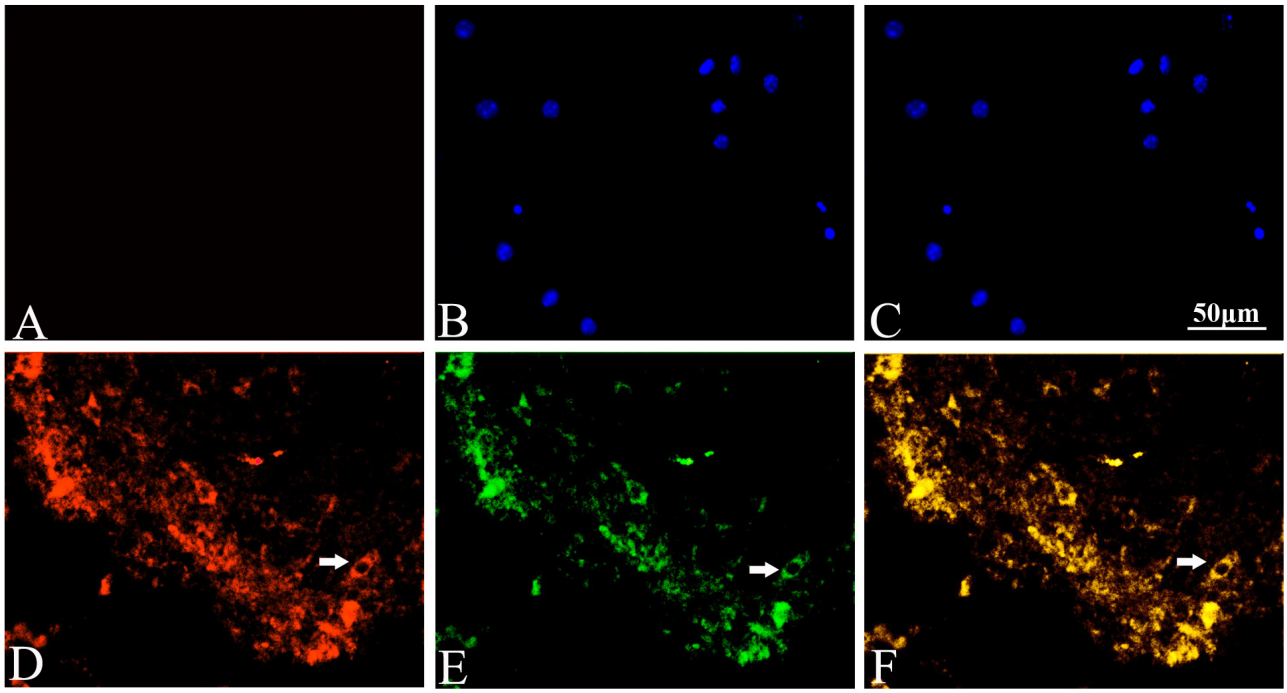

Figure 1 Performance of HSV-BDNF in muscle cells and spinal cord motor neurons. *(A,B,C) showed GFP-positive signals are not showed in muscle cells at 28 days. (D,E,F) showed HSV-BDNF and NeuN was co-expressed in neurons. Scale bar=50μm shown in C.*

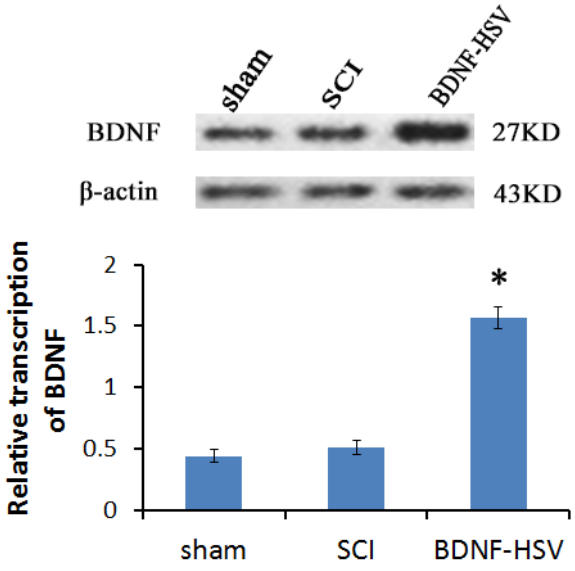

Figure 2 The expression of BDNF in ventral horn neurons in the spinal cord. **P<0.05, compared with no BDNF administrated one.*

sham rats at 3, 7, 14, 28 days. Meanwhile, with the time going on, motor function exhibitor a gradual recovered to some degree althrough this limited. Comparatively, the BBB scale of rats subjected to HSV-BDNF injection gives a significant higher than in the SCI rats at 14 and 28 days (Fig 3), confirming HSV-BDNF administration in skeleton ,muscle is an effective strategy for the repair og SCI .

Discussion

Spinal cord injury, usually encountered in clinic, but the effect for its treatment is far from satisfaction(Grau JW, et al., 2014). A world-wide surgical problem with complex pathological process, novel treatment strategy is waiting to be developed for the treatment of SCI. BDNF, is a crucial factor among the neurotrophins, has showing a positive role in promoting the differentiation and survival of neurons (Hwang DH, et al., 2014). While the concentration of endogenous BDNF is very low in CNS. The usage of BDNF was limited in clinic. In this study, we contributed an efficient vector to transport BDNF into spinal cord from skeleton muscle so as to develop a peripheral therapeutic strategy by using BDNF for repair of SCI.

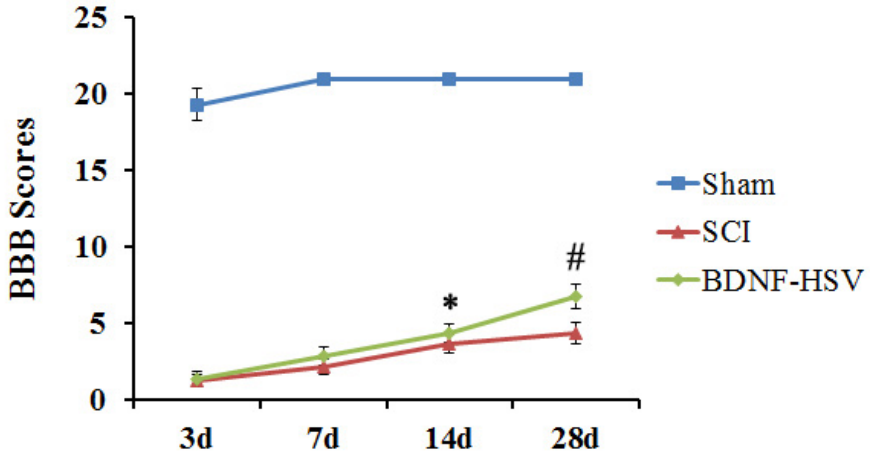

Figure 3 BBB scale of each group rats. **P<0.05, **P<0.01*

BDNF is increased in spinal cord ventral horn neurons after SCT

The BDNF-positive neurons are significantly increased in spinal cord ventral horn neurons after SCI. Anatomically, amounts of motor neurons are located in the ventral horn of spinal cord, and skeletal muscle is the ending of motor reflex arc. Therefor, we injected HSV-BDNF in skeleton muscle and expect HSV-BDNF could be transported into spinal cord. In fact, the HSV recombinant has been absorbed, and reversely transported along with axon of motor neurons. HSV, which showed HIV-BDNF releasing is a optimal technique for the treatment of SCI. As a philic nerve virus (Kelly K, et al., 2008), and HSV has an generous advantage of infection neurons, which provided chance for us to release BDNF by neuronal invasion.

BDNF increase in cord and associatedfunctional implication

Compared with sham one, total BDNF level in SCT is decreased and while it dramatically increased after injected with HSV-BDNF recombinant. These indicates that, HSV has a capacity of infecting the host neurons and the recombinant has been transported from the peripheral environment into the CNS. As an important neurotrophic factor, BDNF exerts a positive influence on the survival of neurons and enhances the remyelination of injured spinal cord(Tolwani RJ, et al., 2004; Han X, et al., 2011). Previously, the BDNF is increased within 7 days in motor neurons after SCI and rapidly decreased(Kasahara K, et al., 2006). In addition, beneficial effects of BDNF depend on the specific receptors-Trk and P75. When BDNF is combined with TrkB, the member of the high-affinity ligand family, it downregulates down-stream signal pathways for cell survival and anti-apoptosis are activated(Proenca CC, et al., 2006; Sweatt JD, 2001; Ying SW, et al., 2002). Therefore, BDNF overexpression is available to recover the cell survival and growth.

In this study, HSV-BDNF has been is used as a optimal strategy to transport BDNF into spinal cord, with better infection efficiency and the beneficial effect on motor function recovery. Therefor, HSV-BDNF injection could be developed to transport BDNF into CNS, so as to d finally promote motor function recovery.

Conclusion

HSV is an efficient vector to transport BDNF into CNS, and importantly, HSV-BDNF bioactivity in improving the motor function recovery is noticed.

Acknowledgement

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Conflicts of interest

There is no conflict interest in this study.

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